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Light-induced cytoplasmic pH changes and their interrelation to the activity of the electrogenic proton pump in *Riccia fluitans*

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In green thallus cells of the aquatic liverwort *Riccia fluitans* light-induced pH changes have been measured, using a turgor-resistant pH-sensitive microelectrode. (1) Light-off/-on causes oscillations of the cytoplasmic pH (pH_c), as well as of the membrane potential difference across the plasmalemma (ψ). Beside the well-known ψ_m changes, the first detectable pH_c change following light-off is a transient acidification of about 0.3 pH units, whereas light-on causes a transient alkalinization of roughly 0.4 pH units. (2) 1 μM DCMU eliminates these transients. (3) In the presence of 0.2 mM procaine, which alkalizes the cytoplasm to over pH 8, the light-induced ψ_m transients are enhanced, but are almost absent, if pH_c is acidified to 6.9 by 1 mM acetate. It is suggested that the transient light-induced changes in pH_c are caused by light-dependent proton translocation across the thylakoid membranes, and it is concluded that the subsequent changes in ψ_m are essentially the result of altered activities of the electrogenic proton pump in the plasmalemma, due to the observed fluctuations of its substrate, the proton.

Introduction

The inner membrane of the chloroplast envelope forms the barrier between the stroma space and the external medium. Upon illumination the stroma of isolated intact chloroplasts becomes alkaline as a consequence of proton translocation across the thylakoid membrane (Jagendorf and Uribe [1], Junge and Witt [2], Witt [3], Heldt et al. [4]). It has been argued that this alkalinization is not transmitted to the external medium, but should give rise to a transenvelope proton gradient (Heber and Heldt [5]). However, the reports on proton movement out of and into chloroplasts upon illumination (or darkness) are contradictory: for isolated chloroplasts Gimmler et al. [6], Demmig and Gimmler [7], Douce and Joyard [8] report light-induced proton release, whereas Walker and Smith [9], Loos [10], and others [11,12] report light-induced alkalinization of the chloroplast-surrounding medium. In order to investigate this basic discrepancy of results on the same issue, continuous pH measurements in the cytoplasm of the liverwort *Riccia fluitans* have been carried out on intact cells by use of a pH-sensitive microelectrode.

In plant cell electrophysiology the phenomenon of light-induced changes in transmembrane potential difference is a well-known and in the past frequently studied phenomenon (for a review, see Bentrup [13]). Upon light-off the first signal to be observed is usually a hyperpolarization which may last from a few seconds up to several minutes, depending on object and external conditions. It has been proved that these changes in potential

Abbreviations: pH_c, cytoplasmic pH; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ψ_m, electric potential difference across the plasmalemma (= membrane potential); Mes, 4-morpholineethanesulphonic acid.

difference are not observed, if either no chloroplasts were present or when photosynthesis was inhibited [13].

The second aim of this study is to find out whether these potential changes are caused by light-induced cytoplasmic changes or by other factors.

Material and Methods

General conditions

Green thalli of the aquatic liverwort *Riccia fluitans* were grown sterile under a 12/12 hour's dark/light regime as described by Felle et al. [14]. 1 or 2 h before the tests the thalli were mounted into a plexiglas perfusion chamber and equilibrated with the test medium. This medium contained 5 mM Tris-Mes/1 mM KCl/1 mM NaCl/ 0.1 mM $CaCl_2$. Deviations from this composition will be given in the legends to the figures. The temperature was kept at 22 ± 1°C, the object was illuminated with white light from a Leitz microscope lamp at roughly 1 W/m^2.

General electrophysiology and pH electrode

Standard electrophysiology has been applied to all measurements of membrane potential as described by Felle [15,16]. The pH-sensitive microelectrodes were fabricated according to Amman et al. [17] and as recently described [18,19]. Briefly: acid cleaned glass capillaries were pulled to roughly 0.4 μm tips. These pipettes were dipped with the blunt end into a 0.1/0.2% dimethyldichlorosilane/benzene solution and baked at 180°C for about about 30 min. In order to resist the turgor of the cells upon impalement, the tips were stabilized with 0.1% polyvinylchloride dissolved in tetrahydrofuran, which was applied by suction from the rear end of the electrode. The proton-ionophore resin (Fluka, Buchs, No. 82500) was backfilled into the tip, the remainder of the capillary was filled with 0.5 M KCl which was buffered with 100 mM Mes-Tris to pH 5.5. These electrodes had typically resistances of $(5-8) \cdot 10^{10}$ Ω, and usually displayed a slope of at least 55 mV/pH unit between pH 7 and 9. Only data from electrodes which had likewise excellent properties after recalibration were used for evaluation in this study. The electrical noise is small enough to allow the detection of pH changes of about 0.02 pH units.

