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Alteration of photosystem II properties with non-photochemical excitation quenching

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Oxygen yield from single turnover flashes and multiple turnover pulses was measured in sunflower leaves differently pre-illuminated to induce either 'energy-dependent type' non-photochemical excitation quenching (q_E) or reversible, inhibitory type non-photochemical quenching (q_I). A zirconium O_2 analyser, combined with a flexible gas system, was used for these measurements. Oxygen yield from saturating single turnover flashes was the equivalent of 1.3–2.0 μmol e^- m^{-2} in leaves pre-adapted to low light. It did not decrease when q_E quenching was induced by a 1 min exposure to saturating light, but it decreased when pre-illumination was extended to 30–60 min. Oxygen evolution from saturating multiple turnover pulses behaved similarly: it did not decrease with the rapidly induced q_E but decreased considerably when exposure to saturating light was extended or O_2 concentration was decreased to 0.4%. Parallel recording of chlorophyll fluorescence and O_2 evolution during multiple turnover pulses, interpreted with the help of a mathematical model of photosystem II (PS II) electron transport, revealed PS II donor and acceptor side resistances. These experiments showed that PS II properties depend on the type of non-photochemical quenching present. The rapidly induced and rapidly reversible q_E type (photoprotective) quenching does not induce changes in the number of active PS II or in the PS II maximum turnover rate, thus confirming the antenna mechanism of q_E. The more slowly induced but still reversible q_I type quenching (photoinactivation) induced a decrease in the number of active PS II and in the maximum PS II turnover rate. Modelling showed that, mainly, the acceptor side resistance of PS II increased in parallel with the reversible q_I.

Keywords: photosynthesis; photosystem II; quenching; photoregulation; photoinhibition

1. QUENCHING EXCITATION

Excess light is a frequent problem for upper leaves of plant canopies. Light is in excess when the next photon arrives before the electron (e^-), produced by the preceding photon, has been removed from the photosystem II (PS II) acceptor Q_A (or, generally, from the terminal acceptor compound of e^- transport chain) blocking the way for the next e^-. Thus, excess light is a result of imbalance between excitation arrival rate in the reaction centre and e^- use rate for CO_2 assimilation, and may appear as a result of too high light intensity or too low photosynthetic rate (Osmond 1994; Anderson *et al*. 1997; Osmond *et al*. 1999). With reduced Q_A, PS II is unable to carry out stable charge separation, i.e. the PS II reaction centres are closed. As a result, the average lifetime of excitation increases from 300–400 ps in the state of oxidized Q_A to 1–2 ns in the state of reduced Q_A (Horton & Ruban 1994). Such long-living high-energy state, a state of high excitation pressure (Gray *et al*. 1996), may be dangerous for the photosynthetic machinery. Nature has worked out ways to quench this state non-photochemically.

(a) *Photochemical quenching*

In photosynthesis photons excite chlorophyll that forms an antenna system around PS II (Dainese *et al*. 1992; Green & Durnford 1996) and the excitation is rapidly transferred to a special pair of chlorophylls P_{680} in the PS II core complex (Krause & Weis 1991; Renger 1992; Van Grondelle *et al*. 1994; Lavergne & Trissl 1995). Within less than 3 ps a primary radical pair $P^+_{680}Pheo^-$ is formed (Wasielewski *et al*. 1989; Jankowiak *et al*. 1989), but this state may reverse, and excitation is rapidly transferred to centre chlorophylls P_{680} and from it to the antenna (Roelofs *et al*. 1992). Excitation travels around the antenna within a picosecond and visits P_{680} again and again. Each time, the P^+_{680} Pheo pair is formed and reversed until finally the separated charges become stabilized when e^- passes from $Pheo^-$ to the primary quinone acceptor (Q_A), which happens within 350 ± 100 ps (Eckert *et al*. 1988). With oxidized Q_A, the lifetime of excitation is determined mainly by the time needed for charge transfer from $Pheo^-$ to Q_A, termed 'charge stabilization'. When Q_A is reduced, charge stabilization cannot happen and excitation continues to travel in the antenna, visiting P_{680} and trying to form the reversible primary radical pair, but failing to because the charged Q_A^- seems to push the next e^- back by its electrical field, formally lowering the average redox potential of the primary pair state (Van Mieghem *et al*. 1995). Thus, in the presence of reduced Q_A, the lifetime of excitation is determined by two other processes competing for excitation quenching: dissipative (thermal) conversion and fluorescence emission. Since these quenchers are slower

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than the photochemical charge separation, the lifetime of excitation lengthens approximately five times to *ca*. 2 ns when Q_A is reduced.

(b) *Chlorophyll fluorescence*

The emission of excitation as fluorescence occurs with an approximately constant probability density, i.e. in each time interval the probability of conversion of the excitation to fluorescence is constant. The longer the lifetime of excitation, the higher the integrated probability of fluorescence emission. Excitation lifetime is the longest when Q_A is reduced and, correspondingly, fluorescence is maximal, being suppressed only by the competing rate constant for thermal conversion. This maximal fluorescence yield is denoted F_m. When Q_A is oxidized, the excitation lifetime is shortest, being determined mainly by the rate constant of charge stabilization from the state of $P^+_{680}/Pheo^-/Q_A$ to $P^+_{680}/Pheo/Q^-_A$, and fluorescence yield is the lowest, denoted F_o. Any intermediate fluorescence yield is a sum of emissions at the F_m and F_o levels from different PS II, dependent on their Q_A reduction state. Thus, steady-state fluorescence yield (F) is an indicator of the average lifetime of excitation before it becomes quenched, either by photochemical charge separation or by thermal conversion, or is emitted as fluorescence (for a review, see Lavergne & Trissl 1995).

(c) *Non-photochemical quenching*

Returning to the situation of excess light, Q_A is reduced in most PS II and the fluorescence yield (F) is expected to be close to F_m. Intuitively, this is probably a dangerous situation because chlorophylls carrying long-living excitation are strong reducers and uncontrolled e^- transfer to lipids or other acceptors may occur. Such a high-fluorescence state was observed by H. Kautsky, who saw strong red fluorescence of the leaf when it was suddenly illuminated with blue light. Unexpectedly, within a minute or two, the fluorescence weakened to a low level, a phenomenon now termed the 'Kautsky effect' (Lichtenthaler 1992; Govindjee 1995). The Kautsky effect shows that when photochemical quenching is insufficient, the excess excitation must be quenched by another, non-photochemical quencher (q_N). Consequently, the fluorescence level (the average lifetime of excitation) stays more or less constant. Since q_N is complementary to the photochemical quencher q_P (Laisk *et al.* 1997), this suggests that these two mechanisms of excitation quenching are similar.

The mechanism of non-photochemical quenching has been subject to numerous studies. At first glance, it seems to be a physiologically important protective mechanism that keeps the excitation lifetime constant independent of the presence or absence of excess light. However, when present for a long time the initially rapidly reversible process ('photoprotection state', Osmond *et al.* 1999) becomes irreversible and continues quenching after the light intensity has decreased, unnecessarily losing quanta. This situation is termed 'photoinhibition' or 'photoinactivation' (Osmond 1994; Osmond *et al.* 1999). Under natural conditions where light is variable, photoinhibition may protect the photosynthetic machinery when light is high, but it causes loss of valuable quanta when light is low. An ideal photoprotective system would relax rapidly, following the time-course of the natural variability of light, but the mechanism of q_N fails to do this. What happens in PS II or in the light-harvesting antenna when q_N is induced? Why is q_N able to follow fast changes in light intensity when excess light has been present for short time but it loses this ability when excess light has been present for longer time? These questions have been the centre of attention for some time. An answer to the first question is contributed here, showing that some types of q_N are accompanied by changes in PS II properties but others are not.

(d) *Non-photochemical 'energy-dependent' quenching*

The general process of non-photochemical quenching q_N seems to have no single mechanism, but is a succession of different processes developing in time. One component of the non-photochemical quenching, termed 'energy-dependent' quenching (q_E), is the fastest. It is induced by the acidification of the thylakoid lumen, dependent on the presence of antheraxanthin and zeaxanthin (Krause & Weis 1991; Horton *et al.* 1996). It does this within 30–60 s and is reversed within 5–15 min, but the component develops and relaxes more slowly than ΔpH itself. This shows that the process is related to some basic rearrangements in the organization of the photosynthetic machinery and the excitation quenching is not a direct consequence of proton accumulation or protein protonation.

Initially it was proposed that non-photochemical excitation quenching occurs in PS II reaction centres that, under the influence of the low surrounding pH, are turned into a form that emits very little fluorescence and has reduced photochemical efficiency (Weis & Berry 1987). The centre-quenching model would explain the complementation between photochemical and non-photochemical excitation quenching if the trapping constant does not change and the trapped excitation is dissipated as heat. However, the centre-quenching model has difficulties in explaining the non-photochemical quenching of 'dark' fluorescence (F_o), the role of zeaxanthin that is located in the antenna (Demmig-Adams *et al.* 1989; Pfündel and Bilger 1994), and several other kinetic phenomena (Ting & Owens 1994; Horton *et al.* 1994; Pfündel & Bilger 1994). A presently widely accepted mechanism explains q_E as excitation quenching in the LHC II complexes (Horton *et al.* 1996) or in the minor CP24 and CP29 complexes (Crofts & Yerkes 1994) of the antenna caused by close interaction between chlorophylls as a result of ligand protonation (Crofts & Yerkes 1994) or between chlorophylls or chlorophyll and zeaxanthin, which are moved into close contact as a result of protein protonation (Horton *et al.* 1994, 1996). The most convincing evidence that q_E is an antenna-based phenomenon and does not involve charge recombination processes is probably the demonstration that q_E was maintained when leaves were cooled to 77 K (Ruban *et al.* 1993). This photoprotective antenna-based mechanism predicts that no changes occur in PS II centres when q_E is induced.

