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Chloride Transport and the Membrane Potential in the Marine Alga, *Halicystis parvula*

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Summary. The relationship between the rate of Cl^- transport and the electrical properties of *Halicystis parvula* was investigated. Three metabolic inhibitors – darkness, cyanide (2 mM), and low temperature (4 °C) – all rapidly and reversibly reduce both the short circuit current (SCC), which is a measure of net Cl^- transport, and the vacuole electrical potential (*PD*). Plotting the *PD vs.* SCC for inhibited cells yields a linear regression with a *y*-intercept of zero. The *PD* is also greatly reduced when the $[Cl^-]$ of the external medium is lowered. Raising the external $[K^+]$ produces an appreciable, but less than Nernstian, depolarization, while increasing the external $[H^+]$ tenfold has no net effect on the *PD*. Decreasing the external $[Na^+]$ by tenfold produces only a slight depolarization. Thus, the outer plasma membrane appears to be moderately selective for K^+ over Na^+ or H^+. The effects of ion substitutions in the vacuolar perfusing solutions on the *PD* reveal that the vacuolar membrane does not discriminate electrically between Cl^- and the much larger anions, isethionate and benzenesulfonate, or between Na^+ and K^+. The data suggest that in internally perfused cells of *H. parvula* generation of the *PD* of -50 to -60 mV by a transport system involving only electroneutral pumps is unlikely and that most of this *PD* is generated by an electrogenic Cl^- pump.

An unusual feature of the giant-celled marine alga, *Halicystis parvula*, is that the Na^+, K^+ and Cl^- concentrations in the vacuolar sap are similar to those in the seawater in which the cell is growing (Graves & Gutknecht, 1976). The electrical potential difference (*PD*) between the vacuole and external solution is -82 mV (inside negative) in nonperfused cells. This *PD* cannot be generated by any combination of diffusion potentials for these ions between the vacuole and external medium, because each of these ions has an equilibrium potential near zero. Thus the origin of the *PD* in *H. parvula* presents an intriguing question.

In their early investigations on the bioelectric properties of *Halicystis*, Blinks and co-workers made several interesting observations which were

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not adequately explained. Raising the external $[K^+]$ produced a large transient depolarization (Blinks, 1932). When the vacuolar $[K^+]$ was raised 50-fold by perfusion in *H. osterhoutii*, which also has a normally low sap $[K^+]$, only a small hyperpolarization occurred, suggesting that vacuolar K^+ was not involved in generating the *PD*. Removing external Cl^- resulted in a large, steady depolarization (Blinks, 1940), but changing the vacuolar $[Cl^-]$ had little effect on the *PD* (Blinks, 1935). From these ion substitution experiments we can conclude only that the ionic selectivity properties of the plasmalemma and tonoplast in *Halicystis* are quite different. The relationship between the metabolic state and the *PD* in *Halicystis* was also studied. The combination of darkness and low oxygen tension caused a large rapid depolarization which was readily reversible (Blinks, 1949; Blinks, Darsie & Skow, 1938). Thus, the *PD* seems closely linked to metabolism.

These results suggest two alternative, although not mutually exclusive, mechanisms for the generation of the *PD* of *H. parvula*. (1) Ionic gradients due to active transport processes at the plasmalemma and/or tonoplast could generate diffusion potentials across these membranes, and the summation of these potentials could produce the *PD*. (2) An active transport system which transports net charge (i.e., an electrogenic pump) could be responsible for some portion of the *PD*. In this paper we describe experiments designed to distinguish between these alternatives. Our approach is based on the fact that under short-circuit current conditions (i.e., no electrochemical driving force for any ion) *H. parvula* develops a short-circuit current (SCC) of 290 peq $\cdot$ cm^{-2} $\cdot$ sec^{-1}, all of which is generated by the net active uptake of Cl^- (Graves & Gutknecht, 1976). Therefore, the Cl^- pump is the most likely candidate for being electrogenic, and to test this hypothesis we have studied the metabolic dependence of both Cl^- transport and the *PD*.

Materials and Methods

Short-Circuit Current Experiments

The techniques and apparatus used for internal (vacuolar) perfusion and measurement of the short-circuit current (SCC) and unidirectional ion fluxes are described in detail by Graves and Gutknecht (1976). Briefly, the unidirectional fluxes were measured by adding tracer to either the perfusing solution or the external bath and then measuring the rate of appearance of the tracer in the solution bathing the other side of the protoplasm. The standard artificial seawater (ASW) used for both perfusing and bathing the cell had the following composition (mM): 450 NaCl, 10 KCl, 10 $CaCl_2$, 25 $MgCl_2$, 26.5 $MgSO_4$, 2.5 $NaHCO_3$, 1.0 NaBr, 0.1 $NaNO_3$, 0.05 Na_2HPO_4, pH 7.9–8.0. In a cell bathed and internally perfused (ca.

2.4 ml · hr^{-1}) with ASW the SCC was determined by recording the current required to hold the cell *PD* at zero with a voltage clamp device. The *PD* in a short-circuited cell was measured by switching the voltage clamp off for about 30 sec, which allowed the *PD* to return to a stable value.

