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Photosynthesis and Fast Changes in Light Emission by Green Plants

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1. PHOTOSYNTHESIS

1.1. Introduction

The present chapter deals with a brief review of the process of green plant photosynthesis and with the fast emission (chlorophyll *a* fluorescence and delayed light) changes that have been used to probe the process of photosynthesis. In order to appreciate the latter, an understanding of overall photosynthesis is presented so that the reader may be able to place the fast-emission changes described in Sections 2 (fluorescence) and 3 (delayed light) in a broader framework.

Green plant photosynthesis involves the uphill transfer of 4 electrons (or hydrogen atoms) from 2 molecules of H_2O to CO_2 to produce 1 molecule of O_2 and a "fragment" of carbohydrate $\{CH_2O\}$ with the aid of at least 8 quanta of light:

$$2H_2O + CO_2 + 8\,h\nu \xrightarrow[\text{(minimum)}]{} O_2 + \{CH_2O\} + H_2O \qquad (1)$$

This process occurs in several steps. Electrons are transferred through two light reactions (I and II) from H_2O to $NADP^+$ (nicotinamide adenine dinucleotide phosphate) reducing the latter to NADPH and oxidizing the former to O_2 [Eq. (2a)]. During this electron flow, protons are translocated from the outside to the inside of thylakoid vesicles, and this proton gradient energy (along with the electrochemical membrane potential) is harnessed to

produce ATP (adenosine triphosphate) from ADP and inorganic phosphate (P_i) through the enzymatic activity of ATP-synthetase (popularly known as the coupling factor, CF_1). ATP and NADPH are then used to convert CO_2 to $[CH_2O]$ [Eq. (2b)] by alternative carbon cycles known as the Calvin-Benson-Bassham cycle or Hatch and Slack pathway.

$$2H_2O + 2NADP^+ + \underset{\text{(minimum)}}{8\,h\nu} \longrightarrow O_2 + 2NADPH + 2H^+ \quad (2a)$$

$$CO_2 + 2(NADPH + H^+) + n\text{ATP} \rightarrow [CH_2O] + 2NADP^+ + n\text{ADP} + n\text{Pi} + H_2O \quad (2b)$$

Details of photosynthesis have been reviewed in many books (Kamen, 1963; Clayton, 1965; Rabinowitch and Govindjee, 1969; Clayton, 1971; Govindjee, 1975; Barber, 1977; Gregory, 1977; Trebst and Avron, 1977).

The two light reactions are sensitized by light absorbed in two functionally separate pigment systems (PSI and PSII) (see the list of abbreviations below). PSI and PSII of green plants differ in having apparently different reaction center chlorophyll (Chl) *a* molecules P700 and P680, respectively. The numbers indicate the locations of the long-wavelength absorption bands, in nm, of their reduced forms, and P stands for Pigment; these are assumed to contain, at least, two Chl *a* molecules joined through water or amino acid group bridges (see Shipman *et al.*, 1976). The major portions of the pigment systems are composed of various spectral forms of Chl *a* and other accessory pigments (e.g., Chl *b* in green algae and higher plants, fucoxanthol in diatoms and brown algae, phycoerythrins in red algae, and phycocyanins in blue-green algae) (see Govindjee and Braun, 1974). Both systems contain carotenoids, with carotenes being predominant in PSI and xanthophylls (carotenols) in PSII. A larger proportion of light energy absorbed by the accessory pigments is channeled to PSII reaction centers, but most of the energy absorbed by the long wavelength absorbing (spectral) forms of Chl *a* is fed to PSI reaction centers. It was this difference that led to the initial discovery of the two pigment systems and two light reaction schemes of photosynthesis (see Emerson *et al.*, 1957; Emerson and Rabinowitch, 1960).

Abbreviations

Chl, chlorophyll; D, an endogenous secondary electron donor to the reaction center Chl *a* of pigment system II; DCMU, 3-(3',4' dichlorophenyl)1, 1 dimethylurea; DLE, delayed light emission, ϕ_f, quantum yield of fluorescence; M, the charge accumulator intermediate involved in O_2 evolution; P680 (P700), reaction center chlorophyll *a* of pigment system II (of pigment system I); Ph, pheophytin; PSI, photosystem I; PSII, photosystem II; Q, the electron acceptor of pigment

system II; R, an electron carrier between Q and the plastoquinone (PQ) pool; S, state of the oxygen evolving system that includes all the carriers from M to Q; τ, lifetime of fluorescence; Tris, tris(hydroxymethyl)aminomethane; W, an auxiliary electron acceptor of system II; X, electron acceptor of system I; and Z (Z_1, Z_2), electron donor(s) to $P680^+$.

1.2. Fates of the Excited State

Light quanta absorbed by pigments located in thylakoid membranes (Chl *a*, Chl *b*, or carotenoids in green algae and higher plants; Chl *a*, carotenoids, and phycobilins in red and blue-green algae; Chl *a*, carotenoids, including fucoxanthol, and Chl *c* in diatoms and brown algae) cause electronic transitions into excited singlet states. Whether the first or higher singlet excited state is reached depends upon the wavelength (or frequency) of light. In any case, the first singlet state is populated in times shorter than a picosecond.

The most general physical picture is as follows. An assemblage of pigments of about 300–400 molecules (antenna) comprises a statistical (if not physical) *photosynthetic unit* with its own reaction center molecule. There must be two types of units (I and II). Light energy absorbed in any one of the accessory pigments leads to excitation energy (or exciton) transfer to Chl *a* molecules. Energy transfer occurs here from the short wavelength to the long wavelength spectral forms of Chl *a* until the energy reaches the reaction center molecules. This transfer process, along with energy migration among the molecules of the same spectral form of the pigment, has to be a very efficient process because the primary photochemical reaction of photosynthesis is extremely efficient. Thus, the major fate of light energy absorbed by various pigments, including most Chl *a* molecules, is excitation energy transfer until the energy is trapped by reaction center chlorophyll (the "trap"). The fate of light energy directly absorbed by or transferred to the trap from other pigment molecules is its utilization in photochemical reactions, i.e., conversion of excitation energy into chemical energy (redox energy). The above events are summarized as:

$$\text{Pigment} + h\nu \rightarrow \text{Pigment}^* \tag{3a}$$

$$\text{Pigment}^* + \text{Chl } a \rightarrow \text{Chl } a^* + \text{Pigment} \tag{3b}$$

$$\text{Chl } a^* + \text{Trap} \rightarrow \text{Trap}^* + \text{Chl } a \tag{3c}$$

$$\text{Trap}^* + \text{Acceptor} \rightarrow \text{Trap}^+ + \text{Acceptor}^- \tag{3d}$$

Most of the existing data appears to be compatible with the belief that

photochemistry occurs from the singlet excited state. There have been some recent suggestions that photochemistry may occur through a charge transfer state that is formed by upconversion of a triplet state (Fong, 1975). However, no experimental data is available *in vivo* that proves this suggestion (Govindjee and Warden, 1977).

Minor fates of excited states in the reaction center molecules are (a) internal conversion; (b) fluorescence; and (c) some transfer back to the antenna pigments. There are no available measurements on internal conversion, but fluorescence has been extensively used as a probe of photosynthetic reactions (see Section 2). Since the concentration of reaction center molecules is very low, most of the observed fluorescence is from antenna pigments. Under conditions when photochemistry is blocked, process (c) may play an important role. In addition, it has been shown that when photochemistry is intentionally blocked, triplet states are formed (Vandermeulen and Govindjee, 1973; Levanon and Norris, 1978). Since there are two photosystems in green plants, we must also consider excitation energy distribution among the two systems. [For a review of fates of excited states, see Govindjee and Govindjee (1974), and for regulation of energy transfer, see Williams (1977).]

Delayed light emission can also occur (see Section 3) if (1) a triplet state is converted into singlet state by thermal quanta; (2) two triplets annihilate and produce one singlet and one ground state; (3) recombination of charges ($-$) and ($+$), created during photosynthesis, leads to the production of a singlet state; and (4) excess electrons and holes in the photosynthetic unit recombine. In photosynthetic systems, delayed light emission does occur with a very small quantum yield.

If triplets are produced, a direct decay to the ground state can occur leading to phosphorescence, which has a longer lifetime and longer wavelengths of emission than fluorescence.

1.3. Reactions Associated with Pigment System II

The present day picture of electron flow from H_2O to $NADP^+$ is summarized in Fig. 1. Light energy absorbed by pigment system II is transferred to its reaction center P680 where the following oxidation reduction occurs: $P680 \cdot Q \xrightarrow{h\nu} P680^* Q \rightarrow P680^+ Q^-$, where Q is the first "stable" electron acceptor of PSII. The cation of Chl *a*, $P680^+$, ultimately recovers electrons from water through a series of steps involving several intermediates (labeled Z_1, Z_2, and M). Oxygen evolution requires four such

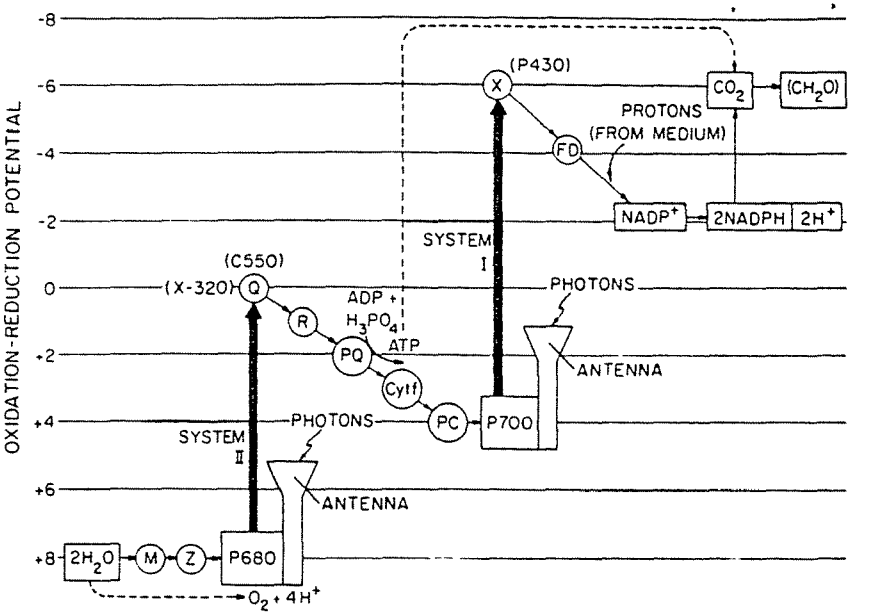


Fig. 1. Electron flow from water to carbon dioxide proceeds against an electrochemical gradient of 1.2 V and requires two photochemical events. The oxidation–reduction potential is given on the ordinate; +2 means +200 mV, and, so on. Four electrons must be transferred, one at a time, to liberate a molecule of oxygen and reduce a molecule of CO_2 to a carbohydrate fragment (CH_2O). The process begins with the absorption of photons by the antennae of the pigment systems. The energy of excitation is conveyed to a chlorophyll *a* molecule in the reaction center of the photosynthetic unit; this molecule is designated P680 for pigment system II because one of the bands in its absorption spectrum is at 680 nm. The excited P680 transfers an electron to the acceptor Q, and subsequently recovers an electron from the donor Z. The real primary acceptor may be at a more negative potential, but the first "stable" acceptor is Q (a quinone, which upon reduction becomes semiquinone and is designated as X-320); C550 is an indicator of changes in Q. Z (Z_1,Z_2) recovers its electron from M. After M has given up four electrons, it regains them by oxidizing $2H_2O$. From Q the electron is passed through a series of carrier molecules, including R (which has also been identified as a quinone), plastoquinone (PQ) and cytochrome *f* (Cyt *f*), to plastocyanin (PC). Plastocyanin injects the electron into pigment system I. The reaction-center chlorophyll of pigment system I, designated P700, is excited through its own antenna pigments, and promotes an electron to the acceptor X. An absorption change at 430 nm (P430) is due to an intermediate after X. Finally, the electron is passed to ferrodoxin (FD) and the enzyme ferredoxin-$NADP^+$ reductase (FNR, not shown) to nicotinamide adenine dinucleotide phosphate ($NADP^+$), which is thereby reduced to NADPH. NADPH is the end product of these reactions. In addition, during two of the electron transfers, adenosine triphosphate (ATP) is generated from adenosine diphosphate (ADP) and inorganic phosphate. One "site" for ATP generation is between plastoquinone and cytochrome *f*; the other is associated with system II but has not yet been located. The NADPH and the ATP drive the process by which CO_2 is incorporated into carbohydrates (multiples of the unit, CH_2O). There is also a cyclic system of photosynthetic, in which electrons pass from P700 to X and then return through various electron carriers to P700; in this system only ATP is produced. Modified after Govindjee and Govindjee (1975).

steps so that four positive equivalents, stored on M, undergo the following reaction: $M^{4+} + 2H_2O \rightarrow O_2 + M + 4H^+$. Electrons on Q^- move downhill through a series of intermediates (R, plastoquinone, Rieske iron protein, cytochrome f, and plastocyanin). Light energy absorbed by pigment system I is transferred to its reaction center where the following reaction occurs: $P700 \cdot X \xrightarrow{h\nu} P700^* \cdot X \rightarrow P700^+ X^-$, where X is the first "stable" electron acceptor of PSI. The Chl *a* cation, $P700^+$, recovers electrons from plastocyanin and cytochrome f and X^- delivers its electrons to $NADP^+$ via several iron–sulfur proteins, ferredoxin-$NADP^+$ reductase, FNR, and ferredoxin; with an additional H^+ present, NADPH is produced.

The following description of the various components of PSII is presented here in detail because both chlorophyll *a* fluorescence (Section 2) and delayed light emission (Section 3) are very closely tied to this system.

