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EVIDENCE FOR AN ELECTROGENIC ION PUMP IN *NITELLA TRANSLUCENS*

I. THE EFFECTS OF pH, K^+, Na^+, LIGHT AND TEMPERATURE ON THE MEMBRANE POTENTIAL AND RESISTANCE

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SUMMARY

1. The effects of external pH and K^+ in the light and dark were investigated to establish conditions in which the membrane potential of *Nitella translucens* was more negative than the diffusion potential for the major ions.

2. At high external K^+ concentrations there is an apparent increase in K^+ permeability and the membrane potential becomes equal to the calculated K^+ equilibrium potential. It is then possible to calculate the K^+ equilibrium potential for individual cells in solutions having low K^+ concentrations.

3. In a solution containing 0.5 mM K^+ at pH 6, the membrane potential in the dark is close to the K^+ equilibrium potential. At a light intensity of 1.0 $mW \cdot cm^{-2}$ the membrane potential is hyperpolarized by 50 mV and there is a decrease in the membrane resistance from 153 $k\Omega \cdot cm^2$ to 17 $k\Omega \cdot cm^2$.

4. In the dark, a decrease in the temperature depolarizes the membrane by 0.97 $mV \cdot °C^{-1}$ and there is no significant effect on the membrane resistance. In the light, the temperature coefficient for the membrane potential is 2.5 $mV \cdot °C^{-1}$ and the membrane resistance is approximately doubled by a 10 °C decrease in temperature.

5. In the light, the current required to reduce the potential across the plasmalemma to the K^+ equilibrium potential was equivalent to a flux of about 20 $pmoles \cdot cm^{-2} \cdot s^{-1}$. This was tentatively identified with the flux through the electrogenic H^+ pump postulated by H. Kitasato (*J. Gen. Physiol.*, 52 (1968) 60).

6. It is suggested that the results cannot be accounted for by the diffusion of the major ions or by an electrogenic pump in combination with a large passive H^+ flux. An alternative analysis in terms of a voltage-dependent, light-stimulated electrogenic pump (S. I. Rapoport, *Biophys. J.*, 10 (1970) 246) is presented.

INTRODUCTION

Initial attempts to explain the origin of the membrane potential in plant cells were based on the most simple hypothesis available, *i.e.* that the potential was due to the passive diffusion of ions across the membranes. The ion pumps were assumed

to be neutral, only contributing indirectly to the potential by maintaining the concentration gradients of the ions across the membrane[1]. This hypothesis has met with limited success. Hope and Walker[2] were able to use the Goldman equation to describe the behaviour of the membrane potential in *Chara corallina* when the external concentrations of Na^+ and K^+ were varied in the range 0.1–2.0 mM. However, this was only possible if the cells were pretreated in 5 mM NaCl to remove some of the Ca^{2+} from the cell wall and the experiments were performed in Ca^{2+}-free solutions; in the presence of Ca^{2+} the membrane potential remained almost constant when the external K^+ concentration was varied [2–4]. A similar situation was found in *Nitella translucens*[5]. Furthermore, in the presence of Ca^{2+}, it was shown that the membrane potential could not be accounted for by the passive diffusion of the major ions. Alternative explanations involve the diffusion of minor ions or presence of electrogenic ion pumps, *i.e.* pumps that contribute directly to the membrane potential by transporting charge across the membrane. The observation by Hope[6] that HCO_3^- produced a hyperpolarization in *C. corallina* led him to postulate the existence of an electrogenic bicarbonate pump. However, it has since been shown that a similar hyperpolarization in *N. translucens* may be produced by using other buffers having the same pH as the bicarbonate solution. The addition of HCO_3^- at constant pH has no effect on the membrane potential[7]. Meanwhile, Kitasato[8], using *Nitella clavata*, demonstrated a strong dependence of the membrane potential on external pH in the range 4–7. With the membrane potential clamped at the K^+ equilibrium potential, the variation of the clamping current with external pH was consistent with a large passive flux of H^+. He therefore postulated the existence of an electrogenic H^+ pump which would balance the net passive influx of H^+ and, in doing so, would polarize the membrane to give the observed membrane potential. The effect of 0.2 mM 2,4-dinitrophenol at pH 6 was consistent with this hypothesis, assuming it inhibited the active H^+ efflux, in that it resulted in a depolarization of the membrane potential. However, the potential remained more negative than would be expected for a H^+ diffusion potential.

Although the evidence points towards the existence of an electrogenic ion pump in the *Characeae*, it is far from conclusive. A basic difficulty is that, in the solutions usually used in this work, the membrane potential is less negative than the Nernst potential for K^+ and is therefore within the range of possible diffusion potentials. This makes it difficult to use inhibitors to demonstrate the presence of a pump since it is not possible to predict the change in potential when the pump is stopped. The situation is much clearer in *Neurospora crassa*[9], certain higher plants[10] and the marine alga *Acetabularia mediterranea*[11] where the membrane potential can be more negative than the Nernst potential for any of the ions and cannot, therefore, be a diffusion potential. By adjusting the external pH, the external K^+ concentration and the incident light intensity, it has been possible to achieve a similar situation in *Nitella*, thus providing further evidence for the existence of an electrogenic pump. The first part of this investigation consists of a systematic study of the effects of these environmental variables.

METHODS

N. translucens was cultured in the laboratory in a solution similar to Medium II of Forsberg[12] except that the concentrations of the major ions were adjusted to be

approximately equal to those in artificial pond water (see below). A sand–soil mixture was added to provide anchorage and improve growth rates.

