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ATP Levels and their Effects on Plasmalemma Influxes of Potassium Chloride in Red Beet[1]

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ABSTRACT

Tissue ATP concentrations in slices of red beet increase progressively with time for up to 7 days after cutting the root. ATP levels are higher in slices taken from stored roots than in slices from fresh roots. ATP is reduced during incubation in salt solutions.

Plasmalemma influxes were measured by 6 minutes incubation with ^{42}K and/or ^{36}Cl under conditions in which cation and anion influxes are independent. Both fluxes showed an approximately linear correlation with ATP level when the latter was varied by addition of CN^-, carbonyl cyanide *m*-chlorophenylhydrazone, or oligomycin, except for Cl influx with oligomycin, where oligomycin had a greater effect on Cl influx than could be accounted for by the reduction in ATP alone.

These results support the view that K^+ and Cl^- influxes are both energized directly or indirectly by ATP, and in addition that the Cl pump may be directly inhibited by oligomycin.

The manner in which metabolic energy is supplied to ion transport processes in plant cells is not well established. It is generally agreed that cation influx can be coupled to ATP hydrolysis (13), although the manner of coupling, and the relationship of cation influx to electrogenic H^+ efflux, remains in doubt. In the present study, the dependence of K^+ influx on ATP concentration has been investigated in more detail. We show in another report (14) that the present relationship between influx and ATP concentration is different from that observed for the electrogenic pump activity.

With regard to anion influx (13), there is some evidence for its dependence on ATP but contrary claims have also been made, especially for storage tissues (2, 11). The present study shows that in storage tissue of red beet Cl^- influx shows a dependence on ATP concentration similar to that observed for K^+ influx. Evidence is also presented that, in addition to its effect on ATP levels, oligomycin inhibits the Cl^- pump at the plasmalemma.

MATERIALS AND METHODS

Plant Material. Disks about 0.8 mm thick and 4 mm diameter were cut from storage tissue of red beet (*Beta vulgaris* L.), and washed in 0.1 mM aerated $CaSO_4$, changed daily, at 22-24 C. The washing was continued for 5-7 days, except where otherwise indicated, in order to allow development of Cl^- transport (12).

Uptake Solutions. Except where different solutions are compared in Table I, ion influx was measured in all cases from a solution of 0.3 mM KCl + 10 mM Tris adjusted to pH 8 with H_2SO_4 after equilibration with air. Metabolic inhibitors were added as indicated below. This influx solution was chosen to ensure that cation and anion influxes were independent of each other (12). In addition, in this ion concentration range, isotope influx is limited by plasmalemma fluxes rather than by tonoplast fluxes (4). Influx was measured after a 1-h pretreatment in the corresponding unlabeled solution. After this pretreatment, it was found that influx rates, ATP levels, and membrane potentials were all steady with time in the presence of various inhibitors.

Influx Measurements. Influx rates were estimated by a 6-min exposure to ^{42}K and/or ^{36}Cl at 25 C followed by a 6-min wash in unlabeled solution, also at 25 C, following which the tissue was immediately frozen in liquid N_2 prior to isotope and ATP measurements. (One g tissue was given three changes of 5 ml isotope solution, 2 min each, followed by three changes of 5 ml wash solution, 2 min each.) With these time periods, the isotope uptake is considered to give a reasonable estimate of influx across the plasmalemma (3).

ATP Assay and Isotope Counting. Extraction and assay of ATP was according to Atkinson *et al.* (1) with modifications as indicated below. One g beet disks frozen in liquid N_2 were disrupted in 40 ml ice-cold 0.4 N $HClO_4$ + 1 mM EDTA disodium salt, in a Virtis homogenizer for 1 min. One-half of the homogenate was taken for isotope counting. This was decolorized with activated charcoal (2.5 ml of a 10% suspension in water), and centrifuged 10 min at 15,000*g*. Eight-ml samples of supernatant + 10 ml Aquasol (New England Nuclear) were counted in a scintillation counter, and then recounted for ^{36}Cl after decay of the ^{42}K.

