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The Role of Protons in Determining Membrane Electrical Characteristics in *Chara corallina*

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Summary. The value of the potential difference between the vacuole and the external medium, ψ_{vo}, and the effects of metabolic inhibitors upon it indicate that, under certain external conditions, there are two main factors determining the potential across the plasmalemma. At all external pH there is a passive diffusion potential and at pH $\geqq 6$ there is an active electrogenic component of potential. Calculations of ionic permeabilities of K^+, Na^+ and Cl^- from their respective passive fluxes and application of the Goldman equation indicate that H^+ permeability is approximately 25 times K^+ permeability at pH 4 and 5 and possibly at pH $\geqq 6$. ψ_{vo} does not respond to increases in $[K^+]_o$ when a large electrogenic component of potential is present, even though the calculated value of P_K under these conditions is such as to predict significant changes in ψ_{vo}. It is suggested that this anomaly may be due to intrinsic properties of the electrogenic pump. There is evidence that the electrogenic pump is an active H^+ efflux which calculations indicate is about 12 nmoles m^{-2} sec^{-1} at pH 5. However the electrogenic potential does not appear to be related to formation of acid and alkali regions in the external solution adjacent to cells of *Chara*.

The potential difference (p.d.) across the plasmalemma of cells of *Chara corallina* has been regarded as a passive diffusion potential determined mainly by the gradient of K^+, and, to a lesser extent, Na^+ (Hope & Walker, 1961; Findlay & Hope, 1964). The relationship between the p.d. and the ionic concentrations is commonly expressed by the equation (Hodgkin & Katz, 1949):

$$\psi_{co}=\frac{RT}{F}\ln\frac{P_K[K^+]_o+P_{Na}[Na^+]_o+P_{Cl}[Cl^-]_c}{P_K[K^+]_c+P_{Na}[Na^+]_c+P_{Cl}[Cl^-]_o} \quad (1)$$

with the first two terms in the numerator and denominator of the logarithm term being most important. ψ_{co} is the p.d. of the cytoplasm with respect to the external solution, $[\,]_o$ and $[\,]_c$ are concentrations of ion j in the external

solution and the cytoplasm, respectively, and P_j is the permeability of ion j which may be calculated from the equations (Goldman, 1943):

$$P_j = -\frac{RT}{z_j F}\vec{\phi}_j \frac{1-\exp(z_j F\psi_{co}/RT)}{\psi_{co} c_j^o} \tag{2}$$

$$P_j = \frac{RT}{z_j F}\overleftarrow{\phi}_j \frac{1-\exp(z_j F\psi_{co}/RT)}{\psi_{co} c_j^c \exp(z_j F\psi_{co}/RT)} \tag{3}$$

where z_j is the valency of ion j, c_j^o and c_j^c are the concentrations of ion j just outside and inside the plasmalemma, respectively, $\vec{\phi}_j$ and $\overleftarrow{\phi}_j$ are the passive influx and efflux of ion j, respectively, and R, T and F have their usual meanings.

Eq. (1) depends upon the assumptions of independence of ion movement and a linear potential gradient within the membrane. Equations of a similar form may be derived from completely different assumptions about potential profiles within the membrane (Coster, 1973). The major problem in applying the Goldman-Hodgkin-Katz theory to *Chara* is that the partial ionic conductances calculated from passive fluxes only partly account for the measured electrical conductance. Possible reasons for the discrepancy are discussed by Walker and Hope (1969), and it seems likely that the permeability coefficients in the above equations are complex parameters which reflect interactions between various ions and between ions and water within the membrane. However, the theory is more satisfactory when used to calculate potential differences in *Chara* and a wide variety of other species and is used in the absence of a more suitable alternative.

Nevertheless, under some conditions, ψ_{co} in *Chara* is more negative than can be accounted for by a potassium diffusion potential. Consideration of other ionic species leads to the conclusion that no known gradient is large enough to yield a Nernst p.d. of greater than about -170 mV across the plasmalemma in the standard external media used. Throughout this paper the occurrence of p.d.'s more negative than any Nernst potentials will be referred to as the hyperpolarized state, and the word "hyperpolarization" will have its usual meaning of a change towards an increased p.d. across the membrane, compared with a previous p.d. In *Chara*, steady p.d.'s of up to -220 mV are observed when the external solution contains 0.1 to 0.5 mM HCO_3^- (Hope, 1965) and, when the external solution is buffered at pH 7, p.d.'s as large as -240 mV have been recorded (Hope & Richards, 1971). Lannoye, Tarr and Dainty (1970) recorded transient hyperpolarized states in cells of *Chara corallina* at pH 8.5. The hyperpolarized state has

also been observed in *Nitella clavata* (Kitasato, 1968; Rent, Johnson & Barr, 1972), *Nitella translucens* (Spanswick, 1970, 1972), and *Nitella flexilis* and *Nitella axilliformis* (Saito & Senda, 1973). Smith and Lucas (1973) have demonstrated a pH-dependence of membrane potential in *Chara* using external electrodes. The finding that the p.d. is a function of external pH and that the hyperpolarized state occurs at high pH has focussed our attention again on the role of H^+ in the ionic relations of charophyte cells.

One explanation of this behavior which has received attention in recent investigations is that of Kitasato (1968). Kitasato concluded that proton conductance is approximately equal to membrane conductance and that passive proton influx is balanced by an active, electrogenic extrusion of protons which adds a component of -100 ± 25 mV to the p.d. at all values of external pH between 4 and 8. More direct evidence for fluxes of protons has come from observations of regions of acid and alkali formation on the outside of *Nitella clavata* (Spear, Barr & Barr, 1969) and *Chara corallina* (Lucas & Smith, 1973). Various aspects of Kitasato's hypothesis have been challenged, particularly the high proton permeability and conductance. Walker and Hope (1969) accounted for 25 and 43% of the plasmalemma conductance in *Chara corallina* and *Nitella translucens*, respectively, by the partial conductances of K^+, Na^+ and Cl^- which were calculated from unidirectional fluxes at pH 5.5. Spanswick (1972), Brown, Ryan and Barr (1973) and Vredenberg (1973) have also questioned Kitasato's conclusions on proton conductance and permeability.

Spanswick (1972) has proposed an alternative hypothesis to explain plasmalemma p.d. as a function of external pH in *Nitella translucens*. He suggests that, when an electrogenic pump is operating, all passive permeabilities, including proton permeability, are very small, and that the major contribution to plasmalemma conductance is a conductance within the electrogenic pump. Spanswick (1972) has also provided evidence that the electrogenic pump in *Nitella translucens* is photosynthetically mediated, and suggests that the external pH may affect the electrogenic pump potential rather than control the passive diffusion potential.

The hyperpolarized state has been observed in several other plant cell systems. In *Acetabularia mediterranea* the p.d. is up to -170 mV in the light with $[K^+]_c$ about 400 mM and $[K^+]_o$ 10 mM (Saddler, 1970). Slayman (1965) found a p.d. greater than -200 mV in *Neurospora crassa* with $[K^+]_o =$ 0.1 mM and $[K^+]_c$ about 180 mM, and concluded (Slayman, 1970) that in many such situations an electrogenic ion transport system is operating. Chloride inward transport seems to be associated with the large negative p.d. in *Acetabularia*. Electrogenic pumps also seem to be responsible for the

hyperpolarized state in *Valonia ventricosa* (Gutknecht, 1967), *Elodea densa* (Jeschke, 1970), *Elodea canadensis* (Spanswick, 1973), the moss *Mnium* (N. Higinbotham, *personal communication*) and some higher plant tissues (Higinbotham, 1970).

