J. Membrane Biol. 56, 1–7 (1980)

The Journal of
Membrane Biology

The *Chara* Plasmalemma at High pH. Electrical Measurements Show Rapid Specific Passive Uniport of H^+ or OH^-

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Summary. Above a critical external pH (about 10.5), the *Chara* membrane acquires new properties. In this state the membrane potential is close to the equilibrium potentials for H^+ and OH^-, hyperpolarizing as external pH increases with a slope of -59 mV/pH unit. The membrane conductance increases by an average factor of 2.4 above the critical pH. These changes are explained by an increase in permeability to OH^- (or H^+). The establishment of a OH^- (or H^+) permeable membrane at high pH suggests that the large fluxes of OH^- (or H^+) which occur in the alkaline band in photosynthesizing cells are passive.

The membrane potential in Charophytes has been shown to exhibit two different states (*Chara corallina:* Hope & Walker, 1961; *C. braunii:* Oda, 1962; *Nitella:* Spanswick, Stolarek & Williams, 1967; *Lamprothamnium:* Bisson & Kirst, 1980). In the depolarized state, the potential is close to the equilibrium potential for K^+ (E_K), is sensitive to the external concentration of K^+ (C_K^o), and is adequately described by equations for passive diffusion. This state is maintained in the absence of external calcium or in the presence of high C_K^o (Hope & Walker, 1975) or of inhibitors such as dicylohexylcarbodiimide (DCCD), diethylstilbestrol (DES), and 2,4-dinitrophenol (DNP) (Keifer & Spanswick, 1978). In a medium containing Ca^{2+} and with a low C_K^o, the membrane potential usually has a value more negative than the equilibrium potential for any ion. This hyperpolarization is believed to be effected by an active, electrogenic H^+-efflux (Spanswick, 1974; Richards & Hope, 1974). In this state the membrane potential is sensitive to external pH (pH^o), showing a maximum negative value near pH 7, and more positive values at lower and at higher pH up to pH 9.0 (Richards & Hope, 1974; Smith & Walker, 1976).

We show here that above an external pH of 10 or 10.5, the *Chara* membrane is in a third state, in which the membrane potential may be described as a passive diffusion potential with H^+ or OH^- as the most permeant ion.

This high permeability at high pH has physiological significance. In an unbuffered solution in the light portions of the membrane are bathed in an alkaline solution which may have a pH above 10 (*see,* e.g., Lucas & Dainty, 1977*a* and *b*). These alkaline bands are formed when the cell is photosynthesizing with CO_2 provided as HCO_3^- (Lucas & Smith, 1973). The fixation of CO_2 from HCO_3^- releases OH^-, which must be either transported out of the cell or neutralized by H^+ brought into the cell, and this transport probably occurs in the alkaline band (Lucas, 1975). The study of the permeability properties of the membrane at high pH may indicate the mechanism of this neutralization transport.

Materials and Methods

Chara corallina Klein ex Willd., em. R.D.W. (=*C. australis* R. Br.) was grown in culture (Hope & Walker, 1975). Isolated internodes were incubated for at least 24 hr in a medium containing 0.1, (in mM) K_2SO_4; 1, NaCl; and 0.5, $CaSO_4$ buffered to pH 7.5 with 5 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid) (pK = 7.5) or HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid) (pK = 7.6). During experiments, the concentration of K_2SO_4 was increased to give 1 mM K^+. This concentration was used to make E_K more positive and to enable us to obtain cells in both depolarized and hyperpolarized states at pH 7.5. The cells were impaled with two glass microelectrodes, and connected with a voltage clamp apparatus (Walker, Beilby & Smith, 1979). Solution at high pH, buffered with the sulfonic acid buffers CHES (2 [N-cyclohexylamino] ethane sulfonic acid) (pK = 9.5) or CAPS (cyclohexylaminopropane sulfonic acid) (pK = 10.4), was flowed past an unclamped cell, and the change in potential was recorded. Conductance (G_m) was measured by clamping the cell at a potential near the open-circuit potential and imposing an alternating staircase of command voltages (Walker et al., 1979).

