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Activity of the Electrogenic Pump in *Chara corallina* as Inferred from Measurements of the Membrane Potential, Conductance, and Potassium Permeability[1]

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ABSTRACT

The effects of various inhibitors on the membrane potential, resistance, and K^+ permeability of *Chara corallina* were measured, providing evidence that there is an electrogenic pump in the membrane. It was found that: (*a*) 5.0 μM carbonyl cyanide *m*-chlorophenyl hydrazone depolarizes the membrane potential and increases the membrane resistance. This inhibition is faster in the dark than in the light but the extent of inhibition is the same in both cases. (*b*) Fifty μM dicyclohexylcarbodiimide increases the resistance and the K^+ permeability and depolarizes the membrane to a diffusion potential mainly controlled by K^+. (*c*) Forty μM diethylstilbestrol and 0.1 mM 2,4-dinitrophenol increase the resistance and depolarize the potential to a value given by the Goldman diffusion equation. (*d*) Both 3-(3,4-dichlorophenyl)-1,1-dimethylurea and darkness (at pH 6) cause the membrane resistance to increase but neither has a large effect on the potential. 3-(3,4-dichlorophenyl)-1,1-Dimethylurea increases K^+ permeability while darkness decreases it.

In all cases, the increase in resistance is interpreted as an inhibition of conductance through the electrogenic pump. As a consequence of this inhibition, the electrogenic component of the membrane potential is reduced, depolarizing the membrane. The electrogenic pump may be an H^+-ATPase in the plasmalemma.

5,5-Dimethyloxazolidine-2,4-dione at 5.0 mM decreases the membrane resistance, by lowering the internal pH providing more substrate for the pump. La^{3+} decreased cation permeability and depolarized the membrane but, since it had little effect on the membrane resistance, it probably does not affect the electrogenic pump.

Cellular membrane potentials have frequently been explained in terms of passive ion diffusion using the Goldman equation:

$$E = \frac{RT}{F} \ln \frac{P_K K_o^+ + P_{Na} Na_o^+ + P_{Cl} Cl_i^-}{P_K K_i^+ + P_{Na} Na_i^+ + P_{Cl} Cl_o^-} \quad (1)$$

where E is the membrane potential, F is the Faraday, and P_K etc. denote the permeability coefficients of the ions K^+ etc. having activities K_o^+ etc. outside and K_i^+ etc. inside the cell. This equation has been of limited utility in explaining the membrane potential of plant cells because the potential does not respond as predicted to changes in the external ionic concentrations. Also, the membrane potential is often more negative than the negative limit of equation 1. For this reason, the membrane potentials that have been observed in many algae and fungi (11, 16, 17, 20, 21, 24, 27) have been explained by postulating the existence of electrogenic ion pumps which transport negative ions into or positive ions out of the cell.

In 1968, Kitasato (11) postulated that *Nitella* has an electrogenic pump for hydrogen ions. Further work on *Nitella translucens* (28) indicated that H^+ was probably the ion pumped electrogenically and that the energy source for the pump was probably ATP, as in *Neurospora crassa* (25).

A point of controversy has arisen concerning the magnitude of the contribution of the pump to the electrical conductance of the membrane. The extreme views may be represented by the equivalent circuits shown in Figure 1. In the first circuit (Fig. 1A), the pump is represented as a current source in which the conductance through the pump is zero. Current flow through the pump is independent of the membrane potential, and the conductance of the membrane is equal to the conductance of the passive channels ($1/R_D$), irrespective of the current flow through the pump. Slayman *et al.* (25) considered that this circuit approximates the situation in *Neurospora*. In the second circuit (Fig. 1B), the pump is represented as a voltage source in series with a conductance ($1/R_P$). The conductance through the membrane is the sum of the passive conductance ($1/R_D$) and the pump conductance ($1/R_P$). The existence of the pump increases the total conductance through the membrane. In this circuit, the current carried by the pump is affected by the membrane potential, thus permitting an operational definition of the pump conductance. In *N. translucens*, the second circuit has been found applicable; in the light the conductance of the pump is greater than that of the passive channels (27). Since the pump contributes to the total membrane conductance, inhibition of the pump should decrease the membrane conductance. This was found to be the case in *N. translucens* (28). Theoretically the conductance of the pump should account for the difference between the conductance calculated from passive ion fluxes and that measured electrically in giant algal cells (2, 33).

Support for an electrogenic pump with conductance has also been obtained by Saito and Senda (21) using *Nitella axilliformis*. Richards and Hope (19), on the other hand, maintained that the electrogenic pump in *Chara corallina* contributes little to the membrane conductance even though the measured conductance is much greater than that calculated from the passive fluxes.

Here we examine the effects of several inhibitors and other agents on the membrane potential, conductance, and K^+ influx in *C. corallina* to determine whether operation of the pump contributes to the membrane conductance and to resolve the relationship between the electrogenic pump and the membrane potential. Some of these data have been reported in preliminary form (10).

MATERIALS AND METHODS

The plant material used in this study was *C. corallina* Klein ex Willd., em. R.D.W. (=*C. australis* R.Br.) (34). The *Chara* was

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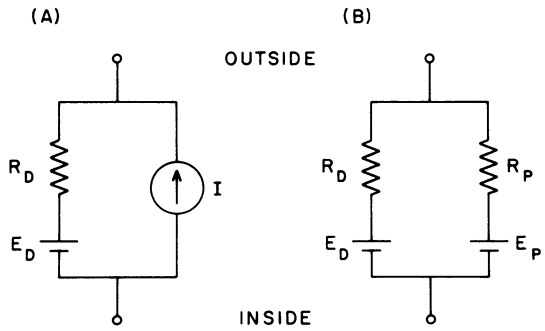

FIG. 1. Two circuits proposed to model a membrane containing an electrogenic pump. R_D: passive diffusion resistance; E_D: passive diffusion potential; I: current produced by constant current source; R_P: resistance of electrogenic pump; E_P: potential of electrogenic pump. A: this circuit models the electrogenic pump as a constant current source; B: this circuit models the electrogenic pump as a constant voltage source with an associated resistance.

cultured in the laboratory, using a method based on the work of Forsberg (5), in tanks containing 180 liters of solution with a solution depth of 38 cm. Fluorescent lamps provided a light intensity at the water surface of 9 to 13 $\mu E/m^2 \cdot sec$ for 15 hr/day. Growth temperature was between 23 and 27 C. The plants were rooted in a 0.5-cm layer of sterile unfertilized soil covered with about 1.5 cm of fine sterile sand. The culture solution initially contained: 0.1 mM $CaCl_2$, 0.04 mM NH_4Cl, 0.1 mM $MgSO_4$, 0.3 mM Na_2CO_3, 0.1 mM KCl, 0.5 mM NaCl, 1.5 μM $FeCl_3$, 0.7 μM $ZnCl_2$, 0.4 μM $NaMoO_4$, 0.35 μM $Na_2B_4O_7$, 10 nM $MnCl_2$, 8.4 nM $CoCl_2$, 23 nM $CuCl_2$, 85 μM NTA,[3] and 0.53 mM MOPS made up in double-distilled H_2O. Cuttings inserted into the sand formed rhizoids and continued to grow vigorously under these conditions.

The solutions used throughout are identified as APW, artificial pond water (27). The label, APW, is followed by a number which indicates the pH of the solution. With addition of 0.4 mM KCl to the APW solution, the final K^+ concentration of all of the solutions was 0.5 mM.

Internodal cells were isolated from the plant the day before the experiment and stored overnight in APW6 + 0.4 mM KCl. For the electrical measurements, the cells were stored in their normal light-dark cycle.