(e) *Very slowly reversible non-photochemical quenching*

When leaves are exposed to the high light for a longer time, the relaxation of q_E becomes slower and, finally, a

rather stable situation is created, termed 'photoinhibition' (q_I) (Horton & Ruban 1994) or 'photoinactivation' (Osmond *et al.* 1999). Photoinhibition needs hours or days for relaxation and it is related to some damage in the PS II centres. A very slow component of q_N, the very slowly reversible photoinhibitory quenching q_I, has been subject to thorough investigation. PS II centres are inactivated in the presence of this type of q_I, as shown by the measurements of O_2 evolution from trains of saturating single turnover flashes in photoinhibited leaves (Anderson *et al.* 1995). There is no clear consensus about the mechanism of photoinactivation. One line of evidence emphasizes the possibility that this q_I originates from damage in the water-splitting mechanism, while others emphasize changes on the acceptor side, probably due to the double reduction of Q_A and the following protonation and dissociation of this e^- carrier (Telfer & Barber 1994; Styring & Jegerschöld 1994; Zer *et al.* 1994; Ohad *et al.* 1994). Both mechanisms are thought to lead to the degradation of the D_1 protein of the PS II core complex, reparation of which needs the transportation of the particular PS II into the non-appressed thylakoid region, but the causal relationships between photoinhibition and D_1 degradation are not completely clear (Ohad *et al.* 1994; Critchley 1994). Repair of D_1 is a slow process and, as the half-time of D_1 degradation is about 1–2 h and repair is even slower (Aro *et al.* 1993), photoinactivation associated with D_1 degradation certainly needs longer than 30 min for relaxation.

The mechanism of q_I may be related to damage on the PS II donor side or acceptor side, but an important feature of this damage is that it causes excitation (fluorescence) quenching. In partially photoinhibited leaves quenching of both, q_E and q_I type is complementary to q_P: as much the quantum yield of q_P decreases as much the quantum yield of q_E or q_I increases. Consequently the lifetime of excitation remains practically constant (Laisk *et al.* 1997). This is unexpected if there are different mechanisms for the two processes, and suggests that any q_I quenched PS II emits fluorescence at a level close to F_o, as well as any q_E quenched PS II and normal q_P quenched PS II. The coincidence of the quenching level in PS II centres which have oxidized P_{680}, which lack Q_A (q_I), which have slightly changed position of chlorophyll in their antenna (q_E) or which quench due to charge separation and stabilization on Q_A (q_P) encourages the search for a single mechanism to explain all three states of PS II quenching. Such a mechanism would be one that is based on charge separation, as for q_P, but with the following recombination of separated charges in the states of q_E and q_I.

(f) *Transition type and reversible inhibitory non-photochemical quenching*

The most difficult to quantify and understand is the transition type quenching, a state that is induced more slowly than the thylakoid energization related q_E but is still reversible, though it relaxes more slowly than q_E. One such component of q_N has been related to state transitions (q_T), during which a part of the PS II antenna detaches and moves to the non-appressed region of thylakoids (Walters & Horton 1991). This process is thought to balance PS II and PS I excitation rates at low absorbed quantum flux density (PAD), when q_E is not yet active enough due to the lower energization level of thylakoids (Allen 1992). The time kinetics of this component of q_N is intermediate between q_E and irreversible q_I, and q_T is specifically activated by limiting PADs. Importantly, the mechanism of q_T predicts no changes either in the number of active PS II or in the PS II turnover rate. To explain the type of q_N that is activated by high light and reverses slower than q_E, it has been proposed that the sustained q_E can last for a longer time after the light is turned off due to the adenylate charge that can reversibly energize thylakoids (Gilmore & Björkman 1994*a*,*b*). Again, the mechanism of this sustained q_E is principally the same as for ordinary q_E and it also predicts no changes in the number of active PS II.

Although this component of q_N has been classified as q_I (Walters & Horton 1991), where the subscript denotes photoinhibition, it still is thought to happen in the antenna, because during this phase of q_N similar changes take place in the antenna as during q_E quenching (Horton & Ruban 1994). However, as Horton and Ruban note, this interpretation may be a problem since the reversible q_I can overlap with the irreversible q_I, a type of quenching known to occur when PS II is damaged (Horton & Ruban 1994). This transient type of quenching is presently the most controversial. Since it cannot be classified as q_E or q_T, it is denoted reversible inhibitory non-phytochemical quenching (q_I), in accordance with (Horton & Ruban 1994), but emphasizing the difference from the irreversible (or very slowly reversible) q_I that can be explained on the basis of possible damages in PS II. The experiments below were aimed to detect whether PS II properties were altered during q_E and reversible q_I.

2. MEASUREMENT OF PS II PROPERTIES

The measurement of the actual functional state of PS II in the presence and absence of different forms of quenching is of crucial importance in the investigation of q_N. Measurements carried out on intact leaves guarantee that no artefacts caused by thylakoid preparation or by interference of inhibitors are introduced into the results. The PS II state can be sensed by O_2 evolution and by chlorophyll fluorescence. During these measurements, care must be taken not to limit the PS II function by blocking e^- transport on the acceptor side, i.e. these measurements must be carried out with (almost) completely oxidized plastoquinone (PQ), the terminal acceptor of e^- from PS II. As the PQ pool is about 8–12 mol per PS II, or about 16–24 e^- per PS II, the amount of transferred e^- is limited to a fraction of this. Thus, only short flashes or pulses of light can be used during which no more than 4–5 e^- per PS II are transferred. A longer exposure would inevitably cause PQ reduction, followed by Q_A reduction and PS II closure. A widely used, but still controversial, approach is applying single turnover flashes, which are so short that every PS II can turn over only once, transporting only one e^- (Chow *et al.* 1989, 1991). The amount of O_2 evolved from a saturating single turnover flash indicates the number of actively O_2 evolving PS II per leaf area. Since one O_2 evolves per four transported e^-, the amount of evolved

O_2 per flash equals a maximum of one-quarter PS II. Multiple turnover pulses allow several PS II turnovers and O_2 evolution from a saturating multiple turnover pulse indicates the maximum rate of PS II turnover. Both these methods require intense light sources and a sensitive method for O_2 recording.

(a) *Flashes and pulses*

For the measurements described below single turnover flashes were produced by a Machine Vision Strobe MVS-7020 (EG&G Optoelectronics, Salem, MA, USA) with 12 or 4 μF discharge capacitors and applied to the leaf via a branch of the fibre-optic light guide. The duration of flashes at half-height was 6 and 3.3 μs, respectively. This flashlamp is equipped with a powerful parabolic mirror, which allows concentration of most of the flash energy into a bundle of fibres. Flash doses of 110 and 60 μmol m^{-2} were obtained in the leaf chamber with the 12 and 4 μF capacitors, respectively. Flashes were attenuated with neutral filters when necessary. The flashes were single turnover, since very little O_2 evolution was recorded from the second flash and maximum O_2 evolution occurred in the third flash from a dark-adapted leaf.

Multiple turnover pulses of up to 15 000 μmol quanta m^{-2} s^{-1} were provided by a Schott KL 1500 light source (H. Walz, Effeltrich, Germany). A computer-triggered spring-operated shutter was constructed (Fast-Est, Tartu, Estonia) that fit into the body of the Schott KL 1500 electronic light source in the slit of the slide filter holder and produced pulses of variable length with edges of 1 ms. The time-course of light intensity during pulses was recorded with a LiCor LI-190SA quantum sensor (LiCor, Lincoln, NE, USA). Light from different sources (actinic background, far-red, fluorescence saturation pulses, single turnover flashes and multiple turnover pulses) was evenly superimposed over the leaf area by a multibranch fibre-optic light guide. A Schott KL 1500 light source was used for background actinic illumination (to vary q_N); for fluorescence saturation pulses the intensity of the same source was electronically turned to 15 000 μmol m^{-2} s^{-1} for 1 s. Another Schott KL 1500, filtered through a 720 nm narrow-band interference filter, was used for far-red (FR) illumination (incident intensity 240 μmol m^{-2} s^{-1}). The absorption coefficient of the leaf for photosynthetically active radiation was measured in an integrating sphere. Irradiation density is expressed as absorbed quantum flux density (PAD).

(b) *Measuring the flash dose*

Single turnover flashes average about 3–6 μs long and are very bright. The intensity of illumination during the flash rises to about 2 mol quanta m^{-2} s^{-1}, brighter than a thousand suns. Photoelectric sensors, such as photodiodes, may become nonlinear at such high quantum flux densities and the flash is so short that its shape can be recorded only by an oscilloscope. However, measurement of flash intensity is not as important as its dose, the integral of the flash in quanta per square metre. The integration can easily be done electrically. For example, in the measurements reported below the LiCor quantum sensor LI-190SA was used. During the flash–dose measurements the photocurrent of the sensor photodiode, connected in the reverse direction, charged a capacitor of 10 μF from a 4.5 V battery. The capacitor was discharged simultaneously through a 0.44 MΩ resistor (time constant 4.4 s). Each flash produced a transient, the peak of which was proportional to the pulse quantum dose. The speed of the transient was such that its peak could be recorded by the data logger along with other signals, without the necessity for a fast recorder.

