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A QUANTITATIVE DETERMINATION OF PHOTOCHEMICAL AND NON-PHOTOCHEMICAL QUENCHING DURING THE SLOW PHASE OF THE CHLOROPHYLL FLUORESCENCE INDUCTION CURVE OF BEAN LEAVES

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Estimations of the changes in the reduction-oxidation state of Photosystem II electron acceptors in *Phaseolus vulgaris* leaves were made during the slow decline in chlorophyll fluorescence emission from the maximal level at P to the steady-state level at T. The relative contributions of photochemical and non-photochemical processes to the fluorescence quenching were determined from these data. At a low photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, non-photochemical quenching was the major contributor to the fluorescence decline from P to T, although large charges were observed in photochemical quenching immediately after P. On increasing the light intensity 10-fold, the contribution of photochemical processes to fluorescence quenching was markedly diminished, with nearly all the P-to-T fluorescence decline being attributable to changes in non-photochemical quenching. The possible factors responsible for changes in non-photochemical quenching within the leaves are discussed.

Introduction

The kinetics of chlorophyll fluorescence emission observed when dark-adapted autotrophic organisms are irradiated contain information about the intrinsic photosynthetic mechanisms of the thylakoid membrane in vivo [1,2]. Recently, the importance of this technique has been emphasized by the demonstration that changes in fluorescence emission during the quenching of maximal fluorescence at P to the steady-state fluorescence level at T (see Fig. 1 for designation of P and T) are intimately related to changes in both the rate of oxygen evolution [3] and carbon metabolism [4,5]. Fluorescence may be quenched by both photochemical and non-photochemical processes. Photochemical quenching occurs as a result of oxidation of the PS II electron acceptors which allows an increase in the rate of dissipation of excitation energy via photochemistry. Increases in the rate of chlorophyll deexcitation via radiationless decay or excitation energy transfer to pigment beds having a lower quantum yield of fluorescence will produce non-photochemical quenching. Although the non-photochemical component of P-to-T quenching has been attributed mainly to the generation of proton gradients across the thylakoid membranes [6,7], the possible involvement of reduced phaeophytin [9,10], cation electrochemical gradients [6,8,11] and phosphorylation of thylakoid polypeptides [12,13] should not be ignored. To realise the full potential of the fluorescence induction curve as a monitor of photosynthetic processes in vivo, it is essential to be able to quantify the photochemical and non-photochemical compo-

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS I, Photosystem I; PS II, Photosystem II; ΔpH, proton concentration gradient; F_{685}, fluorescence emission at 685 nm.

nents of fluorescence quenching. This has been done elegantly for isolated chloroplasts and *Chlorella* by analysis of the fluorescence yield changes observed on addition of DCMU to the experimental systems at different times throughout the fluorescence induction curve [14,15]. Unfortunately, this technique is not applicable to intact leaf tissue, since penetration of DCMU to the total thylakoid population of the monitored tissue is too slow to allow accurate determination of the redox state of the PS II electron acceptors at any given point on the fluorescence induction curve. In this paper we describe a remote, non-destructive technique for the examination and quantitation of the photochemical and non-photochemical components of fluorescence quenching in intact leaf tissue and present a quantitative analysis of the changes in photochemical and non-photochemical fluorescence quenching during the slow phases of fluorescence induction in leaf tissue exposed to low and high excitation intensities.

Materials and Methods

Plants of *Phaseolus vulgaris* cv the Prince were grown from seed in a glasshouse at 20°C for 20–30 days. All fluorescence measurements were made at 21°C from the upper surfaces of intact, fully expanded primary leaves. Leaves were dark-adapted for 30 min prior to excitation from above with 633 nm radiation produced from a 5 mW helium-neon laser (Spectra Physics) through a 633 nm interference filter (Ealing Beck). The kinetics of fluorescence emission at 685 nm were measured from the upper leaf surface using a fibre-optic light guide leading to a photomultiplier tube (Hamamatsu R446) protected by a 685 nm interferece filter (Ealing Beck).