The membrane potential was measured between an internal microelectrode and a large-tipped reference electrode using either a Keithley 603 or a 604 electrometer. The electrodes were filled with 3 M KCl, with 2% (w/v) agar being added to the reference electrode. Electrical contact with the microelectrodes was made using a Ag wire on which a AgCl coating had been deposited. This wire was inserted into the shank of the electrode and sealed with silicone grease. Electrical contact with the microelectrodes was made using a Ag/AgCl wire sealed into the shank of the electrode. The resistance of the cell was measured using the method of Hogg *et al.* (7) in which the current electrode is inserted at the center of the cell and the change in potential is observed with a single electrode located 21% of the cell length from the cell center. The current of 0.5 μamps was generated from square voltage pulses of 2 sec duration given every 100 sec by a Haer Pulsar 4i. The current passed from a Ag/AgCl wire in the bathing solution to the cell and returned from the current electrode through a 100 MΩ resistor used to achieve a constant current pulse. Membrane potential and resistance were recorded on the same trace on a chart recorder. The experiments were performed at 25 C.

For the $^{86}Rb^+$ uptake experiments, the cell surface area was measured the evening before the experiment at the time the cells were cut and trimmed. The cells were kept overnight in the dark in bathing solution (APW6 + 0.4 mM KCl). Ninety min before the start of the experiment the cells were put in fresh bathing solution in the light in a temperature bath at 25 C. At the start of an experiment the control cells were put into bathing solution with $^{86}Rb^+$, at an activity of 0.4 to 1.4 μCi/ml and a concentration of 4 to 9 μM. The treated cells were put into bathing solution containing the appropriate agent and left for a length of time equal to the time for the potential to reach a steady value in the electrical experiments. The cells were then transferred to bathing solution with $^{86}Rb^+$ and the appropriate agent. Following the Rb^+ uptake period of 1.5 ksec, the cells were rinsed in APW5 + 0.4 mM KCl + 10 mM $CaCl_2$ to exchange out the Rb^+ in the cell walls. The low pH and the high level of Ca^{2+} were used to facilitate this exchange. The rinse procedure consisted of a rinse with 5 to 10 ml of flowing solution followed by three changes of 200 ml of stagnant solution for 180, 180, and 600 sec. Following the rinses, the β emission from the $^{86}Rb^+$ was counted by Cerenkov radiation in a Beckman scintillation counter following the basic procedure of Läuchli (12). Preliminary experiments had indicated that the highest counting efficiency (51%) was obtained by putting the cells into scintillation vials containing 10 ml of 5 mM ANDA.

The effectiveness of the rinse procedure was checked by measuring the absorption of $^{86}Rb^+$ into extracted cell walls. The wall extracts were prepared by cutting the cells open with a slit the length of the cell. The cell wall was rinsed free of cell contents and then treated as above for intact cells.

The validity of using $^{86}Rb^+$ as a tracer for K^+ uptake was checked with a double label experiment. The experiment was identical to the procedure described above for $^{86}Rb^+$ uptake, except that the incubation solution contained, in addition to 0.56 μCi/ml of $^{86}Rb^+$, between 0.16 and 0.56 μCi/ml of $^{42}K^+$ depending on when the experiment was done. The concentration of K^+ added as a radiotracer was taken into account and the final concentration of K^+ was maintained at 0.5 mM. The cells were counted twice, once immediately after the uptake and rinse procedure and again 4 to 5 days later when the $^{42}K^+$ had essentially decayed. Since the waveshifter, ANDA, deteriorates with time in solution, these samples were counted in 10 ml of water. This lowered the counting efficiency to 41%. The $^{86}Rb^+$ content of the cells was determined by the second count and was corrected for the decay time from the first count. The corrected $^{86}Rb^+$ counts were subtracted from the first counts to obtain the uptake for $^{42}K^+$. The ratio of uptake of these isotopes into the cells was compared with the ratio of isotopes in samples of the incubation solutions.

The content of K^+ in the cells was determined using 10-μl samples of cell sap from single cells. The diluted cell sap was analyzed using atomic absorption photometry.

Light for these experiments was produced by a Sylvania floodlight operating at 90% of line voltage. This light was located 61 cm from the cell and was passed through 9 cm of water and a glass filter to remove the IR component. The light intensity at the location of the cell was typically 55-60 $\mu E/m^2 \cdot sec$, as measured with a LI-COR quantum radiometer (also measured as 20 w/m^2). Results are presented in the form: mean ± SE (number of cells).

RESULTS

K^+ Influx. The time course of $^{86}Rb^+$ uptake into the cells was determined as outlined under "Materials and Methods," except the cells were incubated in the $^{86}Rb^+$ solution for varying lengths of time. As a control, the time course of absorption into the cell walls was measured. These results both appear in Figure 2A. It

[3] Abbreviations: ANDA: 7 amino-1,3-naphthalenedisulfonic acid; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; DCCD: dicyclohexylcarbodiimide; DES: diethylstilbestrol; DMO: 5,5-dimethyloxazolidine-2,4-dione; DNP: 2,4-dinitrophenol; EDAC: ethyl-3-(3-dimethylaminopropyl)carbodiimide; MOPS: morpholinopropane sulfonic acid; NTA: nitrilotriacetic acid.

will be noticed that the uptake into the cells is not linear and it takes a long time to saturate all of the binding sites in the cell wall. Binding to the cell wall accounts for all of the apparent uptake at very short times, and a large fraction of the apparent uptake at the longer times. To determine actual uptake into the cell, the difference between these two curves was taken and this appears in Figure 2B. There are two linear phases of uptake into the cell. Since interest was in the state of the membrane, a time period for uptake was selected that would be in this first linear portion. Thus the $^{86}Rb^+$ uptake time for all other experiments was 1.5 ksec. All further results in this report have been corrected for binding to the cell wall. In one case this correction yielded a negative value that was not significantly different from zero, and is reported as zero.

The double-labeling experiment using $^{86}Rb^+$ and $^{42}K^+$ indicates that the cell selects for K^+ over Rb^+. The ratio of selectivity of K^+ to Rb^+ is 1.85 ± 0.10 for an average of 42 cells in four separate experiments. Experiments done on extracted cell walls indicate a binding preference of 1.29 ± 0.15 (10). In the presence of the inhibitor DCCD, which drastically increases the membrane permeability to K^+, the selectivity was lowered to 1.13 ± 0.03 (11). Further reports of fluxes, except for those in the presence of DCCD, have been corrected for the selectivity of 1.85. Those fluxes in the presence of DCCD have been corrected by 1.13. The uptake data obtained with Rb^+ are reported as K^+ fluxes.

The K^+ permeability is calculated from the influx values obtained using $^{86}Rb^+$. The K^+ influx in *C. corallina* is assumed to be passive, since a membrane potential (Table I) more negative than the Nernst potential for K^+ produces an inward electrochemical gradient for K^+. Others have also concluded that the K^+ influx is passive (3, 15), although this is not universally agreed upon (19). From the influx, the membrane potential and the external K^+ concentration, the apparent K^+ permeability was calculated (see 19). The potential used in these calculations is the plasmalemma potential. This is obtained by correcting the vacuole potential, observed at the time of the flux measurements, for the tonoplast potential of 16 mv with the cytoplasm more negative than the vacuole (19).

Light and Dark. Table I shows the effect of darkness on the membrane potential, membrane resistance, and K^+ influx at pH 6 in APW6 ± 0.4 mM KCl. Darkness removes the availability of photosynthetic energy but, despite this, the cell is able to keep the membrane potential hyperpolarized. Within experimental uncertainty, the potentials in the light and dark at pH 6 are the same; but it is significant that the membrane resistance doubles in the transition from light to dark and the K^+ permeability is reduced approximately by one-half.

