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Fluorescence Analysis During Steady-State Photosynthesis [and Discussion]

E. Weis, D. Lechtenberg, J. Barber and U. Schreiber

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Fluorescence analysis during steady-state photosynthesis

By E. Weis and D. Lechtenberg

Institute of Botany, University of Düsseldorf, Universitätsstrasse 1, *D*-4000 *Düsseldorf, F.R.G.*

Photosynthetic gas exchange of attached leaves has been measured under steady-state conditions at different light intensities and correlated with simultaneous measurements of chlorophyll fluorescence and oxidized P_{700} (by absorbance changes at 820 nm). The data suggest that during light-saturated assimilation, photosystem II (PSII) photochemistry is mainly controlled by non-photochemical and non-radiative dissipation of excitation energy, rather than by accumulation of reduced acceptor, Q_A, and this could be related to 'high-energy quenching' of fluorescence. The occurrence of oxidized P_{700} at saturating light and low concentration of CO_2 suggests that in the steady state PSI photochemistry is controlled by a shortage of electron donation from the plastoquinone pool (photosynthetic control), rather than by excess electrons at the acceptor side. The significance of the oxidized form of P_{700} as a 'quencher' of excitation energy is discussed. This control of photosystems I and II, both related to the proton gradient across the thylakoid membrane, may serve to match the potential rate of net photochemistry to the demand by the biochemical reactions. However, when light-saturated assimilation is not limited by CO_2, PSI activity is controlled by accumulation of reduced electron acceptors, rather than by photosynthetic mechanisms. Photosynthetic control has been found to determine the redox state of the ferredoxin–thioredoxin system.

Introduction

When light is absorbed by a chloroplast, singlet excited states are created in the antennae of PSI and PSII. At the reaction centres of the two photosystems the excitation energy can be de-excited by photochemical reactions. About one light quantum is consumed for each primary photochemical charge separation. During assimilation, $NADPH + H^+$ and ATP produced by light-dependent reactions are consumed by metabolic reactions such as reduction of 3-phosphoglyceric acid and regeneration of ribulose 1,5-bisphosphate. When assimilation of a C_3-plant is strictly limited by light, about 9.5 quanta (photon flux density, PFD) are absorbed per molecule of O_2 evolved (Björkmann & Demmig 1987), which is close to the theoretical quantum requirement expected for C_3 photosynthesis. Adjustment of the energy distribution between the two photosystems by 'state 1 – state 2 regulation' (for a review see, for example, Briantais *et al.* (1986); Horton (1987)) as well as optimal 'light-activation' of energy-consuming reactions, mediated, for example, by thioredoxin-dependent reductive enzyme activation (Cseke & Buchanan 1986), are assumed to be regulatory mechanisms to optimize the photosynthetic flux under low light and to keep the apparent quantum yield of assimilation high.

When light becomes saturating for biochemical reactions and the potential rate of photochemistry exceeds that of whole-chain electron transport, the apparent quantum yield of assimilation drops below its optimum. Because primary photochemical reactions are almost irreversible processes accompanied by a large free-energy change, large gradients of the primary products of light reactions are expected to be created under high light.

However, photosynthesis may operate efficiently only when the electron transport and photophosphorylation systems are well displaced from the equilibrium (Heber *et al.* 1986). If the redox system were to be forced to an 'over-reduced' state, the photosynthetic flux would collapse, and it is expected that electrons would be delivered to irregular pathways eventually leading to 'photoinhibitory' destruction of the membrane (see Kyle *et al.* 1987). Moreover, the yield of pigment triplet states and related formation of 'reactive' molecular oxygen is expected to increase.

Thus for energetic reasons, optimal rates of assimilation and protection against photoinhibition may be possible only when the potential rate of photochemistry is adjusted to that of biochemical reactions, and this requires feedback control of 'light reactions'. Recent work suggests that plants have indeed developed control mechanisms to 'quench' excess excitation energy from the pigment system by non-photochemical and non-radiative de-excitation (Weis *et al.* 1987; Weis & Berry 1987; Demmig & Björkman 1987; Krause & Laasch 1987; Krause *et al.* 1988; Horton & Hague 1988; Horton *et al.* 1988).

Metabolic reactions can feed back to light reactions by redox equilibria or via the proton gradient across the thylakoid membrane. As we know that both redox feedback to PSII and acidification of the inner-thylakoid lumen are accompanied by characteristic changes in chlorophyll fluorescence emitted from PSII (see Krause & Weis 1984; Briantais *et al.* 1986), fluorescence analysis provides an excellent tool to study feedback during assimilation *in vivo*. Recent progress in a pulse frequency-modulated measuring technique (Schreiber *et al.* 1986) and the 'light-doubling approach' (Bradbury & Baker 1981) facilitates fluorescence measurements during continuous illumination and to separate 'photochemical' and 'non-photochemical' quenching (Schreiber *et al.* 1986).

Accumulation of reduced acceptor, Q_A^-, converts photochemically active ('open') reaction centres into inactive ('closed') centres. Long-lived singlet excited states will populate in antennae connected to centres with Q_A^-, and, as a consequence, the fluorescence yield will increase (for a review, see Briantais *et al.* (1986)). In the absence of other quenching processes, there is a simple complementary relation between photochemical reduction of Q_A and Q_A^--dependent emission of fluorescence. If Q_A-dependent variations in the variable fluorescence are expressed by a coefficient for fluorescence quenching, q_Q ($0 < q_Q < 1$), then

$$\phi_s = \phi_p q_Q \tag{1}$$

and

$$\phi_p = \phi_s / q_Q, \tag{2}$$

where ϕ_s is the apparent quantum yield of electron transport during steady-state assimilation (when part of Q_A is reduced); ϕ_p is a 'potential' quantum yield of photochemistry with all PSII centres in the photochemically active state. In the experiment, ϕ_s can be determined from the whole-chain electron transport, J_e, during steady-state assimilation, as calculated, for example, from photosynthetic gas exchange (von Caemmerer & Farquhar 1981), and the flux of light, I: $\phi_s = J_e/I$. The fluorescence coefficient q_Q indicates the fraction of energy absorbed by PSII that is consumed by electron transport.

Based on equations (1) and (2), values for ϕ_p could be determined in intact plants from photosynthetic gas exchange and q_Q (Weis *et al.* 1987; Weis & Berry 1987). The analysis has revealed that values for the potential quantum yield ϕ_p are not constant, but vary substantially in response to the energetic balance of a leaf. 'High-energy quenching' of chlorophyll fluorescence could be related to changes in ϕ_p and the hypothesis has been developed that PSII

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can be converted to a 'quenching' state with a lowered photochemical yield (Weis & Berry 1987).

