

CHLOROPHYLL FLUORESCENCE AND PHOTOSYNTHESIS: The Basics

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INTRODUCTION

Chlorophyll *a* fluorescence emitted by green plants reflects photosynthetic activities in a complex manner. Recent improvements of fluorescence measuring techniques have made the fluorescence method an important tool in basic and applied plant physiology research. Such methods include the introduction of amplitude modulated fluorescence recording systems and the refinement of lifetime analysis. In particular the use of fluorescence from intact plant leaves has increased as a unique nonintrusive method of monitoring photosynthetic events and judging the physiological state of the plant. The improved techniques have facilitated deeper insight into the mechanism of fluorescence emission and thus have resulted in a more precise interpretation of the emitted signals. However, basic explanations of fluorescence phenomena mostly rely on studies *in vitro* with isolated protoplasts, chloroplasts, thylakoid membranes, and particle preparations. The full interpretation of the complex signals emanating from intact photosynthetic organisms, particularly from leaves of higher plants, is still problematic. Certain measured parameters are of empirical value only.

In the last 15 years a number of reviews on chlorophyll fluorescence have appeared, stressing different aspects of the fluorescence phenomenon and covering most of the relevant literature. An early broad summary of the basic fluorescence characteristics was given by Papageorgiou (124). Fluorescence related to physical and primary photosynthetic events was reviewed by Lavorel & Etienne (94), Butler (22, 23), van Gorkom (159), Moya et al (111), and Holzwarth (67), the two latter dealing predominantly with fluorescence lifetimes. Biochemical and physiological implications of fluorescence are accentuated in the reviews by Krause & Weis (86), Briantais et al (20), Murata & Satoh (114), Renger & Schreiber (130), and Krause & Weis (87). The present article surveys current knowledge about the relationships between fluorescence emission and the physiology of photosynthesis, based on available biophysical and biochemical information. Our review is concerned only with oxygenic organisms, predominantly with higher plants; specific features of fluorescence in lower photosynthetic organisms are not considered. Also the rapidly growing application of fluorescence in detection and analysis of stress effects on plants (see 98, 147a), being highly important to ecophysiology, cannot be covered here.

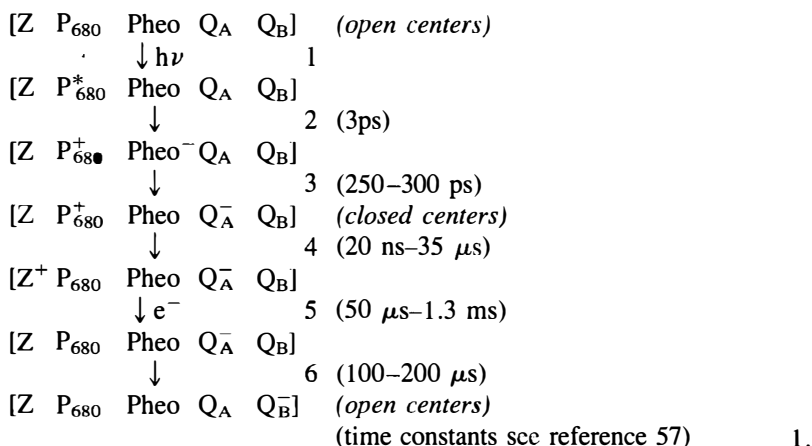
BIOPHYSICAL BASIS OF FLUORESCENCE EMISSION FROM CHLOROPLASTS

Fluorescence as a Reaction Competing in the Deactivation of Excited Chlorophyll

In the photosynthetic apparatus, light is absorbed by the antenna pigments, and the excitation energy is transferred to the reaction centers of the two

photosystems. There the energy drives the primary photochemical reactions that initiate the photosynthetic energy conversion. In low light under optimal conditions, the primary photochemistry occurs with high efficiency. From data of Björkman & Demmig (14) it can be calculated that more than 90% of absorbed light quanta are utilized by photosynthesis. The yield of primary photochemical energy conversion may be even higher. A minor competing process of deactivation of excited pigments is the emission of chlorophyll (Chl)¹*a* fluorescence. At room temperature, most fluorescence is emitted by Chl *a* of photosystem (PS) II. In solution (e.g. in ether) Chl *a* exhibits a high fluorescence yield, ϕF , of about 30%. In contrast, maximum fluorescence yield in the photosynthetic apparatus, ϕF_M (observed when all reaction centers of PS II are "closed"), is only around 3% of the absorbed light (see 8). When all reaction centers are "open," the fluorescence yield, ϕF_o , is about five times lower (approximately 0.6%) owing to the competition with photochemistry.

Before further discussing this competition, we briefly describe the events of photosynthetic electron transport in the PS II reaction center (see 57). In the primary photochemical reaction, one electron is transferred from the pigment P₆₈₀ in the first excited singlet state (P^{*}₆₈₀) to pheophytin *a* (equation 1). From there the electron is transferred to the primary quinone-type acceptor Q_A.



¹**Abbreviations:** Chl: chlorophyll; CP: chlorophyll protein complex; DCMU: 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; LHC II (I): light harvesting chlorophyll *a,b* protein complex of photosystem II (I); PFD: photon flux density; Pheo: pheophytin; PQ: plastoquinone; PS: photosystem; Q_A, Q_B: quinone-type electron acceptors of photosystem II; Z: secondary electron donor of photosystem II

The charge separation creates a highly active oxidant, P_{680}^+ . This receives an electron from the secondary donor Z, which has been identified as a tyrosine residue of the D1 protein. The oxidized donor, Z^+ , is reduced by an electron from the water oxidation system. In a slower reaction, which may involve an iron atom, the electron is transmitted to the quinone Q_B . After receiving two electrons, Q_B binds two protons from the lumen side of the thylakoid membrane and merges into the plastoquinone/plastohydroquinone (PQ) pool.

The rate of fluorescence emission, F , is proportional to the absorbed light flux, I_a , and to the quotient of the rate constant of fluorescence, k_F , over the sum of rate constants, Σk_i , of all competing reactions that result in a return of the Chl molecule to the ground state (equation 2). The most important of those are the photochemical reaction (k_P), thermal deactivation (k_D), and excitation energy transfer to nonfluorescent pigments (k_T)—e.g. to antennae of PS I. From these considerations, the general equation for fluorescence yield is obtained:

$$F = I_a \cdot k_F / \Sigma k_i \quad 2.$$

$$\Phi F = F / I_a = k_F / (k_F + k_D + k_T + k_P) \quad 3.$$

As first proposed by Duysens & Sweers (41) and later elaborated by Butler and coworkers (see 22, 23), fluorescence yield is minimal (ΦF_0) when all reaction centers are in an active, "open" state (see equation 1). The experimentally observed high photon yield of photosynthesis demands that $k_P \gg k_F + k_D + k_T$. However, when Q_A is fully reduced (i.e. all reaction centers are in the state Z P_{680} Pheo Q_A^-), excitation of P_{680} cannot result in stable charge separation ($k_P = 0$). Then the maximum fluorescence yield, ΦF_M , is obtained.

By a similar approach, the potential yield of the photochemical reaction of PS II is obtained as

$$\Phi P_0 = k_P / (k_F + k_D + k_T + k_P) = (\Phi F_M - \Phi F_0) / \Phi F_M = F_V / F_M, \quad 4.$$

where F_M is the maximum total fluorescence and F_V the maximum variable fluorescence emission ($F_V = F_M - F_0$). It should be noted that F_V does not represent an independent fluorescence component; the term only describes the change in fluorescence emission between two defined states. The F_V / F_M ratio has become an important and easily measurable parameter of the physiological state of the photosynthetic apparatus in intact plant leaves. The F_V / F_M ratio was found to stay in a remarkably narrow range (0.832 ± 0.004) among leaves of many different species and ecotypes (14). Under comparable experimental conditions, 0.106 ± 0.001 O_2 are evolved per quantum absorbed

by the leaf (14). For equal distribution of excitation energy between PS II and I, this denotes transport in PS II of $0.85 e^-$ per quantum. Environmental stresses that affect PS II efficiency lead to a characteristic decrease in F_V/F_M .

From equations 3 and 4 an inverse relationship between rate of fluorescence emission and rate of photochemical reaction results. In the fluorescence induction curve, the rise of emission from F_0 to F_M reflects the reduction of Q_A (41). The complex kinetics of the fluorescence rise is discussed below. We also deal with the fact that fluorescence emission is influenced by various nonphotochemical quenching mechanisms.

By introducing rate constants of exciton transfer between antenna Chl and reaction centers and between PS II and PS I units, the bi- and tripartite models were developed (see 22, 23) and later extended by Strasser (152) to the general description of a polypartite model comprising all photosynthetic pigment systems. As discussed below, these models, in which a limitation of primary photochemistry by exciton migration is assumed, have been challenged recently on the basis of fluorescence lifetime analyses.

Lifetimes of Fluorescence

Analyses of fluorescence lifetimes have become increasingly important in the study of the organization and function of the photosynthetic apparatus. In particular, lifetime determinations appear useful for elucidating excitation energy transfer and the kinetics of primary photochemical processes. Application of lifetime measurements to physiological problems has just begun (see e.g. 12, 119) and will certainly increase in the near future. Because fluorescence lifetime analysis has been the topic of several recent reviews (66–68, 111), here we summarize the basic points only.