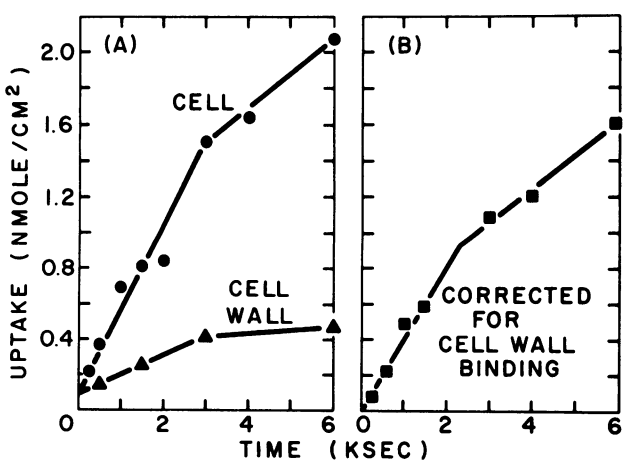

FIG. 2. A: time course of $^{86}Rb^+$ uptake into the cell and binding to the cell wall; B: time course of $^{86}Rb^+$ uptake into the cell corrected for binding to the cell wall.

Uncoupling Agents. Figure 3 shows the time course in the light of the effect of 5.0 μM CCCP, an uncoupler of phosphorylation, on the membrane potential and resistance. The membrane potential depolarizes, the membrane resistance increases, and K^+ permeability decreases (Table II). For these effects there is a lag time of about 3 ksec.

Figure 4 shows the effect of 5.0 μM CCCP in the dark. The differences are substantial. The membrane resistance is initially at a higher value because the cells have been in the dark. CCCP causes the membrane resistance to decrease slightly before increasing. The membrane potential in the dark begins to depolarize immediately after the addition of CCCP. The K^+ influx first increases to six times the control value before decreasing below the control. This represents an almost 10-fold increase in the permeability to K^+, which appears as only a small dip in the resistance curve (since the K^+ conductance is small compared to the total membrane conductance), and contributes to the large depolarization of the membrane potential. Since initially the electrochemical driving force on K^+ ions is inward, the increased permeability will cause an influx of positive charge and thus a depolarization of the membrane potential. If the K^+ permeability has increased to the point of being the dominant passive permeability through the membrane, then the membrane potential should depolarize to a value close to the Nernst potential for K^+ when the electrogenic pump has been inhibited. Close observation of the data for 5.0 μM CCCP and darkness shows a leveling off of

Table I. Measurements of the membrane potential, resistance, and potassium influx in cells of Chara corallina in the light and dark in APW6 + 0.4 mM KCl.

Condition	Membrane potential (mv)	Membrane resistance (kohm-cm²)	Potassium influx (pmole/cm²-sec)
Light	-189 ± 3 (38)	15 ± 1 (37)	0.91 ± 0.13 (21)
Dark	-193 ± 4 (15)	43 ± 9 (12)	0.41 ± 1.11 (19)

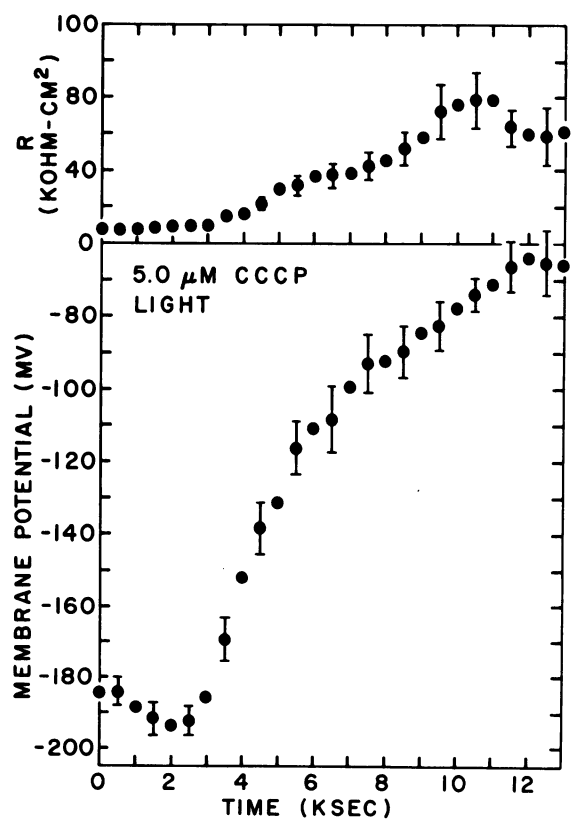

FIG. 3. Time course of inhibition by 5.0 μM CCCP of membrane potential and resistance for cells in the light.

Table II. Measurements of the potassium influx into cells of Chara corallina.

From the influx measurements and the cytoplasmic potential for the corresponding time, the potassium permeability was calculated. The measurements were made on cells that had been in the inhibitor for the given time. The cytoplasmic potential was obtained from the vacuolar potential by correcting for the tonoplast potential. The numbers in parentheses indicate the number of cells measured.

Inhibitor	Time of measurement (ksec)	K^+ influx (p mole/cm^2-sec)	Cytoplasmic potential (mv)	Potassium permeability (cm/sec)
5 μM CCCP light	1 - 2.5	0.53 ± 0.14 (11)	-208 (10)	1.3×10^{-7}
	6.5 - 8	zero (10)	-113 (9)	zero
5 μM CCCP dark	1.5 - 3	3.8 ± 1.1 (11)	-155 (8)	1.3×10^{-6}
	6.5 - 8	0.36 ± 0.19 (11)	-110 (8)	1.6×10^{-7}
50 μM DCCD	4 - 5.5	4.6 ± 0.6 (22)	-160 (14)	1.5×10^{-6}
40 μM DES	3 - 4.5	0.13 ± 0.02 (8)	-115 (11)	0.6×10^{-7}
1.0 mM EDAC	2.5 - 4	0.69 ± 0.10 (22)	-157 (5)	2.2×10^{-7}
2.0 μM DCMU	4 - 5.5	0.76 ± 0.22 (20)	-202 (10)	1.9×10^{-7}
0.1 mM DNP	2 - 3.5	0.58 ± 0.12 (19)	-132 (15)	2.2×10^{-7}
5.0 mM DMO	6 - 7.5	0.33 ± 0.08 (22)	-182 (12)	0.9×10^{-7}
0.5 mM $LaCl_3$	9 - 10.5	0.11 ± 0.07 (21)	-144 (11)	0.4×10^{-7}
Control	--	0.65 ± 0.09 (87)	-205 (38)	1.6×10^{-7}

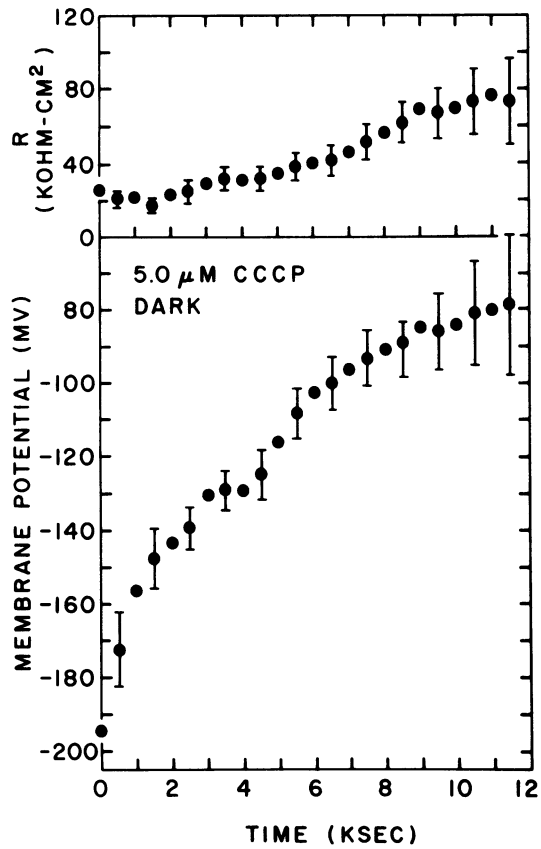

FIG. 4. Time course of inhibition by 5.0 μM CCCP of membrane potential and resistance for cells in the dark.

the potential at −128 mv at a time of 3.5 to 4.0 ksec. This value compares with a Nernst potential for K^+ of −115 to −130 mv for these cells. Later, the K^+ permeability decreases and the membrane potential depolarizes further.

