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The Effects of Bicarbonate Ions and External pH on the Membrane Potential and Resistance of *Nitella translucens*

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Summary. The effects of bicarbonate ions on the membrane potential and resistance of *Nitella translucens* are shown to be primarily due to the change in pH produced by the bicarbonate acting as a buffer, and not due to the presence of an electrogenic anion pump. The mechanism by which pH affects the membrane potential is discussed in the context of recent work by other authors on this effect.

One of the most perplexing results arising from the study of the ionic relations of the Characeae is the fact that, when calcium is present in the external solution, it is not possible to describe the membrane potential in terms of the passive movements of the major inorganic ions (Spanswick, Stolarek & Williams, 1967). Alternative explanations could be based on the passive movements of the minor ions or the presence of electrogenic ion pumps. A case in which the existence of an electrogenic anion pump has been postulated is the effect of bicarbonate ions on the membrane potential. Hope (1965) has shown that the substitution of 0.1 to 1.0 mM bicarbonate ions for an equivalent amount of chloride in artificial pond water (APW) causes a hyperpolarization of about 60 mV in *Chara australis*, the vacuolar potential reaching values of −200 to −220 mV. Poole (1966) has shown that bicarbonate causes a hyperpolarization in beet tissue also. Hope (1965) considers that the existence of an inwardly directed electrogenic bicarbonate pump at the plasmalemma is the most likely explanation of the hyperpolarization and the other electrical effects he has observed.

This paper describes an attempt to test Hope's hypothesis. This endeavour was complicated by the fact that the manner in which the Characeae respond to bicarbonate appears to depend on the conditions under which they are grown. I therefore repeated many of Hope's experiments on *Nitella translucens* before making further electrical measurements.

Materials and Methods

Material

Nitella translucens was collected from a freshwater loch in Perthshire, Scotland and stored in a standard APW solution (*see* Table 1 for composition). For later experiments, the plants were cultured in a medium similar to that described by Forsberg (1965).

Electrical Measurements

Isolated internodal cells were placed in a trough through which a continuous flow of solution could be maintained. One microelectrode, inserted at the center of the cell, could be used to pass a current pulse to a Ag/AgCl electrode placed next to the cell in the external solution and running its entire length. The membrane potential between the vacuole and the external solution, and the change in membrane potential during the passage of a current pulse, were measured using a second microelectrode inserted at a distance 0.42 l from the current microelectrode, where 2 l is the length of the cell. Hogg, Williams and Johnston (1968) have shown that this arrangement of the microelectrodes permits the membrane resistance to be calculated using Ohm's law. Most experiments were commenced at least 12 hr after the insertion of the microelectrodes. This gives time for the cell to recover from the depression of the membrane resistance caused by the microelectrode insertions (Spanswick, *unpublished observations*). A third microelectrode could be inserted close to the potential-measuring microelectrode to measure the potential difference between the cytoplasm and the external solution. This electrode cannot be left in the cytoplasm for long periods because the sealing process (Walker, 1955) increases its resistance and effectively excludes it from the cell. However, such shallow insertions can be repeated without apparently causing significant damage to the cell in most cases. The potential differences were recorded with Keithley 603 Electrometer Amplifiers and chart recorders. Membrane resistances were calculated from measurements of the magnitude of the current pulse and from the resulting change in membrane potential recorded using a Tektronix 502 A oscilloscope and camera. The experiments were performed in a small room which was maintained at an air temperature of 17 ± 0.5 °C and which could be completely darkened.

Solutions

The composition of the solutions used most frequently in the experiments described here (APW, BAPW, BS, and BB) are given in Table 1. The pH of unbuffered APW or BS was usually 5.5, and that of BB or BAPW was 7.8.