(*a*) *P680.* Light reaction II leads to the oxidation of P680 to $P680^+$ and the reduction of a primary electron acceptor which may not be Q, described above: $P680 \cdot O + h\nu \rightarrow P680 \cdot^* O \rightarrow P680^+ \cdot O^-$, where the empty circle (O) represents the real primary acceptor, and $P680^+$ the cation radical. Absorbance decrease due to $P680^+$ formation, decaying with a halftime of ~200 μs was discovered by Döring *et al.* (1967, 1968, 1969). Govindjee *et al.* (1970) (also see Döring, 1975) showed its presence in Tris-washed chloroplasts; this treatment is known to eliminate Chl *a* fluorescence changes under multiple excitation conditions and to inhibit electron donation from H_2O (Yamashita and Butler, 1968). Thus, this absorbance change was not due to fluorescence artifacts, and did not depend upon the activity of the O_2 evolving system. Floyd *et al.* (1971), on the basis of low temperature measurements, and Butler (1972*a*) suggested that P680 is the primary electron donor of PSII. Van Gorkom and Donze (1973) pointed out that some fast decaying signal in the reduction of $P680^+$ must have escaped detection in earlier measurements. *In vitro* studies have shown that production of the chlorophyll cation (Chl^+) should give an absorbance increase around 825 nm (Borg *et al.*, 1970; Fajer *et al.*, 1974). Mathis and Vermeglio (1975) indeed found an increase in absorption at ~825 nm at 100°K and dispelled the idea of fluorescence artifact as there is no significant fluorescence at this wavelength. Gläser *et al.* (1974, 1976) discovered a P680 component that decayed with a halftime of 35 μs. Gläser *et al.* (1976) suggested the existence of a faster decaying (< 1 μs) component; such a component, with a halftime of ~30 ns, has indeed been discovered after the first flash, from absorption changes at 825 nm, by Van Best and Mathis (1978). However, Jursinic and Govindjee (1977a) suggested a component, on the basis of parallel measurements on fast fluorescence rise and delayed light emission decay, with a lifetime of ~6 μs after all the flashes. Such a

component has been found, through absorption changes, by the repetitive flash technique (see Renger *et al.*, 1978).

Van Gorkom *et al.* (1974, 1975) were able to accumulate $P680^+$ under conditions where its reduction was slowed by detergent and ferricyanide treatments; these studies provided confirmation for the existence of P680, and they permitted confidence in the interpretation of data obtained under more physiological conditions.

Since $P680^+$ has an unpaired electron, it is expected to produce an electron spin resonance (ESR) signal; this was discovered by Malkin and Bearden (1975) and Visser (1975). An earlier signal claimed by Malkin and Bearden (1973) to be due to $P680^+$ was due to photooxidation of antenna Chl; it was irreversible, whereas $P680^+$ is reversible at low temperatures (Butler, 1972*b*; Murata *et al.*, 1973; Mathis and Vermeglio, 1974). The doublet nature of the P680 optical signal (Döring *et al.*, 1968) and the 7-gauss linewidth of the ESR signal, which is equivalent to that of a Chl *a* dimer *in vitro* (Norris *et al.*, 1974), suggest that P680 is a Chl *a* dimer. For further discussion of P680, see Sections 2 and 3.

The nature of the real primary electron acceptor (O) is not clear. In analogy with the bacterial system (Fajer *et al.*, 1974; Dutton, 1976), one could speculate that it may be a pheophytin (Amesz and Duysens, 1977; Klimov *et al.*, 1977; Fujita *et al.*, 1978). For further discussion see Sections 2.3.3a and 3.1.1.

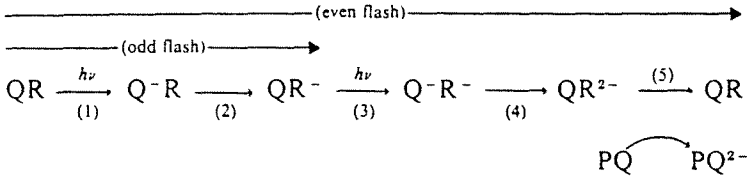
(*b*) *Q(X-320)*. The primary "stable" (in terms of μs scale) electron acceptor is generally accepted to be Q; the symbol Q was initially suggested by Duysens and Sweers (1963) because it acts as a quencher of Chl *a* fluorescence in its oxidized state, but not in its reduced state. Kautsky *et al.* (1960) had earlier interpreted the Chl *a* fluorescence induction in photosynthesizing systems to imply the existence of two light reactions and had suggested that the rise in fluorescence was due to photochemical deactivation of a fluorescence quencher. Govindjee *et al.* (1960), and later Butler (1962), had shown that far-red light (absorbed in system I) caused quenching of Chl *a* fluorescence excited by system II light. Butler (1962) showed that red light (absorbed in system II) caused the recovery of fluorescence. Duysens and Sweers (1963) provided further data on antagonistic effects of light absorbed by PS I and II on Chl *a* fluorescence; they showed that in the presence of 3-(3',4'-dichlorophenyl)-1,1,-dimethylurea (DCMU), which blocks electron flow from PSII to the intersystem intermediates, quenching of fluorescence by PSI light did not occur, and they proposed the Q hypothesis. Chlorophyll *a* fluorescence was high when Q^- was present, and was low when Q was present. Stiehl and Witt (1968, 1969) discovered an absorbance change in the ultraviolet region and proposed that a species, which they called X-320, is the "primary" acceptor of system II; it was

caused by the reduction of a plastoquinone molecule to its semianion. Knaff and Arnon (1969) discovered an absorption change at 550 nm (labeled C550), which was also suggested to be the primary acceptor. Thus, Q, C550, and X-320 competed to be accepted as the primary acceptor. It is now clear (see Butler, 1973) that C550 is only an indicator of changes in the primary acceptor, especially because it appears as an absorption shift (decrease in absorbance at 548 nm, and an increase in absorbance at 543 nm).

Other indicators have been discovered—changes at 680–690 nm—which behave like C550 (Visser, 1975; Van Gorkom, 1974, 1976). It is suggested that these monitor changes in local electric field due to the reduction of Q to Q^-. The presence of Q was, of course, always measured indirectly through changes in Chl *a* fluorescence. Van Gorkom (1974) succeeded in showing the relationship between C550, Q, and X-320. He measured C550 and X-320 in the same system, and showed an excellent correlation between the kinetics of both the changes; Van Gorkom (1976) showed further correlations with Chl *a* fluorescence changes. Thus, Q is now shown to be measured as X-320, and the reduction of Q indeed leads to the production of a semiquinone anion, as measured optically. Its spectrum agrees with that published for the *in vitro* system by Bensasson and Land (1973). Recently, Knaff *et al.* (1977) have succeeded in showing that the primary photochemistry (i.e., oxidation of P680, as measured by ESR signal) stops when Q is extracted, and recovers when quinones are supplied to the Q-free chloroplasts. Thus, the identity of Q as a quinone molecule has been established. No ESR signal has yet been associatd with this species. It must exist, but is perhaps distorted or masked due to the presence of other interfering species. For a discussion of the role of Q^- in the creation of delayed light emission, see Section 3.1.1.

(*c*) *R* (*or B*). It has been known for quite some time that electrons are rapidly ($t_{1/2}$ = 200 to 600 μs) transferred out of Q^- (Forbush and Kok, 1968; Zankel, 1973), or out of X-320 (Stiehl and Witt, 1968, 1969). Where do they go? In the past, it was believed that they were transferred to the plastoquinone (PQ) pool. Bouges-Bocquet (1973) and Velthuys and Amesz (1974), independently reported evidence for the existence of another component called B or R, which they suggested accepts electrons from Q^-. Bouges-Bocquet (1973) measured the reduction of methyl violgen (an electron acceptor for system I only) in system I light, and found that the number of electrons reaching methyl viologen was higher after the 2nd and 4th than after the 1st and 3rd pre-illuminating flashes in system II; this experiment led to the suggestion that a component B is reduced by its first pre-illuminating flash to B^-, and then by the second flash to B^{2-}, and only when B^{2-} was formed were electron transferred to the PQ pool and then to PSI. On the other hand, Velthuys and Amesz (1974) used a system in which

the O_2 evolving system was blocked, and an artificial electron donor was used to donate electrons to PSII. They then, gave the system a series of light flashes, injected dithionite or DCMU (an inhibitor that blocks electron flow out of Q^-) after each flash, and measured the dithionite- or DCMU-induced increase in Chl *a* fluorescence (ΔF). In these experiments, binary oscillations were observed, with maxima after the odd-numbered flashes, and minima after the even-numbered flashes. These data were interpreted in the following scheme:



(4)

Dithionite was suggested to reduce R, Q, and R^- via the PQ pool in a two-electron-transfer reaction. After an odd number of flashes: reactions (1) and (2) occur with the formation of QR^-, and addition of dithionite leads to the formation of PQ^{2-} chemically from PQ. The PQ^{2-} + QR^- produce Q^-R^{2-} + PQ; here, fluorescence is high because of the presence of Q^-. After an even number of flashes, reactions (1) through (5) occur, and after the addition of dithionite, QR^{2-} is produced as PQ^{2-} + QR → QR^{2-} + PQ, and the fluorescence is low because Q is in its oxidized state.

The DCMU effect is explained as follows. After an odd number of flashes, QR^- is produced; the addition of DCMU changes the redox potential of R with respect to Q such that the QR^- → Q^-R reaction occurs and, the fluorescence is high. After an even number of flashes, QR is produced and DCMU cannot produce any changes, and the fluorescence is low. Van Best and Duysens (1975) have confirmed the existence of R in *Chlorella* cells, but the arguments are too complicated to describe here (see Schreiber and Vidaver, 1975). Pulles *et al.* (1976) have obtained data which show that R is a plastoquinone molecule, since they observed an oscillation with a periodicity of 2 in an absorbance change at 320 nm. Govindjee *et al.* (1976) found that in chloroplasts depleted of bicarbonate anions, a capacity to store three electrons exists on the quinone side—one on Q and two on R, because the fluorescence yield is blocked in the high yield state only after 3 flashes. The lifetime for electron donation from R^{2-} to PQ has not been directly measured; indirect evidence indicates that it is of the order of 1 ms.

The experiments of Govindjee *et al.* (1976) also showed that bicarbonate ion (or CO_2) is necessary for the normal rate of reduction of the PQ pool [step 5 in Eq. (4)]; in the absence of CO_2, the halftime of this reaction ($t_{1/2}$ ~ 150 ms) becomes a bottleneck for the electron flow, whereas the

normal bottleneck reaction ($t_{1/2} \sim 20$ ms) is in the oxidation of PQH_2. The conclusion that CO_2 is required for the normal flow of electrons from R to the PQ pool has been confirmed by the biochemical experiments of Khanna *et al.* (1977), and by the absorption measurements of the PQ system by Siggel *et al.* (1977). (For a review on the bicarbonate effects on electron flow in chloroplasts, see Govindjee and Van Rensen, 1978.)

(*d*) *Plastoquinone pool.* Plastoquinone is present in large quantities in chloroplasts; its concentration is 10 to 20 times that of the reaction center P680. There are several types of PQ (PQ-A, PQ-B, PQ-C, etc.) (for a review on PQ, see Amesz, 1973, 1977). The presence of a pool of intermediates, at the above concentration, was shown by two types of measurements.

First, an O_2 burst in chloroplasts without addition of external electron acceptor (Fork, 1963; Kouchkovsky and Briantais, 1963; Kouchkovsky and Joliot, 1967) suggests the presence of a pool of electron acceptors. Kouchkovsky and Briantais (1963) showed that the O_2 burst was reduced if PQ was extracted with hexane (see R. Govindjee *et al.*, 1970).

Second, if chloroplasts are illuminated with continuous light, Chl *a* fluorescence yield increases in a biphasic fashion and reaches a maximum. If DCMU is added, the area bounded by the fluorescence curve and its asymptotes decreases to a value 1/10 or 1/20 that of the control. The larger area over the fluorescence curve is interpreted to be due to the reduction of a pool of intermediates, and the smaller one to be due to Q (see Joliot, 1965; Malkin and Kok, 1966; Malkin, 1966; Forbush and Kok, 1968; Amesz *et al.*, 1972).

The involvement of PQ in photosynthetic electron transport was shown by direct absorption measurements in the 260–300 nm region by Klingenberg *et al.* (1962), Amesz (1964), and Rumberg *et al.* (1964); its concentration was calculated to be ~10 molecules per reaction center molecule. Using long flashes of light, Stiehl and Witt (1968, 1969) calculated 6.5 to 10 electron equivalents on PQ molecules per reaction center molecule. The antagonistic effects of light II and I on the reduction and oxidation of PQ would prove its key role shown in Fig. 1. Q, R, and PQ are all plastoquinones, and therefore absorption changes in the ultraviolet region measure all the species; thus, care should be exercised in future experiments to sort out these changes (Siggel *et al.*, 1977).

(*e*) *Z.* The nature of Z is unknown. It has been suggested (see Sections 2.3.3a, 2.3.3b, and 3.3.4) that there are two Z's (labeled Z_2 and Z_1). The Z_2^+ produced by the flow of positive charge from $P680^+$ (produced as a consequence of light reaction) to Z_2 via Z_1, we believe, is measured by ESR signal II_{vf} (very fast) (Blankenship *et al.*, 1975; Warden *et al.*, 1976). Their chemical nature is not known. However, we consider it likely that they may

involve very tightly bound Mn (Cheniae and Martin, 1971) and, perhaps, another plastoquinone (Okayama, 1974; Sadewasser and Dilley, 1977). The line shape of ESR signal II_{vf} is like that of ESR signal II (discovered by Commoner *et al.*, 1956), which may involve quinone-type species (Kohl, 1972). It is clearly not a semiquinone signal; perhaps, Mn has distorted its electronic configuration. Schmidt *et al.* (1976) have described the preparation of an antibody against a 11,000 molecular weight polypeptide. This antibody inhibits electron flow when benzidine, but not when diphenyl carbazide (DPC) is used as an electron donor. Jursinic (1977) has suggested that DPC does not donate electrons to $P680^+$, but to Z^+, perhaps Z_1^+. Therefore, the 11,000 molecular weight polypeptide may be a constituent of Z_2, as benzidine may donate electrons to Z_2. These are, obviously speculations and need further tests.

(f) The charge accumulator. The Z_2^- receives an electron from the charge accumulator labeled M as: $M^{x+} + Z_2^- \rightarrow M^{(x+1)+} + Z_2$, where x = 0, 1, 2, 3, and 4; the superscripts refer to the number of positive equivalents it can accumulate.

We note here that the most frequently used terminology for the charge accumulator is "S" (Kok *et al.*, 1970); in this review, this term has been reserved for the state of the entire reaction center II complex. That is, S_1 = $M^{1+} \cdot Z_2 \cdot Z_1 \cdot P680 \cdot Q$, which, upon illumination, within 20 ns, forms S_1' ($M^{1+} \cdot Z_2 \cdot Z_1 \cdot P680^- \cdot Q^-$). This, then, relaxes in darkness to S_2 ($M^{2+} \cdot Z_2 \cdot Z_1 \cdot P680 \cdot Q$) through several dark reactions, including transfer of electrons out of Q^- to R and transfer of positive charges all the way down to M.

