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Evidence for an Electrogenic Ion Transport Pump in Cells of Higher Plants

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Summary. Cyanide (CN) and dinitrophenol (DNP) rapidly depolarize the cells o oat coleoptiles (*Avena sativa L.*, cultivar Victory) and of pea epicotyls (*Pisum sativum L.* cultivar Alaska); the effect is reversible. This indicates that electrogenesis is metaboli in origin, and, since active transport is blocked in the presence of CN and DNP, perhap caused by interference with ATP synthesis, that development of cell potential may b associated with active ion transport. Additional evidence for an electrogenic pump is a follows. (1) Cell electropotentials are higher than can be accounted for by ionic diffusior (2) Inhibition of potential, respiration, and active ion transport is nearly maximal, bu a potential of −40 to −80 mV remains. This is probably a passive diffusion potentia since, under these conditions, a fairly close fit to the Goldman constant-field equatio is found in oat coleoptile cells.

In previous work it has been found that dinitrophenol (DNP) reversibl inhibits the cell electropotential (PD) of oat coleoptiles [8, 9], thus sug gesting that the potential is closely dependent upon metabolism. It ha been shown that none of eight major nutrient ions is in a passive electro chemical equilibrium; using the Nernst equation as a criterion, each io appears to be actively transported either outward (e.g., Na^+) or inwar (e.g., all anions) [15]. However, at higher external concentrations (>1 m K^+), the cell PD appears to be greater (more negative) than would b predicted from the known concentration gradients. The passive diffusiona relations are given by an equation based on the assumption of a constan field [10]

$$E=\frac{RT}{F}\ln\frac{P_K[K]_o+P_{Na}[Na]_o+P_{Cl}[Cl]_i}{P_K[K]_i+P_{Na}[Na]_i+P_{Cl}[Cl]_o} \quad (1$$

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where E is the electropotential difference; R, the gas constant; T, the absolute temperature; F, the faraday; P_K, P_{Na}, etc. are the permeability coefficients of the ions designated; and bracketed ions are the concentrations outside, o, or inside, i.

The suggestion that the resting potential is dependent on metabolic energy was made as early as 1935 by Blinks working with *Halicystis* which maintains a PD even when the sap is perfused with a solution identical to the external medium. Somewhat similar evidence was obtained in nerve by Grundfest, Kao and Altamirano [11]. More recently many authors have explicitly adopted the concept of an electrogenic ion pump and have provided data to support such an interpretation.

It is the purpose of this paper to present the evidence from previous studies, and from experiments on cyanide (CN) and DNP inhibition reported here, for the existence of an electrogenic pump in oat and pea tissue.

Methods and Materials

The tissues used were from pea epicotyls, *Pisum sativum L.* cv Alaska, and from oat coleoptiles, *Avena sativa L.* cv Victory. Pea seedlings were grown 7 days in darkness at 25 °C on vermiculite watered with nutrient solution. Oat seedlings were grown similarly for 4 days but were exposed to red light for about 3 hr during the third day. The nutrient solution had the following composition in mM: KCl, 1.0; NaH_2PO_4, 0.904; Na_2HPO_4, 0.048; $Ca(NO_3)_2$, 1.0; $MgSO_4$, 0.25. The pH was 5.8. This solution is designated as 1X and changes in its concentration to 1/10 as 0.1X or 10-fold as 10X, etc. Segments of pea epicotyl 1 cm long were taken at a distance 2 to 4 cm from the apical hook. Oat coleoptile segments 0.5 cm long were taken in the region 0.5 to 3 cm from the apex. Segments were usually pretreated in the nutrient solution 18 to 20 hr prior to use in experiments. In all experiments using CN, the nutrient solution was adjusted to pH 7 by addition of Tris and, after addition of KCN, by use of HCl. When KCN was added, corresponding amounts of KCl were omitted to keep K^+ constant; Cl^- was kept nearly constant at 2 mM by the additions of Tris and HCl. All experiments were done at 20 °C.