Table 1. *Composition of solutions*

Solution	Concentration (mM)					
	Na	K	Ca	Cl	HCO_3	SO_4
APW	1.0	0.1	0.1	1.3	—	—
BAPW	1.0	0.1	0.1	0.3	1.0	—
BS	1.2	0.1	1.0	0.3	—	1.5
BB	1.2	0.1	1.0	0.3	1.0	1.0

Results

Time Course of the Membrane Potential and Membrane Resistance in Solutions Containing 1 mM *Bicarbonate and 1* mM *Calcium Ions*

Apart from one figure showing the time course of the hyperpolarization caused by bicarbonate ions during the first 20 min, Hope (1965) gives no information concerning the duration of the effect. Preliminary experiments on *N. translucens* (Spanswick, 1964) suggested that the effect was largely transient. This observation has now been confirmed. In the presence of bicarbonate ions (solution BB, Table 1), the hyperpolarization reached a peak value of 58 mV (*see* Table 2) after 6 to 12 min, but subsequently declined to a value only 17 mV more negative than the original value of the membrane potential in solution BS. Thereafter, there was a wide variation in the behavior between individual cells: in some there was a slight increase in the hyperpolarization, but in others the membrane potential continued to fall, although even after 9 hr it was always more negative than the original value in solution BS. A representative time course is shown in Fig. 1.

Table 2. *Summary of the effects of bicarbonate on the vacuolar potential and the total membrane resistance (plasmalemma plus tonoplast). The values are expressed as mean ± S.E.M. (no. of cells)*

Solution	Membrane potential (mV)	Membrane resistance (kΩ cm²)
BS	− 95± 8 (10)	113± 9 (10)
BB (maximum hyperpolarization)	−153±11 (10)	102±13 (10)
BB (after 1 hr)	−112± 7 (10)	128±18 (10)
APW	−128± 8 (7)	93±22 (7)
BAPW (maximum hyperpolarization)	−182±10 (7)	93±22 (7)
BAPW (depolarized state)	−108±11 (7)	16± 2 (7)

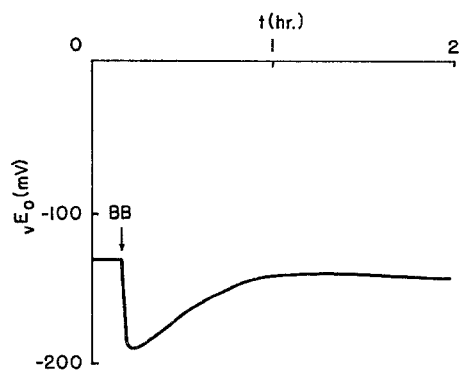

Fig. 1. Time course of the change in vacuolar potential ($_vE_0$) produced by changing from solution BS to BB

The effect on the membrane resistance was even more variable. In most cases, there was a transient decrease during the initial hyperpolarization of the membrane potential, but after 4 hr an increase or decrease compared with the original value in solution BS was equally likely (*see* Table 2).

Location of the Change in Potential Difference (p.d.) Caused by Bicarbonate Ions

That the effect on the membrane p.d. was not due to a change in the p.d. between the cell wall and the external solution was confirmed by observing the p.d. measured by inserting the tip of a microelectrode into the cell wall (Nagai & Kishimoto, 1964; Spanswick *et al.*, 1967). The substitution of bicarbonate for chloride ions had no observable effect on this p.d.

It was possible to confirm Hope's observation that the effect was at the plasmalemma and not at the tonoplast by using two microelectrodes to observe the p.d. between the vacuole and external solution and the cytoplasm and external solution simultaneously. In five experiments, the initial hyperpolarization recorded by both electrodes was identical, indicating that the effect occurred at the outer membrane. Continuation of the experiment was usually prevented by the sealing of the cytoplasm electrode, but in one case it was possible to observe that the two p.d. changed identically during the first hour. In the same experiment, it was observed that the external bicarbonate ions had no effect on the electrical resistance of the tonoplast.

To make certain that the changes in membrane potential after the initial hyperpolarization were not due to changes at the tonoplast, the p.d. and resistance of the tonoplast were measured first in solution BS and again after the cell had been in solution BB for several hours. The data in Table 3 show that there are no large changes in the electrical properties of the tonoplast. It is therefore concluded that the observed changes in p.d. and resistance occur at the plasmalemma.