The present experiments were designed to clarify the relationship between the hyperpolarized state, passive proton permeability and the putative proton pump in *Chara corallina.*

Materials and Methods

Plants of *Chara corallina* were cultured in the laboratory in vessels containing a river sand/mud base and a nutrient solution similar to that described by Hope and Aschberger (1970). Internodal cells of length 20 to 30 mm were cut from the plant and allowed to soak for up to several hours in an artificial pond water consisting of 0.1 mM KCl, 1.0 mM NaCl, 0.05 mM $CaCl_2$ and 2 mM buffer adjusted to pH 6. The same experimental solution with variations in the buffer and the pH was used throughout the experiments unless otherwise indicated. 3,3-dimethylglutaric acid (DMGA) was used as the buffer at pH 4 and 5; MES (2-(N-morpholino)ethanesulphonic acid) was used at pH 6 and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) was used at pH 7 and above. The pH was adjusted with NaOH. During most experiments light intensity at the cells was 1.7 W m^{-2}. For some experiments it was boosted to 12.9 W m^{-2}. Fluorescent tubes were used as a light source.

Electrophysiological techniques for measuring the p.d. and conductance of the cytoplasmic bounding membranes have been previously described (Walker, 1960; Findlay & Hope, 1964; and *see* Hogg, Williams & Johnston, 1968).

Effluxes of Cl^- and K^+ using ^{36}Cl and ^{42}K were measured by the method described by Hope, Simpson and Walker (1966). For Cl^- effluxes the cells were loaded for a week in the experimental solution (pH 6) described above containing ^{36}Cl. The 1 ml of stagnant solution into which the effluxes occurred was replaced every 20 to 30 min. In the case of K^+, cells were loaded for 12 hr and washed in inactive solution for 10 hr before the effluxes were measured, in collection periods of 20 min. For Na^+ influxes cells were soaked in an inactive solution of the appropriate pH before being transferred for 10 min to a solution of the same pH containing ^{22}Na. Cells were then washed for 60 sec in inactive solution of pH 6 containing 5 mM Ca^{++} at approximately 0 °C. Cell nodes and wall were then discarded and the cell contents dried on a planchet for counting. All counting of radioactive samples was done on a Nuclear Chicago Model 4342 gas flow counter.

Measurements of O_2 evolution and consumption were made using a method similar to that described by Lilley and Hope (1971). An oxygen electrode with a small diameter cathode detected the O_2 concentration at the surface of single cells immersed in slowly stirred experimental solutions.

Results

The Effect of External pH on Plasmalemma p.d. and Conductance

The steady potential between the vacuole and the external buffered medium, ψ_{vo}, is shown as a function of the external pH in Fig. 1. ψ_K, the

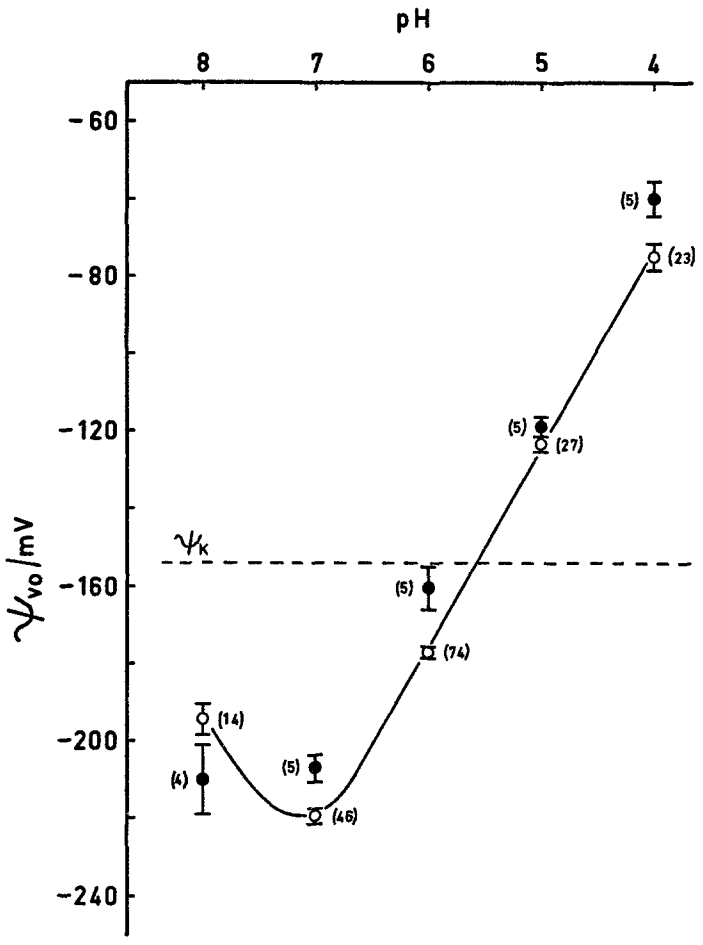

Fig. 1. ψ_{vo} as a function of external pH in the light (○) and dark (●). Each point is the mean ±SEM for the number of cells shown in parentheses. The dotted line shows the Nernst potential for K^+: $\psi_K = 58.1 \log_{10}[0.1/(0.77 \times 59)]$ where 0.77 is the activity coefficient for the vacuolar sap

Nernst potential for K^+, is also shown. Measurements with microelectrodes in both the cytoplasm and the vacuole showed that it was the potential across the plasmalemma which responded to pH. ψ_{vo} in the light changed by an average of 48 mV when the pH was changed from 4 to 5 and by an average of 54 mV when the pH was changed from 5 to 6. Changing the pH from 5 to 4 sometimes elicited a single action potential and very occasionally several action potentials. When the pH was changed from 6 to 7 there was sometimes an initial hyperpolarizing transient in the hyperpolarized state. Apart from this, ψ_{vo} at pH 7 was almost always steady for periods of meas-

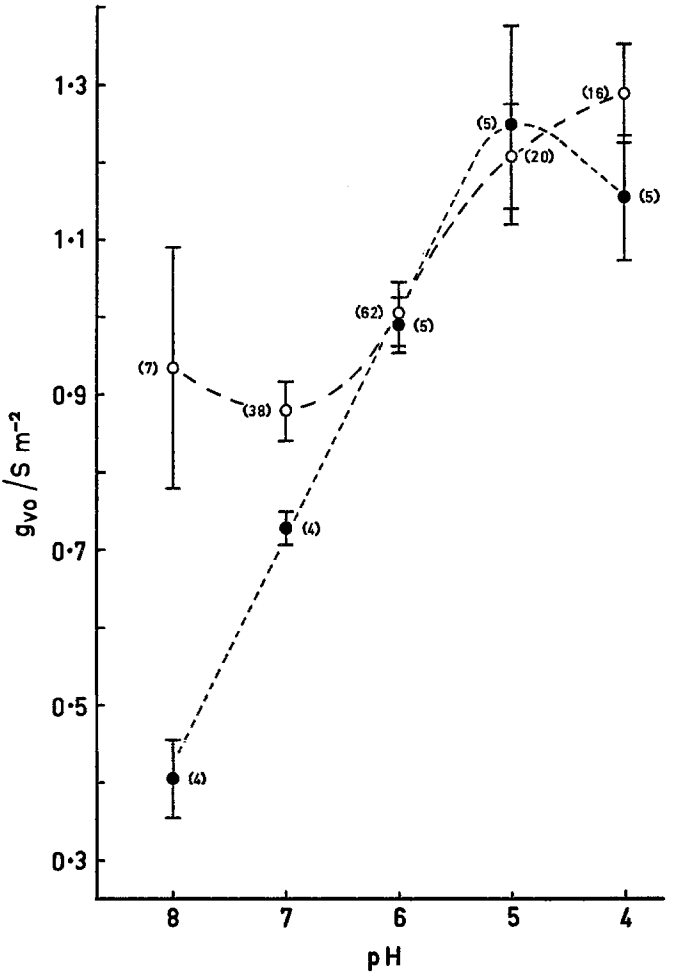

Fig. 2. g_{vo} as a function of external pH in the light (○) and dark (●). Each point is the mean ±SEM for the number of cells shown in parentheses. 1 S m^{-2} ≡ 100 μmho cm^{-2} and corresponds to a resistance of 10 kΩ cm^2

urement of up to several hours. When the pH was changed from 6 or 7 to 8 the hyperpolarized state was commonly transient.