It has been argued previously that the redox state of PS II electron acceptors can be estimated in vivo from analysis of the fluorescence transients generated on exposure of an excited leaf to a second irradiation of saturating intensity [17–19]. Excited bean leaves were exposed to a second continuous irradiation produced from a 5 mW helium-neon laser through a 633 nm interference filter. Fluorescence kinetics were recorded by transient recorders (Datalab DL905) sampling at suitable rates to resolve the minimal and maximal fluorescence levels. The transient recorders were triggered on opening of electronic shutters (Ealing Beck) coupled to the helium-neon lasers. Excitation photon flux densities were attenuated with glass neutral density filters (Ealing Beck). Photon flux densities at the leaf surface were monitored using a quantum sensor (Li 185B, Lambda Instrument Corporation).

Results

The kinetics of 685 nm fluorescence emission from a dark-adapted leaf excited with a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ are shown in Fig. 1 together with the fluorescence transients generated by exposing the leaf to a second irradiation of 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the point of maximal fluorescence, P, or at steady-state fluorescence, T. The second irradiation induces a rapid rise in fluorescence to an initial level, designated F_{O2}, followed by a slower rise to a maximum level,

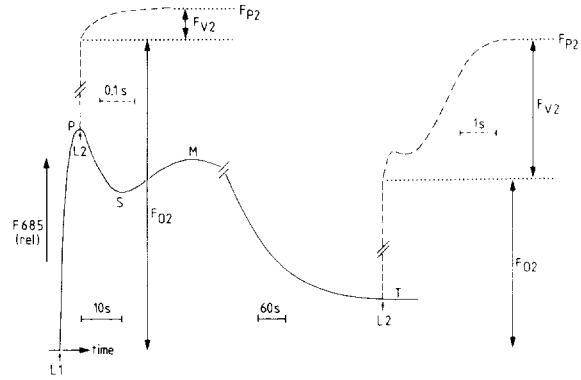

Fig. 1. Kinetics of 685 nm fluorescence emission from a bean leaf. An intact bean leaf dark-adapted for 20 min was excited with a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of 633 nm irradiation, L1. At given points on the fluorescence induction curve, the leaf was exposed to an additional excitation of 500 μmol photons $\cdot m^{-2} \cdot s^{-1}$ of 633 nm irradiation, L2. The fluorescence kinetics induced by L2 (dashed line) are shown when the leaf is given the additional excitation at points P and T of the induction curve. F_{O2} and F_{P2} represent the minimal and maximal levels of fluorescence, respectively, attained on opening of the shutter for the additional excitation, L2. F_{O2} and F_{P2} were resolved by the use of transient recorders, triggered on opening of the shutter, sampling at 20 kHz and 100 Hz, respectively. The redox state of PS II electron acceptors at the point of addition of the second excitation was estimated from the ratio of additional variable fluorescence, F_{V2}, ($F_{V2} = F_{P2} - F_{O2}$), to F_{O2} produced by L2.