Table 3. *Effect of bicarbonate ions on the tonoplast p.d. and resistance*

Experiment no.	Solution BS		Solution BB		Time in solution BB (hr)
	p.d. (mV)	R_m (kΩ cm^2)	p.d. (mV)	R_m (kΩ cm^2)	
83	25	22	24	22	7.5
84	45	19	42	29.5	5.5
87	30	13	27	12.5	7
88	19	11	21	8	4

Effect of Light and Dark in the Presence of Bicarbonate and 1 mM *Calcium*

If the hyperpolarization caused by bicarbonate is due to the activation of an electrogenic pump, the effect should be reduced by conditions such as darkness which inhibit the active fluxes of other ions in this species (MacRobbie, 1965) and most probably the influx of bicarbonate itself (Smith, 1968). Hope (1965) found that darkness generally slowed down or inhibited the hyperpolarization. However, the hyperpolarization was not reversed on reverting from light to darkness, although sometimes the p.d. became about 20 mV more positive.

I have been unable to observe any effect of darkness on the initial hyperpolarization after 1 to 17 hr in darkness as compared to cells kept in continuous light. In eight cells, the average hyperpolarization was 36 ± 3.8 mV in darkness compared to 36 ± 2 mV in the light for the same group of cells.

The effect of changing from light to dark in the presence of bicarbonate is a transient hyperpolarization, i.e., a change in potential opposite in direction to that expected if it were caused by the inhibition of an electrogenic bicarbonate pump. The long-term effect on the p.d. was not studied because of the variability in the control cells. However, an increase in resistance similar to that found by Hope in the absence of calcium was observed, the resistance often rising to values as high as 400 $k\Omega\ cm^2$ (*cf.* Table 2).

The short-term effect of changing from dark to light was a transient depolarization and a decrease in membrane resistance. The short-term effects on the p.d. are similar to but larger than the effects observed in the absence of bicarbonate in this species.

The Effect of Bicarbonate on the Membrane Potential and Resistance in the Presence of Inhibitors

Previous studies have shown that 10^{-6} M dichlorophenyl dimethyl urea (DCMU) inhibits the active chloride influx but has little effect on the potassium influx (MacRobbie, 1965), and, more recently, Raven (1968) has shown that it also inhibits the active bicarbonate influx in *Hydrodictyon africanum*. Hope (1965) found that several inhibitors partially reversed the hyperpolarization caused by bicarbonate, including monuron (CMU) at 10^{-5} M. Because of the transient nature of the hyperpolarization in *N. translucens*, it is not practical to study the effect of DCMU on the membrane potential in the hyperpolarized state. It is possible, however, to observe the initial effect of bicarbonate in the presence of DCMU. In three experiments,

cells were pretreated in APW + 10^{-6} M DCMU for 2 hr and the solution then changed to BAPW + 10^{-6} M DCMU. The maximum hyperpolarizations were 82, 42, and 44 mV; i.e., as one would expect from the results of the dark experiments, the initial hyperpolarization is not abolished by DCMU. The presence of DCMU on its own had little effect on the membrane potential or resistance.

The other inhibitor investigated was carbonyl cyanide, N-chlorophenyl hydrazone (CCCP) at 5×10^{-6} M. MacRobbie (1965) has shown that CCCP at this concentration has only a small effect on the chloride influx but inhibits the active component of the potassium influx, and presumably the sodium efflux. Raven (1968) has shown that it does not affect the active bicarbonate influx in *H. africanum*. Thus, CCCP at this concentration appears to act only on the cation pump and although the Na-K pump would not be expected to be electrogenic because the sodium efflux to potassium influx ratio is approximately unity (MacRobbie, 1962), any excess of active sodium efflux over active potassium influx, as observed in animal cells, could lead to electrogenic effects similar to those expected for an inwardly directed electrogenic anion pump. Investigation of the effect of CCCP is complicated by the fact that the inhibitor itself causes a large transient hyperpolarization of the membrane potential. When BS + 5×10^{-6} M CCCP is substituted for solution BS, the mean peak hyperpolarization for five cells was 54 mV (range, 34 to 86 mV). Within 1 to 2 hr the membrane potential returns to its original level; it was decided to apply bicarbonate ions at this point although by this time the membrane resistance had usually more than doubled. In two experiments, hyperpolarizations of 51 and 27 mV were recorded. Although it is difficult to allow for the changes in membrane permeability caused by the CCCP (as indicated by the increase in the membrane resistance), these results would appear to rule out the perhaps-unlikely possibility that the hyperpolarization is caused by the activation of an outwardly directed electrogenic sodium pump.

