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Review

Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals

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Introduction

In recent years, chlorophyll *a* fluorescence measurements have been increasingly applied to various fields of plant physiology. Chlorophyll can be regarded as an intrinsic fluorescent probe of the photosynthetic system. In the leaf or algal cell, the yield of fluorescence is influenced in a very complex manner by events that are – directly or indirectly – related to photosynthesis. The foregoing article [1] introduces into basic phenomena of fluorescence induction and describes measuring techniques applicable for plant physiological research. The present communication provides a brief review of interpretation of fluorescence signals. Predominantly, fluorescence emission by isolated thylakoids and intact chloroplasts will be considered since our present understanding of chlorophyll fluorescence phenomena is mostly based on studies with these systems in vitro. As will be discussed, the basic interpretations can – with care – be applied to the more complex situation in intact leaves or algae, provided the experimental conditions are clearly defined. To point out the significance of fluorescence interpretation for plant physiology, some examples of effects of the environment, including stress condition, on the different fluorescence parameters are given. A more comprehensive article dealing with stress effects on chlorophyll fluorescence will follow in this series. It should be noted that the present paper does not include fluorescence phenomena in the microsecond and picosecond time scale, which are closely related to primary photophysical and photochemical events.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F, yield of fluorescence emission; F_o, constant (initial) fluorescence; F_v, variable fluorescence; F_m, maximum fluorescence; LHC, light harvesting chlorophyll a/b-protein complex; PQ, plastoquinone; Q_A, primary 'stable' electron acceptor of photosystem II; Q_e, energy (ΔpH)-dependent fluorescence quenching; Q_q, Q_A-dependent fluorescence quenching.

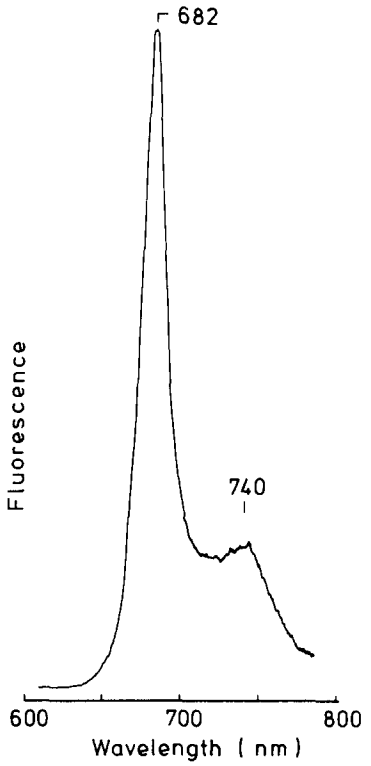

Figure 1. Fluorescence emission spectrum at room temperature of isolated intact chloroplasts in the presence of DCMU ($2 \cdot 10^{-5}$ M). The spectrum was recorded with a band-pass of 1 nm and corrected for wavelength sensitivity of the multiplier. Fluorescence was excited with 480 nm light, band-pass 20 nm.

The physical significance of fluorescence signal

Figure 1 shows the room temperature fluorescence emission spectrum of intact chloroplasts. It exhibits a peak at 682 nm and a broad shoulder at about 740 nm. There is general agreement that at room temperature, contrary to the situation at low temperatures, chlorophyll *a* fluorescence is largely emitted by PS II and by the attached LHC [2]. However, PS I emission may contribute to the long-wavelength band [3].

Chlorophyll molecules in the first excited singlet state have several ways to return to the ground state, namely by fluorescence emission, excitation transfer to neighboring pigment molecules, photochemical reaction, and non-radiative (thermal) de-excitation [for a review see ref. 4]. The experimental fluorescence signal, F, of a sample is therefore determined by the rate of constants of these competing reactions and by the fraction of open reaction centers, A, as only those can contribute to the photochemical de-excitation. Thus F can be given by the equation

$$F = J \cdot \underbrace{\frac{k_F \cdot A}{k_F + k_D + k_T + k_P}}_{\text{open units}} + J \cdot \underbrace{\frac{k_F \cdot (1-A)}{k_F + k_D + k_T}}_{\text{closed units}} \quad (1)$$

where k_F, k_D, k_P and k_T are rate constants for radiative, non-radiative and photochemical de-excitation and for energy transfer, respectively [see ref. 5]. J stands for the absorbed light flux. The fluorescence yield ψ_F is

$$\psi_F = \frac{F}{J} \quad (2)$$

Fluorescence induction at room temperature (Kautsky effect)

1. *Characteristic phenomena*

The term 'induction' is not used uniformly in the literature. It appears practical to include all variations of chlorophyll fluorescence occurring upon dark-light transition until a final stationary emission is reached [see ref. 6]. However, one should be aware that 'induction' often denotes the fluorescence rise to the maximum level only.