F_{P2}. The variable fluorescence produced by the second irradiation is designated F_{V2}. If the second excitation is saturating for the amount of F_{V2} generated relative to F_{O2}, then the ratio of F_{V2}/F_{O2} should be directly related to the fraction of oxidised PS II electron acceptors at the point of addition of the second irradiation. When no variable fluorescence is produced by the second excitation, i.e., $F_{V2} = 0$ and thus $F_{V2}/F_{O2} = 0$, all of the PS II electron acceptors must be reduced. Changes in F_{V2}/F_{O2}, generated by saturating irradiation, observed during fluorescence quenching in isolated thylakoids, have previously been found to correspond to changes in the magnitude of the rapid phase of the light-induced reversal of fluorescence quenching upon addition of DCMU [20]. A strong correlation between the rapid phase of the light-induced reversal of fluorescence quenching by DCMU and additional variable fluorescence has also been observed in higher plant protoplasts [21]. The rapid rise in fluorescence on addition of DCMU to thylakoids and cells is due to the rapid reduction of Q and the level of fluorescence attained at the end of this fast rise is attributed to the fluorescence level that would occur if PS II electron acceptors were maximally reduced [14,15]. The magnitude of F_{V2}/F_{O2} generated by addition of a second excitation to a leaf, initially excited with 100 μmol photons $\cdot$ m$^{-2} \cdot$ s^{-1}, at both the maximal (P) and the steady-state (T) levels of fluorescence, is shown as a function of the excitation intensity of the second irradiation in Fig. 2. Similar intensities of the second irradation, i.e. approx. 500 μmol $\cdot$ m$^{-2} \cdot$ s^{-1}, were required to obtain maximal values of F_{V2}/F_{O2} at both P and T, although it should be noted that the magnitude of the maximal F_{V2}/F_{O2} generated at T was approx. 10-fold greater than at P indicating that the PS II electron acceptors were considerably more reduced at P than at T. Increasing the photon flux density of the second excitation above 500 μmol $\cdot$ m$^{-2} \cdot$ s^{-1} produced a slight but reproducible decrease in F_{V2}/F_{O2}. Plots of the minimal, F_{O2}, and maximal, F_{P2}, levels of fluorescence generated by the second excitation at T as a function of excitation intensity (Fig. 3) show clearly that the decrease in F_{V2}/F_{O2} at high intensities is due to a decrease in the variable component, F_{V2}, and not F_{O2}. The magnitude of F_{O2} is directly proportional to the second

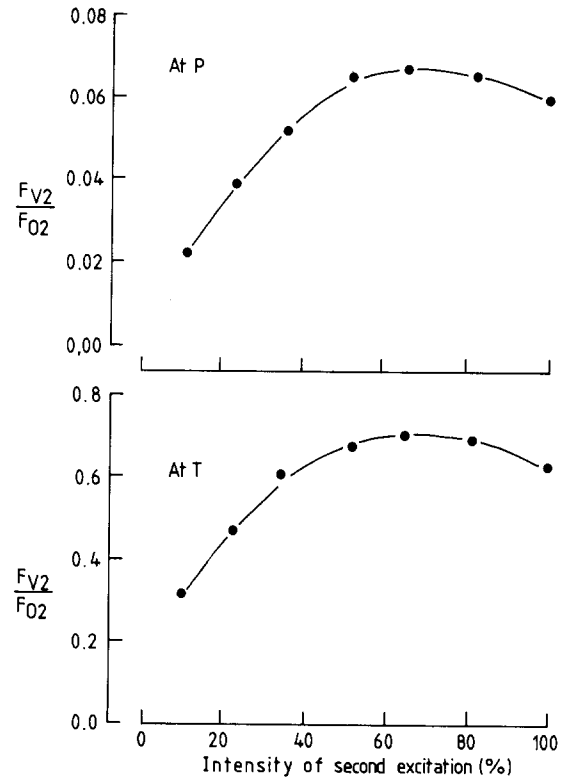

Fig. 2. Changes in F_{V2}/F_{O2} generated by addition of a second excitation to a bean leaf, initially excited with a photon flux density of 100 μmol $\cdot$ m$^{-2} \cdot$ s^{-1}, at both the maximal (P) and steady-state (T) levels of fluorescence as a function of the intensity of the second excitation. Refer to Fig. 2 for definitions of F_{V2}, F_{O2}, P and T.

excitation intensity at all excitation intensities used, while F_{P2} is directly proportional only between 0 and approx. 500 μmol $\cdot$ m$^{-2} \cdot$ s^{-1}. It has been previously demonstrated that photooxidative damage to the photosynthetic apparatus by high light intensities can result in a quenching of F_V but not F_O [22,23], and such a phenomenon may explain the non-linear relationship between F_{P2} and photon flux densities above 500 μmol $\cdot$ m$^{-2} \cdot$ s^{-1}.