The Time Course of the Bicarbonate Effect in the Presence of 0.1 mM *Ca*

The initial hyperpolarization of the membrane potential caused by bicarbonate ions in solutions containing 0.1 mM Ca (BAPW in Table 1) is of similar magnitude to that in solutions containing 1.0 mM Ca (Table 2). Subsequently, however, the membrane potential starts to become more positive at an accelerating rate (Fig. 2), and this may or may not be followed by an action potential. Eventually the vacuole potential comes to a new steady level at -108 ± 11 mV compared with -128 ± 8 mV for the same cells in APW. This change in membrane potential is accompanied by a

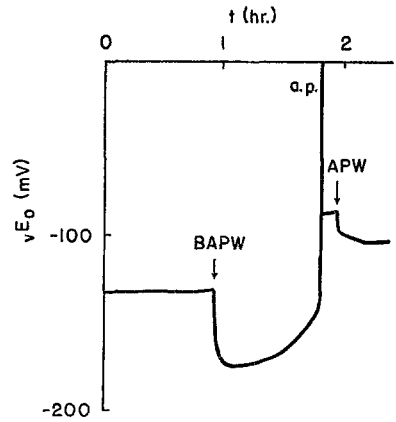

Fig. 2. Time course of the change in vacuolar potential ($_vE_0$) produced by substituting solution BAPW for APW. Note that after the action potential (a.p.) the membrane potential is more positive than the original or subsequent values in APW. This is referred to in the text as the depolarized state

decrease in the total resistance of the two membranes to 16 $k\Omega\,cm^2$, a value not much greater than that of the tonoplast alone for this species (Spanswick & Costerton, 1967). The time it takes for this depolarization and decrease in membrane resistance to occur varies from cell to cell. In some cases it takes place instantaneously, but in other cases it may take 2 hr or longer. Indeed, in cells cultured in a solution with a pH of 7.0, it rarely occurred at all. But in cells cultured at pH 5.4 the change usually took place spontaneously. The effect could be reversed by raising the external calcium concentration to 1 mM.

Effects apparently similar to these have been described by Hope (1965). However, in his experiments, the cells depolarized only when 0.1 mM Na + 1.0 mM K had been substituted for 1.0 mM Na + 0.1 mM K in the external solution and after an action potential had been produced by electrical stimulation. In the hyperpolarized state, the change in potassium concentration from 0.1 to 1.0 mM had little effect on the membrane potential either in the presence or absence of calcium ions. Hope suggests that the change in membrane potential after stimulation is due to an increase in the potassium permeability since the membrane potential is then at a level characteristic of the case when the external potassium concentration is 1 mM and the membrane potential is controlled by sodium and potassium (Hope & Walker, 1961).

This cannot be the case for *N. translucens* since it depolarizes when the external potassium concentration is only 0.1 mM. If the membrane potential

were controlled by the potassium ion, the observed value of the vacuolar potential would be close to −160 mV (Spanswick & Williams, 1964), i.e., about 52 mV more negative than the observed value. This apparent lack of dependence on the external potassium concentration when the cell is depolarized was investigated further by raising the external potassium concentration to 0.5 mM while keeping the external sodium concentration constant. The average change in p.d. for four cells was +4.5 mV. When this is compared with the value of 41 mV for the potassium electrode, it is clear that the membrane potential is not controlled by potassium. It is, of course, possible that the depolarizations in *C. australis* and *N. translucens* are not strictly comparable. However, a spontaneous depolarization has been observed when *C. australis* has been placed in BAPW in this laboratory.