0022-2631/80/0056-0001 $01.40

When the open circuit potentials were very different at neutral and alkaline pH, the membrane was clamped at the same initial potential for the different staircases, to avoid any slow, voltage-dependent changes in G_m. If necessary, the values for G_m were corrected for the cable properties of the cell. Unless otherwise specified, the cell remained at the high pH for the minimum length of time needed to complete the measurements (2–15 min), and was returned to pH 7.5 between all alkaline measurements.

DCCD (dicyclohexylcarbodiimide) was kept frozen as a 50 mM solution in ethanol. It was added to the experimental solution to give a concentration of 50 μM. Addition of ethanol alone had no effect. DCCD was added either at high pH (11.0) or at both high and neutral pH (7.5).

Sulfonic acid buffers were obtained from Calbiochem or Sigma.

Results

Membrane Potential Changes

Cells in the depolarized state (circles, Fig. 1) show no change in membrane potential with increases in external pH (pH^o) up to pH 10. Above a critical pH (pH^o_{cr}) near 10.5, the membrane hyperpolarizes with increasing pH^o. If we assume the cytoplasmic pH (pH^c) to be 8.0, a reasonable value for cells incubated at $pH^o = 7.5$ (Smith & Walker, 1976), the values attained are close to $E_H = E_{OH}$. Hyperpolarized cells (triangles, Fig. 1) show the expected depolarization at moderately high pH^o, but above pH^o_{cr}, again near 10.5, the cells show hyperpolarization with increasing pH^o, as did the cells from the depolarized state. The regression slope of the curve above pH^o_{cr}, for cells with $pH^o_{cr} < 10.5$, is -59 mV (pH unit)$^{-1}$ ($n = 16$; 5 cells). Occasionally, cells do not show a linear hyperpolarization, but cease to hyperpolarize, or depolarize, at very high pH (Fig. 2). Altogether 29 cells were tested, 12 in a hyperpolarized state, 15 in a depolarized state, and 2 which showed both states. All cells showed a highly negative value of ψ_m at high pH^o. Nine cells were tested at 2 or 3 values above pH^o_{cr}; 2 showed the flat response at very high pH.

From the depolarized state, the time course of hyperpolarization at $pH > pH^o_{cr}$ is relatively rapid, smooth, and monotonic (Fig. 3), suggesting a simple change in membrane function. From the hyperpolarized state, the change is initiated as rapidly (Fig. 4), but shows more complex kinetics and may take longer (up to 10 min) to come to steady state. This suggests a more complex change, including turning the H^+-pump off as well as altering membrane permeability. The recovery from the alkaline state is slow, involving a strong depolarization, often triggering an action potential; a fairly rapid repolarization phase; and a slower hyperpolarization, probably representing a slow activation of the H^+-pump.

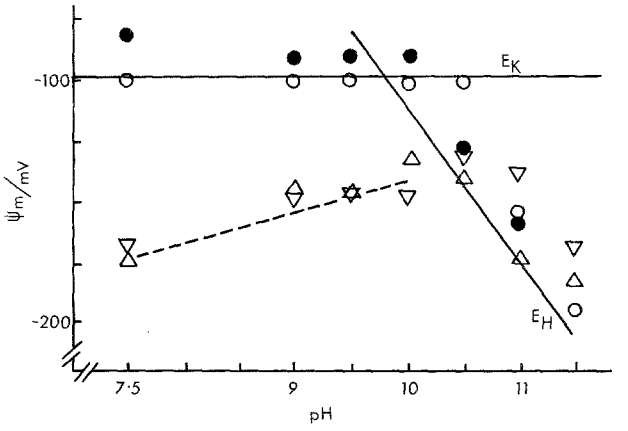

Fig. 1. Effect of pH^o on membrane potential. Each symbol represents a different cell. Solid lines are computed equilibrium potentials for K^+ and H^+ ($=OH^-$). The dashed lines is the computed linear regression through the indicated points