In the measurement of cell potentials, tissue segments were held in a Lucite chamber which could be placed on a microscope stage and which permitted a continuous flow of solution. A multiported valve was used to permit changing the ambient mixture while monitoring the potential of a single cell or after several cells had been measured. Measurements represent the PD between the vacuole and the external solution; evidence suggests that there may be no significant PD between the cytoplasm and the vacuole and thus the major barrier—and site of ion pumps—must be the plasmalemma [9]. All microelectrode insertions were made under microscopic observation. Tip potentials were kept under 15 mV and subtracted from the measurements. In some experiments, PD was measured using a Kintel 204 galvanometer and a shop-made cathode-follower, but the electrometer generally used was a Keithley 610B. It was coupled to capillary microelectrodes via calomel electrodes or Ag-AgCl wires. The microelectrodes used for sticking cells were filled with 3 M KCl and were about 1 μ or less in diameter at the tip; the independent (ground) electrode was filled with 3 M KCl in 2% agar and had a tip diameter of approximately 50 μ. It has been observed that CN may polarize elec-

trodes (R. M. Spanswick, *private communication*); although we have found such an effect with Ag-AgCl wire electrodes directly exposed to CN solution, no influence of CN at 1 mM has been observed on the microelectrode system used here.

Measurements of respiration were made using a Gilson differential respirometer to determine O_2 consumption. In order to maintain a constant CN concentration during CO_2 absorption, the method of Robbie [24] was adopted; this involved use of an appropriate $Ca(OH)_2$-$Ca(CN)_2$ solution in a side arm of the respirometer vessel. Final $Ca(CN)_2$ concentration was determined by the Liebig method [3].

Chemical analyses of K^+ and Na^+ were by flame emission using a Jarrell-Ash Model 82-700 atomic-absorption flame-emission spectrophotometer. Cl^- was assayed using an Aminco-Cotlove titrator. Isotopes were counted with a Nuclear-Chicago planchet counter and gas-flow detector.

Results

Many measurements in this laboratory have shown that at higher external salt concentrations (>1 mM K^+) the measured cell potentials exceed the value predicted by the Nernst or Goldman equations [8, 15]; the discrepancy increases with external concentration. Results with oat seedling tissue compiled from Etherton's work [8] and our own illustrate this point (Table 1); pea seedling roots and epicotyls are similar. Etherton

Table 1. *Relationship in oat seedling tissues of measured and calculated cell electropotential difference to external concentration. (Compiled from Etherton* [8], *Higinbotham et al.* [15] *and Pierce and Higinbotham* [21]*)*

Tissue	External solution concn.[a]	Cell electropotential difference[b] (mV)				
					Using Eq. (1)[c]	
		E_m	E_c	$E_m - E_c$	E_c	$E_m - E_c$
Coleoptiles	0.1X	−109	−144	+35	−116	+ 7
	1X	−105	−109	+ 4	− 89	−16
	3X	−109	− 79	−30	− 67	−42
	10X	−102	− 55	−47	− 44	−58
Root	1X	− 84	− 92	+ 8	− 79	− 5
	3X	− 89	− 71	−18	− 66	−23
	10X	− 71	− 45	−26	− 45	−26

[a] The composition of the solution as given in Materials and Methods is 1X; 0.1X is 1/10 this concentration and 10X is ten-fold, etc.

[b] Subscripts m and c refer to measured and calculated, respectively. $E_c = 58 \log \frac{K_o}{K_i}$.