Flash energy measurements were calibrated in two independent ways. First, the photodiode of the LI-190SA sensor was found to be linear up to 25 μmol quanta m^{-2} (the linearity was tested by checking whether the decay of the flash shape recorded on oscilloscope with the help of the same sensor was still exponential). The integral of the current during the flash was recalculated into μmol quanta m^{-2} using the calibration constant of the sensor from the supplier. Flash intensity had to be attenuated four times for these measurements. Second, a thermoelectric pyranometer was used as an intermediate sensor to compare the high-peak but short (6 μs) single turnover flashes with lower-peak but longer (10 ms) multiple turnover flashes of similar energy. Responses from a series of 10 ms long pulses of different intensity of up to 15 000 μmol m^{-2} s^{-1} were recorded by the LI-190SA quantum sensor (true trace recorded with 40 μs data-logging frequency) and by the thermoelectric pyranometer (a slow bell-shaped integral response recorded with 1 ms data-logging speed). This way the pyranometer was calibrated in units of μmol quanta m^{-2} against the LI-190SA sensor in the range of guaranteed linearity of the latter. Then the same procedure was repeated with single turnover flashes, recording the integral of flashes by the quantum sensor instead of the true trace. As a result, the flash quantum dosage meter was calibrated against the pyranometer, which itself had been calibrated against the same quantum sensor in its linear range. Both methods gave similar results, from which it was concluded that the LI-190SA sensor is a linear meter up to about 25 μmol quanta m^{-2} in single turnover flashes (6 μs long on half-height). For measurement of greater doses, the flashes were attenuated with neutral density filters.

(c) *Oxygen evolution from flashes and pulses*

The highest sensitivity of O_2 evolution measurements is required with single turnover flashes. In leaves the number of active PS II is usually about 1–2 μmol m^2 (Chow *et al.* 1989, 1991; figure 2) and, correspondingly, 0.25–0.5 μmol m^{-2} is the expected maximum O_2 evolution from one saturating flash when s-states of oxidation of the Mn cluster of the oxygen-evolving complex (OEC) are randomized. For reliable measurement of the flash saturation curve, a threshold sensitivity must be 0.005–0.01 μmol O_2 m^{-2}, requiring an analysis system sensitivity of 5–10 pmol O_2 when leaf area is 10 cm^2. Considering that this is still 6.02×10^{11} molecules of O_2, the task is not impossible. The most sensitive for gas phase measurements is a zirconium oxide O_2 analyser. However, its sensitivity meets the above requirements only when the background O_2 concentration is low, such that the O_2 evolution causes a sufficient relative increase over the background O_2 concentration (Laisk & Oja 1998). Low background O_2 concentration is required not only because of the decreasing sensitivity of the analyser but also to keep the background O_2 concentration absolutely

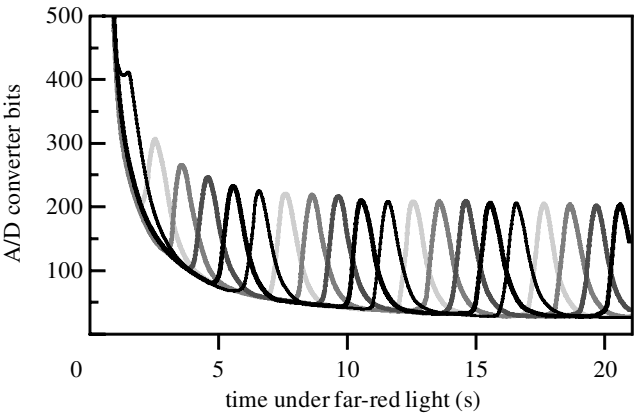

Figure 1. Measurement of changes in the active PS II pool. Trains of flashes, 5 s apart, were given in repeated experiments beginning 2, 3, 4, 5 and 6 s after the actinic light was replaced by far-red light at time zero. The sunflower leaf had been in anaerobiosis for 20 s before. The reference line, showing the response of photosynthesis and the O_2 analyser for the change in light intensity, was measured without flashes. Oxygen evolution was calculated as the difference between traces with and without flashes.

constant. Changes in leaf transpiration rate and even in CO_2 fixation rate will change the dilution ratio of O_2 in the gas stream and cause large variations in its concentration that may exceed the signal from the measured single-turnover flash.

For multiple turnover pulse measurements the constraints are not as severe as for single-turnover flash measurements. In leaves the PQ pool is about 20–30 μmol e^- m^2 (10–15 PQ/PS II). Allowing for the reduction of a maximum of 30% of the PQ pool in one flash, the e^- transport per flash will be 7–10 μmol e^- m^{-2} and the corresponding O_2 evolution will be 2–2.5 μmol m^{-2}.

(d) *Requirements for the gas system*

The high sensitivity of the O_2 analyser requires a very low background O_2 concentration of 10–50 μmol O_2 (mol gas)$^{-1}$. This concentration is too low for normal functioning of leaf respiration, problems with anaerobiosis may appear during the measurements, and leaves can be exposed to the low O_2 environment only for short time. Thus, a gas system that allows fast and reliable manipulation with O_2 concentration is necessary for these measurements.

Leaves were enclosed in a chamber (diameter 3.1 cm, height 0.3 cm) and exposed to a gas flow rate of 0.5 mmol s^{-1} (Fast-Est) (Oja 1983; Laisk & Oja 1998). An open (flow-through) gas system was fed with pure N_2 and O_2 from pressure cylinders and the necessary O_2 concentration was mixed using calibrated capillary flow meters. In N_2 the background O_2 concentration was about 10–50 ppm (dependent on the cylinder of technical grade N_2) and this was used as a background for single turnover flash O_2 evolution measurements. A background O_2 concentration of 0.4% used in some multiple turnover pulse measurements proved to be adequate for stable PS II e^- transport and q_N, but still low enough to maintain sufficient sensitivity of the O_2 analyser. When the very low O_2 background was used, the routine of the experiments was arranged so that

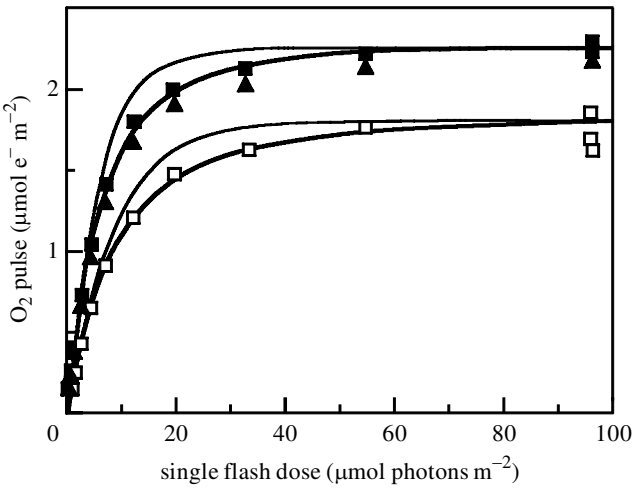

Figure 2. Flash–dosage curves for a sunflower leaf preconditioned 30 min at an absorbed quantum flux density (PAD) of 30.3 μmol m^{-2} s^{-1} (closed squares) and 1700 μmol m^{-2} s^{-1} (open squares). Triangles indicate measurements after reconditioning at the low PAD. Thin lines, exponents calculated for an optically thin object. Thick lines, calculated from a model for a leaf with 10% light transmission.

leaves were in anaerobiosis for no longer than one minute.

3. RESULTS AND CONCLUSIONS

(a) *A routine for the measurement of oxygen yield from single turnover flashes*

A computer-operated routine was used in these measurements. Leaves were preconditioned at different PADs in 2% O_2 and 330 μmol CO_2 mol^{-1} to induce the necessary q_N. PAD and the duration of the preconditioning determined whether q_N was mostly q_E or reversible q_I type. To start the flash measurements, O_2 concentration was decreased to 10–50 μmol mol^{-1} and the following routine was applied: white actinic light was replaced by FR light of 270 μmol m^{-2} s^{-1} (incident) containing about 15 μmol m^{-2} s^{-1} of PS II light (absorbed; background O_2 evolution under FR was the same as under 30 μmol m^{-2} s^{-1} of white light, which equals to 15 μmol m^{-2} s^{-1} PS II light). FR light was necessary to completely oxidize PQ before and between flashes and low PS II light was necessary to mix s-states. Either one single flash or trains of flashes, 5 s apart, were given in repeated experiments beginning 2, 3, 4, 5 and 6 s after the actinic light was turned off (shown with different lines in figure 1; the reference line was measured applying the same routine without flashes). The difference between the recordings with and without flashes was taken as O_2 evolution from the flash. This way it was possible to measure O_2 evolution from the first flash on the drifting reference after 2 s from the moment of turning the actinic light off and after every second further on. Standard deviation of the measured O_2 evolution from one flash was less than ± 1%. Such a measurement routine allowed us to search for changes in the flash O_2 yield during the post-illumination period with time resolution of 1 s beginning from 2 s after the actinic light was replaced by the FR light.

(b) *Quantum dosage response of oxygen yield from a single turnover flash without and with non-photochemical quenching*

In these experiments the phase-shifted flash trains shown in figure 1 were not applied but one flash was given 4 s after actinic light was replaced by FR. Flash intensity was changed to obtain flash–dosage curves of O_2 evolution (figure 2). While the flash–dosage curve for an optically thin layer is exponential, the response of the whole leaf is a complex function, a sum of exponents with different constants. The flash–dosage curves saturate more slowly than single exponents (thin lines), a result of optical thickness of the leaf. Thicker lines that fit the data points were calculated from a computer model that considered exponential attenuation of light in the leaf, assuming that 10% light was transmitted through the leaf. The maximum flash of 95 μmol quanta m^{-2} (absorbed) from the 12 μF capacitor was powerful enough to saturate the response.