The electrical conductance between the vacuole and the external solution, g_{vo}, as a function of pH is shown in Fig. 2. In the light, g_{vo} at pH 6 and 7 was significantly smaller than at pH 4 and 5 and g_{vo} at pH 8 was considerably more variable than at lower pH. Walker (1960) and Findlay and Hope (1964) have shown that the cell, or vacuole-to-medium conductance approximates that of the plasmalemma.

Experiments in which DMGA was substituted for MES at pH 6, and in which unbuffered solution of pH 7 was used, indicated that the buffers

had no effect on ψ_{vo} or g_{vo} other than via the control of pH. Changes in light intensity between 1.7 and 12.9 W m^{-2} at each pH had no effect on the potential or conductance.

The Effects of Changing External K^+ Concentration

Table 1 shows the results of experiments in which external $[K^+]$ was changed (with simultaneous changes in $[Na^+]_o$ such that $[K^+]_o + [Na^+]_o =$ 1.1 mM) at various values of pH, and in which the pH was changed at various values of $[K^+]_o$. The sensitivity of ψ_{vo} to $[K^+]_o$ was dependent upon the pH and the sensitivity of ψ_{vo} to pH was, under certain conditions, dependent upon $[K^+]_o$.

ψ_{vo} in solutions of pH 6 and above was often not responsive to increases in $[K^+]_o$, and in those cells in which ψ_{vo} did respond, it did not do so

Table 1. ψ_{vo} as a function of pH and $[K^+]_o$

pH	ψ_{vo}/mV		
	$[K^+]_o = 0.1$ mM	$[K^+]_o = 0.3$ mM	$[K^+]_o = 1.0$ mM
4.0	−68.0±3.5 (3)	—	−67.5±3.5 (3)
5.0	−123.0±3.5 (7)	−116.0±3.5 (4)	−108.0±2.0 (7)
5.5	−150.5±3.5 (4)	−145.0±3.0 (4)	−113.0±2.5 (4)
6.0	−171.5±2.5 (15)	−173.5±4.0 (7)	−183.5±3.0 (10)
		−137.0 (1)	−107.5±1.5 (7)
7.0	−221.0±4.5 (7)	—	−223.0±6.0 (7)
			−114.0 (1)
8.0	−191.0±5.5 (3)	—	−198.0±5.5 (3)

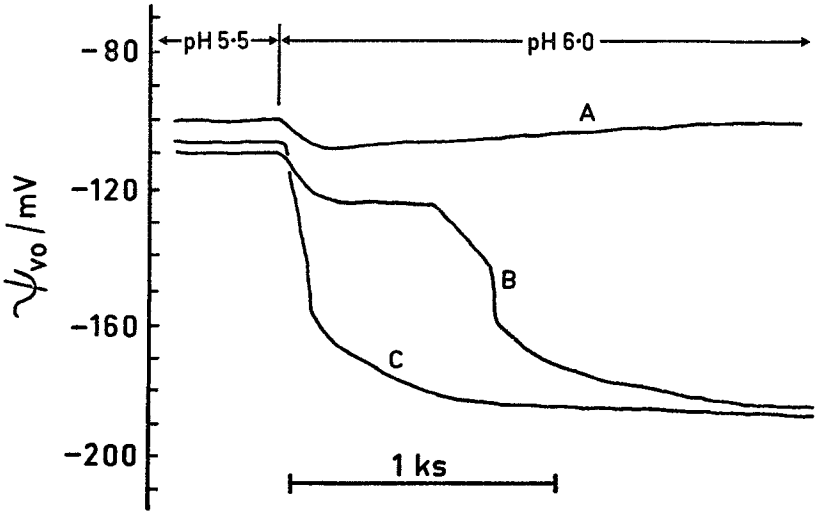

Fig. 3. An example of each of three different responses of ψ_{vo} to a change in pH from 5.5 to 6.0 with $[K^+]_o = 1.0$ mM and $[Na^+]_o = 0.1$ mM

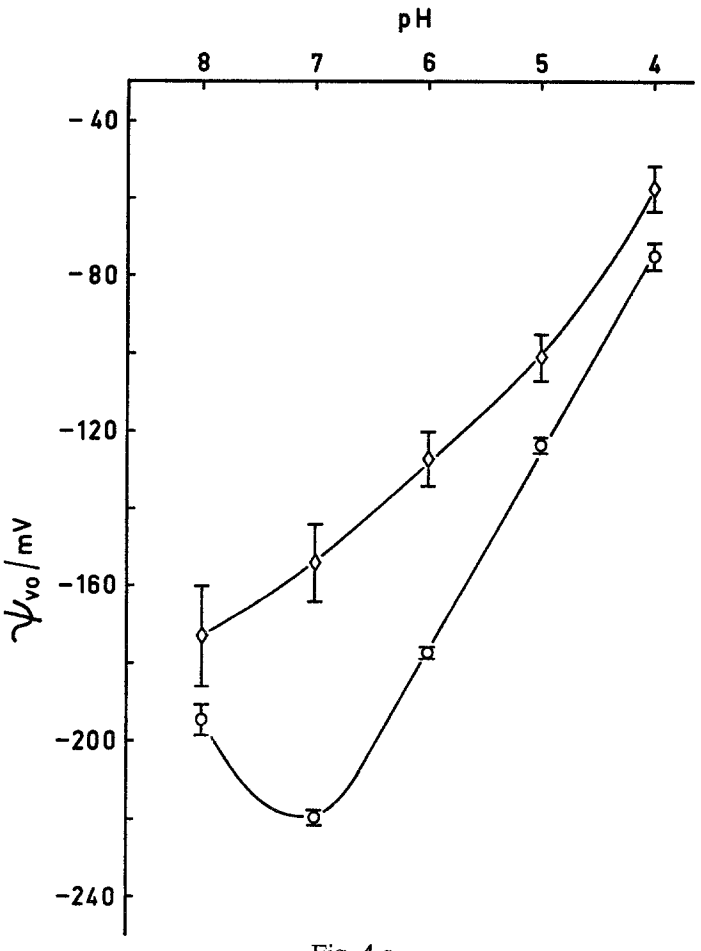

Fig. 4 *a*

Fig. 4. (*a*) ψ_{vo} as a function of external pH at 2.5 °C (◇) and 20 °C (○). Each value of ψ_{vo} at 2.5 °C is the mean ± SEM for 12 cells. The data at 20 °C is replotted from Fig. 1. (*b*) g_{vo} as a function of external pH at 2.5 °C (◇). Each value of g_{vo} at 2.5 °C is the mean ± SEM for 6 cells. These values of g_{vo} are an order of magnitude smaller than g_{vo} at 20 °C (Fig. 2)

consistently. ψ_{vo} always responded to changes in $[K^+]_o$ at pH 5, although the responses were small, about 15 mV depolarization for a 10-fold increase in $[K^+]_o$. At pH 5.5, the corresponding average change was 37.5 mV. However, in the cells tested at pH 5.5, ψ_{vo} at 0.3 mM $[K^+]_o$ was not significantly different from ψ_{vo} in 0.1 mM $[K^+]_o$.