In comparing the data for 5.0 μM CCCP for light *versus* dark, the big difference is in the initial effects, the portions of both time courses for the membrane potential and resistance being identical within experimental uncertainty from 4.5 ksec on. This early difference appears to be due in part to the increased K^+ permeability shortly after the addition of CCCP in the dark. The rapidity of the drop of membrane potential suggests that this permeability change could be occurring very quickly after the addition of CCCP.

DNP has the effect shown in Figure 5. The membrane potential depolarizes to −110 to −112 mv. The membrane resistance, with a slower time course, increases to 60 kΩ·cm^2. The K^+ flux is slightly lowered due to the reduced membrane potential (the membrane permeability increases slightly). The effects of DNP can be reversed by changing back to DNP-free solution.

ATPase Inhibitors. DCCD is a rather unspecific inhibitor of membrane-bound ATPases, and is also known to increase membrane permeability (6, 22). In *Chara* it depolarizes the membrane and increases the electrical resistance (4). The time course for these two events is slightly different (Fig. 6A), the membrane depolarization commencing before the membrane resistance increases. The K^+ influx increases to seven times the control value at which time the K^+ conductance accounts for all of the electrical conductance through the membrane. If the first action of DCCD is on membrane permeability, this could account for the depolarization as K moves down its electrochemical gradient. Then, as DCCD starts to affect the ATPases, cutting off the conductance through the ATPases (pumps), this would increase the electrical resistance to the value determined by the passive permeability of K^+, as observed.

Balke and Hodges (1) have reported DES to be an effective inhibitor of the membrane ATPase in oat roots. In *Chara*, 40 μM DES causes the membrane potential to depolarize to about −100 mv and the resistance to increase (Fig. 6B). The K^+ influx also decreases to a low value. Not only has the K^+ influx decreased, the calculated passive K^+ permeability has drastically decreased. Changing the K^+ concentration from 0.5 mM to 0.1 mM when the cell is inhibited by DES results in a potential change of about −13 mv, further indication of a low K^+ permeability.

The compound EDAC is chemically a carbodiimide as is DCCD. Both are supposed to be inhibitors of ATPases. One significant difference is that EDAC is highly water-soluble, while DCCD is not. Their effects are different, EDAC being the only

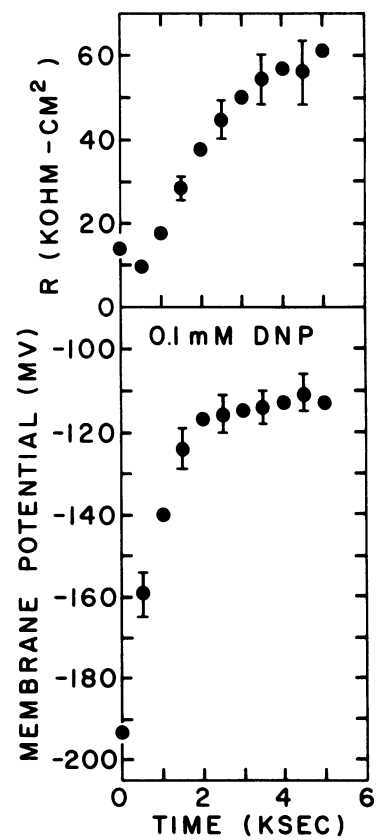

FIG. 5. Time course of inhibition of membrane potential and resistance by 0.1 mM DNP.

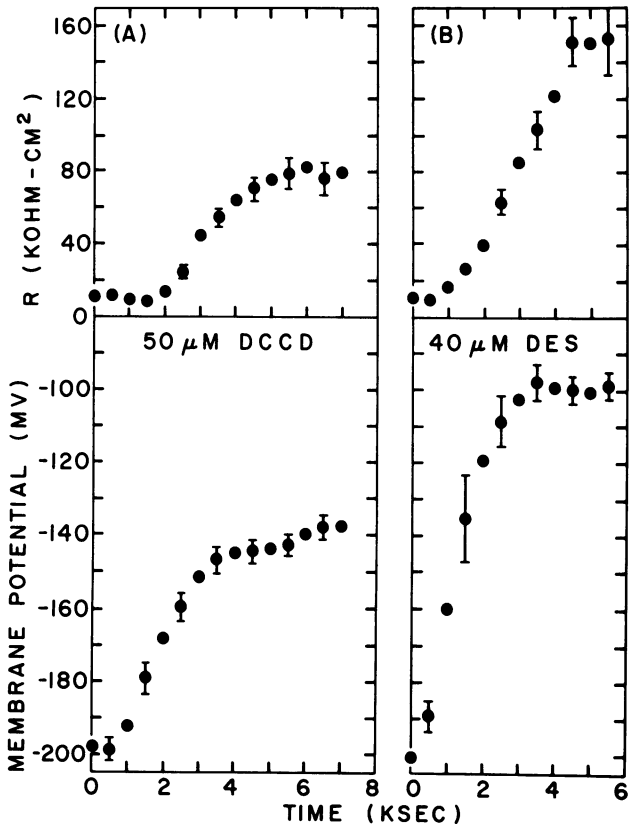

FIG. 6. Time course of inhibition of membrane potential and resistance by: (A) 50 μM DCCD; and (B) 40 μM DES.

inhibitor used that substantially and permanently lowered the membrane resistance (Fig. 7A). The K^+ flux is essentially unchanged and the K^+ permeability is increased only 40% by EDAC. This is insufficient to account for the lowered electrical resistance, since the K^+ permeability contributes only a minor part of the total membrane conductivity. The membrane potential depolarizes to about −135 mv. Changing to EDAC-free solution allows the membrane potential to recover but the membrane resistance does not recover.

DCMU. DCMU is an inhibitor of electron transport between PSII and PSI and thus prevents noncyclic photophosphorylation. Smith and Raven (26) found that DCMU inhibits Cl^-, phosphate, and glucose influxes in *C. corallina* and concluded that either cyclic photophosphorylation does not occur or its energy may not be available. If this is the case, then one would predict that the effect of DCMU in the light should be the same as darkness. In *Chara*, darkness causes a minimal change in potential at pH 6. In the light it is −189 ± 3(38) mv and in the dark −193 ± 4(15)mv, but darkness causes the membrane resistance to increase from 15 ± 1(37) $k\Omega \cdot cm^2$ in the light to 43 ± 9(12) $k\Omega \cdot cm^2$ in the dark. This compares with the effects of 2 μM DCMU illustrated in Figure 7B where the membrane potential depolarizes to −186 ± 9(8) mv and the membrane resistance increases to 44 ± 7(8) $k\Omega \cdot cm^2$. Within the uncertainty of the measurements, at pH 6 the effect of darkness on the membrane potential and resistance is the same as the effect of DCMU. The apparent permeability of the membrane to K^+ in the presence of DCMU has approximately doubled as compared to the dark control. So DCMU not only blocks electron transport in photosynthesis but also increases the membrane permeability.