In this study we shall examine control of PSII in leaves during steady-state assimilation by means of chlorophyll fluorescence in different metabolic states. We shall show, by measuring the oxidation level of P_{700}, how the primary photochemistry of PSI is regulated. A recently developed approach (Weis *et al.* 1987; Harbinson & Woodward 1987) will be used to monitor oxidized P_{700} *in situ* by following absorbance changes at 820–830 nm. It is based on the fact that the photochemically inactive oxidized P_{700} radical shows a broad absorbance band in the far red (Ke 1972). We shall compare steady-state control of PSII with that of PSI, and develop a concept of how both photoreactions are regulated to match the rate at which their products can be consumed by biochemical reactions.

Materials and Methods

Plants were grown in a growth chamber (PFD 500–800 μmol quanta m^{-2} s^{-1}; 25 °C day temperature, 15 °C night temperature). Attached leaves were mounted in a gas chamber with a 10 cm^2 window on the top. The incoming air contained either 2% O_2 plus 300 μbar† CO_2 or 21% O_2 plus 1% CO_2. The outcoming air was analysed either by a CO_2 gas-analyser (Mayhak) or with a laboratory-built Clark-type O_2-electrode (1% CO_2).

The leaf chamber was illuminated from the top with homogeneous white actinic light filtered through heat-mirrors (OCLI). Fluorescence was collected by an optical fibre from the top of the chamber and measured with a PAM 101-fluorimeter (Walz, D-8521 Effeltrich, F.R.G.). After the leaf had reached a steady state (15–30 min) quenching coefficients were determined as described recently (Weis & Berry 1987).

The common arm of a second doubly branched optical fibre was positioned at an angle of approximately 45° to the top window of the chamber and followed changes at 820 nm. One arm of this optical fibre was used to illuminate part of the leaf with a modulated far-red measuring light (820 nm; 3 kHz). The second arm was connected to a photodiode protected with a filter (Schott RG 780) to measure the back-scattered measuring light. Light-dependent absorbance changes at 820 nm (measured from back-scattered measuring light) were followed to determine the redox state of the reaction-centre pigment, P_{700}, during steady-state assimilation, as described recently (Weis *et al.* 1987). A coefficient for light-dependent absorbance at 820 nm, a_{820}, was obtained by comparing the fast (less than 1 s) absorbance change, A_s, observed when the steady-state illumination is interrupted, with the maximal absorbance changes (A_{max}) during a short (1–3 s) saturating far-red (820 nm) light pulse: $a_{820} = A_s/A_{max}$. After a dark period, P_{700}/P^+_{700} may reach the completely non-oxidized form, and the far-red pulse, given after a short dark period, is assumed to cause a transient oxidation of all photochemically active PSI. Because of the high redox potential and the instability of the far-red-absorbing radical form of the PSII pigment, P^+_{680} (see, for example, Schlodder & Meyer 1987), the occurrence of significant amounts of P^+_{680} in the steady state appears rather unlikely, and we relate a_{820}, in a first approximation, to the fraction of PSI centres that are in the photochemically inactive, oxidized form (see also Weis *et al.* 1987; Harbinson & Woodward 1987).

Fluorescence quenching in isolated chloroplasts was measured as described recently (Krause

† 1 μbar = 10^{-1} Pa.

et al. 1988). The steady-state level of 'light activation' of the NADP-dependent malate dehydrogenase (NADP-MDH) in isolated chloroplasts was determined as follows. Chloroplasts were illuminated (300 μmol quanta m^{-2} s^{-1}) in the presence of 2 mM NO_2^- as an electron acceptor and different levels of the uncoupler nigericin. After 5–8 min fluorescence was measured, and the chloroplasts were osmotically ruptured and measured for NADP-MDH activity by a similar method to that described by Heber *et al.* (1979).

With isolated thylakoid membranes the light-induced proton gradient was measured in an isotonic medium (pH 8.0) by means of 9-aminoacridine fluorescence (Schuldiner *et al.* 1972) in the presence of 5 mM $MgCl_2$, 10 μM methyl viologen, 1 mM ADP and P_i as indicated. A thylakoid volume of 25 μl mg^{-1} chlorophyll has been assumed. Non-photochemical quenching of chlorophyll fluorescence was detected by saturating light pulses, essentially as described recently (Krause *et al.* 1988).

Results and discussion

The rate of CO_2 uptake or O_2 evolution, J, was measured under steady-state conditions with attached leaves in slightly reduced (300 μbar) atmospheric CO_2 and low oxygen to suppress photorespiration, or with almost saturating concentration of atmospheric CO_2 (1%). Simultaneously we determined coefficients for 'optical' signals to characterize the photochemical state of PSII and PSI. Figure 1 shows an experiment with sunflower plants, measured in 300 μbar CO_2. In this example, J increased almost linearly with light and the apparent quantum yield, whereas ϕ_s (calculated by dividing the assimilation rate by the incident light flux, $\phi_s = J/I$) remained close to its maximal value, even at high rates of assimilation. It points to a well-balanced production of NADPH and ATP and an optimal 'activation' of the energy consuming biochemical reactions. Considerable decline in ϕ_s was only observed beyond a rather abrupt transition from light-limited to light-saturated assimilation. With other species, we have observed similar light responses, although in some plants (e.g. spinach) the transition from light-limited to light-saturated assimilation was less abrupt (not shown).

In the light-limited range, values for q_Q and a_{820} were close to dark values. High q_Q (i.e. low reduction of Q_A) and low a_{820} (i.e. low oxidation of P_{700}) suggest that, up to high rates of whole chain electron transport, both photoreactions were operating close to their maximum quantum efficiency. This evidently requires an optimal regulation of electron transport and biochemical reactions. Even at very low light fluxes, we usually observed a small non-photochemical quenching of fluorescence (q_N; figure 1). Upon darkening, fluorescence recovered only slowly (more than 10 min), but recovery was faster in a weak far-red light. This far-red sensitivity suggests that it may be related to 'state 1 – state 2' regulation of energy distribution, as mediated by phosphorylation of the light-harvesting complex (LHCII) (for a discussion, see Briantais *et al.* (1986); Horton (1987)). But clearly, more work is required to understand fully this kind of quenching. It was usually not difficult to separate it from the faster 'energy-dependent' quenching, q_E, which presumably is caused by light-induced acidification of the inner thyklakoid space (Krause *et al.* 1983). During light-limited assimilation, q_E was low, but increased in the light-saturated range, almost parallel to the decline in ϕ_s, to maximal values of 0.8 – 0.9. The increase in q_E was accompanied by a large increase in a_{820} (accumulation of P_{700}^+), whereas the decline in q_Q (reduction of Q_A) was rather moderate, similar to results described recently (Weis & Berry 1987; Weis *et al.* 1987). A remarkably high level of oxidized