It is evident from data presented in Figs. 2 and 3 that in order to estimate the redox state of the PS II electron acceptors in the bean leaf from values of F_{V2}/F_{O2}, it is most sensible to generate these parameters with a photon flux density of 500 μmol $\cdot$ m$^{-2} \cdot$ s^{-1}. Fig. 4a shows the changes in F_{V2}/F_{O2} produced by such a photon flux density throughout the 685 nm fluorescence induction

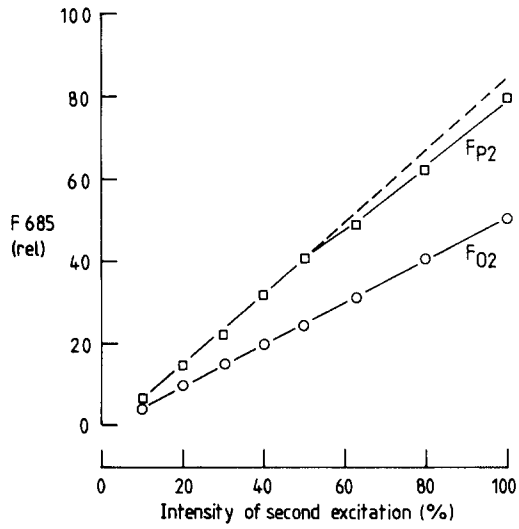

Fig. 3. Changes in the magnitude of F_{O2} and F_{P2} generated from a bean leaf on exposure of the leaf to second irradiation, L2, at the steady-state fluorescence level, T, as a function of the intensity of the second excitation, L2. Refer to Fig. 1 for definitions of F_{O2}, F_{P2}, L2 and T. The steady-state fluorescence at T was generated by a photon flux density of 100 μmol·m^{-2}·s^{-1} of 633 nm irradiation. The maximal (100%) photon flux density of the second irradiation was 830 μmol·m^{-2}·s^{-1}.

curve from a bean leaf excited with 100 μmol·m^{-2}·s^{-1}. The kinetics of 685 nm fluorescence exhibit antiparallel changes to F_{V2}/F_{O2} during quenching of fluorescence from P to T. At any given point on the fluorescence induction curve, the level of fluorescence that would occur if the PS II electron acceptors were maximally reduced, $F_{(Q\,red)}$, can be calculated from the value of F_{V2}/F_{O2} that is produced by the second excitation and the level of fluorescence, $F_{(add)}$, at the point of and prior to addition of the second irradiation, i.e.:

$$F_{(Q\,red)} = F_{(add)} + F_{(add)} \cdot (F_{V2}/F_{O2}) \qquad (1)$$

The level of $F_{(Q\,red)}$ calculated throughout the fluorescence induction curve of the bean leaf exposed to 100 μmol photons·m^{-2}·s^{-1} is shown in Fig. 4b; $F_{(Q\,red)}$ is maximal at P and then decreases to a minimum at T. The reduced level of $F_{(Q\,red)}$ throughout P-to-T fluorescence quenching compared to $F_{(Q\,red)}$ at P can be attributed to quenching of $F_{(Q\,red)}$ by non-photochemical processes. At any point on the induction curve it is possible to