For electrical measurements, an internodal cell was isolated and placed in a bath through which solutions could be passed. Measurements of the membrane potential and resistance were made using the methods described previously[13]. The resistance was measured using the single voltage probe method[14]. Square current pulses were passed between a microelectrode inserted into the centre of the cell and a Ag–AgCl wire lying parallel to the cell in the external solution. The membrane potential was measured between a reference electrode in the external solution and microelectrode inserted in the vacuole at a point 0.42 l from the centre of the cell, where 2 l is the length of the cell. In some experiments a third microelectrode was inserted into the cytoplasm at the same point. The membrane potentials were recorded using Keithley 603 electrometer amplifiers and chart recorders. The applied current used for resistance measurements was passed through a standard 10^4 Ω resistor and the change in potential across this, together with the resulting change in membrane potential, was recorded using a Tektronix 502A oscilloscope and camera or a Brush 220 chart recorder.

Light from an incandescent lamp was passed through water and glass filters to reduce the infrared component. The light intensity incident on the bath containing the cell was 1.0 $mW \cdot cm^{-2}$.

The experiments were performed in a constant-temperature room at 20 °C. Low temperatures were obtained by running precooled solutions rapidly past the cell and measuring the temperature in the bath.

The basic solutions used in this work were designed to have concentrations of the major ions similar to those in the artificial pond water used previously but were buffered to give pH's between 5 and 9. Artificial pond water (pH 5) and artificial pond water (pH 6) were buffered with 2-morpholinoethanesulfonic acid, artificial pond water (pH 7) with morpholinopropanesulfonic acid, artificial pond water (pH 8) with tricine and artificial pond water (pH 9) with tris(hydroxymethyl)methylaminopropane sulfonic acid all at 1 mM. The ionic concentrations were: 0.1 mM K^+; 0.1 mM Ca^{2+}; 0.1 mM Mg^{2+}; 1.0 mM Cl^-; 1.0–1.3 mM Na^+. In addition, artificial pond water (pH 5) contained 0.2 mM SO_4^{2-}. Stock solutions (10 ×) were made up without the addition of NaOH (pH < 5) and stored in a refrigerator. Upon dilution, the pH was adjusted to the desired value with NaOH. The variation in the sodium concentration reflects the mismatch between the pK_A's of the buffers and the specified pH's of the solutions. This small variation in sodium concentration has no significant effect on the electrical properties of the membranes (see below).

RESULTS

The effect of external pH on the membrane potential and resistance in the light and dark

Investigation of the response of the membrane potential to a change in external pH is complicated by the fact that much of the initial change in potential is often transient[7]. This means that an experiment in which the pH is increased stepwise will yield incomplete information since the initial response will not be observed after the first step. Instead, the effect of pH was investigated by returning to artificial pond water (pH 5) before the next change to a higher pH. Where the response was

transient, the peak and steady values of the potential (Fig. 1,a) have been plotted against pH (Fig. 2). Although the initial change in potential is approximately the same in the light and dark[7], the later decline in the potential is much more pronounced in the light (Fig. 1).

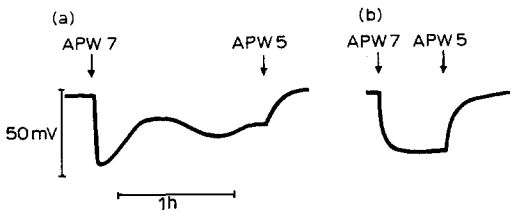


Fig. 1. Time course of the membrane potential on changing from artificial pond water, pH 5, (APW 5) to artificial pond water, pH 7, (APW 7), in the light (a) and in the dark (b).

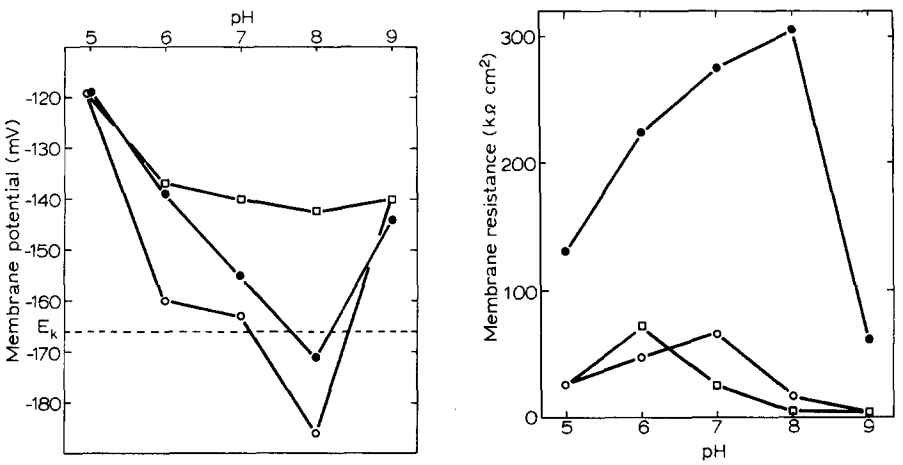


Fig. 2. The effect of external pH on the peak value of the membrane potential in the light (○), the steady value of the membrane potential in the light (□) and the peak value of the membrane potential in the dark (●) which, in this experiment, was the same as the steady level. The external K^+ concentration was 0.1 mM (artificial pond water, pH 5 etc.). Also shown is the value of E_K which was calculated by subtracting 116 mV from the membrane potential in artificial pond water (pH 6) + 10 mM KCl.

Fig. 3. The effect of external pH on the membrane resistance. The measurements were made on the same cell and at the same times as the corresponding measurements shown in Fig. 2, *i.e.* at the peak value of the membrane potential in the light (○), at the steady value of the membrane potential in the light (□) and in the dark (●).

The variation of the membrane resistance with pH in the light and dark is illustrated in Fig. 3. The large increase in resistance in the dark, observed previously[6,7,15] is most pronounced at neutral pH's.