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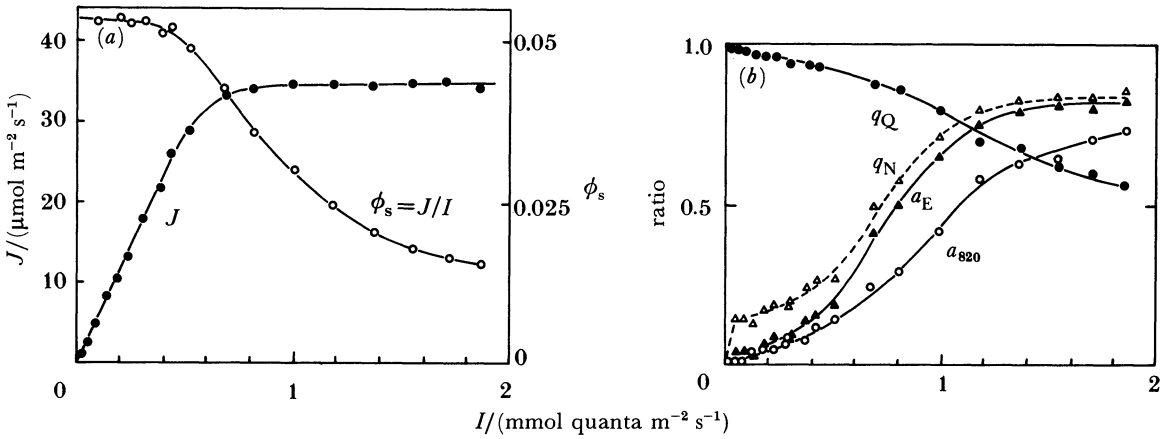

FIGURE 1. (*a*) Light response of net CO_2 uptake, J, and the apparent quantum yield of CO_2 uptake, ϕ_s. The experiment was done with *Helianthus annuus*, at 2% O_2 and 300 μbar CO_2. (*b*) Coefficients for 820 nm absorbance change (a_{820}) and for fluorescence quenching (q), measured simultaneously with J. Non-photochemical quenching, q_N, was obtained by relating the maximum variable fluorescence, F_v, measured in the steady state, to that measured after extended (more than 1 h) dark time. q_E was obtained by relating F_v of the steady state to F_v measured after 15 min illumination with weak light (15 μmol quanta m^{-2} s^{-1}).

Q_A during steady-state assimilation has already been reported by Dietz *et al.* (1985). In the following, we shall analyse the control of PSII and PSI photochemistry of different species under light-saturated assimilation, on the basis of data obtained as shown in figure 1.

Control of photosystem II

In figure 2, Q_A-dependent fluorescence quenching, q_Q, is plotted against the apparent quantum yield of electron transport, ϕ_s (J_e/I). The electron transport, J_e, is calculated from gas exchange. To compare data from different experiments, we normalized ϕ_s, setting the extrapolated maximal value as 1. Normalized values for a quantum yield, in the following

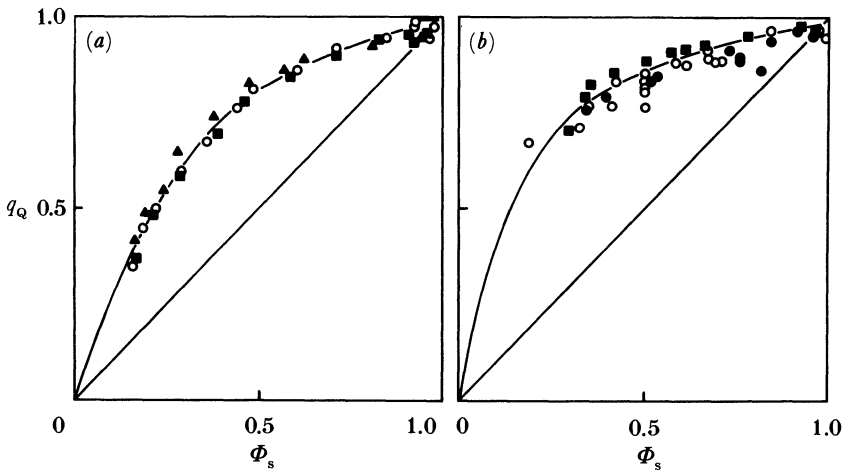

FIGURE 2. Dependency of photochemical fluorescence quenching, q_Q, on the apparent quantum yield of electron transport, Φ_s, calculated from whole chain electron transport, J_e, and incident light, I ($\Phi_s = J_e/I$; values normalized). I has been varied as in figure 1. J_e has been calculated either from CO_2 uptake measured in 2% O_2 and 300 μbar CO_2 (*a*) or from O_2 evolution measured in 1% CO_2 (*b*). Symbols: (*a*) ▲, *Phaseolus vulgaris*; ○, *Gossypium hirsutum*; ■, *Oxalis purpurea*. (*b*) ■, ○ *Phaseolus vulgaris*; ●, *Gossypium hirsutum*.

defined as Φ ($0 < \Phi < 1$), may be used as dimensionless probability coefficients. Φ_s indicates the extent to which energy absorbed by PSII is utilized by photochemistry and 'linear' electron transport, respectively. A nonlinear relation between Φ_s and q_Q was observed. In low CO_2, a decline in Φ_s to 0.5 was accompanied by a decline in q_Q to only about 0.8. In high CO_2, even smaller changes in q_Q, relative to Φ_s were obtained. The result indicates that in addition to Q_A reduction, another process is involved in controlling the photochemical activity of PSII. Following equations (1) and (2), Φ_s has been divided by q_Q to obtain the potential quantum yield for PSII photochemistry, defined as the yield with all centres in the active state. As shown in figure 3, Φ_p is not constant, but varied with Φ_s. In high CO_2, Φ_p declined almost parallel with Φ_s. A strong decline was also observed in low CO_2. However, at Φ_s less than 0.5, values for Φ_p tended to level off and to extrapolate to a minimum value of about 0.3. After lowering the light intensity, Φ_p usually recovered to a high value in the time range of a few minutes (however, see below). The decline in the photochemical yield, not matched by Q_A-dependent fluorescence, indicates that absorbed energy is dissipated by a non-photochemical, non-radiative pathway of de-excitation.

