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The Effect of Transthylakoid Proton Uptake on Cytosolic pH and the Imbalance of ATP and NAPDH/H^+ Production as Measured by CO_2- and Light-Induced Depolarisation of the Plasmalemma

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The light-induced changes of plasmalemma potential and of chlorophyll fluorescence were compared with changes induced by the modulation of O_2- or CO_2-concentration. The fast depolarisation of plasmalemma potential upon illumination as labeled by the time-constant τ_4 (ca. 10 s) is used as a measure of the light-induced alkalinization of the cytosol. Firstly, these experiments on leaves of tabaco, *Agopodium podagraria* and spinach show that transenvelope fluxes of protons and not the uptake of CO_2 mediate the changes in cytosolic pH. Comparing the light-induced depolarisations of plasmalemma potential with chlorophyll luminescence or fluorescence in *Chara* excluded alternative explanations like signal flow via putative thylakoid-chlorolemma connections and alkalinization by PGA-fluxes. Secondly, the occurrence of the pH changes during CO_2 modulation demonstrates the imbalance of ATP and NADPH/H^+ generation by the linear electron flow. Searching for mechanisms furnishing additional ATP was done by measuring heat evolution and O_2-uptake by means of photoacoustic pulse experiments. They show that the Mehler reaction is active under steady-state conditions. It is stimulated when the transthylakoid pH-gradient increases. Further, the time course of heat evolution gave additional evidence that the time-constant τ_4 is related to transthylakoid proton fluxes and alkalinization in the cytosol.

Key words: CO_2 — Mehler reaction — O_2 — Photoacoustic signals — Plasmalemma potential — Thylakoid fluxes.

ATP formation in the chloroplasts of plant cells is driven by a large pH gradient across the thylakoid membrane. The generation of this pH gradient withdraws protons from the stroma (Junge and Polle 1986, Falkner et al. 1976). However, the pH-changes in the stroma are much smaller than those in the lumen. Proton exchange across the envelope may be part of the buffering capacity of the stroma. This suggestion is supported by the finding of light-induced changes in cytosolic pH as shown by several authors making use of pH-sensitive microelectrodes (Davies 1974, Felle and Bertl 1986, Steigner et al. 1987, Remis et al. 1988). In addition, these pH changes can also be monitored by their substrate effect on the electrogenic plasmalemma pump (Sanders et al. 1981) because they are the origin of the fast light-induced depolarisation of plasmalemma potential (Hansen et al. 1987, Vanselow and Hansen 1989, Vanselow et al. 1989a).

Nevertheless, the question remains open whether it is proton uptake into the chloroplasts which causes the light-induced alkalinization in the cytosol. There are three possible mechanisms which could mediate the effect of transthylakoid proton-fluxes on cytosolic pH.

H^+-uptake—Above it is assumed that depletion of protons in the stroma leads to a depletion of protons in the cytosol via uptake across the envelope.

CO_2-uptake—An alternative mechanism accounts for the fact that ATP-supply increases with the transthylakoid

Abbreviations: APO, ascorbate peroxidase; APW, artificial pond water; ATP, Adenosin-5′-triphosphat; ETC, electron transfer chain; F, fluorescence yield; FNR, ferredoxin-NADP-reductase; LED, light emitting diode; LHC, light-harvesting protein complex; MDR, monodehydroascorbate reductase; NADP, nicotinamideadenine-dinucleotidphoshate; PGA, 3-phosphoglycerate; P_i, inorganic phosphate; PS, photosystem; SOD, superoxide dismutase; V, plasmalemma potential.

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pH-gradient. Thus, availability of ATP and consequent stimulation of the Calvin cycle is expected to occur with the same time-constant (τ_4=5 to 40 s) as the increase of pH in the stroma. Increased activity of the Calvin cycle leads to an increased uptake of CO_2 across the envelope. Therefore, this mechanism may result in an increase of cytosolic pH, too.

PGA-uptake—When the chloroplastic pool of PGA is reduced upon illumination, cytosolic PGA^{2-} may be taken up via the phosphate translocator (Laisk et al. 1989). The conversion of PGA^{3-} to PGA^{2-} leads to the withdrawal of one H^+ from the cytosol, and thus to an alkalinization.

As shown below, the third candidate can be excluded because it implies that alkalinization should occur with a time constant faster than τ_4, the time-constant of the transthylakoid pH gradient (e.g. τ_3, according to the nomenclature of Hansen et al. 1991). However, the first two mechanisms could not be distinguished by the kinetic investigations done so far, as the same time-constant (τ_4) plays the crucial role in both of the two mechanisms. An important contribution to this problem is the work of Thaler et al. (1992). They showed that blocking the thylakoid ATP-ase by venturicidin (and consequently CO_2-uptake by the Calvin cycle) did not abolish the light-induced alkalinization of the cytosol.

Further evidence for the dominating role of proton fluxes is provided by the experiments reported here. In addition, the related question of the balance between ATP and $NADPH/H^+$ production is addressed. The studies are based on a comparison of the responses of chlorophyll fluorescence and plasmalemma potential as induced by changes in light intensity and in CO_2-concentration.

Chlorophyll fluorescence and plasmalemma potential show highly complicated kinetic responses. In order to monitor the actions in individual reactions of the photosynthetic apparatus, a mathematical analysis has been applied. As described in Materials and Methods and in previous papers (Dau and Hansen 1989, Hansen et al. 1987, 1991, Vanselow et al. 1989b) curve fitting of the measured time courses of chlorophyll fluorescence can identify the relative contributions of involved processes.

Materials and Methods

Experiments were performed on leaves of *Aegopodium podagraria, Nicotiana tabacum Virginia*, spinach or on single internodal cells of *Chara corallina*, a gift of D. Gradmann, Göttingen. Plants of *A. podagraria* were harvested from a garden. *N. tabacum* and spinach were grown from seeds in the lab. For the measurements, the whole plant was used in order to provide constant nutrition during long-term experiments (up to 1 d). One stem was bent, and the leave fixed in the experimental set-up. *Chara corallina* was grown in basins in the lab on a layer of old beech leaves (according to an advice from Dr. K. Ogata, Fukuoka) and of sand in APW (0.1 mM KCl, 1.0 mM NaCl, and 0.5 mM $CaCl_2$). Plants were kept growing in our lab by virtue of the skill of C. Plieth.

Comparison of changes in chlorophyll fluorescence, luminescence and plasmalemma potential—The experimental set-up enabled simultaneously measuring of plasmalemma potential, chlorophyll fluorescence and -luminescence. Six LEDs (OL-SUR 14180 from Oshino, 660 nm, 3,000 mcd) in a distance of 6 cm provided square-waves of light of up to 30 W m^{-2} with a frequency of 200 Hz. Fluorescence was recorded in the light period and luminescence in the dark period by means of a photodiode (S 1723-04 Hamamatsu). The photodiode (behind a RG 9 filter, Schott) was connected to a current-voltage converter whose sensitivity could be switched by a factor of 500 with a dead time of about 30 μs (Ramm and Hansen, in preparation). The luminescence was averaged over an interval of 70 to 300 μs after light-off.

Membrane potential was measured by conventional microelectrodes filled with 1 M KCl and connected to the amplifier via Ag/AgCl bridges. In the case of *Chara corallina*, the microelectrodes were inserted into the vacuole. Several cells were laid in parallel if luminescence was measured with one cell selected for the electrical measurements.

In the case of leaves, the position of the electrodes could be inside or outside a cell. However, the light-induced responses are well known (Vanselow et al. 1989a, Vanselow and Hansen 1989), and thus polarity could easily be identified. The light-induced responses served as an indicator of polarity when the effects of the modulation of CO_2- and O_2-concentration were investigated.

Comparison of O_2-uptake, O_2-evolution and heat evolution—Gas and heat fluxes were measured by a new photoacoustic technique developed by Kolbowski et al. (1990). Briefly, flashes of light were used as measuring light (4 LEDs, Toshiba, TLRA 180AP, 660 nm, connected to four arms of the light conductor, Schölly Spezial, Mainz, F.R.G.) providing 100 W m^{-2} for 1 ms at the surface of the leave. These pulses were applied repetitively with a temporal distance of 50 ms. The photoacoustic responses were recorded by a microphone (BL 1785, Knowles Electronics, England) with a sampling rate of 10 kHz for 50 ms. The photoacoustic cell was basically identical to that described by Kolbowski et al. (1990). Our gas chamber had a diameter of 6 mm and a volume of 100 μl.