Velthuys (1976) and Etienne (see Velthuys, 1976) have suggested the possible existence of M^{1-} because they were able to observe delayed light emission when the PSII was in the state M^0Q^-. These authors injected 50 μM DCMU after giving 2 flashes that were assumed to have converted M^{1+} to M^{3+}. Then, a third flash was given that converted M^{3+} to M^{4+}, and finally to M^0 in darkness. As DCMU was present, Q^- stayed as Q^-, and no delayed light emission was expected as the system should be in the M^0Q^- state. However, a surge of delayed light was emitted leading to the suggestion that under these conditions, M^0Q^- recombine to yield $M^{1-}Q$ + light. The existence of M^{1-} (also called S^{1-}) has been confirmed by Velthuys and Kok (1978).

In all likelihood, M includes 4–6 atoms of "loosely bound" Mn/P680 (see Cheniae, 1970; Blankenship *et al.*, 1975; Wydrzynski *et al.*, 1975; Den Haan *et al.*, 1976; Govindjee *et al.*, 1977*a*,*b*). Whether a cluster of 4 Mn atoms undergo redox changes from 2+ to 3+ during $M^0 \rightarrow M^{4+}$ transitions, or higher oxidation states of Mn are involved is not yet known. The ESR II_{vf} (Z_2^+ ?) recovers with a halftime of 600–900 μs; this indicates the reaction time for the electron flow from H_2O to Z_2^+.

The actual O_2 evolution step involves reaction of M^{4+} with $2H_2O$ to yield: $M^0 + O_2 + 4H^+$ (M^{4+} is produced after 4 photoacts in PSII have been completed). Govindjee *et al.* (1977*a*) (see also Wydrzynski, 1977; Govindjee, 1978*a*) have presented a theory, based on NMR measurements (Wydrzynski *et al.*, 1976) of the relaxation of water protons, in which H_2O donates electrons to the oxygen evolving system in steps prior to the step mentioned above. Independently, Fowler (1977), Junge *et al.* (1977), and Saphon and Crofts (1977) also suggest that all H^+ are not released in the last step, and that the scheme given earlier by Kok is too simple.

Joliot *et al.* (1969, 1971) and Kok *et al.* (1970) observed oscillations in O_2 evolution per flash as a function of the number of flashes with peaks at the 3rd, 7th, and 11th flashes; a damping of oscillations was observed. These curves were best explained by assuming that in darkness the ratio of S_1 to S_0 is 3:1 and, that there are misses (~10%) in each flash, i.e., the reaction center complex does not always advance forward. Another technical factor, double hits, had to be assumed when long flashes were used; long flashes gave O_2 in the 2nd flash, but shorter flashes gave none (Weiss *et al.*, 1971). Delrieu (1974) has pointed out that O_2 evolution data can be explained also, and perhaps better, by assuming that all misses are in the S_2 state. Lavorel (1976) has questioned the meaning of misses. He has pointed out that more misses should lead to lower quantum yield of O_2 evolution. But, in fact, when misses are high (*Chlorella* cells), quantum yield is high, and when misses are low (chloroplasts), quantum yield is low. He suggests a mechanism by which charges are not lost, but stored on some other component, and used later for photosynthesis. The S states are fairly stable (seconds to minutes), but their deactivation can be accelerated by several compounds (Renger, 1972; Renger *et al.*, 1973). For a further discussion of O_2 evolution, the reader is referred to Mar and Govindjee (1972); Joliot and Kok (1975); Diner and Joliot (1977); Radmer and Cheniae (1977); and Wydrzynski (1977).

1.4. Reactions Associated with Pigment System I

We shall not describe these reactions in any detail because Chl *a* fluorescence changes are usually not associated with the activity of this system. The reader is referred to the various reviews for references on electron carriers involved in system I (Bishop, 1971; Trebst, 1974; Avron, 1975; Bendall, 1977; and Sauer *et al.*, 1978).

Light reaction I leads to the oxidation of P700 and a reduction of the primary acceptor, which may also be a pigment molecule (by analogy with the bacterial system; see Vacek *et al.*, 1977). Electrons are transferred

to one of the several iron-containing compounds and then to a bound ferredoxin, which is suggested to have an absorbance change at 430 nm (labeled P430) (Ke, 1973). From there, the electrons flow to ferredoxin (Fd), and then ultimately to $NADP^+$; this reaction is catalyzed by ferredoxin–$NADP^+$ reductase. A compound labeled FRS (ferredoxin-reducing substance) seems to be needed for the reduction of ferredoxin, but it may not be an intermediate. Oxidized P700 picks up its lost electron from a copper protein plastocyanin via a still unknown intermediate. Whether

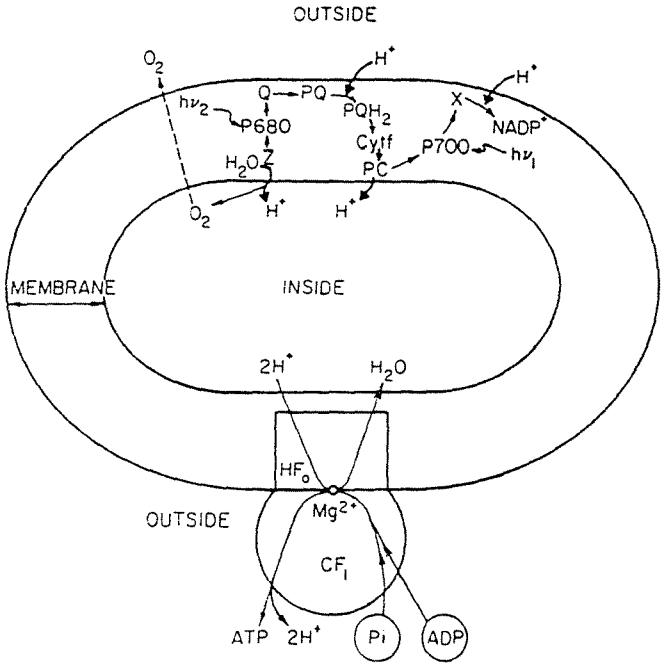


Fig. 2. Diagram of a thylakoid vesicle. Electron transport carriers are located in the membrane. Hydrogen carriers are on the outer side and electron carriers on the inner side. Protons are translocated from the outside to the inner space of the vesicle as a natural consequence of the vectorial electron flow in photosynthesis. During photophosphorylation [conversion of adenosine diphosphate (ADP) and inorganic phosphate (P_i) to adenosine triphosphate (ATP)] energy available from the pH gradient (ΔpH) and the membrane potential [together, proton motive force (pmf), created by light reactions] are utilized through the mediation of ATP-synthetase or coupling factor (CF_1)—the base piece embedded in the hydrophobic interior being labeled as HF_o here. The active surface is the interface region between HF_o and CF_1. Other symbols are: Z = electron donor to the reaction center Chl *a* II (P680); $h\nu_2$ = light absorbed in system II; Q = "primary" electron acceptor of system II; PQ/PQH_2 = oxidized and reduced plastoquinone; cyt *f* = chtochrome *f*; PC = plastocyanin; P700 = reaction center Chl *a* I; $h\nu_1$ = light absorbed in system I; X = "primary" electron acceptor of system I; and $NADP^+$ = nicotinamide adenine dinucleotide phosphate. See Fig. 1 for further details.

cytochrome (cyt) *f* delivers electrons to $P700^+$, in a parallel pathway, needs to be checked. The real bottleneck reaction of the entire chain is in the electron flow from PQ to Cyt *f*; it is of the order of 20 ms. The recovery of $P700^+$ to P700 in darkness is polyphasic, indicating the various stepwise reactions which fill it.

Two cytochromes (Cyt b_{559} and Cyt b_6) are also present in thylakoid membranes. Their possible roles in cyclic electron flow around PSII and PSI, respectively, needs to be explored (Cramer and Whitmarsh, 1977). It is likely that they play a role as electron shunts involved in the transfer of protons across thylakoid membranes, but this needs experimental support.

1.5. Arrangement of Components in the Membrane

A general picture concerning the location of the electron transport intermediates has recently emerged (Trebst, 1974). We present below a similar picture (Fig. 2). Electron donors of PSI and PSII seem to be on the inner side of the membrane, whereas, electron acceptors of PSI and PSII are on the outer side of the membrane. The reaction center chlorophylls (P700 and P680) are not necessarily on the inner side; they could be in the middle of the membrane (accessible from outside by some techniques). This picture is not quite consistent with the earlier ones in which the entire PSI was located more towards the outside than was PSII; the reconciliation between the two pictures is still unclear.

2. VARIATIONS IN CHLOROPHYLL *a* FLUORESCENCE YIELD

2.1. Chlorophyll *a* Fluorescence: Background

2.1.1. Historical

Brewster (1833; see Stokes, 1852) discovered fluorescence when he was measuring light absorption by passing sunlight through "green juices of plants"; he had called this phenomenon opalescence or imperfect transparency, and later, in 1846, internal dispersion. Stokes (1852) also observed the red fluorescence of Chl in alcohol extracts of leaves but, in addition, he also noted the yellow-orange fluorescence of what we now call phycobilins in intact marine algae and in water extracts of the same. The relationship between fluorescence and photosynthesis was suggested by the observations of Kautsky and Hirsch (1931) that, upon exposure to light, dark-adapted leaves show variations in fluorescence intensity complementary to changes

in photosynthesis. Chl *a* fluorescence of living cells, chloroplasts, and subchloroplast particles has now become a powerful tool in the analysis of photosynthetic reactions.

2.1.2. General

Upon illumination, photosynthetic pigments absorb light, reemit only a few percent of this light as fluorescence, and use the largest portion of the absorbed energy for photosynthesis. Chl *a* is the predominant fluorescent pigment. The Chl *b* is either nonfluorescent or very weakly fluorescent, since it transfers its absorbed energy to Chl *a* with almost 100% efficiency. Phycobilins are fluorescent to different degrees depending upon their efficiency of energy transfer to Chl *a*. The Chl *a* fluorescence at physiological temperature originates mainly from the "bulk" pigments, and not from the very few reaction center molecules. Nevertheless, the emitted fluorescence is sensitive to alterations in photochemistry taking place at the reaction centers, particularly that of photosystem II.

Various properties of Chl *a* fluorescence can be measured: excitation spectra, emission spectra, lifetime, degree of polarization, and quantum yield. Historically, the fluorescence yield (ϕ_f) has proven to be the most useful parameter for studying kinetics due to the ease with which the time variations in fluorescence yield can be measured, compared to other fluorescence properties.

On the other hand, shifts in fluorescence maxima provide information about changes in the microenvironment of the chromophore and sometimes about chemical transformations. For example, the disappearance of multiple emission bands and the formation of a single band upon extraction of pigments suggests the presence of the same chromophore *in vivo* in different associations (with other chromophores, with proteins or lipids, or in regions of different solvents); changes in quantum yield and lifetime of excited state indicate closing or opening of the reaction centers (or traps); depolarization of fluorescence indicates excitation energy migration; and polarization indicates the presence of oriented chromophores. Action spectra of fluorescence provide information on the participation of pigments in excitation energy transfer, and emission spectra show the nature of various fluorescence species present in the system. Measurements of these spectra have provided information on the composition of the two pigments systems, and on the excitation energy transfer between the different photosystems (I and II). Thus, those pigments that are coupled to the photochemical reaction of photosynthesis are singled out.

Variations in Chl *a* fluorescence yield following illumination and during illumination have been measured in the range of picoseconds to minutes. In

all time ranges the fluorescence yield shows a dynamic character, the meaning of which is still being investigated.

What is important to emphasize is that we can monitor fluorescence changes on a time scale and with sensitivity not easily approachable by other techniques. Moreover, we can follow changes in photosynthetic process without damaging the system; it is indeed a nondestructive tool.

In the present review, we shall restrict our discussion to (a) the quantum yield and the lifetime of fluorescence (Section 2.2) and (b) the fast fluorescence transient after ns and μs flashes (Section 2.3). For earlier reviews on the use of Chl *a* fluorescence in the study of photosynthesis, including those dealing with slow changes in Chl *a* fluorescence yield with continuous illumination, the reader is referred to Rabinowitch (1956), Weber (1960), Butler (1966), Papageorgiou (1968), Munday (1968), Cho (1969), Mar (1971), Govindjee and Papageorgiou (1971), Goedheer (1972), Mohanty (1972), Govindjee *et al.* (1973), Mohanty and Govindjee (1974), Papageorgiou (1975), Lavorel and Etienne (1977), and Butler (1977, 1978).

2.2. Lifetime and Quantum Yield of Fluorescence

2.2.1. General

Photochemistry of pigment system II seems to be in competition with Chl *a* fluorescence intensity, as evidenced by the antiparallel relationship between the two upon illumination of dark-adapted photosynthesizing systems. On the other hand, system I photochemistry changes do not lead to changes in the already-weak fluorescence of pigment system I Chl *a*. Changes in fluorescence intensity need not mean true changes in the quantum yield of fluorescence, since the photons may be absorbed by increasing or decreasing concentrations of nonfluorescent pigments. Thus, measurements on the lifetime of fluorescence are necessary to measure true changes in the quantum yield of fluorescence (ϕ_f); this is related to the measured lifetime (τ) and the intrinsic lifetime (τ_0) of the excited state as: $\tau = \phi_f \tau_0$, where τ_0 can be calculated from the absorption band of the emitting state. A knowledge of τ provides information on the time needed for the major competing reaction of photosynthesis, the fluorescence. It can also provide information on the number of pigment molecules an absorbed quantum can visit. This sets a limit on the time needed for primary photochemistry.

Simultaneous measurements of τ and ϕ_f can provide information on the organization of a photosynthetic unit (PSU). For example, if τ varies linearly with ϕ_f, we may suggest that several reaction center Chl *a*

molecules (the energy traps) are available to photons absorbed in a "lake" of antenna (light harvesting) pigments; if one trap is closed (i.e., in $P680^+Q^-$, $P680^+Q$, or $P680Q^-$ state), excitons can migrate to another open trap ($P680 \cdot Q$) and be utilized there. The above is also called a multicentral or a dynamic model. On the other hand, if there are separate independent photosynthetic units (each PSU of about 300 Chl molecules with their own reaction centers) without the possibility of energy exchange, then we have to deal with two populations of lifetimes (τ closed and τ open), and a nonlinear convex relationship is expected. This model is called "puddle," independent unit, unicentral, or statistical model. An intermediate model, in which exciton can be exchanged between a few (e.g., 3 or 4) PSU would give an intermediate convex curve between τ and ϕ_f; this model has been dubbed as a connected model, or a pond model. Thus, measurement of τ and ϕ_f could provide information on the topology of photosynthetic units.