Data as shown in figure 3 can be used to calculate coefficients that characterize the distribution of absorbed energy between different pathways for de-excitation at PSII. $\Phi_D = 1 - \Phi_s$ indicates the proportion of energy in the pigment system not consumed by electron transport and biochemical reactions, respectively; $\Phi_e = 1 - \Phi_p$ indicates the proportion of energy, dissipated by non-photochemical and non-radiative de-excitation; $\Phi_r = \Phi_D - \Phi_e$ is the proportion of energy distributed to 'closed' centres and dissipated by 'radiative' de-excitation. Over a wide range of conditions, changes in Φ_D are almost completely matched by changes in Φ_e ($\Phi_D \approx \Phi_e$), while values of Φ_r were found to be much less than 0.1. It suggests that, during light-saturated assimilation, the largest part of 'excess' energy, not used for PSII-electron transport, is dissipated by non-radiative, 'thermal' dissipation of excitation energy. As a consequence, Q_A is kept in a fairly oxidized state, and not more than 10% of the energy absorbed by PSII is delivered to closed centres and dissipated by 'radiative' de-excitation. Only during extreme over-excitation was Φ_r found to increase up to values of 0.3. Under such conditions, assimilation usually started to decline, and after lowering the light intensity, the

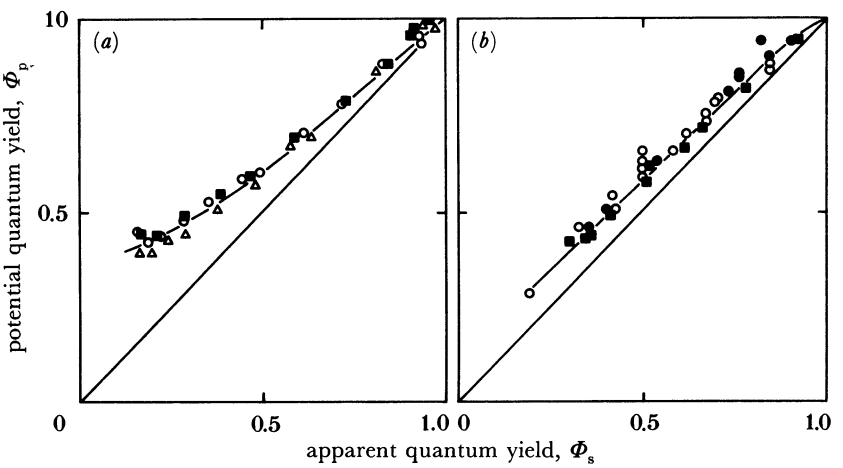

FIGURE 3. Dependency of the 'potential' quantum yield of PSII electron transport, Φ_p, on the apparent quantum yield, Φ_s ($\Phi_p = \Phi_s/q_Q$). (*a*) 300 µbar CO_2, 2% O_2; (*b*) 1% CO_2. Data obtained from experiments shown in figure 2. Symbols as in figure 2.

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recovery of Φ_p to its maximum value was very slow (in the range of hours), indicating that plants were 'photoinhibited' (data not shown). Thus high values of Φ_r may indicate that a plant is under 'light stress'.

As a strong and stable correlation between the decline in Φ_p and energy-dependent quenching of fluorescence, q_E, has been found (Weis & Berry 1987), q_E can be taken as an indicator of non-photochemical, non-radiative 'quenching' of excitation energy. Empirical equations of the form $\Phi_p = xq_E + (1-q_E)$, and, by introducing equation (2), $\Phi_s = q_Q[xq_E + (1-q_E)]$ (where $x \approx 0.35$) were found to describe, to a first approximation, the relation between photochemical yield and fluorescence. Independent of the underlying mechanisms, this empirical relation may allow values of Φ as defined to be calculated from fluorescence. Because the photosynthetic electron flux, J_e, equals $I\Phi_s$, we can calculate rates of electron transport in a leaf from fluorescence and light-flux without measuring gas exchange. The validity of this approach has already been proved in many experiments (Weis & Berry 1987). The comparison of total electron transport, as calculated from fluorescence, with photosynthetic gas exchange may also allow calculation of fluxes of electrons through 'dissipative' metabolic pathways, such as photorespiration.

A model for regulation of photosystem II

Although the above equations may correctly describe the relation between fluorescence and quantum yield of different pathways for de-excitation in an empirical sense, they may not adequately reflect the underlying mechanism. In a modification of the model proposed recently (Weis & Berry 1987), we assume that PSII can exist in three states. A photochemically active state, $PSII_\alpha$, can be converted to an inactive state with closed centres, $PSII_\alpha^-$ (by reduction of Q_A):

$$PSII_\alpha \overset{e^-}{\rightleftharpoons} PSII_\alpha^-. \tag{3}$$

$PSII_\alpha$ can be converted to another photochemically inactive, 'quenching' state, $PSII_\varepsilon$. In the $PSII_\varepsilon$ state ($\phi_p = 0$), excitation energy is dissipated by non-radiative, non-photochemical de-excitation. Although the active form, $PSII_\alpha$, may accumulate during light-limited photosynthesis, its conversion to $PSII_\varepsilon$ may occur upon 'energization' of the thylakoid membrane (formation of a high proton gradient):

$$PSII_\alpha \overset{\Delta pH}{\rightleftharpoons} PSII_\varepsilon. \tag{4}$$

In terms of this model, changes in fluorescence quenching and in the quantum yield of electron transport in going from one steady state to another may indicate changes in the proportion of PSII in these forms: $\Phi_s = PSII_\alpha/(PSII_\alpha + PSII_\alpha^- + PSII_\varepsilon)$, and $\Phi_p = (PSII_\alpha + PSII_\alpha^-)/(PSII_\alpha + PSII_\alpha^- + PSII_\varepsilon)$. An increase in q_E would then indicate, to a first approximation, the proportion of PSII in the $PSII_\varepsilon$ form.