The fluorescence induction exhibits characteristic phases with fluorescence levels usually termed O, I, D, P, S, M, T [7, see also ref. 1, Fig. 1]. The level O (F_o) denotes the 'constant' fluorescence, seen when after a dark period all reaction centers of PS II are open. The fluorescence level above the F_o level is termed 'variable' fluorescence, F_v. From F_o the induction curve rises rapidly to the 'peak', P (Fig. 2). This rise shows two phases. The maximum of the first phase has been termed I ('inflection'), followed by a 'dip', D, of fluorescence emission. From P the fluorescence yield declines, usually with slower kinetics, to a stationary level, S. This may be followed by another maximum, M, and 'terminal' level, T. This general course of induction can however vary substantially, depending on experimental conditions and the subject under study. Certain phases can be missing; several successive SM transients may be observed forming a dampening oscillation of fluorescence emission [see ref. 8].

2. *Constant fluorescence*

The F_o level of fluorescence is thought to represent emission by excited antenna chlorophyll *a* molecules occurring before the excitons have migrated to the reaction centers [4]. Thus the quantum yield of F_o is independent of photochemical events. F_o was termed 'constant' (or 'minimum', 'initial') fluorescence. Emission from PS I antennae may contribute to the F_o level of long-wavelength fluorescence. In the induction signal, the true F_o level is only seen when prior to illumination the first stable acceptor of PS II, Q_A, is fully oxidized. This can be achieved by dark incubation for several minutes

or more efficiently by far-red (700–720 nm) preillumination. In case of isolated chloroplast, addition of a suitable oxidant may be appropriate.

In principle, the yield of F_o emitted from PS II antennae depends firstly on the initial density of excitons within the PS II pigments, which, in turn, is determined by the initial distribution of absorbed energy between PS I and PS II. Secondly, F_o depends on structural conditions that affect the probabilty of excitation energy transfer between antenna pigments and from those to reaction centers of PS II. The distribution of excited states between PS I and PS II is mainly related to the lateral distribution of the three pigment systems, PS I, PS II, and LHC within the thylakoid membrane and can be modulated either by enzymatic phosphorylation-dephosphorylation of the LHC [9, 10, 11, 12] or by variations in the cation concentration around the membrane [13]. By phosphorylation of the LHC both F_o and F_v are lowered with little change in the ratio F_o/F_v [14]. This is most likely due to an increase in the fraction of the absorbed energy initially delivered from LHC to PS I, at the expense of exciton density in the PS II antennae. PS II is thought to be situated almost exclusively in the appressed regions of the grana [15]. It has been discussed that the phosphorylated LHC leaves the appressed regions and becomes more closely associated with PS I, so that the fraction of energy delivered to PS II centers is diminised [10, 14]. When thylakoids are depleted of Mg^{2+} ions by suspending them in a medium of low ionic strength (e.g., 10 to 20 nm monovalent metal cations, absence of divalent cations) F_o is only slightly lowered, whereas F_v becomes drastically diminished in comparison to thylakoid suspensions containing about 5 mM Mg^{2+} [14]

The F_o level is known to be affected by environmental stress that causes structural alterations at the PS II pigment level. Thermal damage of PS II is characterized by a drastic increase in F_o [16, 17, 18]. Photoinhibition of broken chloroplasts [19] may lead to a slight increase in F_o, whereas freezing damage of thylakoids was without detectable effect on the F_o level [20].

3. *Variable fluorescence, F_v; rise of fluorescence from F_o*

Fluorescence induction recorded with a spinach leaf at different intensities of exciting light is depicted in Figure 2. The fluorescence rise from F_o is usually considered to reflect reduction of the electron acceptor Q_A. According to the 'classical' hypothesis of Duysens and Sweers [21], oxidized Q_A acta as a fluorescence quencher, because in the state $P^*_{680}Q_A$ photochemical energy conversion (formation of $P^+_{680}Q^-_A$) is the favored pathway of de-excitation. Once Q_A is reduced, the probability of fluorescence emission from the state $P^*_{680}Q^-_A$ is increased. As suggested by Butler and Kitajima [2]], there is no direct fluorescence emission $P^*_{680}Q^-_A$. Instead a rapid back transfer of excitons from these closed reaction centers to the bulk pigments is assumed to cause an increase in fluorescence emission from chlorophyll *a* antenna molecules.