Comparison of the effects of modulating light intensity and CO_2- or O_2-concentrations—Fig. 1 shows the set-up used for the modulation of the gas concentration and light intensity. Details are given by Hansen et al. (1991). A leaf of *N. tabacum* was mounted in the flat gas chamber and fixed between a curtain of thin wires. Fig. 1 shows the set-up for modulating CO_2-concentration. Two mass flow controllers (FC 260, Tylan, Eching, F.R.G.) provided a con-

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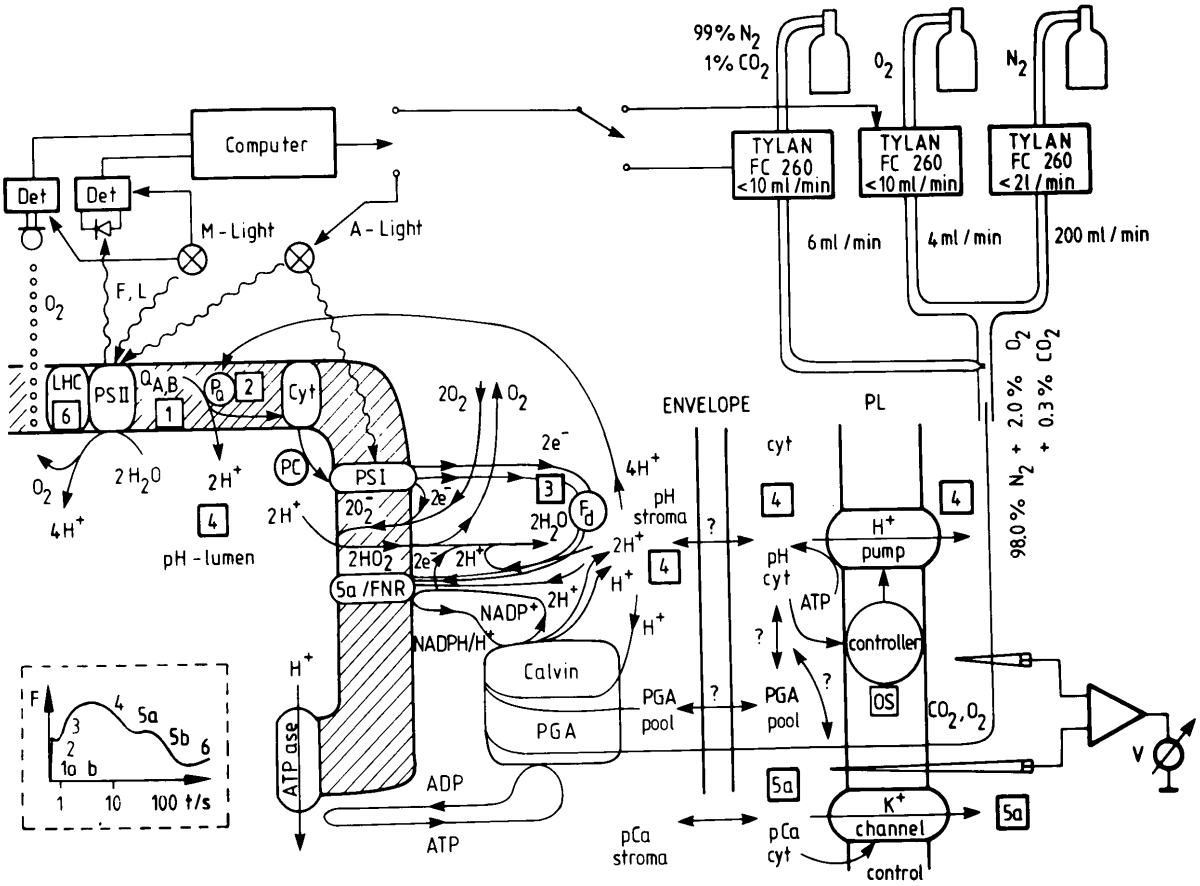

Fig. 1 Experimental set-up and the anticipated interactions between the individual processes in the photosynthetic apparatus. The figure shows the control of the input signals (A-light and modulated streams of O_2 and CO_2) by a computer and the measurement of the output signals. O_2- and heat evolution and O_2-uptake are measured photoacoustically by a microphone (Det 1). The yield of chlorophyll fluorescence (F) and luminescence (L) (Det 2) is the response to the measuring light (M-light). The plasmalemma potential (V) is recorded by a microelectrode. The scheme shows established and hypothetical relationships between those photosynthetical reactions which play a role in the presented investigations. The numbers in the boxes give the time-constants related to the individual reactions. 1: primary acceptor (Q) of photosystem II (PSII), 2: plastoquinone pool (PQ), 3: PSI acceptor pool X, 4: transthylakoid pH-gradient, 5a: unknown, but the results in Fig. 8 may lead to the speculation that it is related to the activation of the FNR and to Ca^{2+}-uptake into the chloroplasts, 5b: unknown, 6: state-1 state-2 transitions via the light harvesting complex (LHC). The reaction scheme of the Mehler reaction at PSI was suggested by Schreiber et al. (1991). cyt=cytosol, PL=plasmalemma.

stant background stream of 200 ml min^{-1} N_2 and 4 ml min^{-1} O_2 resulting in a mixture of 98% N_2 and 2% O_2. A modulated gas stream (changing sinusoidally between 3 and 6 ml min^{-1} of 1% CO_2 in 99% N_2) was added to the background stream 1 cm before reaching the leave resulting in a modulation of the CO_2-concentration between 150 to 300 ppm.

For the modulation of O_2-concentration, the flow of the CO_2-mixture was kept constant at 6 ml min^{-1} (300 ppm CO_2), and the flow of O_2 was modulated between 2 and 4 ml min^{-1} (resulting in sinusoidal changes between 1 and 2% O_2).

For the recording of chlorophyll fluorescence, the measuring light (2 W m^{-2}) and the actinic light were provided by LEDs (OL-SUR 14180 from Oshino, 660 nm, 3,000 mcd) and led through two arms of a light conductor (Schölly Special, Mainz, F.R.G.) to the side of the gas chamber opposite to the gas-inlet. The overall light intensity (including the constant measuring light of 2 W m^{-2}) was changed sinusoidally between 2.5 to 7.5, 10 to 30 and 15 to 45 W m^{-2} as indicated by the mean values of 5, 20 or 30 W m^{-2}, respectively, which are given in the figure legends and tables.

A third arm of the light conductor picked up the fluorescence light and led it to a photodiode detector (Valvo, BPW34 behind a RG9-filter, Schott).

A hole in the gas chamber enabled the insertion of a microelectrode. The reference electrode was connected to the soil in the pot.

Kinetic analysis—Many instruments measuring chloro-

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phyll-fluorescence determine minimum and maximum fluorescence. From these data, photochemical and non-photochemical quenching is calculated (Schreiber and Neubauer 1990). For our investigation a more detailed analysis is required. An approach is wanted which tells quantitatively to what extent the changes in the redox-state of the quencher Q, of the plastoquinone pool and of the acceptor pool of PSI or the changes in transthylakoid pH contribute to the measured changes in chlorophyll-fluorescence.

This can be achieved by curve-fitting of the responses of chlorophyll-fluorescence F and of plasmalemma potential V. The benefit of this analysis is that it provides each reaction in the photosynthetic apparatus (see Fig. 1) with a label (a time constant τ_i). This label enables the evaluation of the contribution of an individual process to the measured responses.

At the present stage, this kind of analysis can be based on curve-fitting routines only if the experiments are done under linearizing conditions (Hansen et al. 1991, Vanselow et al. 1989b). If linearity holds, the responses to a flash (h(t)) or to stepwise change (g(t)) in light intensity (or CO_2- or O_2-concentration) can be described by a sum of exponentials

$$h(t)=\sum_{i=0}^{m} a_i \exp(-t/\tau_i) \quad (1)$$

$$g(t)=\sum_{i=0}^{m} A_i [1-\exp(-t/\tau_i)] \quad (2)$$

$$with \quad A_i=a_i\tau_i \quad (2a)$$

with τ_i being the time-constants, and A_i the amplitude factors which give the relative contributions of the related reactions to the measured overall response.

In our studies, chlorophyll fluorescence serves as a guide line as it displays all of the relevant time-constants, and as it is easy to measure. In the inset of Fig. 1, the response of chlorophyll fluorescence is shown with the time-constants attached to those ranges which are dominated by them.

The numbers in the boxes of Fig. 1 indicate the mechanisms which have been assigned to the individual time-constants according to the results of previous investigations (Dau and Hansen 1989, 1990, Hansen et al. 1987, 1991, Vanselow et al. 1989a, b). In constrast to Hansen et al. (1991), Fig. 1 gives an assignment of τ_2 and τ_3 to individual reactions as Vanselow (1993) could relate τ_3 to the acceptor pool of PSI by virtue of a highly specific effect of nitrate and ammonia on τ_3.