Basic procedures of intracellular pH measurements

Since an ion-selective microelectrode always records the electric signal according to the respective activity across the selective membrane plus the cell membrane potential difference, a conventional ψ_m electrode always has to measure this membrane potential separately, but simultaneously! These two signals are fed into a high-impedance differential amplifier (W.P. Instruments, FD 223, New Haven, CT) which amplifies and immediately subtracts the two signals. An example of such a procedure is given in Fig. 1. In order to obtain the best results, it is desirable that both electrodes be placed within the same cell and compartment, as has been demonstrated before in *Riccia* and *Sinapis* [18,19].

Vacuole or cytoplasm?

The question of whether the sensitive tip is placed within the cytoplasm or the vacuole is a problem of the microelectrode technique in general. Since the cytoplasm of most objects investigated so far seems slightly alkaline (*Nitella* 7.5 [20], *Chara* 7.6 [21], *Chlorella* 7.3 [22], *Riccia* 7.4 [18], *Zea* 7.2, 7.3 [23,24], *Neurospora* 7.2 [25], *Sinapis* 7.4 [19]), whereas the vacuolar sap appears rather acidic (*Phaeoceros* 5.9 [26], *Zea* 5.6 [23], *Acer* 5.7 [27], *Riccia* 4.8 [18]), the impalement of a

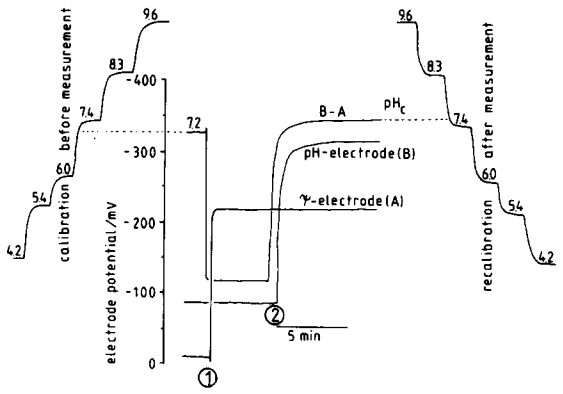


Fig. 1. Example of a routine calibration, impalement of a cell, measurement of cytoplasmic pH, and recalibration of a pH-sensitive microelectrode. A, trace of the conventional voltage-recording microelectrode, impalement at ①; B, trace of the pH-sensitive microelectrode, impalement at ②; B–A, difference trace, displaying the actual voltage, according to the external calibration pH-media (4.2, 5.4,..., 9.6). Please note that the different traces are not on level.

cell with a pH electrode immediately signals the compartment in question. As noted above, the vacuole of *Riccia* is roughly 2.6 pH units (= 146 mV) more acidic than the cytoplasm: all our impalements were carried out within the cytoplasm.

Statistics

The presented results are representative measurements, each from at least 9 equivalent tests. Since the reported data are continuous measurements, errors (± S.E.) are given for ΔpH_c only.

Results

The reaction of cytoplasmic pH and membrane potential to a light/dark regime

Fig. 2A illustrates a typical light-off/on response of a cell with respect to ψ_m and pH_c, as recorded from over 20 cells. Light-adapted green cells react upon total darkness with a transient acidification of about 0.3 pH-units, and a rapid hyperpolarization. The acidification is fully reversed after about 2 min and turned into an alkalinization. Light-on somehow causes the opposite effect with a transient depolarization and an alkalinization of pH_c by about 0.4 pH units; the membrane potential repolarizes and, after a temporary acidification, the pH_c returns to its control value (not shown). When the external pH is lowered from 7.3 to 5.2, the responses to light and dark change: upon light-off both short-term transients, ψ_m and ΔpH_c are smaller than those measured at pH 7.3, whereas the subsequent long-term reactions are clearly more distinct. Light-on causes totally different responses at the pH and ψ_m electrodes, respectively. Whereas the membrane potential slightly depolarizes within the first minute of illumination, the cytoplasmic pH strongly oscillates (Fig. 2B).