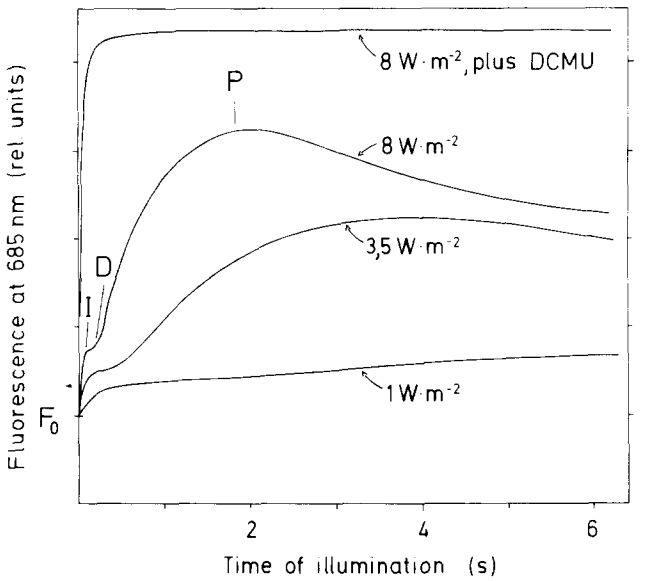

Figure 2. Fluorescence induction curves of spinach leaves, recorded at different light intensities after pre-darkening for 30 min. Fluorescence was excited with a broad band of blue-green light and recorded at 685 nm at room temperature. The signals are normalized at the F_0 level. One leaf was treated with a solution of $5 \cdot 10^{-5}$ M DCMU.

These hypotheses explained the high fluorescence rise by assuming that in the state of $P_{680}Q_A^-$ no photochemical energy conversion can occur. With the existence of an intermediate, most likely phenophytin *a* [23], which acts as an electron mediator between P_{680} and Q_A, this interpretation needs modification, because the 'true' primary photochemical event is the reaction

$$P_{680}^* \text{PheQ}_A \rightarrow P_{680}^+ \text{Phe}^- Q_A .$$

Klimov and Krasnovskii [24] proposed that in the $P_{680}\text{PheQ}_A^-$ state (high-fluorescent state) excitation of P_{680} and related primary charge separation (giving $P_{680}^+\text{Phe}^-Q_A^-$) is followed by fast back reaction of the radical cation and radical-anion (charge recombination), whereby an excitation becomes re-introduced into the PS II antennae. Thus the variable fluorescence is thought to be a 'recombination luminescence':

$$P_{680}^+ \text{Phe}^- Q_A^- \rightarrow P_{680}^* \text{PheQ}_A^- .$$

When reoxidation of Q_A via the electron transport chain is blocked by the herbicide DCMU, the maximum fluorescence level, F_m, is reached. Some fluorescence quenching, however, may take place upon addition of DCMU. This is ascribed to action of oxidized PQ [25], in illuminated DCMU-poisoned chloroplasts, the PQ pool should be fully oxidized. According to Melis and Duysens [26], the proportion of reduced Q_A is linearly related to the area increment A_t/A above the fluorescence induction curve (A denotes

the total area, A_t the area at the time t). The rise of variable fluorescence in the presence of DCMU exhibits a sigmoidal course. This is supposed to indicate cooperation of PS II units [27]. The analysis of the induction indicated a fast nonexponential α-phase and a slow exponential β-phase of A_t/A [28]. This has been interpreted in terms of the existence of connected α-units and disconnected β-units [29]. There is no need to assume two types of PS II centers with different rate constant of photochemistry, k_p, as only different degrees of energetic communication between the antenna pigments may explain the heterogeneity of photosynthetic units, as formulated, e.g., in the connected package model [78] or in the grouping model [72]. A different hypothesis was proposed by Joliot and Joliot [30], who suggested that each reaction center has two electron acceptors, Q_1 and Q_2, which permit two successive photoreactions. Redox titrations [31] seems to support the hypothesis of two types of Q_A. However, the question of different PS II acceptors and PS II heterogeneity is still under discussion [32].

In the absence of DCMU, electrons can be transmitted via Q_B to the PQ pool; Q_A is supposed to stay in a quasi-equilibrium with PQ. Due to the electron transport, the fluorescence rise is much slower than in the presence of DCMU. The biphasic kinetics of the fluorescence rise has been explained by variations in the rates of Q_A reduction and of reoxidation via the electron transport chain [see ref. 6]. In the OI phase, the rate of reduction is supposed to exceed reoxidation. This phase apparently is related to onset of O_2 evolution [see ref. 6]. A subsequent increase in reoxidation *via* PS I delays a further increase of the reduced fraction of Q_A, or even decreases this fraction, thus causing a fluorescence decline (ID phase) [see ref. 6; 33]. The large increase in variable fluorescence in DP phase was found to be correlated to a decline in O_2 evolution and apparently is caused by a transient block of electron transport through PS I [34]. Such block would pile up electrons in the electron transport chain between PS II and I, i.e., leads to reduction of a larger fraction of Q and the PQ pool. The relative height of P depends on the exciting light intensity (Fig. 2). In strong illumination, P may come close to the F_m level, and the ratio F_v/F_o may reach values of 4 to 5. At very low light intensities, the fluorescence emission remains at the F_o level. The transient block of PS I activity that causes the rise to P probably is due to lack of efficiency electron acceptors of PSI, when the $NADP^+$ pool is exhausted. CO_2 as the final substrate of photosynthesis mediates efficient reoxidation of NADPH only after a lag phase during which the carbon reduction cycle is activated. Reduction of O_2, instead of $NADP^+$, may occur (Mehler reaction), but its rate might be insufficient for fast reoxidation of Q and PQ. In fact, when algae or isolated chloroplasts are supplied with an efficient mediator of O_2 reduction such as methylviologen, the fluorescence peak is lowered drastically, i.e., strong transient reduction of electron carriers is avoided [34, 35]. In intact leaves, the height of P