The same parameters can also be evaluated if sine-waves are used for the modulation of the input signals. In that case, the kinetic analysis is based on frequency responses (Dau and Hansen 1989, Hansen 1978, 1985, Hansen et al. 1987, 1991). For this issue, light intensity, CO_2- or O_2-flow was modulated sinusoidally with frequencies between 0.001 to 1 Hz. Fourier analysis of the resulting responses provided the amplitude and the phase of the ground wave.

The frequency dependence of the amplitude and of the phase-shift are combined to give the complex frequency response which was used for the generation of Bode-plots (Figs. 5 and 6). In the case of the light effect on chlorophyll fluorescence (F_L)

$$F_L(p)=A_{LF}(p) \exp(\sqrt{-1}\,\varphi_{LF}(p)) \quad (3)$$

$$with \quad p=2\pi\sqrt{-1}\,f \quad (3a)$$

Curve-fitting of the frequency response of fluorescence is done with the following transfer function

$$F_L(p)=A_0+\sum_{i=1}^{m} \frac{A_i}{1+p\tau_i} \quad (4)$$

Eq. 4 results in the same kinetic parameters τ_i and A_i as Eq. 2 does. Details of this kind of analysis are given by Hansen et al. (1987, 1991).

Using the light-induced depolarisation of plasmalemma potential as a means of measuring changes in cytosolic pH—In order to do a kinetic analysis with a very high resolution, long-term experiments (up to 24 h) have to be done. Thus, a more durable method of measuring cytosolic pH than electrodes or dyes have to be employed. This is provided by the pH-effect on the proton pump of the plasmalemma (Sanders et al. 1981).

In a green plant cell, the action of photosynthetic processes on plasmalemma ion transport is mediated via at least four different pathways (Hansen 1978, 1985, Hansen et al. 1987, 1991, Vanselow et al. 1989a, b, Vanselow and Hansen 1989):

(1) fast (5–40 s) depolarisation due to slowing down of the plasmalemma H^+-pump because of the alkalinization of the cytosol

(2) slow (3–10 min) hyperpolarisation due to increased activity of the H^+-pump, probably because of a decrease in P_i (Takeshige et al. 1992)

(3) slow feed-back loop compensating light-induced changes in cytosolic pH (damped oscillation with a period of 30 to 90 min, Boels and Hansen 1982, Fisahn et al. 1986, Hansen 1990).

(4) fast (10–80 s) closure of K^+-channels probably caused by an increase of pCa (Vanselow and Hansen 1989, Plieth and Hansen 1992).

For the investigation of the involvement of transenvelope H^+- or CO_2-uptake in the light-induced alkalinization, the fast depolarization (first pathway) is used as a sensor for changes in cytosolic pH. Previous investigations have resulted in the model illustrated in Fig. 1. Light drives the photosynthetic electron transfer chain (ETC) via PSII and PSI. This electron flow is coupled to the uptake of protons

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into the inner thylakoid space which leads to a depletion of protons in the stroma. Both, the subsequent uptake of protons from the cytosol or the ATP-driven CO_2-uptake may lead to an alkalinization. This increase in cytosolic pH decreases pump activity via a substrate effect (Sanders et al. 1981).

Evidence for this chain of events has been obtained from a kinetic analysis of simultaneously measured signals: scattering at 535 nm, plasmalema potential and resistance, chlorophyll-fluorescence (Hansen et al. 1987, Vanselow and Hansen 1989, Vanselow et al. 1989a, b), oxygen evolution and heat dissipation (Dau and Hansen 1989, 1990) as measured by photoacoustic signals.

Results

Further experiments relating τ_d of the rapid depolarisation of plasmalemma potential to τ_4 of the transthylakoid H^+-fluxes—As described in the last paragraph of Materials and Methods, the light-induced alkalinization is measured via its effect on the plasmalemma proton pump. Here experiments are presented which provide further support for this relationship.

Firstly, the experiments shown in Fig. 2 deal with an alternative explanation, a putative direct action of electrical thylakoid potential on plasmalemma potential via direct membrane connections (Gynes 1987). Light-induced changes in membrane potential and in chlorophyll-luminescence were measured simultaneously. They were induced by a series of 200-Hz light pulses (24 W m^{-2}) as described above. Typical time-courses of the responses to a stepwise increase in light-intensity of fluorescence (F), luminescence (L) and of potential (V) are shown in Fig. 2.

According to Bilger and Schreiber (1989) the first fast peak of luminescence (L_I) is due to the transient occurrence of electrical transthylakoid potential. The second peak (L_M) is related to the generation of the transthylakoid pH-gradient. Luminescence reaches the peak L_M later than fluorescence obtains its maximum value F_p, because luminescence increases with the transthylakoid pH gradient (Bilger and Schreiber 1989), whereas fluorescence decreases due to energy quenching and stimulation of the Calvin cycle (Schreiber and Neubauer 1990).

The comparison of the kinetics of luminescence with the light-induced changes in plasmalemma potential shows that no phase can be detected in the response of plasmalemma potential which corresponds to the electrical component observed in the luminescence signal. This finding is important as it rules out that a direct action of thylakoid potential on plasmalemma potential is involved in the phenomenon investigated here. Gyenes et al. (1986) have assumed that such a direct action is mediated by connections between thylakoid membrane and the putative chlorolemma via which the electrical signal may spread and contribute to the measured electrical response.

Secondly, Fig. 3 shows that the height of pH-peak in

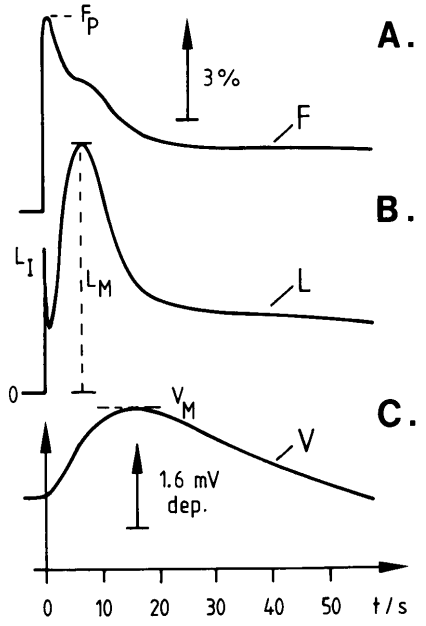

Fig. 2 Responses of membrane potential (V), chlorophyll luminescence (L) and fluorescence (F) from *Aegopodium podagraria* as induced by a series of 200-Hz light pulses (660 nm, 24 W m^{-2}). Illumination time was 40 s. The peak values F_p, L_M and V_M are defined by the graphs above. The y-axis for traces A and B has arbitrary units.

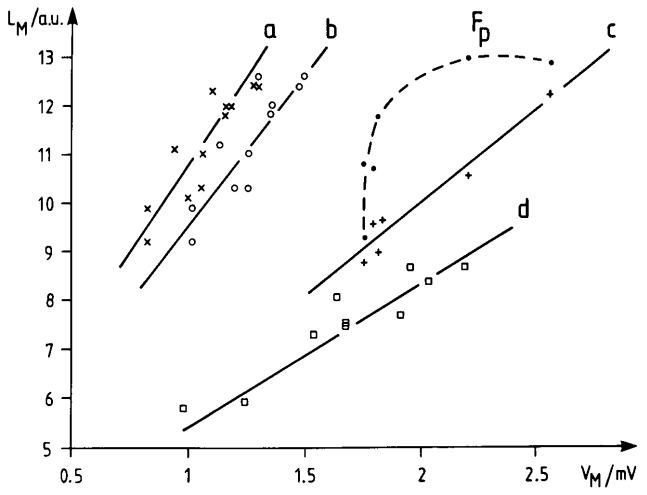

Fig. 3 Evidence for the relationship between proton-uptake into the lumen and the fast depolarisation as provided by the linear dependence of the changes of the peak values in membrane potential (V_M) and of the pH-peak of chlorophyll luminescence (L_M, see Fig. 2) from *Nitella* (a, b, c) and from *Aegopodium podagraria* (d). For the sake of comparison the peak of fluorescence (F_p) measured simultaneously with luminescence and membrane potential of curve c is shown.

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the luminescence (L_M) trace corresponds to the fast depolarisation. The responses in luminescence and plasmalemma potential do not reach their maximum values at the same time. This is not a contradiction to the assumption that both are induced by the generation of the transthylakoid pH gradient. The time of the peak is determined by several additional processes, which are partially different for both signals, especially those responsible for the decaying phase.

