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Membrane Potentials of *Vallisneria* Leaf Cells and Their Relation to Photosynthesis[1]

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ABSTRACT

A study has been made of the effects of the inhibitors carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), and of anoxia on the light-sensitive membrane potential of *Vallisneria* leaf cells. The present results are compared with the known effects of these inhibitors on ion transport and photosynthesis (Prins 1974 Ph.D thesis). The membrane potential is composed of a diffusion potential plus an electrogenic component. The electrogenic potential is about −13 millivolts in the dark and −80 millivolts in the light. The inhibitory effect of DCMU and CCCP on the electrogenic mechanisms strongly depends on the light intensity used, the inhibition being less at a higher light intensity. This is of significance in view of the often conflicting results obtained with these inhibitors. With ion transport in *Vallisneria* the electrogenic pump derives its energy from phosphorylation; however, the process which causes the initial light-induced hyperpolarization and the process that keeps the membrane potential at a steady hyperpolarized state in the light have different energy requirements. The action of photosystem I alone is sufficient to induce the initial hyperpolarization. For continuous operation in the light the activity of photosystem II also is needed.

The membrane potential of photosynthesizing cells often is more negative in the light than it is in the dark. This is the case also in leaf cells of the aquatic plant *Vallisneria spiralis* (3, 15). Previously it has been shown that the light-stimulated Rb^+ and Cl^- fluxes in *Vallisneria* leaf cells depend on photophosphorylation. Under most circumstances cyclic photophosphorylation seems to be the main energy source (15).

The aim of the present investigation was to see whether the same sort of relation exists between the light-dependent hyperpolarization and photosynthesis, and to gain more insight into the nature of this hyperpolarization.

The effect of DCMU, an inhibitor of PSII, and the uncoupler CCCP[5] on the membrane potential and cell membrane electrical resistance was tested at different light intensities. Earlier work of Bentrup *et al.* (3) with *V. spiralis* on the light-dependent hyperpolarization suggested that the energy for this process could be obtained from either PSII or PSI activity. This implies a more direct role of PSII for the mechanism of light-dependent hyperpolarization than was concluded for ion transport.

The present results show that the sensitivity of the membrane potential to inhibitors depends very much on the light intensity used. The meaning of this will be discussed. For *Nitella* it has been shown that the more negative potential in the light is caused by an electrogenic pump (19, 20). Generally, it is assumed that this electrogenic pump is a proton extrusion pump; this may be true for *Vallisneria* also.

MATERIALS AND METHODS

Plants of *V. spiralis* were grown aquatically in a plastic tank on a slightly alkaline soil at room temperature (about 20 C).

Leaf strips of 20 × 4 mm were mounted in a modified Mertz chamber (12), except for experiments on the effect of O_2, in which small (2-mm) leaf strips were used from the margins of the leaves which are devoid of gas-filled intercellar spaces. By doing this a more rapid exchange of O_2 between the cells and the medium was obtained. Cells of the epidermis or outer layer of mesophyll were used. Membrane potentials were measured using 3 M KCl-filled glass microelectrodes. The cells were too small to use the classic two-electrode method for membrane resistance measurements, therefore electrical resistances were measured with the single electrode method of Anderson *et al.* (1), using 10-kHz current pulses between +5 and −5 namp. Over this range the resistance was ohmic and no rectification was observed. Resistances lower than 1.5 MΩ could not be measured accurately with the set-up used.

Despite the recent criticism on this method (6) we still think this method can be used with some precautions. Electrode capacitance compensation was done with the electrode *in situ* as the capacitance mostly changed drastically upon impalement. Only electrodes with a low resistance, equal to or lower than the cell membrane resistance, were used for membrane resistance measurements. Care was taken that the electrodes responded properly and did not show rectification, which was especially important in case of electrode tip sealing. Sealing often occurred during long term experiments and resulted in electrodes with a very high resistance, which often showed rectification and distortion of the waveform and may yield overestimates of the membrane resistance. In a number of experiments we filled the microelectrodes with 3 M KCl acidified with HCl to pH 2 in order to reduce the tip potential (R. M. Spanswick, personal communication). Although no systematic comparison with the normal KCl-filled electrodes was made the results strongly suggested that sealing of the tip was much reduced and that more stable resistance measurements were obtained with the acid-filled electrodes.