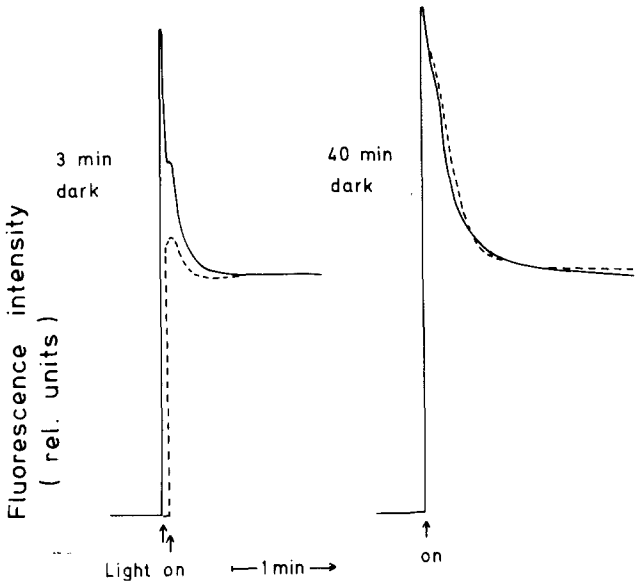

Figure 3. Fluorescence signals of spinach leaves recorded at room temperature in CO_2-free air (———) and air with 300 $\mu l.l^{-1}$ CO_2 (– – – –). A broad band of red light, half-band width 630–680 nm, intensity 50 $w \cdot m^{-2}$, was used for excitation. Fluorescence was recorded in the long-wavelength band at 740 nm.

depends on pre-conditions [36]. At air levels of CO_2, a high fluorescence peak is observed subsequent to long periods of dark incubation (Fig. 3). After a brief dark period (3 min), the peak is low, probably because onset of CO_2 fixation is faster, allowing for fast rates of reoxidation of Q and PQ; accordingly, the peak is high after 3 min darkening, when CO_2 fixation is restricted due to absence of CO_2 in the gas phase. Finally, it should be mentioned that non-photochemical events also seem to influence the kinetics of the fluorescence rise [37, 38].

Environmental stress that causes thylakoid damage usually lowers the variable fluorescence yield, F_v. For instance, this has been described for heat [16, 17, 18, 39] and freezing stress [20] and for photoinhibition [19, 40]. The effects on F_v can be observed in the presence and absence of DCMU, but in the latter case, the causes of F_v lowering may be complex. The lowering of F_v (observed in the presence of DCMU) that is related to photoinhibition has been interpreted to result from transformation of reaction centers to quenchers by destructive action of light [41].

4. *Light-induced fluorescence quenching*

Although the term 'quenching' has an exact physical meaning, it is mostly used in the literature for any kind of decrease in chlorophyll fluorescence emission measured at constant light intensity. Fluorescence quenching is

generally defined as $(F_m - F_t)/F_t$, where F_m is the maximum fluorescence level observed in the high-fluorescent state and F_t is the level at the time t. If known, the yields of variable, instead of total fluorescence can be used in this term. In the fluorescence induction signal, 'quenching' usually denotes the ratio (P–S)/S; one should be aware, however, that frequently P does not come close to F_m.

In intact chloroplasts or in freshly broken chloroplasts supplied with $MgCl_2$ (5 mM), prolonged illumination induces strong fluorescence quenching from P to the stationary level S. A minimum of exciting light intensity of about 5–10 $W \cdot m^{-1}$ is required to obtain the full (or nearly full) extent of quenching. Usually, considerably more than 50% of the maximum variable fluorescence is quenched. Similar quenching may be observed with intact leaves and algae. Intact cells often exhibit faster kinetics of quenching than isolated chloroplasts. In algae, the S level is a transient steady state; the following SMT phase may require several minutes. With leaves, one or several secondary fluorescence maxima may be seen [see ref. 1]; under appropriate conditions, prolonged illumination may induce dampening oscillations of fluorescence emission [8].