In these experiments the following light treatment was applied in order to cause peaks of variable magnitude: Responses similar to those in Fig. 2 were induced by a series of 200-Hz pulses (24 W m^{-2}, 40 s). These series were interrupted by different dark periods ranging from 1 min to 20 min. The magnitude of the 2nd luminescence peak increased after repetitive illumination, the peak of fluorescence decreased. In Fig. 3 the magnitude of the 2nd luminescence peak is plotted versus the magnitude of the fast light-induced depolarisation of plasmalemma potential. A parallel behavior of transthylakoid pH and light-induced changes in plasmalemma potential is indicated in the experiments on the alga *Nitella* (a, b, c) and on the higher plant *A. podagraria* (d).

Exclusion of direct action of CO_2 on cytosolic pH and plasmalemma potential—The following experiments address the question raised in the Introduction, namely whether uptake of protons into the chloroplasts or uptake of CO_2 leads to the transient light-induced alkalinization of the cytosol.

Experiments of the first set were performed on leaves of tabaco still connected to the plant. One of the parameters light intensity, CO_2, or O_2 was changed in a step-wise manner, and the induced changes in plasmalemma potential and in chlorophyll fluorescence were recorded simultaneously. Figure 4 displays the changes induced by CO_2 (traces A, B and C), by O_2 (traces D, E and F) and by light (traces G and H).

Figures 4A and 4D show that CO_2 and O_2 do not induce changes in plasmalemma potential if the leaves are kept in darkness (traces A and D, fluorescence cannot be measured in the dark). However, in contrast to Fig. 4D, the effect of O_2 at concentrations below 1% becomes very complicated in the dark. It increases with decreasing O_2-concentrations. However, these very complicated results at low concentrations will be reported in a subsequent paper.

Secondly, the comparison of the responses to a decrease in CO_2-concentration (Fig. 4B and C) with those induced by an increase in light intensity (Fig. 4G and H) shows that the responses of fluorescence are quite similar in curve-shape. The responses to a change in O_2-concentration very often showed an oscillatory behavior, resembling the oscillations observed by Laisk et al. (1991).

In the case of plasmalemma potential the depolarizing phase is similar in all responses, but there is a strong hyper-

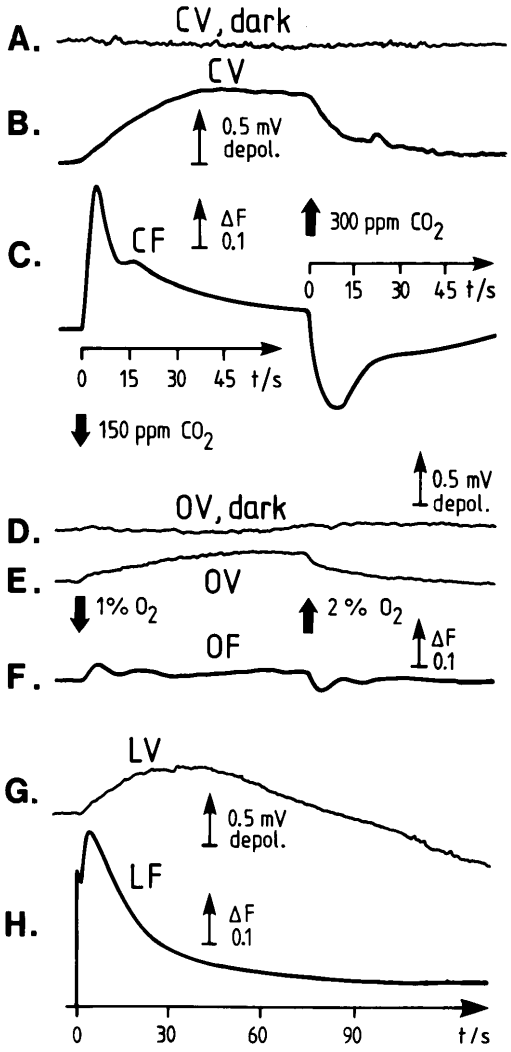

Fig. 4 Responses of plasmalemma potential and of chlorophyll fluorescence to changes in gas concentration and in light intensity. The traces are labelled as follows: the first letter gives the input signal (C=CO_2, O=O_2, L=light), the second one the output signal (V=plasmalemma potential, F=yield of chlorophyll fluorescence). In all experiments, N_2-flux was 150 ml min^{-1}. Traces A to C show the responses to a decrease in CO_2-concentration from 300 ppm to 150 ppm and to an increase back to 300 ppm. O_2-flow was 2% of the N_2-flow. Trace A (CV=effect of CO_2 on plasmalemma potential) was obtained in the dark. Light intensity was 19 W m^{-2} in traces B and C showing the effect of CO_2 on plasmalemma potential (CV) and on chlorophyll fluorescence (CF). In traces D to F, CO_2-concentration was 150 ppm, and O_2-concentration was changed from 2% to 1% and back to 2%. Experiments were done in the dark (D) or at a light intensity of 19 W m^{-2} (E and F). The responses to changes in light intensity (G and H) were caused by a transition from 2 W m^{-2} (measuring light) to 19 W m^{-2} (after adding background light) at 300 ppm CO_2 and 2% O_2. The scaling bars for the y-axis hold for all traces. The leaves of *Nicotiana tabacum* were still connected to the plant.

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polarizing phase in the case of the light-induced responses. The responses to an increase in O_2 are smaller than those to an increase in CO_2, probably reflecting the different affinities of Rubisco.

The records in Fig. 4 provide a rough survey of the involved effects. In order to extract a more detailed information about the putative interactions between various processes of the photosynthetic system, a kinetic analysis based on curve-fitting has to be applied as described in Materials and Methods. In the experiments shown in Figs. 5 and 6, sinusoidal modulation of the input signals was used for linearization. The dependence on frequency of the amplitude of the induced changes describes the attenuation due to the sluggish response of involved reactions. This is illustrated in Figs. 5A, B and 6A, B.

In the case of the light effect on chlorophyll fluorescence, the redox state of the primary quencher Q_a has a major influence. As its time-constant is shorter than 1 s (τ_{1a} or τ_{1b}, Hansen et al. 1991), it can follow the changes in light-intensity over the whole frequency range from 10^{-3} to 1 Hz resulting in a constant (flat) contribution to the measured frequency response F_L shown in Fig. 5A. Other reactions as for instance the generation of the pH-gradient across the thylakoid membrane are slower. They cause the bumps in the F_L-curve of Fig. 5A.

The delay of the action of the input signal due to the involved slow reactions is decribed by the phase-shift which is given in Fig. 5C. The frequency dependence of the amplitude and of the phase-shift are combined to give the complex frequency response (Eq. 3) which is fitted by Eq. 4. The parameters used for fitting the curves in Fig. 5 are given in the legend.

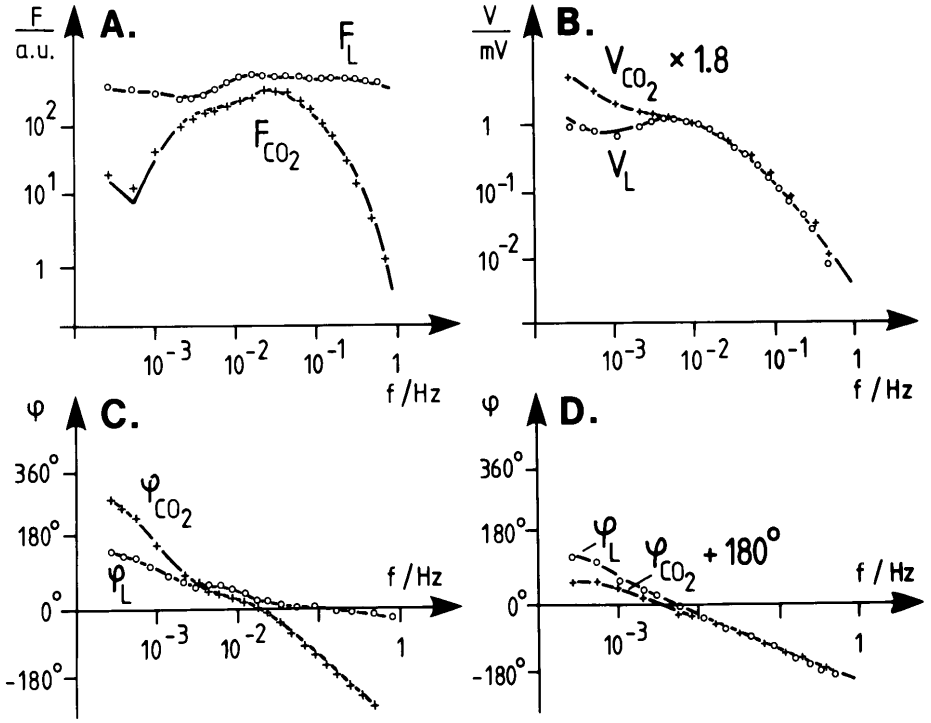

Fig. 5 Frequency responses of the amplitudes (A, B) and phase-shifts (C, D) of the action of CO_2 and light on chlorophyll fluorescence (A, C) and on plasmalemma potential (B, D) in spinach. The data was fitted by Eq. 4 for F and with Eq. 5 for V with the parameters given below. The unit of n (Eq. 5) is s, even though it is in the line of the amplitude factors. $j=\sqrt{-1}$. c, c* gives the two complex values of τ_c and τ_{c^*}. Light intensity was 5 W m^{-2}, Temperature=20°C.

