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Electrogenic Pump Activity in Red Beet: Its Relation to ATP Levels and to Cation Influx

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Summary. In storage tissue of *Beta vulgaris* L., carbonyl cyanide *m*-chlorophenylhydrazone or cyanide + salicylhydroxamic acid reduce cell electropotentials from about −200 to below −100 mV. The relationship between potential and cellular ATP level is examined during treatment with different concentrations of inhibitors. At low ATP levels the potential rises sharply with increases in ATP, but above an ATP level of approximately 50% of the uninhibited level the potential changes very little with ATP concentration. A plot of membrane potential *vs.* $^{86}Rb^+$ influx or of potential *vs.* net K^+ uptake indicates that as the level of inhibition is decreased, the potential tends to reach a limit while cation influx and net uptake continue to increase. Resistance measurements, although subject to difficulties of interpretation, indicate no change in conductance with potential, ion flux, or ATP level. Thus the membrane potential should directly reflect electrogenic pump activity, attributed to active uncoupled H^+ efflux. K^+ uptake can occur against its electrochemical gradient and is attributed to a coupled K^+ influx/H^+ efflux pump. The results show that the electrogenic pump activity is independent of the K^+/H^+ exchange rate. Thus electrogenic H^+ efflux and K^+/H^+ exchange may represent different transport systems, or different modes of operation of a single pump with variable stoichiometry.

Electrogenic pumps, i.e., primary transport processes which contribute directly to the membrane potential, are a conspicuous feature of plant cells [26]. In most cases it appears to be the efflux of H^+ which gives rise to an energy-dependent hyperpolarization. Net H^+ efflux is usually associated with the accumulation of K^+ or other cations, but the link between these ion movements is not yet clear. Slices of storage tissue of red beet (*Beta vulgaris* L.) appear to be a favorable material for investigation of the relation between these transport processes [25] since a large and prolonged efflux of H^+, an equivalent influx of K^+, and a conspicuous hyperpolarization are all evoked by raising the external pH from about pH 6.5 to pH 7.5. Other ion movements such as active ($K^+ + Cl^-$) influx are not sensitive to pH and are much smaller than K^+/H^+ exchange at high pH. In the present study, membrane potentials in beet cells at high pH are investigated in relation to changes in ATP level brought about by metabolic inhibitors, especially cyanide. In addition, membrane potentials are compared with cation influx over various levels of metabolic inhibition, and the results are interpreted in terms of possible schemes of energy coupling for the electrogenic pump and for the accumulation of cations.

Materials and Methods

Tissue Preparation

Roots of *Beta vulgaris* L. were stored in moist vermiculite at 8 to 10 °C to maintain the cells in a dormant state. In preparation for experiments, root tissue, consisting of parenchyma and some vascular tissue, was cut into disks of approximately 0.9 mm thickness and 5 mm diameter. The disks were subsequently washed, usually for five to six days, in aerated de-ionized water at 20 to 25 °C. This procedure releases the tissue from dormancy, stimulates metabolism, and leads to the development of ion absorption processes [36].

Electrical Measurements

The techniques for observing and penetrating individual cells of beetroot have been described elsewhere [22]. The chamber used for holding the tissue under observation was perfused at a rate

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of about 2 ml/min with bathing solutions at 22 to 24 °C. Glass microelectrodes were made from Omega Dot capillary tubing, 1.0 mm OD (Frederick Haer & Co., Brunswick, Me.); and contained 3 M KCl. They generally had tip potentials less than 12 mV and resistances between 10 and 30 MΩ when placed in solutions of 0.3 mM KCl (or 0.15 mM K_2SO_4) + 10 mM Tris $-SO_4$. It is likely that the tip potential would be smaller in the more concentrated intracellular solution. The electrode resistance showed little change on penetration of a cell. The reference electrode was connected to the bathing solution by a polyethylene tube containing 1 M KCl in 3% agar. The signal voltage was amplified by a Model M701 Micro-Probe System (W.P. Instruments, Inc.) and monitored using an oscilloscope and a chart recorder. Measured in this manner, the potential difference between the intracellular and extracellular solutions is taken to represent the electrical potential difference across the plasmalemma [4, 14, 22]. Potential measurements were not considered reliable unless they remained constant for at least 2 min; most of the measurements used in this study were constant for 3 min or longer.

In order to study current-voltage properties and membrane resistance, current was injected through the same microelectrode that was used for measuring electrical potential. The methods of Goldsmith et al. [9] were employed, except that the electrode resistance was not balanced to zero but instead was measured throughout the experiment. In this way, any changes in electrode resistance following cell impalement could be detected. Current pulses generated by a Grass SD9 stimulator were passed through the bridge circuitry of the M701 Micro-Probe. Only electrodes which exhibited a rapid rise-time (0.1–0.5 msec) were used). Following a successful cell penetration, current injection caused a voltage deflection with two clearly distinguishable components: a fast component associated with the electrode resistance and a slower component resulting from the larger *RC* time constant of the membrane (Fig. 1). The electrode and membrane resistances were determined from the amplitude of their associated voltage changes and from the amount of current injected. Values representing total membrane resistance (in MΩ) rather than specific membrane resistance were obtained.