The effect of the external potassium concentration on the membrane potential and resistance in the light and dark

The diffusion potential, E, may be related to the ionic concentrations on either side of the membrane by the Goldman equation:

$$E = 58 \log_{10} \frac{P_K K_o^+ + P_{Na} Na_o^+ + P_{Cl} Cl_i^-}{P_K K_i^+ + P_{Na} Na_i^+ + P_{Cl} Cl_o^-} \text{ mV} \quad (1)$$

where P_K *etc.* are the permeability coefficients for the ions having concentrations K_0^+ *etc.* on the outside, o, and the inside, i, of the membrane. The potential predicted by this equation may vary within limits set by the most extreme values for the Nernst potentials of the individual ions. For *N. translucens* in artificial pond water, the Nernst potential for K^+, E_K, is the most negative[16]. In Fig. 2 it can be seen that the values of the membrane potential in the pH range 6–8 are close to or more positive than E_K as determined by the method described below. An obvious way to establish whether the membrane potential has an electrogenic component is to raise the external K^+ concentration to determine whether E_K can be made more positive than the membrane potential. Preliminary experiments were performed at pH 7 but it was found that the membrane potential in the light was more stable at pH 6 and only experiments at this pH will be described.

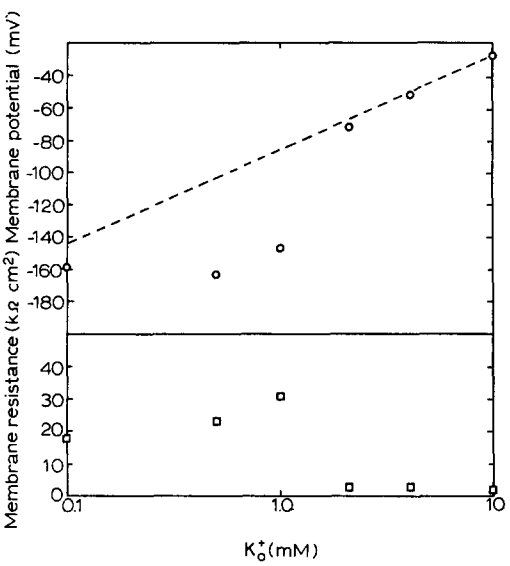


Fig. 4. The effect of external K^+ on the membrane potential and resistance in the light at pH 6.

The effects of increasing the external K^+ concentration in the light are shown in Fig. 4. In this experiment the membrane potential remained almost constant as the concentration was raised to 1 mM. At 2 mM the potential fell sharply and thereafter followed the line with a 58-mV slope which is near the calculated equilibrium potential for K^+ (ref. 16). This and the marked decrease in resistance between 1 and 2 mM (Fig. 4) suggests that there is an increase in P_K at this point. Measurements of the K^+ fluxes, which will be published separately, confirm this hypothesis. The apparent increase in P_K also takes place in the dark (Fig. 5). The experiments in the light and dark were conducted on different cells because exposure to high K^+ concentrations affects any subsequent response to increasing K^+ concentrations (R. M. Spanswick, unpublished). The K^+ concentration at which the increase in permeability

occurs varies from cell to cell but rarely takes place below 1 mM K^+ at pH 6. A concentration of 0.5 mM was chosen as a standard for further work since the membrane potential in the light at this concentration is more negative than E_K.

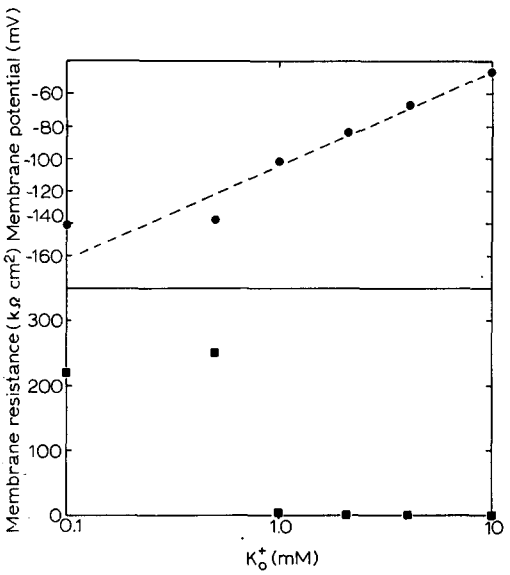


Fig. 5. The effect of external K^+ on the membrane potential and resistance in the dark at pH 6.

The apparent identity between the membrane potential and E_K at high K^+ concentrations provides a convenient method for estimating E_K in other solutions of known concentration. The method has the advantage that, in a particular cell, the error introduced by the microelectrode tip potential will not affect the comparison between the membrane potential and E_K. This method has been used to determine E_K throughout this work.

The effect of external K^+ on the membrane potential was also observed at pH 5 and pH 9. In neither case was there a large effect on the potential at an external concentration less than 1 mM.

The effect of external pH on the membrane potential with $K_0^+ = 0.5$ mM

From the preceeding experiments it is apparent that it should be possible to obtain steady values of the membrane potential in the light which are more negative than E_K when $K_0^+ = 0.5$ mM and the pH is close to neutrality. This was verified using the same series of solutions with the addition of 0.4 mM KCl. With pH 5 as a starting point for each change to a higher pH, the peak and steady values at the higher pH's were recorded (Fig. 6). The only solution in which the steady value of the membrane potential was markedly more negative than E_K was artifical pond water (pH 6) + 0.4 mM KCl. This solution was therefore chosen as the standard for further work on the postulated electrogenic component of the membrane potential.

A complication which is not evident from Fig. 6 should be mentioned here. The membrane potential in artifical pond water (pH 5) + 0.4 mM KCl in the light is

that obtained shortly after the light was first turned on. After repeated exposure to higher pH values, the potential at pH 5 fell to a value close to the dark level. Also, after prolonged exposure to light and many pH changes the steady potential in artifical pond water (pH 6) + 0.4 mM KCl was often close to E_K. The reason for this is not clear at present since the membrane potential in the light has been observed to remain large and negative for many hours if the pH is held constant at pH 6.