The remainder of the tissue homogenate was taken for ATP assays after centrifugation at 10,000*g* for 15 min to sediment debris. Immediately before the ATP assays, 5 ml of the supernatant were added to 5 ml 40 mM glycylglycine buffer (pH 7.4) and the mixture was adjusted to pH 7.4 with 1 M KOH + 0.1 M $KHCO_3$. The extract was then centrifuged at 5,000*g* for 5 min to remove $KClO_4$ and ATP assays were performed without delay. The luciferin-luciferase ATP assays were performed without delay. The luciferin-luciferase ATP assays were performed as described by Schram (15) with a few modifications. The content of one vial of firefly extract (Sigma FLE-50) was dissolved in 5 ml cold distilled H_2O. This reconstitution of the firefly extract gives a final concentration of 50 mM potassium arsenate and 20 mM $MgSO_4$ (pH 7.4). This enzyme solution was agitated occasionally and incubated on ice for 2-5 h. When ready for use, the enzyme solution was centrifuged 10 min at 5,000*g*. The supernatant was either used immediately in the ATP assay or stored frozen for future use.

To scintillation vials containing 700 μl glycylglycine buffer were added 100 μl of the enzyme solution prepared as described above. The vials were swirled vigorously and after 15 s, either 200 μl tissue extract (diluted 5- or 10-fold) or 200 μl ATP standard (5-25

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pmol) were added to each vial. The vials were swirled vigorously for another 15 s, then placed in a scintillation counter set in noncoincident mode to count the samples repeatedly for 0.1-min intervals. The readings for the standard curve and experimental values were taken from the earliest interval after which a smooth decay with time was observed. This was usually the third 6-s interval. In all experiments, residual luminescence due to endogenous ATP in the original FLE-50 was subtracted from the final counts.

As a control, in some cases 20 nmol ATP were added to the surface of the beet tissue immediately before freezing in liquid N_2. Subsequent assay showed complete recovery of this ATP in the tissue homogenate, within the limits of experimental error.

RESULTS

Factors Affecting ATP Levels. Data from a number of experiments are collated in Table I to illustrate the range of ATP concentrations observed in the absence of inhibitors, and some of the factors which affect ATP levels. The values range from about 10–50 nmol ATP/g beet disks. If the ATP is confined to the cytoplasm, and if the cytoplasm occupies about 5% of the tissue volume (10), this corresponds to an ATP concentration of 0.2–1.0 mM. The ATP content increases steadily with the number of days of washing of the tissue after slicing. It is higher in slices from roots which have been stored for some months. Beet disks which have been incubated for 1 h in salt solutions have lower levels of ATP than do disks incubated in water. This decrease in ATP appears to be related to the rate of influx of ions, which was much higher in solution B (2.5 mM KCl + 1.25 mM $CaCl_2$ [pH 6]) than in solution A (0.3 mM KCl + 10 mM Tris SO_4 [pH 8]). For example, in slices from new beets washed for 6 days, K^+ and Cl^- influxes in solution A were 1.62 and 0.53 μeq/g·h, respectively, with an ATP level of 20.6 nmol/g, whereas K^+ and Cl^- influxes in solution B were 9.2 and 8.7 μeq/g·h, respectively, with an ATP level of 13.2 nmol/g. Although these data are consistent with the expectation that ion accumulation consumes ATP, they do not distinguish between cation and anion fluxes or between plasmalemma and tonoplast transport.

Correlation of Influx with ATP Levels in Presence of Inhibitors. Figure 1 shows the plasmalemma influx of Cl^- plotted against tissue ATP concentration in four separate experiments in which the ATP level was varied by the addition of a range of concentrations of CCCP[2] (0–15 μM). The highest value for ATP in each set of points indicates the ATP level in the absence of inhibitor. This control level varies from 34 to 49 nmol/g (Fig. 1). The influx varies even more widely, from 0.27 to 2.22 μeq/g·h in the absence of inhibitor. The variation in influx cannot be attributed simply to the variation in ATP level: the activity of the transport system varies from one batch of tissue to another. No attempt was made to control this variability, since within each experiment influx varied with ATP level in a consistent and approximately linear manner. To compare a number of experiments, the data were therefore normalized by plotting both influx and ATP level as a percentage of the control value in the absence of inhibitor. (The variability of control levels of influx shown in Fig. 1 represents an extreme case. Most of the experiments gave control values for Cl^- influx between 0.5 and 0.9 μeq/g·h, whereas control values for K^+ influx were between 2 and 4 μeq/g·h.)