When the pH was increased from 5 or 5.5 to 6 or 7 while $[K^+]_o$ remained at 1.0 mM, ψ_{vo} responded in one of three different ways. An example of each of these responses is shown in Fig. 3. A response of type *B* was only

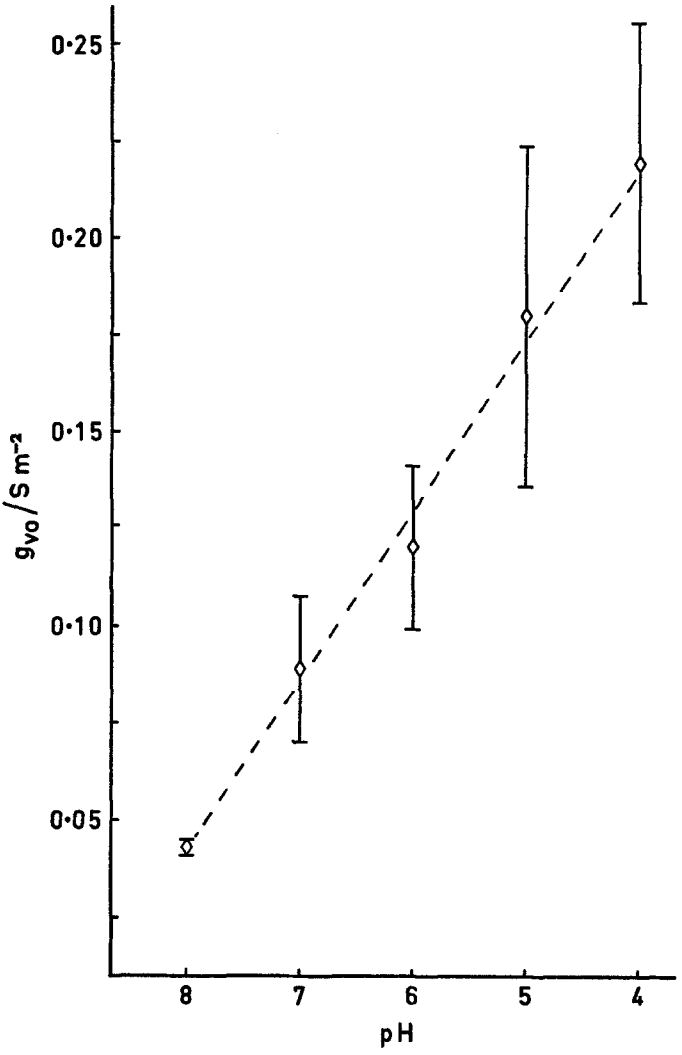

Fig. 4*b*

observed when the change in pH was from 5.5 to 6. Responses of types *A* and *C* were observed for pH changes of 5 to 6, 5.5 to 6 and 6 to 7.

The Effect of Replacing External Cl^- by SO_4^{2-}

ψ_{vo} as a function of pH when Cl^- in the external medium was replaced by SO_4^{2-} for four cells was not significantly different from the results shown in Fig. 1. In another four cells, when the anion in the external medium was changed from Cl^- to SO_4^{2-} and back again at pH 6, 5 and 4, there was no detectable corresponding change in ψ_{vo}.

The Effect of pH on ψ_{vo} and g_{vo} at 2.5 °C

Fig. 4 (*a*) compares ψ_{vo} as a function of pH at 2.5 °C with ψ_{vo} as a function of pH at 20 °C, the latter replotted from Fig. 1. Values of ψ_{vo} at 2.5 °C were measured more than an hour after the change in temperature to allow some slow changes in membrane properties to reach completion (Hope & Aschberger, 1970). The difference between the curves is 17.5 ± 7 (12) mV at pH 4 and increases rapidly at pH > 5 to a maximum of 65 ± 10 (12) mV at pH 7. At pH > 7 the difference between the curves decreases markedly. Fig. 4 (*b*) shows that g_{vo} was less at 2.5 °C than at 20 °C for pH 4 to 8 and that the effect of low temperature on g_{vo} increased with increasing pH (*cf.* Fig. 2). The interpolated values of ψ_{vo} and g_{vo} at pH 5.5 are close to those measured at 2.5 °C by Hope and Aschberger (1970) for *Chara corallina* in FPW (0.2 mM KCl, 2.0 mM NaCl, 0.05 mM $CaCl_2$, pH ~ 5.5, unbuffered).

Effects of 0.2 mM 2,4-Dinitrophenol (DNP)

Table 2 shows the following effects of DNP: (*i*) There was no significant change in ψ_{vo} when pH was changed from 6 to 7. (*ii*) ψ_{vo} at pH 6 and 7 was approximately equal to ψ_K. (*iii*) ψ_{vo} in all cells was reversibly responsive to change in $[K^+]_o$ from 0.1 to 1.0 mM at pH 6 and 7. (*iv*) There was no significant effect of 0.2 mM DNP on g_{vo} at pH 6 or 7. (*v*) There was a significant increase in g_{vo} when the cells were depolarized in 1.0 mM $[K^+]_o$.

At pH 6 and 7, 0.2 mM DNP had no effect on the rate of cytoplasmic streaming and all measured effects of the DNP were reversible. At pH 4 and 5, 0.2 mM DNP caused rapid slowing of cytoplasmic streaming. The effect was reversible at pH 5 if the DNP was removed within about 20 min. However, at pH 4, cells did not recover from a 10-min exposure to 0.2 mM DNP. The initial effect of DNP on ψ_{vo} at pH 5 was a hyperpolarizing transient of 15.5 ± 2.3 (4) mV with the peak from 15 to 18 min after the addition of the DNP. The initial effect on ψ_{vo} at pH 4 was a depolarizing transient of

Table 2. Steady values of ψ_{vo} and g_{vo} in the presence of DNP, with $[K^+]_o$ 0.1 and 1.0 mM

pH	[DNP]/mM	$[K^+]_o$/mM	ψ_{vo}/mV	g_{vo}/S m^{-2}
6.0	0.0	0.1	-178.0 ± 5.0 (4)	0.739 ± 0.286 (4)
6.0	0.2	0.1	-157.0 ± 2.0 (4)	0.539 ± 0.09 (4)
6.0	0.2	1.0	-107.0 ± 1.5 (4)	1.764 ± 0.206 (4)
7.0	0.0	0.1	-213.0 ± 3.5 (4)	0.495 ± 0.194 (4)
7.0	0.2	0.1	-155.0 ± 1.5 (4)	0.292 ± 0.031 (4)
7.0	0.2	1.0	-90.5 ± 0.5 (4)	2.250 ± 0.249 (4)

9.5 ± 1.5 (4) mV with the peak 1 to 2 min after the addition of the DNP followed by a hyperpolarizing transient of 21 ± 7 (4) mV with the peak from 3 to 6 min later.

Effects of Sodium Azide at pH 7

An example of the effect of 1.0 mM NaN_3 at pH 7 is shown in Fig. 5. The depolarization caused by azide had a half time of approximately 20 min and ψ_{vo} reached a steady value of -145 ± 1.5 (4) mV. The mean depolarization was 69 ± 3.5 (4) mV. The concomitant change in g_{vo}, from 0.422 ± 0.029 (4) S m^{-2} to 0.366 ± 0.047 (4) S m^{-2}, was not significant. When 0.7 mM NaN_3 was added at pH 7 the depolarization was 45 ± 7.5 (4) mV and g_{vo} decreased significantly from 0.794 ± 0.128 (4) S m^{-2} to 0.336 ± 0.08 (4) S m^{-2}. These effects were reversible.

Oxygen evolution in the light was totally inhibited by 1 mM NaN_3, the half time for the inhibition being approximately 1 hr. There was no effect on oxygen consumption in the dark.