Only part of the fluorescence decline from P to S (or T) can be explained by enhanced reoxidation of the quencher Q_A due to increased PS I activity. The complexity of quenching was already recognized by Duysens and Sweers [21]. It is now clear that there are several possible mechanisms of non-photochemical quenching, i.e., of fluorescence decline that is not caused by Q_A reoxidation:

a) *ΔpH-dependent ('energy-dependent') quenching.* Fluorescence quenching by the build-up of the 'high-energy state' of the thylakoids has first been observed by Murata and Sugahara [42] with broken chloroplasts illuminated in the presence of DCMU and phenazine methosulfate. Later, energy-dependent quenching in the absence of these reagents was described for intact chloroplasts, broken chloroplasts supplied with Mg^{2+} and intact leaves [36, 43]. This quenching was found to be closely related to the light-induced proton gradient. A linear relationship between intrathylakoid proton concentration and fluorescence quenching was observed [44, 45; see also 46]. Consequently, energy-dependent quenching is reversed by darkening or in the light by addition of uncouplers or inhibitors of electron transport [36, 43, 47]. According to present interpretation [48, 49] the ΔpH-dependent quenching is caused by increased thermal de-excitation (increase in k_D, see equation 1). It is assumed that due to proton pumping in the light, cation exchange processes (H^+ *versus* Mg^{2+} or other cations) occur at the inner thylakoid surface. This cation exchange supposedly causes ultrastructural alterations of the thylakoid membranes which are responsible for the fluorescence decline.

b) *ATP-dependent quenching.* As mentioned above, phosphorylation of the light harvesting complex causes fluorescence quenching [10, 11]. This can be observed when uncoupled chloroplasts (in which ΔpH-dependent quenching is absent) are illuminated in the presence of ATP. The kinetics of this effect is slower (half time about 3 min) and the extent much smaller than that of ΔpH-dependent quenching [10, 11, 50]. Presumably, the phosphorylation of the LHC is regulated by the redox state of the PQ pool [11, 12]; reduced PQ seems to stimulate a protein kinase activity and thus may induce a transition towards state 2. In pea chloroplasts, reversal of the fluorescence quenching is achieved by (apparently non-regulated) phosphatase activity; the reversion can be suppressed by NaF, an inhibitor of phosphatase. Thus, combined action of the kinase and phosphatase is supposed to optimize distribution of incident light energy. For unknown reasons, no reproducible reversion of ATP-dependent quenching could be observed in spinach chloroplasts.

c) *Quenching by Mg^{2+} depletion of thylakoids.* Experimentally, strong lowering of variable fluorescence may be achieved by Mg^{2+} depletion of chloroplasts. Action of cations on the outer thylakoid surface seems to be responsible for this effect [see ref. 44, 51]. Mg^{2+} depletion is thought to cause a large increase in the probability of 'spillover' of excitation energy from PS II to PS I (increase in k_T, see equation 1) leading to the observed decline in F_v, whereas the initial exciton density in the antennae of PS II (and the related yield in F_o) is less affected. Formerly, Mg^{2+} effects have been postulated to play a role in the physiological regulation of energy transfer between the two photosystems [52]. However, in the dark, the Mg^{2+} level in chloroplasts appears to be close to saturation of the cation effect [53]. Since upon illumination, the concentration of free Mg^{2+} in the chloroplast stroma rises [54, 55], a contribution of the Mg^{2+} effect to light-induced fluorescence quenching is implausible. Enzymic phosphorylation of the LHC rather than ionic effects may be involved in the light-dependent regulation of energy distribution in vivo [12, 36]. It should be pointed out that a valid description of the distribution of excited states between different pigment structures require concurrent analysis of low-temperature fluorescence, as will be discussed below.

In suspensions of broken chloroplasts, a high Mg^{2+} level (about 5 mM) is the pre-condition for a high maximum yield of F_v and thus for strong quenching. From isolated chloroplasts with intact envelopes, Mg^{2+} does not leak out, so that addition of Mg^{2+} to the medium is not required.

d) *Quenching related to photoinhibition.* Depending on light intensity and other experimental conditions, during prolonged illumination destructive effects of light may cause a slow fluorescence quenching. For intact chloroplasts an irreversible component of quenching has been described [56] that may represent such a photoinhibitory effect. As mentioned above,

photoinhibition is generally characterized by lowered F_v levels. It should be noted that certain uncouplers, e.g., carbonyl cyanide-4-trisfluoromethoxyphenyl hydrazone (FCCP) and desaspidin, may exert inhibitory side effects; if added in high doses to chloroplast suspensions, these reagents cause strong light-dependent irreversible fluorescence quenching correlated to inhibition of elelctron transport [47].