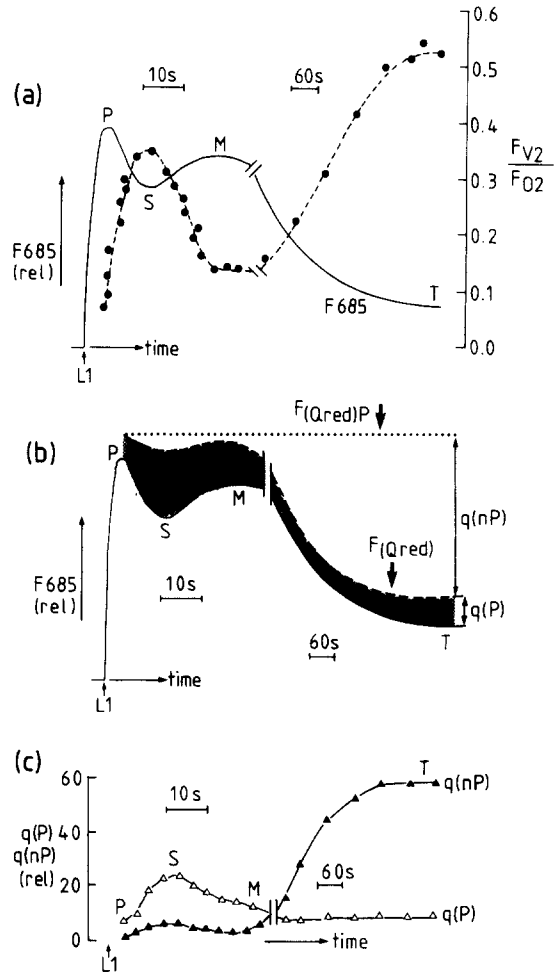

Fig. 4. (a) Changes in F_{V2}/F_{O2} during the fluorescence induction curve from bean leaves. Tissue was initially excited with 100 μmol photons·m^{-2}·s^{-1} of 633 nm irradiation and the fluorescence induction curve produced is shown as a continuous line. At points along the induction curve additional excitations of 500 μmol photons·m^{-2}·s^{-1} of 633 nm irradiation were given. The values of F_{V2}/F_{O2} determined at points on the induction curve are shown as solid circles. (b) Photochemical and non-photochemical components of fluorescence quenching during the slow phase of the induction curve. The solid line represents an induction curve. The level of fluorescence that would occur from maximal closure of PS II traps, $F_{(Q\,red)}$ (dashed line), was obtained by inserting values of F_{V2}/F_{O2} and $F_{(add)}$ into Eqn. 1. Fluorescence quenching from $F_{(Q\,red)}$ observed at P, i.e., $F_{(Q\,red)P}$ (shown by a dotted line), to the level of $F_{(Q\,red)}$ at any point on the induction curve is the result of non-photochemical, q(nP), processes. Fluorescence quenching from the $F_{(Q\,red)}$ level to the observed level of fluorescence is the result of photochemical, q(P), processes (shaded area). (c) Changes in the photochemical quenching, q(P), and non-photochemical quenching, q(nP), throughout the fluorescence induction curve.