Effects of Other Inhibitors

The responses of ψ_{vo} and g_{vo} to light-dark changes showed some variability. Such changes at pH 6 caused depolarizations of as much as

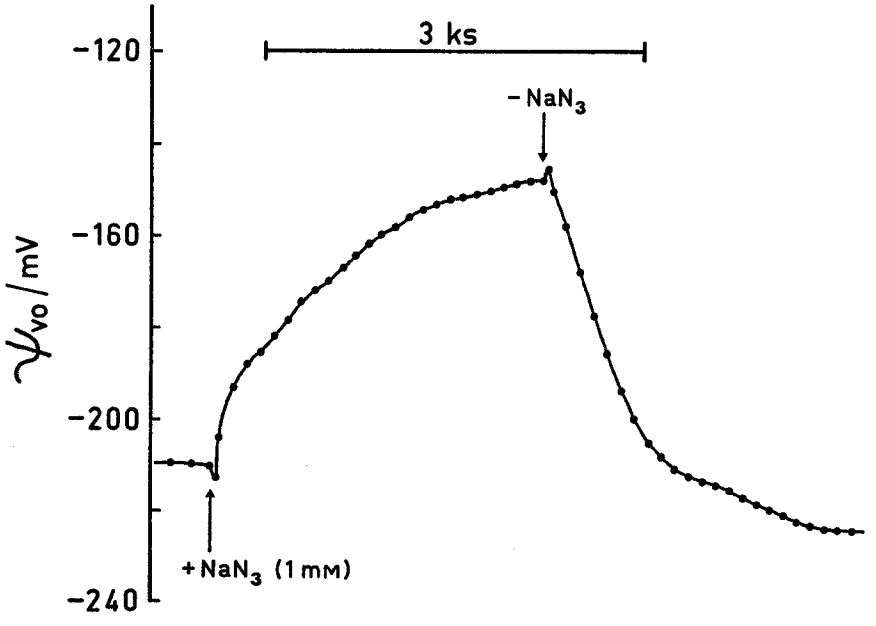

Fig. 5. An example of the changes in ψ_{vo} caused by the addition of 1.0 mM NaN_3 to the medium and its subsequent removal

35 mV, or as little as 5 or 10 mV. The depolarizations of 20 to 30 mV which did occur in the dark at pH 6 were slow, taking at least 15 min to reach 90% completion. The hyperpolarized state was always observed in the dark at pH 7 (Fig. 1) and was steady for at least 30 min. Depolarization in the dark was accompanied by a decrease in conductance of from 10 to 50%. However, even cells which remained in the hyperpolarized state showed conductance decreases of up to 20% in the dark (Fig. 2).

CO_2 and O_2 were removed from the external solution by prolonged and continuous bubbling of the reservoir of bathing solution with pure N_2. This had no effect on ψ_{vo} or g_{vo} at pH 7 in the light. This finding is consistent with that of Findlay, Hope, Pitman, Smith and Walker (1969) who found that bubbling the external medium (FPW) with pure N_2 resulted in a hyperpolarized state since, in an unbuffered solution, removal of CO_2 should lead to an increase in pH. Concentrations of ouabain up to 1.0 mM had no effect on ψ_{vo} or g_{vo} at pH 7.

The Effect of Pretreatment in pH 9

Cells were soaked overnight in the standard solution with pH 9. After electrode insertion, the pH was changed in the sequence 9, 7, 6, 5, 4, 5, 6, 7 (Fig. 6*a*). ψ_{vo} at pH 7 at the beginning of the sequence was 50 ± 4.5 (4) mV more positive than at the end, i.e. after the cells had been exposed to low pH. When the pH was changed from 7 to 6, two different responses were recorded. An example of each, *A* and *B*, is shown in Fig. 6(*b*). A spontaneous hyperpolarization of type *A* was observed in two out of four cells. Fig. 6(*a*) was plotted from responses of type *B*, i.e. in which ψ_{vo} remained constant after the initial change with pH. Measurements were made at each pH for at least 15 min. Responses of type *A* were not observed at any other point in the pH sequence. Despite the divergent values of ψ_{vo} in pH 6 after the second pH change of the sequence, all cells had similar potentials at the subsequent values of pH.

There was no significant difference between g_{vo} in pH 7 at the beginning of the sequence of pH changes (0.635 ± 0.278 (4) S m^{-2}) and at the end (0.388 ± 0.177 (4) S m^{-2}), though in both cases the mean was unusually low and there was considerable variability between the cells.

Some Ionic Fluxes as Functions of pH

Table 3 shows K^+ and Cl^- effluxes, and Na^+ influx as functions of pH between 4 and 8. The Cl^- efflux in SO_4^{2-} medium at pH 6 and Na^+ influx

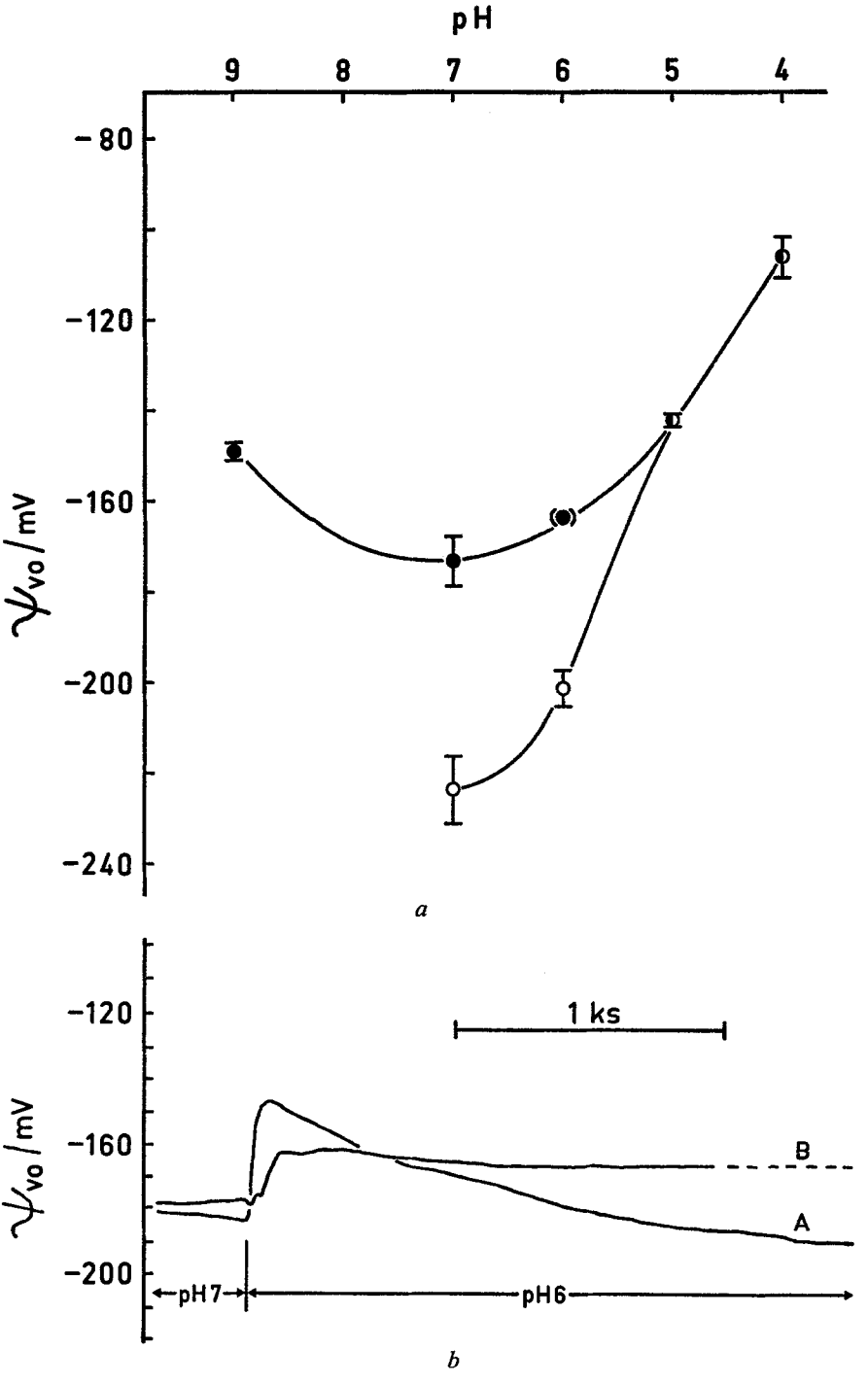

Fig. 6. (*a*) ψ_{vo} as a function of external pH after an overnight pretreatment in pH 9 and before (●) and after (○) a subsequent exposure of the cells to low pH (4 and 5). Each point is the mean ± SEM for 4 cells. (*b*) An example of each of two different responses of ψ_{vo} observed when pH was changed from 7 to 6. Further details of the sequence of pH changes in these experiments are given in the text