In principle, the lifetime of fluorescence is analyzed by measuring the fluorescence decline after brief exciting flashes of light, thus following the decline in exciton density of Chl. The decay can now be resolved to the time scale of a few picoseconds. It can be described by a sum of exponential (first-order) decay reactions:

$$F(t) = A_i \cdot e^{-t/\tau_i}, \tag{5}$$

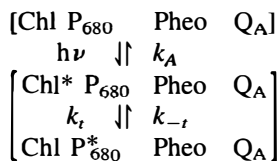
where F is the fluorescence intensity at the time t , A_i the amplitude (pre-exponential factor) and τ_i the lifetime. A_i depends on the wavelength of excitation and emission and is approximately proportional to the number of antenna molecules. The yield of each decay component of fluorescence is given by $\Phi F_i = A_i \cdot \tau_i$, and the total fluorescence yield by

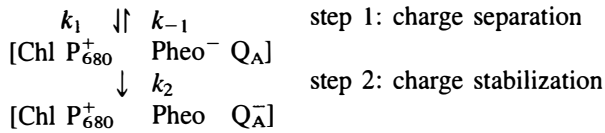
$$\Phi F = \sum \Phi F_i = \sum A_i \cdot \tau_i = \bar{\tau} \cdot \sum A_i, \tag{6}$$

where $\bar{\tau}$ is the average lifetime ($\bar{\tau} = \sum A_i \cdot \tau_i / \sum A_i$).

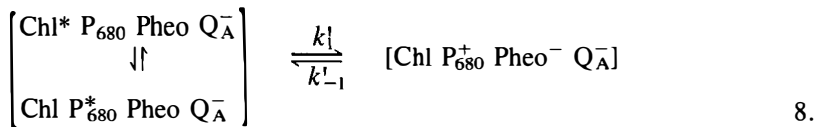
Usually, the decay kinetics can be resolved into three or more exponential components (cf 73). It appears that the number of components that can be distinguished increases with refinement of methods and increased signal-to-noise ratios (see 68). This fact points to the extremely complex nature of the fluorescence decay. Improvements have been achieved by "global" analysis—i.e. measurements of the decay at a set of different wavelengths (64, 66, 73). When the yields ΦF_i or amplitudes A_i of the individual components (characterized by a certain τ_i) are plotted versus the wavelength of emission or excitation, "decay-associated spectra" are obtained. Such methods provide considerably more information than single-decay analysis. The evaluation of data was further refined by so-called "target analysis" (96), in which the data are fitted to sets of rate constants of kinetic models describing the primary processes.

Recently, an ultrafast decay component ($\tau \approx 15$ ps) was resolved (68, 102) that has been attributed to fast reversible excitation equilibration between antennae and reaction centers. This contradicts the earlier "bipartite model" of Butler and coworkers (see 22, 23), which was based on the assumption that the primary photochemical reaction is limited by the rate of exciton migration from the antennae to the reaction center. In Butler's original model the rate constant of energy transfer to the "trap" was supposed to be much higher than the rate constant of back-transfer from reaction centers to the antennae, picturing the PS II unit as a "funnel." In recent years, evidence has accumulated that for oxygenic photosynthesis the photochemical reaction in both photosystems is "trap limited" rather than limited by exciton migration (135–137). Similar conclusions were drawn for the photosystem of purple bacteria (16). This supported the "equilibrium models" assuming quasi-equilibrium of excitation between antenna pigments and reaction center, as well as between singlet excited reaction center Chl and radical pair ($P_{680}^+ \text{Pheo}^-$) in PS II. According to Holzwarth (68), the equilibration of excitons is about an order of magnitude faster than the process of charge separation. In such models, the reaction center appears as a "shallow trap" (135–137). The kinetic model suggested by Holzwarth and coworkers describes the primary photochemical events according to equation 7 (after 136), where Chl denotes antenna chlorophyll, k_A the rate constant of radiationless and radiative deactivation, and k_t and k_{-t} the rate constants for energy transfer between antennae and reaction center:





In the exciton-equilibrated pigment system, the rate of primary charge separation is approximately proportional to the probability of P_{680} excitation. In turn, this probability for a given number of absorbed photons is inversely proportional to the antenna size. Thus, a component of the fluorescence decay can be attributed to the reversible charge separation (step 1, equation 7). The lifetime of this component was shown to be proportional to the number (N) of antenna pigments and is supposed to be about 250 ps for PS II α units having about 180 Chl a and 70 Chl b molecules (102). According to the model of Schatz et al (136), a second component is related to the process of charge stabilization by electron transfer to Q_A (step 2 in equation 7, see also equation 1). The lifetimes of those components that are related to primary photochemistry increase with the closure of the traps, leading to the increased fluorescence yield in the F_M state (see equation 6). In the above model the increase in τ results predominantly from a decreased rate constant of primary charge separation in closed reaction centers (equation 8):



The constant k_1' (for closed reaction centers) was calculated to be lower than k_1 (for open reaction centers, see equation 7) by a factor of about six. This drastic effect is supposed to be caused by the electric field created by the negative charge on Q_A in the state $[\text{P}_{680} \text{ Pheo} \text{ Q}_A^-]$ (cf 73). As a consequence, formation of the primary radical pair, $\text{P}_{680}^+ \text{ Pheo}^-$ is restricted. Schatz et al (136) infer that the primary charge separation even becomes an endergonic reaction in the F_M state. This problem is discussed below with regard to the origin of fluorescence.

By "target" analysis, Holzwarth (68) resolved for cells of the green alga *Scenedesmus* six components for the decay of fluorescence both in the F_O and F_M state. Three of them show a strongly increased lifetime with closure of reaction centers (Table 1). Based on evidence from fluorescence amplitudes and their excitation spectra (68), four components were attributed to revers-

Table 1 Lifetimes of fluorescence from *Scenedesmus obliquus* (from Ref. 68, altered) (τ in ps)

	F_0	F_M	Assignment
τ_1	260–300	700–740	PS II α
	100–130	120–150	PS II β
τ_2	440–550	1510–1520	PS II α
	530–570	2250–2260	PS II β
τ_3	10–20	10–20	exciton equilibration, PS I, PS II
τ_4	94–96	114–118	PS I

ible charge separation (τ_1) and to charge stabilization (τ_2), respectively, of two PS II pools, namely PS II α and PS II β units. The other two components were assigned to ultrafast exciton equilibration in both photosystems, and to PS I emission (Table 1).

It should be emphasized that these assignments must still be viewed as tentative. In recent reports (66, 168) the component with the longer lifetime at F_M ($\tau \approx 2.2$ ns) was attributed to PS II α , whereas the component with $\tau \approx 1.2$ ns was assumed to represent PS II β emission, in contrast to the assignments in Table 1. However, even though results of lifetime measurements from different groups of workers are partly comparable, wide disagreement on interpretation exists. Moya and his coworkers (60, 62–64) concluded from their studies, particularly of *Chlamydomonas* mutants and of various protein/Chl complexes, that there is no need to explain the fluorescence decay on the basis of PS II α /PS II β heterogeneity. Rather, they attribute the different lifetimes to specific domains of antenna pigments, which should exhibit different rates of exciton transfer to the reaction centers. For instance, the two long-lived components at F_M are both attributed to the light harvesting complex of PS II (LHC II). This view, however, presumes a limitation by exciton migration and cannot be reconciled with the trap-limited model discussed above.

In contrast to other workers who postulate at least four decay components of PS II, Keuper & Sauer (73) maintain that the PS II decay of thylakoids is sufficiently described by three exponential decays. The lifetimes of all three components continually increased with trap closure (Table 2). The authors attributed the fast component to the trapping kinetics of open PS II units, because its amplitude tended to approach zero towards F_M conditions. The slow component, which was absent at F_0 is assumed to represent closed PS II units. Keuper & Sauer discuss that the changes in amplitude they observe are in contradiction to the general assumption of exciton transfer (“connectivity”) between PS II α units (72, 105). On the other hand, Holzwarth (67) argues

Table 2 Fluorescence lifetimes of spinach thylakoid membranes and their increase during closure of PS II reaction centers (after Ref. 73).

Component	Lifetime (ps)	Assignment
fast	170–300	open PS II centers
middle	400–1700	Q _B -nonreducing (PS IIβ ?) centers
slow	1700–3000	closed PS II centers

that in the present state, lifetime analysis cannot provide evidence for or against connectivity. The middle component of Keuper & Sauer (73) was suggested to present “Q_B-nonreducing” PS IIβ (see 103, 106), because the amplitude could not be influenced by the redox state of the PQ pool. The contradictions in the interpretations of fluorescence lifetime data await future clarification.