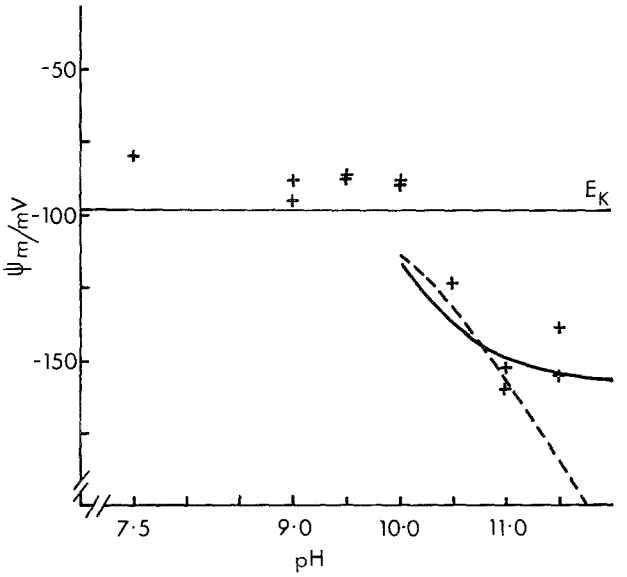

Fig. 2. Effect of pH^o on membrane potential, showing depolarization at very high pH^o. Solid line represents potential expected with high permeability to H^+, and dotted line represents potential expected with high permeability to OH^- (*see* Discussion)

Conductance Changes

The average value for G_m above pH^o_{cr} is 1.15 ± 0.21 S min^{-2} ($n = 20$), and there is no difference in cells from the hyperpolarized or depolarized state. This represents an increase of a factor of 2.38 ± 0.33 (SE; $n = 20$). Again, there is no difference in this factor for cells from the hyperpolarized or depolarized state, providing care is taken to avoid voltage-dependent changes in G_m (*see* Methods).

The absolute value of G_m in the alkaline state has been measured to be as high as 4.0 S m^{-2}.

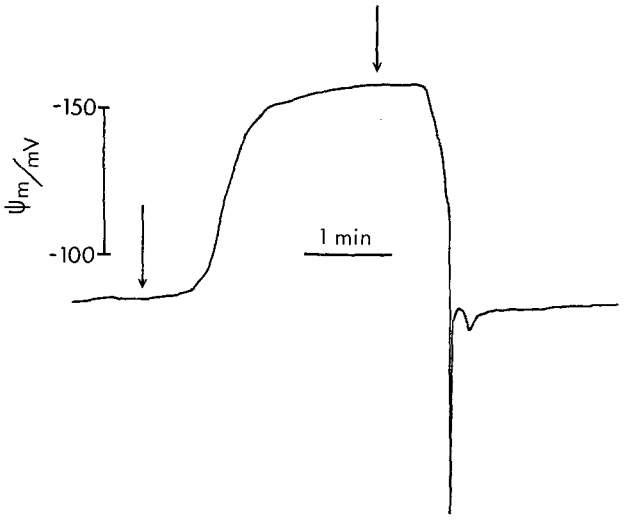

Fig. 3. Change in potential in a depolarized cell when pH^o is changed from 7.5 to 10.5 (arrow at left) and back to 7.5 (arrow at right). Note action potential during depolarization. The lag between the arrow and the onset of the change is partly due to "dead time" in the solution-changing apparatus