Finally, if triplet states are involved in the main pathway of photosynthesis, both τ and ϕ_f may remain unchanged during changes in photochemistry. Moreover, the possibility of delayed fluorescence, by transition of triplet (T_1) to singlet (S_1) followed by its decay may exist. This would give a much longer ($\sim\mu$s) lifetime of measured emission.

2.2.2. Methods

Basically, there are two methods for measuring the lifetimes of excited states: (a) the flash method, and (b) the phase method. The flash method, in principle, employs an ultrafast flash to excite molecules to be followed by direct measurements of the decay of fluorescence. τ is the time at which the fluorescence intensity becomes $(1/e)$th of that at zero time. In the past, the flashes were not short enough (ns), and corrections had to be made for the flash; many improvements have been made in the correction methods; more importantly, ps flashes are available now. The phase method, which assumes a single exponential decay, is useful down to the sub-ns range. One measures the phase delay between fluorescence and incident light, and calculates τ as $\tan \theta/2\pi f$, where θ = phase shift and f = frequency of modulation. By using different frequencies of modulation, one can easily check if the decay is exponential or not.

2.2.3. Experimental Results: Early Measurements

In general, the accepted value for τ for Chl *a in vitro* is $\sim$5 ns, whereas the τ *in vivo* at low light intensities of excitation, where photochemistry is most efficient, is $\sim$0.5 ns. The quantum yield of fluorescence measured as

the number of emitted quanta per number of absorbed quanta (ϕ_f) *in vivo* ranges from 1.5 to 6.0%, whereas it is 30% *in vitro* (Weber and Teale, 1957; Latimer *et al.*, 1956). This shows an efficient trapping of energy *in vivo*, if other routes are not important.

Brody and Rabinowitch (1957) and Dmetrievsky *et al.* (1957) independently measured, for the first time, the lifetime of the excited state of Chl *a in vitro* and *in vivo* by flash and phase delay methods, respectively. During the succeeding years, several investigators confirmed and extended the measurements on τ as a function of light intensity, concentration of open traps, and temperature (Tomita and Rabinowitch, 1962; Butler and Norris, 1963; Murty and Rabinowitch, 1965; Nicholson and Fortoul, 1967; Tumerman and Sorokin, 1967; Müller *et al.*, 1969; Singhal and Rabinowitch, 1969; Govindjee *et al.*, 1972; Mar *et al.*, 1972; Borisov and Godik, 1972; Briantais *et al.*, 1972; Borisov and Il'iana, 1973; Moya, 1974; Hervo *et al.*, 1975; Govindjee *et al.*, 1975; Moya *et al.*, 1977). Table I shows a summary of some of the results.

Merkelo *et al.* (1969) devised a mode-locked He-Ne laser that had the capability to measure lifetimes (τ) down to 80 ps, in order to check the earlier values. The τ for Chl *a* in the green alga *Chlorella* at high light intensity was in the range of 1.4 ± 0.05 (phase) to 1.6 ± 0.2 (direct flash) ns—in fair agreement with the earlier measurements. In the presence of an inhibitor of electron flow, the maximum value of τ was measured to be 1.94, in good agreement with 1.97 ± 0.03 measured by Müller *et al.* (1969). Mar *et al.* (1972), using the phase shift method and the mode locked laser, measured the lifetime of fluorescence in several algae under conditions when photosynthesis was not operative to obtain the τ_{max} and the rate constants (k) of deactivation (including transfer to nonfluorescent Chls) and of trapping; the estimated quantum yield of fluorescence (ϕ_f) for three algae are shown in Table 2.

The ϕ_f, calculated from τ values, is the yield of the fluorescent species only. If there are absorbing species that do not fluoresce then ϕ_f, measured by the usual method of counting the number of emitted quanta per number of absorbed quanta, will be lower than ϕ_f calculated from τ values. One can make the transformation by defining $\phi_f = a\,(\tau/\tau_0)$, where a = fraction of absorbing molecules that fluoresce, $1-a$ = fraction that do not fluoresce. Quenching of fluorescence yield (ϕ_f) could then be due to (1) decrease in a; (2) decrease in τ, or (3) increase in τ_0 (which is usually assumed to be constant). If, in an experiment, τ is found to remain unchanged, but fluorescence intensity decreases, then we know that a has changed (perhaps, by formation of nonfluorescent complexes), and we call this process *static* quenching. However, if a = constant, then fluorescence intensity is $\propto \tau$, and a decrease in τ means a decrease in ϕ_f, and thus an increase in rate

TABLE 1. Lifetime Measurements: Nanosecond Flashes or Phase Method[a]

Reference	Sample	τ, ns
Brody and Rabinowitch (1957) (Flash)	*Chlorella*	1.6
	Porphyridium	1.5
	Anacystis	1.2
Müller *et al.* (1969) (Phase)	*Chlorella* (5×10^2 ergs $cm^{-2} s^{-1}$)	0.46–0.61
	Chlorella (1.5×10^4 ergs $cm^{-2} s^{-1}$)	1.15
	Chlorella + 5 μM DCMU (1.5×10^4 ergs $cm^{-2} s^{-1}$)	1.91–2.01
	Chlorella + 1 μM hydroxylamine (1.5×10^4 ergs $cm^{-2} s^{-1}$)	0.67
	Chloroplasts (4.4×10^2 ergs $cm^{-2} s^{-1}$)	0.58
	Chloroplasts (1.5×10^4 ergs $cm^{-2} s^{-1}$)	0.99
Briantais *et al.* (1972) (Phase; mode-locked laser)	*Chlorella* ("0" level)	0.6
	Chlorella ("P" level)	1.7
Moya *et al.* (1977) (Phase)	Chloroplasts (cation-depleted; "0" level)	0.46
	Chloroplasts (cation-depleted; "P" level)	1.36
	Chloroplasts (+10 mM Na^+; "0" level)	0.38
	Chloroplasts (+10 mM Na^+; "P" level)	0.94
	Chloroplasts (+10 mM Na^+ + 10 mM Mg^{2+}; "0" level)	0.46
	Chloroplasts (+10 mM Na^+ + 10 mM Mg^{2+}; "P" level)	1.36

[a] DCMU = 3-(3',4' dichlorophenyl)-1,1 dimethyl urea; "0" level = fluorescence when all the traps are open; "P" level = fluorescence when all the traps are closed.

TABLE 2. Lifetime and Quantum Yield of Fluorescence[a]

Algae	$\tau_{max.\ ICF}$ (ns)	τ, 77°K F685 (ns)	τ, 77°K F735 (ns)	$k_{radiationless}$ (RT, 77°K) (s^{-1})	$k_{trapping}$ (s^{-1})	$\phi_{trapping}$	$\phi_{fluorescence}$ PSII (closed center)	ϕ_f predicted for intact cells at low intensity (open centers)
Chlorella	1.7	1.4	2.3	6×10^8	1.2×10^9	66%	10%	2–3%
Porphyridium	1.0	0.9	1.2	9×10^8	2×10^9	68%	6–7%	1–2%
Anacystis	0.7	0.8	0.8	13×10^8	2×10^9	60%	5%	1%

[a] $k_{radiationless}$ = rate constant for radiationless loss; $k_{trapping}$ = rate constant for trapping; RT = room temperature. λ excitation = 632.8 nm; assumed τ_0 = 15.2 ns. Brody and Rabinowitch, 1957 (after Mar *et al.*, 1972)

constants of other deactivation processes, since $\phi_f = k_f/\sum ks$, where k_f = rate constant of fluorescence, and $\sum ks$ = the sum of all the rate constants of deactivation (k_f, k_r, k_{chem}, where k_r is for the radiationless loss and k_{chem} for chemistry).

It has been known since the time of Brody and Rabinowitch (1957) that a is not 1.0 in photosynthetic cells, because measured τ gave higher ϕ_f than the ϕ_f measured by Latimer *et al.* (1956) and Weber and Teale (1957). Later data by others (Tomita and Rabinowitch, 1962; Nicholson and Fortoul, 1967; Singhal and Rabinowitch, 1969) support this conclusion. These data suggest that a fraction of Chl *a* exists that is nonfluorescent or only weakly fluorescent.

If PSU organization is such that each unit is served by its own reaction center, and there is no energy transfer among these units, then the lifetime of fluorescence from those units that are closed will be different from those that are open. Measured τ, in the first approximation, may be expressed as:

$$\tau = \frac{\alpha\phi_1\tau_1 + (1 - \alpha)\phi_2\tau_2}{\alpha\phi_1 + (1 - \alpha)\phi_2}$$

where α = fraction of open traps. Thus τ/ϕ would be affected by changes in α. Choosing appropriate values for τ_1, ϕ_1 etc., it can be shown that τ/ϕ would first rise and then decrease as α is increased, i.e., a convex curve for τ vs. ϕ is expected. However, if excitation energy absorbed in closed units can be freely transferred to open units, only one value of τ and a linear relationship between τ and ϕ are expected. Tumerman and Sorokin (1967) were the first ones to show that τ was almost linear with ϕ. Briantais *et al.* (1972) provided additional data, and showed that within an error of ± 0.1 ns, τ was linear with ϕ in *Chlorella* suspensions. It was clear that energy migration occurred among different PSII units (Robinson, 1967). However, the number of units over which this transfer occurred could not be estimated, due to the errors involved. Moya (1974) later showed that there is a slight convexity in τ vs. ϕ curves, in agreement with the conclusions of Joliot *et al.* (1968) that the probability of this energy transfer is ~0.6, and the transfer may involve only 3 or 4 units. Such a connected model was discussed by Lavorel and Joliot (1972).

Two points should be emphasized here. τ increases almost linearly with fluorescence intensity suggesting that $\tau = \tau_0 \phi_f$ relationship holds in the first approximation; moreover, an almost linear relationship between τ and ϕ has been taken to mean excitation energy exchange between different PSII units. Secondly, as the excitation intensity is increased from a low value (open centers; high rate of photochemistry) to high value (closed centers; low rate of photochemistry), τ increases in intact *Chlorella* cells from a

value of ~0.6 to 1.9 ns, a threefold increase (Briantais *et al.*, 1972). In chloroplasts, τ increases from ~0.4 to ~1.7 ns, a 4-fold increase (Moya *et al.*, 1977).

Picosecond measurements will be discussed below. We note here that the τ values obtained by Campillo *et al.* (1976a) with low-intensity ps pulses agree with the earlier values of Briantais *et al.* (1972) for *Chlorella*.

2.2.4. Picosecond Flash Illumination

In order to appreciate picosecond measurements the reader is referred to reviews by Alfano and Shapiro (1973), Netzel *et al.* (1973), Eisenthal (1975), Busch and Rentzepis (1976), Seibert (1977), Campillo and Shapiro (1978), and Holten and Windsor (1978).

2.2.4a. Methods

The basic design of a picosecond experiment is as follows. The ultrashort, intense light pulse from the mode-locked laser perturbs the system such that a nonequilibrium condition is forced upon it. The system's return from the nonequilibrium to the normal state is then monitored by one of three basic techniques: the optical Kerr gate method, the probe-beam technique, or the streak camera approach. The bases of these monitoring techniques are summarized below.

The *optical Kerr gate method* can be used to investigate the intensity profile of light emitted from a sample as a function of time. The technique is based on the fact that intense laser pulses can induce short-lived birefringence in carbon disulfide solutions. Thus, if light emitted from a sample in response to a laser pulse perturbation is directed through a carbon disulfide cell situated between a crossed polarizer, no light is transmitted through the "gate," except for that portion of the emitted light that arrives at the carbon disulfide cell simultaneously with the pulse generating birefringence. Hence, by varying the time at which the "gate" is open it is possible to construct the emitted light's time variations. For further details the reader is referred to Seibert and Alfano (1974), and Pellegrino *et al.* (1978).

The *probe-beam technique* allows for the examination of light absorption or light scattering phenomena as a function of time after excitation of the sample. In essence, however, this technique is the simplest of those applied to measure the system's relaxation following picosecond perturbation, the principle being that a weak probe beam derived from the intense pulse (used to excite the sample) via second harmonic generation or stimulated Raman scattering is used to interrogate the sample during the time in

which both the probe beam and the exciting pulse are coincident on the sample. Hence, by varying the path followed by the probing pulse, the parameter being interrogated can be measured as a function of time.

Light emitted from a sample (e.g., fluorescence) can also be investigated by the *streak-camera approach*. The streak-camera relies for its operation upon the fact the electrons are released from a photocathode in response to the light falling on it. The electrons so generated are then accelerated in an electric field, and then subsequently deflected by an applied voltage. This results in a "streaking" of the electrons across a phosphorescent screen. The overall result is that electrons, released at different times in response to the light, strike the screen at different positions. The streaking of electrons is captured photographically, and the photograph is then analyzed to obtain the lifetime of the event. For a review of the applications of the streak camera to τ measurements, see Campillo and Shapiro (1978).

2.2.4b. *Experimental Results*

Seibert *et al.* (1973) used the optical Kerr gate in conjunction with a laser capable of giving ps flashes. Seibert *et al.* (1973) and Seibert and Alfano (1974) characterized the fluorescent emission kinetics of isolated spinach chloroplasts; they found two components with (1) a lifetime of less than 10 ps (risetime, 10 ps) and (2) a lifetime of 270 ps. They speculated that the first one was due to PSI while the second one, which appeared after a 90 ps delay, was due to PSII.

Yu *et al.* (1975) studied the fluorescence emission kinetics of particles prepared from spinach chloroplast fragments enriched in PSI or PSII activity. They reported decay times of 60 $\pm$ 10 ps and 200 $\pm$ 20 ps for PSI and PSII, respectively. Furthermore, the risetimes of both PSI and PSII fluorescence emissions were found to be $\sim$5 ps. Thus, besides the fact that the 60-ps lifetime found for PSI particles did not correlate with the 10-ps component found earlier in chloroplasts, the fast risetimes found for both PSI and PSII made it difficult to visualize the significance of the rise and the dip found in the time dependence of the fluorescence from chloroplasts by Seibert and Alfano (1974). Furthermore, these lifetime values were much lower than those obtained earlier by the phase method or by ns flashes.