The experiment was carried out with two preconditioning PADs: 26 and 1700 μmol quanta $m^{-2} s^{-1}$ and exposures of 30 min. The flash O_2 evolution saturated at 2.2 and 1.6 μmol e^- $m^{-2} s^{-1}$ in the low- and high-light conditioned states, respectively. For the high q_N state induced by a 30 min exposure to the high PAD the number of active PS II centres decreased from 2.2 to 1.6 μmol PS II m^{-2} or to 72.7% from the initial state at the low PAD. However, the initial slope of the flash–dosage curve (flash quantum yield in mol e^- per mol photons) decreased from 0.35 to 0.19 or to 53% of the low-light adapted value. This shows that under the preconditioning routine the number of active PS II centres decreased in response to the induced q_N, but, additionally, antenna efficiency also decreased in each PS II centre that remained active. This state of reduced PS II number and lower antenna efficiency was mostly reversible, as seen from the curve measured after re-adaptation to the low PAD for 30 min at the end of the experiment (figure 2, triangles).

(c) *Changes in PS II accompanying energy-dependent and reversible inhibitory non-photochemical quenching*

Though the q_N induced by the 30 min exposure reverted almost completely within 30 min at the low PAD, the relaxation kinetics were two phase, suggesting that two different processes were involved. In order to see whether the number of active PS II was reduced during the faster or the slower phase of q_N, the above experiments were repeated but the time of preconditioning at PAD of 1700 μmol quanta $m^{-2} s^{-1}$ was reduced to 1 min. Such a short exposure induced only q_E type (photoprotective) quenching that reversed completely within 5 min. The experiment was carried out according to the routine of figure 1, preconditioning the leaf at PADs of 51 μmol $m^{-2} s^{-1}$ for 30 min and of 1700 μmol $m^{-2} s^{-1}$ either for 1 min or 30 min. The low-light preconditioned leaf showed a constant O_2 yield of 1.3 μmol e^- m^{-2} from repeated flashes (figure 3, open squares). After the 1 min conditioning at the high PAD the flash yield increased rapidly and approached the low-light preconditioned level within 10 s (figure 3, open triangles). After the longer exposure (30 min) to the high PAD the flash O_2 yield had decreased to 1 μmol e^- m^{-2} and increased very slowly (figure 3, closed triangles). When the leaf was re-conditioned at the low PAD for 30 min again, the flash yield increased to almost the initial level (not shown). After brief or prolonged exposure to high PAD, F_m was quenched to 40% of its initial value. During the first 10 s under FR there was very little change in F_m, which showed that the fast post-illumination relaxation of the PS II inhibition was not related to the q_E quenching of F_m. Currently it is not known whether this very rapidly reversible downregulation of PS II activity was a real regulatory event or it reflected the speed of plastoquinol oxidation under FR during the first 10 s after the high PAD was turned off. However, at the same F_m quenching, the short exposure to high light did not induce changes in the number of active PS II while the long exposure induced a decrease of about 25%, which was reversed within 30 min under low PAD.

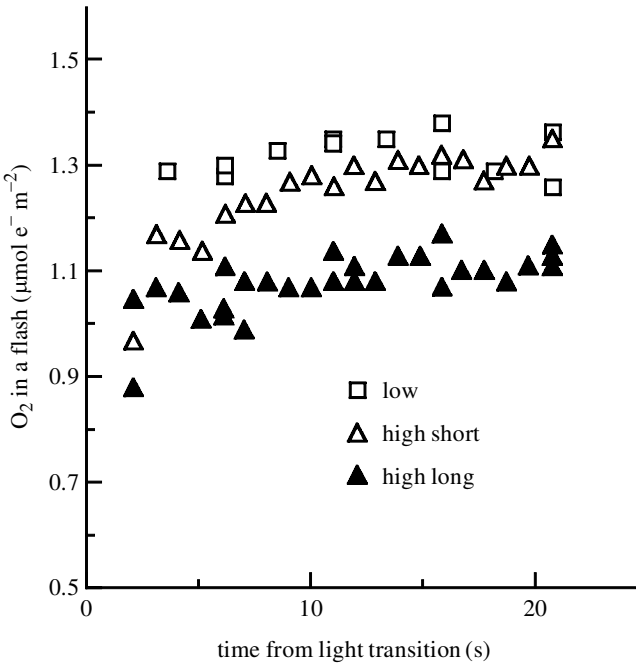

Figure 3. Changes in the pool of active PS II during the relaxation of q_E. A sunflower leaf was exposed at low and high absorbed quantum flux density (PAD) and the flash O_2 yield was measured under far-red light in repeated phase-shifted flash trains, as shown in figure 1. Open squares, after an exposure at 51 μmol quanta $m^{-2} s^{-1}$ for 30 min. Open triangles, after an exposure at 1700 μmol $m^{-2} s^{-1}$ for 1 min. Closed triangles, after an exposure at 1700 μmol quanta $m^{-2} s^{-1}$ for 30 min.

These experiments showed that no changes in the number of active PS II centres occurred that could be directly related to the rapidly reversible, q_E type non-photochemical quenching, while the longer exposure at the high PAD induced a more slowly reversible component of q_N and a parallel decrease in the number of active PS II centres by about 25%. The fast increase of the active PS II pool after the short exposure to the high PAD shows that the activity of PS II is a dynamic parameter that may change during photosynthesis, but these changes are not directly related to the q_E-type quenching of F_m.

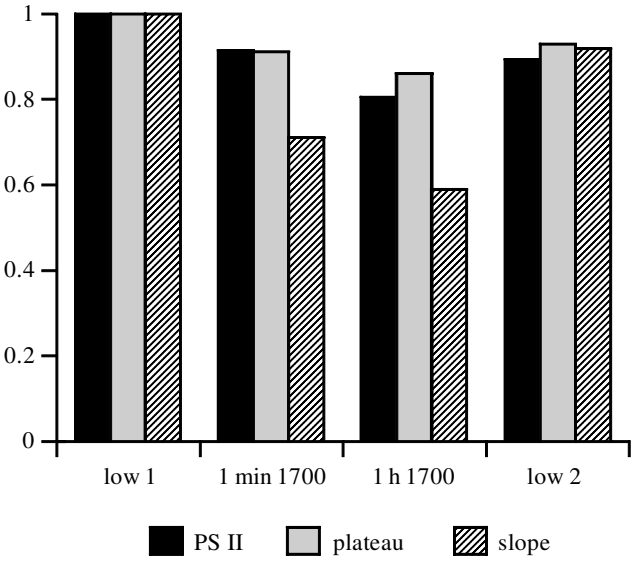

Figure 4. Comparison of O_2 yields from saturating single turnover flashes with O_2 yields from saturating and limiting multiple turnover pulses. Oxygen yields from a saturating single turnover flash, from an 8.6 ms pulse of 12 750 μmol quanta $m^{-2}s^{-1}$ and a 38.6ms pulse of 850 μmol quanta $m^{-2}s^{-1}$ were measured after the leaf was preconditioned at 37 μmol quanta $m^{-2}s^{-1}$ for 1h (low 1) at 1700 μmol quanta $m^{-2}s^{-1}$ for 1min (1 min 1700), at the same absorbed quantum flux density (PAD) for 1h (1 h 1700) and again at 37 μmol quanta $m^{-2}s^{-1}$ for 15 min (low 2). The initial slope and maximum rate V_m (plateau) of the hyperbolic PS II light-response curve were calculated from the pulse O_2 yields and plotted relative to the same values measured after the initial conditioning at the low light.

(d) *Comparison of single turnover flashes and multiple turnover pulses*

Saturating single turnover flashes detect the number of O_2 evolving PS II centres. Oxygen yield from high intensity (saturating) multiple turnover pulses reflects the maximum e^- transport rate through active PS II centres, while O_2 evolution from low intensity pulses reflects the intrinsic quantum yield of PS II. As described in § 2, PQ was pre-oxidized in these experiments and the number of e^- transported during the pulse did not exceed 8 μmol e^- m^{-2}, i.e. four e^- per PS II. For better comparison of measurements with high- and low-intensity pulses the number of e^- transported in comparable experiments was made approximately equal by changing the duration of the pulse. It was therefore ensured that the acceptor side limitation due to PQ reduction did not exceed 15% on average and was equal in comparable experiments.

Comparable measurements applying a saturating flash, a high-intensity multiple turnover pulse and a low-intensity multiple turnover pulse were carried out 5 s after the white actinic light was replaced by FR. The preceding exposure at 1700 μmol quanta $m^{-2}s^{-1}$ was either 1 min or 60 min. Subsequent to the 1 min preconditioning at 1700 μmol quanta $m^{-2}s^{-1}$, O_2 yield from flashes decreased by 9% compared with the preconditioning at PAD of 37 μmol $m^{-2}s^{-1}$ (figure 4). As in the experiment shown in figure 3, this small decrease detected in the active PS II pool was not related to the q_E-type quenching of F_m. Pulse O_2 yield from the high-intensity multiple turnover pulses of 12 800 μmol $m^{-2}s^{-1}$ (absorbed) and 8.6 ms duration decreased slightly more than the flash O_2 yield (by 16%), while the pulse yield from low-intensity pulses (850 μmol $m^{-2}s^{-1}$, 38.6 ms) decreased by 27% after 1 min exposure under 1700 μmol quanta $m^{-2}s^{-1}$. The maximum pulse did not completely saturate the PS II yield. Knowing that the PS II light-response curve is a rectangular hyperbola (see figure 9), the initial slope (quantum yield) Υ and the maximum rate V_m was calculated and the relative changes of these parameters is given in figure 4. After the 1 min exposure under the PAD of 1700 μmol quanta $m^{-2}s^{-1}$ the maximum PS II turnover rate decreased in proportion to the pool of active PS II, while the quantum yield of PS II at low light, represented by the initial slope of the light-response curve, decreased more.