Three metabolic inhibitors were employed in these experiments, i.e., darkness, cyanide and low temperature. The cell was darkened by shrouding the faraday cage with black cloth and by turning out the room lights. During periods of normal lighting the light intensity around the cell was 110 J · m^{-2} · sec^{-1} as measured with a YSI-Kettering Radiometer, while during periods of darkness the light intensity was below the sensitivity of this instrument (i.e., <0.2 J · m^{-2} · sec^{-1}). Artificial seawater containing 2 mM NaCN was prepared freshly for each experiment, and the pH was adjusted to 8.0 with HCl. The cell was exposed to cyanide by replacing the external bath with ASW plus NaCN. At the end of the exposure cyanide was removed by rinsing the chamber twice with ASW. A low temperature bath was achieved by manually exchanging the bath with ASW which had been pre-cooled to 2–3 °C. The temperature was maintained at approximately 4 °C by replacing aliquots of the gradually warming bath with the precooled ASW.

Cl^- Efflux Measurements in Intact Cells

In order to measure Cl^- efflux from intact cells, 5–6 cells were incubated in $^{36}Cl^-$-labeled ASW (0.5–0.7 μCi/ml) for 3–5 days, during which time isotopic equilibrium was achieved. These cells were rinsed briefly in nonradioactive ASW, drained on a tissue and placed in a Lucite chamber containing 1.5 ml ASW. This chamber had a perforated "false bottom" so that the ASW could be stirred magnetically without damaging the cells (*see* Graves, 1974). At 30-min intervals the chamber was perfused with fresh ASW for about 30 sec in order to obtain a 5.0 ml sample of perfusate. This sample was then dried in a 2-inch planchet and counted with an end window counter (Beckman Wide Beta II). After each experiment the dimensions of the cells were measured and the intracellular contents were extracted with hot H_2O. Aliquots of this extract were dried in planchets containing 5 ml of ASW and counted to determine the radioactivity remaining in the cells. Separate aliquots were assayed for Cl^- by coulometric titration to determine the intracellular $[Cl^-]$.

A semilogarithmic plot of the intracellular $^{36}Cl^-$ remaining vs. time was constructed by successively adding back the radioactivity washed out during each sampling period. The slope of such a plot yields the rate constant (k) (*see* Kotyk & Janacek, 1970), and with this value the efflux (J^{out} in mole · cm^{-2} · sec^{-1}) is calculated by the relation:

$$J^{out} = \frac{k \cdot V \cdot C_i}{A} \tag{1}$$

where V is the cell volume, A is the surface area and C_i is the internal $[Cl^-]$.

Ion Substitution Experiments

External ion substitution experiments were conducted on nonperfused cells which were impaled with microelectrodes (*see* Graves & Gutknecht, 1976). Experimental solutions were perfused into the chamber by gravity flow, and the solution changes were complete within 2–3 min. In all such experiments the cell was exposed only to control conditions prior to changing the solution. The major substitute for Cl^- was isethionate ($HOCH_2CH_2SO_3^-$) (Eastman Kodak Co.). In preparing these solutions the appropriate amount of NaCl was replaced by Na isethionate. The osmotic coefficients for these two salts are similar, and the

final solutions had an osmolality of 990 ± 10 mosmoles/kg (measured by freezing point depression). To obtain ASW with 20 mM Cl^-, the NaCl was replaced by Na isethionate, the $MgCl_2$ and KCl were replaced by $MgSO_4$ and K_2SO_4, and 50 mM sucrose was added to adjust the osmolality. Isethionate solutions more than 36 hr old produced large transients in *PD* and therefore were not used. Other presumably impermeant anions, i.e., propionate, pyruvate and benzene-sulfonate, were also used as Cl^- substitutes. Of these, only pyruvate required the addition of sucrose to adjust the osmolality. In solutions in which the $[K^+]$ was altered, the $[Na^+]$ was changed in a converse manner, and $([Na^+]+[K^+])$ was therefore constant. Choline Cl and LiCl were both used as NaCl substitutes. The osmotic coefficients of these salts are similar, and no adjustments in osmolality were necessary. All solutions used in these external ion substitution experiments were buffered at pH 8 with 4.0 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES).

To determine the effect of changing the pH on the *PD* we used ASW buffered at pH 7 with 4.0 mM HEPES. However, this change in pH is accompanied by a change in either $[HCO_3^-]$ or pCO_2, and to distinguish between the effect of changing pH and that of changing $[HCO_3^-]$ or pCO_2, we prepared the pH 7 solutions by two methods. In one method the $[HCO_3^-]$ was kept constant by equilibrating the ASW with 0.3 % CO_2 in air. This solution was freshly prepared and was kept in a sealed glass container before use. In the alternative method the pH 7 ASW was equilibrated with room air which kept the pCO_2 constant while $[HCO_3^-]$ was allowed to decrease by a factor of ten.

The solutions used for internal ion substitution experiments were similar to those used in external substitutions except that no buffer other than HCO_3^- was added. The cell was perfused with the ion-substituted ASW for 40–60 min while the open circuit *PD* was recorded.