Origin of Fluorescence Emission

Whereas most agree that fluorescence at F_0 is an emission by antenna Chl *a* molecules, the origin of variable fluorescence has been the subject of controversy. Butler and coworkers (see 22, 23) postulated that all fluorescence originates in the antennae. Variable fluorescence was assumed to arise owing to back-transfer of excitation energy from the closed reaction centers. Klimov et al (74, 75) established that Pheo is the primary electron acceptor of PS II and transmits electrons to the quinone Q_A. According to their hypothesis, variable fluorescence results from recombination of the radical pair P₆₈₀⁺Pheo⁻ in closed reaction centers (see equation 8) and either arises from emission by P₆₈₀^{*} after charge recombination, or from emission by antennae Chl *a* following exciton transfer from P₆₈₀^{*}. It was also suggested that Pheo emits fluorescence from closed reaction centers. Breton (18) attributed the emission band at 695 nm observed at 77 K to emanation from Pheo*. However, recent progress both in time-resolved fluorescence spectroscopy and in preparation of functional reaction centers of PS II provided strong evidence against the hypotheses of Klimov et al (74, 75) and Breton (18).

As discussed above, Schatz et al (136) deduced from the picosecond fluorescence decay in thylakoid membranes that in closed reaction centers the primary charge separation forming P₆₈₀⁺Pheo⁻ is strongly restrained by the electrostatic effect of Q_A⁻. Similar conclusions were drawn by Schlodder & Brettel (138) from flash-induced absorbance changes related to the primary radical pair in PS II particles. Thus, the quantum yield of primary charge separation and therefore of recombination appears to be low in the presence of Q_A⁻. It should be noted that the kinetic equilibrium model of Schatz et al

(136), in fact, includes the possibility that charge recombination contributes to fluorescence emission both in the F_0 and F_M state. However, the rate constants calculated for this model (96) would favor prompt fluorescence emission. Also Hodges & Moya (62, 63) concluded that their results from lifetime measurements cannot be explained by the charge-recombination hypothesis. They found in PS II-enriched preparations three fluorescence decay components with variable τ_i between F_0 and F_M [similar to Keuper & Sauer's finding (73) for thylakoids] in the range of 20 ps to 2 ns. The authors argue that the variable nature of τ_i , starting in the picosecond range, contradicts the assumption of a slow (nanosecond) recombination luminescence. Furthermore, a *Chlamydomonas* mutant lacking functional PS II reaction centers exhibited lifetime components very similar to those of the wild type in the F_M state (63). This also speaks against the origin of the long-lived components at F_M from charge recombination.

Further information on this topic has been obtained from fluorescence lifetime studies of isolated reaction centers. Such particles have recently been isolated by Nanba & Satoh (115) and Barber et al (7). The preparations are free of antenna chlorophyll protein complexes and of bound PQ and nonheme iron. The centers consist of five polypeptides: the D1/D2 heterodimer, cytochrome b-559 with one heme and two protein subunits, and a further chloroplast-encoded 4.8-kDa protein (see 6). The complex binds 4 Chl *a*, 2 Pheo, and 1 β -carotene. The reaction centers are able to perform the primary photochemical reaction but proved very unstable when illuminated in the presence of O₂. Considerable stabilization was achieved by applying anaerobic conditions (32). The centers emitted fluorescence (44% of total yield) of a very long lifetime (around 36.5 ns). This long-lived component was attributed to recombination of the radical pair $P_{680}^+ \text{Pheo}^-$. Similarly, Govindjee et al (48) assigned a very slow decay component (5–20 ns) observed with isolated reaction centers to the charge recombination process (cf 108). They demonstrated that this component disappears when the centers are prereduced ($P_{680}\text{Pheo}^-$); in the state $P_{680}^+ \text{Pheo}^-$ the exciton cannot initiate the charge separation/recombination process.

At first sight, these results are in agreement with Klimov's hypothesis (74, 75), showing that recombination of the radical pair $P_{680}^+ \text{Pheo}^-$ can indeed give rise to light emission. However, the long lifetime (in the range of 5–35 ns) speaks against its role in emanation of variable fluorescence in the intact PS II. There is no indication of a significant contribution of such long-lived components in intact thylakoids (see 134 and Tables 1,2).

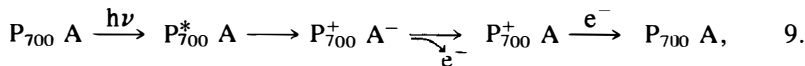
Obviously, charge recombination as a major deactivation pathway may take place only in the isolated reaction center complex which lacks bound quinones. In the intact system, the presence of Q_A favors electron transfer to this acceptor. When Q_A has been reduced, the rate of charge separation is

supposedly strongly diminished, as outlined above. The 695-nm band of low-temperature fluorescence does not occur in the emission spectrum of the isolated reaction center complex (8, 158) and thus cannot result from Pheo*, as had been suggested by Breton (18) and van Dorssen et al (157). It has been shown (158) that this band originates from the CP47 core antenna complex. Thus, present evidence supports the assumption that most of the chlorophyll fluorescence in vivo (both at F_0 and F_M levels) emanates from the antenna system.

Fluorescence of PS II and PS I at Ambient and Low Temperatures

EMISSION AT ROOM TEMPERATURE In fluorescence emission spectra of dilute suspensions of thylakoid membranes or intact isolated chloroplasts (see 86), a sharp peak around 685 nm with a broad shoulder at about 740 nm is observed. In leaf tissue with densely packed chloroplasts, light scattering and reabsorption effects strongly lower the 685-nm band relative to 740-nm emission (see 132). At room temperature, most of the fluorescence in both spectral regions is emanated from PS II. There is a minor contribution of emission by PS I, which is larger at the longer wavelengths than at 685 nm. According to data of Holzwarth (68), PS I (in *Scenedesmus* cells) contributes around 5% at 720 nm and 1–2% at 685 nm to the total fluorescence yield in the F_M state. From lifetime studies of fluorescence emitted at 5° C by PS I particles, Holzwarth et al (69) concluded that the primary photochemical reaction in PS I—like that in PS II—is trap limited.

Closure of PS I reaction centers does not contribute to variable fluorescence (see 20). This can be explained by the relative stability of P_{700} in the oxidized state, whereas P_{680}^+ is readily returned to the ground state by electron donation (cf equation 1). The reaction sequence in PS I probably is



where A denotes the primary PS I electron acceptor. Depending on the rate of electron transfer, most of the “closed” PS I reaction centers are either in the state $P_{700}^+ A^-$ or $P_{700}^+ A$. The oxidized pigment P_{700}^+ (like P_{680}^+) still acts as a trap for excitation energy (which is dissipated as heat) and therefore represents a fluorescence quencher—i.e. upon closure of PS I reaction centers, the fluorescence yield remains low (118).

EMISSION SPECTRA AT LOW TEMPERATURES Cooling of green leaf tissue, or of chloroplast or thylakoid suspensions drastically alters the emission spectra. A most conspicuous effect is the strong increase in PS I fluorescence

Table 3 Assignments of fluorescence emission bands at low temperatures (77–4 K).

Band	Assignment	References
F ₆₈₀	LHC II	see 146
F ₆₈₅	PS II core (CP43)	see 146
F ₆₉₅	PS II core (CP47)	158
F ₇₂₀	PS I core ("PS I-65")	see 146
F ₇₃₅	PS I (LHC I?)	112, 113

in the far-red region (720–740 nm). When light scattering and reabsorption of fluorescence are minimized (see 20), three distinct bands are seen at 77 K, termed F₆₈₅, F₆₉₅, and F₇₃₅ after their approximate emission maxima. The broad F₇₃₅ band can be resolved into two emissions showing peaks at 714–725 nm (F₇₂₀) and 735–745 nm (F₇₃₅) (see 114). Further spectral changes, particularly in the relative peak sizes, occur upon cooling from 77 to 4 K. At about 4 K, a shoulder at 680 nm appears. Even though these temperature-induced changes are presently not well understood, the various low-temperature bands can be attributed to specific Chl/protein complexes (Table 3). Evidence for these assignments is now largely consistent thanks to studies by numerous workers using isolated particles and mutants devoid of certain complexes (see 20, 112–114, 146).

VARIABLE FLUORESCENCE AT LOW TEMPERATURES PS II fluorescence at 77 K (F₆₉₅) exhibits a very similar F_V/F_M ratio (about 0.8) as at room temperature. The rise from F_O to F_M is supposed to reflect reduction of Q_A in the photochemical reaction (see 20). However, in contrast to the case at ambient temperature, reoxidation of Q_A^- is extremely slow.

In the PS I band (F₇₃₅), the F_V/F_M ratio is much lower (below 0.3). According to Butler's hypothesis (22, 23), variable fluorescence (and part of F_O) emanating from PS I is a result of exciton transfer from PS II to PS I, termed "spillover." If the fluorescence yield of PS I is plotted vs that of PS II during the rise from F_O to F_M , a straight line is obtained. The slope of this line is assumed to be proportional to the rate of spillover. From Butler's model it follows that there are no fluorescence changes related to the photochemical reaction in PS I (see above). The relationship between PS I and PS II fluorescence at 77 K has often been used to study the distribution of excitation energy between the two photosystems (for references see 20). However, interpretation of the spillover phenomenon appears problematic, as PS II is predominantly located in the appressed grana regions and PS I in the stroma lamellae of the thylakoids and border regions of grana (2). Efficient energy

transfer from PS II to I appears possible only in the nonappressed membrane domains, where besides PS I supposedly PS II β units are present (see the following section). Thus, spillover might be dominated by energy transfer from PS II β to PS I and is expected to vary with changes in the ratio of PS II α to PS II β .