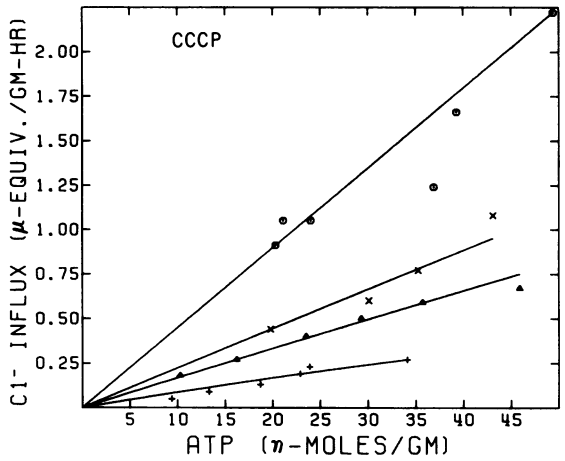

FIG. 1. Relationship of Cl^- influx to ATP content in four experiments, each using a range of concentrations of CCCP (0–15 μM). Each experiment is represented by a different symbol. The point with the highest level of ATP in each case represents the treatment without inhibitor.

Figure 2 combines data from a number of experiments showing influx *versus* ATP concentration, as a percentage of the uninhibited values, for K^+ and for Cl^- in the presence of CN^- (0–20 μM), CCCP (0–15 μM), and oligomycin (0–15 μM). For each ion, a straight line was fitted to the CN^- values by the least-squares method, and the same (CN^-) line was then drawn through the points for the other inhibitors, to facilitate comparisons between inhibitors. In the presence of CN^-, K^+ influx varies with ATP level in a linear manner within the limits of experimental error. K^+ influx in CCCP solutions may be a little lower than in CN^- for comparable levels of ATP, but the difference may not be significant. In the presence of oligomycin, the correlation between K^+ influx and ATP level follows closely that observed with CN^-. Since these two inhibitors have different presumed modes of action, the similar correlation with ATP levels supports the widely held view that cation influx from low external concentrations is dependent on ATP hydrolysis. The fact that K^+ influx is approximately linear with ATP level suggests that the normal (uninhibited) level of ATP (30–50 nmol/g in these experiments) is not sufficient to saturate the transport ATPase.

Cl^- influx in Figure 2 also varies with ATP level in a roughly linear manner, in the presence of either CN^- or CCCP, and there is no apparent difference between the effects of these inhibitors. The data for CN^- and CCCP thus provide evidence for the dependence of Cl^- influx on ATP rather than, for instance,

Table I. *Factors Affecting ATP Content of Beet Disks*

"New beet" refers to material used within 1 month of harvesting. "Stored beet" refers to roots stored for 3–6 months in moist Vermiculite at 7 C. ATP levels were measured in extracts from beet disks frozen in liquid N_2 after 1 h of incubation at 25 C in the solutions indicated. Solution A is 0.3 mM KCl + 10 mM Tris SO_4 (pH 8). Solution B is 2.5 mM KCl + 1.25 mM $CaCl_2$ (pH 6).

Time of Washing after Slicing	ATP Content					
	New Beet			Stored Beet		
	H_2O	Solution A	Solution B	H_2O	Solution A	Solution B
days	*nmol/g beet disks*					
1		11.8			21.3	
3		15.1			26.7	
4	31.9	19.5	19.1	47.9	32.9	18.7
		18.7			27.8	
6		20.6	13.2		41.2	
		24.5			46.6	
7		33.0			47.9	

[2] Abbreviation: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone.