comp. no.		1	2	3	4	5a	5b
cf, lf	τ_i/s	1.3	6.0	9.2	14.5	45.4	161
cf	A_i	−4.6	44.9	−63.4	26.8	−5.9	1.7
lf	A_i	2.6	−13.0	29.2	−27.3	12.9	−13.0

comp. no.		h	c, c*	n	d=4	dd	5a
cv, lv	τ_i/s	2,400	220 ±1,150 j		14.5	1.2	45.4
cv	A_i	34.6	7.3	5,600	−1.8		0.2
lv	A_i	13.0	6.7	4,363	6.0		−5.9

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In the case of the CO_2-effect on chlorophyll fluorescence, a chain of slow reactions is located between the Calvin-cycle and the PSII antenna. Thus, chlorophyll fluorescence does not immediately follow the changes in gas concentration. Consequently, the responses to fast frequencies are much more attenuated and result in much smaller amplitudes than the slow ones do. This leads to the high-frequency slope of F_{CO_2}.

The responses of plasmalemma potential to modulation of light intensity or CO_2-concentration are shown in Fig. 5B and D. In order to demonstrate the similarity at high frequencies, the amplitude responses of plasmalemma potential to light and to CO_2 are scaled by a constant factor which makes the high-frequency slopes coincide. The phase-shifts differ by exactly 180°. This corresponds to the similarity of the depolarizing phases in Fig. 4 as caused by a decrease in CO_2 and by an increase in light intensity, respectively. The differences in the low frequency behavior seem to resemble the different behavior of the slow hyperpolarizing phase in Fig. 4. (The temporal changes in the time-domain in Fig. 4 are faster by a factor of 2π than those in the frequency domain of Figs. 5 and 6). However, the investigation of this effect is not the issue of this paper. Without very time-consuming experiments (due to the required durations of the sine-waves up to 4 h) it cannot be stated whether these differences are within the normal scatter, or whether they indicate a real difference in the action of the two input signals.

For the problem investigated here, the coincidence of the high-frequency slope is important. Fig. 5B and D show that CO_2-modulation does not result in a faster action on plasmalemma potential than modulation of light intensity does. A faster CO_2-response would be expected if there were a direct action of CO_2 on cytoplasmic pH.

This finding obtained from a visual inspection of Fig. 5 is supported by the results provided by curve-fitting. Curve-fitting of the frequency responses of fluorescence can be done by Eq. 4 with A_0 being omitted in the case of CO_2 as there is no fast direct action of CO_2 on chlorophyll fluorescence as in the case of light.

The responses of light- and CO_2-action on plasmalemma potential are fitted by the following transfer function.

$$V(p)=\frac{V_h}{(1+p\tau_h)(1+p\tau_{hh})}+\frac{V_c(1+pn)}{(1+p\tau_c)(1+p\tau_{c'})(1+p\tau_{cc})}+\frac{V_d}{(1+p\tau_d)(1+p\tau_{dd})}+\frac{V_k}{(1+p\tau_k)(1+p\tau_{kk})} \quad (5)$$

This equation (which can be rearranged to a form like Eq. 4) accounts for the results from previous investigations (Hansen 1978, 1985, Martens et al. 1979, Vanselow and Hansen 1989) that light acts on plasmalemma potential via the four pathways which are described in Materials and Methods, and which are presented by the four terms in Eq. 5. They are related to light-induced hyperpolarisation (index h), control of cytosolic pH (index c), light-induced depolarisation (index d) and light-induced changes in the activity of the K^+-channel (index k). The new indices in Eq. 5 replace the nomenclature used by Hansen (1978, 1985) and Martens et al. (1979) as the old indices may lead to confusion with those of chlorophyll fluorescence. The correspondence is: $\tau_h=\tau_{1V}$, $\tau_{hh}=\tau_{6V}$ (slow and fast time-constant of a pathway hyperpolarizing with light), τ_c, $\tau_{c'}=\tau_2$, τ_3, $\tau_{cc}=\tau_{7V}$ (two complex time-constants and a fast time-constant related to the oscillations of the pH-regulating system, Hansen 1985, 1990). $\tau_d=\tau_{4V}=\tau_4$, $\tau_{dd}=\tau_{5V}$ (slow and fast time-constant of the depolarizing pathway). The index V (not shown explicitely in previous papers) is related to changes in potential V. n in the numerator accounts for the fact that the pH of the cytosol is disturbed by the depolarizing and the hyperpolarizing pathway (Hansen 1978, 1985, Martens et al. 1979).

In order to reduce the number of parameters when fitting the data, we used $\tau_{hh}=\tau_{cc}=\tau_{kk}=\tau_{dd}$ as these time-constants were faster than τ_{dd} and outside the range of investigated frequencies (Martens et al. 1979).

The reliability of curve fitting of kinetic data can be improved if background information is incorporated. In the case of the investigations presented here, we can make use of the following knowledge:

(1) Light and CO_2 stimulate from different input ports, but the same system, the ETC, is involved. Thus, all of the photosynthetic reactions shown in Fig. 1 are expected to be involved in the kinetics of the responses of chlorophyll-fluorescence to light or to changes in CO_2-concentration. Hansen et al. (1991) have shown that the same time-constants τ_i do occur in both signals, and that they differ by the amplitude factors A_i.

(2) τ_4 (Eq. 4) of fluorescence is equal to τ_d (Eq. 5) of the light action on plasmalemma potential and τ_{5a} is equal to τ_k (Hansen et al. 1987, Vanselow et al. 1989a, Vanselow and Hansen 1989).

(3) It is expected that τ_4 of the CO_2-action on fluorescence is equal to τ_d of its action on plasmalemma potential, because CO_2 causes changes in the transthylakoid pH gradient (Hansen et al. 1991) which is expected to cause an influence on plasmalemma potential as in the case of the light-induced changes of transthylakoid pH.

Because of this preinformation, the frequency responses like those in Fig. 5 were fitted in "global" or "joint" fit. These means that the same set of τ_i was used for the light- and for the gas-induced changes in chlorophyll fluorescence ($\tau_{iL}=\tau_{iC}$, L=light, C=CO_2), and that the computer had to use the constraints $\tau_4=\tau_d$ and $\tau_{5a}=\tau_k$. The amplitude factors A_i of Eq. 4 and V_i (i=h, c, d, k) of Eq. 5 (including the zero n) were allowed to take different values. What remained to be shown by curve-fitting is that no additional fast time-constant is required which would indicate a direct action of CO_2 on cytosolic pH.

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However, before doing these joint fits, it was tested whether the above assumption no. 3 is correct. In order to show that $\tau_4=\tau_d$ holds also for the responses to changes in CO_2 of fluorescence (cf) and of plasmalemma potential (cv), 10 frequency responses (obtained under conditions similar to those in Fig. 5) of the action of CO_2 on fluorescence and on plasmalemma potential were fitted independently. This resulted in the values of $\tau_{4,cf}=26.6\,s+/-2.9\,s$ and of $\tau_{4,cv}=24.7\,s+/-3.4\,s$, showing the expected coincidence.

The final curve fitting by the joint fit as specified above resulted in the parameters given in the legend of Fig. 6. As already shown by Hansen et al. (1991), the amplitude factors A_{iL} (i=1 to 6, L=light) of the action of light on chlorophyll fluorescence have the opposite signs of the A_{iC} (C=CO_2) of the action of CO_2. We ignore the results related to the hyperpolarizing pathway (index h) and the pH-controller (index c) as the range of applied frequencies should have been extended to lower frequencies (ca. 1 cycle/4 h) for studying these components. Here, we concentrate on the depolarizing pathway and have a short glance at the K^+-pathway.