FLUORESCENCE INDUCTION AND PS II HETEROGENEITY

Fluorescence Transient from F_0 to F_M

In the dark-adapted state of the photosynthetic apparatus, Q_A normally is fully oxidized. If continuous illumination is started, a fluorescence rise from F_0 is observed related to Q_A reduction (41). The induction signal represents a complex polyphasic process which in detail depends on experimental conditions. As discussed below, the fluorescence rise kinetics is influenced by: 1. PS II cooperativity, 2. PS II heterogeneity, 3. size of the PQ pool and rate of its reoxidation, 4. rate of electron transport beyond PS I including carbon metabolism, and 5. rate of electron donation to P^{+}_{680} .

When the actinic PFD is high enough to warrant a rate of Q_A reduction that is faster than reoxidation of Q_A (see equation 1), the F_M state is reached, representing full reduction of Q_A . However, at a given time during the rise from F_0 to F_M the variable fluorescence is not directly proportional to the redox state of Q_A . For a mixed population of open and closed centers, the variable fluorescence is lower than expected from the proportion of oxidized Q_A , presumably because of excitation energy transfer from closed to open centers (72). Moreover, heterogeneity of PS II influences the shape of the rise curve, as energy transfer seems possible between PS II α but not between PS II β units (see the next section).

As electrons are passed on from Q_A^- via Q_B to PQ, the fluorescence rise is also related to the reduction of these electron carriers and has, in fact, been used to determine the size of the PQ pool (see 20). Oxidized PQ has been shown to act as a fluorescence quencher (160a). Thus, the true F_M can only be reached when the PQ pool becomes reduced. The rate of its reoxidation depends on electron transfer via PS I and on the final consumption of reducing equivalents in carbon metabolism and other metabolic reactions. In addition, PQH₂ reoxidation is controlled by the transthylakoid ΔpH (see below). The typical fluorescence induction signal exhibits a relatively fast rise from F_0 to F_I (I = "inflection") followed by a plateau or "dip" (F_D) and a slower rise to the "peak" (F_P) or fluorescence maximum (F_M). F_P is lower than F_M when full reduction of Q_A is not achieved.

REOXIDATION KINETICS OF Q_A^- The "dark decay" of variable fluorescence back to the F_0 state can be used to study the kinetics of electron transfer from Q_A^- to Q_B (and PQ) or more generally, the reoxidation kinetics of Q_A^- (24, 93, 133). In this method, by means of high-intensity single-turnover flashes approximately all Q_A (but not Q_B and PQ) is reduced. In the following dark period, the decline of fluorescence (induced by a weak modulated "measuring" light) is recorded and reflects the reoxidation of Q_A^- . The decay proceeds in three exponential phases with lifetimes in spinach thylakoids of about 500 μ s, 10 ms, and 2 s (24). The fast phase (amplitude about 62%) is attributed to electron transport from Q_A^- to Q_B in centers that possess bound Q_B . The middle phase (18%) is thought to represent those centers that had no bound Q_B before the flash; thus this phase may indicate the kinetics of equilibration of PQ binding to the Q_B site of the D1 protein. The third, very slow component (20%) seems to represent the decay of the F_I level and has been attributed to PS II centers that are unable to transmit electrons to the PQ pool. The slow Q_A^- reoxidation apparent from this phase is thought to result from recombination between Q_A^- and the water oxidation system in the S_2 state.

The F_I Level and Inactive PS II

In recent years, evidence has accumulated for the interpretation first given by Lavergne (93) that the rise from F_0 to F_I (in low-incident light) reflects reduction of Q_A in centers that are not (or are only loosely) connected to Q_B and PQ (24, 26, 27, 103, 106). Convincing proof for the existence of inactive PS II centers *in vivo* comes from Chylla & Whitmarsh (27, see 121), who measured in intact leaves the dark "recovery" of the rapid electrochromic absorbance shift (ΔA_{518}). This signal is supposed to indicate charge separation in PS II and PS I reaction centers. It was found that about 20% of the amplitude of ΔA_{518} required a very long recovery time ($t_{1/2} = 1.7$ s). The kinetics of this slow phase was virtually identical with that of the F_I to F_0 fluorescence decay, indicating that the inactive centers belong to PS II. Their turnover rate was calculated to be only $0.3 \text{ e}^- \text{ s}^{-1}$, as compared to the $200\text{--}300 \text{ e}^- \text{ s}^{-1}$ of active centers. The fraction of inactive PS II was estimated as about 30% in spinach leaves. Inactive PS II centers were detected in all of a series of higher plant species studied.

The concept of " Q_B -nonreducing" centers is further supported by the observation that artificial electron acceptors such as ferricyanide (106) or 2,5-dimethyl-p-benzoquinone (24), which cause rapid reoxidation of the PQ-pool, lower the variable fluorescence of thylakoids only to the F_I level. In contrast, 2,6-dichloro-p-benzoquinone can lower the variable fluorescence below F_I and virtually to F_0 , thereby leading to enhanced photosynthetic O_2 evolution (24). It is assumed that the latter quinone accepts electrons directly

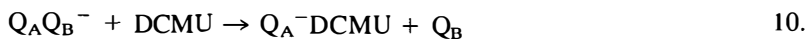
from Q_A^- . This effect indicates that the inactive centers possess a functional water oxidation system but, owing to their inability to reduce PQ, do not significantly contribute to net electron transport.

Fluorescence Induction in High Light

It should be emphasized that the F_I level can serve as an indicator of inactive PS II only in relatively low actinic light. The slower rise from F_I to F_M then represents closure of active reaction centers concomitant with reduction of PQ. In higher light, the two rise phases overlap. Very high continuous light induces a fast rise (in about 800 μ s) to a first high F_{I1} level, followed by a dip (F_D) and biphasic rise via F_{I2} to F_M in a total time of about 200 ms (116, 139). At F_{I1} (in saturating light) all Q_A is in the reduced state. The further fluorescence rise is supposed to be of nonphotochemical nature ("thermal phases"). At the F_{I1} level, an initial limitation of electron donation to P_{680}^+ is assumed. Accumulation of P_{680}^+ quenches fluorescence (see the next section). During the rise from F_D to F_{I2} this limitation then would be overcome. The slower phase from F_{I2} to F_M apparently reflects reduction of PQ (which quenches in the oxidized form; see 160a). This rise phase is prevented by electron acceptors that keep PQ oxidized or by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) which prevents electron transfer to PQ. These findings have practical implications, as they show that F_M cannot be reached in a shorter time than about 200 ms even in the highest actinic light. When fluorescence methods (e.g. the amplitude-modulated techniques) are applied, in which a high fluorescence level is reached by saturating light pulses (143), the peculiar rise kinetics in high light should be considered.

Rise in the Presence of DCMU and α/β Heterogeneity

DCMU blocks electron transfer from Q_A^- to PQ, probably by binding to the Q_B site of the D1 protein. Therefore, in the presence of DCMU the fluorescence rise from F_0 to F_M is much faster than in the absence of the inhibitor. The apparent F_0 level often is slightly increased by addition of DCMU. It appears that a small part of Q_A^- is not reoxidized even during dark periods of several minutes. A further effect of DCMU binding is a back-transfer of electrons in a fraction of the centers that are in the state $Q_A Q_B^-$ (see 159):



Also the true F_M level normally is not reached in the presence of DCMU. At least part of this quenching may be related to oxidized PQ (160a).

If these complications are neglected, the "complementary area" above the fluorescence induction curve (in other words, the integrated fluorescence deficit) at a given time is assumed to be proportional to the fraction of reduced

Q_A . The kinetics of "area growth" represents the closure of PS II reaction centers. The rate of Q_A reduction obtained from the area growth is proportional to PFD, antenna size, and quantum yield of the photochemical reaction. The maximum size of the area, A_{\max} (obtained at a given PFD) is a measure of the quantity of centers photochemically active in Q_A reduction.

Melis & Homann (107; see also 106) found that the area growth can in semilogarithmic plots be resolved into two phases, α and β . Based on such analyses, the existence of two types of PS II units was postulated. The fast nonexponential α phase is attributed to connected PS II α units, which are supposed to reside in the appressed grana membranes (2). The slow exponential β phase is assumed to represent PS II β units, located in the nonappressed membrane regions. The exponential kinetics of this phase indicates separate units without exciton migration between them. The β phase normally comprises about 20–35% of A_{\max} . The biphasic kinetics of Q_A reduction was confirmed by absorbance measurements in the wavelength region of 250–350 nm (105, 155).