Addition of 1 μM DCMU causes a short and transient acidification of pH_c by little more than 0.1 pH units (Fig. 3). Subsequently, in the presence of DCMU, the early transient responses of both, pH_c and ψ_m, to light-off/on fail to appear, whereas the long-term reactions (Fig. 2A) still exist.

The light-/dark responses as varying pH_c

Procaine ($pK = 9.1$) and acetic acid ($pK = 4.75$) change cytoplasmic pH according to their dissociation properties, if added at the appropriate external pH and concentration (cf. Sanders and Slayman [25], Bertl et al. [18]). As Fig. 4A demonstrates, 0.2 mM procaine massively alkalinizes the pH_c from 7.5 to 8.1, while the membrane potential drops to a niveau known as the so-called diffusion potential [15]. In the presence of 0.2 mM procaine light-off causes a fast and constant acidification of pH_c by about 0.3 pH units and a substantial

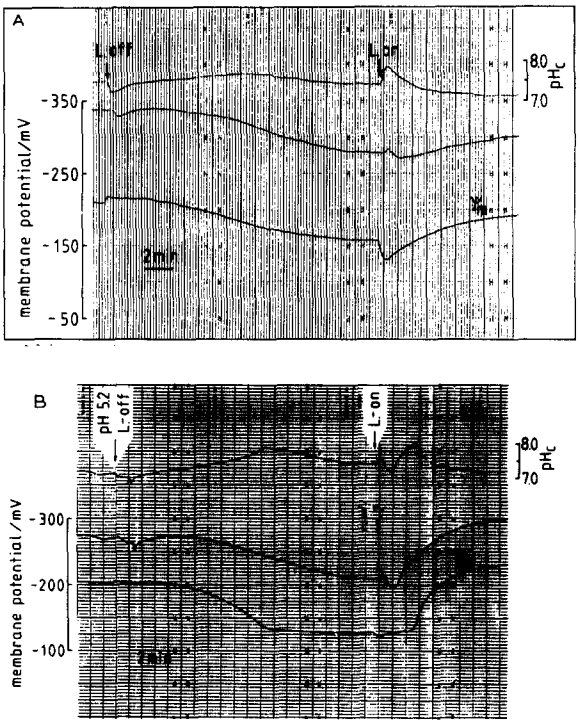


Fig. 2A and B. Representative example ($n = 14$) of light- and dark-induced changes in membrane potential (ψ_m) and cytoplasmic pH (pH_c), recorded from green thallus cells of *Riccia fluitans*. Before turning off the light, the cells were light-adapted for approx. 2 h in the test chamber in a test solution of pH 7.3 (A), and pH 5.1 9B). The middle trace is the direct recording from the pH-sensitive microelectrode. The difference trace of the pH-electrode minus the voltage-electrode is given in the most upper trace. Since the recordings are photographed originals, no correction has been made for the spatially transposed traces due to the chart recorder pens. Therefore, the ψ_m trace appears 12 s ahead of the difference trace and 36 s ahead of the pH electrode. This will be in all figures with originals. Zero mark of difference trace at −300 mV (A), at −250 mV (B). ΔpH (light-off) = 0.31 ± 0.04 S.E., ΔpH (light-on) = 0.39 ± 0.05 S.E. (A). ΔpH (light-off) = 0.12 ± 0.02 S.E. (B).

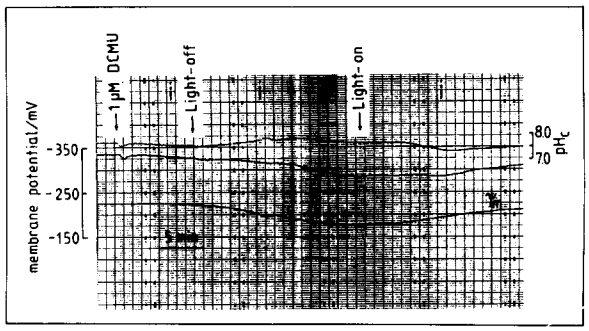
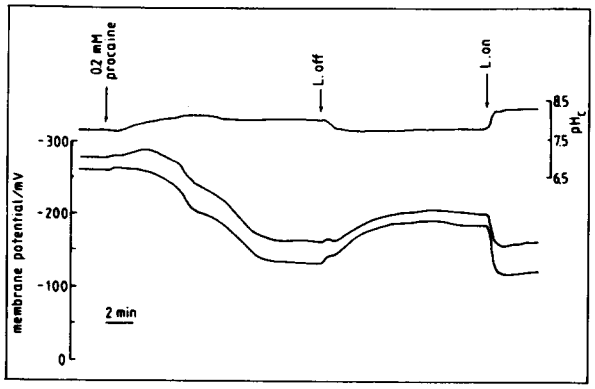


Fig. 3. Representative photographed original trace of the action of 1 μM DCMU and of light/dark in the presence of DCMU on pH_c and ψ_m of green thallus cells of *Riccia fluitans*. Traces as in Fig. 2. External pH 7.3. Zero mark of difference trace at −250 mV.