DMO. DMO is a weak acid that has been used at low concentration to determine internal pH of cells through its distribution across the plasmalemma, since it dissociates according to pH and only the neutral form permeates the membrane rapidly. When added at a high concentration it is likely that it will lower the internal pH of the cell as the DMO dissociates inside. Spanswick and Miller (29) have shown in *N. translucens* that 5 mM DMO lowers the internal pH by more than a pH unit, but does not affect the membrane potential unless the cell has previously depolarized, in which case the DMO hyperpolarized the membrane back to its usual value. Figure 8A shows that DMO does have a small effect on the membrane resistance. Its effect on the potential is not immediate and is not the expected hyperpolarization, but is a delayed gradual depolarization. The K^+ permeability decreased slightly after application of 5 mM DMO.

La^{3+}. La^{3+} has been found by Takata *et al.* (31) and others to

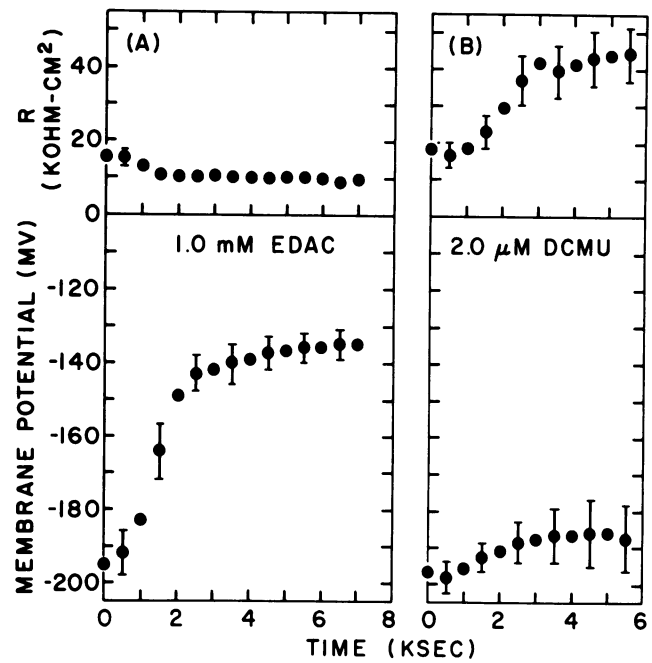

FIG. 7. Time course of inhibition of membrane potential and resistance by: (A) 1.0 mM EDAC; and (B) 2.0 μM DCMU.

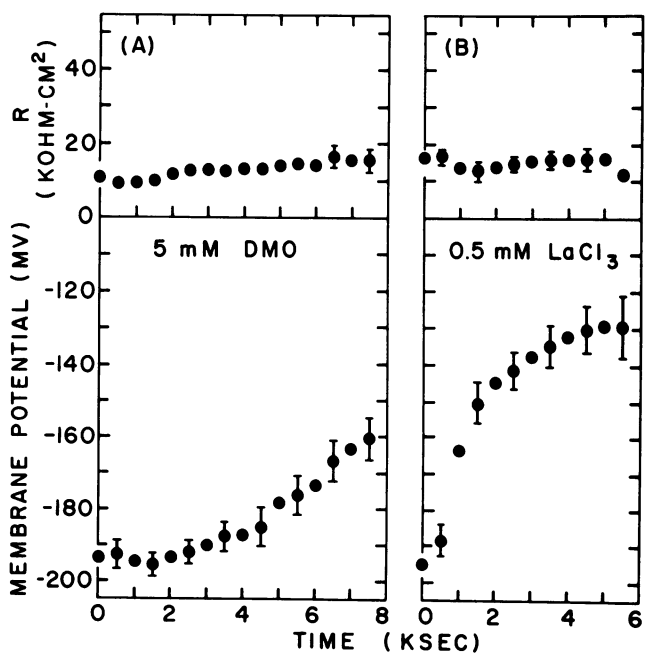

FIG. 8. Time course of inhibition of membrane potential and resistance by: (A) 5.0 mM DMO; and (B) 0.5 mM $LaCl_3$.

lower the passive permeability of membranes. As seen in Figure 8B, La^{3+} at 0.5 mM has essentially no effect on membrane resistance, but does depolarize the membrane to −129 mv. The K^+ permeability, which is 4×10^{-8} cm/sec in the presence of $LaCl_3$ as compared to a control value of 1.6×10^{-7} cm/sec, has indeed been lowered by La^{3+}. If the other parameters remain constant, a change in K^+ permeability of this magnitude would not be observed electrically as a change in the resistance since K^+ contributes only a small fraction of the total conductance. In order to examine the effects of La^{3+} without the complication of having the electrogenic pump operating, the inhibitor 40 μM DES was added along with 0.5 mM $LaCl_3$. This combination reduced the membrane potential to −70 to −82 mv. The electrical resistance increased in the presence of both $LaCl_3$ and DES just as it does in the presence of DES alone.

Effect of External pH. The steady membrane potentials and resistances found at different external pH are shown in Figure 9. The most negative membrane potential in the light is at pH 7. In the dark the membrane potential is significantly more negative than in the light for pH 7 and above. The membrane resistance in the dark is much larger than that in the light over the pH range 5 to 10. The significant change in membrane resistance in the light is a fall in resistance at pH 9 and above. The associated K^+ permeabilities are shown in Table III. This extends the work done previously by Richards and Hope (19) to higher pH values at which *Chara* frequently grows in laboratory cultures. The plants will raise the pH of the growth medium, even when buffered, to pH values of 9 to 10. Lucas (13) has shown that in this upper pH range an active HCO_3^- uptake and fixation mechanism is operating. For the range where these measurements overlap with Richards and Hope (19), there are some differences. We found that the membrane potential in the dark is the same or more negative than the potential in the light over the whole pH range and that in the dark the membrane resistance is always larger than the resistance in the light.

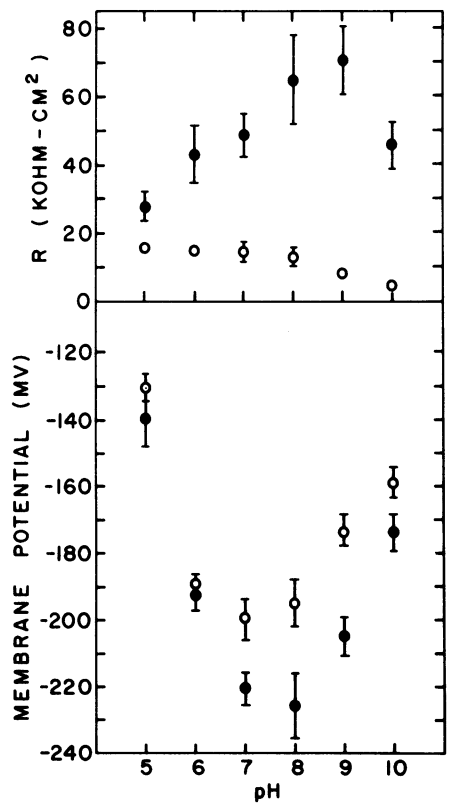

FIG. 9. Effect of pH on membrane potential and resistance. ○: cells in the light; ●: cells in the dark.

Table III. Measurements of the potassium influx into cells of Chara corallina at various pH's in the light and dark.