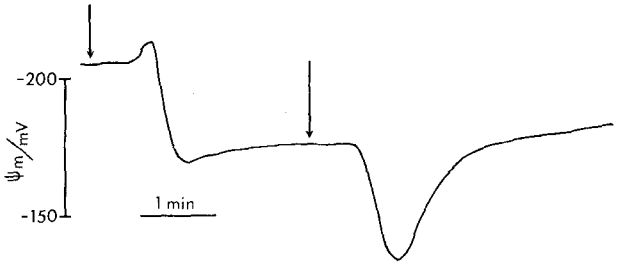

Fig. 4. Change in potential in a hyperpolarized cell following a change in pH^o from 7.5 to 10.5 (left arrow) and back to 7.5 (right arrow). The strong depolarization after a change from high pH to 7.5 is often accompanied by an action potential, although none is seen in this example. The full repolarization took about 30 min

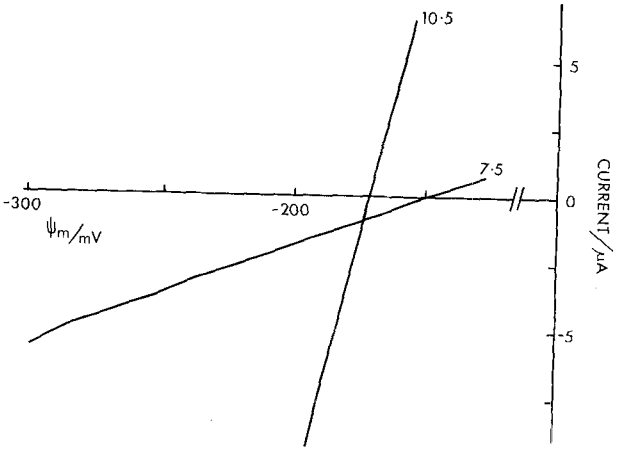

Fig. 5. Current-voltage curves for a cell at pH 7.5 and at 10.5. Cell is in the depolarized state at $pH^o=7.5$. Clamp for measuring G_m was initially −150 mV

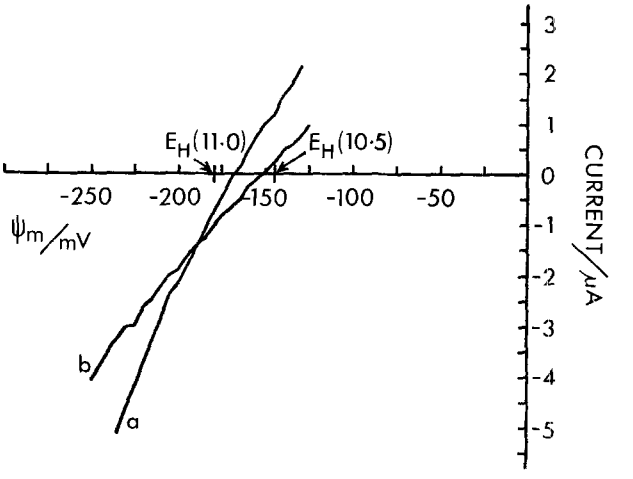

Fig. 6. Difference $i-\psi$ curves for the transition from the depolarized to the alkaline state. (*a*): pH 11.0. (*b*): pH 10.5

The values are underestimates of the plasmalemma conductance, since they are not corrected for the series tonoplast conductance, which may be as low as $10\,S\,m^{-2}$, but may be higher (Walker et al., 1979). In the worst case error, a measurement of $4.0\,S\,m^{-2}$ could represent an actual conductance of $6.7\,S\,m^{-2}$.

Current-Voltage Curves

The current-voltage ($i-\psi$) curve (Fig. 5) above pH^o_{cr} is linear between the limits of action potential (about −100 mV) and punch through (about −300 mV). It intersects with the control $i-\psi$ curve (pH 7.5) at a value near the calculated $E_{OH}=E_H$. The control currents at each potential value may be subtracted from those obtained at high pH to obtain a difference $i-\psi$ curve. If the change in pH involves a change in only one transport system, this kind of curve can give information about this transport system. This is most likely to be true of the transition from the depolarized to the alkaline state. Difference $i-\psi$ curves for this change for pH 10.5 and 1.0 are seen in Fig. 6. Each difference curve is linear and intersects the voltage axis near $E_{OH}=E_H$, suggesting that the change between the two states involves an increase in passive transport of OH^- or H^+. The slope of the curve for pH 10.5 is slightly less than that at pH 11.0 (0.786 and $1.27\,S\,m^{-2}$, respectively) suggesting that the permeability is slightly less at pH 10.5. Although the change from the hyperpolarized state is probably more complex (*cf.* Figs. 3 and 4), the difference $i-\psi$ curves are quite similar (Fig. 7). Again the slope at