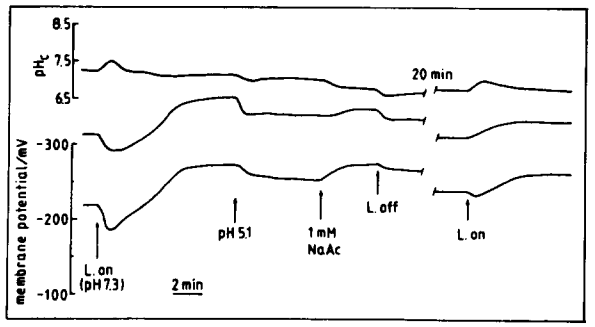


Fig. 4. A and B. Effect of 0.2 mM procaine (A) and 1 mM acetate (B) on pH_c, ψ_m, and light/dark-reactions of green thallus cells of *Riccia fluitans*. The procaine is added at pH_0 = 9.5, the acetate at pH_0 = 5.1. Zero mark of different trace at −300 mV. ΔpH (procaine) = 0.6 ± 0.1 S.E. ΔpH (light-off) = 0.37 ± 0.05 S.E., ΔpH (light-on) = 0.41 ± 0.04 S.E. (A). ΔpH (acetate) = 0.2 ± 0.03 S.E., ΔpH (light-off) = 0.18 ± 0.03 S.E., ΔpH (light-on) = 0.26 ± 0.05 S.E. (B).

hyperpolarization of about 55 mV. Light-on even exceeds these responses with a depolarization of over 60 mV and an alkalinization to pH 8.3.

Fig. 4B illustrates that at pH_0 = 5.1, 1 mM acetate acidifies the cytoplasm by 0.2–0.3 pH units and hyperpolarizes the plasmalemma by 25–30 mV. Under these conditions light-off or light-on only yields relatively small changes of both, pH_c and ψ_m.

Light- and dark-responses in the presence of cyanide

Cyanide is frequently used to separate active (electrogenic pump) and passive transport elements (e.g., Refs. 15 and 28). The current rationale inherent to this procedure is that cyanide is supposed to inhibit the electron-transfer chain of oxidative phosphorylation and therefore ATP synthesis. This ATP deficit is effective at the pump that leads to a reduction in transport activity and subsequently to a depolarization of the plasmalemma. In Fig. 5 the jump in external pH from 7.3 to 9.5 leads to an increase in pH_c from 7.4 to 7.6. Addition of 1 mM NaCN at pH 9.5 causes a rapid depolarization to −126 mV, the so-called diffusion potential [15]. This depolarization is evidently too fast for the pH electrode to follow, which is why the difference trace yields an apparent transient. In the presence of 1 mM cyanide light-off

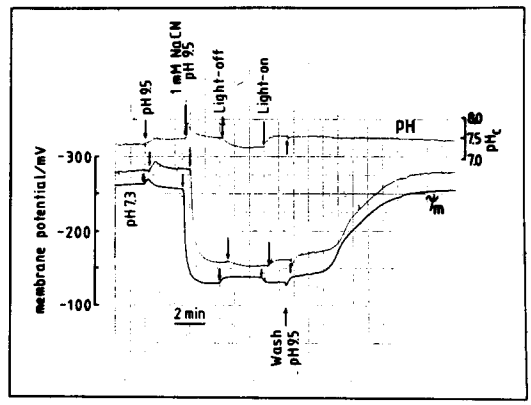


Fig. 5. Representative photographed original traces of light- and dark-induced changes in ψ_m and pH_c in the presence of 1 mM NaCN, recorded from green thallus cells of *Riccia fluitans*. External pH 9.5; wash: removal of the CN^-. Zero mark of the difference trace (pH) at −300 mV. ΔpH (light-off) = 0.25 ± 0.07 S.E., ΔpH (light-on) = 0.31 ± 0.06 S.E.