The two- to three-fold slower kinetics of primary photochemistry that is obvious from the β phase was first ascribed to less efficient quantum conversion. Later, this view was revised on grounds of the finding of a lower absorption cross section of PS II β (104, 105, 154). For spinach chloroplasts, the antenna size was estimated to be 250 ± 40 Chl $a+b$ for PS II α and 120 ± 20 Chl $a+b$ for PS II β (104). Similar values were calculated for tobacco chloroplasts (154). Further studies (49, 55, 103, 109) indicated that PS II α units possess besides their core antennae an inner and a peripheral Chl a/b LHC II. The peripheral LHC II antenna appears to be absent in PS II β . From recent lifetime analyses it was concluded that α and β centers also differ in their rate constants of primary charge separation (and recombination) and in their F_V/F_M ratio (96). Interconversion between PS II β and PS II α is viewed as a dynamic property of the thylakoid membrane (53, 54, 101, 153). Reversible phosphorylation of part of the LHC II transforms α to β units, but transformation independent of phosphorylation (e.g. induced by heat stress) also seems possible (see the next section).

The concept of α/β heterogeneity is, however, still controversial (see also the section on fluorescence lifetimes, above). Based on the observation that high concentrations of DCMU diminish the β phase of fluorescence induction, it was argued that this phase merely reflects a type of PS II centers less sensitive to DCMU (59, 142). This conclusion was disputed (106) in view of chaotropic side effects of DCMU at high concentrations. More recently (147), the effect of high [DCMU] on fluorescence rise kinetics could not be reproduced.

A further problem presently under discussion is the relationship between PS II α/β and Q_B -nonreducing (inactive) units. The observation of an exponential

fluorescence rise from F_0 to F_I (attributed to inactive centers) and indications of a smaller antenna system of inactive units (27) make it very likely that the Q_B -nonreducing units are of β type. This is supported by studies of Guenther et al (55), who found that PS II β in the green alga *Dunaliella salina* occurs in both an active and an inactive state. It was postulated that Q_B -nonreducing units represent a subpopulation of PS II β . In spinach thylakoids, PS II β and Q_B -nonreducing units appeared to be identical (106). Presumably, PS II α and active and inactive PS II β are interconvertible (54, 56). Localization of active PS II β in the appressed thylakoid regions has been suggested (4). Recently the role of the inactive units has been proposed to be that of a reserve pool for assembly of active PS II (56), related either to development or to turnover of the D1 protein in the light and repair of "damaged" centers (53).

It should be noted that several further heterogeneities, deduced from fluorescence and absorbance studies and redox titrations, have been reported. Those phenomena might be related to the above inactive and active forms of PS II (for discussions see 4, 15).

FLUORESCENCE QUENCHING

The typical fluorescence induction signal of chloroplasts *in vivo* in continuous light is known as the Kautsky phenomenon (see 20): A decline in fluorescence yield follows the peak, F_P , or maximum, F_M , of emission. Normally, a polyphasic "quenching" is observed, sometimes interspersed by one or several secondary peaks, until a final steady-state level of fluorescence, "terminal" fluorescence, F_T , is reached in the span of minutes. In a more general sense, the term "quenching" denotes all processes that lower the fluorescence yield below its maximum. Commonly, the extent of quenching is expressed by quenching coefficients ($0 \leq q \leq 1$) indicating the quenched proportion of maximum fluorescence. If not defined otherwise, q refers specifically to quenching of maximum *variable* fluorescence, F_V (143): $q = (F_V - F_V')/F_V$, where F_V' denotes the variable fluorescence in the quenched state. But the quenching coefficients may also be used to describe lowering of fluorescence yield of F_0 or F_M . Alternatively, quenching can be expressed by the normalized "remaining" fluorescence level. For variable fluorescence emission, this is $F_V'/F_V = 1 - q$. Another definition of quenching (21, 37, 85), based on the Stern-Volmer equation, expresses the ratio of "quenched" to "remaining" fluorescence, $\Delta F/F' = (F - F')/F' = F/F' - 1$. Various mechanisms contribute to the quenching observed. The major routes to the lowering of fluorescence yield have been briefly reviewed recently (87). Resolution of quenching components provides important information on the functional state of the photosynthetic apparatus, and more specifically on the efficiency of PS II.

As already outlined (see equations 2, 3), the fluorescence yield is lowered owing to competition of the photochemical reaction with other pathways of de-excitation. Such *photochemical quenching* depends on the presence of Q_A in the oxidized state. The coefficient for photochemical quenching, q_P (or q_Q) denotes the proportion of excitons captured by open traps and being converted to chemical energy in the PS II reaction center. The reoxidation of Q_A^- thus causes quenching. It should be emphasized that q_P is supposedly larger than the proportion of oxidized Q_A because of energy transfer from closed to open units (except for the extreme cases $q = 0$ and $q = 1$).

However, the fluorescence yield can also be lowered by mechanisms not directly related to the redox state of Q_A . Such *nonphotochemical quenching*, q_N (or q_{NP}), may be caused in vivo under physiological conditions by three major mechanisms:

1. "energy-dependent" quenching (q_E) caused by the intrathylakoid acidification during light-driven proton translocation across the membrane;
2. quenching related to "state 1–state 2" transition (q_T) regulated by phosphorylation of LHC II;
3. "photoinhibitory" quenching (q_I) related to photoinhibition of photosynthesis.

Besides, several other nonphotochemical ways of quenching have been elucidated, which are probably not of great physiological significance.

Resolution of Quenching Components

In principle, the contribution of various components to total fluorescence quenching can be resolved by means of their relaxation kinetics. In isolated chloroplasts, photochemical and nonphotochemical quenching were first resolved by addition of DCMU in the light (85). This causes reversion of quenching in two phases ($t_{1/2} \approx 2$ s and 15 s). The fast phase represents relaxation of q_P due to reduction of the proportion of Q_A that was oxidized at the time of DCMU addition. The slower phase indicates reversion of q_N (most of it representing q_E) following the decay of the transthylakoid ΔpH . Alternatively, the application of saturating pulses (11, 17, 143; see 141) allows one to determine q_P and q_N at any point of the fluorescence induction signal, as well as during dark relaxation. By means of the pulses (duration about 1 s) all Q_A connected to active centers and the PQ pool become reduced.

More detailed studies using either technique revealed three distinct phases of relaxation of q_N (61, 71, 163; cf 36). The fastest phase ($t_{1/2} < 1$ min) is attributed to q_E , the second phase ($t_{1/2} \approx 8$ min) to q_T and the slow phase ($t_{1/2} \approx 40$ min) to q_I . It should be noted that the resolution of q_N can be problematic. Depending on the material, the relaxation of q_I is sometimes

quite fast (97, 149) and may occur in several phases (71). Separation from q_T is not always possible (see 151). Also, the kinetics of q_E relaxation can vary. A slowly relaxing q_E component has been observed recently in *Dunaliella* (95).

Mechanism of Energy-Dependent Quenching

Energization of the thylakoids because of the build-up of a transmembrane Δ pH may lead to quenching of up to about 90% of F_V . Also some quenching of F_0 (up to 15–25%) has been reported. Such F_0 quenching in leaves, originally attributed to state transition (143), may rather be related to q_E in view of the fast dark relaxation observed (165). As demonstrated in Figure 1, the extent of the Δ pH- or energy-dependent quenching is linearly related to the intrathylakoid H^+ concentration (21, 91). From 77 K fluorescence analyses it was concluded (78) that the quenching is based on an increased rate constant of thermal deactivation in PS II (k_D in equation 3). The molecular mechanism of this quenching is still unknown. An ultrastructural change induced by intrathylakoid acidification and concomitant cation exchange has been postulated (for discussion of earlier literature see 20, 86). It should be

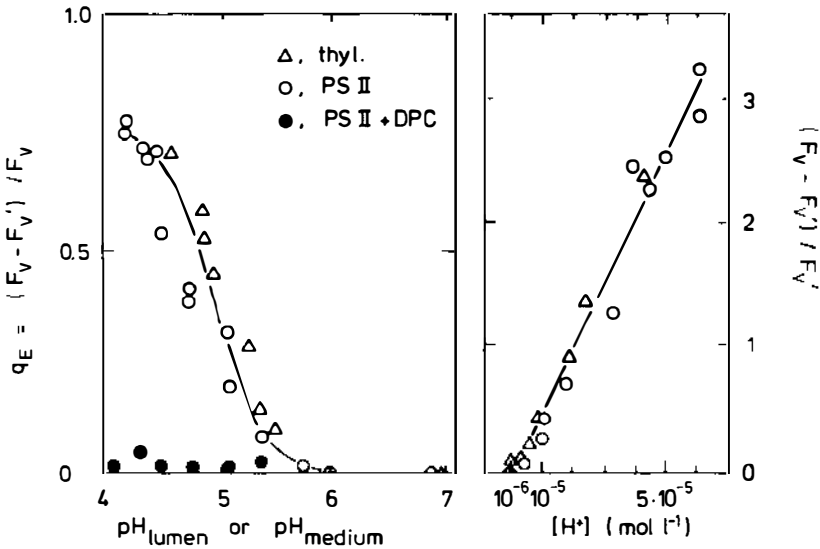


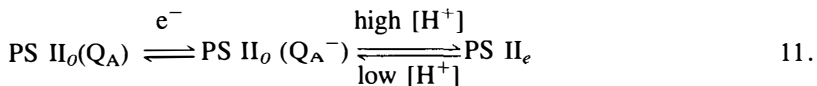
Figure 1 Relationship between energy-dependent fluorescence quenching and pH (left) or proton concentration (right). Quenching was induced in thylakoids (Δ) by light-dependent H^+ uptake into the lumen or by suspending PS II particles in media of different pH (\circ). Inhibition of quenching by an artificial electron donor to PS II (diphenylcarbazide) is shown (\bullet , left). Quenching is expressed as the fractional coefficient q_E (left) or according to the Stern-Volmer equation (right). F_V = maximum variable fluorescence, F_V' = variable fluorescence in the quenched state. Experiment by A. Krieger and E. Weis, unpublished.

noted that changes in high-energy quenching are usually slower than changes in the intrathylakoid $[H^+]$ (10).