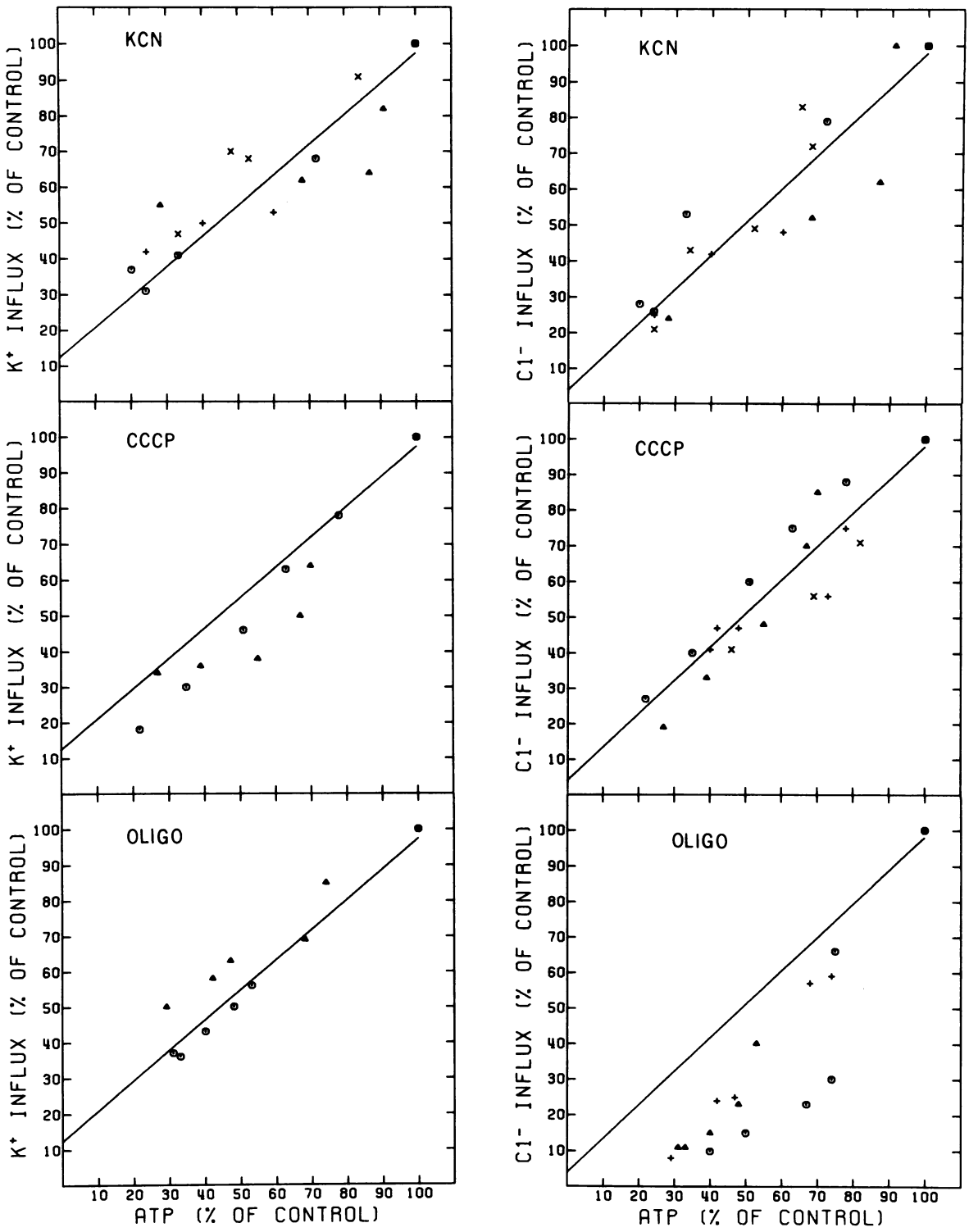

FIG. 2. Combined data on influx of K^+ and of Cl^- *versus* ATP content, in the presence of various concentrations of CN^- (0–20 μM), CCCP (0–15 μM), or oligomycin (0–15 μM), expressed as a percentage of the values in the absence of inhibitor. Within each graph, each symbol represents a different experiment. For each ion, a straight line was fitted to the CN^- values by the least-squares method, and the same (CN^-) line was drawn on the graphs for the other inhibitors.