The finding that the small epidermis cells have higher resistances than the larger parenchyma cells and the observation that

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[5] Abbreviations used: CCCP: carbonylcyanide *m*-chlorophenylhydrazone; DNP: 2,4-dinitrophenol; $_{L}E_{vo}$: vacuolar electrical potential in the light; $_{D}E_{vo}$, vacuolar electrical potential in the dark; PD: potential difference; $_{L}R_{vo}$: plasmalemma plus tonoplast electrical resistance in the light.

5 μM CCCP after prolonged treatment, which has been shown to increase ionic permeabilities (15), drastically decreases the measured resistance (see under "Results") indicate that the method is useful to monitor gross changes in resistances even though the absolute values may be underestimated (6).

The solution used in the experiments had the following composition (mM): K^+, 0.1; Na, 10.5; Ca^{2+}, 1.0; Cl^-, 12.1; Mes, 1.0 (pH 6.2). When DCMU and CCCP, dissolved in ethanol, were added, the same amount of ethanol (not more than 1 ml/l) was added to the control. After cutting, the leaf strips were kept overnight, 16 h, in the test solution before the experiment was started.

In all cases the tip of the intracellular electrode must have been in the vacuole. Therefore, the values given here are potential differences and electrical resistances between vacuole and medium, designated as E_{vo} and R_{vo}, respectively.

RESULTS

The membrane potentials of epidermal and mesophyll cells approached the same value under all circumstances. Also, the sensitivities for inhibitors etc. were similar. The parenchyma cells in general showed a somewhat more stable potential. After impalement the membrane potential normally stabilized at the resting potential in a few minutes. The membrane resistance very often showed a tendency to increase during the course of the experiment (about 2 h). This has been found by others also (1, 18). The mean resting potential in the light was 194 ± 2 mv (n = 100)[6] and in the dark 126 ± 2 mv (n = 45). In Figure 1 typical "light-on" and "light-off" reactions are shown. The induction pattern was comparable with that found before (15) although the transients were less pronounced. The typical shoulder seen in the "light-off" reaction sometimes was absent. In the light the membrane potential stayed hyperpolarized for at least several hours.

In a series of 18 cells the membrane resistance was measured both in the dark and under light-saturating conditions (> 16.4 × 10^3 ergs cm^{-2} s^{-1}). Resistance was 4.1 ± 0.7 MΩ in the dark, and 3.7 ± 0.4 MΩ in the light. There was no significant difference between the cell membrane resistance in light and dark as has been found in some other cells (7, 19, 20, 22). Typical values for the membrane resistance were 8 MΩ for epidermal cells and 2 MΩ for parenchyma cells; the latter have a lower resistance because of their larger size and greater surface area.

Electropotentials in Darkness, $_DE_{vo}$. In the dark E_{vo} seems to consist of a passive component of −113 mv and an active component of −13 mv, as indicated by the effects of anoxia (Table I) and of CCCP (Table II). Both 2 μM CCCP and anoxia depolarized $_DE_{vo}$ by about 13 mv. CCCP at this concentration had no effect on R_{vo} (Table III).

A higher CCCP concentration, 5 μM, caused a further depolarization of $_DE_{vo}$ (Table II) especially after a prolonged, 4-h treatment. Earlier it was concluded that such a treatment increases the ionic permeability of the cell membranes (15). This has now been confirmed by resistance measurements; after such a treatment the resistance of the epidermal cells was less than 1.5 MΩ compared with 7 MΩ in the control cells (Table III).

Electropotentials in Light, $_LE_{vo}$. The relationship between light intensity and hyperpolarization ($_LE_{vo}$ - $_DE_{vo}$) yielded a sigmoid curve and low light intensities (about 1–4 × 10^3 ergs cm^{-2} s^{-1}) had no effect on E_{vo} (Fig. 2). Complete light saturation was obtained at about 16.4 × 10^3 ergs cm^{-2} s^{-1}. No points of the highest light intensity used are shown here, but a large number of experiments, done at 16.4 × 10^3 ergs cm^{-2} s^{-1} and 440 × 10^3 ergs cm^{-2} s^{-1}, invariably showed that the hyperpolarization was similar. For the inhibitor experiments three different light intensities were used: a limiting light intensity of 7.6 × 10^3 ergs cm^{-2} s^{-1}, a transitional saturation intensity of 16.4 × 10^3 ergs cm^{-2} s^{-1}.

[6] Results are given in the form: mean ± SE (n = number of cells).