Results

Effects of Metabolic Inhibitors on Cl^- Transport and PD

In order to characterize the metabolic dependence of the Cl^- pump and the membrane electrical properties, we first investigated the effects of darkness. Figure 1 shows the rapid and reversible inhibitory effects of darkness on the SCC, Cl^- influx and *PD* in a single cell bathed and perfused with ASW. The apparent differences between the time-courses of these parameters in Fig. 1 are exaggerated by the manner in which the data are recorded. Although the SCC is essentially a continuous trace, the *PD* is measured periodically by opening the circuit for 30 sec. The influx values are averages over 15-min intervals, and thus do not provide a truly accurate measure of the flux under nonsteady-state conditions. Table 1 shows that darkness inhibits both the SCC and Cl^- influx by the same fractional amount, and this observation is consistent with our earlier conclusion that the SCC is an accurate measure of the rate of net active Cl^- transport (Graves & Gutknecht, 1976).

Having established that the SCC is an accurate measure of active Cl^- transport in both normal and inhibited cells, we studied further the

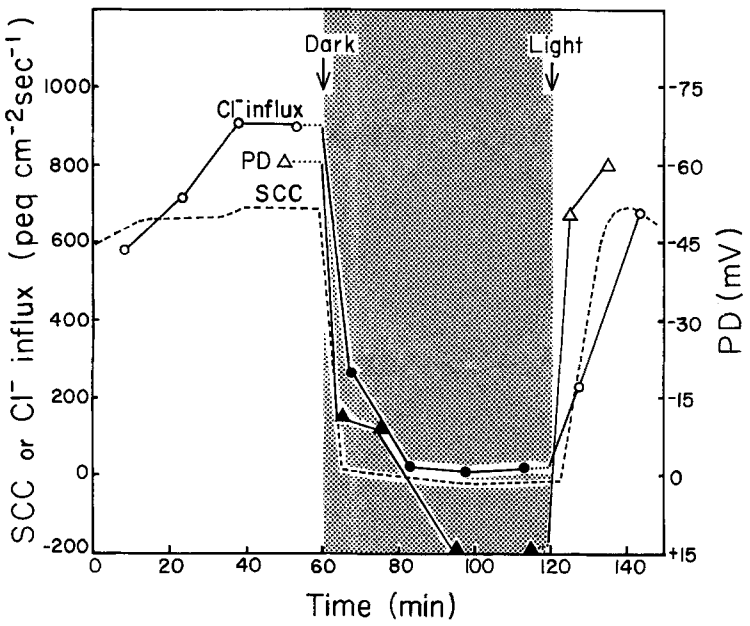

Fig. 1. Time-course of the response of SCC, *PD* and Cl^- influx to darkness in a perfused cell. Changes in illumination are shown at arrows (shaded area). Cl^- influx values are corrected for the wash-out time of the system and are plotted at the middle of the sampling period

Table 1. Effect of darkness on the SCC, *PD* and Cl^- influx in internally perfused cells of *H. parvula*

Cell number[a]	SCC			Cl^- influx[b]		
	($peq \cdot cm^{-2} \cdot sec^{-1}$)			($pmole \cdot cm^{-2} \cdot sec^{-1}$)		
	Light	Dark	% inhib.	Light	Dark	% inhib.
1	221	41	81	262	55	79
2	292	−10	103	322	22	93
3	664	−8	101	779	21	97

[a] Single experiments.
[b] This is total unidirectional influx from which the passive component has not been subtracted. In the light the passive component of the influx is about 60 $pmoles \cdot cm^{-2} \cdot sec^{-1}$ (Graves & Gutknecht, 1976). In the dark the passive component is not known.

metabolic relationship between the *PD* and SCC using the metabolic inhibitors, cyanide and low temperature. The effect of 2.0 mM NaCN on the SCC and *PD* in a perfused cell is shown in Fig. 2. When the external ASW was replaced by ASW plus cyanide, the SCC and *PD* were both reduced to near zero within 2 min and recovered with a slower time-course. Low

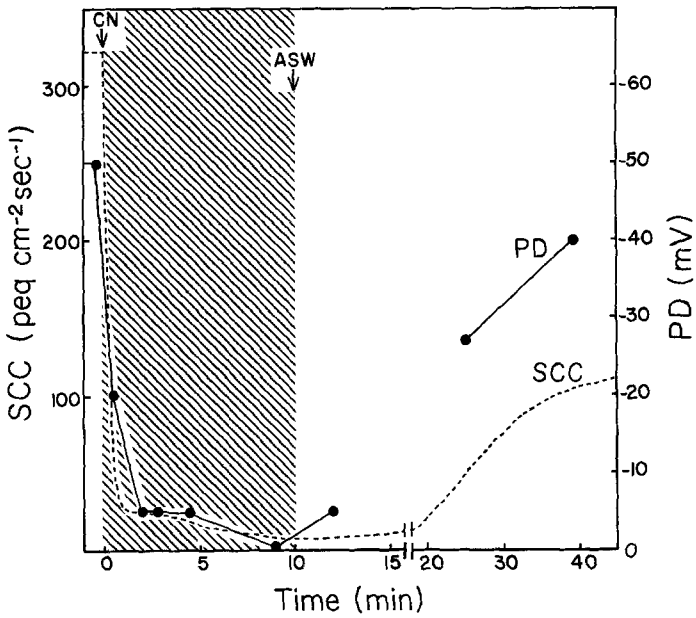

Fig. 2. Time-course of the inhibition of SCC and *PD* by 2.0 mM cyanide in a perfused cell. Application and removal of cyanide are shown at arrows (shaded area). Note scale-break in time axis