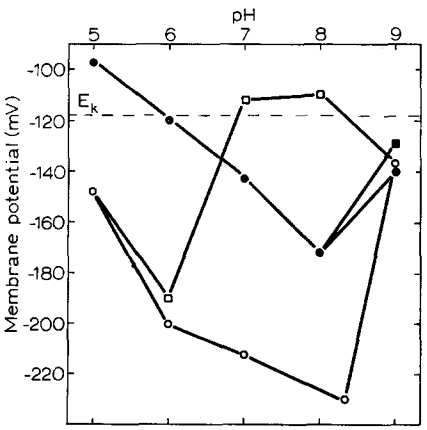


Fig. 6. The effect of pH on the membrane potential with $K_0^+ = 0.5$ mM. Shown are the peak (○) and steady (□) values in the light and the peak (●) and steady (■) values in the dark. Also shown in the value of E_K as calculated by subtracting 75 mV from the membrane potential in artificial pond water (pH 6) + 10 mM KCl.

The effect of the external Na^+ concentration on the membrane potential and resistance at pH 5.

It has been suggested[17,18] that the depolarization produced by low pH in *C. corallina* is due to an increase in the permeability coefficient for Na^+ rather than high permeability to H^+ (ref. 8). Lannoye *et al.*[17] supported their hypothesis with flux measurements but implicit in their argument is the assumption that the membrane potential is determined by the passive fluxes of Na^+ and K^+. A simple test of their hypothesis may be performed by varying the external Na^+ concentration at low pH. If P_{Na} (Eqn 1) becomes as large as they suggest and the membrane potential is controlled by Na^+ and K^+ the change in membrane potential for a 10 × increase in Na_0^+ should approach +58 mV and the membrane resistance should decrease several-fold. The results of the three experiments in Table I clearly demonstrate that this hypothesis does not hold for *N. translucens* either in the light or the dark since the changes in both the membrane potential and resistance are small.

The effect of light and dark on the membrane potential at pH 6 with $K_0^+ = 0.5$ mM

A typical time course for the membrane potential of a cell in artificial pond water (pH 6) + 0.4 mM KCl is shown in Fig. 7. After a delay of 5 min, during which there is a small depolarization, the membrane potential rises to a maximum value which is often followed by a small decline, although the potential remains hyperpolarized. When the light is switched off the potential returns to the dark level. Results for several similar experiments are collected in Table II. The average hyper-

TABLE I

EFFECT OF Na^+ ON THE MEMBRANE POTENTIAL AND RESISTANCE AT pH 5

Expt	*Light (L) or dark (D)*	*Artificial pond water (pH 5) plus*		
		—	*3 mM NaCl*	*10 mM NaCl*
		(a) Membrane potential (mV)		
L359	L	−114	−110	−107
L360	L	−105	− 92	− 87
L361	L	−118	−112	−109
L359	D	− 98	−105	−106
L360	D	−101	− 94	− 86
L361	D	−117	−114	−113
		(b) Membrane resistance ($k\Omega \cdot cm^2$)		
L359	L	16.2	13.8	12.8
L360	L	9.0	8.5	7.4
L361	L	13.2	11.9	10.6
L359	D	53.4	73.5	64.5
L360	D	54.5	54.5	64.6
L361	D	26.6	45.0	41.2

polarization is about 50 mV. The value of the potential in the dark is very close to E_K but, as can be seen from Fig. 5, this does not mean that K^+ then controls the membrane potential.

This effect of light on the membrane potential should not be confused with the short term transients studied by Vredenberg[19]. These are visible in Fig. 7 immediately after switching the light on or off, and are in the opposite direction to the larger changes. A similar effect was observed by Nishizaki[15] in *Chara braunii* after exposure of the cell to darkness for 24 h, but the hyperpolarization was transient. Under intermittent illumination, light produced a depolarization. By contrast, the hyperpolarization described here has been observed for periods of up to 17 h and the potential returns to the hyperpolarized level after short periods of darkness. However,

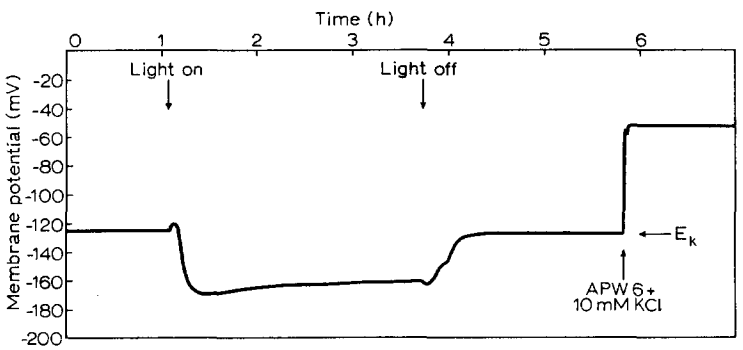


Fig. 7. The effect of light on the membrane potential of a cell in artificial pond water, pH 6 (APW6) + 0.4 mM KCl. Also shown is the effect of APW6 + 10 mM KCl and the value of E_K in APW6 + 0.4 mM KCl calculated from the potential in APW6 + 10 mM KCl.

a similar hyperpolarization in artificial pond water (pH 7) + 0.4 mM KCl which was maintained for long periods during the Spring of 1971 later became transient.

The effect of temperature on the membrane potential and resistance in the light and dark

Changes in permeability coefficients have formed the basis of previous attempts to explain the effects of temperature on the membrane potential and resistance[20,21] in cells pretreated in NaCl to make them responsive to external Na^+ and K^+ (refs 2 and 5). However, under the present conditions the membrane potential is not under the control of the passive movements of Na^+ and K^+ (Figs 4 and 5, Table I) and such an explanation would not be appropriate. An alternative explanation for large temperature effects would be the presence of an electrogenic pump associated with chemical reactions having a high Q_{10}. For example, a temperature-sensitive component of the membrane potential in *Anisodoris nobilis* can be abolished by ouabain and has been attributed to an electrogenic Na^+–K^+ pump[22].

TABLE II

EFFECT OF LIGHT AND DARK ON THE MEMBRANE POTENTIAL OF CELLS IN APW6 (ARTIFICIAL POND WATER, pH 6) + 0.4 mM KCl AND THE VALUE OF E_K CALCULATED FROM THE MEMBRANE POTENTIAL IN APW6 + 10 mM KCl.