electron transport (*cf.* 3, 11). In the case of Cl^- influx in the presence of oligomycin, however, the pattern is clearly different than with the other inhibitors. Oligomycin inhibits Cl^- influx to a greater extent than can be accounted for by its effect on ATP levels.

In Figure 3, the additional effect of oligomycin on Cl^- influx is illustrated by plotting influx in the presence of oligomycin as a percentage of the influx in CN^- or CCCP solutions giving the same level of ATP. In this way, we have subtracted the component of the oligomycin effect which is due to changes in ATP level. The remaining component of oligomycin inhibition, seen in Figure 3, is attributed to a direct effect of oligomycin on the plasmalemma

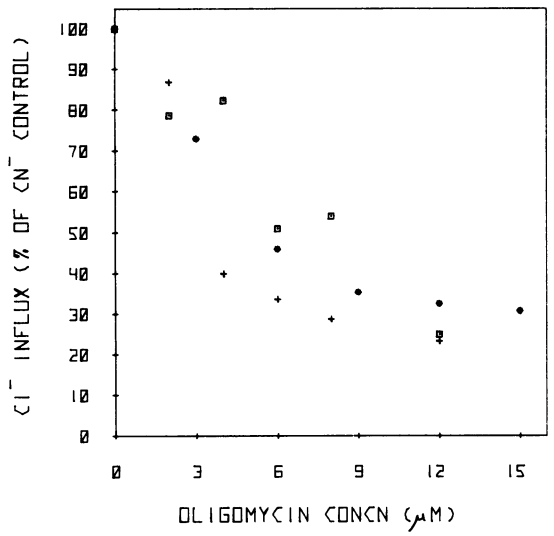

FIG. 3. Direct inhibition of Cl^- influx by oligomycin. Influx of Cl^- in the presence of oligomycin (0–15 μM) is plotted as a percentage of the influx in CN^- or CCCP solutions giving the same level of ATP. Data are taken from Figure 2.

Cl^- transport system. The inhibition increases with increasing oligomycin concentration, and the oligomycin concentration giving 50% inhibition is about 6 μM. No such effect is observed on K^+ transport, even though in some experiments both fluxes were measured simultaneously by double-labeling with ^{42}K and ^{36}Cl.

DISCUSSION

Validity of Measurements. The conditions of these experiments were chosen to ensure that cation and anion uptake are independent of each other, and that the isotope uptake is mainly determined by the rate of influx at the plasmalemma. Another question is whether, during the 1-h pretreatment in the uptake solutions, the plasmalemma influxes could be influenced by ATP-dependent changes in tonoplast transport. In an accompanying paper on the effects of anoxia (9) we show that: (*a*) inhibition of cation and anion influx under N_2 atmosphere is entirely accounted for by the reduction in ATP level; and (*b*) that the inhibition occurs within approximately 1 min, which is too short a time for changes in tonoplast transport to affect the plasmalemma, at least via changes in cytoplasmic ion concentrations.

Dependence of Influx on ATP. The present results indicate that the plasmalemma influxes of both K^+ and Cl^- are dependent on ATP, presumably as an energy source, but give no direct evidence for the manner of coupling to ATP hydrolysis. The relationship between influx and ATP level is approximately linear for both ions, suggesting that the normal cytoplasmic level of ATP, estimated at 0.6–1 mM, is not sufficient to saturate the transport ATPase. The K^+-stimulated ATPase in membrane preparations from oat roots has a K_m value for ATP which varies according to experimental conditions, but is approximately 1 mM (5).