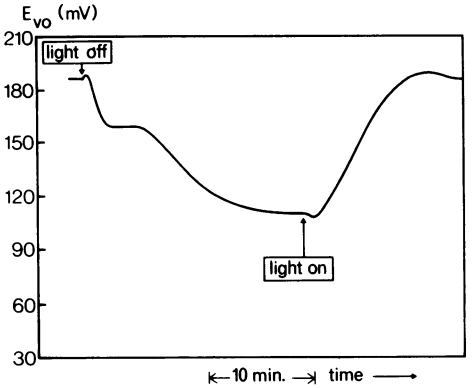

FIG. 1. Effect of light and dark on E_{vo}. A recorder tracing of a typical experiment is given. Mean values for $_DE_{vo}$ and $_LE_{vo}$ are given in the text.

Table I. *Effect of Anaerobiosis on E_{vo} in the Dark*

The leaf tissue was bathed in streaming experimental solution either aerated with normal air ($_DE_{vo}$ in air) or with pure nitrogen ($_DE_{vo}$ with N_2). The electrical potential was recorded continuously.

$_DE_{vo}$ in Air	$_DE_{vo}$ with N_2	Depolarization by Anaerobiosis
	mv	
−132 ± 2 (n = 12)	−121 ± 2 (n = 13)	+12 ± 1 (n = 11)

Table II. *Effect of CCCP on E_{vo} in the Dark*

$_DE_{vo}$ first was measured in the absence of any CCCP (control); thereafter, the experimental solution was replaced by a solution containing CCCP. $_DE_{vo}$ was recorded continuously except in the case of prolonged treatment. In these latter experiments with 5 μM CCCP the tissue was pretreated for 4 h after which the cells were impaled.

	Control	2 μM CCCP	5 μM CCCP	5 μM CCCP Prolonged (4 h) Treatment
		mv		
Experiment A	−117 ± 3 (n = 8)	−103 ± 2 (n = 13)		
Experiment B	−129 ± 3 (n = 4)		−97 ± 4 (n = 4)	−46 ± 5 (n = 12)

Table III. *Effect of CCCP on R_{vo}*

The experimental procedure was the same as described in Table II.

	Resistance				
	Control		2 μM CCCP		5 μM prolonged (4 h) Treatment (Epidermis)
	Epidermis	Parenchyma	Epidermis	Parenchyma	
			MΩ		
Light	7.0 ± 0.5 (n = 6)	1.8 ± 0.3 (n = 8)	7.9 ± 1.1 (n = 8)	3.0 ± 0.7 (n = 6)	<1.5 (n = 12)
Dark		2.0 ± 0.5 (n = 4)	6.9 ± 0.9 (n = 5)	2.7 (n = 2)	

During the experiments the solution was exposed to normal air; thus CO_2 was present in the medium. Due to the presence of CO_2, DCMU inhibits not only PSII activity but also, indirectly, cyclic photophosphorylation at low light intensities but not under high light conditions (17). The hyperpolarization was inhibited by 2 μM DCMU at 7.6 × 10^3 and 16.4 × 10^3 ergs cm^{-2} s^{-1} (Table IV). This concentration of DCMU causes complete inhibition of photosynthetic O_2 production and maximal inhibition of ion transport (15). At the highest light intensity, however, there was a normal hyperpolarization by light in the presence of DCMU. The normal "light-on" and "light-off" induction pattern was observed in the

presence of DCMU provided the light intensity was high enought (Fig. 3). Contrary to the findings of Bentrup *et al.* (3) no specific effects of DCMU on the transients were observed.

In four experiments the hyperpolarization in the presence of DCMU was recorded over a long period of time. Invariably the membrane potential depolarized slowly in the light, after the maximal hyperpolarization had been reached, to a level somewhere between the normal stable level in the light and the dark potential (Fig. 3). DCMU caused no decrease of the membrane resistance but did give an insignificant rise in $_{L}R_{vo}$ (Table IV).

At the two lowest light intensities 2 μM CCCP was an effective inhibitor of the light reaction. At the highest light intensity 2 μM CCCP had no effect on the hyperpolarization in the light (Table V). At this concentration CCCP had no effect on R_{vo} (Table III). CCCP at 5 μM was more effective (Table V) and inhibited the light-driven hyperpolarization even at the highest intensity. As was mentioned above, however, at this concentration CCCP also has an effect on the ionic permeability of the cell membrane and cell membrane resistance (Table III).