The fluorescence lifetime for the blue-green alga, *Anacystis*, and for *Chlorella* cells were studied by Kollman *et al.* (1975) who used a streak camera. The values were 75 and 47 ps for *Anacystis* and *Chlorella*, respectively. This work also indicated that fluorescence was completely depolarized within 10 ps after the flash excitation of the *Anacystis* sample; hence, a strong case for the existence of energy transfer was made. Kollman *et al.* (1975) further showed that it was possible to generate fluorescence

decay curves *in vitro* that mimic the *in vivo* curves by mixing pigment molecules in concentrations comparable to those found in algae. Measurements with the streak camera were extended by Shapiro *et al.* (1975), Beddard *et al.* (1975), and Paschenko *et al.* (1975). Most of the lifetime values obtained by these authors were also lower than those obtained earlier by ns flashes or the phase method.

Beddard *et al.* (1975) reported the following τ values: 108 ± 10 ps (*Chlorella*), 92 ± 10 ps (red alga *Porphyridium*), and 130 ± 10 ps (isolated chloroplasts). They also decreased the light intensity over an order of magnitude and concluded that their intensities were not high enough to cause nonlinear effects (such as exciton annhilation that would reduce τ) because their τ values remained independent of intensity. As we will see later, this conclusion is no longer valid.

Mauzerall (1976*a*, 1978) pointed out the difficulties with the use of high intensities—multiple excitations in photosynthetic units during a brief light period leading to a decrease in both ϕ_f' and τ. Mauzerall (1976*a*) demonstrated his point by showing a decrease in ϕ_f' with 7-ns laser pulses with increasing light intensity. This was confirmed for ps pulses by Campillo *et al.* (1976*b*). Soon thereafter Campillo *et al.* (1976*a*) showed clearly that decreasing the intensity of ps laser flashes increases the lifetimes. Campillo *et al.* (1976*a*) estimated a value of 650 ps (raw data value was still too low: 350 ± 50 ps) for low intensity excitation (10^{14} photons cm^{-2}/20ps flash). At 3×10^{14} photons cm^{-2}/flash, τ was 175 ± 25 ps, and at 3×10^{15} photons cm^{-2}/flash, it was 50 ± 10 ps. Thus, τ at low intensity measured with ps lasers is in the same range as τ measured earlier with ns flashes (see Section 2.2.3).

The low τ values with high intensity lasers are, perhaps, due to singlet–singlet or singlet–triplet annihilation. Possibility of (a) conversion to higher excited states exists in a multiquanta process, and (b) trapping in closed reaction centers occurs, which does not lead to fluorescence from the first excited singlet state. Kung and DeVault (1978) and DeVault and Kung (1978) have indeed obtained evidence for fluorescence from higher excited states in photosynthetic bacteria supporting possibility (a). Recently Hindman *et al.* (1977) have observed lasing action in Chl *a* solutions at extremely high intensities ($\sim 10^{25}$ photons cm^{-2}/s, or 10^{17} photons cm^{-2}/10 ns flash). The τ at extremely high intensities could be, in principle, lower simply because stimulated emission has a low τ. It is doubtful, however, that this phenomenon plays any significant role in the lower value of τ observed *in vivo*. The singlet–singlet or singlet–triplet annihilation theory seems adequate to us at the present time. No evidence for a Chl *a in vivo* laser has yet been reported. Also, the extremely high intensities needed for the lasing action have not been used in the experiments discussed here.

The problem of annihilation of singlet states at high light intensities has

been dealt with, among others, by Swenberg *et al.* (1976, 1978). Geacintov and Breton (1977) and Geacintov *et al.* (1977*a*) observed a dramatic decrease in the fluorescence emission at 735 nm at 100°K with increasing light intensity or the number of ps laser pulses; fluorescence at 685 nm was also affected, but to a lesser extent. At 2×10^{18} photons cm^{-2}/pulse train, 735-nm emission was almost absent. This suggests that exciton annihilation may be more sensitive in pigment system I than in pigment system II; this may be due to annihilation of singlets by triplets. However, using single ps pulses, Geacintov *et al.* (1977*b*) have shown equal sensitivity of 735- and 685-nm emissions to light intensity. (Obviously, triplets were not allowed to be formed here!)

It is now generally accepted that τ measured with low-intensity, single ps pulses are "true" τ. Also, high-intensity, single ps pulses produce singlet–singlet annihilation processes (which must be experimentally proven, however), and high-intensity, multiple ps pulses produce, in addition, singlet–triplet annihilation processes (which must also be experimentally proven). Beddard *et al.* (1975) observed intensity independence of τ (see above) because triplets, produced with the early ps pulses, must have lasted throughout their experiment with multiple ps pulses.

Low-intensity ps pulses can be successfully used to monitor the relevant ϕ_f and true τ, and high-intensity ps pulses to study the topology of PSU, because exciton annihilation may provide information on the Chl–Chl interactions and the nature of interactions in the PSU.

For further details, the reader is referred to the following publications: Breton and Geacintov (1976), Harris *et al.* (1976), Beddard and Porter (1977), Campillo *et al.* (1977), Paschenko *et al.* (1977), Porter *et al.* (1977), Searle *et al.* (1977), Yu *et al.* (1977), and Govindjee (1978*b*).

Finally, one might question: Why should one use ps methods if the true τ are in the ns range? Among the advantages, the two that stand out are: (a) true kinetics of fluorescence rise and decay can be measured, and (b) information on the time of energy transfer from one pigment to the other can be measured. Thus, it is expected that the ps method will continue to provide new information.

2.3. Transients After ns or μs Flash Illumination

2.3.1. Methods

Measurement of these changes in Chl *a* fluorescence yield demands certain equipment characteristics not required for slower transient experiments. First, the detecting element—in most cases a photomultiplier—must

have a properly designed dynode chain and anode circuit to allow submicrosecond rise with linear response. Second, the most desirable actinic light source must be capable of delivering one quantum per PSU in a time span less than the lifetime of the most rapid reaction of interest. Low intensity light with pulse widths of many microseconds complicates data analysis since the observed changes depend on the photon flux rather than on the reaction rates. However, at high light intensities, the possibility of multiphoton hits at the reaction center exists.

In practice, the fluorescence yield is determined using the following relationship:

$$F(t) = \phi_f(t)\, \sigma(t)\, I \tag{5}$$

where $F(t)$, $\phi_f(t)$, and $\sigma(t)$ are the fluorescence intensity, fluorescence yield, and optical cross section at time t, respectively, and I is the incident analytic light intensity. Fluorescence is normally described in relative units as compared to the zero level fluorescence, the fluorescence yield in weak light prior to strong excitation. The relationship is as follows:

$$\frac{F(t)}{F_0} = \frac{\phi_f(t)\, \sigma(t)\, I}{\phi_0 \sigma_0 I} = \frac{\phi_f(t)\, \sigma(t)}{\phi_0 \sigma_0} \tag{6}$$

where F_0, ϕ_0, and σ_0 are the fluorescence intensity, fluorescence yield, and optical cross section in weak light prior to actinic excitation. In all the recent literature on fast fluorescence, except for Mauzerall (1972), $\sigma(t)$ and σ_0 have been tacitly assumed to be identical by operationally defining $\phi_f(t)/\phi_0 = F(t)/F_0$. The assumption that the optical cross section is time invariant after an excitation flash may lead to an erroneous assignment of intensity changes to yield changes. This possible deficiency of the method must be kept in mind when reading literature in this field.

The two general techniques for measuring rapid changes are: (a) the double illumination method in which an excitation flash is followed by a variable-delay weak analytic flash, the latter causing little activation of photocenters; and (b) the single illumination method in which the same illumination flash is used for both actinic and analytic purposes.

The double illumination method was used by Mauzerall and Malley (1971), with measurements made up to 100 ns after an excitation flash that had a pulse width at half-height of 10 ns. Within 100 ns after a single saturating excitation, a doubling in fluorescence yield was observed. Identical measurements over an extended time range from 10 ns to 50 ms (Mauzerall, 1972) showed the fluorescence yield changes to be quite complex (Fig. 3): the maximum occurs at about 20 μs after the flash, but only following the first flash after 10 min of dark adaptation did the yield show an inter-

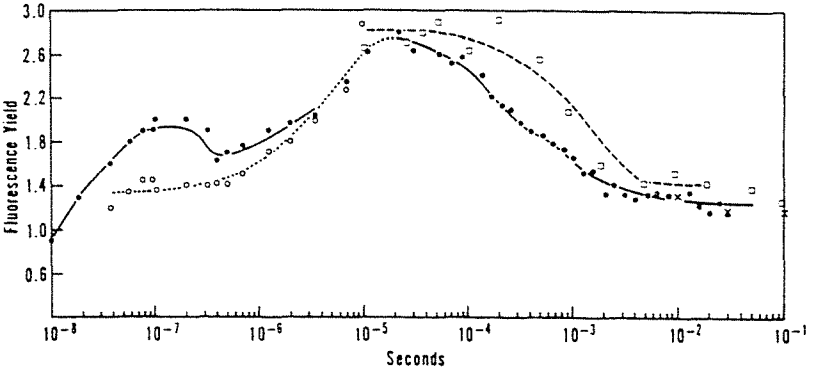


Fig. 3. The Chl *a* fluorescence yield at 685 ± 25 nm of fully dark-adapted green alga *Chlorella* at 25°C is plotted (●) against time after a single saturating 10-ns pulse of light at 337.1 nm. The delayed or test flash was a portion of the same 10-ns laser flash for the time range 10–100 ns, and an electronic flash filtered through a Corning 4-72 glass for 100 ns and longer times. Cooling the algae to 5°C produces no change in the rise at 25 ns, but has a large effect on the decay in the ms time range (□). The fluorescence yield for short times after the fourth flash in a series, each separated by 50 ms, is also plotted (○). Light intensity for the first main flash is about 3×10^{14} light quanta/cm² From Mauzerall (1972).

mediate maximum at about 100 ns. The rise in the 1–20 μs range had only a small sensitivity to flash number, and only the decay after 20 μs showed any temperature effects.

Den Haan *et al.* (1974) used the single illumination method. Excitation and analytic light were provided by a xenon flash, which had a width at half height of 13 μs and an extended tail. Fluorescence rise data observed using this method is shown in Fig. 4. Comparison with the 5–20 μs range of Fig. 3 shows similarity of the results. However, in the 1–5 μs range, the

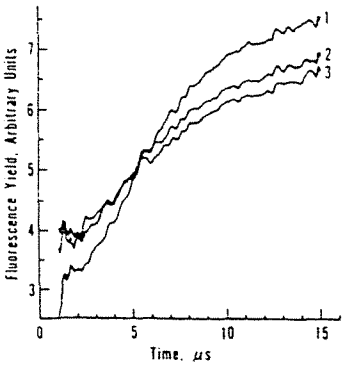


Fig. 4. Chlorophyll *a* fluorescence yield rise in *Chlorella* after 10 min of dark adaptation. The flash intensity was just able to saturate oxygen evolution and flashes were given as numbered. Data taken from Den Haan *et al.* (1974).

fluorescence yield is lower in the first flash in the single, as opposed to the double, illumination technique. This difference is most probably a complication generated by the single illumination method since the excitation flash delivers quanta over a period of time (many μs) that is much longer than the relaxation time of fluorescence yield altering the reactions. In other words, the data at $t < 5$ μs with the single illumination method are affected by both the time variation in quanta delivery and the actual reactions.

Delosme (1967) used an ingenious method to measure rapid changes in chlorophyll fluorescence yield: a 1000-W xenon lamp as a light source and a bullet driven shutter. The opening time of the shutter was about 5 μs, and increases in fluorescence as rapid as 50 μs were observed using this continuous light technique. Interpretation of results on the μs scale was not meaningful with this technique since, on the average, only one quantum was being absorbed per PSU every 15 μs.

2.3.2. Interpretations of the Fast Transient

2.3.2a. The Q' Hypothesis

One of the earlier attempts to measure rapid changes in Chl *a* fluorescence yield was that by Sybesma and Duysens (1966) (Table 3). Using a low intensity flash lamp for excitation in a single illumination-type experiment, a 10-μs rise in fluorescence yield was observed, followed by a slower rise during the tail of the flash-lamp emission. In order to explain

TABLE 3. Microsecond Chlorophyll *a* Fluorescence Rise Components

Component	Method	Reference
25–35 ns (after 1st flash only)	Double illumination	Mauzerall (1972)
≤ 1 μs (after 1st flash only)	Single illumination	Duysens *et al.* (1975)
3–10 μs	Double illumination	Mauzerall (1972)
		Jursinic and Govindjee (1977*a*)
	Single illumination	Duysens and Sybesma (1966)
35–50 μs	Observed with μs-flashes	Zankel (1973)
		Joliot (1974)
		Joliot (1977)
		Joliot and Joliot (1977)
	Not observed with ns-flashes	Mauzerall (1972)
		Duysens *et al.* (1975)
		Jursinic and Govindjee (1977*a*)

this result, two light reactions were proposed, the faster one driving Q, the primary acceptor of PS II, into its nonquenching form Q′ and the slower one regenerating Q from Q′, a precursor of Q (Duysens and Sweers, 1963) that quenches fluorescence but is not involved in the electron transport reaction. This particular theory for fast change in fluorescence yield has the least acceptance since later experiments in which Q-related changes were blocked by DCMU (Zankel, 1973) or low temperature (Joliot, 1974) still showed the same rapid rise in fluorescence yield.

2.3.2b. *The Fluorescent State Hypothesis*

This theory was introduced by Mauzerall (1972) to explain rapid changes in fluorescence from *Chlorella* following a 10-ns laser pulse (Fig. 3). The most rapid change observed by Mauzerall (1972) had a risetime of 25 ns that was seen only after the first flash following dark adaptation of *Chlorella* cells (Table 3). Also, a 3-μs rise component, which reached a maximum at 20 μs, occurred after all other flashes. It was argued that since these changes in fluorescence yield were much slower than the Chl excited singlet-state lifetime of about 1.5 ns (Brody and Rabinowitch, 1957), a direct coupling of the photochemical reaction to the singlet state could be ruled out! (However, see next section.) Between a few tens of ns and ms not only fluorescence yield, but optical cross section, fluorescence emission spectrum, and temperature sensitivity changed, and to explain all this, six different fluorescent states were proposed (Mauzerall, 1972). Except in a descriptive sense, the relationship between the various fluorescent states and the photosynthetic mechanism was not defined. Thus, it has been very difficult to test this theory.