The potassium permeability was calculated using the influx measurements and the cytoplasmic potential at that pH. The cytoplasmic potential was obtained from the vacuole potential by correcting for the tonoplast potential. The numbers in parentheses indicate the number of cells measured. The measurements of influx were made 5.4 ksec after exposure to the given conditions. The potential measurements were made when a steady value was obtained but at least 1.8 and less than 5.4 ksec after exposure to the given conditions.

	pH	K^+ influx (pmole/cm²-sec)	Cytoplasmic potential (mv)	Potassium permeability (cm/sec)
Light	5	0.46 ± 0.12 (22)	-147 (15)	1.6×10^{-7}
	6	0.65 ± 0.09 (87)	-205 (38)	1.6×10^{-7}
	7	1.12 ± 0.19 (22)	-215 (12)	2.7×10^{-7}
	8	1.15 ± 0.19 (22)	-211 (10)	2.8×10^{-7}
	9	1.24 ± 0.22 (21)	-189 (16)	3.4×10^{-7}
	10	1.31 ± 0.29 (21)	-175 (14)	3.8×10^{-7}
Dark	5	0.27 ± 0.09 (22)	-156 (6)	0.9×10^{-7}
	6	0.41 ± 0.08 (19)	-208 (15)	1.0×10^{-7}
	7	0.74 ± 0.17 (21)	-237 (7)	1.6×10^{-7}
	8	1.04 ± 0.24 (20)	-242 (4)	2.2×10^{-7}
	9	1.38 ± 0.22 (21)	-221 (7)	3.2×10^{-7}
	10	1.03 ± 0.24 (22)	-190 (10)	2.8×10^{-7}

DISCUSSION

Under noninhibited conditions at pH 6 in either the light or the dark (Table I) the membrane potential is more negative than any possible value for a diffusion potential. The Goldman equation predicts a potential of −106 mv under these conditions. (P_K from Table III; $P_{Na} = 0.93 \times 10^{-7}$, $P_{Cl} = 8.1 \times 10^{-10}$ [19], K_i = 74 mM, Na_i = 47 mM, Cl_i = 106 mM [8]). The observed hyperpolarization beyond this level is strong evidence for the existence of an electrogenic ion pump.

It is likely that there are at least two electrogenic pumps in the *Chara* membrane. Work by Lucas (14) on the HCO_3^- and OH^- transport mechanism indicates that there is probably an electrogenic HCO_3^- pump in this system since the two fluxes are not directly coupled and do not seem to be directly coupled to any other fluxes. This prediction has received support from the electrical measurements of Walker and Smith (32). This paper provides evidence for a second electrogenic pump that operates in the dark or in CO_2-free conditions (unpublished data) when the HCO_3^- uptake mechanism is not operating and keeps the membrane potential hyperpolarized beyond the range of any possible diffusion potential. For the majority of experiments reported here, it is this second system that is being observed as most of the experiments were done at pH 6. The concentration of HCO_3^- in solution at pH 6 is 5 μM when in equilibrium with air. Information from Lucas (13) for the kinetics of HCO_3^- fixation (K_m = 0.59 mM; and V_{max} = 60 pmol/cm² · sec) indicates that the fixation of HCO_3^- present at a concentration of 5 μM will be about 0.25 pmol/cm²·sec. This is equivalent to a conductance of 1.0 μmho/cm² (or a resistance of 10^3 kΩ·cm²), which is small compared to the total conductance through the membrane of 67 μmho/cm² in the light without an inhibitor. Thus the electrogenic pump being examined here is not the HCO_3^- pump. Nor is it the OH^- efflux pump as the OH^- efflux is in the wrong direction to account for a hyperpolarization of the membrane potential.

Richards and Hope (19) used Kitasato's (11) postulate of an electrogenic H^+ pump to explain the hyperpolarization of the membrane potential but rejected the idea of the large passive H^+ influx which Kitasato used to account for the high membrane conductance and the effect of pH on the membrane potential. They also rejected the hypothesis that the electrogenic pump has a high conductance even though there is a large discrepancy between magnitudes of the major ion fluxes and the membrane conductance.

Findlay *et al.* (3) indicated that the magnitudes of the fluxes, whether active or passive, in *C. corallina* at the plasmalemma in

the light are: K^+ = 0.5 to 3.5 pmol/cm^2·sec; Na^+ = 0.4 pmol/cm^2·sec; and Cl^- = 0.5 to 5.0 pmol/cm^2·sec. Fluxes of the divalent ions in *C. corallina* have not been measured but are likely to be smaller than the fluxes of the monovalent ions (30). In the dark where all of these fluxes are significantly reduced and no HCO_3^- uptake is observed, the membrane conductance is still 23 μmhos/cm^2. This is equivalent to a flux of 14 pmol/cm^2·sec of monovalent ions, several times larger than the sum of the maximum fluxes of K^+, Na^+, and Cl^-. Richards and Hope (19) based their conclusion that the pump has a low conductance on measurements made with the inhibitors DNP and sodium azide. They concluded that these inhibitors had no significant effect on the conductance although it decreased in all of their experiments. Because of the large variability and the small number of their measurements, they could attach no significance to this change. This may be contrasted with the results presented in this paper, where apparent inhibition of the electrogenic pump is accompanied by a marked decrease in the membrane conductance. Since the agents used can have multiple effects, the results must be interpreted carefully.

DCCD inhibited the electrogenic ion pump either directly or indirectly and also increased the K^+ permeability. Under such conditions the membrane potential should fit the potential given by the Goldman equation. In solutions containing 0.5 mM K^+ the predicted membrane potential is −123 mv, and in 0.1 mM K^+ it is −149 mv. The difference in potential predicted for these two solutions, 26 mv, is quite close to the measured value of 24 mv. This is not equal to the 41 mv differences expected if this were strictly a K^+ diffusion potential, but does indicate a larger sensitivity to K^+ than found in the control (6 mv change). The measured value of −138 mv for the membrane potential in 0.5 mM K^+ is close to the predicted value considering the uncertainty in the measurements of the various parameters.

Figure 10 shows the data for 40 μM DES plotted in a different format. Time, the independent variable, has been eliminated and the membrane conductance (which equals the reciprocal of the resistance) is plotted as a function of the membrane potential. The relationship in this case between the potential and the conductance is a linear one. This suggests that as the electrogenic pump is inhibited the voltage depolarizes to lower values and the conductance drops simultaneously.

The dashed line in Figure 10 represents the approximate value of membrane conductivity associated with nonelectrogenic movements of the ions K^+, Na^+, and Cl^-. It is located at 3.5 μmho/cm^2 and assumed to be independent of the membrane potential. The inhibition by 40 μM DES drops the conductance from 90 μmho/cm^2 to about 6.6 μmho/cm^2, essentially removing the electrogenic conductance. With the electrogenic component removed, the potential is −100 mv. To predict the potential from the Goldman equation, the same ion concentrations are used as above. The measured permeability for K^+ under DES inhibition is reduced from the control by a factor of 0.28. If the other permeabilities have been reduced by the same factor, the Goldman equation gives a value for the potential of −106 mv in 0.5 mM K^+, and a change in potential of −7 mv for a K^+ change from 0.5 mM to 0.1 mM. This compares with a measured membrane potential of −100 mv and a change in potential of −13 mv for the K^+ concentration change. All of this indicates that the major conductance through the membrane is due to the electrogenic pump and that with the pump inhibited, the membrane potential is determined by the diffusion potential.