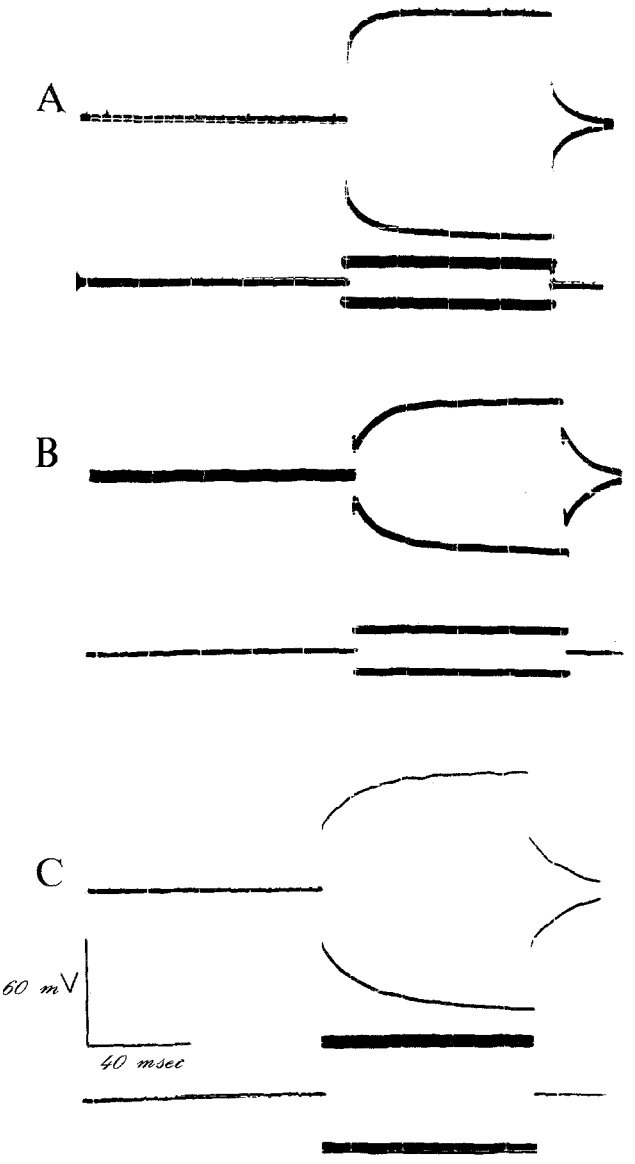

Fig. 1. Current injection pulses. Upper traces in *A*, *B*, and *C* are oscilloscope recordings of membrane potentials in three different cells. The recordings show the displacement of voltage in response to injected currents of 10^{-9} A in either the depolarizing (upward) or hyperpolarizing (downward) direction. Lower traces in all cases are monitors of the injected current. The voltage scale in the lower left of the figure applies to the membrane potential recordings; the time scale applies to all traces. (This figure was prepared with the photographic assistance of Guy L'Heureux.)

Solutions

All bathing solutions contained 0.3 meq/liter of K^+ ions (mainly as K_2SO_4 or as KCl) and 10–15 mM Tris at 22 to 24 °C. HCl was used to adjust the pH of KCl solutions; H_2SO_4 was used for adjusting the pH of K_2SO_4 solutions. Fresh stock solutions of metabolic inhibitors were prepared just prior to each experiment. The following compounds were dissolved in ethanol before being added to bathing solutions: carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), salicylhydroxamic acid (SHAM), and valinomycin. In all cases, the final ethanol concentration in the bathing solution did not exceed 1% (vol/vol). Control experiments indicated that the membrane potential was not affected by the presence of 1% ethanol or by varying the Tris concentration between 10 and 15 mM.

$^{86}Rb^+$ Influx and ATP Measurement

The influx of cations across the plasmalemma was estimated using a short-term influx procedure [2] similar to that described elsewhere [20]. One-gram samples of disks were pretreated for 1 hr in solutions containing various concentrations of metabolic inhibitors. This allowed time for influx rates as well as cellular ATP to reach steady levels (T. Petraglia, *unpublished results*). The solutions were renewed at regular intervals to ensure that uptake by the tissue would not deplete the extracellular K^+ concentration by more than 15–20%. Following pretreatment, the samples were bathed for 6 min in the appropriate bathing solutions with the addition of 0.01 microcuries/ml of ^{86}RbCl. Subsequently, they were bathed for 6 min in unlabeled solutions to remove isotope from the cell walls and were frozen in liquid nitrogen to prevent metabolic recovery [20]. The samples were prepared for counting by homogenization for 1 min in 40 ml of 0.4 N perchloric acid + 1 mM EDTA. The homogenate was centrifuged with activated charcoal for 10 min at 15,000 × *g* to remove cell debris and to clarify the extract. Duplicate samples of extract were mixed with Aquasol scintillation fluid and were counted in a Beckman scintillation

counter with a window setting of 3–10. An external standard was used to correct for quenching. In each experiment, the values of $^{86}Rb^+$ influx were expressed on a percent basis, with 100% corresponding to the influx rate of control samples (which had been pretreated with solutions that did not contain inhibitors). This was done in order to compensate for variations in transport rates, metabolic activities, etc., between the beets used in different experiments.

Cellular ATP was measured from 1-g samples which either were pretreated for 72–75 min in experimental solutions or were pretreated for 1 hr and subjected to the short-term labeling technique above. The tissue was frozen in liquid nitrogen and homogenized in perchloric acid and EDTA as above. ATP was assayed by the luciferin-luciferase method as described elsewhere [20]. Samples were compared with ATP standard solutions ranging from 1 to 50 pmol ATP per ml assay mixture. In each experiment, the ATP levels were expressed on a percent basis, with 100% corresponding to the level of control (noninhibited samples). In some experiments, an internal standard, consisting of 10 or 20 pmol of ATP, was added to 1-g sample of tissue prior to freezing. Assay of the sample indicated complete recovery of the ATP with little or no quenching.

Net Uptake and Intracellular K^+

Net uptake of K^+ ions was measured following a 1-hr pretreatment in experimental and control solutions. Potassium loss from the solutions was measured with a flame photometer. The intracellular K^+ concentration was estimated by flame photometric assay of perchloric acid + EDTA extracts of the tissue. It was assumed that 65% of the tissue comprised live cells [22].

Results

Effect of pH on the Membrane Potential

Mean intracellular potentials in the standard bathing solutions (pH 8) without inhibitors are shown in Table 1, together with measurements at lower pH values for comparison. As in earlier work [22] where intracellular potentials were measured in the presence of bicarbonate but in the absence of Tris buffer, the potential becomes more negative with increasing pH. A large effect of pH on K^+ uptake but not Cl^- uptake [17] and the balancing of the excess cation influx at high pH by a stoichiometric efflux of H^+ [24] have been previously documented.

Table 1. Effect of pH on membrane potential[a]

A) K_2SO_4 solutions					
pH	K_2SO_4 (meq/liter)	Tris (mM)	H_2SO_4 (mM)	Mean potential (mV)	No. of cells
6.0	0.3	10	4.0	-167 ± 6	10
7.0	0.3	10	3.0	-197 ± 6	18
8.0	0.3	10	0.5	-206 ± 3	43
B) KCl solutions					
pH	KCl (meq/liter)	Tris (mM)	HCl (mM)	Mean potential (mV)	No. of cells
5.6	0.3	0	0	-149 ± 4	14
8.0	0.3	10	0.3	-191 ± 2	22

[a] Membrane potentials were recorded after 50 to 180 min in the solutions indicated.

Effects of Inhibitors on Membrane Potential

Figures 2 to 4 illustrate the effects of different metabolic inhibitors on membrane potentials recorded from individual cells. While membrane potentials were measured, the chamber holding the tissue was perfused continuously with bathing solution containing inhibitors as indicated.