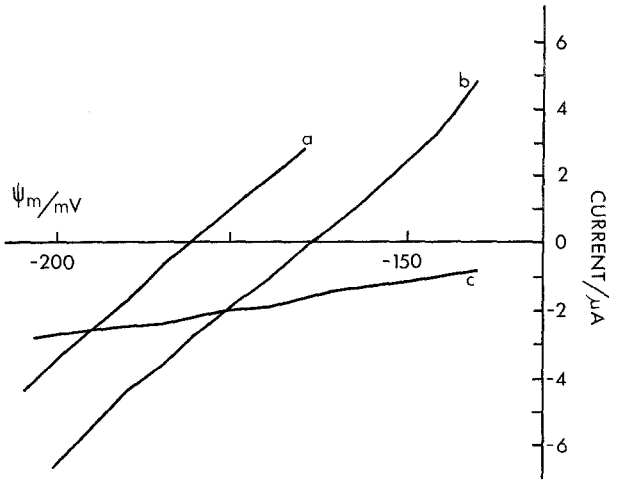

Fig. 7. Difference i-ψ curve for the transition from the hyperpolarized to the alkaline state. (*a*): pH 10.5 ($E_H = -206$ mV). (*b*): pH 11.0 ($E_H = -177$ mV). (*c*): pH 11.5 ($E_H = -148$ mV)

pH 10.5 (0.572 S m^{-2}) is less than that at pH 11.0 (3.03 S m^{-2}), but remains the same at pH 11.5 (2.97 S m^{-2}). This suggests that the change in membrane permeability to H^+ or OH^- is not discrete, but that near pH^o_{cr} the permeability may take on values intermediate between those of the alkaline and neutral states. This was seen in 3 cells of the 9 tested. The average of all slopes for such difference curves is 1.49 ± 0.29 S m^{-2} (15). There is no significant difference between transition from the hyperpolarized or depolarized state.

DCCD Effects

The ATPase inhibitor DCCD shifts a cell at neutral pH from the hyperpolarized to the depolarized state (Keifer & Spanswick, 1978). DCCD affects both membrane potential and membrane conductance at high pH (Fig. 8). The response to DCCD is usually much slower than that which occurred in the experiment shown in Fig. 8. In order to avoid alterations in the cell caused by prolonged exposure to high pH, further experiments were done by adding 50 μM DCCD at $pH^o = 7.5$, allowing the DCCD to act, then changing to pH 11.0 + 50 μM DCCD. In hyperpolarized cells, the effectiveness of the action of DCCD could be measured by the depolarization at $pH^o = 7.5$. When care was taken to ensure that DCCD did have full effect, an inhibition of the changes at high pH was seen consistently. As shown by the examples in Table 1, with DCCD there is no difference in ψ_m or G_m above and below the critical pH. Like the effect on ATPase activity, the effect is usually slow (requiring up to 60 min for full effect) and irreversible.