In the case of the action of CO_2, the data leads to the following statements:

CO_2 reaches the photosynthetic apparatus quite rapidly within less than a few seconds. This holds, because the kinetics of the CO_2-induced and of the light-induced changes in plasmalemma potential do not show major deviations at high frequencies up to 1 Hz.

Fitting of the CO_2-induced changes in plasmalemma potential can be done without introducing an additional pathway which would indicate a direct action of CO_2 on plasmalemma potential (and thus on cytosolic pH accord-

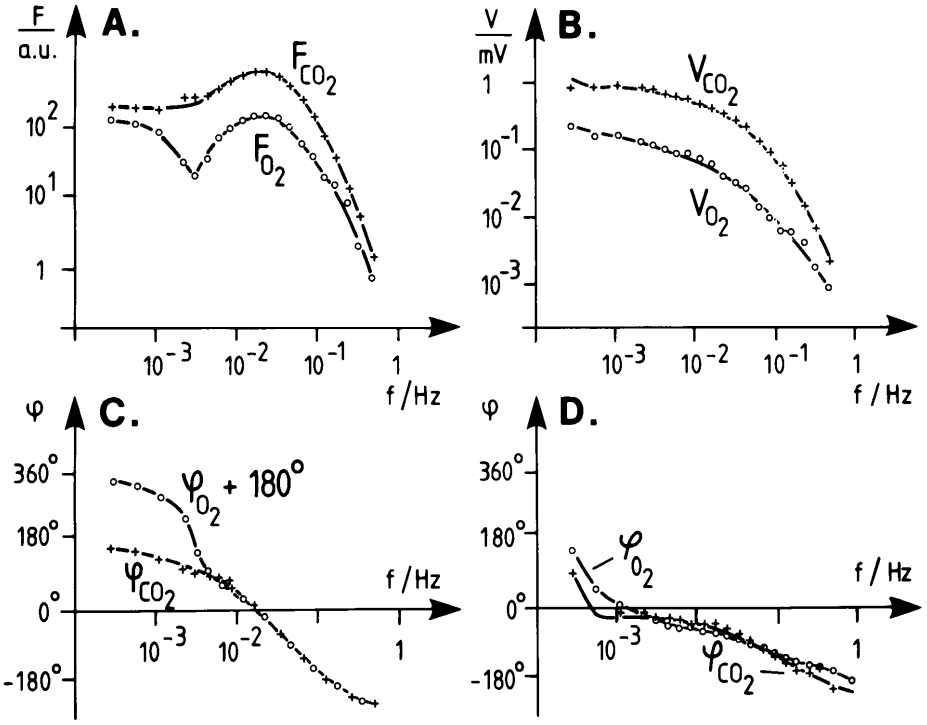

Fig. 6 Frequency responses of the amplitudes (A, B) and phase-shifts (C, D) of the action of CO_2 and of O_2 on chlorophyll fluorescence (A, C) and on plasmalemma potential (B, D) in spinach. The data was fitted by Eq. 4 for F and with Eq. 5 for V with the parameters given below. The unit of n (Eq. 5) is s, even though it is in the line of the amplitude factors. $j=\sqrt{-1}$. Light intensity was 20 W m^{-2}, Temperature=20°C

comp. no.		1	2	3	4	5a	5b
cf, of	τ_i/s	1.0	3.0	6.0	8.6	43.9	102
cf	A_i	−0.2	3.4	−12.4	9.5	−0.3	0.2
of	A_i	−0.04	+0.7	−2.4	+1.7	−0.2	+0.4

comp. no.		h	c, c*	n	d=4	dd	5a
cv, ov	τ_i/s	2,000	240 ±1,400 j		8.6	1.5	42.9
cv	A_i	−36.0	−52.7	643	−2.2		−1.5
ov	A_i	+31.2	4.7	3,221	−0.3		−0.6

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ing to its assumed action of the plasmalemma H^+-pump, Hansen et al. 1987, Vanselow and Hansen 1989a).

The fastest action on plasmalemma potential is in either case (light or CO_2) mediated by the mechanism labelled by τ_4, the generation of the transthylakoid pH-gradient.

The effect of O_2 on chlorophyll fluorescence and on plasmalemma potential—O_2 is expected to exert the same influence on the Calvin cycle (and thus on the thylakoid proton fluxes) as CO_2 does, but it is not assumed that there is a direct effect of O_2 on cytosolic pH. Fig. 6 shows the frequency responses of the action of CO_2 and of O_2 on chlorophyll fluorescence and on plasmalemma potential. The generation of reliable data becomes more difficult as the responses to O_2-modulation are small and are more sensitive to spurious signals. The amplitude response of fluorescence to CO_2 and to O_2 seems to be similar. The similarity of the high-frequency behavior becomes more obvious in the phase responses which should be regarded to be more reliable. The phase-shifts coincide (Fig. 6C and D). Again, the differences in the low-frequency behavior are not discussed here. For a statistically reliable statement much more data have to be collected. Nevertheless, the notch in the amplitude of the O_2-action on fluorescence is in line with the occurrence of oscillations in Fig. 4F, as a notch may indicate the involvement of complex time-constants which are related to oscillations (Hansen 1978, 1990).

Curve fitting by means of Eqs. 4 and 5 reveals that τ_4 and τ_d are identical for the CO_2- and the O_2-action. Averaged data are shown in Table 1.

The dependence on light-intensity—Hansen (1978), Fisahn et al. (1985) and Vanselow et al. (1988) have shown that τ_4 depends strongly on light intensity. This feature can be used as additional support for the statement that the mechanism related to τ_4 (transthylakoid H^+-fluxes) and not a direct action of CO_2 on cytosolic pH mediates the light effect on plasmalemma potential.

The experiments in Figs. 5 and 6 were done at different mean light intensities (5 and 20 W m^{-2}, respectively). The data in the legends shows that all time-constants, especially $\tau_4=\tau_d$, become faster at higher light intensities. Averaged data for τ_4 and for τ_{5a} from additional experiments like those in Figs. 5 and 6 are shown in Table 1. This dependence on light intensity is not expected if the dominating time-constant of the fast changes in plasmalemma potential, τ_d, were related to an effect originating from a direct action of CO_2 on cytosolic pH.

The imbalance of ATP and NADPH/H^+ generation and the involvement of the Mehler reaction—The activity of the Calvin cycle has two counteracting effects on ATP balance. Firstly, it supports linear electron flow through the ETC and generates the transthylakoid pH-gradient. Secondly, it consumes ATP and thus decreases the pH-gradient. The question is discussed of whether the first effect, especially if supported by the Q-cycle (Haehnel et al. 1990), results in a sufficient number of translocated protons for an adequate supply of ATP. In Fig. 4. the effect of the modulation of CO_2- and O_2-concentrations on plasmalemma potential reveals a hyperpolarisation (net flow of protons out of the lumen) with increased activity of the Calvin cycle. This shows that CO_2-fixation (or photorespiration) consumes more ATP than is generated by the flow of electrons through the ETC to $NADPH^+$. Consequently, additional processes have to be involved in order to provide additional ATP. One candidate is the Mehler reaction (Schreiber and Neubauer 1990).

Table 1 Dependence of $\tau_4=\tau_d$ (upper part) and of $\tau_{5a}=\tau_k$ (lower part) on the mean value of light intensity

Light intensity	5 W m^{-2}	20 W m^{-2}	35 W m^{-2}
$\tau_4=\tau_d$			
cf*	42.5 (1.5)	15.7 (1.5)	6.1 (1.3)
cf	22.8 (1.5)	11.4 (1.9)	8.1 (1.1)
of	28.6 (1.1)		6.9 (1.2)
lf	26.3 (1.3)	4.6	
cv	20.9 (1.5)	12.0 (1.8)	8.1 (1.1)
ov	28.4 (1.1)		8.2 (1.1)
lv	26.2 (1.3)	4.6	
No. of exp.	6	4	3
$\tau_{5a}=\tau_k$			
cf	43.7 (1.5)	25.3 (2.0)	15.2 (1.6)
of	52.2 (1.1)		12.6 (1.4)
lf	53.6 (1.1)	25.6	
cv		24.6 (2.2)	13.8 (2.8)
ov	45.7 (2.0)		
lv	57.8 (1.1)	30.4	
No. of exp.	6	4	3

cf, lf, etc.: 1st letter: input, 2nd letter: output. cf*: data from Hansen et al. (1991). The unit of the τ_i is s. The numbers in parenthesis give the scatter factors, (resulting from averaging in a logarithmic scale, Hansen 1978). For instance, if the scatter factor is 1.5 the range given by the standard deviation is from "mean value/1.5" to "mean value $\times$ 1.5".