In agreement with inhibitor studies on other plant material [7, 13, 34, 32], rapid depolarizations were observed when cells were treated with CCCP or with cyanide (Fig. 2*A* and *B*). When the inhibitors were removed by changing the bathing solution back to one which did not contain inhibitors, the inhibition was reversed and the cells repolarized. 0.01 mM CCCP produced a small depolarization (Fig. 2*A*), while CCCP concentrations of 0.03 mM (not shown) or 0.05 mM (Fig. 2*A*) depolarized cells by approximately 100 mV. Depolarizations of about 10 and 35 mV, respectively, occurred in response to 0.005 and 0.01 mM cyanide (not shown); 0.05 mM cyanide typically depolarized cells by approximately 100 mV as in Fig. 2*B*.

When cells were exposed to cyanide for longer than 15 min, significant repolarization in presence of the inhibitor was usually observed (Fig. 3). This did not result from depletion of cyanide from the bathing solution, since the tissue was superfused continuously. The time course and degree of the repolarization were somewhat variable. Generally, however, membrane potentials repolarized from approximately -90 to between -140 and -195 mV within 25 to 40 min of treatment in 0.05 mM cyanide. After about 40 min, the potential tended to reach a steady level, increasing only slightly with time. As shown in Fig. 3, removal of the cyanide at this point caused a further repolarization, raising the potential approximately to the level of control (noninhibited) cells (-206 mV, shown in Table 1*A*). Re-addition of the same concentration of cyanide depolarized the cell to about the potential that was recorded just prior to cyanide removal.

It was possible to reduce the membrane potential further and to minimize the repolarization by treating cells with a combination of cyanide and salicylhydroxamic acid (SHAM), an inhibitor of the cyanide-resistant respiratory pathway of various plants [30]. Fig-

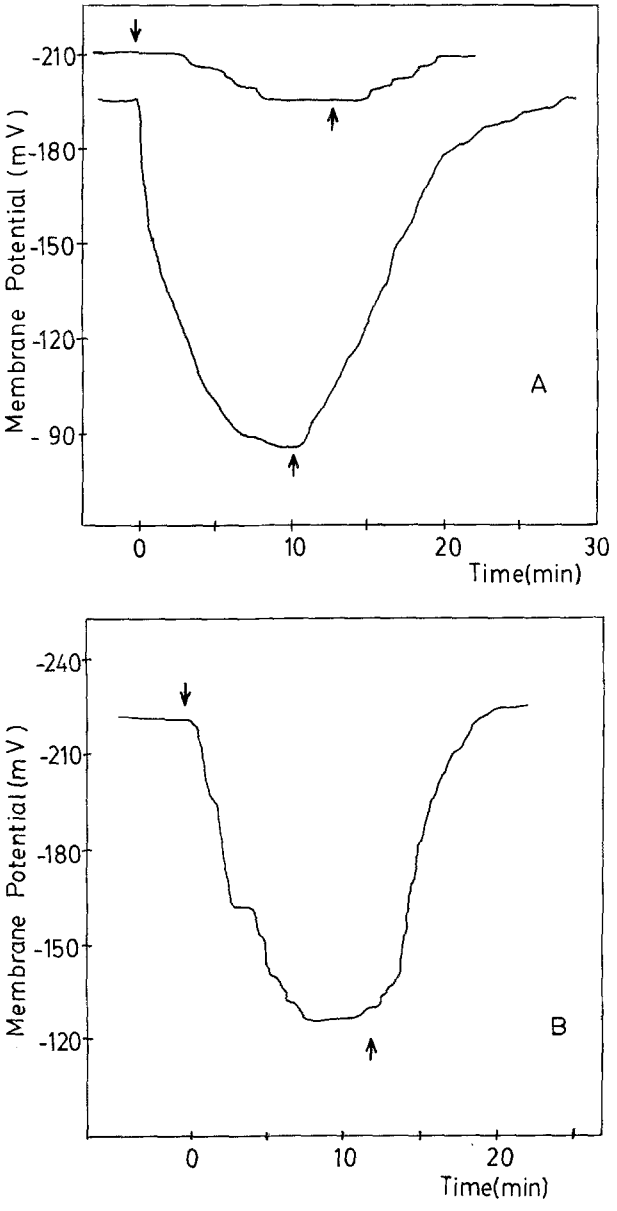

Fig. 2. Effects of CCCP and cyanide on membrane potentials. The figure shows tracings of membrane potentials recorded continuously over time after beginning treatment with inhibitors. (*A*): Effects of 0.01 mM CCCP (upper trace) and 0.05 mM CCCP (lower trace). (*B*): Effect of 0.05 mM cyanide. Downward arrows indicate the introduction of inhibitors in the bathing solution; upward arrows indicate the removal of inhibitors. Bathing solutions contained KCl + 10 mM Tris (pH 8.0) in *A* and K_2SO_4 + 10 mM Tris (pH 8.0) in *B*. In this and all other figures, the total K^+ concentration in each bathing solution was 0.3 meq/liter.

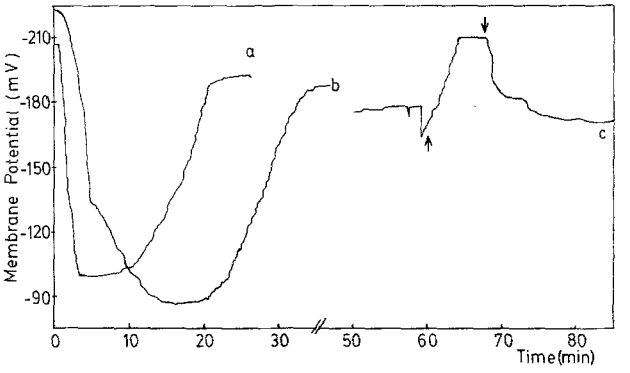

Fig. 3. Prolonged exposure to 0.05 mM cyanide. Membrane potentials of two cells, *a* and *b*, recorded over time after treatment in cyanide was commenced. (*c*): Removal (upward arrow) and replacement (downward arrow) of 0.05 mM cyanide following a 1 hr treatment with the inhibitor.