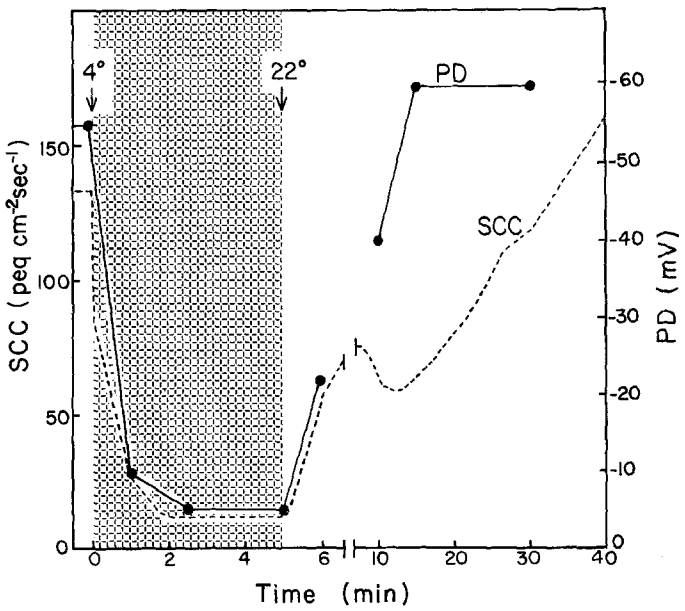

Fig. 3. Time-course of the response of SCC and *PD* to low temperature in a perfused cell. Temperature changes are shown at arrows (shaded area). Note scale-break in time axis

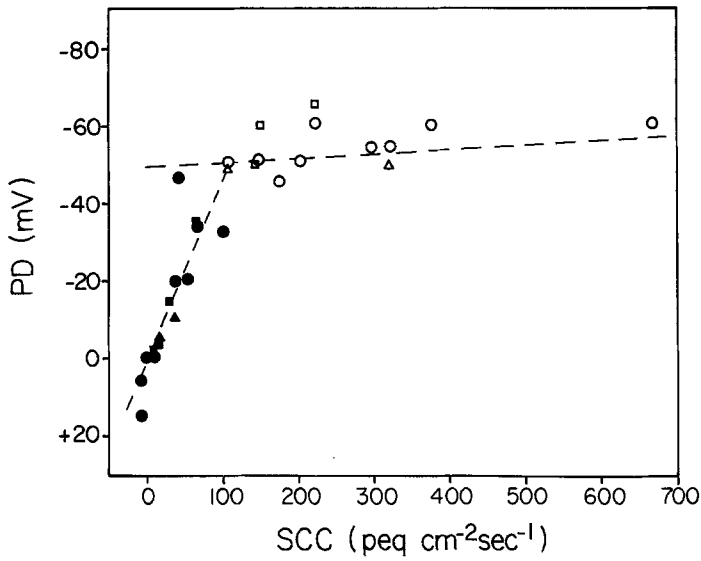

Fig. 4. Relationship between *PD* and SCC before (open symbols) and after (closed symbols) inhibition by darkness (circles), 2.0 mM cyanide (triangles) and low temperature (squares). Dashed lines represent least squares regression with the characteristics: Before inhibition: slope = 0.02 mV/peq · cm^{-2} · sec^{-1}, *y*-intercept = −49.2 mV, $r = 0.67$. After inhibition: slope = 0.47 mV/peq · cm^{-2} · sec^{-1}, *y*-intercept = −0.4 mV, $r = 0.84$

temperature (ca. 4 °C) also inhibited the SCC and *PD* rapidly and reversibly (Fig. 3). Possible physiological causes of the dissimilarities in the time-courses of *PD* and SCC during recovery in Figs. 2 and 3 will be discussed below.

The reduction of *PD* and SCC by the three inhibitors used was somewhat variable, as shown in Fig. 4. The points during metabolic inhibition fall on a regression line with a slope of 0.47 mV/(peq · cm^{-2} · sec^{-1}) ($r = 0.84$) and a *y*-intercept of −0.4 mV. This *y*-intercept is not significantly different from zero ($p \gg 0.1$), and this suggests that the *PD* and SCC have a common origin, i.e., the Cl^- pump. In contrast to the relationship which exists during inhibition, the linear regression through the points before inhibition has a slope 0.02 mV/(peq · cm^{-2} · sec^{-1}) ($r = 0.67$). A "test for independence" (Dixon & Massey, 1957) indicates that this slope is only marginally significantly different from zero ($0.1 < p > 0.05$).