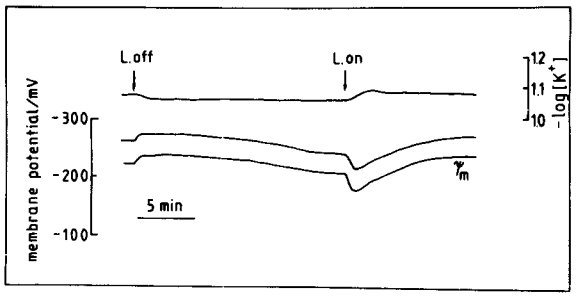


Fig. 6. Light- and dark-induced changes in ψ_m and K^+ activity (upper trace), recorded simultaneously in green thallus cells of *Riccia fluitans*. The *V* scale fo K^+ trace is not identical with *V* scale of ψ_m trace.

results in an immediate and constant acidification of 0.2–0.3 pH units, accompanied by a likewise constant hyperpolarization. Light-on reverses the just described effects, while washout of the cyanide readily repolarizes the membrane potential, without significant changes in cytoplasmic pH.

Light-induced changes in cytoplasmic K^+-activity

Although determinations of intracellular absolute potassium activities carried out by means of K^+-sensitive microelectrodes may not be too accurate because of the high K^+-background, changes of cytoplasmic K^+ activities in the millimolar range can be detected and are shown in Fig. 6. Light-off within 1 min causes an increase in cytoplasmic K^+ activity by 2–3 mM, light-on fully reversed these changes. There are evidently no oscillations as observed with the light-induced pH changes (Figs. 2–5).

Discussion

The light-dependent changes in pH_c

This study demonstrates that, independently of the boundary conditions, light-off is always followed by a fast and transient acidification of pH_c, whereas light-on always results in an alkalinization of pH_c (Fig. 2). DCMU, which is believed to inhibit Photosystem II, eliminates these fast light reactions, but not the subsequent long-drawn pH and ψ_m changes (Fig. 3). Additional experiments with the chloroplast-free rhizoid cells of *Riccia* (not shown) prove that the observed effects are indeed closely related to the presence and the functioning of chloroplasts. According to the Mitchell hypothesis [29] and the related later work of Junge and Witt [2], light causes active H^+ transport from the stroma into the inner thylakoid space, leaving a proton deficit in the stroma. It seems still a matter of dispute, whether this H^+ deficit is compensated by protons from the cytoplasm, or if a membrane potential is built up across the chloroplast envelope, in order to preserve the pH gradient across the thylakoid membrane. This, however, does not seem to be necessary, since the built-up of $\Delta\mu_{H^+}/F$ is a dynamic and continuous process. In suspensions of dark-adapted isolated chloroplasts there are reports on both light-induced acidification and alkalinization. Fig. 2A gives the answer: it seems a matter of justice when the test takes place. If the pH_c is measured within the first two minutes after illumination, an alkalinization can be expected, but an acidification upon longer illumination times. This apparently splits the light reactions into two portions. The one which occurs immediately after the change from light to dark, or vice versa, is very likely related to the light-triggered proton translocation at the thylakoid membrane, sicne DCMU not only inhibits this reaction, but seemingly to a certain extend mimics a dark reaction by causing transient acidification (Fig. 3). Another possibility would be that these pH_c transients are overshoots, caused by the light/dark-induced sudden changes in CO_2 fixation. The origin of the long-drawn pH changes is less clear. It seems possible that the steady-state pH in the light is reached quite slowly, since the conversion of the potential gradient across the thylakoid membrane into a pH-gradient is likewise a slow process. Although this would be consistent with our data, it is difficult to be sure of this conclusion, since changes in metabolism, e.g., ATP-synthesis and pH-regulatory processes (see below) may account for at least some of the observed pH changes.

The nature of the light-triggered K^+ movements (Fig. 6) give also rise to speculations. Whether these reflect an ATP-dependent H^+/K^+ exchange at the chloroplast envelope, as proposed by Barber [30], or are reactions quite independent of each other (the K^+ fluxes could also occur at the plasmalemma, according to the altered driving forces), cannot be decided right away without additional evidence.