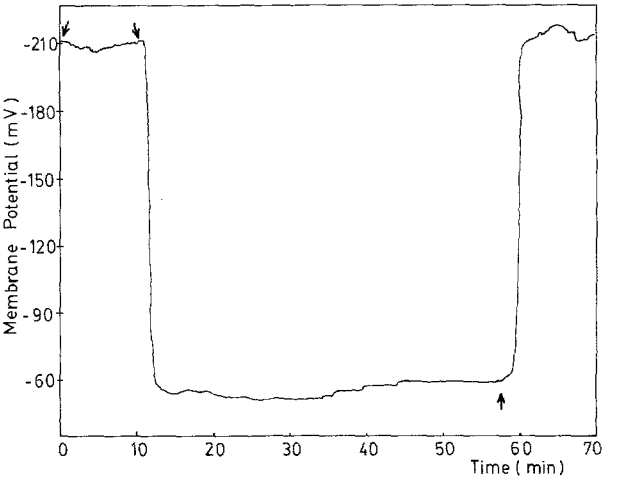

Fig. 4. Membrane potential during prolonged exposure to cyanide and SHAM. The cell was bathed initially in 0.3 meq/liter K_2SO_4 + 10 mM Tris (pH 8.0). The first downward arrow indicates the introduction of 5 mM SHAM to the bathing solution. The second downward arrow indicates a further introduction of 0.05 mM cyanide. The upward arrow marks the removal of cyanide (but not SHAM) from the bathing solution.

ure 4 illustrates the effects of the two inhibitors. Introduction of only 5 mM SHAM to the bathing solution had little or no effect on the potential within the first 9 min. Subsequently, treatment with 5 mM SHAM + 0.05 mM cyanide reduced the potential to less than −60 mV, and a repolarization of only 10 mV was recorded. Removal of only the cyanide increased the potential to about the level obtained prior to inhibition. Thus, SHAM greatly enhanced the inhibition produced by 0.05 mM cyanide and diminished the repolarization significantly, but it had little or no effect on the potential by itself.

Membrane Potentials and ATP

Membrane potentials and ATP levels were measured from cells treated with various concentrations of cyanide and SHAM. Disks of tissue used for measuring potentials were pretreated for 40–90 min in flasks containing the inhibitor and control solutions, so that

membrane potentials could reach approximately steady levels (Figs. 3 and 4). Disks from the same beet were pretreated in the solutions for 72–75 min before being frozen for ATP measurement (*see* Materials and Methods). Table 2 summarizes the results of two such experiments. As stated above, SHAM enhanced the depolarizations observed in the presence of cyanide but exerted very little effect on the potential by itself. Cyanide (in the concentrations tested) reduced the cellular ATP level to about 50% of the value obtained for control samples. The combination of cyanide and SHAM reduced the ATP level by about 10% more than did cyanide alone. SHAM, by itself, lowered the ATP level by 20%.

The results from Table 2 and two other experiments conducted in the same manner are combined and plotted in Fig. 5. No significant change in membrane potentials was observed when the ATP level was reduced to about 62% of control level. Large depolarizations (approximately 125 mV) corresponded to the reduction of ATP level from 62 to 40% of control. (The ATP content of the control samples of Fig. 5 varied from 36 to 44 nmol per g tissue, or 0.7 to 0.9 mM, assuming that the ATP is contained in the cytoplasm and that the cytoplasmic volume is 5% of the tissue volume [21].) The results suggest that an ATP level of approximately 50% of control level is critical for the generation of the large, negative potential by the electrogenic pump, and that the membrane potential tends to reach a limit when the ATP level exceeds 60% of control level. Results similar to those of Fig. 5 were obtained in another series of experiments conducted at pH 7.0 [27].

Table 2. Effects of cyanide and SHAM on ATP level and membrane potential[a]

	KCN (mM)	SHAM (mM)	ATP (% of control)	Mean potential (mV)	No. of cells
Expt. A	0	0	100	−216 ± 2	4
	0	5.0	80	−188 ± 7	6
	0.05	0	46	–	–
	0.05	5.0	37	−73 ± 3	4
Expt. B	0	0	100	−198 ± 14	2
	0.10	0	50	−127 ± 8	5
	0.10	1.0	43	−77 ± 7	3

[a] The external solutions were buffered to pH 8 with 10 mM Tris and H_2SO_4, and enough K_2SO_4 was added to give a total K^+ concentration of 0.30 meq/liter in each case. ATP level was measured in duplicate 1-g samples of tissue and expressed as % of control (uninhibited) samples.

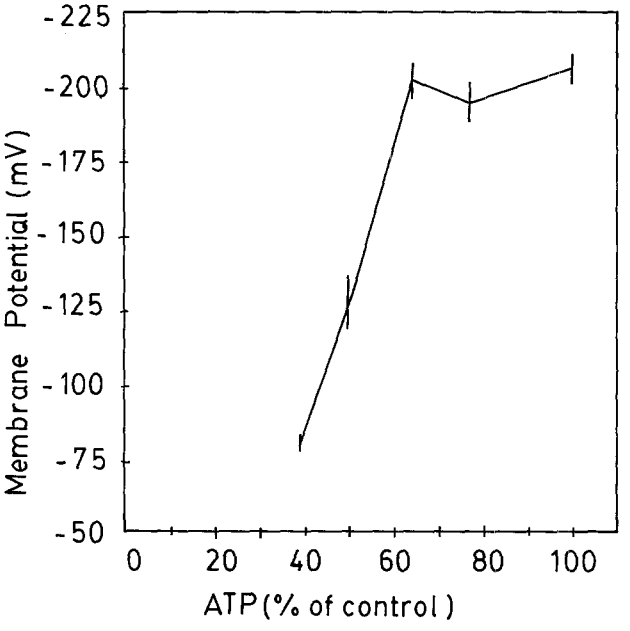

Fig. 5. Membrane potentials *vs.* ATP level. Cells were treated with solutions containing K_2SO_4, 10–15 mM Tris (pH 7.8–8.0), and the following concentrations of inhibitors: 0.01 mM KCN, 0.02 mM KCN, 0.10 mM KCN, 5 mM SHAM, 0.05 mM KCN + 5 mM SHAM, 0.10 mM KCN + 1 mM SHAM, and 0.10 mM KCN + 5 mM SHAM. The point corresponding to 100% ATP was obtained from control samples which were treated without inhibitors. Membrane potentials represent the means (± SEM) of 13, 5, 9, 11, and 13 measurements, respectively, for the points from left to right.

Membrane Potentials and $^{86}Rb^+$ Influx

Experiments were conducted to compare membrane potentials with cation influx during treatment in cyanide and SHAM. Samples of tissue used for membrane potential measurement were pretreated as in the ATP experiments; disks of tissue from the same beet were pretreated for 1 hr before being subjected to a short-term influx measurement using $^{86}Rb^+$ (*see* Materials and Methods). The results of five experiments are combined and plotted in Fig. 6. Changes in membrane potentials and cation influx do not appear to be closely parallel over the various levels of metabolic inhibition. The potential rises very steeply as influx increases from 30 to 50% of control, but thereafter the potential tends to reach a limit while the decrease of inhibition permits a large increase in the influx rate. As in the case of Fig. 5, similar results were obtained in other experiments conducted at pH 7.0 [27].