2.3.2c. *The $P680^-$ Quencher Hypothesis*

In this theory the rise in fluorescence yield actually corresponds to the decay of the oxidized form of the PSII reaction center Chl, $P680^+$, which is a quencher of Chl *a* fluorescence. This explanation was proposed by Butler (1972*b*) and has been used by others to explain μs changes in fluorescence yield (Den Haan *et al.*, 1974; Etienne, 1974; Joliot, 1974; Duysens *et al.*, 1975). The suggestion that $P680^+$ was a quencher of Chl *a* fluorescence was based on fluorescence measurements made at 77°K (Butler, 1972*b*; Okayama and Butler, 1972). The Chl *a* fluorescence yield remained low if Cyt b_{559} (electron donor at 77°K) was oxidized prior to illumination. Upon illumination, $P680^+$ is formed and is stable since Cyt b_{559} is in its oxidized form. It was therefore suggested that $P680^+$ was a

quencher of Chl *a* fluorescence. The validity of this hypothesis in normal chloroplasts presumes that extrapolation to room temperate is correct. Furthermore, those who have measured fluorescence changes have not simultaneously measured the formation and decay of $P680^+$, precluding any proof that this is the main reason for fluorescence changes in their system.

2.3.2d. Z^+ as a Fluorescence Quencher

Zankel (1973) observed the μs rise in fluorescence yield in a single-illumination-type experiment using a flash lamp having an extended tail. He suggested that Z, a donor of electrons to $P680^+$, in its oxidized form (Z^+) quenched Chl *a* fluorescence, and the rise in fluorescence yield reflected the reduction of Z^+. This hypothesis may be equivalent to that of $P680^+$ if, during the tail of the excitation flash, reaction centers are re-excited to form $Z^+P680^+Q^-$, and the fluorescence is quenched by $P680^+$. A more detailed discussion of this possibility will be given later.

2.3.2e. The Carotenoid Triplet Hypothesis

It has been suggested (Duysens *et al.*, 1972; Zankel, 1973) that, during excitation, photochemical generation of carotenoid triplets occurs, which quench Chl *a* fluorescence. The formation of this quencher is most easily observed if normal photochemistry is blocked by DCMU and weak background illumination (Zankel, 1973; Den Haan, 1977). Quencher formation can also be observed in normal chloroplasts and algae that have been illuminated with flashes a few μs in duration as the fluorescence yield declines by 3 to 5 μs after initiation of the flash (Delosme, 1971; Zankel, 1973) (Fig. 5.). This low fluorescence yield has been suggested to be due to quenching by carotenoid triplets (Zankel, 1973); this phenomenon is barely perceptible when the flash intensity is just able to saturate oxygen evolution (Den Haan *et al.*, 1974), but becomes increasingly pronounced at higher intensities (Duysens *et al.*, 1975). Illumination with saturating flashes of a few tenths to many μs in duration is capable of generating triplets in algae and chloroplasts (Mathis and Galmiche, 1967; Wolff and Witt, 1969), and the decay characteristics of these triplets seem to correspond well with the rise in fluorescence yield observed with the single illumination technique (Zankel, 1973; Duysens *et al.*, 1975). However, those who have measured fluorescence changes have not simultaneously measured the formation and the decay of triplets, thus precluding any proof that this is the main reason for the decline and rise of fluorescence yield in their system.

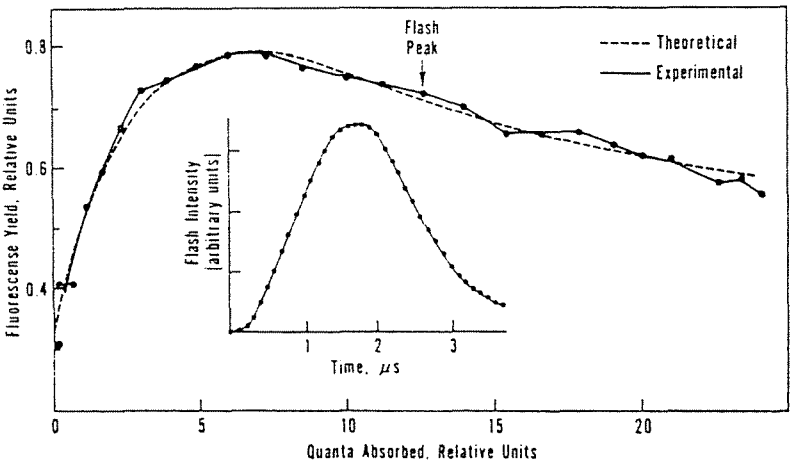


Fig. 5. Variable part of the fluorescence yield as a function of quanta absorbed during the fifth, brief (2 μs) flash given to dark-adapted chloroplasts (●——●). The quanta absorbed at time t was calculated from the time course of the intensity of the flash (insert) assuming that quanta are proportional to $\int_0^t I\,dt$, where I is flash intensity. The peak of the flash is indicated by the arrow. From Zankel (1973).

2.3.3. Experimental Results

The theories that have been put forth in the literature to explain the rapid changes in fluorescence yield have been presented above and we will now discuss a variety of experimental results.

2.3.3a. Microsecond and Nanosecond Flash Illumination

Throughout the μs time range different experimental results are observed depending on the method of illumination, i.e., μs or ns flashes. Mauzerall (1972) observed (Fig. 3) a 25–35 ns rise in fluorescence yield only after the first 10-ns excitation flash. This was interpreted as a change in the coupling between antenna Chl molecules and reaction center traps in PSU experiencing only single hits (Mauzerall, 1976*b*). In other words, the transfer of excitation energy from the antenna Chl (and carotenoids) to the reaction center is decoupled with a 35-ns halftime, and the decoupled state has an enhanced yield of Chl *a* fluorescence (Herron and Mauzerall, 1972; Mauzerall, 1972). Monger *et al.* (1976) have suggested that, in bacterial chromatophores, bacteriochlorophyll triplets are formed and quench bacte-

riochlorophyll fluorescence, and the 35-ns rise observed there is a removal of these bacteriochlorophyll triplets by β-carotene! Since Mauzerall's (1972) analysis associates the 35-ns rise with single hit units only, the bacteriochlorophyll triplet concept extrapolated to Chl triplets seems unlikely.

Data from μs illumination experiments are different and lead to another hypothesis for fluorescence yield changes, with lifetimes of a μs or less. Zankel (1973) observed that the risetime of fluorescence yield was inversely proportional to the intensity of a μs flash. A similar component in the fluorescence yield rise was observed by Duysens *et al.* (1975); however, this component was only seen after the first flash and was interpreted to be due to a rapid donation of electrons by Z to the quencher $P680^+$, although a direct correlation between fluorescence yield rise and the re-reduction of $P680^+$, as measured by absorption changes, is still lacking. It has recently been suggested (Joliot and Joliot, 1976; Joliot, 1977) that the electron donation time to $P680^+$ is 35 ns. Of course, the 35-ns rise was not observed by Zankel (1973) or Duysens *et al.* (1975) due to the slow response times of their equipment and the slow risetimes of the μs flashes. Van Best and Mathis (1978), measuring the rate of $P680^+$ re-reduction by observing the change in absorption at 820 nm, observed a 30-ns component following the first flash after dark adaptation of chloroplasts. Since these measurements were made after the first flash, it is not known if this 30-ns component occurs after other flashes, but, it would appear to be due to a special priming reaction associated with the 25–35 ns rise in fluorescence yield observed by Mauzerall (1972).

All the theories concerning the 1 μs or less rise component of fluorescence have not been specifically addressed to the uniqueness of occurrence only after the first flash. It is also not clear why the coupling between the antenna molecules and the trap (Mauzerall, 1976*b*) would occur only on the first flash. This still needs to be answered. The charge transfer theory is adaptable since the rate of charge movement between Z and $P680^+$ may vary with flash number and the consequent storage of charge in the oxygen evolution mechanism. Duysens *et al.* (1975) observed a decreased rate in the fluorescence yield rise to a few μs on flashes other than the first and, perhaps, this reflected a different rate of electron donation to $P680^+$.

The rise in fluorescence yield following a single ns flash, which provides on the average two photons per PSU, is essentially exponential in character (Jursinic and Govindjee, 1977*a*) with lifetime of 6 μs and reaching a maximum at about 20 μs after the flash (Mauzerall, 1972; Duysens *et al.*, 1975; Jursinic and Govindjee, 1977*a*). This portion of the rise in fluorescence has been interpreted to be either due to (1) decay of carotenoid triplets (Duysens *et al.*, 1975; Mauzerall, 1976*b*), or to (2) charge transfer to

$P680^+$ (Den Haan *et al.*, 1974; Jursinic and Govindjee, 1977*a*). The risetime in fluorescence yield is close to the 5 μs value of Wolff and Witt (1969) for carotenoid triplet decay in air-saturated buffers; however, the rise in fluorescence yield, measured using the double illumination method with a 10 ns excitation flash, did not become significantly slower as expected of triplet decay under low oxygen concentration (Fig. 6). We consider it likely that this 6-μs rise in fluorescence yield reflects both carotenoid triplet decay and a slow charge transfer to $P680^+$. Renger *et al.* (1978) have observed a change in absorption at 690 nm, having a 4.5-μs halftime, under repetitive flash illumination. They have interpreted this absorption change to be due to electron donation to $P680^+$ with a 4.5-μs halftime (6.5-μs lifetime).

An additional complication becomes apparent when the rise in Chl *a* fluorescence yield at times greater than 20 μs after the flash are considered. Under conditions that cause Q^- decay to be slow, such as the presence of DCMU (Zankel, 1973; Joliot, 1977) and low temperature (Joliot, 1974), the fluorescence yield continues to rise with a halftime of 35 μs, and reaches a maximum between 100 and 500 μs after a μs flash. This slow rise is fluorescence yield is not observed when ns illumination is used (Mauzerall, 1972; Duysens *et al.*, 1975; Jursinic and Govindjee, 1977*a*,*b*); instead, a maximum in fluorescence yield is reached at 20 μs in samples with or without DCMU present and at 0°C or room temperature (Table 3).

It was suggested by Joliot (1974) that this slow increase in fluorescence yield may be due to movement of electrons from secondary donors as follows: $Z_2 \rightarrow Z_1 \rightarrow P680$, where Z_2 and Z_1 are charge carriers between the oxygen evolution system (M) and the PSII reaction center P680. Upon illumination $P680^+$ is formed and electron donation from Z_1 takes place in a few μs or less and from Z_2 to Z_1 with a halftime of 35 μs. This proposal is also in agreement with measurements of a 20 $\pm$ 10 μs half-risetime in EPR signal II_{vf} (Babcock *et al.*, 1976; Blankenship *et al.*, 1977) if the Z_2^+ species

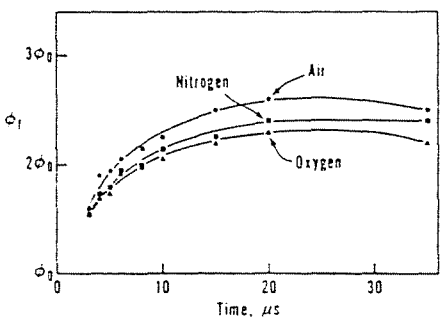


Fig. 6. Plot of the rise in Chl *a* fluorescence yield ϕ_f in terms of ϕ_o, the level of fluorescence yield prior to excitation. The fluorescence yield rise was observed after the final flash in a series of flashes given at a rate of 1 flash/s using the technique of Jursinic and Govindjee (1977*a*). Alaska pea chloroplasts were used at a 5 μg/ml Chl concentration and were bubbled for 5 min with the gas indicated. From Jursinic and Govindjee (unpublished data).

is responsible for signal II_{vf}. Presuming $P680^+$ is a quencher of fluorescence in order to explain the 35 μs rise in fluorescence, Joliot (1977) concluded that the equilibrium constant between $P680^+$ and Z_1 was high such that Z_1 remained in the oxidized state, and, therefore, the rate of reduction of $P680^+$ to P680 was determined by electron flow from Z_2 to Z_1^+. A high equilibrium constant favors stabilization of the $P680^+$ charge by movement to the donor Z_1, spatially separated from Q^-. A charge stabilization, of course, is consistent with PSII having a quantum yield of electron transport close to unity (Sun and Sauer, 1971).

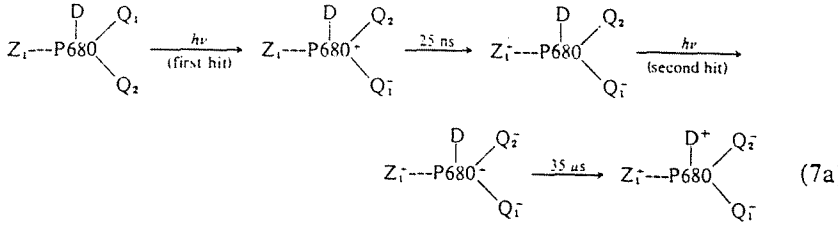
Another possible explanation for the 35-μs rise in fluorescence, which also accounts for it only being observed with μs flash excitation, is given below. Perhaps during the tail of the flash, which lasts tens of μs, some of the reaction centers are excited a second time, an expected occurrence in view of double hits observed in oxygen evolution with μs flashes (Forbush *et al.*, 1971). Also, with DCMU present as in the experiments of Zankel (1973) and Joliot (1977), double hits are increased during the flash tail (Diner, 1974). For a double hit, the following reaction takes place during the second excitation:

$$Z_2Z_1^+\ P680\text{-}Q^-\ \overset{Q_{aux}}{\underset{h\nu}{\longrightarrow}}\ Z_2\,Z_1^+\ P680^+\ \overset{Q_{aux}^-}{Q^-} \qquad (7)$$

where Q_{aux} is an auxiliary one-electron acceptor (Diner, 1974); electron acceptors other than Q have been proposed by Gläser *et al.* (1976) and by Van Best and Duysens (1977). The acceptor side of PSII now seems even more intriguing as recent absorption change measurements in PSII particles suggest that pheophytin may act as an intermediate prior to Q (Klimov *et al.*, 1977). In the $Z_2Z_1^+P680^+$ form the fluorescence is quenched by $P680^+$ and the rise in yield could be dependent on the 35-μs $Z_2Z_1^+ \rightarrow Z_2^+Z_1$ reaction, not the more rapid $Z_1P680^+ \cdot Z_1^+P680$ reaction. When ns-excitation flashes are used, double hits do not occur, the $Z_2Z_1^+P680^+$ condition of the reaction center does not arise, and the 35-μs component is not observed. (See Table 3.)