Expt	*Membrane potential (mV)*		$(E)_{light} - (E)_{dark}$ *(mV)*	E_K *(mV)*
	Dark	*Light*		
L359	−119	−164	−45	−119
L360	−107	−159	−52	−111
L361	−121	−162	−41	−122
L362	−108	−146	−38	−113
L363	−105	−174	−69	−124
L364	−117	−163	−46	−124
L365	−113	−166	−53	−115
L366	−102	−162	−60	−111
Mean	111.5	162.0	50.5	117.4
± S.E.	± 2.3	± 2.6	± 3.4	± 1.8

The effect of temperature on *N. translucens* in artificial pond water (pH 6) + 0.4 mM KCl in the light, assuming a linear response to a change from a temperature in the range 19.0–19.8 °C to a temperature in the range 8.9–11.0 °C, was 2.5 ± 0.5 mV·°C^{-1} (mean ± S.E., 7 cells). In the dark, over a similar temperature range, the response was 0.97 ± 0.19 mV·°C^{-1}. Similar results were obtained in an earlier set of experiments at pH 7. These results are consistent with the hypothesis that an electrogenic pump controls the membrane potential in the light. Even in the dark the effect of temperature is greater than would be predicted from Eqn 1 on the basis of constant permeability coefficients (approx. 0.5 mV·°C^{-1}). The difference could indicate some residual activity of the pump or a change in the ratio of the permeability coefficients.

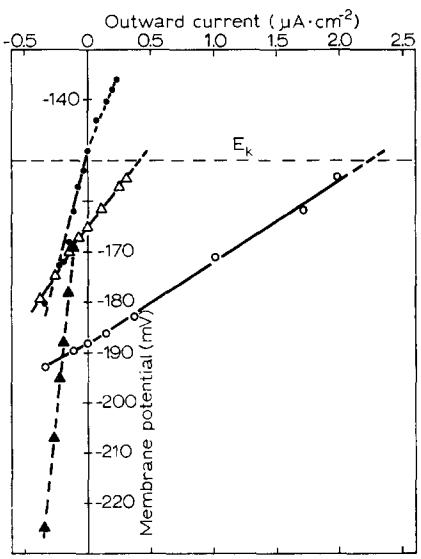
The difference between light and dark is also reflected in the effect on the membrane resistance. For seven cells, an average temperature change of 9.4 °C in the light produced an increase in resistance from 17.2 ± 2.5 kΩ·cm^2 at the higher temperature to 35.2 ± 5.8 kΩ·cm^2 at the lower temperature. In the dark, for an

average temperature change of 9.0 °C, the corresponding change in membrane resistance was only from 153 ± 32 kΩ·cm² to 160 ± 44 kΩ·cm².

Current–voltage characteristics of the plasmalemma and recovery from the action potential

The membrane resistances presented above have been calculated on the assumption of a linear current–voltage relationship. Measurements of the current–voltage relationship for the plasmalemma were made with a microelectrode inserted in the cytoplasm (Fig. 8). They show that this assumption is correct for cells in the light but in the dark there is a non-linear response similar to that observed by Bradley and Williams[23] but with a lower threshold (Fig. 9). The time course of the change in response to a square pulse of current is similar to the "hyperpolarizing response" observed by Kishimoto[24] for cells in 100-mM solutions of monovalent cations. This hyperpolarizing response has also been observed in *N. translucens* after the apparent increase in P_K at high external K^+ concentrations (R. M. Spanswick, unpublished). One property observed by Kishimoto, and in the present work, is that the delay before the onset of the hyperpolarizing response decreases with repeated current pulses until it may not be possible to observe the switch to the high resistance state as an event distinct from the initial response to the applied current. From Fig. 8 it can be seen that this could lead to an approximate doubling of the apparent resistance and emphasizes the importance of using small changes in potential for resistance measurements.

The current–voltage relationship for cells in the light is linear and the depolarizing current, in the case shown in Fig. 8, was raised to a value sufficient to bring



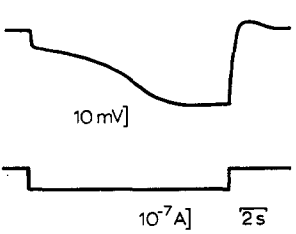


Fig. 8. The current–voltage characteristics of the plasmalemma of a cell in the light at 19.5 °C (○), in the light at 9.0 °C (△) and in the dark at 19.0 °C before (●) and after (▲) the hyperpolarizing response.

Fig. 9. Time course of the membrane potential in the dark in response to a hyperpolarizing square pulse of current.

the membrane potential close to the value of the dark resting potential and E_K. The significance of the value of this current will be dealt with in the Discussion.

The conditions under which the present experiments were conducted also affect the action potential. In the dark (Fig. 10a), the recovery from the action potential is normal, but in the light the recovery in the light in artificial pond water (pH 6) + 0.4 mM KCl is in two stages. First the potential returns to a value close to E_K and then there is a slower return to the hyperpolarized state (Fig. 10b). This behaviour is consistent with an increase in P_K being associated with the recovery phase of the action potential. The return to the hyperpolarized state would then be associated with a subsequent decrease in P_K. The effect of lowering K_o^+ to 0.1 mM can be seen in Fig. 10c and d. The shoulder in the recovery phase at about 2 s after stimulation is more negative by 15–20 mV. For a K^+ electrode the effect would be 41 mV. Thus, even when P_K is transiently increased, K^+ is not the major factor controlling the membrane potential.