However, even though the ΔpH is a strict requirement for q_E formation, other factors are involved. Antimycin A eliminates q_E without affecting the ΔpH (122). The inhibitor is known to block PS I-driven cyclic electron transport, possibly by affecting a ferredoxin-quinone reductase (110). This observation suggests that q_E is subjected to a redox control. A role of zeaxanthin in the thylakoids has been postulated for q_E . From studies of correlation between quenching capacity and zeaxanthin content (1, 38; see 37) it was concluded that q_E consists of two components. The major component in leaves, characterized by a decrease in F_O , is eliminated by dithiothreitol, an inhibitor of zeaxanthin formation. The second component (without F_O quenching) was suggested to be independent of zeaxanthin (cf 46). This component was found to be minor in intact leaves but a substantial effect in isolated chloroplasts. The mechanism of zeaxanthin-related quenching is seen in an interaction of the xanthophyll with antenna Chl causing an increase in k_D of equation 3 (see also discussion on q_I , below). According to Rces et al (129), the presence of zeaxanthin in isolated chloroplasts enhances q_E at intermediary ΔpH values, but the maximum q_E is not altered.

The relationship between $[H^+]$ and q_E , indeed, is not necessarily constant. It can be substantially influenced by stress factors such as heat (91) or freezing/thawing (151). In presence of tertiary amines such as dibucaine, the proton gradient and rate of photophosphorylation in thylakoids are lowered, indicating uncoupling, while photosynthetic control of electron transport and q_E are not affected (92).

Whether the quenching originates in the reaction center or in the antennae is still controversial. In a model of Weis & Berry (165), deduced from detailed data on q_P and q_E in leaves, and in a modified version of this model (166, 167), a photochemically active form (PS II_o, with open or closed centers) and an "energized", photochemically inactive and nonfluorescent form of PS II (PS II_e) are postulated. The populations of the two states are assumed to depend on the intrathylakoid H^+ concentration and the redox balance at the centers:



A strongly increased dissipative process in the reaction centers is suggested for PS II_e which would be responsible for decline both in the net photochemical yield and in fluorescence. A negative correlation between q_E and photon yield of open centers [calculated as $\phi_s q_p$, where ϕ_s is the measured apparent

quantum yield of electron transport at steady state (165)] has been confirmed by several groups (71, 125, 127, 160, 167). Since most of the quenching occurs in the range between pH 5.5 and 4.5 in the thylakoid lumen, an apparent pK value of 4.5–5.0 has been assumed for the interconversion between PS II_o and PS II_e suggested in the model (88, 167; see also Figure 1).

However, this concept of pH-dependent control of PS II activity by conversion of centers to a “quenching state” was opposed by Horton and his coworkers. Mainly from the differential effect of antimycin A on fluorescence quenching and Δ pH they concluded that in addition to nonphotochemical decay processes, PS II efficiency is controlled by alternative cyclic photochemical processes, not necessarily related to fluorescence quenching (117, 123, 128).

An alternative model that again relates the quenching to decreased PS II efficiency was proposed by Genty et al (44, 45). This model presumes that fluorescence quenching occurs in the antennae. The ratio of the fluorescence parameters in the quenching state, $F_V'/F_M' = (F_M' - F_0')/F_M'$, is taken as a measure of quantum efficiency of open centers. Such assumption would not be valid for two populations of centers, as proposed by Weis & Berry (165). Though partially contradictory in their theoretical implications, both models allow full quantification on a purely empirical basis and can be applied to evaluation of complex photosynthetic behavior. Thus, both models can be used to calculate electron transport rate or efficiency of PS II from fluorescence signals (44, 45, 165, 167).

The concept of “antenna quenching,” developed on the basis of Butler’s model of energy transfer processes (see 22, 23), has often been used to explain fluorescence quenching under physiological conditions (see also 34, 128a). It is, however, in conflict with recent concepts based on time-resolved fluorescence spectroscopy, which assume that energy transfer within the antenna is very fast and fluorescence is mainly determined by photochemical events in the center (see the section above on lifetimes).

As a possible mechanism of Δ pH-dependent quenching at centers, a limitation of electron donation to P₆₈₀ caused by intrathylakoid acidification has been suggested (139, 141). Evidence for a donor-side limitation of PS II at low pH comes from time-resolved spectroscopic studies of reduction of P⁺₆₈₀ (138). In fact, quenching at low pH seems to depend on the redox balance on the donor side of PS II: Under moderately reducing condition, or in the presence of specific electron donors to the donor side of PS II, no quenching was seen at low pH in PS II particles (31, 88; see also Figure 1). When electron donation from the water splitting side is slowed down, excitation energy trapped by PS II may be dissipated by fast internal charge-recombination processes. An increased yield of recombination processes at low pH is indicated by μ s-luminescence (140). From time-resolved fluores-

cence studies it has been concluded that in the high energy state, centers are indeed kept in an open state (with oxidized Q_A) even in high light (A. Krieger, I. Moya, E. Weis, unpublished).

Quenching Related to State Transition

Phosphorylation of part of the LHC II—leading to a transition from “state 1” to “state 2”—is supposed to cause detachment of this complex from the core antenna of PS II. Thereby the absorption cross section of PS II relative to PS I is lowered, resulting in decreased fluorescence emission expressed as q_T . F_V and F_0 are quenched in the same proportion. The phosphorylation is controlled via the redox state of the intersystem electron chain. Detached phosphorylated LHC II was found to move from appressed to nonappressed membrane regions. Changes in the physical state of the thylakoid membranes (e.g. by mild heat treatment) may also cause detachment of the LHC II from the core antennae, and the fraction of PS II β increases. In this case, PS II units containing the core antennae and inner LHC II were found to move from appressed to nonappressed regions, leaving the peripheral LHC II in the grana region (153). Details about these well-studied phenomena have been reviewed elsewhere (20, 169).

Originally it was assumed that in “state 2,” light energy absorbed by phosphorylated LHC II could be transferred to PS I, but this concept is still debated (169). Remarkable enhancement in PS I activity was found, however, upon phosphorylation, when PS II was converted to its β -form by exposure to moderately elevated temperature (156). Spillover may occur in a complex [PS II β -LHC-P-PS I] (65), but whether such a complex is formed under physiological conditions is not clear. Perhaps state regulation and spillover depend on a dynamic interaction between the physical state of the membrane (as affected, for example, by cation relations or temperature) and the metabolically controlled enzymatic phosphorylation of the LHC II.

The extent of q_T is usually much lower than that of q_E and q_I ; maximum values of q_T for barley protoplasts seen in low light were around 0.2 (71). It has been reported that conditions of high light (and high ΔpH) that promote formation of q_E and q_I tend to suppress q_T (35, 42, 71). The physiological significance of state changes and their impact on regulation of photosynthetic electron transport are still not well understood.

Photoinhibitory Quenching

Photoinhibition of photosynthesis, observed within minutes to hours in response to excessive irradiation, is consistently related to quenching of F_V , whereas changes in F_0 may vary. This effect has recently been reviewed (76). Low-temperature fluorescence spectroscopy indicates that q_I (like q_E) results from increased nonphotochemical de-excitation of pigments. Reversal of the

quenching is correlated with recovery from photoinhibition. The quenching is often expressed as a decrease in the ratio F_V/F_M , recorded after a dark period of several minutes following high light exposure. (The dark interval is required to relax q_E and q_T , while q_I is not readily reversible in the dark.). The decrease in F_V/F_M in leaves was found in numerous studies to be linearly correlated with a decline in simultaneously measured optimal quantum yield of photosynthesis (e.g. 13, 34, 39, 148) or to loss of PS II photosynthetic activity of isolated thylakoids (84). A linear relation between quantum yield and F_V (not F_V/F_M) was, however, observed with kiwifruit leaves (52). One should keep in mind that light gradients within the leaf (162), causing gradients of photoinhibition, can influence such relationships.

HYPOTHETICAL MECHANISMS Various hypotheses regarding the mechanism of photoinhibitory quenching are presently under discussion. One relates the major component of q_I (as well as of q_E ; see above) to formation of zeaxanthin from violaxanthin in the xanthophyll cycle. High light is known to induce this reaction. Indeed, a close correlation between degree of fluorescence quenching (or calculated k_D values) and concentration of zeaxanthin in the thylakoids has been observed in various investigations reviewed lately (37). Zeaxanthin is suggested to facilitate increased thermal deactivation in the PS II antennae in a reversible fashion. On the other hand, it is unknown so far whether this specific xanthophyll species could cause quenching of singlet excited states. Several explanations have been discussed (see 37). There are also reports showing that a correlation between quenching and level of zeaxanthin is missing (43, 126).