[c] P_K, P_{Na}, and P_{Cl} were estimated in oat coleoptile tissue in the 10X solution [21], where P_K is 1, P_{Na} is 0.68 and P_{Cl} is 0.037. Here it is arbitrarily assumed that the ratios of the permeability coefficients remain constant at different external concentrations. Some estimates of concentration gradients are unpublished, but all are within a well-established range.

concluded, on this basis, that K^+ may be pumped out when the outer concentration is high since it does not accumulate to the degree expected from the measured electropotential gradient. It may be argued that the concentration in the cytoplasmic layer is much higher than in the vacuole and that this would account for the potentials observed. However, calculations show that with coleoptiles in 10X solution the concentration required in the cytoplasm to give a potential of -102 mV is 0.98 M; a concentration of this magnitude seems highly unlikely and would be several-fold higher than the estimates obtained using compartmental analysis techniques [21]. Therefore, using the Nernst equation or the Goldman equation as a criterion, the evidence strongly suggests that the observed potential does not arise from the diffusion of K^+, Na^+, and Cl^- [8] or from other major nutrient ions, namely Ca^{++}, Mg^{++}, NO_3^-, $H_2PO_4^-$ or SO_4^{--} [15].

Inspection of Eq. (1) shows that when K^+ is the more permeable ion, the potential predicted for the K^+ gradient alone (E_K) is higher than that calculated for the sum of the three ions (Table 1). Where the specific ion permeabilities P_{Na} and P_{Cl} increase relative to P_K, the predicted potential decreases; conversely, decreases in P_{Na} and P_{Cl} lead to larger E_c values but none greater than E_K. Consequently, changes in the permeability of these ions relative to one another within any reasonable limits does not alter the general conclusion that the observed potential is higher than predicted. We believe that the measured potential has its origin not from diffusion but rather from metabolically driven transport.

Effect of CN and DNP

An electrogenic pump may be defined as net active transport of a charged particle leading to an electropotential gradient. This could occur where the sum of cation transport differs only slightly from the sum of anion transport. Inhibition of metabolic steps which constitute the source of energy for the process should result in a rapid depolarization; if membrane permeability is not impaired, the effect should be reversible. On the other hand, blocking ion transport which is nonelectrogenic or inducing a partial impairment of the membrane would lead to a slow loss of PD as concentrations in and out tend to become equal. Treatment with CN produces a dramatic reversible depolarization of pea epicotyl cells as shown in Fig. 1. Recovery is not as rapid as depolarization; it is somewhat slower than the recovery of potential blocked by azide *Neurospora crassa* [27]. However, it is possible in the multicellular tissue used here either that the CN is not readily removed by the flowing solution, or that the supply of energy, once blocked, requires time to be renewed. Results similar to those with

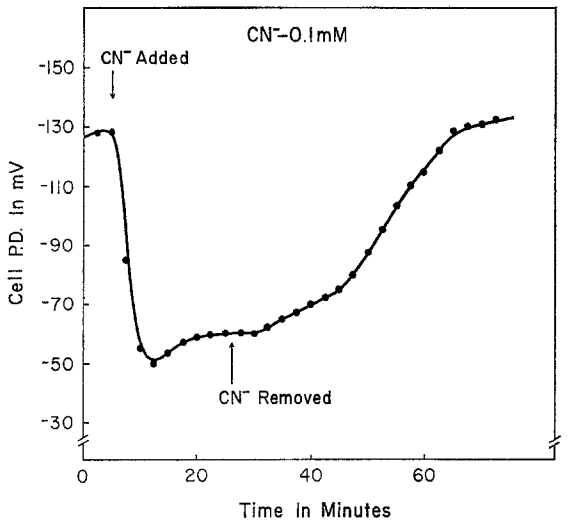

Fig. 1. The time course of cell potential inhibition by CN and recovery. Pea epicotyl tissue

Table 2. *Inhibition by CN or DNP and subsequent recovery of cell potential after removal of inhibitor.* 1X *nutrient solution*

Inhibitor	Concn. (mM)	Tissue	No. of cells	Cell potential[a] (mV)			% Inhibition
				Initial	Inhibited	Recovery	
CN	10	Oat coleoptile	6	102±19	33±22	76±28	67 ±13
	1		5	119±25	42±12	114±22	65.5± 7
	1	Pea epicotyl	2	120± 0	63±33	117± 3	47 ±28
	0.1		8	142±10	80±23	143±11	44 ±14
	0.1		10	140±10	64± 8	138± 8	54 ± 5
DNP	0.1	Oat coleoptile[b]	6	120±19	90±20	113±22	25
	0.1	Pea epicotyl[c]	10	117±19	—	—	
			9	—	32± 7	—	73
			5	112± 9			
			10	—	26± 7	—	77

[a] Mean ±SD.