If some portion of the *PD* is generated by an electrogenic pump, the membrane resistance will influence the magnitude of that *PD* (Rapoport, 1970; and *see Discussion*). However, interpretation of the resistance data in

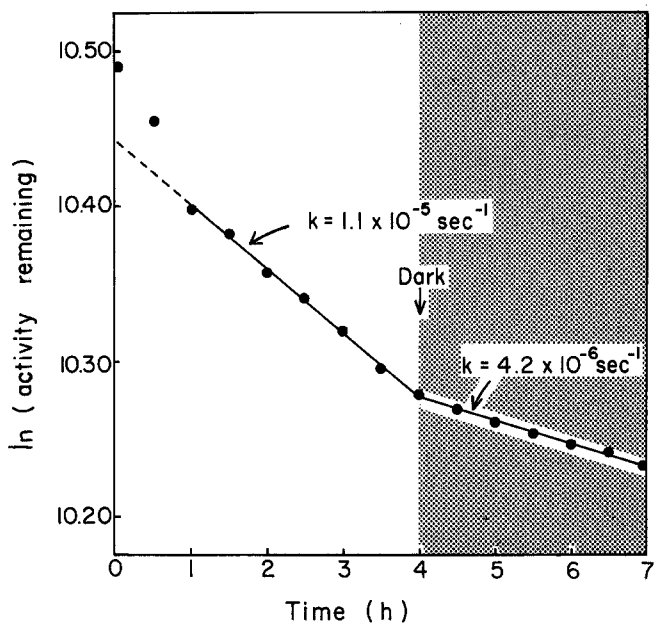

Fig. 5. Effect of darkness on the rate of wash-out of $^{36}Cl^-$ from a group of six intact cells

Halicystis is complicated by the fact that the Cl^- pump is voltage sensitive and thus contributes directly to the membrane conductance (Graves & Gutknecht, 1977). Therefore, even though darkness causes the measured resistance to increase from about 700 to 2400 $\Omega \cdot cm^2$ (Graves & Gutknecht, 1977), the degree to which this reflects a change in the resistance to passive ionic flow is not known. One way of indirectly estimating the effect of darkness on passive ionic flow is to measure the effect of darkness on the Cl^- efflux. The Cl^- efflux in perfused cells is the dominant dissipative flux and is highly sensitive to the electrochemical driving force (Graves & Gutknecht, 1976, 1977), which suggests it is largely due to simple diffusion. Figure 5 shows the effect of darkness on the Cl^- efflux from a group of six intact cells. The rate constant in the dark is about 40% of that in the light. For intact cells in which the *PD* is about -80 mV, the Cl^- efflux in the light is 278 ± 34 pmoles $\cdot cm^{-2} \cdot sec^{-1}$ (mean $\pm$SE, 6 experiments), which is in contrast to the dark-inhibited value of 111 ± 38 pmoles $\cdot cm^2 \cdot sec^{-1}$ (3 experiments). In one experiment with a perfused and short-circuited cell the Cl^- efflux was inhibited 43% by darkness. Collectively these data suggest that the true passive resistance across the protoplasm of *H. parvula* increases by a factor of 2–3 in the dark.

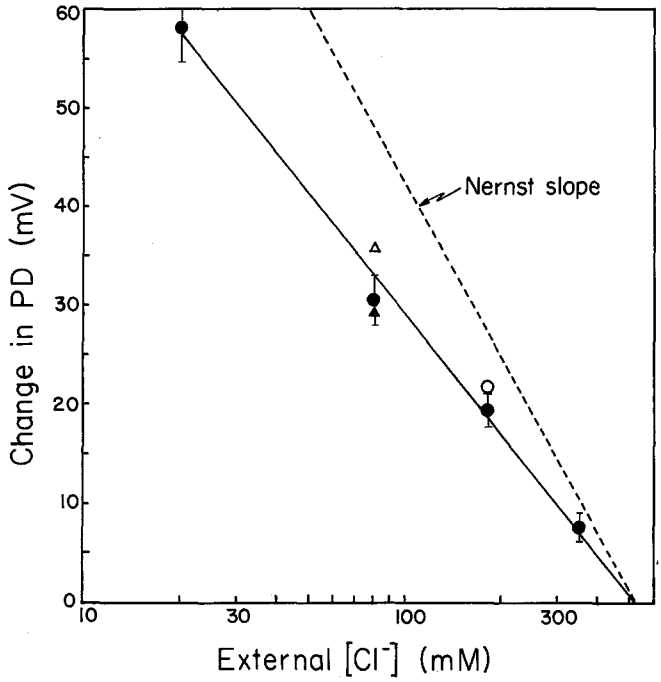

Fig. 6. A semilogarithmic plot of the change in *PD* (i.e., amount of depolarization) as a function of the external Cl^- concentration. Isethionate substitution points (●) and bars represent mean ±SE for 5–6 cells. Other points are means of 3–4 cells with propionate (○), pyruvate (▲) or benzenesulfonate (△) substitutions. Error bars are omitted for the sake of clarity. Line through isethionate points is a least squares regression, slope = 40 mV per 10-fold change in $[Cl^-]$, $r = 0.97$. Dashed line represents 59 mV per 10-fold change in $[Cl^-]$

Effects of External Ion Substitutions on the PD

The hypothesis of an electrogenic Cl^- pump predicts that the *PD* should become less negative when the external $[Cl^-]$ is reduced (i.e., as the pump is substrate-limited). Replacing a large portion of the Cl^- in the bathing ASW with a large organic anion caused the cell to depolarize with a half-time of about 3 min from the beginning of the solution change, and this depolarization was reversible with a similar time-course. (The time required for 50% completion of the solution change was about 1 min.) The compiled results of reducing the external Cl^- to various levels are shown in Fig. 6. In computing these points we have used the maximum depolarization observed in each experiment. The similarity between the effects of isethionate and other substitute anions (i.e., propionate, pyruvate and benzenesulfonate) verifies that the change in *PD* is a function of external $[Cl^-]$ rather than a direct effect of the replacement anion. The extent to