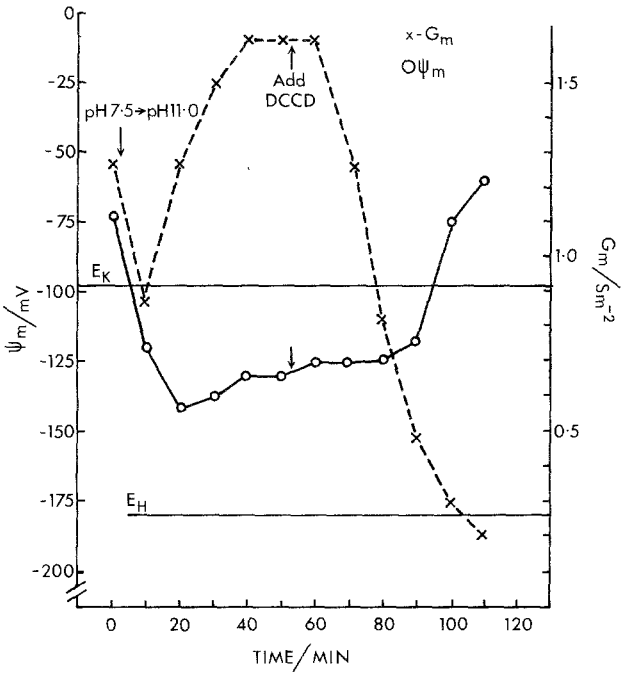

Fig. 8. The effect of 50 μM DCCD on membrane potential (ψ_m) (solid line) and conductance (G_m) (dashed line) at high pH^o. Cell is initially in the depolarized state at $pH^o = 7.5$. The initial fall in conductance at $t = 10$ min occurs because G_m was measured at the open circuit ψ_m, and voltage-dependent changes in G_m are occurring as well as pH-dependent changes

Table 1. The effect of DCCD (50 μM) on the alkaline response in *Chara*[a]

	pH 7.5		pH 11.0	
	ψ_m	G_m	ψ_m	G_m
Cell 1				
Control	−174	0.32	−153	0.57
+DCCD	− 88	0.95	− 98	0.77
Cell 2				
Control	−170	0.32	−164	2.02
+DCCD	− 68	0.20	− 67	0.24

[a] ψ_m: membrane potential (mV). G_m: conductance (S m^{-2}).

Discussion

The Passive Permeability of the Membrane

Our data show that above a critical pH, near 10.5, the cell membrane in *Chara* behaves as though highly permeable to H^+ or OH^-. This is indicated by the fact that the membrane potential (Fig. 1) depends on pH in a manner described by an equation for passive diffusion potential (*see* below) and by the shape of

the i-ψ curves (Figs. 5, 6, 7), which show zero current near E_{OH}.

Exposure of the whole cell surface to external pH up to 11.5 does not seem to harm the membrane. In all data shown, there was little change in ψ_m and $i-\psi$ curves at pH 7.5 before and after exposure to high pH. In one case, exposure to pH 11.5 damaged a cell, as judged by an irreversible change in ψ_m and $i-\psi$ curve at $pH^o = 7.5$.

We have assumed in calculating E_{OH} that $pH^c = 8.0$, the value found for cells in pH 7.5 (Smith & Walker, 1976). During the short periods for which the cells are exposed to high external pH, it seems possible that the cytoplasmic pH does not shift by much. During long exposures to high pH, cells do tend to depolarize slightly (e.g., from -142 to -125 over 50 min (Fig. 8)). This depolarization can be explained by an increase in pH^c of 0.29 units. Extrapolation of the correlation between pH^c and pH^o (Smith & Walker, 1976) past the limits within which it was measured suggests that cytoplasmic pH would ultimately increase to 8.7, so an increase to 8.3 in 50 min is not unlikely.

The Transported Ion

We have not shown whether the changes above pH^o_{cr} are caused by a permeability increase to H^+ or to OH^-. If we assume that K^+ is the only other ion to have a significant permeability, the membrane potential might be described by Eq. (1) or (2):

$$\psi_m = \frac{RT}{F} \ln \frac{a^o_H + P_K/P_H a^o_K}{a^c_H + P_K/P_H a^c_K} \quad (1)$$

$$\psi_m = \frac{RT}{F} \ln \frac{a^c_{OH} + P_K/P_{OH} a^o_K}{a^o_{OH} + P_K/P_{OH} a^c_K} \quad (2)$$