Joliot and Joliot (1977) have observed a lag at low light intensities in the light saturation curve for the 35-μs fluorescence rise component. This lag has also been interpreted to mean that the 35-μs component results from double hits. However, instead of it being due to the reaction rate of the Z_2Z_1 reaction, as described above, it was associated with the movement of electrons from an unidentified electron donor, D, to $P680^+$. In normal chloroplasts, this electron donation by D would occur only if the reaction center received two photons within a few microseconds.

In the present day picture, where Q aux is labeled as Q_2 and Q as Q_1, the double hit process (after the first μs flash) can be expressed as follows:



(7a)

The existence of a fast Q_1 and a slow Q_2 and of a slow donor D had been proposed earlier (see R. Govindjee *et al.*, 1970, for Q_1 and Q_2, and Den Haan *et al.*, 1974, for D).

2.3.3b. Effects of Tris Washing

The rise in fluorescence yield is expected to be sensitive to changes in charge flow between the oxygen evolving system and the PSII reaction center, particularly, if $P680^+$ is a quencher of fluorescence. One method of altering electron flow between the oxygen evolving system and PSII is washing chloroplasts with Tris at pH 8.0 (Yamashita and Butler, 1968), which completely stops electron flow from water to PSII. Various exogenous reductants such as ascorbate, phenylenediamine, hydroquinone, and benzidine become effective donors of electrons to PSII after Tris washing (Yamashita and Butler, 1968; Yamashita and Butler, 1969).

A 6-μs rise in fluorescence yield following a 10-ns laser pulse was studied by Jursinic and Govindjee (1977*a*) in Tris-washed chloroplasts. On the first flash following dark adaptation, no Tris effect was observed, on the second flash a small decrease in the fluorescence rise occurred, but on the third and succeeding flashes, given at a rate greater than 2 Hz, only a small rise in fluorescence yield occurred by 30 μs. Thus, two pre-illumination flashes were required before the full extent of Tris washing on the μs-fluorescence yield was observed, and these Tris effects could be reversed by the addition of electron donors mentioned above. The requirement for two pre-illumination flashes for the full Tris effect was interpreted to indicate the existence of two charge carriers, described earlier as Z_1 and Z_2, between the site of Tris block and P680. The Tris effect was absent if the dark time between excitation pulses was greater than a few seconds, the time required for Z_1 and Z_2 (or D?) to lose their charge. This was in agreement with decay times estimates based on ESR signal II_{vf} measured by Babcock and Sauer (1975) in Tris-washed chloroplasts.

The small fluorescence yield rise in Tris-washed chloroplasts was explained by Jursinic and Govindjee (1977*a*) in the following manner. On the first two flashes Z_1 and Z_2 were oxidized by donating electrons to $P680^+$ formed during the flash, and $P680^+$, a quencher of fluorescence, was reduced in a normal manner, and the rise in fluorescence yield was unaffected by Tris. On the third flash Z_2 and Z_1 were already oxidized, so $P680^+$ remained high in concentration and fluorescence was quenched, and the Tris effect appeared.

It was assumed by Jursinic and Govindjee (1977*a*) that the normal 6-μs rise in fluorescence yield reflected the rate of electron transfer from Z_1 to $P680^+$. This is supported by observations mentioned in Section 2.3.3a. However, this is not essential for explaining the data. If the electron transfer from Z_1 to $P680^+$ took place in 1 μs or less (Section 2.3.3a) and the 6-μs rise in fluorescence yield reflects the decay of triplets, then on the third flash in Tris-washed chloroplasts the 1-μs Z_1 to $P680^+$ charge transfer could not take place, since Z_1 would be oxidized, the fluorescence yield would be low due to the high $P680^+$ concentration, and the 6-μs triplet decay would be unobservable as a rise in fluorescence yield.

The Tris effect on fluorescence yield (Jursinic and Govindjee, 1977*a*) does suggest that a capacity for the storage of two charges exists between the Tris block and P680. This conclusion appears to be in agreement with the absorption increases at 820 nm due to $P680^+$ (Haveman and Mathis, 1976) and at 320 nm due to Q^- (Mathis *et al.*, 1976), and the recent ESR signal II_{vf} data (Blankenship *et al.*, 1977). Contrary to this, Velthuys and Amesz (1974), observing changes in fluorescence after rapid additions of dithionite, had concluded that Tris-washed chloroplasts only store a single positive charge on the donor side of PSII. Renger *et al.* (1978) did not observe a 6.5-μs lifetime component in the 690-nm absorption change of Tris-washed chloroplasts. This is consistent with the above hypothesis, since the 690-nm absorption measurements were made under repetitive illumination, a condition that would fill the charge carriers between the Tris block and P680.

2.3.3c. *Effects of Hydroxylamine*

The inhibitory effects of hydroxylamine (NH_2OH) on photosynthesis have long been known (Rabinowitch, 1951), with the specific effect being on the PSII charge flow between the reaction center and the oxygen evolving site (Cheniae and Martin, 1971) as hydroxylamine removes the manganese pool (Cheniae and Martin, 1970) involved in O_2 evolution. The inhibition of normal PSII charge transfer reactions by hydroxylamine has led to its use

in a number of experiments where the μs changes in Chl *a* fluorescence yield were measured.

Zankel (1973) observed, using the single illumination technique, that a rise in fluorescence yield occurred between 80 and 100 μs, after initiation of illumination, with a half-risetime of 35 μs at 22°C and 63 μs at 4°C. This component of fluorescence yield rise was most clearly seen if hydroxylamine was present. Etienne (1974) found that the maximum level of fluorescence that was attained during a 5-μs flash was lowered by hydroxylamine, and proposed that a fast rise in the fluorescence yield must have been slowed down by hydroxylamine. Measurements made in the 0.5 to 100 μs range (Den Haan *et al.*, 1974) revealed that the normal halftime of Chl *a* fluorescence yield rise of a few μs was changed to 25 μs by the presence of 1–10 m*M* hydroxylamine. Den Haan *et al.* (1974) interpreted the fast rise in fluorescence yield as an electron transfer from Z to $P680^+$; the latter is believed to be a quencher of fluorescence. It was suggested that hydroxylamine inhibits this normal transfer of charge and, instead of Z, a secondary donor D (Den Haan *et al.*, 1974) donates charge to $P680^+$ with a 25-μs halftime. No identification of D was made, but it was distinguished from hydroxylamine since the 25-μs rise persisted after hydroxylamine was washed out. D was found (Den Haan *et al.*, 1976) to be only capable of donating two electrons in rapid succession if hydroxylamine was washed out of the sample, but in the presence of hydroxylamine, D acts as an intermediate carrier of charge to $P680^+$ with a 25-μs transfer time.

Den Haan *et al.* (1976) measured the time dependence of the onset of the hydroxylamine effect on oxygen evolution and the fluorescence yield rise during a flash. The inhibition of oxygen evolution and appearance of the slow (25 μs) fluorescence yield rise proceeded at the same rate in agreement with the hypothesis that they were both related to the inhibition of the $ZP680^+ \rightarrow Z^-P680$ charge transfer reaction by hydroxylamine. For both oxygen evolution and fluorescence yield the time of onset of the hydroxylamine effect was inversely proportional to hydroxylamine concentration.

Joliot (1977), for times greater than 20 μs after initiation of illumination, reported a 70-μs rise in fluorescence yield following a flash in control chloroplasts at 2°C. It was emphasized that this same rise exists with hydroxylamine present, but a fast component, sampled at 1 μs during the flash, was largely inhibited by hydroxylamine. A similar finding concerning the existence of a slow-rising component of fluorescence yield in the presence of hydroxylamine was reported earlier by Zankel (1973), based on a restricted observation time beginning 80 μs after the flash. Besides the secondary donor hypothesis of Den Haan *et al.* (1974), Joliot (1977) suggests two other explanations for the hydroxylamine effect. First, Z, the

primary donor to $P680^+$, is not eliminated when hydroxylamine is present, but a chemical or structural modification of the donor site occurs causing the normally rapid $ZP680^+ \cdot Z^- P680$ (1 μs) charge transfer reaction to become slow (35 μs). Second, the reaction centers are heterogeneous with some centers having $P680^+$ reduced rapidly by Z and others slowly by D. Hydroxylamine would specifically inhibit the ZP680 type centers. Joliot (1977) pointed out that the theory of heterogeneous centers would explain the closeness in values of the slow fluorescence yield that was observed in samples with and without hydroxylamine; however, as pointed out in Section 2.3.3a, a slow fluorescence yield rise at times greater than 20 μs after the flash is not a universal phenomena. At the present time no one theory for the action of hydroxylamine on the rise of fluorescence yield seems clearly better than any other, and only future studies will resolve which model is best.

Most of the studies on the hydroxylamine effect on the fast fluorescence rise have been conducted on samples incubated with hydroxylamine in the dark. As pointed out by Cheniae and Martin (1971) dark incubation only eliminates oxygen evolution activity, while incubation in the light eliminates this activity as well as the ability of hydroxylamine to donate electrons to PSII. Jursinic (1977) observed no fluorescence rise in samples incubated with hydroxylamine in light, whereas those incubated in the dark showed the usual 35-μs rise. It was suggested that the incubation in light caused all electron donation to $P680^+$ to cease, leaving the Chl *a* fluorescence quenched.

2.3.3d. Comparison of Changes in P680 Absorption and Chlorophyll a Fluorescence Yield

If the concept that $P680^+$ is a quencher of Chl *a* fluorescence is correct, then the rise in fluorescence yield following a flash would suggest possible lifetimes for $P680^+$ reduction of ≤ 1 μs, 6 μs, and 35 μs. A comparison of changes in P680, measured directly by 690-nm absorption kinetics with fluorescence yield changes does not show a direct correlation in all cases.

Döring *et al.* (1967) found that a reversible bleaching occurred at 690 nm, following flash illumination, and identified it with light reactions of the PSII reaction center Chl, P680. Döring *et al.* (1969) suggested that the 690 nm absorption change reflected a sensitizer reaction for PSII charge flow, but now it is generally believed that it reflects a light-induced redox reaction involving P680 (Döring, 1975). By measuring 690-nm absorption changes, after single turnover repetitive flashes, the reduction of $P680^+$ was observed to occur with 50- (Gläser *et al.*, 1974) and 260-μs lifetimes (Döring *et al.*, 1969; Govindjee *et al.*, 1970) (Fig. 7). The 260-μs component is not observed

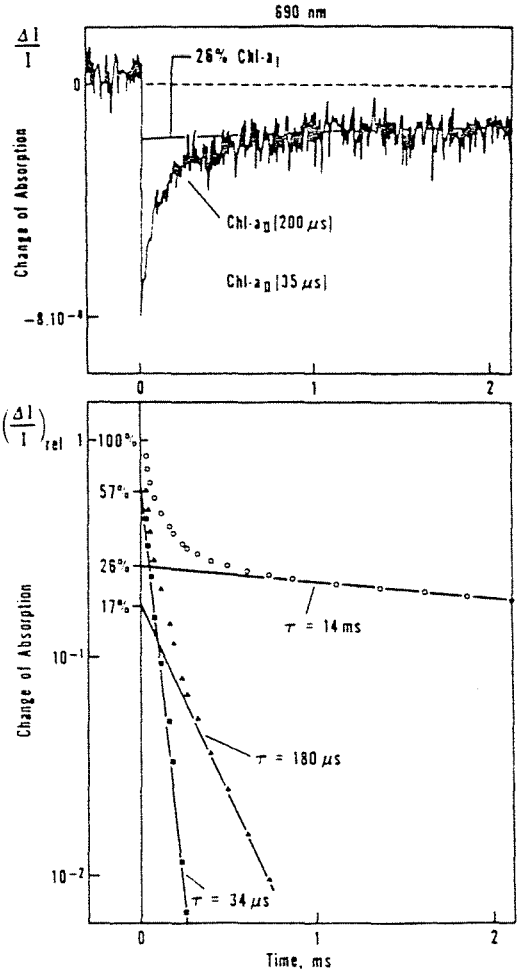


Fig. 7. *Top:* Absorption change at 690 nm as a function of time in a suspension of spinach chloroplasts. At $t = 0$ a single turnover flash (0.4 μsec) of half saturating intensity was fired. *Bottom:* Relative absorption changes as function of time plotted on a log scale. The values for τ are decay halflife values. From Gläser *et al.* (1974).

in the fluorescence yield rise. This may be due to this component being a very small portion (perhaps as low as 10%, see later discussion) of the total 690-nm absorption change. Alternately, the 260-μs component of the 690-nm absorption change may reflect a recombination reaction between $P680^+$ and Q^-; this type of reaction cannot be observed by changes in Chl *a* fluorescence yield since both $P680^+Q^-$ and P680Q have low fluorescence yields. (However, this should be observed in delayed light emission, see Section 3.3.) If a recombination reaction were responsible for the 260-μs decay of $P680^+$, then a parallel rate of change in Q^- should be observed. However, in normal chloroplasts, Renger and Wolff (1976) observed a 500-

μs halftime decay of Q^-, as indicated by the 320-nm absorption change. The 50-μs lifetime of 690-nm absorption change decay corresponds to the 50-μs lifetime rise in Chl *a* fluorescence, which is observed using the single illumination method. Of course this correlation is expected if both phenomena reflect changes in the $P680^+$ concentration. As discussed earlier, ns excitation does not produce an observable 50-μs fluorescence rise, and it would be interesting to determine if the 50-μs component of the 690-nm absorption change would occur following a ns flash instead of the 500-ns duration, 4-Hz repetitive flash conditions of Gläser *et al.* (1974).

A 6-μs component in 690-nm absorption change has been reported by Renger *et al.* (1978), as noted earlier and as expected from Chl *a* fluorescence rise data of Jursinic and Govindjee (1977*a*), presuming that $P680^+$ is a quencher of fluorescence and that the triplet quencher may not have played a significant role. However, it is likely that the coincidence may have been fortuitous. The 6-μs component in the absorption change was observed under repetitive flash illumination; in addition, the 50- and 260-μs components were also observed as previously reported.

