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Phloem Loading of Sucrose

INVOLVEMENT OF MEMBRANE ATPase AND PROTON TRANSPORT[1]

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ABSTRACT

p-Chloromercuribenzenesulfonic acid markedly inhibited sucrose accumulation into sugar beet source leaves without inhibiting hexose accumulation. The site of inhibition is proposed to be the plasmalemma ATPase, since the ATPase-mediated H^+ efflux was completely inhibited by p-chloromercuribenzenesulfonic acid under conditions where intracellular metabolism, as measured by photosynthesis and hexose accumulation, was unaffected. Fusicoccin, a potent activator of active H^+/K^+ exchange, stimulated both active sucrose accumulation and proton efflux in the sugar beet leaf tissue. These data provide strong evidence for the phloem loading of sucrose being coupled to a proton transport mechanism driven by a vectorial plasmalemma ATPase.

Inasmuch as the cellular events operating in exporting leaves govern translocation (3, 7, 8, 12), their elucidation at the mechanistic level is of considerable importance in understanding assimilate transport. Phloem loading, the process by which photosynthate enters the sieve tubes prior to export, is one aspect of source leaf physiology that has recently received attention (8–10). These studies have dealt mainly with the cellular transport pathway and sugar selectivity of the loading process. There is compelling evidence that photosynthetically derived sucrose, synthesized in the mesophyll cytoplasm, enters the apoplast prior to its metabolic and selective accumulation into the phloem (8–10). The coupling mechanism between the sucrose transporter and metabolism remains unresolved. Based on the proton dependence of sucrose accumulation and translocation, and the characteristics of the sieve tubes, we have proposed that the phloem loading of sucrose is coupled to the co-transport of protons (9–11). The driving force was proposed to be the electrochemical potential gradient of protons generated by a vectorial plasmalemma ATPase (11). This hypothesis is attractive for phloem loading not only because of the sieve tube characteristics (low H^+/K^+ ratio, membrane ATPase) but also because sugar transport coupled to proton fluxes has been documented in bacteria, algae, fungi (10 and refs. therein), and most recently in higher plants (16–19, 24). The sucrose/H^+ model may also explain, in part, the observed ATP stimulation of phloem loading (8) and the acquisition of sieve tube ATPase activity during the onset of export (13).

Several recent studies have substantiated the existence of a sucrose-specific/proton co-transport system in higher plants. Racusen and Galston (24) have reported sucrose-dependent membrane depolarizations and proton fluxes in pulvinar cells. Komor (18) and Hutchings (16, 17) have characterized in detail a sucrose/H^+ co-transport in *Ricinus* cotyledons and Malek and Baker (21) have recently proposed such a mechanism for sugar uptake into *Ricinus* phloem.

This study further investigates phloem loading of sucrose in sugar beet source leaves in relation to ATPase-mediated proton transport. Evidence is presented that PCMBS[2] selectively inhibits sucrose transport by inhibiting plasmalemma ATPase-proton transport. FC, a stimulator of active H^+/K^+ exchange, stimulated both net proton efflux and sucrose accumulation. These data are discussed in terms of a sucrose/H^+ co-transport involved in phloem loading.

MATERIALS AND METHODS

Sugar beet plants (monogerm hybrid, size 3) were grown for 8 to 10 weeks in a controlled environment under conditions described previously (9). Accumulation of ^{14}C-sugar was measured into 0.5-cm^2 discs cut from fully expanded exporting leaves (1 dm^2) which were abraded with carborundum to enhance solute entry (10). Discs, in triplicate, were incubated in ^{14}C-sugar (1 μCi/μmol) for 30 min with specific conditions given in the table and figure legends. After removal of free space label by washing, the tissue radioactivity was determined by liquid scintillation counting (10). ^{14}C-Sugar uptake was also measured into source leaves of an intact plant by supplying ^{14}C-sugar to an abraded area of the leaf for 30 min and then collecting discs from the area for ^{14}C counting (8). The results from both the disc and intact leaf systems were quantitatively and qualitatively similar.