where a^o_j and a^c_j represent the external and cytoplasmic activities of the indicated ion, and P_j represents the permeability to that ion. The data in Fig. 1 can be described adequately by Eq (1) if $P_K/P_H \simeq 10^{-8}$, and by Eq. (2) if $P_K/P_{OH} \simeq 10^{-3}$, assuming $a^o_K = 75$ mM (Hope & Walker, 1975). It might be argued that 10^{-8} is an unreasonably small number, and that therefore it is more likely that the permeant ion is OH^-. However, it must be remembered that the permeation of ions like H^+, K^+ and OH^- through lipid membranes is not likely to be by a simple diffusive process described only by a diffusion coefficient. It is more likely to be by specific mechanisms whose apparent permeability is dominated by a partitioning effect. If this can be expressed by a K_M for binding and a V_m for subsequent transport, the limiting apparent permeability will be

$$P = V_m/K_M. \quad (3)$$

We can see that a permeability ratio of 10^{-8} might be the result of a V_m-ratio of 1 and K_M-ratio of 10^8. There are indications that K^+ transport in *Nitella* exhibits a K_M of 11 mM (Walker, 1980), so that the required ratio could be achieved with a K_M for H^+ binding of 10^{-10} M – corresponding to a pK_a of 10. An amine site could readily have such a pK_a. We cannot therefore dismiss H^+ passive transport on the grounds that it requires P_K/P_H to be 10^{-8}.

At very high pH, Eqs. (1) and (2) predict different behavior of ψ_m as a function of pH (solid and dotted lines in Fig. 2). Since in this region some cells seem to obey one equation, and others the other, we are so far not able to distinguish H^+ from OH^- permeation on this basis.

Inhibition of this effect of high pH by DCCD is interesting but inconclusive. DCCD inhibits ATP synthesis (Beechey, 1974) and reduces the ATP concentration in *Chara* (Keifer & Spanswick, 1979), although the change shown in Fig. 8 is faster than the decline in ATP is usually (R. Reid, *personal communication*). However we do not need to postulate active transport of an ion to explain the present results, and suggest instead that DCCD is acting directly to inhibit H^+ (or OH^-) transport, as it does in chloroplasts (Sigrist-Nelson, Sigrist & Azzi, 1978; Pick & Racker, 1979*a*), in mitochondria (Sebald, Graf & Lukins, 1979; Pansini, Guerrier & Papa, 1978), in *Escherichia coli* (Fillingame, 1976), and cytochrome oxidase (Casey, Thelen & Azzi, 1979). Another activity known for DCCD other than blocking proton or hydroxyl channels is inhibition of the Ca^{2+}-ATPase in sarcoplasmic reticulum (Pick & Racker, 1979 b); here DCCD is believed to act on the carrier site for Ca^{2+}. The inhibition of the increase in permeability by DCCD is quite consistent with its interaction with a proton or hydroxyl translocator.

Thus the present data do not allow us to distinguish between H^+ and OH^- as the permeant species. Ferrier, Greenleaf and Lucas (1980) argue that from a solution at pH 9 or higher, outside the cell, H^+ could not be supplied by (diffusion or) water-splitting at a rate sufficient to explain the observed currents (~ 0.1 A m^{-2}). However, catalyzed water-splitting is a known phenomenon in desalination membranes, and Simons (1979) has proposed a mechanism by which it might occur. He has, further, noted that this phenomenon might well occur in the electric fields present in biological membranes. We cannot therefore rule out fluxes of H^+ until a great deal more is known of the molecular mechanism in

the membrane. Although we refer below to OH^- transport, equivalent statements can be made about H^+ transport.