It has been proposed (37) that the q_I and q_E mechanisms are in fact identical, as far as the zeaxanthin-related components are concerned. Their only difference would lie in the kinetics of formation and relaxation of quenching. However, this hypothesis neglects the fact that q_E relaxes readily in the dark or upon dissipation of the ΔpH by the action of uncouplers. In contrast, relaxation of q_I is controlled by light (see below). Thylakoids isolated from photoinhibited leaves show decreased PS II activity in the uncoupled state (30, 84, 120, 149). Even if the two mechanisms appear alike in terms of increase in energy dissipation, substantial differences in underlying mechanisms must be assumed.

A number of authors favor the hypothesis that the photoinhibitory quenching is initiated in the PS II reaction center. A transformation (of unknown type) of a fraction of centers to "quenchers" has been postulated (29). Such transformed centers would still act as energy traps, but they would be incapable of the normal photochemical reaction and would convert the excitation energy to heat. The fluorescence level of these centers would stay (approximately) at F_0 , whereas the remaining centers are supposed to possess

largely unaltered activity. There is, in fact, evidence against “antenna quenching”: Increased k_D in the antennae should slow down the rate of Q_A reduction (indicated by the fluorescence rise in the presence of DCMU). Such an effect was not seen upon photoinhibition of thylakoids or intact chloroplasts (29, 84). Moreover, quenching in the antennae should result in inhibition of PS II-driven electron transport measured in low light, while little or no inhibition should be seen in high, saturating light. In contrast, thylakoids isolated from photoinhibited spinach leaves exhibited PS II activities lowered to about the same degree when measured in limiting or saturating light (84, 149).

Quenching in the antennae—expressed as a gradual increase in k_D (equation 3)—would be in agreement with the linear correlation between F_V/F_M and quantum efficiency of photosynthesis (13). However, a simple model (Ch. Giersch, G. H. Krause, unpublished), presuming two populations of centers—either photoinhibited (“quenched”) or active—also results in a quasi-linear relationship between quantum yield and F_V/F_M , if one infers (a) α/β heterogeneity with predominant photoinhibition of α units, which probably is due to larger antennae (28, 84, 100), and (b) excitation energy transfer from active α units in the F_M state to photoinhibited α units. The variable response of F_0 to photoinhibition (e.g. 34, 148, 149) is obtained in this model from differences in k_D of the inhibited population.

In earlier studies, it has been inferred that the formation of q_I is directly related to damage and degradation of the D1 protein in the reaction center (see 90). Turnover of this protein in the light is obviously involved in photoinhibition. However, more recent data indicate that photoinhibition and concomitant quenching precede the dysfunction and degradation of D1 (25, 161; S. Schöner, G. H. Krause, unpublished). Moreover, it was demonstrated that degradation of D1 in *Chlamydomonas* (in low light in the presence of chloramphenicol, which suppresses resynthesis of D1) does *not* lead to the quenching of F_M that is characteristic for photoinhibition (19).

Molecular oxygen and/or reactive species derived from it may be involved in formation of q_I (e.g. 9, 131, 145). Also, presence of O_2 seems to be required for D1 degradation (e.g. 3, 89). However, anaerobiosis strongly enhances q_I formation. This effect is related to a conspicuous increase in F_0 , indicating that photoinhibition under anaerobiosis is caused by a mechanism different from that in the presence of O_2 (80). The F_0 increase was ascribed by Setlik et al (145) to “permanently closed” reaction centers with a stable Q_A^- . In C_3 plants, high O_2 levels indirectly diminish photoinhibition because they enhance photorespiration, which increases the drain of photosynthetic energy (see 79).

REVERSIBILITY OF PHOTONHIBITION Recovery from photoinhibition, as seen by relaxation of q_I , has been suggested to proceed *via* a “PS II repair

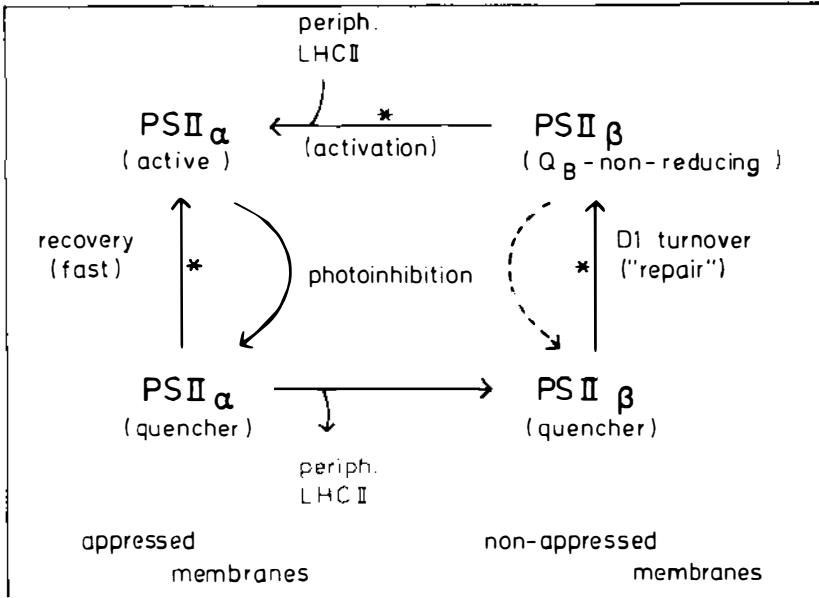


Figure 2 Scheme of hypothetical mechanism of photoinhibition and recovery (after 53, 54, altered). Stars denote possible points of light regulation.

cycle” involving transformation of “damaged” PS II α to PS II β units, in which form the reaction center is restructured by D1 degradation and replacement by the freshly synthesized protein (53). In view of the fast initial phase of recovery ($t_{1/2} \approx 15$ min) that can be found, depending on material and conditions (97, 149), we speculate that photoinhibited (“quenching”) centers may, in part, be reversed to the active (“fluorescent”) state without D1 replacement. Another fraction (possessing damaged D1?) would follow the path of D1 turnover known to occur in the light (see scheme of Figure 2). It is interesting to note that the recovery is light-regulated in a complex manner. Recovery proceeds very slowly in darkness and is optimal in rather low light. There is evidence (51, 97, 150) that moderate light (much below the level causing detectable photoinhibition) restricts recovery. Thus the hypothesis (50) that “damage” and “repair” proceed simultaneously in high light, and that photoinhibition becomes apparent only when the rate of damage exceeds the capacity for repair, may not be valid under all circumstances.

In summary, the present evidence, though providing considerable insights, does not support a unifying hypothesis of photoinhibitory quenching. It cannot be excluded that both discussed mechanisms—quenching in the antennae and in the reaction center—operate *in vivo*, depending on conditions and the physiological state of the plant.

Further Quenching Mechanisms

THE MAGNESIUM EFFECT The fluorescence emanating from thylakoid suspensions, particularly F_V , is strongly influenced by the cation concentration of the medium. This phenomenon is based on the lateral segregation of the two photosystems (at high Mg^{2+} levels) related to the stacking of membranes, which controls energy transfer from PS II to PS I. The effect has been reviewed previously (20). It seems that quenching due to lack of Mg^{2+} , a large and conspicuous effect *in vitro*, is not important *in vivo*.

QUENCHING BY PIGMENT RADICALS Excitation energy captured by the radicalic or excited states of photosynthetic pigments is converted to heat, and fluorescence is quenched. Such molecular species are, for example, $Pheo^-$, P_{680}^+ , Chl^* , and Car^+ , which show a shift of their absorbance bands towards the far-red region of the spectrum. However, since these molecular species are usually very unstable, it seems unlikely that they significantly contribute to fluorescence quenching *in vivo*. Under anaerobic or strongly reducing conditions, in high actinic light a reversible fluorescence quenching is observed, which has been attributed to photoaccumulation of $Pheo^-$ (58, 74, 145). The explanation given for this effect is that fast electron donation to P_{680}^+ in the presence of prerduced Q_A leads to the state $P_{680}Pheo^-Q_A^-$, in which excitons will be converted to heat by $Pheo^-$.

It has been suggested that the radical P_{680}^+ is an efficient quencher of variable fluorescence (see 22). To explain the fluorescence induction kinetics in very high light, transient quenching by P_{680}^+ has been suggested (139). Because of its extremely high redox potential, P_{680}^+ is very short-lived, even when electron donation is inhibited, and normally it may never accumulate in the light. Photoaccumulation of oxidized carotene and the possible significance of this pigment species as a quencher in chloroplasts have been discussed by van Gorkom (159).

QUENCHING BY OXIDIZED PLASTIQUINONE In the oxidized state, the PQ pool exerts a "static" quenching (160a). This is usually a rather minor effect, but quenching is enhanced by detergents [e.g. when used in particle preparations (see 159)].

Physiological Aspects of Quenching

Chlorophyll fluorescence quenching has to be seen in close context with regulation of photosynthesis and adjustment to external factors. Quenching phenomena are strongly influenced by environmental stresses. A complex interrelation between the various quenching components became apparent in numerous recent studies (e.g. 34, 36, 44, 45, 61, 71, 81, 83, 85, 165–167).