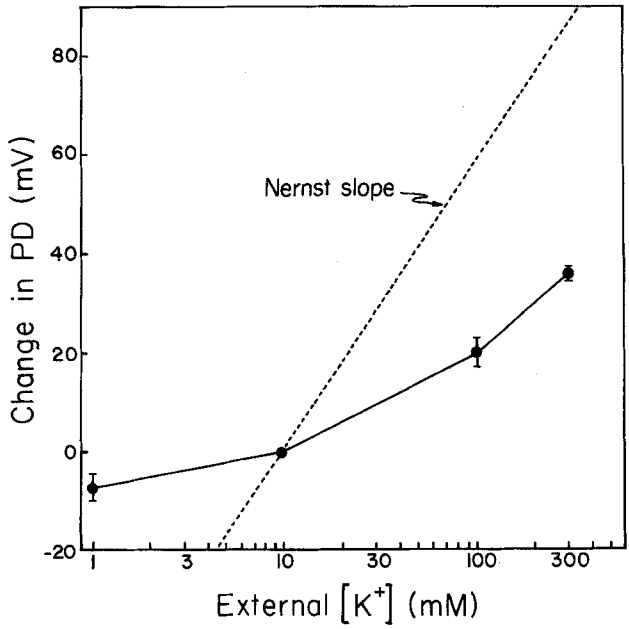

Fig. 7. A semilogarithmic plot of the change in *PD* as a function of the external K^+ concentration. Points and bars represent the mean ±SE for 5 cells. Dashed line represents 59 mV per 10-fold change in $[K^+]$

which the depolarization at low external $[Cl^-]$ is due to a change in a Cl^- diffusion potential is not known, but the effect is qualitatively consistent with the existence of an electrogenic Cl^- pump with a K_m within this range of concentrations.

In order to ascertain the possible contributions of Na^+, K^+ or H^+ to a diffusion potential across the plasmalemma, we studied the effects on the *PD* of altering the external $[K^+]$, $[Na^+]$ and $[H^+]$ in nonperfused cells. Altering the $[K^+]$ in the bath caused the changes in *PD* shown in Fig. 7. At the higher concentrations of K^+ the cell usually depolarizes smoothly to a new steady *PD* within 10 min, which is unlike the large transient depolarization observed in similar experiments with *Halicystis* by Blinks (1932). A peculiar phenomenon occurred when the external $[K^+]$ was decreased to 1 mM. Under this condition the *PD* anomalously depolarized by 5–10 mV before reaching a slightly hyperpolarized level after 30–90 min. The cause of this early depolarization is not known, but it was also observed when *H. osterhoutii* was exposed to K^+-free ASW (Blinks, 1932). Although the effects of varying external $[K^+]$ are rather complex, the data suggest that the selectivity for K^+ relative to other monovalent cations is

appreciable and that K^+ diffusion would contribute to any diffusion potential across the plasmalemma.

A tenfold reduction in the external [Na^+] produced no appreciable change (i.e., less than 3 mV) in the *PD*'s of three cells when either choline or Li^+ was used as a substitute for Na^+. This result indicates that the relative Na^+ conductance must be small.

Lowering the ASW pH from 8 to 7, with both solutions buffered with 4.0 mM HEPES and with the [HCO_3^-] kept constant, caused a transient depolarization of about 5 mV (two cells), after which the original *PD* was restored within 10–15 min. Similar results were obtained in a second experiment in which pH was lowered from 8.0 to 7.0 at constant pCO_2 and with [HCO_3^-] reduced tenfold. The small depolarization observed with a tenfold change in [H^+] suggests that the relative conductance for H^+ across the plasmalemma must also be small.

Effects of Internal Ion Substitutions on the PD

In a series membrane system such as *Halicystis* it is theoretically possible for the Cl^- pump to be electroneutral and for the cell to exhibit the simultaneous changes in *PD* and SCC, as well as the depolarization due to low external [Cl^-]. Such a model, like that proposed by Koefoed-Johnsen and Ussing (1958) for the frog skin, requires that the membrane at which the pump resides have a high relative permeability to the co- or counter-ion, and that the opposite membrane have a high relative permeability to Cl^-. To test these possibilities we studied the effects of vacuolar ion substitutions on the *PD* to determine the ionic selectivity properties of the tonoplast.

The effect of a low internal [Cl^-] on the *PD* was determined by perfusing the vacuole with 80 mM Cl^- ASW after perfusion with normal ASW (i.e., 530 mM Cl^-). Substituting the Cl^- with either isethionate or benzenesulfonate produced no consistent change in the *PD*. Therefore, the tonoplast appears to have no electrical selectivity among these anions. The cationic selectivity of the tonoplast was also tested by vacuolar ion substitutions. The *PD* in two cells was unaffected when either 300 mM K^+ or 4.5 mM Na^+ (Li^+-substituted) solutions were perfused. Perfusing the vacuole with K^+-free ASW also had no effect on the *PD*, which agrees with the observations of Blinks (1935) on *H. osterhoutii* and *H. ovalis*. Thus, the tonoplast appears to be electrically nonselective with respect to Na^+ and K^+.