Table 3. Ion fluxes (nmoles m^{-2} sec^{-1}) as functions of pH

pH	K^+ efflux	Na^+ influx	Cl^- efflux (SO_4^{2-} external medium)
4.0	154.1 ± 20.2 (5)	7.2 ± 1.9 (8)	4.3 ± 0.7 (5)
5.0	24.5 (3)	5.4 ± 0.9 (8)	1.7 ± 0.3 (5)
6.0	13.9 ± 1.5 (16)	6.9 ± 0.7 (8)	1.2 ± 0.2 (39)
7.0	10.8 ± 0.8 (5)	7.5 ± 1.0 (8)	1.4 ± 0.3 (5)
8.0	12.9 ± 1.9 (5)	5.0 ± 0.6 (8)	2.6 ± 0.9 (5)

10 nmoles m^{-2} $sec^{-1} \equiv 1$ pmole cm^{-2} sec^{-1}.

at pH 6 in Table 3 are similar to those measured by Findlay *et al.* (1969) in FPW, though the K^+ effluxes at pH 6 reported here are somewhat larger than they measured.

Discussion

pH and the Electrogenic Potential

The high negative values of potential in the pH range 6 to 8 (Fig. 1) suggest the presence of an electrogenic pump in *Chara* under these conditions. The results shown in Fig. 4(*a*) also indicate an electrogenic component of potential between pH 6 and 8 and suggest there may be a smaller electrogenic potential at pH 4 and 5. However, Hope and Aschberger (1970) have provided evidence that some of the depolarization in *Chara* with decreasing temperature is the result of an increase in the ratio P_{Na}/P_K at pH 5.5 (the pH of unbuffered FPW at 2.5 °C). It is likely therefore that there is little or no electrogenic component of potential at low pH and that the potential in this pH range is a passive diffusion potential. The effects of 1.0 mM NaN_3 and 0.2 mM DNP (Fig. 5 and Table 2) also indicate an electrogenic component of potential of 60 to 70 mV at pH 7, and 15 to 25 mV at pH 6 but none in the pH range 4 to 5, since in this range the initial effect of DNP was either a hyperpolarization or a small depolarization followed by a larger hyperpolarization. The consistent responses of ψ_{vo} to changes in $[K^+]_o$ at pH 7 and 6 in the presence of DNP, and at pH 5 provide further evidence that, when the electrogenic component of potential is absent or inhibited, ψ_{vo} is a function of the ionic concentrations and passive permeabilities.

Membrane Conductance

The measured electrical conductance is greater at all values of pH than would be predicted from the ionic conductances calculated from the passive

fluxes. It is possible that there are "pump conductances" as suggested by Spanswick (1972, 1973) for *Nitella* but the results do not indicate that a conductance in the electrogenic pump makes a major contribution to total membrane conductance in *Chara*. The changes in g_{vo} when the electrogenic component of potential is inhibited by DNP or NaN_3 are not consistent and may be direct effects of these inhibitors on passive ionic permeabilities, or on interactions between ions or ions and water within the membrane. The very low conductances observed in *Nitella* in the dark (Spanswick, 1972, 1973) were observed in *Chara* at 2.5 °C only. However, the markedly low values of conductance at 2.5 °C in *Chara* occurred even at low pH where the membrane potential is apparently due to passive diffusion. This suggests that the low values of g_{vo} at 2.5 °C at all pH are due to a large extent to decreases in passive ionic permeabilities and changes in interactions between both ions and water in the membrane, rather than to a decrease in a pump conductance.

Cell Metabolism and the Electrogenic Potential

No simple direct link between the electrogenic potential and some particular aspect of metabolism has been established. However, the present results eliminate some possibilities. The occurrence of a stable hyperpolarized state in the dark (Fig. 1) suggests the electrogenic pump in *Chara* is not directly linked to photosynthesis. However, the variable slow depolarizations observed in the dark at high pH suggest that some of the energy for the electrogenic pump may be supplied via an indirect pathway from photosynthesis. Saito and Senda (1973) concluded that the hyperpolarized state in *Nitella flexilis* and *Nitella axilliformis* was closely related to photosynthesis but found that in some cells of both species darkness caused no inhibition of the hyperpolarized state. Spanswick (1972) has provided some evidence for a light-stimulated electrogenic pump at pH 6 in *Nitella translucens*. However, he also observed potentials considerably more negative than ψ_K in the dark at pH 7 and 8 (Fig. 6 in Spanswick, 1972). Spanswick (1970) also found that the magnitude of the transient hyperpolarization caused in *Nitella translucens* by the addition of bicarbonate, i.e. an increase in the pH, was the same in light and dark.

The occurrence of the hyperpolarized state in anaerobic solutions suggests the pump is not directly linked to respiration and the absence of an effect of ouabain on the electrogenic potential suggests the electrogenic pump is not the ouabain-sensitive $Na^+ - K^+$ exchange pump found in *Nitella* by MacRobbie (1962) and in *Hydrodictyon* by Raven (1967). Azide apparently

does not act as a respiratory inhibitor in *Chara* and the difference between the half times of the effects of azide on the hyperpolarized state and on O_2 evolution in the light suggests these two effects are independent. DNP is known to be an uncoupler of respiration though apparently it is not acting in this capacity, because of the absence of effect of anaerobic conditions on the hyperpolarized state. DNP is known to inhibit acid secretion in animal tissues (Keynes, 1969) but the mechanism of action is unknown.

Passive Fluxes and Ionic Permeabilities

Lannoye *et al.* (1970) measured fluxes of Na^+, K^+ and Cl^- in *Chara* at three different values of pH. Their values for Na^+ and Cl^- fluxes were significantly different from those reported here and from those of Findlay *et al.* (1969). Lannoye *et al.* (1970) found little if any increase in K^+ or Cl^- effluxes at low pH. Our findings agree with those of Kitasato (1968) who found considerable increases in these two fluxes at low pH in *Nitella clavata.* A possible explanation of this discrepancy lies in the fact that Lannoye *et al.* (1970) used unbuffered solutions. Effluxes are usually measured in small volumes of stagnant solution and both *Chara corallina* and *Nitella clavata* are capable of causing alkalinization of the external medium (Spear *et al.*, 1969; Rent *et al.*, 1972; Lucas & Smith, 1973).

In *Chara* both Cl^- efflux in SO_4^{2-} external medium and K^+ efflux are believed to be passive fluxes since they are down measured electrochemical potential gradients (Findlay *et al.*, 1969). A proportion of the Na^+ influx is light-stimulated (Hope & Walker, 1960; Findlay *et al.*, 1969). However, for an initial consideration of ionic permeabilities, the Na^+ influxes in Table 3 have been considered as passive. The passive ionic permeabilities of the plasmalemma (Table 4) can be calculated from Eqs. (2) and (3) with

Table 4. Passive ionic permeabilities as functions of pH

pH	P_K/m sec^{-1}	P_{Na}/m sec^{-1}	P_{Cl}/m sec^{-1}
4.0	1.28×10^{-8}	2.06×10^{-9}	6.17×10^{-11}
5.0	9.11×10^{-9}	1.02×10^{-9}	1.60×10^{-11}
6.0	3.08×10^{-8}	9.31×10^{-10}	8.10×10^{-12}
7.0	1.05×10^{-7}	8.25×10^{-10}	7.98×10^{-12}
8.0	5.23×10^{-8}	6.17×10^{-10}	1.63×10^{-11}

P_K and P_{Cl} were calculated from Eq. (3) and P_{Na} was calculated from Eq. (2) using the mean values of their respective fluxes given in Table 3, and the mean values of ψ_{vo} from Fig. 1. The values of cytoplasmic concentrations used were $[K^+]_c = 100$ mM and $[Cl^-]_c = 20$ mM.