[b] Cells exposed to DNP for short periods of time (3–13 min) to observe recovery. Longer periods of inhibition gave a greater effect but less complete recovery.

[c] Reversal of inhibition in single cells was not attempted. The period of inhibition was about 0.5–1 hr.

pea have been obtained with oat coleoptiles. DNP, as previously reported [9, 14], also reduces the cell potential but the recovery is less rapid than with CN. There is evidence that DNP may impair the membrane [20, 25]. However, at a concentration of 0.1 mM, DNP rapidly inhibits the PD, and this is reversible (Table 2). This suggests that there is no irreversible effect

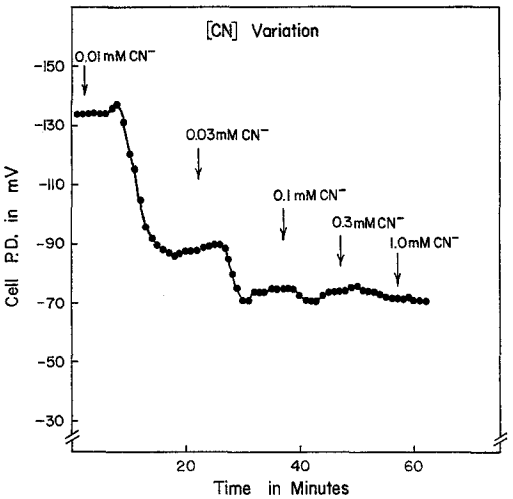

Fig. 2. Increasing the concentration of CN depolarizes a cell, but the effect is maximal (or nearly so) at 0.1 mM. Pea epicotyl tissue

on the membrane and that ATP may be involved in electrogenesis since DNP also blocks ion accumulation [13, 25]. It is possible that the leakiness observed with DNP may result, at least in part, from its effect on the electropotential gradient.

Relation of CN Concentration to Potential

The results suggest that the measured cell potential is the sum of a diffusion potential and a potential arising from an active transport process. Inhibition of an electrogenic active transport – without affecting semipermeability – should block the potential arising metabolically but not the portion of the potential arising from diffusion. Therefore, with increasing concentration over a suitable range, CN should show a saturation effect; at this point increasing the concentration should give no further decrease in potential arising from diffusion. Figs. 2 and 3 indicate that the predicted response occurs. In typical experiments, the maximum inhibition in pea epicotyl cells was (mean $\pm$SD) $53.8 \pm 5.3\%$ (at 0.1 mM CN) and in oat coleoptile cells $65.5 \pm 6.9\%$ (at 1.0 mM CN). The results of several tests showing reversibility are summarized in Table 2.

Effect of CN on Ion Uptake and Respiration

It is well known that CN blocks respiration and ion accumulation. Here we have attempted to relate this effect to the inhibition of potential in pea

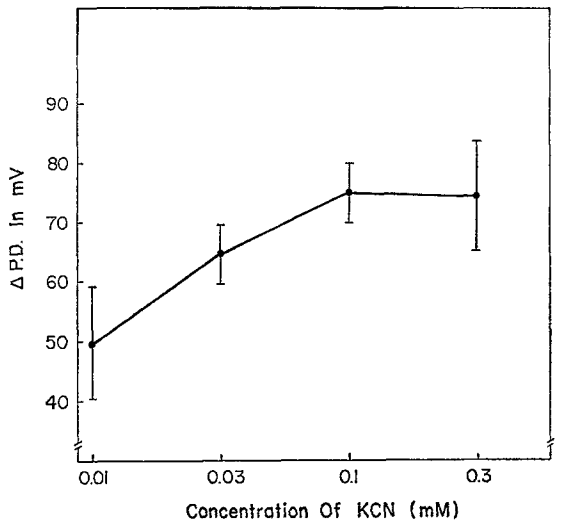

Fig. 3. The relationship of CN concentration to the amount of depolarization (*ΔPD* in mV) induced. The points represent means and the vertical bars indicate standard deviations. Pea epicotyl tissue