Treatment of source leaf tissue with PCMBS prior to sugar accumulation or photosynthesis measurements consisted of incubating the tissue in 20 mM K-phosphate (pH 7.0) containing various PCMBS concentrations. The tissue was treated only 5 to 10 min followed by several washings in 0.5 mM $CaCl_2$ to remove the unbound modifier. Photosynthesis was measured in 1-cm^2 source leaf discs essentially as described by Chollet (2). Control and PCMBS-treated tissue were incubated in 1 ml of H_2O in 50-ml side armed flasks (two discs/flask, upper surface down) for 10 min at 25 C and 3,500 ft-c. During the illumination the flasks were purged with a humidified gas mixture (340 μl/l CO_2, 21% O_2 [v/v], and N_2 balance) at a flow rate of 10 flask volumes/min. The flasks were sealed and immediately injected with 0.2 ml $^{14}CO_2$ (about 0.2 μCi) giving an initial CO_2 concentration of 340 to 345 μl/l. After a 2-min fixation, illumination was ceased and the discs were processed for liquid scintillation counting (2).

Net proton efflux from sugar beet leaf tissue was measured by a modification of the technique described by Lin and Hanson

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[2] Abbreviations: PCMBS: p-chloromercuribenzenesulfonic acid; DNP: dinitrophenol; FC: fusicoccin; NEM: N-ethylmaleimide.

(20). About 2 g fresh weight of 0.5-cm^2 leaf discs were distributed into three cheesecloth bags and incubated in 100 ml of 0.2 mM KH_2PO_4, 0.2 mM $CaCl_2$ (pH 6). After about 15 to 20 min the net proton efflux rate was measured with a Beckman Expandomatic pH meter-recorder assembly. Proton flux rates were quantitated by titrating with standard NaOH and HCl. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

Previous studies have shown that ^{14}C-sucrose supplied to exporting leaves is accumulated into the minor veins and can be translocated at rates comparable to photosynthetically derived sucrose (8). Several characteristics of the accumulation process, such as sucrose specificity, energy, and pH dependence, and saturation kinetics, strongly indicate a metabolic carrier mechanism during phloem loading from the apoplast (8–11). The following data attempt to provide further insight into the driving force of the sucrose-loading process.

Selective Inhibition of Sucrose Uptake. Earlier, we employed the relatively nonpermeant sulfhydryl reagent, PCMBS, to provide evidence for the apoplastic route of sucrose transfer to the sieve tubes (9). This reagent markedly but reversibly inhibited sucrose uptake in sugar beet source leaves. We have since found that PCMBS selectively inhibits sucrose uptake. PCMBS treatment of tissue prior to sugar uptake markedly inhibited sucrose uptake (>80%) without inhibiting hexose accumulation (Fig. 1). The insensitivity of hexose uptake to PCMBS is not attributed to hexose uptake being nonmetabolic. Table I shows that both sucrose and hexose uptake are equally sensitive to inhibitors of metabolism such as low temperature, DNP, and the lipophilic sulfhydryl reagent, NEM. In the same experiment, PCMBS caused a 75% inhibition of sucrose uptake with no inhibition of hexose uptake. Sucrose and hexose uptake showed a similar sensitivity to various DNP concentrations indicating that a differential inhibition was not obscured by high DNP concentrations (Fig. 2).

Importantly, a 5-min PCMBS pretreatment followed by removal of excess PCMBS failed to inhibit photosynthesis under conditions where sucrose uptake was inhibited by 70% (Fig. 3 and legend). NEM, the penetrating —SH group alkylating reagent, markedly inhibits photosynthesis, indicating that photosynthesis is sensitive to —SH modification. PCMBS treatment of abraded regions of leaves on an intact plant caused a 69% reduction in sucrose uptake into the intact leaf with no inhibition of hexose uptake. These results agree with our previous interpretation that the PCMBS acts at the membrane level and not with intracellular metabolism directly (9). It should be cautioned that many of the so-called nonpenetrating reagents, including PCMBS, are nonpenetrating only in a relative sense. Long treatment times and/or high inhibitor to tissue ratios decrease the selectivity of PCMBS by adversely affecting intracellular metabolism.

Since these results show that PCMBS can selectively inhibit sucrose accumulation, elucidating its mechanism of inhibition may provide insight into the mechanism of phloem loading. The following data suggest that one site of inhibition is the plasmalemma ATPase.