$\psi_{co} = (\psi_{vo} - 16)$ mV where ψ_{vo} is the experimentally determined value and 16 mV is taken as the potential across the tonoplast.

From the conclusion that ψ_{vo} at pH 4 is a passive diffusion potential, P_H at pH 4 may be calculated by substitution into Eq. (1) including terms for H^+ concentration and permeability and using the permeabilities in Table 4 and a value of $[H^+]_c$ of $10^{-6.3}$ M (Rent *et al.*, 1972). The value of P_H calculated on this basis is 3.2×10^{-7} m sec^{-1} ($\simeq 25\ P_K$).

Fig. 7 shows ψ_{co} as a function of pH calculated from Eq. (1) with terms for H^+ and using the relationship $P_H = 25\ P_K$. There is a good fit with the experimental data in the pH range 4 to almost 5.5 and a better agreement with the experimental data would be obtained if P_H/P_K decreased between pH 5 and 6. The theoretical values of ψ_{co} at pH 6 and 7 are in approximate agreement with measured values of ψ_{co} in the presence of low temperatures, 0.2 mM DNP and 1.0 mM NaN_3 at these values of pH. However, the external concentrations of the various ions during the flux experiments and the results of the electrical measurements described earlier indicate that an electrogenic component of potential would have been present during flux measurements when pH was $\geqq 6.0$. Thus, ionic permeabilities calculated from fluxes measured when the electrogenic pump is operating predict with reasonable accuracy the potentials measured when the electrogenic pump is inhibited, under all experimentally applied conditions. This is in contrast to the hypothesis of Spanswick (1972, 1973) for *Nitella* that the membrane potential is determined entirely by the electrical properties of the electrogenic pump.

The value of P_H calculated above is considerably less than that proposed by Kitasato (1968) for *Nitella*. However, Kitasato's conclusion that proton conductance is approximately equal to membrane conductance is based on two assumptions: (*i*) that all changes in ψ_{vo} with changes in pH are due to changes in a passive diffusion potential, and (*ii*) that individual ionic conductances do not change with pH. For a cell under steady-state conditions in which only passive diffusion is occurring,

$$\sum_{j=1}^{n} J_j = 0 \tag{4}$$

where J_j is the passive current carried by ions j, and

$$J_j = g_j(\psi_m - \psi_j) \tag{5}$$

where g_j is the conductance of ions j, ψ_m is the membrane potential and ψ_j is the Nernst equilibrium potential for ions j. From Eqs. (4) and (5) it can

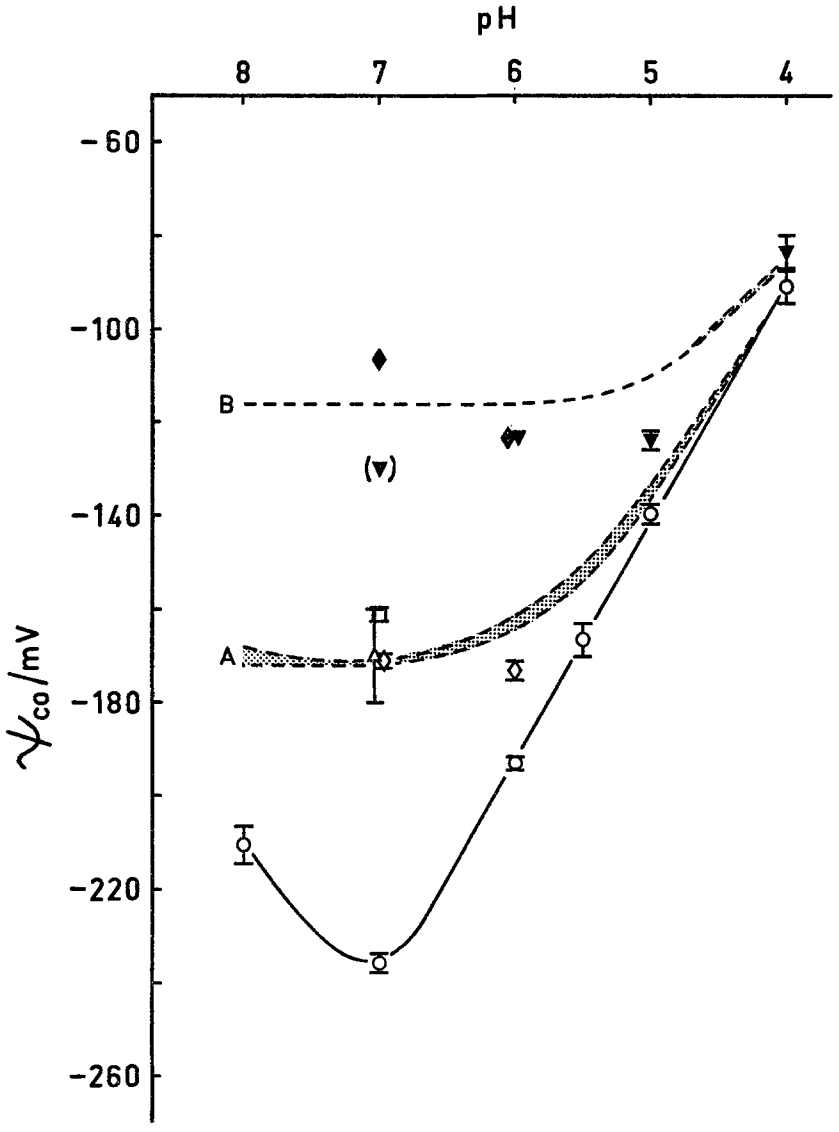

Fig. 7. A comparison of experimental and theoretical values of ψ_{co} as functions of pH. Experimental $\psi_{co}(=(\psi_{vo}-16)$ mV) have been replotted from the data in Figs. 1 and 4 (*a*), Tables 1 and 2, and the data on the effects of 1.0 mM NaN_3 given in the text. Theoretical ψ_{co}, for $[K^+]_o=0.1$ mM with $[Na^+]_o=1.0$ mM (curve *A*), and $[K^+]_o=1.0$ mM with $[Na^+]_o=0.1$ mM (curve *B*), have been calculated from Eqs. (1), (2) and (3) using ± 1 SEM for the passive fluxes and potentials and the relationship $P_H=25\,P_K$. The key to the symbols is as follows — (○) 20 °C, 0.1 mM $[K^+]_o$; (△) 2.5 °C, 0.1 mM $[K^+]_o$; (◇) 0.2 mM DNP, 0.1 mM $[K^+]_o$; (□) 1.0 mM NaN_3, 0.1 mM $[K^+]_o$; (▼) 20 °C, 1.0 mM $[K^+]_o$; (◆) 0.2 mM DNP, 1.0 mM $[K^+]_o$. Standard errors of the mean are shown where they are large enough to be visible. The point in parentheses is a single value

be shown that at 20 °C, if all g_j remain constant,

$$g_j/g_m = \Delta\psi_m/58.2 \tag{6}$$

where g_m is the total membrane conductance; i.e. $g_m = \sum_{j=1}^{n} g_j$ and $\Delta\psi_m$ is the change in ψ_m when the concentration of j is changed 10-fold. Eq. (6) is the general form of Eq. (18) in Kitasato (1968). Since ψ_{vo} in *Chara* and *Nitella* changes by almost 58 mV when the pH is changed from 5 to 6, Eq. (6) predicts $g_H \simeq g_m$. However, the results presented here for *Chara* show that neither of Kitasato's assumptions holds: some of the p.d. changes are due to changes in activity of the electrogenic pump; and permeabilities, and hence ionic conductances do change with pH. In fact, Kitasato's own data for K^+ and Cl^- effluxes show that P_K decreases and P_{Cl} increases at low pH (*see* Table 2 and Fig. 9 in Kitasato, 1968).