In figure 4, q_E values obtained from a large number of independent experiments conducted with intact plants and isolated chloroplasts are plotted against Φ_p. In chloroplasts, q_E quenching and related decline in Φ_p was completely abolished when an uncoupler was present (see also experiments shown in Krause *et al.* (1988)). Apparently, the data do not show the simple linear relation predicted by the model developed above. This may partly arise from 'intrinsic' artifacts related to the saturating light-pulse approach. In the steady state most centres are supposed to be in a 'quenching' state ($PSII_\alpha$ or $PSII_\varepsilon$), whereas during the

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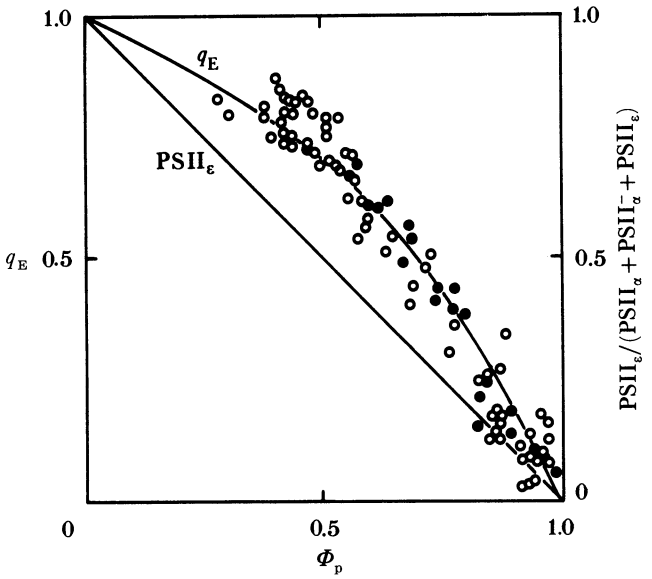

FIGURE 4. Dependency of non-photochemical fluorescence quenching. q_E, on the potential quantum yield, Φ_p ($q_E = \Phi_p(1-c)/(1-c\Phi_p)$; $c = 0.5$). Values obtained from six independent experiments with intact plants (light responses, conditions as in figure 1; open circles), and from experiments done with isolated intact chloroplasts (light responses with NO_2 as an electron acceptor; closed circles). In the chloroplast experiments, q_E was less than 0.1, when nigericin was present.

saturating light pulse, all centres in the $PSII_\alpha$-form are closed. If PSII units are 'connected', quanta can escape from a closed centre and be trapped by another connected quenching centre (Joliot *et al.* 1973). Energy transfer will generally increase the efficiency of any kind of quenching centre to absorb energy. During the light pulse, a certain fraction of quanta may thus escape from $PSII_\alpha^-$ to be quenched by $PSII_\varepsilon$. Therefore, values for q_E determined by a light pulse may actually be higher than the related proportion of $PSII_\varepsilon$. As shown in figure 4, a hyperbolic relationship is obtained on the basis of an equation of the form $q_E = \Phi_p\,(1-c)/1-\Phi_p c$, where c ($0 < c < 1$) denotes the probability that quanta trapped by $PSII_\alpha^-$ can escape to $PSII_\varepsilon$. Overestimation of q_E may also arise from difficulties in closing all centres during the 'saturating' light pulse. Under certain conditions, e.g. at high fluxes of light, the light pulse may not be saturating. In addition, electron donation to PSII centres may become limiting during the high light pulse (Schreiber & Neubauer 1987; see also Schreiber, this symposium). Evidently, further investigations are required to evaluate the 'true' relation between fluorescence quenching and Φ_p.

Energy-dependent quenching and proton gradient

From the work of Krause and co-workers (Krause *et al.* 1982, 1983), we know that q_E quenching of fluorescence is caused by light-driven proton uptake into the inner thylakoid lumen. The proton gradient across the thylakoid membrane is part of the driving force for photophosphorylation (Mitchell 1974). Changes in the metabolite level and in P_i can feedback and affect this gradient and the proton concentration within the thylakoid lumen, respectively. An increase in the proton gradient can be caused by a shortage of ATP consumption and related increase in the ATP/ADP ratio. In intact plants, this may occur at limiting concentrations of CO_2. An increase in the proton motive force may also be caused by a decline

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in the stromal P_i concentration and related restriction of ATP synthesis. Recycling of P_i from the cytosol into the stroma may be a major regulatory factor in assimilation (Laisk & Walker 1986; Sivak & Walker 1986; Walker & Osmond 1986; Stitt 1986; for a discussion, see Woodrow & Berry 1988), and with isolated chloroplasts, it could be demonstrated that P_i limits assimilation at a point where it also limits ATP synthesis (Giersch & Robinson 1987; Furbank *et al.* 1987). In intact plants, P_i limitation is expected to occur during light- and CO_2-saturated assimilation (as, for example, in the presence of 1% CO_2).

At $[P_i] \leqslant 1$ mM, the rate of photophosphorylation has been found to be proportional to $[P_i]\,[H^+(in)]^2$, where $[H^+(in)]$ is the proton concentration within the thylakoid lumen (Tran-Ahn & Rumberg 1987). From this it can be predicted that, in the steady state, pH-dependent q_E-quenching is directly related to the free inorganic phosphate concentration. This is essentially confirmed by the experiment shown in figure 5. We suspended isolated thylakoid membranes at pH 8.0 in presence of 1 mM ADP with methyl viologen as an electron acceptor. In the light we measured the pH in the inner thylakoid lumen (by means of 9-aminoacridine fluorescence quenching) and q_E-quenching of fluorescence, in dependence of the inorganic phosphate concentration. At $[P_i] > 1$ mM fluorescence quenching was low, and also the potential photochemical yield, Φ_p, was close to its maximum value. The maximum value for Φ_p has been determined in presence of an uncoupler, where no q_E occurs. The ΔpH was 2.6–2.7. At $[P_i] < 1$ mM, a sharp rise in q_E and a related decline in Φ_p were observed, accompanied by a drop in the inner-thylakoid pH from 5.3 to 4.8 and related to an increase in the ΔpH to 3.2.