Again, these experiments show that the rapidly inducible and rapidly relaxing q_E-type quenching influences neither the number of active PS II centres nor decreases the maximum turnover rate of the centres at saturating PAD, but considerably decreases the quantum yield of PS II at low PAD.

After the 60 min exposure from a PAD of 1700 μmol $m^{-2}s^{-1}$ the number of active PS II detected from the flash O_2 yield decreased by 20% and this downregulation reversed within 15 min to 89% of the initial value (rightmost bars in figure 4) and continued to recover. After this exposure, the calculated PS II maximum turnover rate V_m decreased slightly less than the number of centres, but the difference is not considered meaningful because V_m was extrapolated. The initial slope (quantum yield) decreased to 0.58 of the initial value. After 15 min of q_N relaxation, both V_m and quantum yield recovered to 90% of the initial value and continued to recover. The results showed that the 1 h exposure under 1700 μmol $m^{-2}s^{-1}$ induced a reversible photoinhibitory effect that decreased flash yield and high- and low-intensity pulse O_2 yield (all by 10%). In parallel with the photoinhibition, in active PS II centres the q_E quenching caused a remarkable decrease of the low-light quantum yield, but only a less than 10% decrease in the maximum PS II turnover rate. In PS II centres that were not photoinhibited the effect of q_E quenching was similar before and after the long exposure under high PAD, causing a *ca.* 30% decrease of the quantum yield and a 10% decrease of V_m and the number of active PS II, but the latter effect was not related to F_m quenching and reversed within 10 s.

(e) *Oxygen evolution from multiple turnover pulses: dependence on pulse length*

In these studies the kinetics of O_2 evolution and electron transport through PS II during the illumination with multiple turnover pulses were analysed in greater detail. As already emphasized, the short pulses were necessary in order to avoid PQ reduction and to detect acceptor side unlimited PS II kinetics.

Using the specially designed shutter fitted to the KL 1500 light source, the time kinetics of O_2 evolution were resolved by measuring how the total O_2 evolution per pulse increased with increasing pulse length (figure 5). The slope of the graphs obtained by increasing pulse length at a constant pulse PAD represents the O_2 evolution rate as a function of time during the pulses.

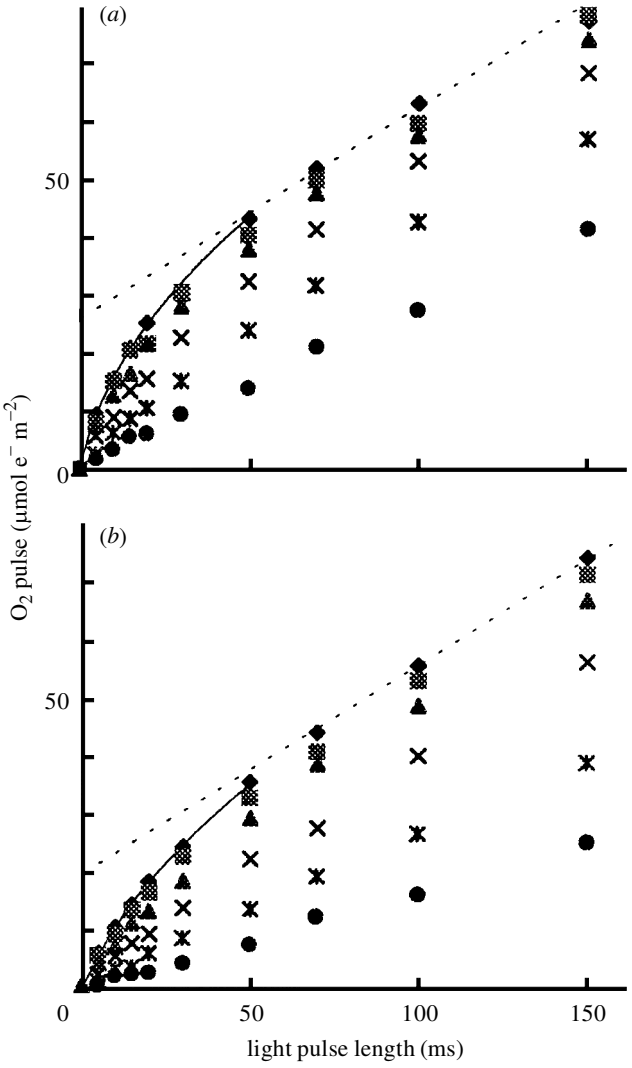

Figure 5. Total O_2 evolution from a light pulse dependent on the pulse length and intensity for a sunflower leaf pre-conditioned (*a*) at low light and (*b*) at high light. Pulse absorbed quantum flux densities (PADs) were (downward for curves) 13 500, 10 200, 6800, 3600, 1720 and 880 μmol $m^{-2} s^{-1}$. Approximation with a hyperbola is shown for the uppermost curve. The slope of the dotted line represents O_2 evolution rate 50 ms after the beginning of the pulse (360 μmol e^- $m^{-2} s^{-1}$).

Experimental data points were approximated by non-rectangular hyperbolic relationships, as shown for the maximum pulse PAD. Clearly, the slope of the hyperbola was high at short pulse length and it decreased rapidly with increasing pulse length as PQ became more reduced. At pulse lengths longer than 50 ms electron transport rate (temporarily) stabilized because PQ reduction had reached an equilibrium steady state determined by the balance between reduction and oxidation rates. The pool of reduced PQ in this state was estimated from the extrapolation of the dotted line to the ordinate axis. In the low-light adapted state this estimate was 26 μmol e^- m^{-2} and in the high-light adapted state it was 20 μmol e^- m^{-2}. This method of estimation of the reduced PQ pool considered that the oxidation rate saturated at very low levels of reduced PQ, being practically independent of the pulse length. Considering that the pool of PS II in these leaves was about 2 μmol m^{-2}, there were about ten to 13 doubly reduced PQ molecules per PS II.

The initially high O_2 evolution rate (up to 2000 μmol $m^{-2} s^{-1}$ in the low-light conditioned state) decreased to 360 μmol e^- $m^{-2} s^{-1}$ with fully reduced PQ (rate calculated from the slope of the dotted line at pulse length longer than 50 ms), and this rate was similar in the low- and high-light conditioned states. This rate evidently characterized the maximum rate of PQ oxidation by cytochrome b_6f in the absence of proton gradient, a kind of uncoupled electron transport rate in an intact leaf. After a few seconds this rate further decreased to 225 μmol e^- $m^{-2} s^{-1}$, evidently because a proton gradient was generated. Finally, the rate declined to an acceptor-limited value of 120 μmol e^- $m^{-2} s^{-1}$ when the pre-accumulated 3-phosphoglyceric acid (PGA) pool was consumed by reduction and CO_2 diffusion became rate limiting (not shown). These rates demonstrate the temporal interplay between sequential rate limiting processes in photosynthetic e^- transport, the next becoming active after the pool of the preceding intermediate was consumed.

(f) *Oxygen evolution during pulses: dependence on pulse PAD*

The initial rates of O_2 evolution with completely oxidized PQ pool were calculated from the slope of the curves after 3 μmol e^- m^{-2} were transported. Such a threshold value was used because fluorescence measurements (below) indicated that the first one to two e^- were transported at a higher speed than the following e^-, probably because the first e^- did not exchange with the free PQ pool but stayed on the bound quinones on PS II acceptor side. These O_2 evolution rates were plotted as light-response curves for PS II e^- transport from the OEC to PQ (figure 6, open symbols). The light-response curves of PS II electron transport fit well to rectangular hyperbola, as seen from the match of the experimental points of O_2 evolution (open symbols) with calculated hyperbolic curves. The half-saturation PAD (K_m) of the function was 7600 μmol $m^{-2} s^{-1}$ in the low-light adapted and 7400 μmol $m^{-2} s^{-1}$ in the high-light adapted state, showing that the maximum rate and initial slope decreased rather proportionally. The highest experimentally available PADs were nearly twice the K_m and the very good fit of the recorded data to rectangular hyperbolae allowed us safely to extrapolate the maximum (plateau) values for PS II electron transport. Both, the initial slope (intrinsic quantum yield Y_m) and the plateau V_m of the hyperbolic light-response curve of the PS II electron transport decreased when the preconditioning PAD was increased and q_E plus the reversible q_I increased from the minimum to the maximum value. The values of the extrapolated maximum PS II electron transport rate reached 2860 μmol e^- $m^{-2} s^{-1}$ in the low-light conditioned state of the leaf and decreased to 1450 μmol $m^{-2} s^{-1}$ in the high-light conditioned state. The intrinsic quantum yield Y_m decreased from 0.41 to 0.23. In these experiments the plateau of PS II light-response curves decreased almost proportionally with the initial slope. Respectively, the initial slope decreased to 0.58 and the maximum rate to 0.51 of the low-light adapted value. These data allow us to