Also plotted in Figure 10 are the data for inhibition by 5.0 μM CCCP in both the light and the dark. The fit to the straight line is not as good as with DES. There are two possible reasons for the lack of fit at the ends of the curves with CCCP. Unpublished work in this laboratory on *Nitella* shows that CCCP causes an increased efflux of Cl^-. If CCCP shuts off the electrogenic ion pump and lowers the potential to a diffusion potential, then the effect of a change in the Cl^- permeability will be very distinct. In comparison with the diffusion potential obtained with DES, this increased Cl^- permeability along with a low K^+ permeability will, when the Goldman equation is examined, cause the diffusion potential to be depolarized. As predicted, the lower end of the curve departs from the straight line of DES and approaches a more depolarized membrane potential and a higher conductance value (Fig. 10). The other end of the curve for 5.0 μM CCCP in the dark departs from the straight line because in the dark the passive permeability of the membrane decreases to about one-half the value in the light. This allows the electrogenic pump to run at about one-half its normal rate and still maintain the membrane potential at the same value as in the light.

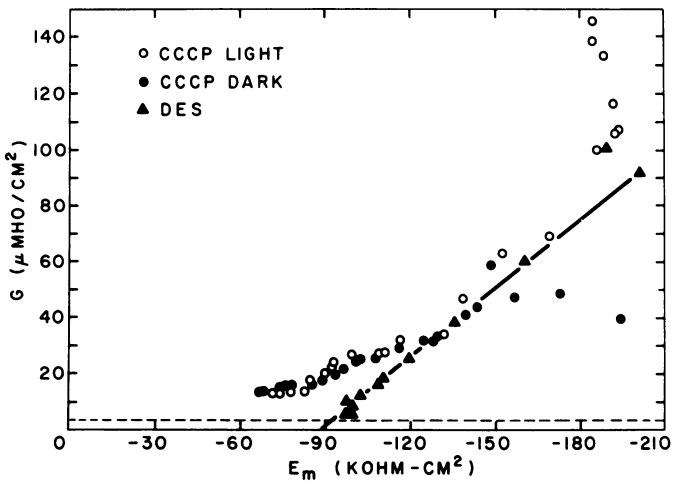

FIG. 10. Variation of membrane conductance with membrane potential for cells inhibited by 40 μM DES (▲), by 5.0 μM CCCP in the light (○), and by 5.0 μM CCCP in the dark (●). Dashed line represents approximate conductance due to passive ion movements.

DNP shuts off the electrogenic pump, probably by decreasing the energy supply from the mitochondria and chloroplasts. Putting the appropriate values of the K^+ permeability into the Goldman equation gives a value for the potential of −109 mv which is very close to the measured value of −110 to −112 mv.

The effect of EDAC cannot be explained by inhibition of an electrogenic pump with conductance. The potential depolarizes, which could suggest that the operational level of the electrogenic pump has been reduced, but probably does not because there is no increase in the membrane resistance corresponding to the shutting down of the pump. Instead the membrane resistance decreases. This inhibitor may be causing a permeability change to some ion other than K^+. Prochaska and Gross (18) showed that EDAC causes inhibition of binding of Ca^{2+} to membranes. Exchanging the Ca^{2+} out of the binding sites causes the membrane potential to behave as a diffusion potential (9). The potential observed is −135 mv which is within the range of possible diffusion potentials but is not as depolarized as the value of −110 mv calculated from the Goldman equation. The increased permeability through the membrane may be short circuiting the electrogenic pump reducing the potential to a value intermediate between the normal potential and the diffusion potential.

DMO is expected to lower the internal pH of the cell. If there is a H^+ pump that regulates the internal pH, then the H^+ efflux should increase. The resistance does change initially by an amount equivalent to a conductance increase of 21 μmho/cm^2. This is an increase of 23% in the conductance which probably indicates an increased flux through the pump. At later times the conductance decreases and the membrane potential starts to depolarize. This may indicate that the cell has a limited capacity to deal with a sustained influx of H^+ carried by the large influx of DMO.

The effect of La^{3+} by itself is difficult to explain quantitatively.

However, with the interference from the electrogenic ion pump removed by 40 μm DES, the effect of La^{3+} can be shown to be due to the decreased passive permeabilities. As measured, the K^+ permeability decreases from the control by a factor of 0.22 due to La^{3+}. The postulated action of La^{3+} (32) indicates that it should exert a similar effect on the permeabilities of all cations. Thus the Na^+ permeability probably should be decreased by a factor of 0.22. To obtain the Goldman diffusion potential for this case with both DES and La^{3+}, the values used earlier for DES were used again with one change, the permeabilities to K^+ and Na^+ being reduced by a factor of 0.22. The predicted Goldman diffusion potential is -86 mv corresponding to a measured value of -70 to -82 mv. This supports the idea that La^{3+} affects only the passive permeabilities and not the electrogenic pump.

In conclusion, there must be at least two electrogenic pumps in the membrane. The operational level of these pumps is indicated by the membrane electrical conductance. A conductance of 3 to 5 μmho/cm^2 or less indicates that the electrogenic pumps are not in operation. For conductances above that level there is a direct relation between the operational availability of the pumps and the membrane conductance: the larger the conductance the more pump sites are available for operation.

Values cannot be estimated for a number of the membrane parameters. The diffusion conductance of the membrane is 3 to 5 μmho/cm^2 (or a resistance of 200–300 k$\Omega \cdot$cm^2). The maximum conductance of the HCO_3^- pump is 100 μmho/cm^2 from the data of Lucas (13) for a maximum HCO_3^- influx of 60 pmol/cm$^2 \cdot$sec. The other electrogenic pump, the H^+ pump, has a conductance of 67 μmho/cm^2. The typical combined conductance through these two pumps is 67 μmho/cm^2 at pH 6 because, in the absence of added HCO_3^-, the HCO_3^- pump is not carrying significant current at this low pH. At higher pH both pumps may be operating and the conductance could be the sum of that for the two pumps or 167 μmho/cm^2. This is a resistance of 6.0 k$\Omega \cdot$cm^2 and is close to the observed resistance at pH 9 to 10. While the solutions used at pH 9 and 10 should contain significant amounts of HCO_3^-, it cannot be guaranteed that there was sufficient HCO_3^- in solution to saturate the operation of the HCO_3^- uptake system.

These values for the membrane conductance indicate that the constant current pump model (Fig. 1) is not appropriate for the *Chara* membrane. The conductance through the electrogenic pump in *Chara* is an order of magnitude larger than the conductance through the passive channels; so in this respect the alternate model (Fig. 1B), in which the pump conductance is put explicitly into the model, is better.

It is often asked why a pump with such a high conductance does not produce an easily observed H^+ efflux. One explanation is that the pump may be stalled under normal conditions if the energy input to the pump is equal to the energy required to move H^+ against its electrochemical gradient. During measurement of the membrane conductance the membrane potential is changed, allowing the pump to run in either the forward or backward direction. This should result in a flux of H^+ through the pump. However, we have not been able to demonstrate a net flux of H^+ under these conditions.