The lack of electrical selectivity of the tonoplast among anions or between Na^+ and K^+ is not unique to *Halicystis*. Kishimoto (1965)

demonstrated a similarly nonselective tonoplast in *Nitella*. This apparent lack of selectivity makes it unlikely that an electroneutral Cl^- pump at either membrane is responsible for the SCC. Furthermore, it is unlikely that a large negative potential would be developed across the tonoplast by a combination of diffusion potentials for Na^+, K^+ and Cl^-, because the equilibrium potential Cl^- across that membrane is probably positive and the equilibrium potentials for Na^+ and K^+ oppose each other (Graves & Gutknecht, 1976).

Discussion

Metabolic Dependence of the Cl^- Pump

In perfused cells of *H. parvula* the SCC and Cl^- influx are both rapidly inhibited to a similar extent by darkness (Table 1 and Fig. 1), which indicates that photosynthesis is involved in the metabolic support of the Cl^- pump. An obvious mechanism by which photosynthesis can directly provide energetic support for ion transport is by the production of ATP via photophosphorylation. However, in fresh-water algae spectral and inhibitor studies on the Cl^- fluxes suggest that the photosynthetic electron transport chain, rather than photophosphorylation, may in some way drive the inward Cl^- transport (MacRobbie, 1970). The large and rapid inhibition of the SCC by 2.0 mM NaCN (Fig. 2), which is presumably an inhibitor of oxidative phosphorylation, suggests that the Cl^- pump of *H. parvula* relies on ATP. This would implicate photophosphorylation as the more likely mechanism of light-stimulation. However, this suggestion must be viewed with caution for several reasons. Holm-Hansen (1970) found that darkness produces only a small transient decline in the intracellular ATP concentration in green algae. Also, inhibitor studies on Cl^- transport in the marine alga, *Griffithsia*, suggest that the Cl^- pump requires photosynthetic ATP, but the ATP concentration is little affected by the same inhibitors reduce Cl^- transport (Lilley & Hope, 1971*a*, *b*). In addition, the presumed inhibitors of oxidative respiration, cyanide and azide, inhibit O_2 evolution but *not* O_2 uptake in leaf slices of the higher plant *Tradescantia* (Johansen & Lüttge, 1974). Finally, Ouitrakul and Izawa (1973) showed that in isolated chloroplasts photosynthetic electron transport is inhibited by cyanide. Therefore, the use of such inhibitors must be coupled with direct measurements of ATP or other suspected energy intermediates in order to verify the presumed action of the inhibitor, and that has not been done on

our study. Thus, we can state only that the activity of the Cl^- pump is related to the photosynthetic activity of the cell.

Relationship between PD and SCC

We have shown that the metabolic inhibitors, darkness, cyanide and low temperature, all rapidly and reversibly inhibit the *PD* and SCC (Figs. 1, 2 and 3). Although the inhibitory phases in these experiments have approximately similar time-courses, the time-courses for *PD* and SCC during the recovery phases, particularly for cyanide and 4 °C, are not parallel. Although the dissimilarities during recovery might mean that the *PD* and SCC are not closely coupled, there are at least three physiological properties of this system which could cause the observed dissimilarities. Firstly, in a series membrane system the SCC is equal to net active transport only when it is constant with time (Ginzburg & Hogg, 1967). If the electrochemical gradients for the ions between the cytoplasm and other compartments are changing, there will be changes in the passive currents which will affect the magnitude and rate of change of the SCC during transient phases. Secondly, there are several other ion pumps in *H. parvula* [e.g., Na^+ and K^+ pumps at both the plasmalemma and tonoplast (Graves & Gutknecht, 1976)] which could influence the SCC during transient periods, especially if there are different rates of recovery from inhibition. Finally, when an electrogenic pump is responsible for the SCC, the resulting *PD* depends on the membrane resistance (Rapoport, 1970), and thus the changing *PD* need not be proportional to changes in SCC if the resistance of the membrane system is also changing. This phenomenon will be discussed more thoroughly later. In view of these considerations we find the qualitative similarity of the changes in *PD* and SCC during inhibition and recovery to be consistent with the idea that the SCC and *PD* are related in a cause-effect manner. Furthermore, the zero *y*-intercept for the curve relating the SCC and *PD* under inhibitory conditions (Fig. 4) suggests that these two parameters have a common origin which relies on cellular energy metabolism, i.e., the Cl^- pump.