The Effect of Changes in pH in the Absence of Bicarbonate

Preliminary experiments on the effect of the membrane potential of raising the external pH (Spanswick, 1964) indicated that the effects were much smaller than those obtained using bicarbonate at the same pH. In these experiments, the pH was raised by adding small amounts of NaOH, a method previously used by Kishimoto (1959) with similar results. These experiments provided the main reason for testing the hypothesis that there existed an electrogenic anion pump, an hypothesis which has received little support from the results presented above. It was later found that the pH of the APW-NaOH solutions changed as the solution flowed through the bath containing the cell. Thus, a solution with an initial pH of 9.0 could have a pH as low as 7.0 after flowing through the bath. This discovery was prompted by the observation that imidazole at a concentration of 10^{-4} M causes a hyperpolarization of the membrane potential. At the time it was the property of imidazole as an inhibitor of the potassium influx which was being investigated, but imidazole also acts as a buffer and this suggested that possibly it was the same property that enabled bicarbonate to bring about the hyperpolarization. Experiments with a variety of buffers at a concentration of 1 mM (including citrate, phosphate, tricine, Tris and imidazole in the pH range 7 to 8) all gave large transient hyperpolarizations similar to those observed with bicarbonate. The example given in Fig. 3 is for a change of solution from APW (pH 5.5) to APW + 1 mM Tris (pH 6.85). This example also illustrates the fact that the transition to the depolarized state takes place in the same manner as in BAPW. Other similarities between the effect of bicarbonate and other buffers which have been observed are: (1) the hyperpolarization takes place in the dark; (2) the effects of light-dark changes are similar; and (3) transition to the depolarized state

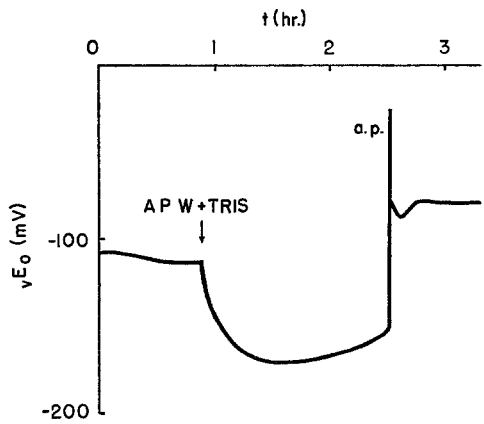

Fig. 3. Time course of the change in membrane potential produced by substituting APW+Tris (pH 6.85) for APW. Note that the cell remained depolarized after the action potential (a.p.)

may take place with or without an action potential. Similar effects have since been observed by MacRobbie, Bendall and Smith (*personal communication*) using external electrodes.

The Effect of Bicarbonate Ions at Constant pH

Clearly, the correct control experiment to determine if the bicarbonate ion is affecting the membrane potential in any way other than in its capacity as a buffer is to apply the bicarbonate solution after the cell has been pretreated in another buffer at the same pH. Any observed changes should then be due to some special property of the bicarbonate ion and not the result of a change in pH at the membrane surface.

The effects on the membrane potential of changing from BS+tricine (pH 7.8) to BB at the same pH were small, but they were difficult to assess quantitatively because the membrane potential varies fairly rapidly in a random manner at this pH. At pH 7.1, the membrane potential is more stable once the initial transient has been completed; the experiment was therefore repeated at this pH. The mean change in membrane potential 10 min after changing from APW+1 mM N-Tris-(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES) to APW+0.1 mM $NaHCO_3$, both at pH 7.1, was 0.2 ± 1.0 mV (mean for six cells $\pm$ S.E.M.). Furthermore, it was observed that the initial hyperpolarization caused by APW+TES was, on the average, the same as that caused by APW+0.1 mM $NaHCO_3$, i.e., 31 mV. An example of the effect of bicarbonate at constant pH is given in Fig. 4.