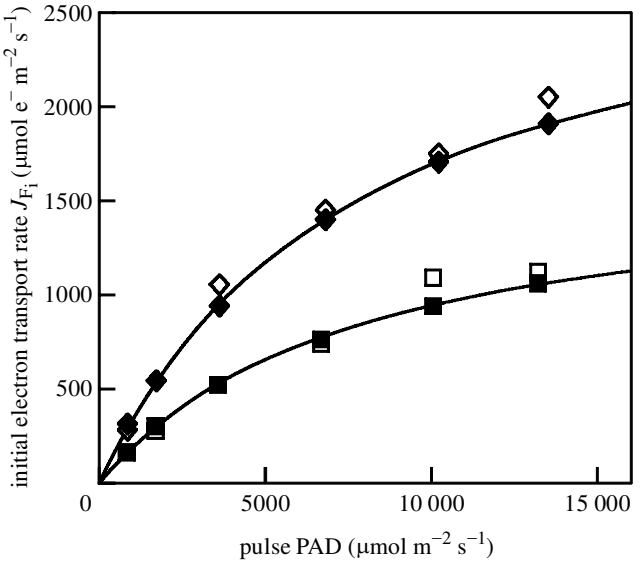

Figure 6. Light-response curves of the initial electron transport into the oxidized plastoquinone pool, calculated from the O_2 evolution measurements in figure 5 (open symbols) and from the fluorescence measurements in figure 9 (filled symbols). Diamonds, low-light preconditioned leaf. Squares, high-light preconditioned leaf. Lines are rectangular hyperbolae calculated from the mathematical model of PS II electron transport with constants given in table 1.

state with certainty that both the initial slope and the plateau of the PS II light-response curve decreased in the presence of q_N that was mostly of the reversible q_I type.

In the above experiments many pulses were applied while the leaf was preconditioned to either the low or high PAD (six different pulse PADs and a series of nine different pulse lengths at each pulse PAD). Correspondingly, it was not possible to recondition the leaf at 2% O_2 after each pulse but the leaf was permanently at 0.4% O_2, this compromise being the lowest possible O_2 concentration at which the q_E and q_I quenching were stable and no indications of anaerobiosis were seen. To complete the whole routine, the time of exposure of the leaf at 0.4% O_2 extended to 90 min. Though reconditioning at the low PAD showed that the PS II inhibition reverted and the yields approached 85–90% of the initial values, the reversible q_I-type quenching evidently dominated in the total q_N in these experiments. Thus, these experiments clearly showed that PS II e$^-$ transport capacity decreased in parallel with the reversible q_I.

(g) *Fluorescence induction and oxygen evolution during multiple turnover pulses: the effect of PS II donor side resistance*

Chlorophyll fluorescence is a reliable quantitative indicator of steady state e$^-$ transport rate. Since processes leading to fluorescence emission are completed within nanoseconds, there is no reason to doubt that fluorescence should not be as good a quantitative indicator of e$^-$ transport during multiple turnover pulses of ms duration. An important difference between the transient and steady-state process is that e$^-$ transport in pulses is extremely fast compared with the steady state. Under this condition the accumulation of P^+_{680} may become important. In the following experiments the total O_2 yield from pulses were

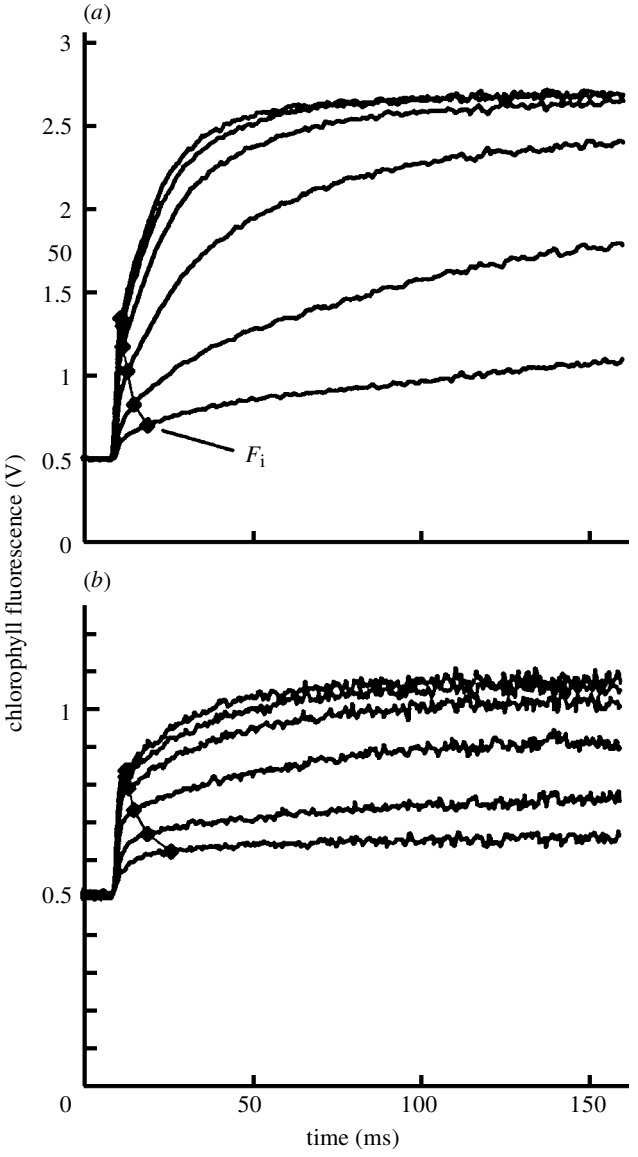

Figure 7. Fluorescence-induction curves in (*a*) a low-light and (*b*) a high-light adapted sunflower leaf. At the beginning of the traces, fluorescence yield corresponds to F'_o and the upper-limit of the plot area corresponds to F'_m (= 3.06 V in (*a*) and 1.27 V in (*b*)). The fast increase of fluorescence corresponds to the beginning of the light pulse. Marked points correspond to fluorescence (F_i) when 3 μmol e$^-$ m^{-2} have been transferred to reduce bound acceptors. Further on, free plastoquinone is reduced. Pulse absorbed quantum flux densities (PADs) were (downward for curves) 13 500, 10 200, 6800, 3600, 1720 and 880 μmol m^{-2} s^{-1}.

compared with the integral of e$^-$ transport calculated from fluorescence induction during the same pulses. Fluorescence was measured with a PAM 101 fluorometer (H. Walz, Effeltrich, Germany). Electron transport rate was calculated using the formula (Genty *et al.* 1989):

$$\mathcal{J}_F = a_{II} Q \frac{F'_m - F(t)}{F'_m}, \qquad (1)$$

where F'_m is light-saturated and $F(t)$ is time-dependent fluorescence yield during the pulse. The fraction of light absorbed by PS II antenna, a_{II}, was 0.5 in the low-light adapted state but 0.43 gave a better fit in the high-light adapted state.

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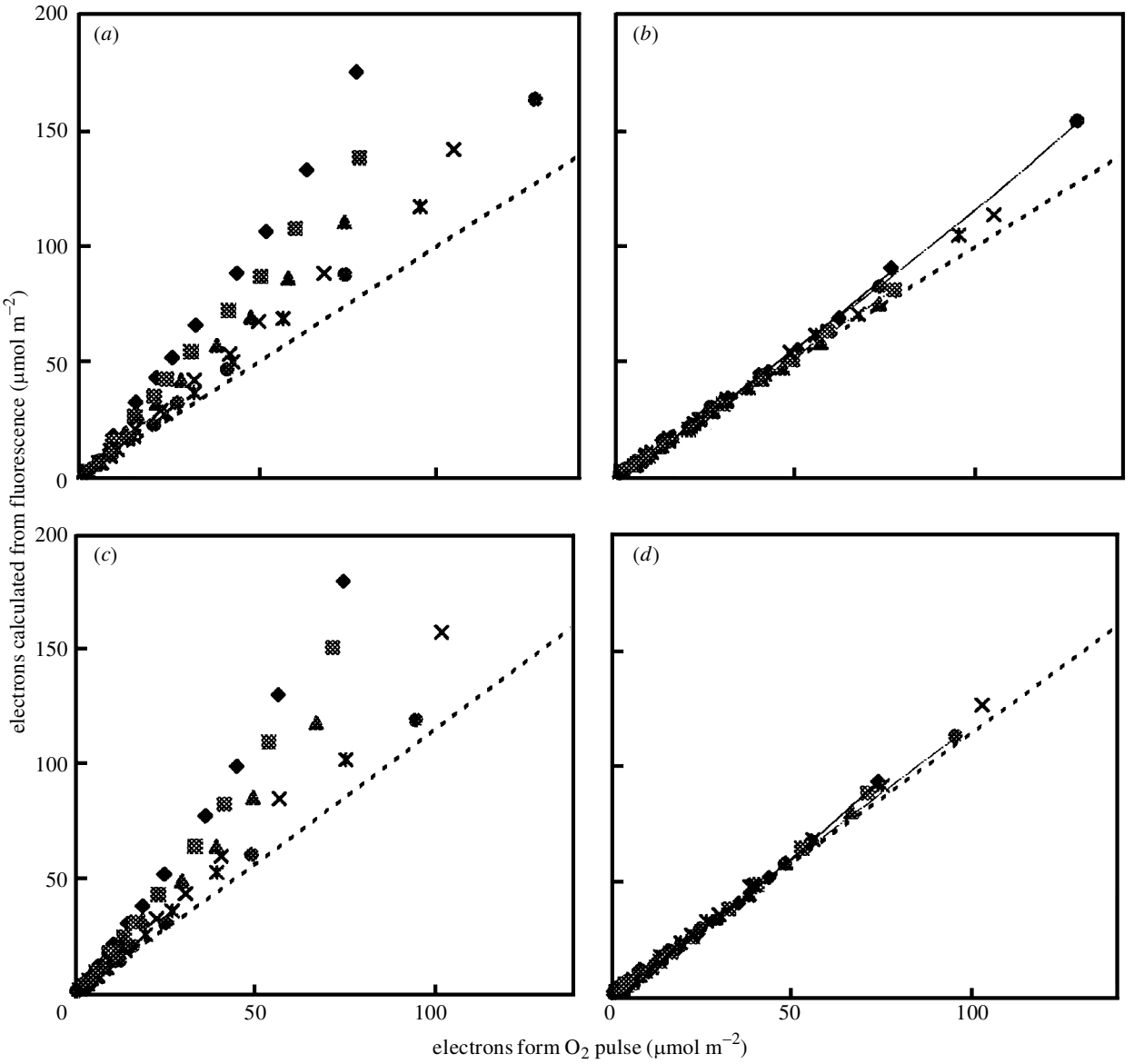