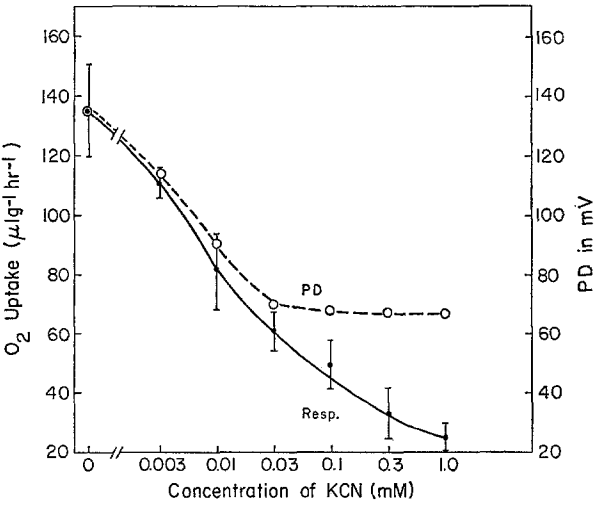

Fig. 4. The effect of CN concentration on PD and respiration. Vertical bars represent standard deviations

epicotyl tissue. As can be seen in Fig. 4, the degree of inhibition is about the same for potential and respiration over the concentration range 0.003 to 0.03 mM CN. However, at higher CN concentrations, cell PD is not further depressed but respiration is. The actual amount taken up by control tissue (apparent uptake measured by tracer) over the 4-hr period was, in μequiv g^{-1} hr^{-1}, 7.5 K^+ and 0.36 Cl^-.

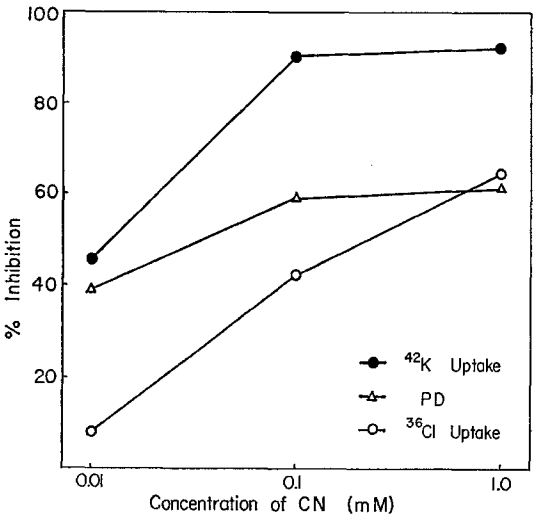

Fig. 5. Percent inhibition of labeled K^+ and Cl^- uptake and of cell potential as related to CN concentration. Vertical bars represent standard deviations. In the 4-hr period, the average apparent net influx of labeled K^+ and Cl^- in tissue not treated with CN was 1.9 and 0.21 μequiv g^{-1} hr^{-1}, respectively. In this series, prior to being treated with CN, the cells had an average potential of 139 ± 10 mV ($N=25$). The PD measurements were on pea epicotyl segments comparable to those used in tracer uptake

The depolarization induced by CN might possibly be explained by a marked increase in anion permeability. However, if this occurred, it is reasonable to expect faster rates of tracer Cl^- exchange unless the anion influx is preponderantly pump-controlled. The evidence here fails to indicate any increase in P_{Cl} but rather only a reduction of Cl^- uptake as CN concentration is raised. In view of the data showing that CN at moderate concentrations does not result in leakage of ions [25], we are disinclined toward the view that depolarization is a result of selective permeability. The effect on Cl^- uptake seems explicable as interference with active absorption.