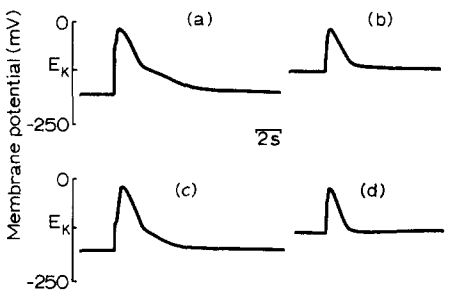


Fig. 10. Time course of the action potential (a) in the light in artificial pond water, pH 6 (APW 6) + 0.4 mM KCl, (b) in the dark in APW6 + 0.4 mM KCl, (c) in the light in APW6 and (d) in the dark in APW6

DISCUSSION

Conditions have been established in which the membrane potential is hyperpolarized in the light to a value that is more negative than any diffusion potential that would be attributed to the major ions. Control of the potential by passive diffusion of the minor ions would require unrealistically high values for the permeability coefficients for HCO_3^- (ref. 25) or OH^- or a low value (< 3) for the pH of the cytoplasm. An acid pH for the cytoplasm is generally thought to be unlikely and preliminary measurements indicate that in *C. corallina* it is close to neutrality (R. M. Spanswick, unpublished). The only alternative is to postulate the existence of an electrogenic pump. This is consistent with the large effect of temperature on the membrane potential in the light.

Parallels to several of the observations reported here may be recognized in other work in the literature. The effect of light on the membrane potential of green cells is usually transient[26]. Nagai and Tazawa[27] observed a sustained hyperpolarization in the light for *Nitella flexilis* in a solution of 10^{-4} M KCl or NaCl. However, the membrane potential remained within the range of possible diffusion potentials for cells in these solutions. This is not the case for experiments in which the external K^+ concentration was raised under fixed lighting conditions. In the presence of Ca^{2+}, Oda[28] showed that the membrane potential of *C. braunii* would remain polarized at K^+ concentrations

up to 100 mM but would depolarize after an induced action potential. Similarly, Hope[6] showed that in the presence of HCO_3^- the K^+ concentration could be changed from 0.1 to 1.0 mM with little effect on the membrane potential. Again, induction of an action potential depolarized the membrane potential to a level close to E_K. In both cases the membrane potential before stimulation was at a more negative level than any possible diffusion potential for the major ions. To account for these results, Oda[4] postulated the presence of a "non-ion equilibrium potential" in series with a diffusion potential while Hope[6] favoured the idea of an electrogenic HCO_3^- pump. The first postulate lacks specificity and the second appears to be invalid since the addition of HCO_3^- at constant pH has no effect on the membrane potential when the solutions are buffered[7]. Nevertheless, the presence of an electrogenic ion pump appears to be the hypothesis most consistent with the available evidence.

The large effect of light on the membrane resistance may account for the variation to be found in the literature[13,29] since the light intensity is often unspecified.

Although the postulated electrogenic pump can account qualitatively for the electrical properties of the membrane, a quantitative explanation calls for a more detailed examination of the data presented here in relation to current hypotheses. I shall consider three main hypotheses which have the following distinguishing features: (a) Modification of the "classical" theory to take into account ion depletion effects at the surface of the membrane[18]. (b) An electrogenic H^+ pump balanced by a passive H^+ influx[8]. (c) A membrane with a low permeability to H^+ (ref. 30) containing a potential-dependent electrogenic pump[31].

Walker and Hope[18] found a discrepancy between the measured membrane conductance and that calculated from the fluxes of Na^+, K^+ and Cl^- during voltage-clamp experiments. They sought to account for this discrepancy on the basis of errors in the flux measurements due to back-fluxes of labelled ions. For example, during efflux measurements they proposed that the specific activity of K^+ in the cell wall would rise and result in a back-flux of labelled ions. This would lead to an apparent decrease in the efflux. However, experiments with isolated cell walls[32] show that the wall is a highly permeable structure, the rate of diffusion of KCl being limited partly by the unstirred layers of solution on either side of the wall. Furthermore, K^+-efflux measurements (R. M. Spanswick, unpublished) show that, when P_K remains low at concentrations up to 10 mM, the apparent K^+ efflux remains constant with increasing concentration. According to Hope and Walker[18] the K^+ efflux should increase because the increased concentration of unlabelled ion in the wall would lower the specific activity there and hence lower the back-flux also. Thus, without further information, the seriousness of the errors in the flux measurements remains open to question.

As an alternative to the large passive flux of hydrogen ions[8], it was suggested[18] that the effect of decreasing pH on the membrane potential could be explained by (i) a decrease in P_K (Eqn 1), (ii) an increase in P_{Na} or (iii) an increase in P_{Cl}. The first alternative is inconsistent with the efflux measurements of Kitasato[8] in which he showed that the K^+ efflux remained approximately constant when the membrane potential was clamped at E_K and the pH was changed. The second is inconsistent with the small effect of external Na^+ on the membrane potential at pH 5 (Table I) and the third is inconsistent with Kitasato's measurements of the Cl^- efflux which yield an approximately constant value for P_{Cl} at acid pH.

To account for the light-stimulated hyperpolarization (Fig. 7) using Eqn 1, it would be necessary to assume that an active influx of K^+ at the plasmalemma caused a reduction in K_o^+ at the surface of the membrane. However, it would require an approximately 10-fold reduction. This seems unlikely in view of the high permeability of the cell wall, referred to above, and the small response of the membrane potential to external K^+ under these conditions (Figs 4 and 5). Walker and Hope[18] also suggest that it is possible that "Ca^{2+} is adsorbed to the plasmalemma in such a way that K^+ within the conduction regions of the membrane are screened and hence the p.d. and resistance are prevented from changing". Although they give no definition of "screening", it is apparent that it involves the reduced movement of K^+ at the outer surface of the plasmalemma. If the adsorbed Ca^{2+} also has a low mobility this would give this layer a high resistance and, since it is in series with the "conduction regions of the membrane", would confer a high resistance on the membrane as a whole, thus effectively reducing P_K. The depletion effects observed by Barry and Hope[33] can undoubtedly account for the transient responses of the membrane potential to large applied current densities, but the K^+ flux is smaller by more than an order of magnitude than the current densities used in their experiments. Finally, depletion of the K^+ concentration at the outer surface or the plasmalemma in the light would give rise to an increase in the membrane resistance rather than the dramatic decrease that is observed[6] (Figs 3 and 8). Thus, even with the introduction of such arbitrary concepts as 'screening', it does not seem possible to account for the electrical properties of *Nitella* by using the classical theory. Indeed, the sharp transition to K^+-dependence above 1 mM and the small effect of K^+ on the recovery of the action potential (Fig. 10) emphasize the fact that the electrical properties of the plasmalemma are independent of K^+ below 1 mM.