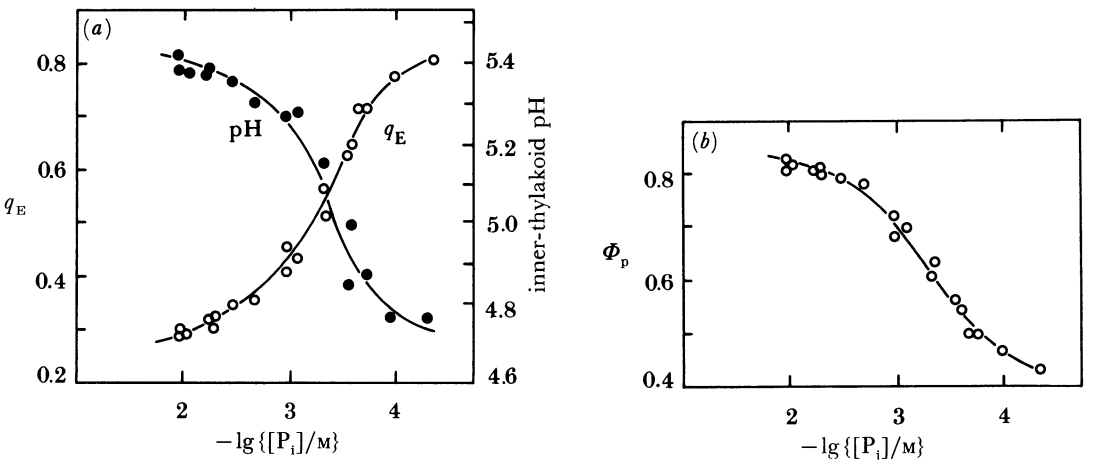

FIGURE 5. Dependency of the potential quantum yield, Φ_p, non-photochemical fluorescence quenching, q_E, and the pH of the inner-thylakoid lumen (as measured by means of 9-aminoacridine fluorescence) on the concentration of P_i in the medium. Isolated thylakoid membranes were suspended in an isotonic medium at pH 8.0, and 20 µM methyl viologen was present as an acceptor.

In figure 6, q_E is plotted against the inner-thylakoid pH. The values have been obtained from P_i-response experiments, as in figure 5, but it should be noted that we have obtained the same relation when we changed the ΔpH by varying the ATP/ADP ratio, at constant P_i (not shown). The figure demonstrates that a large change in q_E (and related regulation of PSII) may occur within a small range of pH. On the basis of the 9-aminoacridine approach, an apparent pK value of about 5.0 for pH-dependent quenching could be determined. This is

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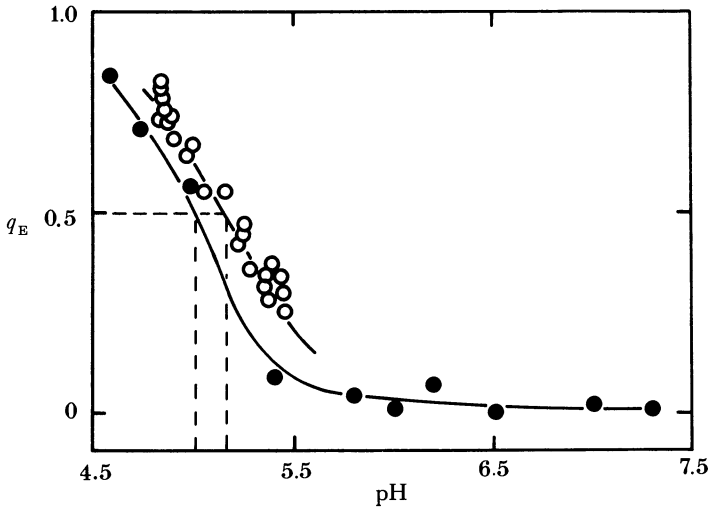

FIGURE 6. Dependence of non-photochemical fluorescence quenching on the pH in the thylakoid lumen (open circles: values from figure 5), or on the pH in the suspension medium (closed circles). In this event, thylakoid membranes were suspended in the absence of an acceptor at different pH and measured from the maximum variable fluorescence, F_v. F_v at pH 8.0 was set as 1 and $q_E = F_v(\text{pH } x)/F_v(\text{pH } 8)$.

close to a value obtained from a direct 'pH titration' of fluorescence quenching, done by suspending thylakoid membranes in the dark in media with different pH (solid circles). If we assume that the conversion of $PSII_\alpha$ to $PSII_\varepsilon$ is related to a protonation reaction, then the equilibrium between the postulated two states of PSII can be related to an apparent pK_α value, obtained from figure 6:

$$PSII_\alpha + H^+ \rightleftharpoons PSII_\varepsilon; \quad pK_\alpha \approx 5.0. \tag{5}$$

Photosynthetic control and regulation of photosystem I

From the above data it follows that during light-saturated assimilation more than 50% of the energy absorbed by PSII can be 'quenched' by the pH-dependent q_E-quenching mechanism. This energy dissipation at PSII would cause a drastic change in the distribution of excitation, in favour of PSI. As a consequence, the plastoquinone pool (PQ) would be over-oxidized. However, the moderate decline in q_Q as q_E increases (see figures 1 and 2) suggests reduction of Q_A. Because of its redox potential, PQ is expected to be even more reduced than Q_A. On the other hand, oxidized P_{700} accumulated as the light flux was increased and q_E-quenching developed (increase in a_{820}; figure 1), suggesting that PSI is controlled by a shortage of electron donation (see also Weis *et al.* 1987). This redox crossover points to a control step between PQ and PSI. Feedback control of the photosynthetic electron transport has been referred to as 'photosynthetic control' (West & Wiskich 1968). Because electron transport from the PQ pool through the cytochrome *b/f* complex is coupled to transport of protons into the thylakoid lumen, it thermodynamically relates the net flux to the proton gradient. Photosynthetic control may only occur above a critical threshold ΔpH, similar to q_E-quenching.

It is important to understand that a shift of P_{700} to the oxidized state, caused by photosynthetic control of electron donation, does not simply 'close' PSI centres. Because of its absorbance properties, P^+_{700} traps excited states from the antenna as efficiently as photochemically active centres, but converts the absorbed energy by a fast internal decay (Nuijs *et al.* 1986). Thus formation of oxidized P_{700} may serve to deliver excitation energy into

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a pathway for non-photochemical de-excitation, thereby adjusting the potential photochemical activity to the rate of biochemical reactions, similar to the scheme proposed for q_E-quenching. It may also protect PSI against photoinhibition.

In low CO_2, a_{820} was almost linearly related to Φ_s (figure 7), suggesting that PSI was almost completely controlled by electron donation and related formation of oxidized centres, rather than by an excess of electrons on the acceptor side. As an important consequence of this pH-dependent feedback, ferredoxin and NADP/NADPH are expected to be in a rather oxidized state, even when light is in excess and CO_2 is strictly limiting for assimilation. Because decline in Φ_p (PSII) and accumulation of P^+_{700} occur in an almost parallel way, PSI and PSII excitation are kept in balance, even under photosynthetic control (compare figure 3 and figure 7). A clearly different situation is observed in 1 % CO_2 (figure 7), where the increase in a_{820} was less than proportional to the decline in Φ_s, suggesting that PSI was only partly controlled by electron donation. To some extent an excess of electrons at the acceptor side may 'close' PSI centres, and PSI may be controlled by both electron 'backpressure' and photosynthetic control. A higher reduction level on the acceptor side of PSI is expected, compared with the situation at low CO_2.