PCMBS Inhibition of Net Proton Efflux. If sucrose uptake and

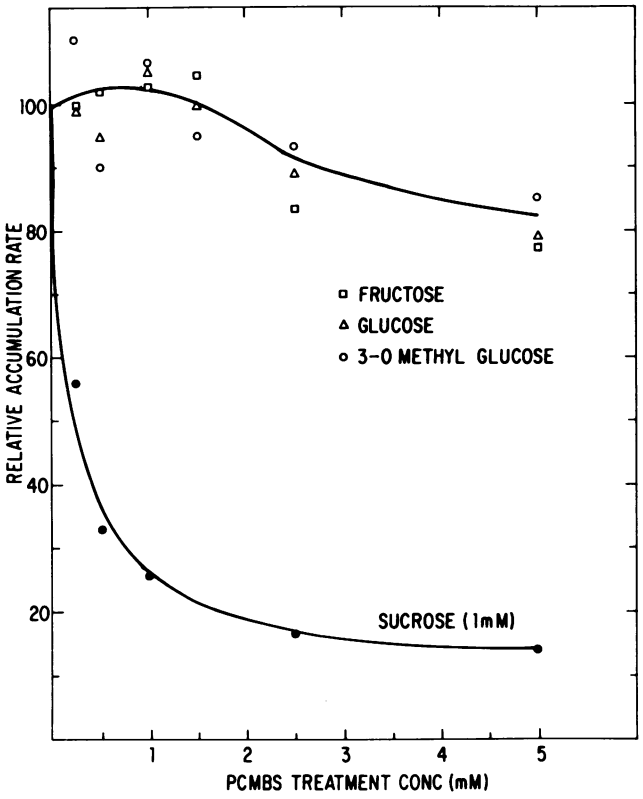

FIG. 1. Differential effect of PCMBS pretreatment of sucrose and hexose accumulation. Source leaf tissue was incubated in 1 mM ^{14}C-sugar for 30 min.

TABLE I. Effect of Various Inhibitors on Sugar Accumulation into Source Leaf Tissue of Beta vulgaris

	Accumulation Rate							
	Sucrose		Glucose		Fructose		3-O Methyl Glucose	
	μmoles/hr·dm^2 (% Inhibition)							
Assay Condition								
Control 18 C	1.4		1.0		1.0		0.9	
3 C	0.37	(76)	0.27	(67)	0.25	(75)	0.25	(70)
Q_{10}	2.42		2.39		2.52		2.35	
Dinitrophenol	0.18	(87)	0.18	(82)	0.15	(85)	0.16	(80)
N-ethylmaleimide	0.37	(76)	0.4	(51)	0.28	(75)	0.31	(65)
PCMBS	0.35	(75)	0.9	(10)	1.06	(0)	0.88	(2)

2.5 mM ^{14}C sugar; 2 mM Inhibitor.

ATPase-mediated proton transport are related (11) then alterations of ATPase function should be reflected in similar changes in sucrose transport. It is generally accepted that plant cells display an electrogenic proton extrusion system driven by a membrane ATPase (15, 17, 25). This net proton efflux in a medium containing K^+ and $CaCl_2$ was used as an indicator of membrane ATPase activity. Table II shows that PCMBS virtually abolished the proton efflux from source leaf tissue at a concentration where intracellular metabolism and hexose uptake were unaffected. This reveals that the inhibition of sucrose uptake by PCMBS is accompanied by an inhibition of ATPase-mediated proton efflux. That PCMBS markedly inhibits ATPase-mediated K^+ uptake into pea stem segments without affecting tissue respiration (14) along with the observation that PCMBS markedly inhibits *in vitro* (K^+-Mg^{2+})-stimulated ATPase (TK Hodges, personal communication; Lin and Giaquinta, unpublished data) provide further support that PCMBS is acting at the ATPase level. Interestingly, PCMB abolished the cytochemical localization of ATPase activity in sieve