Oligomycin Inhibition of Anion Transport. In addition to its effect on ATP levels, oligomycin has an inhibitory effect on Cl^- influx at the plasmalemma, which is not seen with K^+ influx. A number of other reports offer evidence for a specific effect of oligomycin on anion transport at the plasmalemma of plant cells. Jacoby and Plessner (6) show that oligomycin inhibits Cl^- influx more than K^+ influx in excised barley roots. Lin and Hanson (7) showed that oligomycin inhibits phosphate influx in corn roots to a greater extent than can be accounted for by its effect on ATP levels, and Malone *et al.* (8) gave histochemical evidence for an oligomycin-sensitve ATPase at the plasmalemma of corn roots.

The K^+-stimulated fraction of ATPase activity of plant membranes is not sensitive to oligomycin (5), and when oligomycin sensitivity is found it is usually attributed to mitochondrial contamination. Our results suggest, however, that an oligomycin-sensitive anion-ATPase may be a true component of the plasmalemma. Since the mitochondrial ATPase can show sensitivity to anions as well as to oligomycin (C. Grubmeyer, personal communication) it remains a problem for the future to distinguish these enzymes.

LITERATURE CITED

1. ATKINSON MR, G ECKERMANN, M GRANT, RN ROBERTSON 1966 Salt accumulation and adenosine triphosphate in carrot xylem tissue. Proc Nat Acad Sci USA 55: 560–564
2. CRAM WJ 1969 Respiration and energy-dependent movements of chloride at plasmalemma and tonoplast of carrot root cells. Biochim Biophys Acta 173: 213–222
3. CRAM WJ 1969 Short term influx as a measure of influx across the plasmalemma. Plant Physiol 44: 1013–1015
4. CRAM WJ, GG LATIES 1971 The use of short-term and quasi-steady influx in estimating plasmalemma and tonoplast influx in barley root cells at various external and internal chloride concentrations. Aust J Biol Sci 24: 633–646
5. HODGES TK 1976 ATPases associated with membranes of plant cells. *In* U Lüttge, MG Pitman, eds, Encyclopedia of Plant Physiology, Vol 2A. Springer-Verlag, Heidelberg, pp. 260–283
6. JACOBY B, OE PLESSNER 1970 Oligomycin effect on ion absorption by excised barley roots and on their ATP content. Planta 90: 215–221
7. LIN W, JB HANSON 1974 Phosphate absorption rates and adenosine 5′-triphosphate concentrations in corn root tissue. Plant Physiol 54: 250–256
8. MALONE CP, JJ BURKE, JB HANSON 1977 Histochemical evidence for the occurrence of oligomycin-sensitive plasmalemma ATPase in corn roots. Plant Physiol 60: 916–922
9. PETRAGLIA T, RJ POOLE 1980 Effect of anoxia on ATP levels and ion transport rates in red beet. Plant Physiol 65: 973–974
10. PITMAN MG 1963 The determination of the salt relations of the cytoplasmic phase in cells of beetroot tissue. Aust J Biol Sci 16: 647–668
11. POLYA GM, MR ATKINSON 1969 Evidence for a direct involvement of electron transport in the high-affinity ion accumulation system of aged beet parenchyma. Aust J Biol Sci 22: 573–584
12. POOLE RJ 1976 Transport in cells of storage tissues. *In* U Lüttge, MG Pitman, eds, Enclopedia of Plant Physiology, Vol 2A. Springer-Verlag, Heidelberg, pp 229–248
13. POOLE RJ 1978 Energy coupling for membrane transport. Annu Rev Plant Physiol 29: 437–460
14. POOLE RJ, AJ MERCIER, T PETRAGLIA 1979 Hyperpolarization and cation influx have different ATP requirements in red beet. *In* RM Spanswick, WJ Lucas, J Dainty, eds, Plant Membrane Transport. Elsevier/North Holland, Amsterdam. In press
15. SCHRAM E 1970 Use of scintillation counters for bioluminescence assay of ATP. *In* ED Bransome, ed, The Current Status of Liquid Scintillation Counting. Grune & Stratton, New York, pp 129–133