The new photoacoustic technique developed by Kolbowski et al. (1990) enables the separate determination of O_2-evolution, -uptake, and of heat evolution. The photoacoustic responses f(t) to a short flash (measuring light, 1 ms, 100 W m^{-2}) were recorded during 50 ms and were splitted into these three components by curve fitting

$$f(t)=\sum_{i=0}^{3} k_i s_i(t) \qquad (6)$$

s_1, s_2, and s_3 are model curves of heat evolution, O_2-evolution and of O_2-uptake, respectively. They have been obtain-

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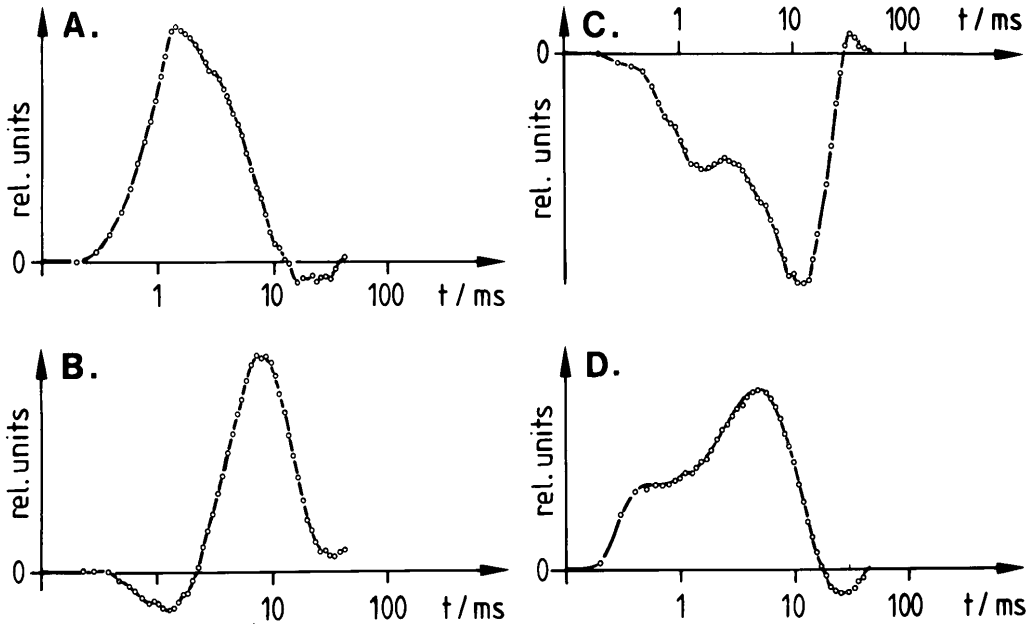

Fig. 7 Splitting of measured photoacoustic responses from leaves of *N. tabaccum Virginia* into three components by means of curve-fitting with a sum of three model responses. The model responses are obtained under conditions as proposed by Kolbowski et al. (1990). Measuring light was provided by pulses of 1 ms (100 W m^{-2}, 660 nm) with a repetition rate of 50 ms. 10 pulse responses are averaged. A: s_1: heat evolution measured under saturating (1,000 W m^{-2}) background light. B: s_2: O_2-evolution measured after preillumination with 1,000 W m^{-2} for 10 min. C: s_3: O_2-uptake (after dark adaptation, 6 h). D. Response measured under normal conditions (dots) and theoretical curve obtained from curve-fitting (Eq. 6).

ed by means of the protocols proposed by Kolbowski et al. (1990): saturating background light (s_1), preceding saturating illumination (s_2), preceding dark adaptation (s_3). Fig. 7 shows the three model curves and an overall response fitted by these model curves by means of Eq. 6. It is obvious that measured photoacoustic pulse responses can be fitted very well by model curves.

In order to study the kinetic behavior of the three components (k_i in Eq. 6) light pulses (measuring light) were given every 50 ms for about 200 s. The responses (similar to Fig. 7D) were recorded with a sampling rate of 10 KHz. Groups of 50 of these responses were averaged on line, and the averaged curves were stored in the computer. After the experiment, the averaged responses were subject to curve-fitting by means of Eq. 6. Each fit provided three data: k_1, k_2, and k_3. This provided a temporal resolution of 2.5 s for the changes in the k_i.

Changes in the k_i were induced by different experimental protocols. In the first group, the measuring light (pulses) also served as actinic light, as photoacoustic responses were recorded after switching on the measuring light (group 1). In other experiments, the measuring light was on throughout the experiment, and the actinic light was provided by switching on background light (group 2), or switching off background light (group 3). Time-courses of the individual components (k_i) of Eq. 6 were plotted ver-

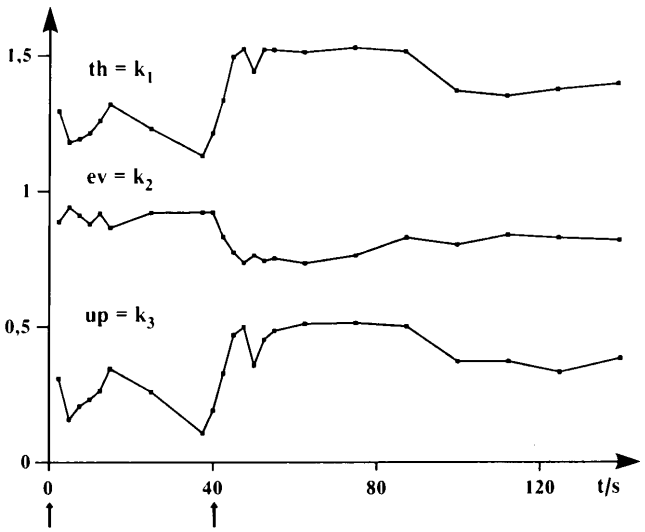

Fig. 8 Time-courses of the individual components k_i (relative contribution of the model curves of Fig. 7 A to C, th=heat production, ev=O_2-evolution, up=O_2-uptake) of the photoacoustic responses (Fig. 7D). Each data point was obtained from curve fitting (Eq. 6) of the average of fifty 50-ms responses similar to those in Fig. 7. First arrow at t=0: group-1 response: the measuring light was switched on, acting also as actinic light. Second arrow at t=40 s: group-2 response: actinic background light (10 W m^{-2}) was switched on. Conditions as in Fig. 7.

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Table 2 Mean values of the time-constants τ_i and amplitude factors k_i as obtained from curve-fitting (Eq. 2) of the time-courses of the photoacoustic responses shown in Fig. 8

Component no.	2	3	4	5a	5b
Time-constant τ_i/s					
Group 1, $0 \rightarrow 2$ W m^{-2}	1	7.0	40.3	57.7	121.8
Group 2, $2 \rightarrow 10$ W m^{-2}	1	7.3	30.1	57.0	109.5
−Group 3, $10 \rightarrow 2$ W m^{-2}	1.4	7.9	26.9	60.8	140.3
Scatter factor	1.1	1.1	1.5	1.1	1.3
Amplitude factors k_i of heat evolution					
Group 1	2.1	−1.9	17.4	−27.1	13.6
Group 2	3.0	−4.6	15.3	−26.4	27.7
−Group 3	3.0	−4.0	8.4	−10.5	6.2
Scatter factor	2.5	3.0	2.2	1.8	2.3
Amplitude factors k_i of O_2-uptake					
Group 1	3.4	−2.3	20.1	−32.8	17.7
Group 2	4.9	−7.0	21.7	−31.9	29.4
−Group 3	4.0	−3.7	4.3	−5.4	3.9
Scatter factor	5.0	3.5	1.5	1.4	1.5
Amplitude factors k_i of O_2-evolution					
Group 1	−2.1	0.1	2.0	−2.5	0.7
Group 2	−3.1	3.6	−12.4	19.6	−18.3
Group 3	−1.2	0.6	3.8	−3.9	0.2
Scatter factor	2.3	3.6	2.4	2.1	2.2

Data sets were obtained from 3 sets of experiments according to the three protocols mentioned in the text. The scatter factor is the averaged scatter factor of the three data in the related column. *Nicotiana tabacum Virginia.*

sus time (Fig. 8), thus establishing three curves of the behavior of each component after a change in light conditions.

The following features are important.

(1) The responses of O_2-evolution (ev) were badly reproducible, and not evaluated here.

(2) The time-courses of heat evolution (th) and O_2-uptake (up) look very similar.