Membrane Potentials and Net K^+ Uptake

Data from three experiments in which membrane potentials and net K^+ uptake were measured in the presence of inhibitors are shown in Fig. 7. The data are insufficient to determine the significance of the gradual change in membrane potential observed at

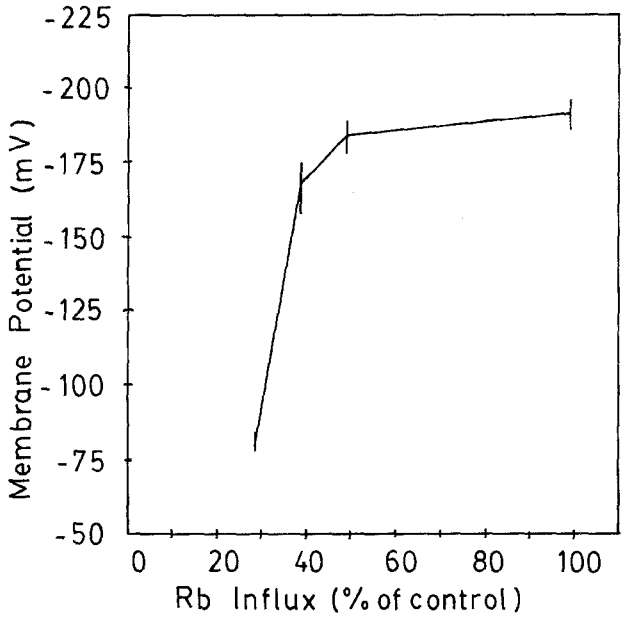

Fig. 6. Membrane potentials *vs.* $^{86}Rb^+$ influx. Disks of cells were treated with solutions containing K_2SO_4, 10–15 mM Tris (pH 8.0), and the following concentrations of inhibitors: 0.01 mM KCN, 0.02 mM KCN, 0.05 mM KCN, and 0.10 mM KCN +5 mM SHAM. The point corresponding to an influx rate of 100% was obtained from control samples which were treated without inhibitors. Membrane potentials represent mean values (±SEM) of 6, 9, 18, and 13 measurements, respectively, for the points from left to right.

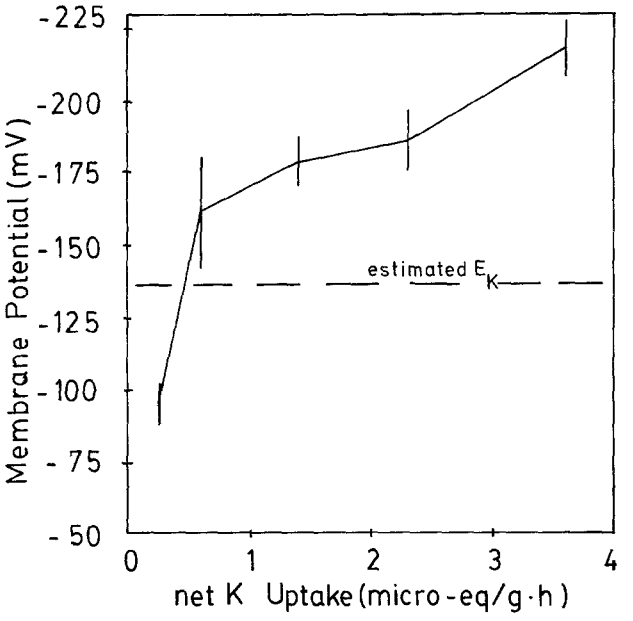

Fig. 7. Membrane potentials *vs.* net K^+ uptake. Cells were treated with solutions containing K_2SO_4, 10–11 mM Tris (pH 8.0), and the following concentrations of inhibitors: 0.01 mM KCN, 0.02 mM KCN, 0.10 mM KCN, and 0.10 mM KCN +1.0 mM SHAM. Control samples which were treated without inhibitors are included. Membrane potentials represent mean values (± SEM) of 11, 5, 8, 10, and 12 measurements, respectively, for the points from left to right.

the higher uptake rates, which is more noticeable than in Fig. 6. In any case, over the lower range of uptake rates there is a nonlinear relationship between electrical potential and net K^+ uptake, with approximately 50% of the change in potential being associated with only 10% of the change in uptake rate.

Under severe metabolic inhibition (Fig. 7) the mean electrical potential fell to 30 mV below the potassium equilibrium potential (E_K), estimated from the average intracellular K^+ concentration. Under this condition, a low but significant net uptake of K^+ was measured, indicating transport of K^+ against its electrochemical gradient. Five μM valinomycin produced transient hyperpolarizations of 2 to 14 mV in cells treated with 0.1 mM cyanide + 5 mM SHAM (results not shown). This effect of valinomycin provides further evidence that the electrochemical gradient for K^+ was in the direction favoring outward movement of K^+. It was also found that during treatment with 0.1 mM cyanide +5 mM SHAM, increasing the external K^+ concentration successively from 0.3 to 3.0 and to 30 meq/liter resulted in depolarizations of only 15 to 25 mV per 10-fold change in K^+ concentration.

Current Injection Experiments

A variety of experiments were conducted by injecting pulses of current into the cells and measuring the resulting displacement of membrane potential (*see* Materials and Methods). There have been no reports of membrane excitability in beetroot cells, and in the present study the voltage responses to injected current showed no signs of membrane excitability. Therefore, no attempt was made at voltage-clamping.

Figure 8 illustrates the current *vs.* voltage relationships obtained for two cells treated with cyanide +SHAM. The inhibitors reduced the membrane potentials from −210 to −128 mV (cell *A*) and from −218 to −107 mV (cell *B*). Hyperpolarizing pulses of 180 msec duration produced a linear change in potential with injected current, up to potentials higher than the uninhibited levels. Depolarizing pulses (data not shown) also gave a linear change in potential with approximately the same slope. The membrane resistance of the cells in Fig. 8 remained fairly constant at about 6 MΩ (cell *A*) and 27 to 32 MΩ (cell *B*). The electrode resistances for *A* and *B*, respectively, were 13.5 and 10 MΩ, and these values were constant to within 1.5 MΩ throughout the experiments.