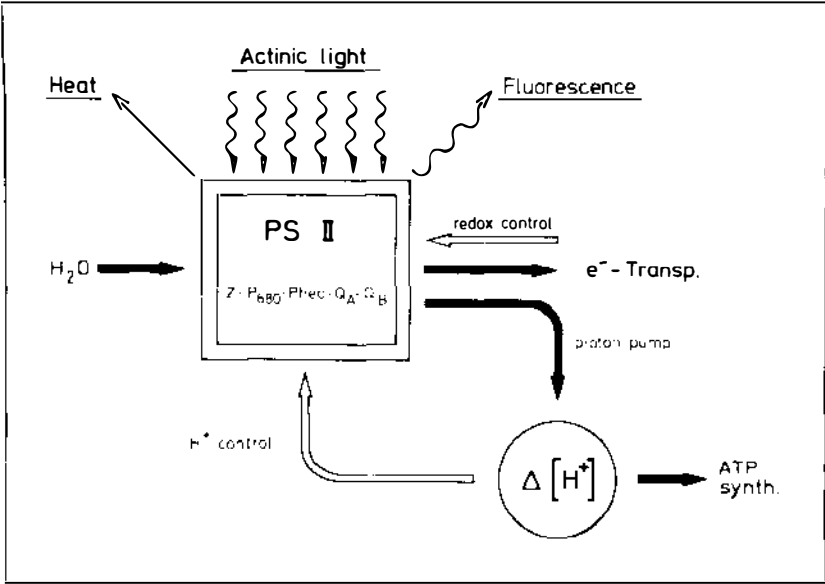


Figure 3 Scheme of PS II-dependent energy flux showing control by the proton gradient and redox state. Open arrows denote feedback control.

In leaves kept in low light, q_P is high (> 0.9), allowing optimal trap function (165). Maximum q_T may develop at low PFD, indicating transition to “state 2”. The related change in absorption cross section is viewed as a regulatory mechanism that alters the distribution of excitation energy between PS II and PS I according to the requirement for optimal function of photosynthetic energy conversion. Such balance of excitation is important in low, limiting light (71). With increasing PFD, a growing proportion of absorbed light energy is not used in the photosynthetic process. An increased ΔpH may control q_T and promote q_E ; a strong increase in q_N (mainly q_E) is observed. The response of q_E , indicating increased nonphotochemical energy dissipation, has been suggested to represent a dynamic property of the thylakoid membrane (81, 82, 83, 165). The q_E mechanism is supposed to open a pathway of controlled and harmless deactivation of excessively excited pigments, serving a protective function against adverse effects of high light (see scheme of Figure 3). It has been experimentally proven that in the presence of a high q_E photoinhibition is diminished (77, 82, 123).

Photoinhibition of PS II appears to be enhanced in the state of reduced Q_A . Thermal deactivation achieved at high q_E presumably increases the fraction of open centers present in the steady state (164, 165). Only when the PFD exceeds light saturation of photosynthetic CO_2 assimilation and q_E is also saturated does Q_A become largely reduced (q_P strongly declines). Under such

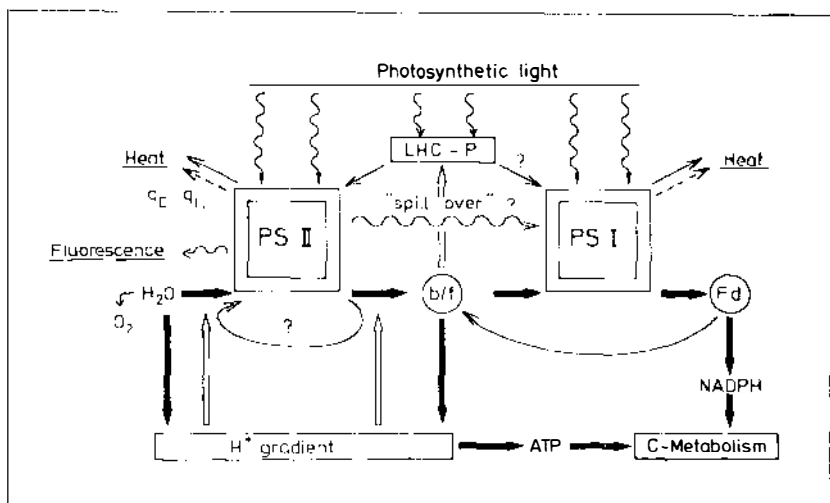


Figure 4 Scheme of the hypothetical relationships between photosynthetic energy conversion, PS II regulation, and fluorescence emission. Closed arrows denote photosynthetic energy conversion, open arrows regulation, broken arrows controlled increase in heat emission.

conditions, q_I is enhanced (71). This concept has been confirmed in a study with spinach leaves on effects of freezing/thawing and subsequent high light stress (151). Freezing stress was found to affect, besides CO₂ assimilation, the formation of q_E in response to the ΔpH . This caused under steady-state conditions in moderate light a decrease in q_P and corresponding increase in q_I ; an inverse linear relation between q_P and q_I was seen. Formation of q_I is generally promoted by conditions that limit energy utilization in carbon metabolism or slacken "repair" processes. Then even moderate light leads to strong photoinhibitory quenching (52, 148; see 76).

Formation of q_I (like that of q_E) may be viewed as a protective mechanism of thermal energy dissipation, insofar as the effect is reversible (see scheme of Figure 2). It has been suggested (13, 34, 76, 87, 150) that the q_I mechanism, although it reduces photosynthetic activity, prevents gross destruction of the photosynthetic apparatus. Photoinhibitory quenching becomes effective within minutes in excessive light, when the capacity for q_E is exhausted. This would stabilize the photosynthetic system and allow long-term acclimatory processes to proceed. Notably, these regulatory phenomena occurring in PS II might also contribute to stabilization of PS I under extreme conditions (see 166, 167, 167a). The hypothetical role of the discussed mechanisms to optimize electron flow and energy balance in contrasting environmental conditions is schematically presented in Figure 4. In addition to the different quenching processes, this regulation may include direct control of linear

electron transport *via* the ΔpH (167, 167a); it may also include dissipation of excess, potentially harmful energy by a futile cycle of electrons around PS II (70, 117, 128, 139, 140) and by accumulation of P^+_{700} in the reaction center of PS I (164, 167, 167a).

From the above consideration it follows that the quenching phenomena in intact leaves are strongly related to regulation and the steady-state condition of photosynthetic carbon metabolism. We refer to a recent review by Seaton & Walker (144) of this well-studied topic (see also 20).

CONCLUSIONS AND PERSPECTIVES

Our understanding of the physical and structural basis of fluorescence in relation to primary processes of photosynthesis has progressed in recent years together with our knowledge about the molecular architecture of the photosynthetic apparatus. Improved time-resolved spectroscopy has particularly helped this development. We have also made substantial progress in the characterization of various types of nonphotochemical fluorescence quenching and its relation to control of photosynthesis. Nevertheless, the molecular mechanisms of photoinhibitory and high-energy quenching, and of fluorescence quenching in various types of PS II β units, are still not well understood. Despite open questions regarding underlying mechanisms, progress in techniques to separate nonphotochemical and photochemical quenching *in vivo* has opened new applications of fluorescence spectroscopy in basic and applied plant biology. In combination with other techniques such as photosynthetic gas exchange and far-red spectroscopy that detects the redox state of PS I, fluorescence spectroscopy can be used to study regulation and control parameters of photosynthesis in leaves. The analysis of complex fluorescence transients (e.g. fluorescence oscillations) may help us to understand the network of various feedback interactions between primary processes of energy conversion and the biochemical reactions of carbon metabolism in intact plants (see 70, 144).

More work is required to improve the reliability of these approaches, and to simplify their application in the field. The various expressions that have now been developed to predict the quantum efficiency of PS II, photosynthetic electron transport, and gas exchange from fluorescence quenching in intact plants are still empirical. Obviously, more general expressions are required on a biophysical and/or biochemical basis. There is still disagreement with respect to the value and physical meaning of empirical parameters, yet these expressions may be regarded as first steps towards using fluorescence spectroscopy for small- and large-scale scanning of biochemical activity and photosynthetic fluxes in intact plants or canopies. Digitized video techniques have recently been introduced for detecting fluorescence. Images of fluores-

cence quenching have been obtained from small leaf areas (33). Small-scale fluorescence scanning may be used in the future to investigate photosynthesis in relation to various physiological processes at the whole-plant level, to address the source-sink problem in plants, and to study canopy photosynthesis. For large-scale scanning and for vegetation analyses first attempts have been made to use sunlight- or laser-induced fluorescence detected from the ground, as well as from aircraft (see 40, 99, 130). Extension of fluorescence measurements to large-scale spectroscopy may be useful in basic and applied environmental research, such as mapping of the photosynthetic activity of terrestrial and marine vegetation. In future developments, chlorophyll fluorescence will certainly remain an ever-refined instrument for basic photosynthesis research, but we can also expect a wide diversification of practical applications in the plant sciences.

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