Under the present experimental conditions the diffusion potential of the cell is about -106 mv, although it can be altered by the effects of various inhibitors on the membrane permeabilities. The diffusion potential will be masked by the operation of the electrogenic pump unless the pump has been inhibited. When this pump is inhibited the membrane potential no longer has an electrogenic component and depolarizes to a diffusion potential. In summary: (*a*) 5.0 μM CCCP inhibits the electrogenic pump, depolarizing the membrane to a diffusion potential and increasing the resistance. This inhibition is faster in the dark than in the light but the extent of the responses is the same in both cases. (*b*) Fifty μM DCCD inhibits the electrogenic pump, increasing the membrane resistance, but, because it increases the K^+ permeability, the potential only depolarizes to a diffusion potential controlled mainly by K^+. (*c*) Forty μM DES and 0.1 mM DNP both inhibit the electrogenic pump. This increases the resistance and depolarizes the membrane potential to a value predicted by the Goldman diffusion equation. (*d*) DCMU has an effect similar to that of darkness, increasing the membrane resistance, but neither causes much change in the potential. (*e*) DMO, by lowering the internal pH, provides more substrate for the pump and decreases the membrane resistance as a result. (*f*) La^{3+} decreases cation permeability and depolarizes the membrane, but this effect is related to its action on passive diffusion as it has no effect on the electrogenic pump.

The evidence presented here implicates H^+ as the ion carried by this electrogenic pump. Further work needs to be done to confirm this. Perhaps observations of the internal pH under different conditions would provide more evidence of the role of the H^+ ion in controlling the membrane potential.

As with the electrogenic pump in *Neurospora* (25), it is probable that the electrogenic pump in *Chara* is also an ATPase. Shimmen and Tazawa (23), by perfusing tonoplast-free cells of *Chara australis* with various combinations of cations and adenine nucleotides, have concluded that the plasmalemma contains an electrogenic Mg^{2+}-activated ATPase. Of the chemicals which have been shown in this paper to inhibit the electrogenic pump, the uncouplers DNP and CCCP remove the energy supply for ATPases, and DCCD (6, 22) and DES (1) have been shown to inhibit ATPases directly. This also provides evidence that the electrogenic pump is an ATPase. Further support for this conclusion would be provided by determining the effect of these inhibitors on the intracellular ATP levels in the cells of *Chara*. Future work on this system will include measurement of the ATP levels to elucidate the operation of these inhibitors and identify the energy source for the electrogenic pump.

LITERATURE CITED

1. Balke NE, TK Hodges 1977 Inhibition of ion absorption in oat roots: comparison of diethylstilbestrol and oligomycin. Plant Sci Lett 10: 319–325
2. Dainty J 1962 Ion transport and electrical potentials in plant cells. Annu Rev Plant Physiol 13: 379–402
3. Findlay GP, AB Hope, MG Pitman, FA Smith, NA Walker 1969 Ionic fluxes in cells of *Chara corallina*. Biochim Biophys Acta 183: 565–576
4. Fliss PA, RM Spanswick 1975 Effects of DCCD on the membrane potential and resistance in *Chara corallina*. Plant Physiol 56: S-41
5. Forsberg C 1965 Nutritional studies of *Chara* in axenic cultures. Physiol Plant 18: 275–290
6. Harold FM, JR Baarda, C Baron, A Abrams 1969 Inhibition of membrane-bound adenosine triphosphatase and cation transport in *Streptococcus faecalis* by *N,N'*-dicyclohexylcarbodiimide. J Biol Chem 244: 2261–2268
7. Hogg J, EJ Williams, RJ Johnston 1968 A simplified method for measuring the membrane resistances in *Nitella translucens*. Biochim Biophys Acta 150: 518–520
8. Hope AB, NA Walker 1960 Ionic relations of cells of *Chara australis*. III. Vacuolar fluxes of sodium. Aust J Biol Sci 13: 277–291
9. Hope AB, NA Walker 1961 Ionic relations of cells of *Chara australis* R.Br. IV. Membrane potential differences and resistances. Aust J Biol Sci 14: 26–44
10. Keifer DW, RM Spanswick 1977 Membrane potential and resistance of *Chara australis*. Plant Physiol 59: S-85
11. Kitasato H 1968 The influence of H^+ on the membrane potential and ion fluxes of *Nitella*. J Gen Physiol 52: 60–87
12. Läuchli A 1969 Radioassay for β-emitters in biological materials using Cerenkov radiation. Int J Appl Radiat Isot 20: 265–270
13. Lucas WJ 1975 Photosynthetic fixation of ^{14}carbon by internodal cells of *Chara corallina*. J Exp Bot 26: 331–346
14. Lucas WJ 1976 Plasmalemma transport of HCO_3^- and OH^- in *Chara corallina:* non-antiporter systems. J Exp Bot 27: 19–31
15. MacRobbie EAC 1970 The active transport of ions in plant cells. Q Rev Biophys 3: 251–294
16. Murdoch S, J Sinclair 1976 The electrical potential difference and resistance of *Chara vulgaris*. Can J Bot 54: 2187–2192
17. Pickard WF 1973 Does the resting potential of *Chara braunii* have an electrogenic component? Can J Bot 51: 715–724
18. Prochaska LJ, EL Gross 1975 The effect of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide on calcium binding and associated changes in chloroplast structure and chlorophyll-*a* fluorescence in spinach chloroplasts. Biochim Biophys Acta 376: 126–135
19. Richards JL, AB Hope 1974 The role of protons in determining membrane electrical characteristics in *Chara corallina*. J Membr Biol 16: 121–144
20. Saddler HWD 1970 The membrane potential of *Acetabularia mediterranea*. J Gen Physiol 55: 802–821
21. Saito K, M Senda 1974 The electrogenic ion pump revealed by the external pH effect on the membrane potential of *Nitella*. Influences of external ions and electrical current on the pH effect. Plant Cell Physiol 15: 1007–1016
22. Schoner W, H Schmidt 1969 Inhibition of ($Na^+ + K^+$)-activated ATPase by N,N'-dicyclo-

hexylcarbodiimide. FEBS Lett 5: 285–287
23. SHIMMEN T, M TAZAWA 1977 Control of membrane potential and excitability of *Chara* cells with ATP and Mg^{2+}. J Membr Biol 37: 167–192
24. SLAYMAN CL 1965 Electrical properties of *Neurospora crassa:* effects of external cations on the intercellular potential. J Gen Physiol 49: 69–92
25. SLAYMAN CL, WS LONG, CYH LU 1973 The relationship between ATP and an electrogenic pump in the plasma membrane of *Neurospora crassa.* J Membr Biol 14: 305–338
26. SMITH FA, JA RAVEN 1974 Energy-dependent processes in *Chara corallina:* absence of light stimulation when only photo-system one is operative. New Phytol 73: 1–12
27. SPANSWICK RM 1972 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. Biochim Biophys Acta 288: 73–89
28. SPANSWICK RM 1974 Evidence for an electrogenic ion pump in *Nitella translucens.* II. Control of the light-stimulated component of the membrane potential. Biochim Biophys Acta 332: 387–398
29. SPANSWICK RM, AG MILLER 1977 The effects of CO_2 on the Cl^- influx and electrogenic pump in *Nitella translucens.* Colloque du CNRS Echanges Ioniques Transmembranaires Chez Les Vegetaux 258: 239–245
30. SPANSWICK RM, EJ WILLIAMS 1965 Ca fluxes and membrane potentials in *Nitella translucens.* J Exp Bot 16: 463–473
31. TAKATA M, WF PICKARD, JY LETTVIN, JW MOORE 1966 Ionic conductance changes in lobster axon membrane when lanthanum is substituted for calcium. J Gen Physiol 50: 461–471
32. WALKER NA, FA SMITH 1977 Circulating electric currents between acid and alkaline zones associated with HCO_3^- assimilation in *Chara.* J Exp Bot 28: 1190–1206
33. WILLIAMS EJ, RJ JOHNSTON, J DAINTY 1964 The electrical resistance and capacitance of the membranes of *Nitella translucens.* J Exp Bot 15: 1–14
34. WOOD RD, K IMAHORI 1965 A Revision of the Characeae, 2 vols. J Cramer, Weinheim, Germany