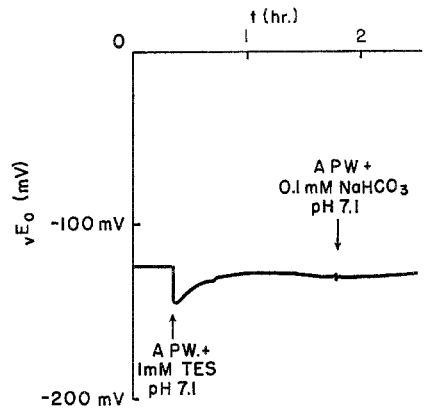

Fig. 4. The effect of bicarbonate ions on the membrane potential at constant pH. At the beginning of the experiment, the cell was in APW

Discussion

Because the electrical effects resulting from the addition of bicarbonate ions to the solution can also be produced by the addition of a variety of other buffers giving a similar change in pH, and because bicarbonate ions have no effect when the cell has previously been in a buffered solution at the same pH, it may be concluded that bicarbonate exerts its effect on ion transport through its ability to control the pH of the external solution. This conclusion is supported by the fact that the experiments designed to test Hope's hypothesis of an electrogenic anion pump produced negative results.

Although this conclusion is somewhat negative, it does raise the question as to what role the hydrogen ion plays in controlling the membrane potential. A dependence of the membrane potential on pH has also been observed by Slayman (1965) in *Neurospora crassa*. He found that the membrane potential varied with the external pH when, as was the case in the present experiments, the membrane potential is not controlled by the external sodium and potassium concentrations (Spanswick *et al.*, 1967). The impossibility of measuring the partial fluxes of hydrogen ions and the uncertainty about the cytoplasmic pH make it difficult to design experiments to test if hydrogen ions contribute directly to the membrane potential and conductance. However, these problems appear to have been circumvented by Kitasato (1968), who showed not only that the membrane potential of *Nitella clavata* was pH-dependent but also that there was a large net passive flux of hydrogen ions across the membrane. To do this, he kept the membrane potential clamped at the potassium equilibrium potential so that the contribution of

potassium ions to the clamping current was zero. In addition, he was able to show that the contribution of sodium and chloride to the clamping current was negligible. However, when he decreased the external pH, there was an increase in the clamping current in a direction consistent with the current being carried by hydrogen ions. Indeed, hydrogen ions made the major contribution to the membrane conductance.

A major difficulty, which apparently caused Hope (1965) to reject this possibility, arises from the fact that the membrane potential of the plasmalemma is normally far more negative than would be expected if it were controlled by the passive diffusion of hydrogen ions, unless the cytoplasm has a pH of about 3.2. This is generally thought to be unlikely. However, because of the high permeability to hydrogen ions and because they will tend to diffuse into the vacuole down their electrochemical potential gradient, it is necessary to postulate that there is a pump transporting them back out of the cell. Kitasato (1968) suggested that if this pump were electrogenic it could account for the large negative value of the membrane potential. Supporting evidence for this hypothesis was obtained by poisoning the cell with 0.2 mM 2,4-dinitrophenol (DNP) and observing the effect on the membrane potential and resistance. He found that the membrane potential became more positive, although not enough to be solely due to the passive diffusion of hydrogen ions. However, the resistance increased at the same time, and he suggested that the magnitude of the membrane potential could be explained if the increase in membrane resistance were the result of a decrease in hydrogen ion permeability, since potassium would then make the membrane potential more negative.

The effects of uncoupling agents on *N. translucens* are more difficult to interpret because the initial effect of DNP, at both 0.2 and 0.01 mM, is a hyperpolarization of the membrane potential (Spanswick, 1964) that is similar to the effect of CCCP reported here. In the case of CCCP, the hyperpolarization is not always accompanied by an immediate increase in resistance, so it seems unlikely that the hyperpolarization is caused by a decrease in hydrogen ion permeability. This suggests that the results of experiments involving inhibitors should be treated with caution and that, in order to investigate this problem further, it will be necessary to determine the effect of each inhibitor on the permeability coefficients of the individual ions.

However, even on the basis of the small effects on the membrane potential of darkness which inhibits the active potassium and chloride influxes, and of 10^{-6} M DCMU which inhibits the active chloride influx, it may be concluded that the energy source of the postulated hydrogen ion pump must

be different from those of the other ion pumps or the pump must have a much higher affinity for its substrate.

Explanations of the transient nature of the pH effect and of the subsequent depolarization in the presence of 0.1 mM Ca will also need to take into account changes in permeability coefficients. However, the experiments described above show that, if the depolarization is the same phenomenon as that described by Hope (1965), an increase in the permeability to potassium is not a sufficient explanation.

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