e) *Fluorescence quenching related to high temperatures.* A reversible decrease in F_m observed after exposure of leaves to high but not injurious temperatures has been interpreted in terms of a temperature-induced change in excitation energy distribution in favor of PS I [57, 58]. Heat-induced damage of the water splitting apparatus causes a decrease in the variable fluorescence due to limitation of electron donation to PS II centers i.e. increases the steady-state fraction of open reaction centers, A [16, 39]. Such quenching can be reversed by addition of an artificial electron donor, such as hydroxylamine [39]. More severe heat treatment results in an increase in F_o and a decrease in F_m which is accompanied by an inhibition of PS II activity and cannot be reversed by addition of hydroxylamine [18, 39]. The mechanism of this heat-induced quenching is not yet fully understood [18]. As a possible explanation, disconnection between chlorophyll molecules within the antennae and/or between antennae and reaction centers affects the overall fluorescence lifetime and the related F_m level. The probability of an exciton to reach a functioning reaction center is lowered (decrease in k_p, see equation 1), and thereby F_o is increased [18].

f) *Quenching states under physiological conditions.* In intact systems such as plant leaves, algal cells, isolated protoplasts, and isolated intact chloroplasts, the different types of fluorescence quenching may occur simultaneously, depending on experimental conditions. In the induction signal measured with intact chloroplasts or *Chlorella* cells, two major components seem to be involved in the fluorescence decline, namely Q-dependent and energy-dependent quenching, termed Q_q and Q_e, respectively. This has been deduced from the biphasic reversion of quenching seen when DCMU is added during illumination [48]. The fast phase of reversion, ($t_{1/2} \approx 1$ s) is attributed to reversal of Q_q, the slow phase ($t_{1/2} \approx 15$ s in intact chloroplasts and 5 s in cells of *Chlorella pyrenoidosa*) to reversal of Q_e. This approach has recently been confirmed by Horton [59]. Like the height of P, the fluorescence decline and transient secondary maxima M are strongly influenced by the physiological state of the chloroplast and the whole cell [36, 43, 48, 59]. Accordingly, the proportion of the two major components may vary greatly. Predominant Q-dependent quenching is expected under conditions of fast reoxidation of NADPH and utilization of ATP in Calvin cycle activities. Restricted electron transport (due to $NADP^+$ exhaustion) diminishes Q_q, and low rates of ATP turnover (low Calvin cycle activity) will promote energy-dependent quenching. A transient increase in fluorescence in the SM phase

might be caused by a decrease in NADPH reoxidation (causing partial reversal of Q_q), while utilization of ATP by phosphorylation of ribulose-5-phosphate continues (causing partial release of Q_e). Walker et al. [8] have tentatively interpreted the SMT phase in such terms and regard it as the simplest case of the dampening oscillations of fluorescence emission that are observable in intact leaves. These oscillations were found to be related to anti-parallel oscillations of O_2 evolution [8] and CO_2 uptake [60] and have been attributed to overreactions of the regulatory system of carbon metabolism. The reason, why the oscillations of fluorescence and O_2 evolution are slightly out of phase (the fluorescence increase anticipating the decline in O_2 evolution) is not clear at present. Strasser explained oscillations of fluorescence occurring upon sudden changes in environmental conditions in terms of non-equilibrium thermodynamics [79].

It appears difficult to detect a contribution of ATP-dependent quenching in the normal induction signal of coupled chloroplasts. Usually, the change in energy distribution caused by phosphorylation of LHC lowers the quantum yield of fluorescence by only about 10–30% and thus probably contributes only a minor component to the PS decline. However, changes in excitation energy distribution may be monitored by means of the different responses of fluorescence emission, seen when the actinic beam is switched from light 2 (exciting predominantly PS II) to light 1 or vice versa. In most cases, this requires an experimental setup with modulated measuring beam [see ref. 1]. Fluorescence phenomena related to this 'state 1–state 2 transition' have first been observed in *Chlorella pyrenoidosa* [61] and later have been studied with red algae [62, 63] and intact leaves [64].

Changes in energy distribution should influence the rate of electron transport and thereby affect Q_q and Q_e, as portrayed in the scheme of Figure 4. Phosphorylation of LHC that causes weak ATP-dependent quenching, enhances photoreaction I relative to photoreaction II. This would increase Q_q, and due to higher energization of the thylakoid system might also strengthen Q_e. The state transition must be viewed as a mechanism of fine control that is integrated into a network of photosynthetic regulation that maintains a balance between Calvin cycle activity, stromal energy charge and photosynthetic electron transport. The fluorescence level may reflect this complex regulation, but careful studies are needed to distinguish between different types of quenching. Light-induced light scattering changes measured simultaneously with fluorescence have been found to be useful to monitor relative changes in thylakoid energization in situ [65] and therefore help to identify energy-dependent fluorescence changes.