DISCUSSION

A resumé of the main results, depicted schematically in Figure 4, gives ample evidence that the membrane potential (E_{vo}) of *V. spiralis* leaf cells consists of two components: a metabolically controlled portion sensitive to light and inhibitors, and a passive portion not significantly affected by light or inhibitors, providing that the latter do not affect membrane permeability as is the case at high CCCP concentrations. For photosynthetically active cells the most generally held view has been that E_{vo} is built up by a diffusion potential which can be described by the Goldman equation and by a light-dependent electrogenic potential generated by a proton extrusion pump. A true electrogenic origin of part of E_{vo} has been demonstrated only in a few cases by comparison of the predicted diffusion potential with the membrane potential actually measured (8, 9, 19, 20). In other studies, *e.g.* in *Vallisneria,* the evidence for electrogenicity is mostly indirect. No ionic gradients are known which may generate the observed high potentials in the light (up to −240 mv). Also, the rapid response of the membrane potential to "light-off" and to inhibitors points to the electrogenic nature of this response.

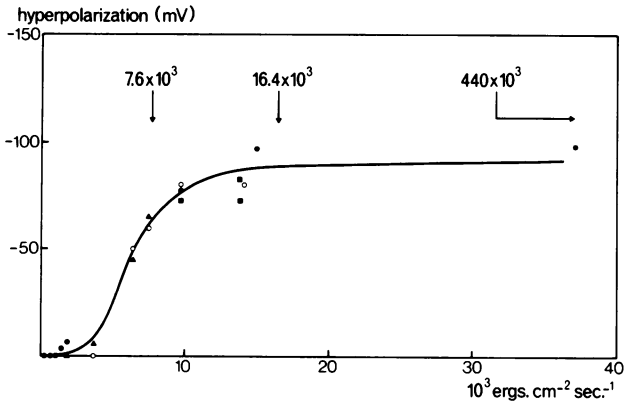

FIG. 2. Relation between light intensity and light-dependent hyperpolarization of E_{vo}. The three different light intensities used in the inhibitor experiments are indicated.

Table IV. *Effect of 2 μM DCMU on the Light-dependent Hyperpolarization and on R_{vo}*

E_{vo} and R_{vo} were measured in the absence and presence of 2 μM DCMU in three separate experiments at different light intensities (Experiments A, B and C). R_{vo} and E_{vo} were measured before and after addition of DCMU. The mean values for R_{vo} obtained in Experiment A are from mesophyll cells and in Experiment C from epidermal cells and thus may not be compared with one another.

Light intensity	Control		+ DCMU	
	Hyperpol.	Resistance	Hyperpol.	Resistance
ergs cm⁻² s⁻¹	*mv*	*MΩ*	*mv*	*MΩ*
Experiment A	−77 ± 7	3.1 ± 0.7	−2 ± 2	3.7 ± 0.6
7.6 × 10³	(n = 5)	(n = 5)	(n = 10)	(n = 5)
Experiment B	−76 ± 5		−3 ± 2	
16.4 × 10³	(n = 10)		(n = 11)	
Experiment C	−78 ± 3	6.4 ± 1.0	−59 ± 5	8.4 ± 2.4
440 × 10³	(n = 10)	(n = 4)	(n = 17)	(n = 5)

Table V. *Effect on CCCP on the Light-dependent Hyperpolarization*

The experimental procedure was the same as described for Table III, except that both $_{D}E_{vo}$ and $_{L}E_{vo}$ were determined both in the absence or presence of CCCP.

Light intensity	Light-induced Hyperpolarization		
	Control	+ 2 μM CCCP	+ 5 μM CCCP
ergs cm⁻² s⁻¹		*mV*	
7.6 × 10³	−65 ± 6	−6 ± 3	
	(n = 4)	(n = 7)	
16.4 × 10³	−62 ± 5	−11 ± 5	
	(n = 7)	(n = 12)	
440 × 10³	−65 ± 4	−70 ± 5	−6 ± 5
	(n = 8)	(n = 11)	(n = 7)