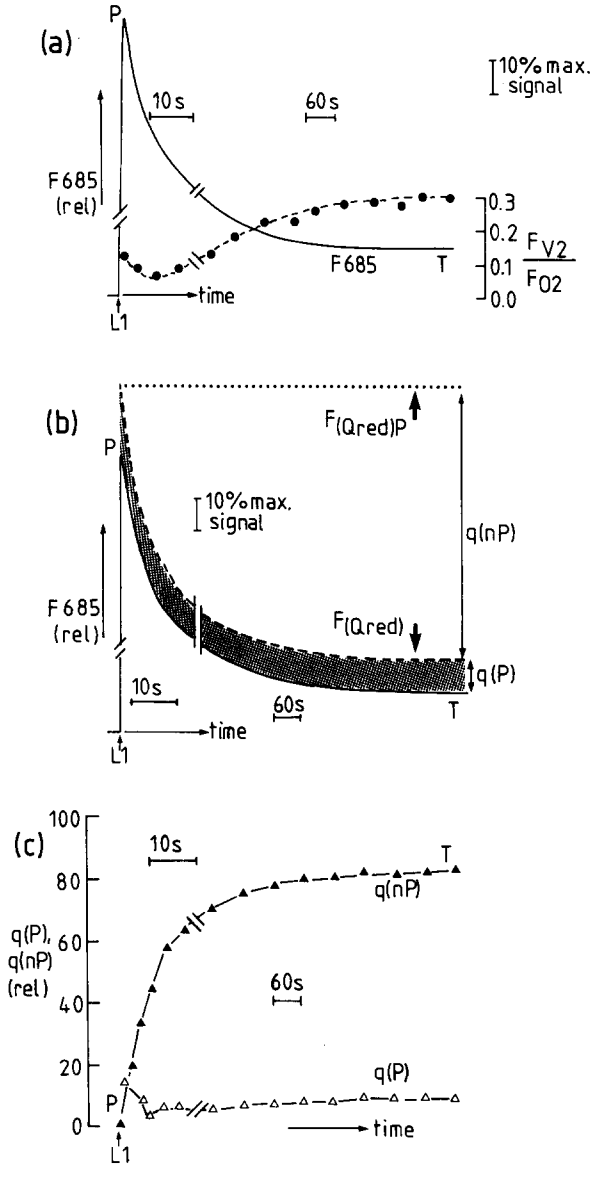

Fig. 5. Fluorescence quenching in bean leaves excited with a photon flux density of 1000 μmol·m^{-2}·s^{-1} of 633 nm irradiation. (a) Fluorescence induction curve (solid line) and changes in F_{V2}/F_{O2} determined by addition of 500 μmol photons·m^{-2}·s^{-1} of 633 nm irradiation (●). (b) Photochemical and non-photochemical components of fluorescence quenching. The solid line represents the induction curve. The level of fluorescence that would occur from maximal closure of PS II traps, $F_{(Q red)}$ (dashed line) was obtained by inserting values of F_{V2}/F_{O2} and $F_{(add)}$ into Eqn. 1. Fluorescence quenching from $F_{(Q red)}$, observed at P, $F_{(Q red)P}$ (shown by a dotted line), to the level of $F_{(Q red)}$ at any point on the induction curve, is the result of non-photochemical, q(nP), processes. Fluorescence quenching from the $F_{(Q red)}$ level to the observed level of fluorescence is the result of photochemical, q(P), processes which are represented by the shaded area. (c) Changes in photochemical quenching, q(P) and non-photochemical quenching, q(nP), throughout the induction curve.

quantify the amount of quenching of fluorescence by photochemical processes from the level of fluorescence which would be attained at P if PS II electron acceptors were maximally reduced, i.e. $F_{(Q red)P}$, by subtracting $F_{(add)}$ from $F_{(Q red)}$. Thus, the contribution made to the total fluorescence quenching, q(Tot), from the $F_{(Q red)P}$ level by photochemical quenching, q(P), throughout the induction curve can be determined (Fig. 4b). The amount of q(Tot) attributable to non-photochemical quenching processes, q(nP), can then be calculated from the expression:

$$q(\text{Tot}) = q(\text{P}) + q(\text{nP}) \quad (2)$$

Calculated values of q(nP) and q(P) throughout fluorescence quenching from P to T are shown in Fig. 4c. Changes in fluorescence yield between P and M can be seen to be mainly attributable to changes in the amount of photochemical quenching; the non-photochemical component of the total fluorescence quenching is relatively small during this period. However, from M to T it can be seen that there is a large increase in q(nP) and little change in q(P), implicating that fluorescence yield decreases from M to T mainly as the result of an increase in non-photochemical quenching. Although it may appear incongruous that q(P) exhibits only a small change from M to T (Fig. 4C) whilst F_{V2}/F_{O2} (Fig. 4a) shows an increase, the increase in F_{V2}/F_{O2} is insufficiently large to compensate for the decrease in $F_{(add)}$ produced by the incrase in q(nP) and thus $F_{(Q red)}$ decreases, almost in parallel with $F_{(add)}$, from M to T with the consequence that q(P) shows little change.

On irradiating leaf tissue with a considerably increased photon flux density of 1000 μmol·m^{-2}·s^{-1}, we have previously shown that the S-M transient is lost and a monotonous quenching from P to T is observed with the PS II electron acceptors being maintained in a more highly reduced state throughout the major portion of the quenching than is the case when the leaf is excited with 100 μmol·m^{-2}·s^{-1} [18]. Examination of F_{V2}/F_{O2}