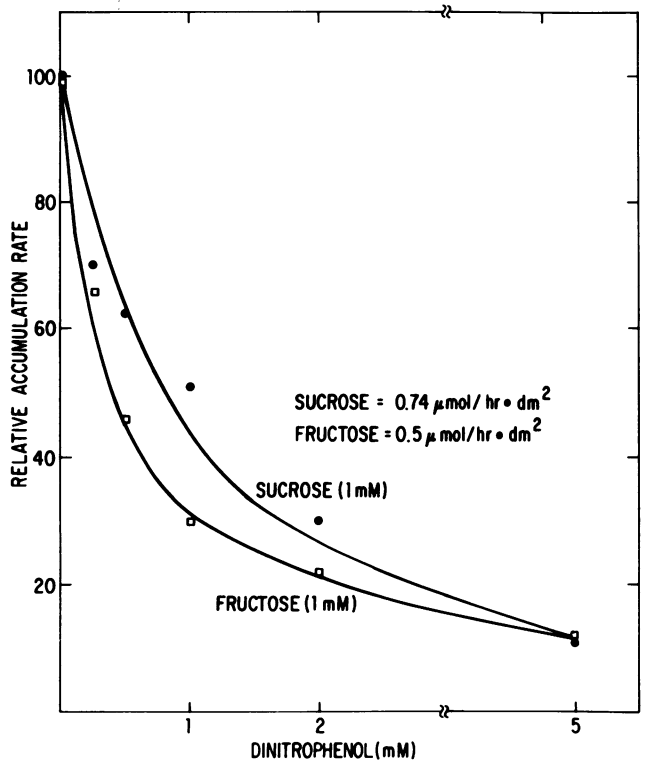

FIG. 2. Effect of DNP on sucrose and fructose uptake.

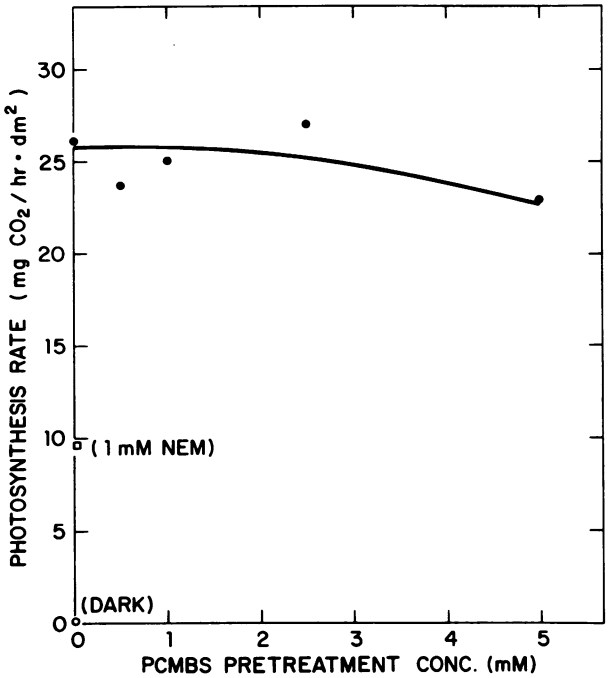

FIG. 3. Effect of PCMBS pretreatment on leaf disc photosynthesis. NEM treatment consisted of a 5-min pretreatment in 1 mM NEM followed by washing prior to photosynthesis measurements. CO_2 fixation in the dark was 0.1% of the light contro.. In parallel experiments on tissue from the same leaf, 2.5 mM PCMBS pretreatment inhibited sucrose uptake (from a 1 mM ^{14}C sucrose) by 70% (1.41 *versus* 0.42 μmol/h·dm²) while glucose uptake was not inhibited (1.48 *versus* 1.55 μmol/h·dm²).

TABLE II. Effect of PCMBS and Fusicoccin on H^+ Efflux from Sugar Beet Source Leaf Tissue

	Rate of H^+ Efflux (μmol/hr·gram fr. wt.)
Exp. 1.	
No Tissue	n.d.
+ Tissue	1.46
+ PCMBS (0.5 mM)	n.d.
Exp. 2.	
No Tissue	n.d.
+ Tissue	0.98
+ Fusicoccin (10 μM)	3.1
+ PCMBS (0.5 mM)	2.1
+ PCMBS (1 mM)	1.8
+ FCCP (10 μM)	n.d.

n.d. = none detected. Addition of 0.5 mM PCMBS increased the buffering capacity of the medium twofold. H^+ efflux rates were corrected for this difference. Additions refer to order of reagents to the same assay.