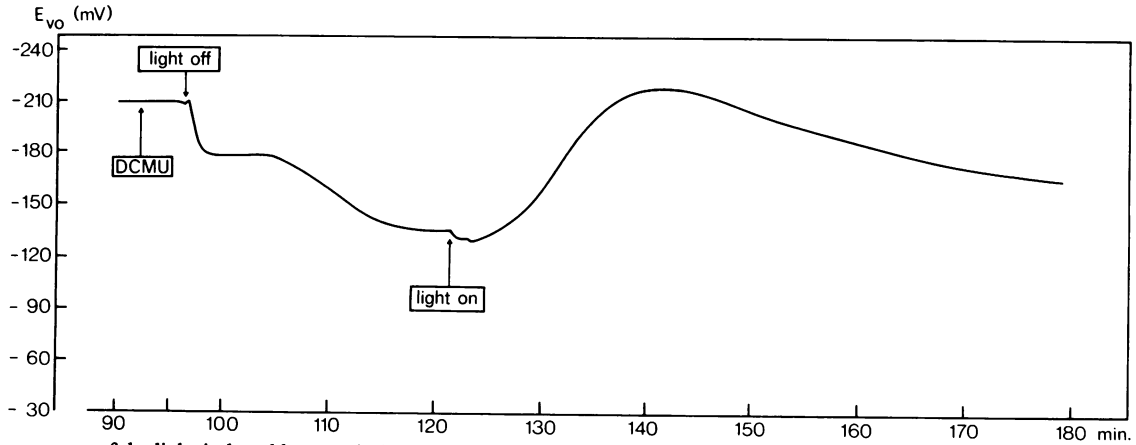

FIG. 3. Time course of the light-induced hyperpolarization of E_{vo} in the presence of 2 μM DCMU. The light intensity used in this experiment was 440 × 10^3 ergs cm^{-2} s^{-1}.

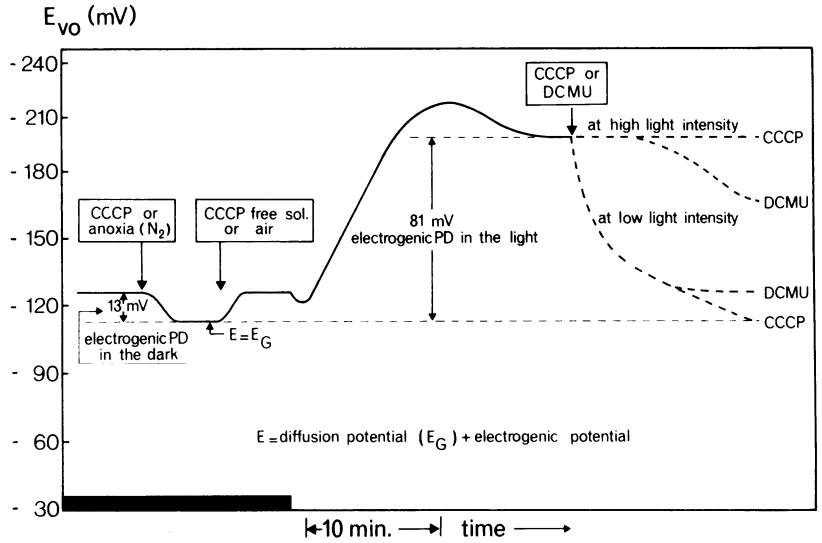

FIG. 4. Main effects of DCMU, CCCP (2 μM), and anoxia are schematically depicted in this figure. E_G = Goldman or diffusion potential. Black bar on x axis indicates duration of dark period.

For the dark potential the situation is less clear since the value of ${}_DE_{vo}$ is close to values known for diffusion potentials. The rapid depolarizing effect of anoxia and CCCP indicates that in the dark also there is an electrogenic potential. For a number of different types of plant cells it has been shown that anoxia eliminates the electrogenic component of E_{vo} (4, 8, 13).

Therefore we assume that ${}_DE_{vo}$ also consists of a diffusion potential of about -113 mv and an electrogenic component of about -13 mv.

Contrary to our findings, Bentrup *et al.* (3) concluded that only ${}_LE_{vo}$ in *Vallisneria* was electrogenic and that ${}_DE_{vo}$ was a diffusion potential. This conclusion was based merely on the finding that in the light the membrane potential was sensitive to a change of the pH while in the dark it was not. We found, however, that E_{vo} was depolarized at low pH in the light as well as in the dark (results not shown here). The depolarization in the dark was small (8 ± 2 mv [n = 11] with a pH change from 5 to 4) and it was less than in the light. It could be measured only when ${}_DE_{vo}$ was recorded continuously while the pH was changed since the depolarization was less than the variation from leaf to leaf. This small depolarization of ${}_DE_{vo}$ indicates also a small electrogenic potential in the dark.