The Function of the Alkaline Band

The alkaline band in Charophytes is the site of effective net outward movement of OH^- during photosynthetic assimulation of HCO_3^- (Arens, 1939; Spear, Barr & Barr, 1969; Lucas & Smith, 1973). Its high pH has been seen as the effect of hydroxyl transport outward (e.g., Lucas, 1975). We suggest that the alkalinity is not only an effect of transport but also a causal factor. When the pH of the alkaline band is above pH^o_{cr}, that portion of membrane will become permeable to OH^-, allowing a large passive flux of OH^- out of the cell, reducing the pH of the cytoplasm, and increasing the pH of the outside medium. This provides a positive feedback mechanism maintaining large, sharp and stable bands. The fluxes required to maintain an alkaline band are quite large, >1 μmol m^{-2} sec^{-1} (Lucas, Ferrier & Dainty, 1977). These values are comparable with the current densities which flow into the cell at the alkaline zone (Walker & Smith, 1977), consistent with the hypothesis of an electrogenic uniport. Since, as we show, these large fluxes can occur passively, they do not require the energy input postulated by Lucas et al. (1977). Since they can occur near equilibrium, they do not involve much dissipation of energy.

Two objections may be raised against this hypothesis. The first is that alkaline bands in some cells have a pH^o less than 10.0, i.e., below pH^o_{cr}. However, in the alkaline zone the cytoplasmic pH is probably higher than normal, and this may depress pH^o_{cr}. Also the cell wall offers a diffusion resistance to the movement of ions, so that the measurement of pH outside the cell wall may underestimate the pH at the plasmalemma during net OH^- efflux.

The second possible objection is that hydroxyl ions are postulated to diffuse passively outwards at the alkaline band, whereas our data indicate that in a whole cell buffered at high pH the direction of passive movement is inward. The difference $i-\psi$ curves suggest that the hydroxyl conductance in the alkaline state is 1.5 S m^{-2} (*see* Results). Figure 1 indicates that the cells are about 20 mV more positive than E_{OH}. The result should be an initial passive influx of OH^- of about 20 mA m^{-2} or about 2×10^{-7} mol m^{-2} sec^{-1}, when the external pH is changed from 7.5 to 11.0. In a cell in unbuffered medium with acid and alkaline bands, however, at least half of the cell membrane is outside the alkaline band, and presumably is in the hyperpolarized state. In these portions of the membrane active electrogenic H^+ efflux would maintain the membrane potential more negative than E_{OH}, despite the depolarizing tendency in the alkaline bands, and thus passive movement of OH^- would be outward. To generate a passive flux of 75 mA m^{-2}, as measured in the alkaline band (Walker & Smith, 1977), given a conductance of 1.5 S m^{-2}, the membrane potential would have to be about 50 mV more negative than E_{OH}. At $pH^o=10$, E_{OH} is -110, or more positive if $pH^c>8$ in the alkaline band, as suggested above. Since a cell in buffered medium at pH 7 often has $\psi_m=-200$ mV, the membrane potential in the alkaline band could easily be brought to -160 mV despite the depolarizing tendency of the OH^- efflux. This is possible because the resistivities of the medium and of the cytoplasm are low. Walker and Smith (1977) measured a potential difference of 5–10 mV in the external medium near the cell between the acid and alkaline bands. They calculated that current flow in the cytoplasm implied a potential difference between the bands of 15 mV. This suggests that the difference in membrane potential between the acid and alkaline bands would be about 20–25 mV, i.e., that the membrane potential in the alkaline band could be as negative as -175 mV.

Chemiosmotic Considerations

Our view of the alkaline band, as a region of high H^+ permeability and in consequence of low $\Delta\mu_H$, means that transport processes dependent on $\Delta\mu_H$ cannot take place there. It will be of interest to check the postulate (Walker, 1980) that such processes as proton-chloride symport are inactivated rather than allowed to run in the direction opposite to their normal one.

This research was supported by grants from the Australian Research Grants Committee; M.A. Bisson was a Queen's Fellow in Marine Biology. We thank C.W. Slayman for helpful discussions, and G.O. Kirst for critical reading of the manuscript.

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Received 6 November 1979; revised 8 February 1980