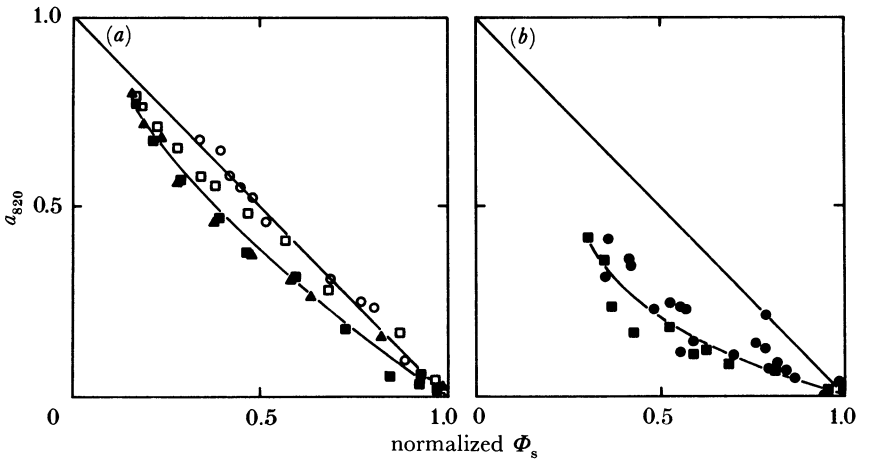

FIGURE 7. Steady-state level of light-dependent absorbance change at 820 nm, a_{820}, dependent on the apparent quantum yield, Φ_s. Experiments were done in 300 µbar CO_2 and 2 % O_2 (*a*) or in 1 % CO_2 (*b*). (*a*) ■, *Oxalis purpurea*; ▲, ○, *Phaseolus vulgaris*; □, *Valerianella locusta*. (*b*) ■, ● *Phaseolus vulgaris*.

Photosynthetic control and thioredoxin activation

Because feedback from the carbon metabolism does not result in a reduction of the electron-transport system, and even PSI seems to be controlled by a shortage of electron donation, a rather low reduction level on the acceptor side of PSI is expected. This may include the ferredoxin–thioredoxin system, which functions in photosynthesis by linking light energy to the reductive activation of selected regulatory enzymes (Cseke & Buchanan 1986). As the activation state of the NADPH-dependent malate dehydrogenase (NADPH-MDH) may, to a first approximation, reflect the activation state of the thioredoxin system (Scheibe 1987), we tested the activation state of this enzyme to examine the thioredoxin system. Isolated intact chloroplasts were illuminated with saturating light in the presence of NO_2^- as an electron acceptor. Under such conditions, no ATP is consumed and a strong pH-dependent feedback is expected.

Indeed, electron transport was low ('controlled') and q_E-quenching high. Addition of different concentrations of an uncoupler (nigericin) released photosynthetic control (enhancement of electron transport) and decreased q_E. After osmotic lysis of chloroplasts, the activation state of NADPH-MDH was determined and related to the level of q_E (figure 8). An inverse relation between q_E and thioredoxin-dependent NADPH-MDH activation was found. The figure shows that in the coupled state, where electron transport was low (not shown) and q_E was high, the enzyme was almost completely inactivated, i.e. close to its dark level of activation, indicating an oxidized state of the ferredoxin–thioredoxin system. In the uncoupled state ($q_E = 0$), where all the feedback was released, the activation state was close to its maximum level, suggesting an excess of electrons at the acceptor side of PSI.

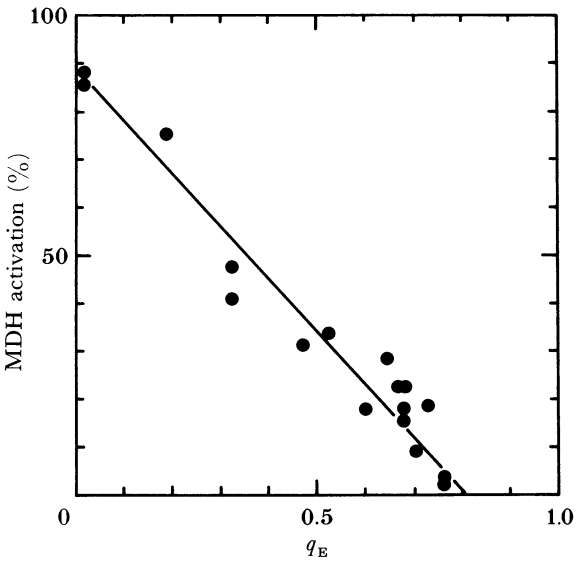

FIGURE 8. Steady-state level of activation of the $NADP^+$-dependent malate dehydrogenase (MDH), dependent on non-photochemical fluorescence quenching, q_E. Experiments were done with isolated intact chloroplasts. The NADP-MDH activity obtained from chloroplasts previously illuminated in the presence of 1 μM nigericin and in the absence of an electron acceptor was set as 100%. Illumination was 300 μmol quanta m^{-2} s^{-1}, and NO_2^- was present as an electron acceptor.

CONCLUSIONS

The analysis of photosynthetic gas exchange, fluorescence quenching and measurements of P_{700} leads us to conclude that plants have developed a feedback mechanism to adjust the potential rate of photochemistry of both photosystems to the energy demand by the carbon metabolism. It is an important conclusion of the analysis presented here and in a recent study (Weis & Berry 1987) that the 'intrinsic' quantum efficiency of PSII is not a constant system parameter, but is highly variable and adjusted to the energetic balance of the leaf. As a consequence of this control mechanism, the electron transport chain is kept in a fairly oxidized state, even under conditions where the rate of light absorption exceeds the energy demand by biochemical reactions.

We relate energy-dependent quenching of chlorophyll fluorescence to the regulation of PSII, which may be mediated by the proton concentration within the inner thylakoid lumen. A

model is proposed that is based on the assumption that fluorescence quenching during steady-state assimilation reflects the equilibrium between three different states of PSII; $PSII_\alpha$ is the photochemically active state. This state will accumulate in the light-limited range, where the overall quantum yield of assimilation is high. It is converted to the closed state, $PSII^-_\alpha$, by reduction of the acceptor Q_A. Because such centres cannot 'trap' quanta, energy is dissipated from the antenna by 'radiative' de-excitation. $PSII_\alpha$ can be converted, possibly by protonation, to a quenching form, $PSII_\varepsilon$.

Changes in the equilibrium between the postulated different states of PSII function to regulate the net rate of PSII photochemistry. We assume, however, that this regulation is adjusted in such a way that reduction of Q_A and related closure of centres is of minor importance in controlling PSII. Photochemistry is mainly adjusted by non-radiative energy dissipation, as indicated by the decline in the potential quantum yield for photochemistry, Φ_p.