It was not possible to measure cell dimensions during the resistance measurements, due to the thickness of the leaf, and thus to calculate the resistance per unit area for each cell. For a number of epidermal cells we calculated the mean surface area to be 8×10^{-5} cm^2, thus for a typical epidermal cell with a membrane resistance of 8 MΩ the resistance per unit area will be about 0.64 kΩ cm^2. This may seem a rather low value; however, epidermal cells of *Vallisneria* are transfer cells, that is, their cell membrane surface area is greatly increased by cell wall ingrowth (14). The precise increase of membrane area is unknown but values up to 20-fold are estimated, thus yielding a very high value of about 13 kΩ cm^2. Inhibition of the electrogenic pump often results in an increased cell membrane resistance (19, 20, 22), which, however, was not observed in the described experiments. Inhibition by CCCP and DCMU resulted in a slight but not significant increase of R_{vo}, indicating that the resistance for passive ion movement was low compared with the parallel resistance of the electrogenic pump (16).

The inhibitory effect of DCMU on the light-induced hyperpolarization and the countereffect of high light intensity hereupon were also observed in *Elodea* (10) and in ion transport experiments with *Vallisneria* demonstrating that the electrogenic pump can be driven by cyclic photophosphorylation. In accordance with this observation a hyperpolarization in the light under anaerobic (N_2) conditions was observed, although this hyperpolarization seemed to be transient as was also the case for DCMU. Apparently, the activity of PSI is sufficient to generate a transient hyperpolarization but the activity of both systems seems to be necessary to maintain a steady-state of the hyperpolarization, as is normally observed.

According to Bentrup *et al.* (3) DCMU did not influence the stationary potential but it changed the induction pattern that occurs after "light-on" or "light-off." What these authors call the stationary level is the maximum hyperpolarization that occurs in the light. We also observed that this level was not affected by DCMU (at 2 μM) in light at high intensity, but Bentrup *et al.* (3) did not continue their experiments long enough to observe the transient nature of the hyperpolarization in the presence of DCMU. If the light intensity was made high enough a normal induction pattern was observed in the presence of DCMU in our experiments (Fig. 3). The induction pattern for the "light-on" as well as the "light-off" reaction of the PD depends upon the light intensity. There are sharper peaks at higher light intensities even if we compare two saturating light intensities which both bring the membrane potential to the same final level. If the light is turned on after a dark period the stable light level is reached in a shorter time at the higher light intensity. Therefore, the effect of DCMU the induction course, found by Bentrup and co-workers, was not due to a specific effect caused by inhibition of PSII but resulted from the fact that light is less effective in the presence of DCMU.

The depolarizing effect of CCCP below 2 μM and of anoxia in the dark also points to a dependence of the electrogenic pump on phosphorylation. The further depolarizing effect of CCCP at concentrations higher than 2 μM apparently is caused by the increased ionic permeabilities, as found earlier in ion transport studies (15) and as indicated by the sharp decrease of R_{vo} under such conditions. It is also conceivable that CCCP interferes with the electrogenic mechanism itself presumably acting as a proton carrier as was, *e.g.* concluded for *Riccia* (7), since uncoupling of phosphorylation seemed to occur in this plant only at high CCCP concentrations and the depolarization caused by CCCP was accompanied by a decrease of the membrane resistance (7). The lack of effect of CCCP below 2 μM on R_{vo} in our experiments does not indicate such a direct effect of CCCP on the electrogenic pump, the less so since we know, from photosynthesis and respiration measurements, that CCCP at low concentrations is a very efficient uncoupler in *Vallisneria* (15). Also, the suppression of the inhibi-

tory action of CCCP by high light intensities indicates that CCCP acts on a site close to the light-dependent photosynthetic reactions (2, 11, 21).

As for the finding that PSII activity is needed for the steady-state hyperpolarization we suggest that the electrogenic mechanism depends on a continuous supply of protons. PSII may play a role for this; from CO_2 fixation experiments it is known (R. J. Helder, personal communication) that CO_2 is fixed in *Vallisneria* for a large part as malic acid, as is the case in *Elodea* (5).

The observation that the electrogenic pump depends on metabolism in very much the same way as ion transport does may reflect a common dependence on phosphorylation or may indicate that a functioning electrogenic proton pump is a basic requirement for active cation and anion transport across the plasmalemma. It seems worthwhile to consider the possibility that electrogenicity is not limited to one particular pump but that it may be a common feature of all or most ion pumps.

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