TABLE III. Fusicoccin Stimulation of Sucrose Accumulation into Sugarbeet Source Leaf Tissue

Treatment	Sucrose Accumulation μ mol/hr·dm^2	% Stimulation
Control	0.50	-
+ Fusicoccin (10 μM)	0.73	46

Tissue was preincubated in 10 μM fusicoccin, for 30 min prior to addition of 0.5 mM ^{14}C-sucrose (1 μCi/μ mol). The uptake time was 30 min. In 21 experiments, fusicoccin gave a 20-50% stimulation of sucrose uptake. The range of stimulation agrees with that reported for 3-O-methyl glucose uptake in various tissues (4).

tube membranes (13) further suggesting that the PCMBS inhibition of phloem loading of sucrose is caused by inhibition of the plasmalemma ATPase. The data are also consistent with PCMBS inhibiting the sucrose carrier as well as the ATPase, assuming they are separate entities. Sucrose uptake into sugar beet source leaf tissue has a K_m for protons near 0.01 μM, indicating that the carrier is half-maximally protonated near pH 8 (10). It is interesting that the pK of —SH groups is also near pH 8.

FC Stimulation of Sucrose and Proton Transport. Further correlation between H^+ transport and sucrose uptake is indicated by the effect of FC on these processes. Marrè and co-workers (23) have shown that the fungal toxin, FC, is a potent activator of an active H^+/K^+ exchange system in many plant species. That radiolabeled FC has been shown to bind to a plasmalemma-enriched membrane fraction (5) and that FC stimulates *in vitro* ATPase activity (1) may suggest that FC acts on the plasmalemma ATPase, although this question has not been resolved. Recent studies have shown that FC enhances hexose uptake into plant tissue and this stimulatory effect was consistent with a hexose/H^+ co-transport system (4). FC stimulated sucrose uptake in sugar beet source leaf tissue (Table III). Optimum FC concentration was 10 μM with higher concentrations (>100 μM) being inhibitory. FC stimulated sucrose uptake over a wide range of sucrose concentrations (0.1–100 mM) and the stimulation was independent of added K^+. Colombo *et al.* (4), reported FC enhancement of 3-*O*-methyl glucose uptake into maize root and coleoptile and pea stems. They showed a 26 to 74% stimulation of hexose uptake by FC which was independent of hexose concentration and exogenous K^+, results similar to those reported here.

The stimulation of sucrose uptake was also reflected in an increased net proton efflux (Table II). Interestingly, although PCMBS inhibited the basal proton efflux rate (Table II, exp. 1), it reduced the FC-stimulated rate only about 40% (Table II, exp. 2). The proton-conducting uncoupler, FCCP (and inhibitor of sucrose uptake [9]) completely inhibited both basal and FC-stimulated proton efflux indicating that both H^+ effluxes are metabolic. The observation that PCMBS and diethylstilbesterol markedly inhibited the basal proton efflux and *in vitro* plasmalemma ATPase but only partially inhibited the FC-stimulated proton efflux suggests that the FC-enhanced proton efflux is in part not ATPase-mediated (Lin and Giaquinta, unpublished data).

SUMMARY

The data presented indicate that PCMBS selectively inhibits sucrose transport into exporting sugar beet leaf tissue. The site of PCMBS inhibition is probably at the plasmalemma ATPase level, since ATPase-mediated H^+ fluxes are markedly inhibited at a PCMBS concentration where intracellular metabolism is not affected. FC stimulates both sucrose and proton transport indicating that the metabolic and selective loading of sucrose into the phloem is coupled to proton co-transport probably mediated by a vectorial plasmalemma ATPase. The ATPase-mediated proton extrusion can be coupled to K^+ influx (17) thereby explaining the high K^+ concentration of the sieve tubes and, in part, the K^+ stimulation of phloem loading and translocation (6, 21). Addition of 10 mM KCl to sugar beet source leaf tissue incubated in 0.2 mM $CaCl_2$ (pH 6) caused a marked H^+ efflux (Giaquinta, unpublished) and is consistent with this hypothesis. Microelectrode measurements of the phloem are needed to characterize phloem loading further.

During the preparation of this manuscript, Malek and Baker (22) also found that FC stimulated sucrose uptake into the phloem of *Ricinus* petioles.