The relation of cell potential to tracer uptake is shown in Fig. 5. The inhibition of tracer K^+ uptake over a 4-hr period is nearly parallel to the effect of PD and is greater than the depression of tracer Cl^- uptake. In the tracer uptake experiments, HCN in the gas phase was not controlled (as in the respiration measurements); thus there may have been some loss of CN concentration. Nonetheless, these data suggest that K^+ uptake is largely related to cell potential whereas Cl^- uptake seems more nearly parallel to the effect on respiration. The chemical content of K^+, Na^+, and Cl^- in the tissue at 0.1 mM CN remained nearly steady over a 4-hr

period; at lower CN concentrations there was some net gain, but at higher CN concentration net loss occurred. After 4 hr in 0.1 mM CN (in 1X solution), the content of K^+ in the tissue was 43.5 ± 0.5 μequiv g^{-1} (fresh wt) compared to 48.4 ± 0.5 μequiv g^{-1} in the control; Cl^- was 2.93 ± 0.13 μequiv g^{-1} compared to 3.09 ± 0.02 μequiv g^{-1} in the control. In view of the low concentration of Cl^- in the tissue, it seems likely that there may be a greater effect of CN on other anions, e.g., NO_3^- and $H_2PO_4^-$, which are at higher internal concentrations. These data do suggest that there is no pronounced leakage of Cl^- and thus, presumably, no large change in P_{Cl}. In the case of K^+, the net uptake rate chemically assayed was 3.7 μequiv during the period of the experiment so that reduction of influx could account for most of the difference, again not necessarily requiring a change in permeability.

Discussion

The evidence presented here strongly supports the hypothesis that electrogenesis in pea and oat shoot cells is a result, in part, of a metabolically driven ion pump and, in part, of diffusion. If this is correct, then, with the active transport blocked, Eq. (1) should be satisfied. It is possible to test the fit approximately, since, for oat coleoptile tissue – in a 10X nutrient solution (10-fold that used here) – the permeability coeffcients of K^+, Na^+, and Cl^- have been estimated. For the plasmalemma, taking P_K as unity, P_{Na}/P_K is 0.68 and P_{Cl}/P_K is 0.038 [21]. Similar values for NO_3^- and $H_2PO_4^-$ are not available, but it seems unlikely that they are far different from P_{Cl}. Ca^{++}, Mg^{++}, and SO_4^{--} probably have quite low permeabilities and are omitted. We then have the following relationship:

$$E_D = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}\Sigma([Cl]_i + [NO_3]_i + [H_2PO_4]_i)}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}\Sigma([Cl]_o + [NO_3]_o + [H_2PO_4]_o)} \quad (2)$$

where E_D is the diffusional potential, i.e., the cell potential remaining after maximum inhibition with CN; the other symbols are as before. For oat coleoptiles, the average E_D for five cells in 1 mM CN was -42 mV. Tissue concentration values for the 10X solution at 24 hr under conditions approximating those for which permeabilities were estimated are available from previous work [15]. In 10X solution, the internal concentrations were, in mM, $K^+ = 79$, $Na^+ = 24$, $Cl^- = 42$, $NO_3^- = 61$, and $H_2PO_4^- = 19$. Using these values, and correcting for activities, Eq. (2) predicts a diffusion potential of -39 mV which is close to the potential under CN inhibition.

In a 1X solution (as used here), the internal concentrations were, in the same order, 42, 4, 15, 18, and 9, and a diffusion potential of -66 mV is predicted; i.e., $E_c(=E_D)$ exceeds E_m in contrast to the results previously reported (Table 1). In these calculations, it is assumed that the ions are equally distributed between the cytoplasm and vacuole. Higher concentrations in the cytoplasm, which are likely [21], would tend to increase E and could explain the cases in which E_m (e.g., under CN inhibition) exceeds the calculated E_D. It is unlikely that the permeabilities remain constant over a wide range of external concentrations; Cram [5] reports evidence with carrot tissue suggesting that P_{Cl} increases with external concentration of Cl^-. Thus rigorous testing of Eq. (2) will require measurement of all the parameters under identical conditions. However, the present data, incomplete as it is, strongly support the hypothesis that only part of the PD can be explained by passive diffusion and that the remainder is metabolic in origin.