The origin of the light-triggered changes in ψ_m

It has been shown elsewhere that fluctuations in cytoplasmic ATP are not the cause for light-induced $\Delta\psi_m$ [31]. Lüttge and Pallaghy [32], and others [13,33,34] have discussed a possible role of protons and other cations in a variety of plants, such as *Atriplex, Nitella, Chenopodium, Riccia,* etc., with respect to the well-known light-triggered changes in membrane potential and concluded that these plus other factors (e.g., permeability changes) determined $\Delta\psi_m$ through a feed back system. Jeschke [35] argued for *Elodea* that changes in pH_c were not sufficient to explain $\Delta\psi_m$ via a passive mechanism, i.e., the diffusion potential, and favours changes in ion-permeability and directly light-activated pumps. We think that the data presented in this study allow the conclusion that the electrogenic H^+-extrusion pump has at least part in the observed $\Delta\psi_m$. Not through a direct stimulation by light, but because protons are substrate for this pump. This has been demonstrated in detail before *Neurospora* [25], *Sinapis* [19], and *Riccia* [36], but is also shown in Fig. 4A, where 0.2 mM procaine is shown to alkalinize pH_c and to inhibit severely the pump. Fig. 4B illustrates that 1 mM acetate acidifies pH_c and hyperpolarizes the plasmalemma, and thus evidently stimulates the pump. Fuji et al. [31] do not agree to such an interpretation. For *Spirogyra* they concluded that the assumed changes in pH_c could not explain the light-induced hyperpolarization, because their crucial test failed: they argued correctly that, if the pump were involved in the above-suggested way, at very alkaline pH_c (8.7) the light-induced reactions should become more prominent, whereas at acidic pH_c (5.1) these reactions should be weaker, but found significant light-induced reactions in the range pH 6.2–7.9 only. As we know now, the plasmalemma proton pump has a very narrow optimum curve [37], so the chosen pH values of 8.7 and 5.1, respectively, were far out of range. On the other hand, Fig. 4A clearly proves that the light-reactions in the presence of procaine, which alkalinizes pH_c by about 0.4 pH units, are indeed much more prominent than the control, whereas those in the presence of acetate, which acidifies the cytoplasm by 0.2 pH units, are almost absent (Fig. 4B). So, it is evident to us that the pump does react to the light-induced pH_c changes with a $\Delta\psi_m$.

The pump as part of the pH_c-regulation?

Hansen [38], and Boels and Hansen [39] have put forward a model that discusses the pump as part of a feed-back loop which is integrated in pH_c regulation. According to this model, the observed light-triggered oscillations of ψ_m and pH_c would be the expression of that loop which attempts to restore a light-disturbed parameter (pH_c) through the control of one or more transport systems which contribute either to the generation of the membrane potential (e.g., the electrogenic H^+ pump) or dissipate it (e.g., H^+ co-transport). From this rationale one would expect considerable changes in cytoplasmic pH, if either pump or co-transport are activated. To our knowledge this has never been found. On the contrary, it has been demonstrated for *Neurospora* [25], *Sinpais* [19], and *Riccia* [36] that any stimulation or inhibition of the proton extrusion pump at the plasmalemma does not considerably drain the H^+ pool of the cytoplasm, so it rather appears that the pump itself is regulated by cytoplasmic pH. Similar arguments hould for H^+ co-transport. From yet unpublished experiments we know that in *Riccia* neither amino acids nor hexoses, if added in concentrations around their respective k_m values have any effect on pH_c. The reason for this pH insensitivity may be found in the cytoplasmic buffer capacity. For *Riccia* cells we do not know yet the exact buffer capacity of the cytoplasm, but experiments with weak acids and bases of different kinds and concentrations [19,36] signal a capacity in the millimolar range, as has also been demonstrated for *Neurospora* [25]. Therefore it is quite remarkable that simply turning the light off or on can cause changes of more than 1 pH unit (e.g., Fig. 2B)! This must mean that the proton movements out of and into the chloroplasts are quite substantial. Considering the all-over area of the involved thylakoid membranes, such H^+ fluctuations appear not unreasonable.

Being focussed on the cytoplasmic pH one easily overlooks a possible role of the extracellular pH. It is well known that changes in extracellular pH have strong effects on the electrogenic pump as well as on co-transport. As Prins and Helder [40], and Walker et al. [41] suggested, extracellular pH could well be involved in, and may even be target of, a cellular pH regulation. The strong light-induced oscillations in cytoplasmic pH at low exter-

nal pH (Fig. 2B) support this idea, which, however, for the lack of more precise data cannot be discussed in more detail at this point.

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