Fluorescence emission spectra and induction at low temperature (77K)

At low temperatures, characteristic emission by chlorophyll-protein complexes such as PS I, PS II and the LHC can be distinguished. Measurements were done at temperatures as low as 4K [66], but in most studies

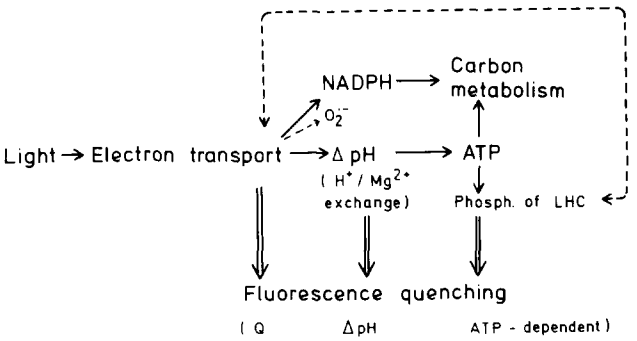

Figure 4. Scheme demonstrating the interrelation between different ways of fluorescence quenching and light-driven processes in intact photosynthetic systems.

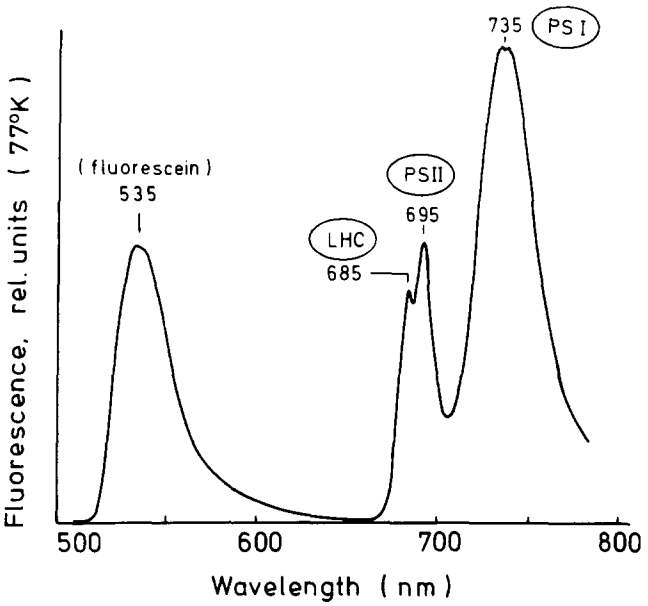

Figure 5. Flourescence emission spectra at 77 K of intact isolated spinach chloroplasts in the presence of 2 μM fluorescein (sodium salt) as internal standard. After 4 min dark incubation at room temperature, the sample was frozen with liquid nitrogen. The fluorescence spectrum was recorded as for Figure 1.

liquid nitrogen (77 K) was used for cooling. Emission spectra [see ref. 67 for a review), excitation spectra [for refs. see 66] and fluorescence induction at different wavelengths were investigated. Figure 5 depicts a fluorescence emission spectrum of intact chloroplasts recorded in the presence of fluorescein as internal standard. The bands near 685, 695 and 735 nm have been attributed to emission predominantly by the LHC and antenna pigments of PS II and PS I, respectively [68, 69]. There is, however, some overlap of emission by these complexes.

Emission spectra are influenced by reabsorption of fluorescence which is strongest in the short-wavelength bands. The extent of reabsorption depends on the chlorophyll concentration, thickness of sample, light scattering properties, geometrical and other factors. In chloroplast suspensions (curvette diameter 1.8 mm) minimal reabsorption was seen at chlorophyll concentrations of 5–10 μg/ml (own observation). In leaves, extremely high reabsorption strongly lowers the emission recorded at the short wavelengths, as compared to the 735 nm band. A method to minimize the reabsorption effect by preparing a 'diluted' suspension of cell fragments from frozen leaf tissue was developed by Weis [57]. The absolute amplitude of bands depends on the geometry of the frozen samples, and this may vary considerably; therefore, comparison of emission between different chloroplast samples is facilitated by addition of an internal fluorescent standard, such as fluorescein (see Figure 5). Spectra then may be normalized at the fluorescence of the standard [49, 50].

The fluorescence induction curve measured at 77 K exhibits an F_o level from which the emission rises to F_m. At 690 nm, the F_o level is attributed to emission by antenna chlorophyll *a* of LHC and PS II that occurs before excitation energy is trapped by open reaction centers [22]. The rise to F_m is caused by reduction of Q_A, i.e., by return of excited states to the pigment bed when the PS II reaction centers are closed. Like at room temperature, the F_v/F_m ratio is about 0.8 in intact dark-adapted chloroplasts [50]. At 77 K no appreciable Q_A reoxidation is possible. It was established by Butler and co-workers that the donor of PS II at 77 K is high-potential cytochrome b-559. As P^+_{680} acts as a quencher, the rate of donation of electrons to P^+_{680}, i.e., the rate of cytochrome b-559 oxidation, is supposed to influence the fluorescence rise [for a review see ref. 70].