Figure 8. Total per pulse of electrons calculated from fluorescence related to total per pulse of electrons calculated from O_2 evolution when pulse length was varied from 5 to 160 ms in (*a*,*b*) a low light and (*c*,*d*) a high light preconditioned sunflower leaf. Donor side resistance (r_d in equation (2)) was assumed to be 0 (*a*,*c*) or 0.00014 s m^2 μmol^{-1} (*b*,*d*). Data from figure 5 (O_2 evolution) and figure 7 (chlorophyll fluorescence). Pulse absorbed quantum flux densities (PADs) were 13 500 (diamonds), 10 200 (squares), 6800 (triangles), 3600 (crosses), 1720 (asterisks) and 880 (circles) μmol m^{-2}s^{-1}.

Fluorescence induction transients recorded during long pulses of different intensities in leaves preconditioned to low and high PAD are shown in figure 7. In dark-adapted leaves the induction was a complex curve with a transient minimum (data not shown) but adaptation to PAD of 60 μmol m^{-2}s^{-1} or higher eliminated the minimum and the induction became approximately exponential. Electron transport during the pulses ($\mathcal{J}_F$, equation (1)) was calculated using simultaneously recorded data points of fluorescence and pulse PAD and an F'_m-value measured at the end of a separate 1 s pulse of 13 500 μmol quanta m^{-2}s^{-1}, corrected for the presence of electron transport rate (ETR) and donor side resistance. This F'_m (3.06 V in figure 7*a* and 1.27 V in figure 7*b*, corresponds to the upper limit of the plot area), was higher than that reached at the end of 160 ms pulses in figure 7, because ETR through PS II was still fast during the pulse.

The calculated values of $\mathcal{J}_F$ were integrated point by point to find the total e$^-$ transport during a pulse of a given length, which was then compared with the measured total O_2 evolution during the same pulse. Pulse totals of the calculated e$^-$ transport and measured O_2 evolution were proportional to one another when pulse length was increased at constant PAD, but the slope of the relationship (e$^-$ from fluorescence/e$^-$ from O_2 evolution, $\mathcal{J}_F/\mathcal{J}_O$) was dependent on pulse PAD (figure 8*a*,*c*). In pulses of low PAD, where ETR was slow, $\mathcal{J}_F/\mathcal{J}_O$ was close to one independent of pre-adaptation conditions, but in pulses of high PAD that caused fast e transport $\mathcal{J}_F/\mathcal{J}_O$ was much higher than one. Thus, equation (1) well describes the relationship between fluorescence and PS II e$^-$ transport during fluorescence induction at physiological ETR values, but it progressively overestimates $\mathcal{J}_O$ when PAD becomes much higher than physiological and

ETR increases during short pulses. These results suggest that there is a fraction in $F'_m - F(t)$ that is quenched, but not photochemically, because it is not accompanied by corresponding e^- transport. Quite evidently, this fraction is quenched by P^+_{680}, the PS II donor pigment that accumulates in oxidized form in the presence of the very fast e^- transport from OEC to PQ during the high-intensity pulses. An empirical formula that describes the relationship between fluorescence and e^- transport in the presence of P^+_{680} can be found from the data presented here, considering that the amount of P^+_{680} is proportional to e^- transport rate and the proportionality constant may be expressed as PS II donor side resistance:

$$\mathcal{J}'_F = \mathcal{J}_O = a_{II} Q \frac{F'_m - F(t)}{F'_m} \times \frac{1}{1 + a_{II} r_d Q}. \quad (2)$$

Compared with equation (1), equation (2) contains an additional parameter r_d, which characterizes the donor side resistance of PS II per unit leaf area. The quality of equation (2) for the calculation of fast ETR through PS II is demonstrated in figure 8*b*,*d*, where one and the same donor side resistance r_d of 0.00014 $\mu mol^{-1} m^2 s$ was applied for all pulse lengths and PADs and for both low- and high-light adapted states.

(h) *Time-course of PS II electron transport calculated from fluorescence*

Time-courses of electron transport were calculated from fluorescence induction curves applying equation (2) considering the donor side re-reduction time. In figure 9 the data are plotted against the cumulative amount of e^- transported into the PQ pool, an integral of $\mathcal{J}_F$ calculated from all recorded data points. After the rising edge of the pulse passed, fluorescence increased immediately and continued to increase (figure 7). Correspondingly, the calculated e^- transport rate decreased from the beginning of the pulse. This was unexpected since it did not agree with the assumption that PQ reduction had a small reverse effect on the Q_A reduction due to the more negative redox potential of the latter, but, rather, indicated that in the light-adapted state the medium point redox potentials of Q_A and free PQ are almost equal. The initial e^- transport rate from OEC to a completely oxidized PQ pool, $\mathcal{J}_{Fi}$ was obtained as the electron transport rate after the first 3 $\mu mol\, e^-\, m^{-2}$ were transported (shown with a dotted line in figure 9). The first 2–3 $\mu mol\, e^-\, m^{-2}$ were transported at a higher rate, as seen clearly in figure 9*b*, evidently because they reduced Q_A and Q_B and were not exchanged with the free PQ pool. The initial rate of PS II electron transport $\mathcal{J}_{Fi}$ reached about 2000 $\mu mol\, m^{-2} s^{-1}$ for the maximum pulse PAD in the low-light adapted state, but was clearly lower (1000 $\mu mol\, e^-\, m^{-2} s^{-1}$) in the high-light adapted state.

PS II light-response curves were obtained by plotting $\mathcal{J}_{Fi}$ values from figure 9 against pulse PAD (figure 6, closed symbols). The light-response curves of PS II electron transport were rectangular hyperbola, and fit well with the O_2 evolution data. Scattering of $\mathcal{J}_{Fi}$ calculated from fluorescence, however, was considerably less than the scattering of $\mathcal{J}_{Oi}$ calculated from O_2 evolution. Since there were 2 μmol PS II m^{-2} (see figure 2), and using the

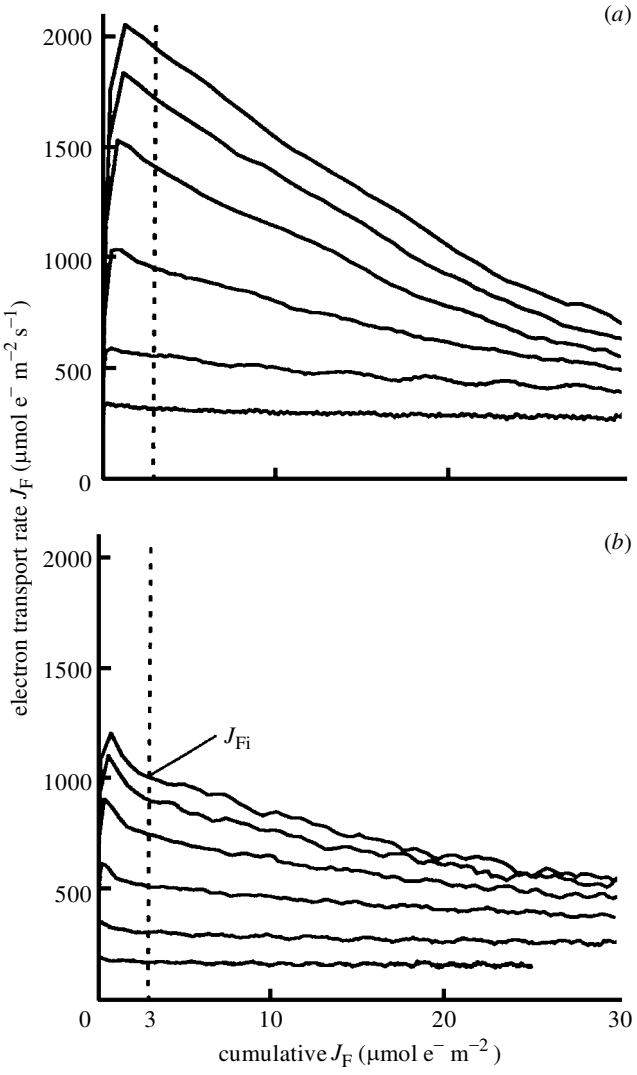

Figure 9. Electron transport rate during light pulses calculated from equation (2) as a function of cumulative electron transport in a sunflower leaf preconditioned at (*a*) 60 μmol quanta $m^{-2} s^{-1}$ and (*b*) 2000 μmol quanta $m^{-2} s^{-1}$. Pulse absorbed quantum flux densities (PADs) were (downward for curves) 13 500, 10 200, 6800, 3600, 1720 and 880 $\mu mol\, m^{-2} s^{-1}$. The initial electron transport rate ($\mathcal{J}_{Fi}$) was read after 3 $\mu mol\, e^-\, m^{-2}$ were transported (at the dotted line).

extrapolated $\mathcal{J}_{Fm}$-values, the average electron transfer time from OEC through PS II to PQ were calculated to increase from 700 to 1380 μs when q_N increased from the minimum to the maximum.