generated at P and T as a function of the photon flux density of the second exciting beam for leaf tissue exposed initially to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ demonstrated that maximal F_{V2}/F_{O2} values were produced by an additional excitation of 500 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (data not shown, but similar to those shown in Fig. 2) as was the case for leaf tissue initially excited with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Values of F_{V2}/F_{O2} produced by a second excitation of 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ throughout the high-intensity induction curve are shown in Fig. 5a. The considerably reduced values of F_{V2}/F_{O2} observed at steady-state, T, on the curve induced by the high light intensity compared to those determined at T for the leaf excited with a 10-fold lower photon flux density (see Fig. 4) demonstrate that the increased light intensity maintains the PS II electron acceptors in a more reduced state at T. The changes in the calculated values of $F_{(Q\,red)}$, q(P) and q(nP) during quenching from P to T at the high photon flux density are shown in Fig. 5b and c. It is evident from these data that at high light intensities non-photochemical processes are the major factors responsible for decreasing the fluorescence yield from P to T.

Discussion

This study has shown that changes in the amount of fluorescence quenching produced by both photochemical and non-photochemical processes occur during the fluorescence quenching from P to T of the induction curve from bean leaves. However, at both high- and low-light levels non-photochemical processes were the major factors in reducing the maximal level of fluorescence at P to the steady-state level at T. Under low light the rapid fluorescence quenching from P to S is due to increase in both q(P) and q(nP), with q(P) exhibiting the greatest change (Fig. 4). Similarly, the fluorescence rise from S to M is attributable mainly to a decrease in q(P), only a relatively small change in q(nP) is observed. The large increase in q(P) from P to S reflects a reoxidation of PS II electron acceptors and implies an increase in non-cyclic electron transport. Similarly, the decrease in q(P) from S to M is the result of a rereduction of PS II electron acceptors and implies a decrease in non-cyclic electron flow. Studies of the fluorescence transients from reconstituted chloroplast systems have shown that NADP, ADP, phosphate and ferredoxin levels as well as light intensity can determine the rate of non-cyclic electron transport and thus fluorescence yield [24]. Clearly, the stromal concentration of such metabolites may play an important role in determining rates of electron transport in vivo. If one accepts the hypothesis that the major contributor to non-photochemical quenching is the proton gradient across the thylakoid membrane [6,7,25,26,27], then it can be presumed that only small changes in ΔpH are occurring during the PSM transient. However, during the slow quenching from M to T in bean leaves, little change is observed in q(P) and the majority of the large fluorescence decline can be attributable to a large increase in ΔpH quenching. This conclusion would argue against the hypothesis that the large M-to-T quenching in leaves is associated with Q oxidation as the rate of photosynthetic carbon assimilation increases [24]. However, it should be stressed that the relative contributions of q(P) and q(nP) to fluorescence quenching during the induction curve are likely to be dependent upon the metabolic status of the leaf tissue and thus are likely to be extremely variable unless determined under very well defined conditions.

Interestingly, at P under high light the PS II electron acceptors are not as highly reduced as is the case under low light, as demonstrated by the higher F_{V2}/F_{O2} value at P for the high light treatment (see Figs. 4 and 5). This is presumably due to high light producing a greater increase in the rate of electron flow between plastoquinone and PS I than between H_2O and plastoquinone. After P, unlike the situation in low light, there is little change in q(P) and the majority of fluorescence quenching from P to T is the result of increased quenching by non-photochemical processes (Fig. 5). This would imply, using the hypothesis that q(nP) is caused by ΔpH, that there is a considerable build up of ΔpH during this period. However, it is possible that other non-photochemical processes may be significant at such high light levels. It should be emphasised that although the technique described in this paper may estimate quantitatively the amount of fluorescence quenching attributable to non-photochemical processes, it

cannot determine the nature of these processes. It has been widely speculated, as discussed above, that the major contributor to non-photochemical quenching is ΔpH. However, if this is the case for the normal, physiologically active chloroplast, it may not be true for chloroplasts in leaves exposed to stress conditions such as high light intensity. Besides ΔpH, non-photochemical quenching can be induced by increases in reduced phaeophytin [9,10] and increases in the distribution of excitation energy to PS I relative to PS II [11,28,29]. These factors may only play a minimal role in quenching during fluorescence induction in the mature, healthy leaf; however, there has not yet been any quantitative analysis of their actual contributions.

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