In the PS I band (735 nm), the variable part of fluorescence is smaller (F_v/F_m about 0.3 in intact dark-adapted chloroplasts, see ref. 50). It has been proposed [69] that at 77 K a long-wavelength form of chlorophyll, C-705, acts as an energy trap of PS I antenna pigments and gives rise to the 735 nm emission. These pigments act as an intrinsic probe indicating the overall excitation density within the PS I antennae whereas the PS I reaction centers have no effect on fluorescence. The F_o level of PS I fluorescence reflects the energy coming from direct absorption of PS I pigments and from the fraction of energy *initially* delivered from LHC and PS II antennae to PS I. Variable fluorescence at 735 nm is attributed to excitons that have migrated from closed PS II centers *via* PS II antennae to PS I (spillover). These relationships were formulated by Butler and Kitajima [22] and Butler and Strasser [71] in their 'tripartite' model which includes rate constants for energy migration from LHC to PS II and PS I, and from PS II to PS I antennae. This was modified later to the 'grouping model' by Strasser [72].

Low-temperature fluorescence measurements were applied to study changes in excitation energy distribution between PS II and PS I [e.g., refs.

50, 73, 74, see also ref. 70], to characterize phenomena of fluorescence quenching [e.g., refs. 44, 48, 49, 75], and to analyze alterations of the photosynthetic pigment system due to environmental factors [e.g., refs. 18, 58, 76, 77]. In isolated chloroplasts, quenching at room temperature induced by Mg^{2+} depletion, by the 'high-energy state' or by phosphorylation of the LHC results in different characteristics of 77 K fluorescence [49, 50]. Mg^{2+} depletion leads to a strong increase in F_{735} and decrease in F_{695}, giving a large increase in the F_{735}/F_{695} ratio in accordance with a redistribution of excitation energy in favor of PS I. By Mg^{2+} depletion, F_{v690} is strongly, F_{o690} only slightly decreased [22, 49]. ΔpH-dependent quenching is related to a much smaller, but significant increase in the ratio F_{735}/F_{695} at 77 K. However, in constrast to the effect of Mg^{2+} depletion, emission in both bands is strongly decreased. From these and other data it was concluded that the ΔpH effect is based on increased rates of thermal de-excitation rather then on changes in energy distribution. An apparently similar change in low-temperature spectra, termed 'state 2–state 3' transition was observed in the red algae *Porphyra perforata* [75]. Lowered emission bands in both regions of the spectrum at 77 K concurrent with a slight increase in the F_{735}/F_{695} ration were also seen in photoinhibited leaves and samples of chloroplast [77, 19].

ATP-dependent quenching at room temperature is related to an increase in the band at 735 nm and a decrease in the 695 nm band at 77 K, as expected for changed energy distribution. In constrast to the Mg^{2+} effect, at 690 nm F_o was lowered to a similar extent as F_v (the ratio F_v/F_m remained about constant), as reported in ref. 50. Different findings of Haworth et al. [73] were discussed by Horton and Black [14] who investigated interferences between Mg^{2+} and ATP effects. The low-temperature fluorescence analysis [50] supports the conclusion that phosphorylation of the LHC causes mainly an increase in initial energy distribution to PS I (change in the absorption properties, J), whereas Mg^{2+} predominantly affects the energy transfer ('spillover') from PS II to PS I (change in the rate constant for energy transfer, K_T; see equation 1).

Concluding remarks

The present survey demonstrates the manifold information on photosynthetic processes that can be obtained from chlorophyll fluorescence analysis. This should encourage application in plant physiology. In view of the complexity of fluorescence phenomena, unambiguous interpretation of measured signals is however often difficult, if not impossible, particularly when intact leaves are concerned. Valid information can be obtained when *several* approaches of fluorescence analysis are combined. For instance, conclusions drawn from room temperature fluorescence may be corroborated – or disproved – by fluorescence at low temperatures, where photosynthetic processes other than

primary light reactions are inhibited. Moreover, fluorescence phenomena should be viewed – and often are only interpretable – in context with biochemical parameters, such as pigment composition, photosynthetic CO_2 fixation, pool sizes of carbon cycle intermediates, energy charge, and O_2 evolution. Photometric analysis of electron transport components, such as cytochromes, might aid the interpretation of fluorescence signals. If pitfalls of interpretation are avoided by multiple cross-checking, the fluorescence technique may, indeed, prove a versatile tool in plant physiology.

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