The evidence supporting the hypothesis of an electrogenic pump is as follows. (1) Measured cell potentials exceed the values predicted from the diffusion gradients of any ions measured when external concentrations are above 1 mM. (2) CN and DNP cause a rapid but reversible depolarization in accord with predictions if active ion transport is electrogenic. (3) Inhibitions of cell potential and respiration are nearly parallel over a wide range of CN concentration. (4) Inhibition of cell potential is maximal at about 0.1 mM CN, but a residual potential remains. (5) At maximal inhibition of the cell potential, there is a reasonable fit to the Goldman constant-field equation for the few cases in which it can be tested.

The rapidity of depolarization, even at low CN concentration (0.003 mM), suggests that the process affected is in the plasmalemma. A comparison of the rapid time course of respiration and depolarization could provide evidence on this point. If depolarization is faster than inhibition of respiration, the site affected must be in the plasmalemma; this would also suggest that the pump is dependent upon an ATPase and, perhaps, a cytochrome system.

In recent years, several other reports of electrogenic pumps have appeared. Slayman [27] found that the cell potential of *Neurospora* is rapidly depolarized by azide, DNP, CO, and anoxia; the effect is reversible and electrical resistivity is not affected. The percentage inhibition in azide was about 65%, which is close to the value found here for oat cells in CN solution. Hope [16] has produced evidence with *Chara australis* for an electrogenic anion influx pump which is dependent on photosynthesis and is reversibly inhibited by agents blocking photosynthetic processes. Barr [2]

has concluded that, under normal conditions, the potential of *Nitella clavata* arises from an electrogenic chloride pump. Other evidence for electrogenic ion pumps in giant celled algae has been cited [12]. In higher plants, the potential difference between the xylem exudate of an excised root (of corn) and the external medium is partially abolished by CN or DNP; in the presence of CN, depolarization with increasing concentration of KCl is in accord with predictions of the Nernst equation [7]. Cummins and Vaughan [6] found evidence for an electrogenic pump in rat stomach tissue; the potential is linearly dependent on Na^+ concentration and sensitive to DNP and ouabain. In snail nerve cells, Kerkut and Thomas [17] report an electrogenic pump with hyperpolarization being dependent upon the inner Na^+ concentrations and on the presence of external K^+; it is inhibited by ouabain and parachloromercuribenzoate. CN and DNP abolish action potentials in rat nerve [26], and, in the mollusc *Aplysia*, synaptic actions may activate a Na^+ electrogenic pump [22]. Other reports of electrogenic pumps have appeared [1, 18, and others]. The evidence for coupling of electrogenesis and metabolically driven ion pumps is now broadly based.

In animal tissue, the Na ion is frequently the subject of the electrogenic pump [1, 6, 17, 18, 22]. In plants a Na efflux pump exists [15, 21, etc.] but is probably not essential physiologically since many plants can be grown in the absence of Na; however, in nature Na^+ is almost universally present and the efflux pump may explain the high selectivity for K^+ relative to Na^+ in salt accumulation by most plants. There is evidence that HCO_3^- transport may be electrogenic [16, 23]; however, Spanswick [28] has provided data indicating that the hyperpolarization induced by HCO_3^- is an effect of pH and is essentially abolished with effective buffering. Kitasato [19] provides evidence that H^+ fluxes are appreciable in *Nitella* and that H^+ may be actively extruded. Higinbotham *et al.* [15] found that in oat and pea seedling tissues, each of four anions (NO_3^-, Cl^-, $H_2PO_4^-$, and SO_4^{--}) is actively accumulated; the process could be electrogenic. This does not preclude the existence in higher plants of an electrogenic H^+ extrusion mechanism or similar process for Na^+, Ca^{++}, and perhaps other cations. Future work should be directed toward quantifying the active transport term, identification of the ion pumped electrogenically, and investigation of the chemical nature of the site.

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