These time-courses were fitted by a sum of exponentials (Eq. 2) in order to reveal the involved components as is done above in the case of the frequency responses of Figs. 5 and 6. Again, the time-constants τ_2 to τ_4, τ_{5a}, τ_{5b} are employed for fitting the data, because they label the most important reactions in the photosynthetic apparatus (Fig. 1). These reactions all participate whenever the system is stimulated by a signal. This signal may be light or changes in gas concentration. Thus, differences are expected to occur in the amplitude factors, but not in the time-constants. The assignment of the measured time constants to those in Fig. 1 can be done by identifying τ_4 first. From previous studies (Dau and Hansen 1989, 1990) it is known that the amplitude factor related to τ_4 of heat production has a positive sign and is the first time constant with a major amplitude factor.

Averaged values are displayed in table 2. The components related to the time-constants τ_4 and τ_{5a} are maximum. This may also hold for τ_{5b}. However, this time constant is at the edge of the investigated time-range, and thus conclusions should be left to investigations which will concentrate on the long-term range.

The finding that in heat evolution the component related to τ_4 is much stronger than those related to τ_2 or τ_3 (table 2) is in agreement with previous investigations of Dau and Hansen (1990). This is expected as energy quenching (related to τ_4) increases thermal deactivation in the antenna (Schreiber and Neubauer 1990, Horton et al. 1991, Ramm and Hansen 1993). Dau and Hansen (1990) showed that the amount of energy related to the increase in the thermal signal is identical to that related to the decrease in fluorescence.

The interesting feature is that τ_4 determines also the increase in O_2-uptake, and that τ_{5a} is related to the decay of both signals.

Discussion

The conclusions of these investigations are based on previous findings that the measured depolarisations of the

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plasmalemma in the light result from an alkalinization of the cytosol via a substrate effect on the H^+-pump (Hansen 1978, 1985, Hansen et al. 1987, 1991, Sanders et al. 1981, Vanselow et al. 1989a, Vanselow and Hansen 1989).

The results presented here provide additional support that the action of light on plasmalemma potential is related to the time-constant τ_4 which labels the generation of transthylakoid pH (Fig. 3 and Fig. 8). The involvement of a direct action of electrical potential across the thylakoid membrane via membrane connections to the putative chlorolemma (Gyenes 1987) can be excluded because of the results of Fig. 2. However, this holds only for the τ_4-component investigated here, as a small light response of the measured electrical potential which is very similar to the luminescence induction curve of Fig. 2 can be observed in *Nitellopsis obtusa* treated with tentoxin (Gyenes et al. 1986).

Also, transenvelope fluxes of PGA do not play an important role in the light-induced changes in cytosolic pH. According to Laisk et al. (1989), ATP is not immediately available after an increase in light-intensity (delayed by τ_4), and the PGA-Pool is depleted by $NADPH^+/H$. This may lead to uptake of PGA^{2-} from the cytosol and to alkalinization as mentioned above. If this would have a significant effect of cytosolic pH, a faster time constant than τ_4, probably τ_3, would dominate the light-induced depolarisation of plasmalemma potential. However, this has never been found.

The verification of the relationship between τ_4, Mehler reaction (Fig. 8), acidification of the lumen (Fig. 3) and plasmalemma depolarisation per se does not answer the question of whether proton- or CO_2-fluxes mediate the light-induced changes in the cytosol, as both fluxes are related to the generation of the transthylakoid pH-gradient, directly or via the supply of ATP. The answer is obtained from the CO_2- and O_2-effect on plasmalemma potential. The action of CO_2 or O_2 shows the same kinetics as the light effect does. If there were a direct effect of CO_2 on cytosolic pH, a component faster than the τ_4-component should become obvious in the CO_2-curves of Fig. 5. It cannot be argued that the CO_2-fluxes from the outside and those into the chloroplast occur under different conditions, thus leaving room for a suggestion that fluxes into the chloroplasts do have an effect and those from the outside do not. The experiments are done under linearizing conditions. This implies that there is a steady-state concentration of CO_2 in the cytosol which is increased and decreased during a period of a sine-wave by each of the two fluxes. Because of this, the cytosol cannot find out whether a change in CO_2-concentration is brought about by an increase of the flux from the outside or a decrease of the efflux to the chloroplasts. As the fluxes from the outside are the cause of the modulation of photosynthetic activity, its effect should be greater than that of the uptake related to the τ_4-component. Such a component has not been observed.

The conclusion from these experiments and considerations is that the proton fluxes across the envelope resulting from H^+-uptake into the lumen are the origin of the alkalinization of the cytosol. Thaler et al. (1992) have come to a similar conclusion when studying the effect of venturicin (a blocker of the thylakoid ATPase) on the light-induced changes in cytosolic pH. The question remains open which transporters mediate the H^+-fluxes across the envelope.

According to the above results (and also to Thaler et al. 1992), transenvelope fluxes of protons result in changes of the cytolic pH. Because of this, the CO_2-induced hyperpolarisation of the plasmalemma with the time-constant τ_4 indicates a release of protons from the lumen into the stroma and finally into the cytosol. The occurrence of the CO_2-induced changes in plasmalemma potential (Fig. 4, 5 and 6) is an indication of the imbalance of ATP and $NADPH/H^+$ production by the linear electron flow to the Calvin cycle. The outflow of protons results from the degradation of the transthylakoid pH gradient when additional CO_2 consumes more ATP than is generated by the concomitant increase in linear electron flow. As these experiments are done under linearizing conditions, the demand for additional mechanism providing additional ATP is shown to hold also under steady state conditions.

In the photoacoustic experiments, τ_4 is not only the dominating time-constant of heat evolution (thus verifying again the relationship between τ_4 and transthylakoid pH gradient) but also of O_2-uptake, indicating the involvement of the Mehler reaction. The occurrence of τ_4 in O_2-uptake supports the model of Schreiber et al. (1991) and Reising and Schreiber (1992) implying that the Mehler reaction is stimulated by acid pH in the lumen and alkaline pH in the stroma. The mechanism assumed by these authors is shown in Fig. 1. O_2 gets an electron from PSI. The negative surface charge at the stroma site pushes O_2^- to the inside and takes up a proton. The superoxide dismutase (SOD) convertes 4 HO_2 to 2 O_2+2 H_2O_2. The monodehydroascorbate reductase (MDR) and the ascorbate peroxidase (APO) mediate the conversion $H_2O_2+2\,e^-+2\,H^+ \rightarrow 2\,H_2O$.

Actually, there is no net O_2-uptake by this reaction as water splitting for the electrons and dismutation generate as many O_2 as the O_2-reduction at PSI consumes. However, as already suggested by Reising and Schreiber (1992) the photoacoustic technique seems to see a net O_2-uptake at PSI because the evolution of O_2 from dismutation comes later and does not compensate the uptake during the first 50 ms after the light flash. Since the pH optimum of the SOD is at pH 5.5 in the lumen and that of the MDR is at pH 8.5 in the stroma, the generation of the transthylakoid pH stimulates the Mehler reaction and thus the photoacoustically measured O_2-uptake with the time-constant τ_4.

The decrease of O_2-uptake with the time-constant τ_4 after switching off preillumination implies that the Mehler-

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reaction was active during the preceding long-term illumination. Thus it participates in the generation of additional ATP under steady-state (linearizing) conditions. The question remains open whether it supplies enough ATP. Katona et al. (1992) argue that cyclic electron transport is more important than the Mehler reaction in supporting a high transthylakoid pH gradient. However, their experiments were done under conditions of water stress.

The data in Fig. 8 may also contribute to the identification of τ_{5a}. The identity of this time-constant is still unknown. The τ_{5a}-component has the opposite sign of the τ_4-component in heat evolution and in O_2-uptake. Vanselow and Hansen (1989) found that τ_{5a} was the time-constant of the light-effect on the K^+ channel in the plasmalemma probably mediated by the light-induced uptake of Ca^{2+} into the chloroplasts (Heimann et al. 1987, Kreimer et al. 1985, Muto et al. 1982, Miller and Sanders 1987). The expected action of this Ca^{2+} uptake on cytosolic streaming has been measured by Plieth and Hansen (1992). There is the speculation that Ca^{2+} is required for the stimulation of the NAD-kinase (Miller and Sanders 1987, Jarret et al. 1982). The results in Table 1 may fit into this hypothesis.

The decrease in the activity of the Mehler reaction labelled by τ_{5a} may be caused by the activation of the FNR as this would withdraw electrons from the Mehler reaction and lead it to the FNR. In addition, heat evolution related to energy quenching would decrease if the supply of $NADPH/H^+$ would stimulate the Calvin cycle and diminish the transthylakoid pH-gradient. However, these suggestions remain to be tested.

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