We have presented several other forms of evidence that are consistent with the hypothesis of an electrogenic Cl^- pump. The rapid time-course of inhibition, particularly with cyanide and low temperature, is similar to the effect of metabolic inhibitors on the *PD* in the fungus, *Neurospora crassa*, for which there is other independent evidence for an ATP-driven electrogenic

pump (Slayman, 1970; Slayman, Long & Lu, 1973). Also, the fast response to inhibitors implies that the change in *PD* is not due to the dissipation of ion concentration gradients across the plasmalemma or tonoplast. The substantial depolarizations caused by reducing the external [Cl^-] (Fig. 6) are consistent with and form a necessary criterion for the existence of an electrogenic Cl^- pump. Finally, we have shown that the tonoplast has no electrical selectivity among Cl^-, isethionate, and benzenesulfonate or between Na^+ and K^+. The generation of such a large SCC by an electroneutral Cl^- pump is unlikely when the tonoplast has such non-selective properties for these major ions. Therefore, the simplest interpretation consistent with all the data is that an electrogenic Cl^- pump generates a *PD* of -50 to -60 mV in perfused cells of *H. parvula.*

The early observations of Blinks *et al.* (1938, 1940) that the *PD* of *Halicystis* was sensitive to anaerobiosis and that the *PD* was abolished when the external Cl^- was removed are consistent with the proposal of an electrogenic Cl^- pump. Saddler (1970) has shown that a *PD* of similar magnitude (i.e., -70 to -80 mV) is generated by an electrogenic Cl^- pump in *Acetabularia mediterranea.* While electrogenic pumps appear to play an appreciable role in generating the *PD* in several other plant cells, the ion(s) which is transported electrogenically is usually not known (Higinbotham, 1973). In a few systems an H^+ efflux pump is strongly suspected to be electrogenic (Spanswick, 1972; Slayman *et al.*, 1973; Richards & Hope, 1974), but the inability to conduct H^+ tracer experiments makes this idea difficult to test directly.

Effect of Membrane Resistance on the Magnitude of the PD

For a cell in which an electrogenic ion pump is operating the total *PD* may be described by the following equation:

$$PD = \Sigma T_j \cdot E_j + I_p \cdot R_m. \tag{2}$$

The first term on the right side of Eq. (2) results from a derivation by Staverman (1952) for a membrane potential generated by ionic diffusion, where E_j is the equilibrium potential for ion j, and T_j, the transport number for j, is defined by:

$$T_j = g_j / G_t \tag{3}$$

in which g_j is the partial ionic conductance and G_t is the total conductance of the membrane system. The second term in Eq. (2) describes the potential

generated according to Ohm's law by the electrogenic pump (*see* Rapoport, 1970), where I_p is the net ionic current through the pump and R_m is the resistance of the membrane system. It is this involvement of the membrane (i.e., transprotoplasm) resistance in the pump-generated *PD* which may provide the explanation for the variable reduction of the *PD* by metabolic inhibitors (Fig. 4). Graves and Gutknecht (1977) show that the Cl^- pump probably lowers the measured R_m, and it is therefore not meaningful to use the changes in electrical resistance during inhibition to predict the *PD* which would be generated by a residual SCC. However, the reduction in the Cl^- efflux in the dark (Fig. 5) suggests an increase in the true R_m (i.e., resistance to passive ionic flow) of over two-fold. This increased resistance would "amplify" the *PD* resulting from any SCC remaining. Thus, only when the SCC is totally abolished would the *PD* fall to near zero, and when an appreciable, though small, SCC remains, the inhibition of the *PD* may be much less and more variable.

In principle Eq. (2) further allows us to estimate the magnitudes of the components of the *PD*. However, this calculation is complicated by the probability that the Cl^- pump contributes to the membrane conductance. For example, using $700\,\Omega \cdot cm^2$ for R_m in perfused cells (Graves & Gutknecht, 1977) and the mean SCC of $29\,\mu A \cdot cm^{-2}$ (Graves & Gutknecht, 1976), we calculate that -20 mV is the upper limit for the potential produced electrogenically, whereas the inhibitor studies suggest that the electrogenic pump contributes -50 to -60 mV. The discrepancy may lie in the underestimation of the true R_m due to a contribution to the conductance by the electrogenic pump. We may be able to estimate the true R_m under normal conditions and, thus, the pump contribution to the *PD* (i.e., V_p), by using the data on the effects of darkness on the *PD*, SCC and Cl^- efflux. The relationship between *PD* and SCC in the dark (Fig. 4) is essentially a current-voltage plot, and the slope of the linear regression through these points is $0.46\ mV/peq \cdot cm^{-2} \cdot sec^{-1}$ ($r = 0.84$). This slope gives an estimate for R_m in the dark of about $4700\,\Omega \cdot cm^2$. If we now assume that the reduction of Cl^- efflux in the dark reflects a general increase in resistance to passive ionic flow, we can estimate the true R_m in the light. The ratio of Cl^- efflux in the light to that in the dark is about 2.5. Dividing $4700\,\Omega \cdot cm^2$ (i.e., R_m in the dark) by 2.5 yields $1880\,\Omega \cdot cm^2$ as an estimate of R_m in the light. Using this value for R_m and the mean SCC of $29\,\mu A \cdot cm^{-2}$, we calculate V_p from Ohm's law to be -54.4 mV. This theoretical value of V_p compares favorably with the mean of all the uninhibited *PD*'s presented in this paper, which is -55.2 mV, and this agreement suggests that the estimated value of R_m in the light is reasonable. Furthermore, Graves and Gutknecht (1977) obtain a

similar value for R_m in the light by considering only the voltage sensitivity of the Cl^- efflux in perfused cells. Thus, both experimental and theoretical evidence support the hypothesis that an electrogenic Cl^- pump is responsible for virtually all of the *PD* in perfused cells of *H. parvula.*

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