The current-voltage properties of three other cells were studied as in Fig. 8. In each case, no decrease in membrane resistance was observed in response to increasing amplitudes of hyperpolarizing current. When the inhibitors were removed from the bathing

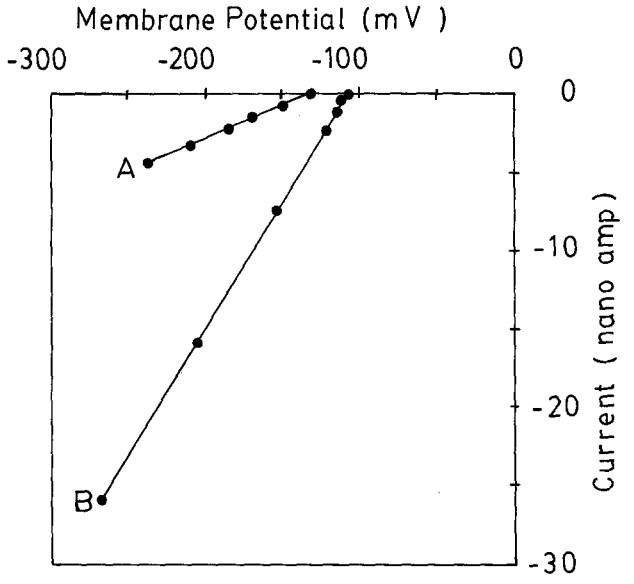

Fig. 8. Current-voltage relations for two cells (*A* and *B*) bathed in a solution containing 0.20 meq/liter K_2SO_4, 11 mM Tris (pH 8.0), 0.10 mM KCN, and 1.0 mM SHAM. The membrane potentials recorded before inhibitors were introduced to the bathing solution were −210 mV and −218 mV, respectively. Current pulses were 180 msec in duration.

Table 3. Membrane resistances measured with depolarizing and hyperpolarizing current pulses[a]

Membrane potential (mV)	Electrode resistance (MΩ)	Membrane resistance (MΩ)	
		Depolarizing pulse	Hyperpolarizing pulse
−162	18	23	26
−177	14	28	34
−213	25	16	16
−215	12	52	46
−227	16	3	3
−228	9	16	15

[a] The resistance of each of 6 cells is shown, measured with 80–100 msec pulses of 1 nA current, injected first in the depolarizing and then in the hyperpolarizing direction. The cells were bathed in 0.3 meq/liter of K_2SO_4 and 10 mM Tris (pH 8.0 with H_2SO_4).

solution, the cells repolarized to within 10 mV of the potentials obtained prior to inhibition.

Some experiments were attempted using pulse durations of 1.2–1.4 sec. These produced variable results. Apparently the sequence of long pulses caused damage to the cells, which all appeared abnormally pale under miscroscopic observation, and failed to repolarize following removal of the metabolic inhibitors from the bathing solution.

Table 3 lists the membrane resistances of uninhibited cells measured with depolarizing and hyperpolarizing current pulses of 80–100 msec duration. A current amplitude of 1 nA was used, so that the potentials were displaced by 1 mV for each MΩ of membrane resistance. For each cell, depolarizing and hyperpolarizing currents gave nearly the same resistance value; only small differences (0–6 MΩ) were observed. In addition, hyperpolarizing pulses did not give consistently lower (or higher) resistance values than depolarizing pulses.

Table 4. Membrane potentials and resistances of cells before and during cyanide inhibition[a]

No cyanide (a)		5×10^{-5} M CN^- (*b*)		Electrode resistance (MΩ) (*a*, *b*)
E (mV)	*R* (MΩ)	*E* (mV)	*R* (MΩ)	
−222	34	−126	30	22,26
−186	8	−128	5	15,18
−222	8	−87	8	23,27
−189	20	−87	18	24,24
−215	8	−150	9	18,16
−181	12	−120	14	29,33
−167	32	−94	36	20,18

[a] The resistance (*R*) of each cell and the value of the membrane potential (*E*) when the resistance was measured are shown for seven cells before (*a*) and during (*b*) treatment with 5×10^{-5} M cyanide. The electrode resistance at the time of each measurement is also indicated. Measurements were made with 80-msec pulses of 10^{-9} A of injected, hyperpolarizing current.

The resistances of several cells before and during treatment with 0.05 mM cyanide are shown in Table 4. In this case, resistance measurements were made within a few minutes of inhibition, while the membrane potentials were low. There was no significant change in membrane resistance in response to treatment with cyanide. These results differ somewhat from those of Anderson et al. [1], who reported a threefold increase in membrane resistance of root cells of *Pisum sativum* L. during treatment with 1.0 mM cyanide.

Discussion

Effect of pH on the Potential and on K^+/H^+ Exchange

The membrane potential measurements of Table 1 confirm the pH response previously observed in bicarbonate solutions [22]. In addition, extension of the pH range to pH 8 shows that, although the potential becomes more negative by 30 mV between pH 6 and pH 7, there is relatively little further change (some 9 mV) between pH 7 and pH 8. In fact, comparison with the previous data [22] suggests that the potential may reach a maximum at about pH 7.2. On the other hand, K^+/H^+ exchange may show its greatest sensi-

tivity to pH above pH 7.2 [17, 24]. Thus, there is not a close parallel between hyperpolarization and increase in K^+/H^+ exchange with increasing pH. We have shown previously [24] that the electrogenic properties of the cell membrane seem to vary with pH independently of the transport properties, but changes in membrane resistance with pH have not been ruled out.

The tendency (Table 1) for the membrane potential of storage tissues to be somewhat lower in chloride solutions than in sulfate solutions has been observed previously [3, 22], but its significance is not yet clear.

Relation of Membrane Potential to ATP Level

The large, rapid and reversible depolarizations induced by CCCP or cyanide (Fig. 2), as observed in other plant material [7, 13, 32, 34] provide evidence for an ATP-dependent electrogenic pump. More direct evidence for such a pump has been obtained in plasma membranes of *Neurospora* [28] and *Chara* [31].

A plot of membrane potential *vs.* ATP concentration (Fig. 5) shows that there is a critical range of concentrations of ATP, between 40 and 60% of the uninhibited level, at which the potential is sharply dependent on ATP concentration. At higher ATP concentrations there is little further increase in membrane potential. Slayman et al. [32] interpreted the relation between potential and ATP in *Neurospora* in terms of Michaelis-Menten kinetics. There is insufficient data to attempt this for beet cells, but the present results (Fig. 5 and ref. [27]) favor a sharper regulation of pump activity at a critical concentration of ATP, estimated at about 0.4 mM in the cytoplasm. The apparent K_m for the electrogenic pump of *Neurospora* was estimated at about 2 mM [28] but that in *Chara* appears to be saturated even at 0.05 mM ATP [31].