(i) *Modelling PS II electron transport*

Was this increase related to changes on the PS II donor or acceptor side? Since donor and acceptor side resistances are in series, without applying a mathematical model it is difficult to differentiate where and to what extent the rate constants for e^- transfer changed. A mathematical model of PS II electron transport and fluorescence (Laisk *et al.* 1997) considers mutual conversions of the four states of PS II, $A = D^-A^-$, $B = D^-A^+$, $C = D^+A^-$, $D = D^+A^+$, where D is donor and A is acceptor. The experimental data were used for the identification of the parameters of the model. For an extreme case of infinitely high PAD and completely oxidized PQ

Table 1. *Model parameters for low- and high-light adapted leaves, calculated assuming that PS II with P^+_{680} emits fluorescence either at a level of F_o or 0*

	$F(P^+_{680}) = F_o$		$F(P^+_{680}) = 0$	
adaptation PAD[a]	low	high	low	high
PS II, μmol m^{-2}	2	2	2	2
a_{II}, PS II relative cross-section	0.5	0.436	0.5	0.436
k_P, rate constant for photochemistry	1	1	1	1
k_F, rate constant for fluorescence	0.24	0.24	0.24	0.24
k_N, rate constant for q_N	0	0.62	0	0.62
J_m, maximum e transport rate, μmol m^2 s^1	2856	1448	2856	1448
τ_m, throughput time constant, μs	700	1381	700	1381
τ_d, donor side time constant, μs	361	615	289	329
τ_a, acceptor side time constant, μs	559	1169	607	1315
F_A, fluorescence of fraction A	1	0.279	1	0.279
F_B, fluorescence of fraction B	0.194	0.129	0.194	0.129
F_C, fluorescence of fraction C	0.194	0.129	0	0

[a] Absorbed quantum flux density.

the system of budget equations given in Laisk *et al*. (1997) reduces to the following equation, describing the maximum rate of e^- transport through a PS II complex:

$$\frac{\tau_m}{\tau_d + \tau_a} = 0.75 + 0.25\left(\frac{\tau_d - \tau_a}{\tau_d + \tau_a}\right)^2, \quad (3)$$

where τ is an exponential time constant, d is donor, a is acceptor and m is maximum throughput, the latter determined from experiments. In terms of time constants, a light-response curve expresses as

$$\tau_j = \tau_q + \tau_m, \quad (4)$$

where j is the PS II e^- transport rate and q is the PS II excitation rate. Equation (4) represents a rectangular hyperbola in rates (it is equivalent to the double-reciprocal plot used in enzyme kinetics). It states that e^- transport rate is proportional to PAD at low PADs but hyperbolically saturates at a rate of $1/\tau_m$ at high PADs. The hyperbolic shape of PS II light-response curves is seen from figure 6, where experimental data are approximated by rectangular hyperbolae. The maximum rate (plateau) of the light-response curves is symmetrically dependent on the donor and acceptor time constants (equation (3)) and without additional information it would be impossible to resolve both time constants separately. This additional information was available from fluorescence measurements, where excitation quenching by oxidized donor could be found and the donor side time constant was calculated independently from the acceptor side time constant (see figures 7 and 8). However, interpretation of those fluorescence data was critically dependent on the level of fluorescence emitted from PS II with P^+_{680}. Two possible cases were considered: when PS II with P^+_{680} either did not emit fluorescence or emitted it at a level close to F_o. The donor side resistances obtained from these two cases were considered separately in the calculations of the acceptor side resistances (time constants) per PS II centre (table 1).

Data in table 1 show that PS II acceptor side time constants increase approximately twofold when q_N is induced: from 607 μs to 1315 μs (2.16 times) when PS II in P^+_{680} does not emit fluorescence and from 559 μs to 1169 μs (2.09 times) when it emits fluorescence at a level close to F_o. Thus, the PS II acceptor side resistance increased about twofold in the presence of predominantly reversible q_I-type quenching. Assuming no fluorescence from P^+_{680} the donor side time constant increased only marginally when q_N obtained its maximum value (from 289 μs to 329 μs, or 1.14 times), but it almost doubled with the onset of q_N when P^+_{680} was assumed to emit fluorescence at a level close to F_o (from 361 μs to 615 μs, or 1.70 times). Thus, for the interpretation of these fluorescence data the level of fluorescence emitted in P^+_{680} is of crucial importance. If this level was close to F_o, the calculations showed that then the donor side resistance increased in parallel with the acceptor side resistance, but if the fluorescence was close to zero, then no changes on the donor side of PS II were detected in parallel with the reversible q_I.

4. CONCLUSIONS

Oxygen yield from single turnover flashes and multiple turnover pulses is an informative parameter that characterizes the activation–inactivation state and electron transport rates through PS II in intact leaves. The zirconium O_2 analyser, combined with a flexible gas system, is an appropriate tool for these measurements. Parallel recording of chlorophyll fluorescence and O_2 evolution allowed detection of PS II donor side resistance separately. Interpreted with the help of a mathematical model of PS II electron transport, these data reveal a full picture of rate-limiting processes at PS II.

These experiments showed that PS II properties depend on the type of non-photochemical quenching present. The rapidly induced and rapidly reversible q_E type (photoprotective) quenching induces changes neither in the number of active PS II nor in the PS II maximum turnover rate (a very rapidly reversible process was still detected, but the relaxation time was faster than that of F_m). The antenna mechanism of the q_E-type quenching is therefore confirmed. The more slowly but still reversible q_I-type (photoinactivation) quenching induced a decrease in the number of active PS II and in the maximum PS II turnover rate. The latter parameter was an average over

the leaf area and its decrease could result from the decrease in the number of e^- transporting PS II, while the properties of active PS II did not change. This result shows that the type of quenching termed here as reversible q_I type is different from the state transition related q_T type, and from the sustained q_E-type quenching, both of which cannot induce changes in PS II properties.

In the introduction it was pointed out that there is a surprisingly good complementation between photochemical and non-photochemical quenching of excitation, which, independent of the type of quenching, results in an almost constant excitation lifetime. Such tight interrelationship between the seemingly different processes encouraged us to look for a single mechanism to explain all three types of PS II quenching, one that is based on charge separation. However, the results of this work did not support the idea of a unique quenching mechanism for q_E and q_I, because changes in PS II properties were detected that paralleled q_I but no changes in PS II centres accompanied q_E.

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Discussion

C. Critchley (*Department of Botany, University of Queensland, Australia*). How do you explain the extremely high (e.g. 10) O_2 evolution rates compared to the CO_2 dependent rates? What is the relative merit of J. Whitmarsh's proposal of limited access by PS II to the PQ pool against your argument of regulating this possibility, i.e. no limitations or access to the PQ pool?

A. Laisk. The fast O_2 evolution rates were possible in these experiments because electrons were transported only from H_2O to completely oxidized plastoquinone (PQ). The slowly turning around cytochrome b_6f complex was not yet rate limiting. Plastoquinone was oxidized before a pulse was applied and the pulse was so short that PQ became only partially reduced during these pulses. The measured O_2 evolution rates characterize the rate limitations imposed by water splitting and PQ diffusion.

Our multiple turnover pulse experiments detected the rate of electron transport supported by those PS II that had access to the PQ pool. The rather good fit of the PS II light response to a rectangular hyperbola allows one to conclude that the e^- transport times on the PS II donor and acceptor side were rather similar at all PS II. Thus, there was no wide distribution of e^- transport times at individual PS II. If there was a significant portion of PS II with strictly limited access to PQ, these could be detected by fluorescence rise. The fluorescence induction curves, however, corresponded well with the model that considered only a homogenous PS II population. The presence of a very small fraction of PS II with different kinetic characteristics was still possible.

C. B. Osmond (*Research School of Biological Sciences, Australian National University, Australia*). Is the rate of development of fast and slow components of O_2 flash yield determined by PS II efficiency?

A. Laisk. The fast component of non-photochemical quenching (q_N) is usually termed q_E (energy-dependent) and it develops within 30–60 s. This component of q_N does not induce changes in the number of active PS II, as determined from saturating single turnover flashes, nor in the maximum PS II turnover rate, as determined from saturating multiple turnover pulses. The slower component of q_N that was termed reversible inhibitory quenching (q_I) develops within 30–60 min of illumination and reverses within about 15–30 min. This component of q_N is accompanied by a decreased number of active PS II and a decreased maximum PS II turnover rate.

M. Richter (*Universität Mainz, Germany*). Nonphotochemical energy dissipation at the PS II antenna and at the PS II reaction centre including enhanced donor side resistance has been distinguished by the related changes in the light intensity dependence of oxygen evolution. This is also possible through the measurement of the F_o fluorescence that is exclusively quenched by antenna related dissipation but not by processes operating at the reaction centre or by enhanced donor side resistance. Have F_o measurements been performed and are the data consistent with the results from oxygen measurements?

A. Laisk. F_o measurements were carried out but their interpretation is difficult because of the additional PS I fluorescence component in F_o. We are presently working on quantification of the PS I fluorescence by measuring emission spectra at different quenching states.