The Sensitivity of ψ_{vo} to Changes in $[Na^+]_o$ and $[Cl^-]_o$

The model of ψ_{vo} as a function of pH outlined above predicts a decrease in ψ_{vo} of about 25 mV if $[Na^+]_o$ is increased from 1 to 10 mM at pH 5. The measured decrease in ψ_{vo} under these conditions is about 5 mV in *Chara* (Hope & Richards, 1971) and averages about 7 mV in *Nitella translucens* (Spanswick, 1972). The most likely explanation of this discrepancy is that, as mentioned above, a proportion of Na^+ influx is not passive and independent. Smith (1967) found that Na^+ influx in the dark in *Nitella* was from 0.1 to 0.25 of the Na^+ influx in the light. A new theoretical curve calculated using values of P_{Na} of one-tenth those shown in Table 4 would be no more than 5 mV more negative than the theoretical curve of Fig. 7 and at the extremes of pH the difference between the two curves would be only 1 or 2 mV. With such a decreased value of P_{Na} the calculated decrease in ψ_{vo} at pH 5, when $[Na^+]_o$ is increased from 1 to 10 mM, is 5 mV and the theoretical curve fits the experimental curve more closely around pH 5. Thus, without affecting our conclusions about the value of P_H, the experimental data may be explained by the reasonable assumption that Na^+ influx in the light is considerably greater than passive Na^+ influx.

When $[Cl^-]_o$ becomes zero (SO_4^{2-} medium) the model described above predicts a maximum change in ψ_{vo} of less than 1 mV at pH 4, where P_{Cl} is greatest. This is in agreement with the data.

The Sensitivity of ψ_{vo} to Changes in $[K^+]_o$

Fig. 7 also shows the relationship between the calculated passive diffusion potential and pH for $[K^+]_o = 1.0$ mM, $[Na^+]_o = 0.1$ mM. The per-

meabilities used for these calculations were those calculated from fluxes measured with $[K^+]_o = 0.1$ mM (Table 3). This is a possible source of error since Spanswick (1972) has provided evidence that P_K increases as $[K^+]_o$ is increased. It can be seen that there is a reasonable agreement between theoretical and experimental values of ψ_{vo} in high $[K^+]_o$ and that, if a higher value of P_K were used for the calculations the agreement between theoretical and experimental points would be improved at pH 5.

From Fig. 7 and the complex responses of ψ_{vo} to changes in $[K^+]_o$ and pH as described in Results, a number of conclusions can be drawn. (*i*) When cells are depolarized in high $[K^+]_o$ there is no electrogenic component of potential, regardless of pH. (*ii*) The smaller the electrogenic component of potential, the more sensitive ψ_{vo} becomes to increases in $[K^+]_o$. (*iii*) In the absence of the electrogenic component of potential, ψ_{vo} is described by the Goldman equation with terms for H^+ and with permeabilities calculated from fluxes measured under conditions in which an electrogenic pump is operating.

The explanation of the absence of depolarization of ψ_{vo} when $[K^+]_o$ is increased while the electrogenic pump is operating must lie in the properties of the pump itself. Factors such as the dependence of the "electrogenicity" of the pump on both the specific ionic conductances and the passive ionic equilibrium potential may be involved. It is possible that when the electrogenic pump is operating, potential is controlled by a feedback loop. An idea of the complex nature of the pump may be obtained from Fig. 3. A detailed understanding of the mechanisms of electrogenic pumps is obviously required. Present mathematical models of active transport and electrogenic pumps (Finkelstein, 1964; Rapoport, 1970) may serve as bases for this understanding.

The complex effects of various combinations of pH and $[K^+]_o$ reported here are very similar to those observed in *Chara braunii* by Oda (1962) and in *Nitella flexilis* and *Nitella axilliformis* by Saito and Senda (1973).

The Electrogenic Potential, Passive Proton Flux and Internal pH

The permeability of the plasmalemma to H^+ and the inwardly directed electrochemical potential gradient for protons indicate that if cytoplasmic pH is to remain constant, passive H^+ influx must be balanced by an active H^+ efflux at all pH's. There are two pieces of evidence which are consistent with the idea that an active H^+ efflux causes the electrogenic potential. The first is that the electrogenic component of ψ_{vo} is stimulated by increasing pH (Fig. 7). In this respect active H^+ extrusion balancing passive H^+

influx is analogous to the electrogenic $Na^+ - K^+$ exchange pump observed in a molluscan neurone by Marmor and Gorman (1970). As the pH is increased beyond 5.5 the difference between the passive diffusion p.d. and the Nernst potential for H^+ across the plasmalemma decreases; e.g., $\psi_H - \psi_{co}$ is 210 mV at pH 5 and 70 mV at pH 8. If there is not an immediate accompanying decrease in active H^+ efflux, the resultant imbalance of H^+ fluxes is in such a direction as to create an electrogenic potential. Such an electrogenic p.d. would cause a larger passive H^+ influx than would the passive diffusion p.d. and thereby maintain to some extent the proton flux balance. Thus the electrogenic potential becomes part of a feedback control of cytoplasmic pH.

The second piece of evidence is the effect of prolonged exposure to very low $[H^+]_o$, on ψ_{vo} as a subsequent function of pH (Fig. 6*a* and *b*). Continued active H^+ efflux at high pH should lead to a decrease in $[H^+]_c$ and, if the H^+ pump is analogous to the electrogenic Na^+ pump in animal tissue (Carpenter & Alving, 1968; Marmor & Gorman, 1970), to a consequent decrease in pump activity through feedback control. Subsequent treatment with low pH should lead to a stimulation of the pump and a return of the electrogenic potential. The results in Fig. 6(*a*) and (*b*) are consistent with this interpretation.

Fluxes of H^+

From Eqs. (2) and (3) and using the relationship $P_H = 25\ P_K$ the net passive fluxes of H^+ can be calculated for each pH (Table 5). There is a net passive proton influx for all values of pH between 4 and 8. It has been found that cells of *Chara* are irreversibly damaged by prolonged exposure to pH 4, probably because the active H^+ efflux is not great enough to balance the large passive influx at this pH. Cells do survive at pH 5 which suggests the active H^+ efflux is about 12 nmoles $m^{-2}\ sec^{-1}$ at this pH

Table 5. Theoretical net passive H^+ flux as a function of pH

pH	Net passive H^+ flux (nmoles $m^{-2}\ sec^{-1}$)
4.0	111.4
5.0	12.1
6.0	5.7
7.0	2.4
8.0	0.1

Net passive H^+ flux was calculated from Eqs. (2) and (3) using the relationships $P_H = 25\ P_K$. The net passive H^+ flux is inwards.

(Table 5). It is not possible to attach much significance to the calculated passive net influxes at pH > 6 since in this pH region the term $P_H[H^+]_o$ in the Goldman equation becomes small making it difficult to draw conclusions about the relationship between P_H and P_K.

The acid and alkali bands which form on the outside of *Chara corallina* (Lucas & Smith, 1973) and *Nitella clavata* (Spear *et al.*, 1969) do not appear to be related to the electrogenic pump in *Chara*. Unlike the electrogenic potential, both acid and alkali formation are consistently light-stimulated and Lucas and Smith (1973) have shown that the alkalinization is a result of HCO_3^- uptake followed by CO_2 fixation and OH^- efflux.

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