The sharp dependence of the potential on ATP concentration within a critical range provides a probable explanation for the spontaneous repolarization frequently observed in the presence of 0.05 mM cyanide (Fig. 3). This concentration of cyanide brings the ATP concentration within the critical range. Under these conditions, quite small increases in intracellular ATP level with time in cyanide would be sufficient to repolarize the cell.

Relation of Membrane Potential to Cation Influx

The hyperpolarization observed in beet cells at high external pH has been thought to arise from H^+ efflux, on the basis of its pH dependence and its apparent association with the net active transport of H^+ [24]. Since the uptake of K^+ is stoichiometrically related to H^+ efflux at high pH [24], it might have been expected that the potential would change in parallel with the influx of K^+ under the influence of factors affecting the activity of the H^+ pump. However, the present results, showing that the potential tends to reach a limit at ATP levels below the normal intracellular concentration, differ from the relationship between K^+ influx and ATP. Petraglia and Poole [20] found potassium influx to be linearly related to ATP level with no sign of approaching saturation at maximal ATP levels. The relationship of membrane potential to ^{86}Rb influx is shown in Fig. 6. Although ^{86}Rb cannot be regarded as a quantitative tracer for K^+ [18, 19, 37], it is believed to be transported on the same carrier in plant cells [35], and in Fig. 6 it serves to confirm the nonlinearity between the potential and the influx of cations.

Net flux of K^+ (Fig. 7) also shows a nonlinear relationship to the potential. The mechanism of K^+ transport will be considered below, but it may be noted here that its relationship to the potential is not what would be expected for K^+ diffusion. Not only are the curves of Fig. 6 and 7 in the opposite direction (concave downward) to that predicted by the Goldman equation [16, *cf.* 22] but uptake of K^+ can be observed against its electrochemical gradient (Fig. 7).

Membrane Resistance, and Relation of Membrane Potential to Electrogenic Pump Activity

The membrane potential will quantitatively reflect the activity of the electrogenic pump (i.e., the current flow through the pump) only to the extent that the passive membrane resistance is constant. This was examined in Fig. 8 and Tables 3 and 4. There is no detectable change in membrane resistance with potential (Fig. 8) in inhibited cells; no significant differences are found between the resistance of uninhibited cells measured with hyperpolarizing *vs.* depolarizing current pulses (Table 3), and there is no significant effect of 5×10^{-5} M cyanide on the membrane resistance (Table 4). These results, taken at face value, suggest that the membrane resistance does not vary in response to changes in potential, ion flux, or ATP level, and thus the membrane potential can be taken as a direct measure of electrogenic pump activity.

It is difficult, however, to accurately assess the validity of this conclusion. One question concerns the unknown intracellular location of the electrode and the possible contribution of the vacuole mem-

brane (tonoplast) to the resistance measurements. While a number of studies [6, 12, 15] indicate that the tonoplast typically has a relatively low resistance, a recent study [8] on oat coleoptile cells reports a high tonoplast resistance. If the latter were the case in beet, and if the electrode were in fact in the vacuole, the present experiments would not provide a sensitive test for changes in plasmalemma resistance.

The passive resistance properties of the membrane could also be obscured if the pump conductance were large, and were not decreased by cyanide. However, the large changes in membrane potential with rather small changes in ATP level (Fig. 5) are difficult to account for in terms of a change in the equilibrium potential of the pump, which would seem to imply an effect of ATP on pump conductance. The fact that this is not reflected in the present resistance measurements suggests that the pump conductance does not make a major contribution to the membrane conductance in beet cells.

Etherton et al [5] have pointed out that membrane resistance measurements made with a single microelectrode may be adversely affected if the electrode resistance increases on penetrating the cell, resulting in an increase in the *RC* time constant of the electrode. This can be a problem when the membrane resistance is very small compared to that of the electrode, or when the change in the electrode resistance is large. In the present study, the values of membrane resistance and electrode resistance are well within an order of magnitude, and in many instances the membrane resistance exceeds that of the electrode. Moreover, in all cases reported (Tables 3 and 4, Fig. 8) the electrode resistance did not increase by more than 5 MΩ on penetration, and in most instances the change in resistance was less (0–2 MΩ). Variations in the electrode resistance, therefore, are generally small compared to the membrane resistance and probably do not introduce significant error in the measurements. The average resistance value ($\pm$SEM) for all the cells reported in this study is 19.8 ± 3.4 ($N=14$), which compares favorably with data obtained from other higher plant material using two microelectrodes [33].

Other questions relate to the magnitude of the measured conductance and the role of intercellular coupling through plasmodesmata. By comparison of the betacyanin content of isolated protoplasts with that of beet slices, we estimate [29] that one gram of beet disks contains 4×10^6 cells. If this figure is used to estimate the ion flux in a single cell, for comparison with the measured conductance, it appears that the conductance is greater by about 100-fold. This may indicate a high degree of intercellular coupling, about which little is known [10]. Intercellular coupling would tend to invalidate the current-voltage curves of Fig. 8, but would not invalidate the comparison of membrane resistance in the presence and absence of cyanide (Table 4). Current leakage at the point of insertion of the microelectrode can also be a problem, although many of the present resistance measurements are comparable with the highest values recorded in higher plant cells [10]. We conclude that our resistance measurements are at least consistent with the assumption that the membrane potential in beet, as in *Neurospora* [11, 32], is an approximately linear measure of the electrogenic pump activity.

The Electrogenic Pump in Relation to Net Proton Efflux and Cation Influx

The present results indicate that the activity of the electrogenic pump in beet, which is thought to transport protons, shows a different pattern of dependence on ATP level and on pH than does the rate of cation/proton exchange across the cell membrane. There is evidence that K^+ influx is carrier-mediated [23] and that both net K^+ influx (Fig. 7) and net proton efflux [22] can occur against their respective electrochemical gradients. K^+ influx shows no positive correlation with the pH gradient or the proton-motive force across the plasmalemma, so there is no evidence for K^+-H^+ cotransport. It therefore seems reasonable to postulate a K^+/H^+ exchange pump. The fact that this putative K^+/H^+ pump shows a different pattern of activity than does the electrogenic pump may indicate that they are mediated by different ATPases. A similar separation of functions of these two transport processes has been observed in the yeast *Rhodotorula gracilis*, in which triphenyl tin was found to inhibit K^+/H^+ exchange without influencing the potential (M. Höfer, *personal communication*). On the other hand, K^+ uptake and the electrogenic pump are both sensitive to fusicoccin and to a number of other treatments [26]. A possible explanation of the differences and similarities between K^+/H^+ exchange and electrogenic H^+ efflux is that they may represent different modes of operation of a single pump with variable H^+/K^+ stoichiometry. The testing of this hypothesis may have to await the isolation and reconstitution of the transport activities *in vitro*.