If the permeability to the major ions is low, as suggested above, the evidence for a high passive permeability to H^+ becomes correspondingly stronger. Positive evidence for a large passive flux of H^+ is provided by (i) the dependence of the membrane potential on pH[7,8] (Figs 2 and 6) and (ii) the variation of the clamping current with pH when the potential is clamped at E_K[8].

Kitasato[8] modified Eqn 1 to take into account the passive diffusion of H^+:

$$E = \frac{RT}{F} \ln \frac{P_K K_o^+ + P_{Na} Na_o^+ + P_H H_o^+ + P_{Cl} Cl_i^-}{P_K K_i^+ + P_{Na} Na_i^+ + P_H H_i^+ + P_{Cl} Cl_o^-} \qquad (2)$$

where P_H is the permeability coefficient for H^+ and H_o^+ and H_i^+ are the external and internal concentrations of H^+, respectively. To explain the strong dependence of the membrane potential on external pH in the range 4–6 it is necessary that $P_H H_o^+ \gg P_K K_o^+$ and the other terms in the numerator. If the internal (cytoplasmic) pH is close to neutrality, the membrane potential predicted by Eqn 2 would be too positive. Kitasato[8] suggested that this problem could be overcome if the equation were modified to take into account the flux, J, through an electrogenic pump which would cause a current JF to flow through the membrane conductance, g_m, to maintain electrical neutrality. Then

$$E = (E_m)_o + \frac{JF}{g_m} \qquad (3)$$

where $(E_m)_0$ is the potential given by Eqn 2 and is the value of E when $J = 0$.

Kitasato[8] suggested that at high external pH the activity of the pump would decrease due to an increase in the internal pH resulting from the extrusion of H^+. Under these conditions, $P_H H_i^+$ and $P_H H_0^+$ would be small compared with the values of $P_K K_i^+$ and $P_K K_0^+$, respectively. E should then be approximately equal to E_K. This was the case when K_0^+ was 0.1 mM but not when it was 1.0 mM since the potential was only 5 mV more positive. The small effect of K^+ in the range 0.1–1.0 mM was also observed for *N. translucens* at all pH values.

There are similar problems relating to the membrane resistance. Kitasato interpreted the change in applied current on changing from pH 5 to pH 6, with the membrane potential clamped at E_K, as a change in the passive H^+ flux. In *N. translucens* in the light there is little difference in the steady values of the membrane potential at pH 5 and pH 7 (Fig. 2) and therefore there should be a large decrease in the passive H^+ influx at pH 7 due to the decrease in H_0^+. This should be reflected in a large increase in the membrane resistance but this is not case (Fig. 3). Another point that is difficult to account for is the large difference between the resistance in the light and the dark at, for example, pH 7 when the steady values of the membrane potential are very close but the resistances differ by a factor of 10. Is it necessary to postulate a light-dependent value of P_H or is the low value of the membrane resistance in the light due to something other than a passive H^+ flux?

As an alternative to these two hypotheses I shall develop a third which differs from the others in that the permeability of the membrane to all ions, including H^+, is low, and the electrical properties of the membrane reflect those of the postulated electrogenic pump. Smith[30], though not primarily interested in the electrical properties of the cell membrane, has incorporated the H^+ pump into an hypothesis to account for the effect of pH on the Cl^- influx. Briefly, the pump sets up a pH gradient which drives the Cl^- influx through a Cl^-–OH^- exchange mechanism. A reasonable efficiency for this process depends on the passive influx of H^+ being low. Is it, then, possible to account for the electrical properties of the membrane in terms of an electrogenic pump? The membrane potential in the presence of an electrogenic pump may be represented formally by Eqn 3. Using this equation, it is possible to make an estimate of the minimum flux through the electrogenic pump from the information in Fig. 8 for the current–voltage characteristics of the plasmalemma in the light. The important quantity is the applied current required to bring the membrane potential to the same level as E_K (which is only 2 mV more negative than the membrane potential in the dark in this case). At this point $E \approx (E_m)_0$ and the net passive flux through the passive channels in the membrane must be close to zero. Thus the applied current is a measure of the active electrogenic flux, J. If there have been any changes in the permeability coefficients on switching on the light and $(E_m)_0$ is now more positive, then the applied current required to make $E = E_K$ will be an underestimate of J. For the case in Fig. 8 this current is 2.2 $\mu A \cdot cm^{-2}$ which corresponds to a flux of approximately 22 $pmoles \cdot cm^{-2} \cdot s^{-1}$. This is much greater than the measured fluxes for the major ions and will therefore be tentatively identified with the active H^+ efflux which has been estimated to be 5–20 $pmoles \cdot cm^{-2} \cdot s^{-1}$ by Spear et al.[34]. A change in current of the same magnitude was measured by Kitasato when the membrane potential was clamped at E_K and the external pH changed from 5 to 6. He interpreted the change in current as a decrease in the passive influx of H^+. An alter-

native interpretation would be that the current was an increase in the efflux through the electrogenic pump. This will be considered explicitly below.

Two related measurements have been reported by Strunk[35] for *Nitella clavata*. He perfused the cell vacuole with salt solutions and found that with the same solution on the outside the short-circuit current was 1.7 $\mu A \cdot cm^{-2}$. He obtained the same value from the difference in the currents required to reduce the membrane potential to zero in the light and in darkness *plus* ouabain. While reservations may be expressed about the effects on the permeability coefficients of perfusion or the effect of taking the membrane potential through the region of excitation, the similarity between these values and the value obtained here reinforces Strunk's conclusion that the current is a measure of the flux through the electrogenic pump. It should be noted that if Smith's hypothesis[30] is correct it will be difficult to measure the H^+ efflux through the pump because the OH^- efflux from the $Cl^- - OH^-$ exchange mechanism will partly neutralize it.