The relation between energy-dependent q_E-quenching and the pH in the inner-thylakoid lumen (figure 6) suggests that a high ΔpH can be built up in the light, without feeding back to PSII. Actually, a high ΔpH is a prerequisite for efficient photophosphorylation (Gräber *et al.* 1987). Feedback, as indicated by q_E-quenching, may only occur above a critical concentration of protons within the thylakoid lumen. Beyond this threshold, only a small increase in the pH is required to shift PSII to the photochemically inactive quenching state, i.e. no large thermodynamic constraint is required to control PSII.

The molecular mechanisms linking the interconversion between different quenching states of PSII to the energization state of the membrane, and the mechanism of energy quenching, are not yet known. We assume that reaction centres of PSII itself are converted to a state where singlet excited states are de-excited by a fast internal decay. It has also been proposed that excess energy can be dissipated by a 'futile' cycle of electrons around PSII (Schreiber & Neubauer 1987; see also Horton, this symposium). We may not exclude the possibility that several quenching mechanisms could contribute to 'high-energy quenching' in plants. Evidently, more work is required to elucidate the mechanisms of this quenching process.

Independent of mechanisms and functions of the quenching mechanisms under investigation, we could relate changes in fluorescence to changes in the quantum yield of net electron transport by empirical equations. This provides a means of conducting measurements of the photochemical efficiency, and even the rate of electron transport *in situ*, from fluorescence data (see also Weis & Berry 1987). Furthermore, the comparison of total electron transport, as calculated from fluorescence, with photosynthetic gas exchange allows calculation of the partitioning of photosynthetic fluxes between carbon assimilation and 'dissipative' metabolic pathways, such as photorespiration, to be made. Coefficients can be derived that characterize the distribution of absorbed energy between the different pathways for de-excitation for each steady-state condition.

The use of absorbance changes at 820 nm to monitor the formation and steady-state level of oxidized P_{700} presented in this and a previous study (Weis *et al.* 1987; see also Harbinson & Woodward 1987) provides a new way to examine the control of PSI photochemistry *in situ*. The high level of oxidized P_{700} observed during CO_2-limited assimilation suggests that PSI photochemistry is controlled by shortage of electron donation to PSI, caused by a concerted control of both PSII (q_E-quenching) and electron transport at the plastoquinone pool. As the P^+_{700} radical is a highly efficient 'quencher' of singlet excited states, quanta absorbed by

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antennae connected to oxidized PSI centres will be trapped and dissipated, at the expense of photochemical conversion. PSI centres closed by reduced acceptors have no trapping efficiency (Nuijs *et al.* 1986).

The simultaneous occurrence of oxidized P_{700} and energy quenching at PSII, especially at low CO_2 (figure 1, compare also figures 3 and 7, 300 μbar CO_2), as well as the relative stability of the redox state of Q_A in changing from light-limited to light-saturated assimilation, suggests that the balance between both photoreactions is not disturbed when photosynthetic control and related quenching mechanisms come into effect. Clearly, photosynthetic control and high-energy quenching serve to match the rate at which electrons enter the electron-transport system to the electron demand by biochemical reactions. There are conditions, however, where PSI can be controlled by an excess of electrons at the acceptor side. Then, photosynthetic control may not come into effect, even when the proton gradient (and q_E) is high. This may occur in high light and saturating CO_2 (compare figures 3 and 7, 1% CO_2) and during photosynthetic induction (not shown). One may speculate that under such conditions electrons are delivered from the reduced acceptor side of PSI into a cyclic pathway around the photosystem.

pH-dependent feedback of the light-reaction presumably may also affect regulation of the carbon cycle. Strict limitation of electron donation to PSI by q_E-quenching and photosynthetic control will shift the thioredoxin system to a more oxidized state. Thus enzymes of carbon metabolism could be down-regulated, even in high light. This feedback loop could play a role in controlling assimilation during strictly CO_2-limiting conditions.

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Discussion

J. Barber *AFRC Photosynthesis Research Group, Imperial College, London, U.K.*). Is it possible that the non-photochemical trapping state of photosystem II that Dr Weis calls $PSII_\varepsilon$ is either of the fluorescence quenching states discussed in Barber (this symposium), namely P^+_{680}Pheo Q_A or $P_{680}Pheo^-Q_A^-$? Such states may be induced when the thylakoid lumenal pH drops to a critical value.

E. Weis. The far-red absorbing, radical form of pigments in the reaction centres may well represent the quenching state I proposed for the 'high-energy state'. In PSI the oxidized form of the pigment P_{700}, which I demonstrated to accumulate in leaves under conditions of photosynthetic control, may function as a non-photochemical quencher of excitation energy. We do not know the state of PSII centres in the quenching state. P^+_{680} is known to be very unstable and may not be an efficient quencher in the steady state. $Pheo^-$ is a more likely candidate. In this context, it may be worthwhile to remember that PSII centres contain two pheophytin molecules, only one being involved in photochemistry. One may speculate that the second pheophytin, in its radical form, may function as a non-photochemical trap. The photo-oxidized carotene at the reaction centre may also function as a quencher.

U. Schreiber (*Universität Würzburg, F.R.G.*). One may consider that $PSII_\varepsilon$ corresponds to a fraction of PSII that is cycling electrons and in this way dissipates energy. Such centres will be characterized as open centres, by showing a large q_Q, but they will not contribute to linear electron transport. This is best demonstrated by an experiment with chloroplasts to which the ADRY reagent ANT-2p is added. We know that this substance deactivates H_2O-splitting. However, upon addition there is a marked stimulation of q_Q (from 0.2 to 0.9) accompanied by a smaller stimulation of q_N (from 0 to 0.2). This is to be expected if the PSII cycle becomes largely suppressed by saturating light.

E. Weis. I agree with Dr Schreiber that electrons can cycle around PSII and such a cycle may even play a role in regulation. However, a cyclic reaction may not necessarily be identical with the postulated dissipative process. From our experiments, it appears that the dissipative process causing 'high-energy quenching' cannot be 'saturated', even if the light intensity used in the experiment exceeds several times that of full sunlight. This raises the question, whether the *capacity* of the cyclic electron transport postulated by Dr Schreiber could be high enough to explain this quenching process. Certainly, we need more experiments to give a final answer to this question. However, I currently think it more likely that the sort of high-energy quenching that I discussed in my paper is caused by an internal decay process at the PSII pigment, by which excitation energy is directly converted to heat, with no photochemical reaction involved.

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