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References

1. Anderson, W.P., Hendrix, D.L., Higinbotham, N. 1974. The effect of cyanide and carbon monoxide on the electrical potential and resistance of cell membranes. *Plant Physiol.* **54**:712
2. Cram, W.J. 1969. Short term influx as a measure of influx across the plasmalemma. *Plant Physiol.* **44**:1013
3. Cram, W.J. 1975. Relationships between chloride transport and electrical potential differences in carrot root cells. *Aust. J. Plant Physiol.* **2**:301
4. Etherton, B., Higinbotham, N. 1960. Transmembrane potential measurements of cells of higher plants as related to salt uptakes. *Science* **131**:409
5. Etherton, B., Keifer, D.W., Spanswick, R.M. 1977. Comparison of three methods for measuring electrical resistances of plant cell membranes. *Plant Physiol.* **60**:684
6. Findlay, G.P., Hope, A.B. 1964. Ionic relations of cells of *Chara australis*. VII. The separate electrical characteristics of the plasmalemma and tonoplast. *Aust. J. Biol. Sci.* **17**:62
7. Gardner, J.M., Scheffer, R.P., Higinbotham, N. 1964. Effects of host-specific toxins on electropotentials of plant cells. *Plant Physiol.* **54**:246
8. Goldsmith, M.H.M., Cleland, R. 1978. The contribution of tonoplast and plasma membrane to the electrical properties of a higher plant cell. *Planta* **143**:261
9. Goldsmith, M.H.M., Fernandez, H.R., Goldsmith, T.H. 1972. Electrical properties of parenchymal cell membranes in the oat coleoptile. *Planta* **102**:302
10. Goldsmith, T.H., Goldsmith, M.H.M. 1978. The interpretation of intracellular measurements of membrane potential, resistance, and coupling in cells of higher plants. *Planta* **143**:267
11. Gradmann, D., Hansen, U.-P., Long, W.S., Slayman, C.L., Warncke, J. 1978. Current-voltage relationships for the plasma membrane and its principal electrogenic pump in *Neurospora crassa:* I. Steady-state conditions. *J. Membrane Biol.* **39**:333
12. Greenham, C.G. 1966. The relative electrical resistances of the plasmalemma and tonoplast in higher plants. *Planta* **69**:150
13. Higinbotham, N., Anderson, W.P. 1974. Electrogenic pumps in higher plant cells. *Can. J. Bot.* **52**:1011
14. Higinbotham, N., Graves, J.S., Davis, R.F. 1970. Evidence for an electrogenic ion transport pump in cells of higher plants. *J. Membrane Biol.* **3**:210
15. Higinbotham, N., Hope, A.B., Findlay, G.P. 1964. Electrical resistance of cell membranes of *Avena* coleoptiles. *Science* **143**:1448
16. Hodgkin, A.L., Katz, B. 1949. The effect of sodium on the electrical activity of the giant axon of the squid. *J. Physiol. (London)* **108**:37
17. Hurd, R.G. 1958. The effect of pH and bicarbonate ions on the uptake of salts by disks of red beet. *J. Exp. Bot.* **9**:159
18. Jacoby, B. 1975. Light sensitivity of ^{22}Na, ^{86}Rb, and ^{42}K absorption by different tissues of bean leaves. *Plant Physiol.* **55**:978
19. Jacoby, B., Nissen, P. 1977. Potassium and rubidium interaction in their absorption by bean leaf slices. *Physiol. Plant* **40**:42
20. Petraglia, T., Poole, R.J. 1980. ATP levels and their effects on plasmalemma influxes of K^+ and Cl^- in red beet. *Plant Physiol.* **65**:969
21. Pitman, M.G. 1963. The determination of the salt relations of the cytoplasmic phase in cells of beetroot tissue. *Aust. J. Biol. Sci.* **16**:647
22. Poole, R.J. 1966. The influence of the intracellular potential on potassium uptake by beetroot tissue. *J. Gen. Physiol.* **49**:551
23. Poole, R.J. 1969. Carrier-mediated potassium efflux across the cell membrane of red beet. *Plant Physiol.* **44**:485
24. Poole, R.J. 1974. Ion transport and electrogenic pumps in storage tissue cells. *Can. J. Bot.* **52**:1023
25. Poole, R.J. 1976. Transport in cells of storage tissues. *In:* Encyclopedia of Plant Physiology, New Series. Vol. 2*A*, p. 229. Springer-Verlag, Heidelberg
26. Poole, R.J. 1978. Energy coupling for membrane transport. *Annu. Rev. Plant Physiol.* **29**:437
27. Poole, R.J., Mercier, A.J., Petraglia, T. 1980. Hyperpolarization and cation influx have different ATP requirements in red beet. *In:* Plant Membrane Transport. R.M. Spanswick, W.J. Lucas, and J. Dainty, editors. p. 499. Elsevier-N. Holland.
28. Scarborough, G.A. 1976. The *Neurospora* plasma membrane ATPase is an electrogenic pump. *Proc. Nat. Acad. Sci. USA* **73**:1485
29. Schmidt, R., Poole, R.J. 1980. Isolation of protoplasts and vacuoles from storage tissue of red beet. *Plant Physiol. (in press)*
30. Schonbaum, G.R., Bonner, W.D., Storey, B.T., Bahr, J.T. 1971. Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiol.* **47**:124
31. Shimmen, T., Tazawa, M. 1977. Control of membrane potential and excitability of *Chara* cells with ATP and Mg^{2+}. *J. Membrane Biol.* **37**:167
32. Slayman, C.L., Long, W.S., Lu, C.Y.-H. 1973. The relationship between ATP and an electrogenic pump in the plasma membrane of *Neurospora crassa*. *J. Membrane Biol.* **14**:305
33. Spanswick, R.M. 1972. Electrical coupling between cells of higher plants: A direct demonstration of intercellular communication. *Planta* **102**:215
34. Spanswick, R.M. 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
35. Sze, H., Hodges, T.K. 1977. Selectivity of alkali cation influx across the plasma membrane of oat roots. Cation specificity of the plasma membrane ATPase. *Plant Physiol.* **59**:641
36. Van Steveninck, R.F.M. 1975. The "washing" or "aging" phenomenon in plant tissues. *Annu. Rev. Plant Physiol.* **26**:237
37. West, K.R., Pitman, M.G. 1967. Rubidium as a tracer for potassium in the marine algae *Ulva lactuca* L. and *Chaetomorpha darwinii* (Hooker) Keutzing. *Nature (London)* **214**:1262

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