I now wish to examine the terms g_m and J in Eqn 3 in greater detail. g_m is the passive ion conductance and is usually identified with the measured membrane conductance. However, Finkelstein[36] pointed out that an electrogenic pump itself has the property of electrical conductance and the flux through the pump will be voltage-dependent. Thus an electrogenic pump can be represented by an equivalent circuit consisting of an EMF and a conductance placed in series. Unfortunately the electrochemical equations which he derived are not easily applied to a practical situation, because they contain terms for the carrier concentrations just inside the membrane surfaces and these variables are not amenable to experimental determination.

An alternative approach, based on irreversible thermodynamics, has recently appeared[31]. In effect, it translates the theory put forward by Finkelstein into equations containing measurable variables. However, it is limited by the assumptions of linearity and nearness to equilibrium on which irreversible thermodynamics is based. For instance, the electrical conductance of the electrogenic pump is proportional to L_{rr}, the thermodynamic conductance coefficient relating the rate of the chemical reaction driving the pump to the free-energy change of the reaction. Any variation in L_{rr} is not predictable from the theory and can only be determined by experiment. This contrasts with Finkelstein's prediction of a variable conductance due to changes in the carrier concentrations at the surface of the membrane. For the present, I would suggest that the pump conductance, g_p, for *Nitella* in the light is much larger than g_m, as calculated from the major ion fluxes, and this accounts for the discrepancy that has always existed between the measured and calculated conductances[13]. If this is the case, most of the current injected during resistance measurements will pass through the active channel and it should be possible to change the active H^+ flux by voltage clamping. An equivalent circuit is shown in Fig. 11. When $g_p \gg g_m$, $E_m \approx E_p$. An expression for the "pump EMF", E_p, can be derived by adapting the formulation of Rapoport[31] to the present case. Assuming H^+ is the only ion involved in the electrogenic system, the free-energy change for active transport is:

$$\Delta F_r = \Delta \bar{\mu}_p - \nu_H \Delta \bar{\mu}_H \qquad (4)$$

where $\Delta \bar{\mu}_p$ represents the free-energy change for the non-transported components of the reaction, ν_H is the stoichiometric coefficient and $\Delta \bar{\mu}_H$ is the electrochemical

potential difference for H^+ across the membrane. The rate of the chemical reaction is given by:

$$J_r = L_{rr}(-\Delta F_r) \tag{5}$$

Now consider the case when the passive permeability coefficients for all ions tend to zero. The chemical reaction will continue until the membrane becomes sufficiently polarized to halt it. At this point $J_r = 0$, hence $\Delta F_r = 0$ and therefore

$$\begin{aligned}\Delta\bar{\mu}_p &= \nu_H \Delta\bar{\mu}_H \\ &= \nu_H[RT \ln(H_i^+/H_o^+) + FE]\end{aligned} \tag{6}$$

$$i.e. \quad E(=E_p) = \frac{\Delta\bar{\mu}_p}{F\nu_H} - \frac{RT}{F} \ln \frac{H_i^+}{H_o^+} \tag{7}$$

Thus the EMF of the pump is clearly dependent on many variables and this permits an alternative explanation of several of the present observations if $g_p \gg g_m$. The hyperpolarization in the light would be attributed to a more negative value for $\Delta\bar{\mu}_p$ and the effect of external pH would be an effect on E_p rather than on $(E_m)_o$. The initial hyperpolarization in the light on changing to pH 7 would be due to the change in H_o^+ and the later decline could be due to a decrease in H_i^+ as the pH of the cytoplasm is raised by the active extrusion of H^+.

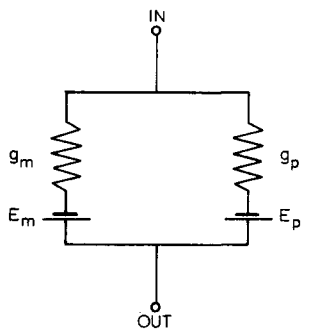


Fig. 11. An equivalent circuit showing the pump *EMF*, E_p, and conductance, g_p, in parallel with the diffusion potential , E_m,and the passive conductance, g_m.

The large effect of light on the membrane conductance is more difficult to explain. If the effect is on g_p there must be some effect on L_{rr} since

$$g_p = F^2 L_{rr} \nu_H \tag{8}$$

It is not possible to derive an explanation for this effect from irreversible thermodynamics. However, Finkelstein has shown that the pump conductance in his model is proportional to the concentration of carriers in the membrane. It is conceivable that the carriers require activation by some metabolic reaction and thus the effective concentration in the light could be greater than that in the dark, giving the observed effect on the conductance. The effect of temperature on the membrane resistance in the light (Fig. 8) would be accounted for in a similar way.

As pointed out by Rapoport, the membrane potential acts as a regulator on the

ion pump. If the pump rate in Eqn 3 were constant and g_m low, the membrane could be polarized to the extent that dielectric breakdown would occur. However, in the present case, hyperpolarization of the membrane would reduce the flux through the pump due to the inclusion of E in ΔF_r in Eqn 5.

Further tests of this hypothesis will require measurements of the cytoplasmic pH, the H^+ fluxes and identification of the reaction driving the pump. For the latter, the effects of inhibitors are of interest. Kitasato[8] suggested that the depolarization caused by 2,4-dinitrophenol at pH 6 was a result of its effect on the electrogenic H^+ pump and that the potential then tended towards E_H. However, he used a medium containing 1 mM K^+ and the potential actually approached E_K. This has been confirmed for *N. translucens* using other inhibitors and the results will be presented in the next paper in this series.

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