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LITERATURE CITED

1. Beffagna W, S Cocucci, E Marrè 1977 Stimulating effect of fusicoccin on K-activated ATPase in plasmalemma preparations from higher plant tissues. Plant Sci Lett 8: 91–98
2. Chollet R 1978 Evaluation of the light/dark ^{14}C assay of photorespiration. Tobacco leaf disk studies with glycidate and glyoxylate. Plant Physiol 61: 929–932
3. Christy WL, CA Swanson 1976 Control of translocation by photosynthesis and carbohydrate concentrations of the source leaf. *In* IF Wardlaw, JB Passioura, eds, Transport and Transfer Processes in Plants. Academic Press, New York, pp 329–337
4. Colombo R, MI DeMichelis, P Lado 1978 3-*O*-Methyl glucose uptake stimulation by auxin and fusicoccin in plant materials and its relationships with proton extrusion. Planta 138: 249–256
5. Dohrmann U, R Hertel, P Pesci, SM Cocucci, E Marrè, G Randazzo, A Ballio 1977 Localization of "*in vitro*" binding of the fungal toxin fusicoccin to plasma-membrane-rich fractions from corn coleoptiles. Plant Sci Lett 10: 291–299
6. Doman DC, DR Geiger 1978 Effect of exogenous potassium on phloem loading and carbohydrate metabolism in leaves of *Beta vulgaris*, L. Plant Physiol 61: S–71
7. Fisher DB 1970 Kinetics of C-14 translocation in soybean I. Kinetics in the stem. Plant Physiol 45: 107–113
8. Geiger DR 1975 Phloem loading *In* MH Zimmermann, JA Milburn, eds, Transport in Plants. I. Springer-Verlag, New York, pp 395–431
9. Giaquinta RT 1976 Evidence for phloem loading from the apoplast. Chemical modification of membrane sulfhydryl groups. Plant Physiol 57: 872–875
10. Giaquinta RT 1977 Phloem loading of sucrose. pH dependence and selectivity. Plant Physiol 59: 750–755
11. Giaquinta RT 1977 Possible role of pH gradient and membrane ATPase in the loading of sucrose into the sieve tubes. Nature 267: 369–370
12. Giaquinta RT 1978 Source and sink leaf metabolism in relation to phloem translocation. Carbon partitioning and enzymology. Plant Physiol 61: 380–385
13. Gilder J, J Cronshaw 1973 The distribution of ATPase activity in differentiating and mature phloem cells of *Nicotiana tabacum* and its relationship to phloem transport. J Ultrastruct Res 44: 388–404
14. Hendrix DL, N Higinbotham 1974 Heavy metals and sulfhydryl reagents as probes for ion uptake in pea stem. *In* U Zimmermann, J Dainty, eds, Membrane Transport in Plants. Springer-Verlag, New York, pp 412–417
15. Higinbotham N, WP Anderson 1974 Electrogenic pumps in higher plant cells. Can J Bot 52: 1011–1021
16. Hutchings VM 1978 Sucrose and proton co-transport in *Ricinus* cotyledons. I. H^+ influx associated with sucrose uptake. Planta 138: 229–235
17. Hutchings VM 1978 Sucrose and proton co-transport in *Ricinus* cotyledons. II. H^+ efflux and associated K^+ uptake. Planta 138: 237–241

18. KOMOR E 1977 Sucrose uptake by cotyledons of *Ricinus communis* L.: characteristics, mechanism and regulation. Planta 137: 119–131
19. LICHTNER FT, RM SPANSWICK 1978 Sucrose transport in developing soybean cotyledons. Plant Physiol 61: 5–28
20. LIN W, JB HANSON 1974 Increase in electrogenic membrane potential with washing of corn root tissue. Plant Physiol 54: 799–801
21. MALEK F, DA BAKER 1977 Proton co-transport of sugars in phloem loading. Planta 135: 297–299
22. MALEK F, DA BAKER 1978 Effect of fusicoccin on proton co-transport of sugars in the phloem loading of *Ricinus communis* L. Plant Sci Lett 11: 233–239
23. MARRÈ E 1977 Effects of fusicoccin and hormones on plant cell membrane activities: observations and hypotheses. *In* E Marrè, O Ciffurri, eds, Regulation of Cell Membrane Activities in Plants. Elsevier North Holland, Amsterdam, pp 185–202
24. RACUSEN RH, AW GALSTON 1977 Electrical evidence for rhythmic changes in the co-transport of sucrose and hydrogen ions in *Samanea* pulvini. Planta 135: 57–62
25. SPANSWICK RM 1972 Evidence for an electronenic ion pump in *Nitella translucens*. I. The effects of pH, K^+ Na^+, light and temperature on the membrane potentials and resistance. Biochim Biophys Acta 288: 73–89