It had been suggested (Butler, 1972*a*), based on the amplitude of the 690-nm absorption change being much smaller than expected (Butler, 1972*b*), that the reported 690-nm absorption change was only a portion of the $P680^+$ decay actually occurring, because most of the $P680^+$ decay was too rapid for observation by the techniques in use at that time. This now seems to have been demonstrated as the correct interpretation by Gläser *et al.* (1976), who observed a twofold increase in the extent of 690-nm absorption changes in Tris-washed samples. To explain their results Gläser *et al.* (1976) suggested that in normal, but not in Tris-washed chloroplasts, a rapid reduction (< 1 μs) of $P680^+$ occurred, which their technique was unable to detect. The effect of Tris washing occurs only after two or more excitation flashes (Jursinic and Govindjee, 1977*a*), and Gläser *et al.* (1976) had indeed used repetitive flashes. Based on the 690-nm absorption data of Gläser *et al.* (1976) and Renger *et al.* (1978), and assuming the extinction coefficient for P680 to be the same as that of Chl *a in vitro* (Döring *et al.*, 1969), we calculate [Chl]/[P680] = 1000. If the 690-nm absorption change observed for Tris-washed chloroplasts (Gläser *et al.*, (1976) and normal chloroplasts (Renger *et al.*, 1978) reflect the turnover of every P680, then the [Chl]/[P680] ratio is expected to be 500 to 600, presuming 250 to 300 Chl molecules (Govindjee and Govindjee, 1975) are associated with each reaction center. These values are quite close considering the experimental errors involved and the estimates for the P680 extinction coefficient at 690 nm. Based on the data of Gläser *et al.* (1974, 1976) and Renger *et al.* (1978), the original 260-μs component of the 690-nm change observed by Döring *et al.* (1969) only represents 1/7 to 1/10 of the total 690-nm change and the

50- and 6-μs components make up the remainder of the 690-nm absorption change. The existence of a $P680^+$ decay component more rapid than 6 μs has not been demonstrated by 690-nm absorption change due to equipment's risetime limitations. A 30-ns component is, however, expected following the first flash after dark adaptation, and has been shown to exist by 820-nm absorption measurements (Van Best and Mathis, 1978). We believe that it is necessary to seek correlation, in parallel measurements, with Chl *a* fluorescence rise and delayed light emission decay in this time range after the first flash as well as after all other flashes.

Measurements made at 77°K in *Chlorella* and spinach chloroplasts have shown that Chl *a* fluorescence yield rises to a maximum by 40 μs following a flash (Den Haan *et al.*, 1973). At 77°K, absorption changes at 682 nm, assumed to reflect changes in P680 concentration, show decays with halftimes of 30 μs and 4.5 ms (Floyd *et al.*, 1971). The 40-μs rise in fluorescence yield and the 30-μs lifetime bleaching recovery at 682 nm are in accordance with the $P680^+$ fluorescence quencher hypothesis; however, the lack of a 4.5-ms change in fluorescence yield at 77°K is a difficulty. Also, it has been suggested (Butler, 1972*a*) that the 30-μs component of the 682 nm absorption decrease could possibly be artifactual, and could be due to the delayed light emission excited by the flash. Thus, it is necessary that these measurements be repeated under parallel conditions and with improved instrumentation before the hypothesis of $P680^+$ being a quencher of Chl *a* fluorescence is considered proven.

Chl *a* fluorescence yield data and 690-nm absorption data at room temperature seem to be reconciled for components having lifetimes of 260, 50, and ≤ 1 μs; however, the existence of a 6-μs component needs to be resolved. Furthermore, the interpretation of these various components awaits further experimentation and analysis. We consider it likely that the rate of reduction of $P680^+$ is a variable parameter depending upon (a) the donor (Q^-, D, Z_1) donating electrons to it; (b) the physical proximity of the donor to $P680^+$; and (c) the rate determining step in the flow of electrons.

2.3.3e. Reduction of $P680^+$ and Electrochromism

The primary donor (P680) and acceptor (Q) of PSII are suggested to be vectorially arranged in the thylakoid membrane with P680 toward the inside and Q toward the outside (Trebst, 1974; Zilinskas, 1975). Upon illumination, charge separation occurs and produces an electric field in the thylakoid membrane that can be detected spectroscopically as electrochromic band shifts in the absorption spectra of membrane-bound pigments (Junge, 1977*a*,*b*). Charge separation associated with PSI also

contributes to the electrochromic absorption change. The most prominent electrochromic absorption changes occur at 480 and 515 nm and have been attributed to Chl *b* and the carotenoids (Govindjee and Govindjee, 1965; Schmidt *et al.*, 1971; Witt, 1975).

After the initial $P680^+ Q^-$ charge separation, the positive charge on $P680^+$ is stabilized by transfer to Z, as explained earlier. This shift in charge location may affect the kinetics of electrochromism. Evidence of a similar change in photosynthetic bacteria does indeed exist. Jackson and Dutton (1973), using chromatophores of *Rhodopseudomonas sphaeroides*, found that flash-induced electrochromic shifts in the carotenoid band were multiphasic. A very rapid initial phase ($\tau_{\frac{1}{2}} < 1\ \mu s$) was correlated to the initial charge separation ($P870^+ X^-$), where P870 is the primary electron donor and X the acceptor of the bacterial system, and a slower phase ($\tau_{\frac{1}{2}} \simeq 150\ \mu s$) to the reduction of $P870^+$ by cytochrome *c*. The primary charge separation was believed not to span the entire thylakoid membrane, and movement of charge to cytochrome *c*, which is located closer to the surface of the membrane than P870, was believed to result in an increased charge separtion and a larger membrane potential.

When a similar effect on the electrochromic shift, due to the movement of charge between Z and $P680^+$ in green plants, was looked for, multiphasic behavior was not observed (Joliot and Joliot, 1976). However, when the samples were treated with hydroxylamine, which eliminates the flow of the positive charge to the PSII secondary donor Z, the electrochromic shift was greatly reduced. The Joliots concluded that $P680^+ Q^-$ may not span the entire width of the thylakoid membrane, and hydroxylamine may inhibit further charge movement to secondary donors located close to the membrane surface. It was suggested that the lack of biphasic behavior in the electrochromic shift in control samples due to the $P680^+ Q^-$ charge separation followed by further charge movement from $P680^+$ to Z was missed, since the first event occurred much more rapidly than the time response of their instrument. It was suggested that the $ZP680^+ \rightarrow Z^+P680$ reaction had a lifetime of less than 1 μs; however, since their instrument response time was 4 μs, a lifetime of about 4 μs could also be possible. The lack of biphasic changes in the electrochromic shift might also be interpreted to mean that Z is located laterally, not tangentially, from P680, with respect to the thylakoid membrane surface. The reduction in electrochromic shift by hydroxylamine would then be some secondary effect and would not be due to blockage of charge flow between Z and $P680^+$. Additional experiments with apparatus having a faster time response are needed before data of this type can provide conclusive information on the thylakoid structure or the rate of the $ZP680^+ \rightarrow Z^+P680$ stabilization reaction.

3. DELAYED LIGHT EMISSION (DLE)

3.1. Background Information

While attempting to measure the production of ATP in the green alga *Chlorella*, Strehler and Arnold (1951) discovered a weak light emission from the intact algal cells after the termination of illumination (also see Strehler, 1951). This delayed light emission (DLE) has an emission spectrum almost identical to Chl *a* fluorescence (Arnold and Davidson, 1954; Arnold and Thompson, 1956; Lavorel, 1969), suggesting that DLE comes from the deexcitation of the singlet excited state of Chl *a*. DLE is observed from nanoseconds to many minutes following termination of illumination, and is suggested to reflect a temporary storage of high-energy photosynthetic products, which back react and repopulate the singlet excited state of Chl. Thus, the main difference between fluorescence and DLE is the means by which the Chl *a* singlet excited state is populated. Fluorescence originates from relaxation of all the antenna Chl *a* that have absorbed quanta, while DLE originates, perhaps, from Chl *a* molecules close to the reaction center; the metastable products of the latter recombine making energy available for the generation of the Chl *a* singlet excited state. Various aspects of DLE have been reviewed by Arnold (1965, 1977) Mar (1971), Fleischman and Mayne (1973), Mar and Roy (1974), Lavorel (1975), Malkin (1977*a*,*b*), Jursinic (1977), and Amesz and Van Gorkom (1978).

DLE in green plants is found to originate predominantly from PSII as is shown by the absence of DLE in algal mutants that lack PSII (Bertsch *et al.*, 1967; Lavorel, 1969; Haug *et al.*, 1972), the action spectra for PSII activity and DLE being almost identical (Arnold and Thompson, 1956; Lavorel, 1969), and the 60- to 90-fold higher level of DLE in PSII versus PSI enriched particles (Lurie *et al.*, 1972; Vernon *et al.*, 1972; Itoh and Murata, 1973; Gasanov and Govindjee, 1974). DLE does originate from PSI (Shuvalov, 1976), however, with significantly lower yield than from PSII. Antagonistic effects of light absorbed in pigment system II and in pigment system I on DLE was demonstrated by Bertsch (1962) and Goedheer (1962); these results were taken, at that time, to support the emerging theory of two light reactions and two pigment systems in photosynthesis.

Theories

The mechanism for the generation of DLE in photosynthetic systems remains unsettled. The theories that have been proposed to explain the

origin of DLE can be grouped into the following three categories: Charge recombination, electron–hole recombination, and triplet fusion.

In the *charge recombination theory* (Arthur and Strehler, 1957; Strehler and Lynch, 1957, Lavorel, 1969; Van Gorkom and Donze, 1973; Lavorel, 1975) DLE is generated by the back reaction of oxidized and reduced products of the photoreaction that occurs at the reaction center for PSII; this can be written as:

$$ZP680Q \xrightarrow{h\nu} ZP680^{+}Q^{-} \rightarrow ZP680^{*}Q \rightarrow ZP680\,Q + h\nu_{DLE} \qquad (8)$$

where $P680^{+}$ and $P680^{*}$ are the reaction center Chl in the oxidized and singlet excited state, respectively; Q is the primary electron acceptor; Z is the first electron donor to the reaction center; and $h\nu_{DLE}$ is the DLE quantum. It is generally believed that the exciton on $P680^{*}$ is transferred to antenna Chl *a* molecules close to it before it is emitted as a quantum. If the positive charge is on Z, and this charge flows from it to produce $P680^{+}$, then the latter's recombination with Q^{-} may also generate DLE (Van Gorkom and Donze, 1973). The charge recombination theory is consistent with the largest portion of experimental results and has the widest acceptance of all the theories (Amesz and Van Gorkom, 1978). When the positive charge is on Z^{+}, not $P680^{+}$, and DLE is due to the above mechanism, then the decrease in DLE intensity by hydroxylamine addition is interpreted to support the charge recombination theory, as hydroxylamine donates electrons to Z^{+} and thus eliminates one of the partners (see Bennoun, 1970; Stacy *et al.*, 1971; Mohanty *et al.*, 1971). A similar decrease in DLE intensity by the addition of silicomolybdate, which accepts electrons from Q^{-}, also supports the charge recombination theory (Zilinskas and Govindjee, 1975; Jursinic, 1977). The observation of DCMU-induced luminescence in dark-adapted chloroplasts, observed by Etienne and Lavorel (1975), can also be explained in terms of the charge recombination theory.

The charge recombination theory may have to be altered to accommodate the possible role of pheophytin in the reaction-center primary reactions. Recent absorption spectra measurements (Klimov *et al.*, 1977) indicate that in PSII particles, in which the redox potential is maintained below −50 mV to keep Q chemically reduced, photoreduction of pheophytin (Ph) takes place. If this photoreaction is found to take place in whole cells and chloroplasts, then a different scheme should be written as follows:

$$\begin{array}{l} ZP680\,PhQ \xrightarrow{h\nu} ZP680^{*}PhQ \xrightarrow{1} ZP680^{+}Ph^{-}Q \xrightarrow{2} ZP680^{+}PhQ^{-} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad ZP680\,PhQ + h\nu_{DLE} \end{array} \qquad (9)$$

ere, charge recombination between $P680^+$ and Ph^- gives rise to DLE. cosecond spectroscopy has not yet been carried out on PSII, but by ıalogy to the bacterial systems, one might expect reaction 1 to occur ithin 10 ps after a flash (Dutton *et al.*, 1975), reaction 2 within 200 ps Rockley *et al.*, 1975), and reaction 3 within 6 ns (Shuvalov and Klimov, 976; Klimov *et al.*, 1977). DLE at times longer than 6 ns would reflect the :versal of reactions, such as reaction 2.

In the *electron–hole theory* of DLE, the PSU is thought of as a semi-onductor having a conduction band with associated charge traps of various epths. Two sites for charge trapping in the PSU of PSII are proposed, one or electrons and the other for holes (Arnold and Azzi, 1968; Bertsch, 1969), ach having its own reaction center. In the PSU a photon causes an electron nd hole to be generated. The electron is trapped at a reaction center and ıe hole remains free, while at the other type of reaction center, a hole is apped and the electron remains free. The recombination of free electrons nd holes, or free holes with untrapped electrons and free electrons with ntrapped holes will generate DLE. This theory is not generally accepted, as o evidence exists for the presence of free electrons and holes, or the semi-onductor pigment complex. Arnold (1976) has recently published a new etailed scheme for the semiconductor description of photosynthesis and)LE. Although not disproven, this theory of DLE is also not widely ccepted, since no evidence exists for the various details of the theory; it will ot be described here as it has been recently reviewed by Arnold (1977).

Upon receiving excitation energy or by Z^+ and Q^- recombination, the 'SII reaction center may produce triplet states by intersystem crossing Stacy *et al.*, 1971). These triplet excitons may decay by radiationless transi-ions, or two triplets may undergo fusion and produce an excited singlet and ı ground state (*triplet theory*). This excited singlet then decays to the ground state giving rise to DLE. The production of triplet states in green olants has been observed only when normal photochemical reactions have been saturated or somehow inactivated. At high light intensities, where the eaction center is closed to normal photochemistry, Chl *a* fluorescence is believed to be quenched by the proposed formation of triplets (Zankel, 1973; Den Haan *et al.*, 1974; Mauzerall, 1976*b*). The presence of triplets was shown by the existence of phosphorescence (Krasnovsky *et al.*, 1975) and by microwave-induced changes in Chl *a* fluorescence yield at 2°K (Hoff and Van der Waals, 1976; Hoff *et al.*, 1977). Certain thermoluminescence glow peaks have been suggested to arise from metastable triplet states, but only when other reactions are saturated (see Sane *et al.*, 1977). The generation of triplets in PSI subchloroplast fragments, in which the normal photochemical reactions have been blocked, has been followed by observing Chl phosphorescence at 77°K (Shuvalov, 1976), and it has been suggested

that these triplets give rise to a weak DLE from these PSI particles. Although triplet states can exist in green plant photosystems, there is no evidence at this time that triplet–triplet fusion is responsible for DLE in normal plants exposed only to saturating light.

Keeping an open mind about the triplet theory seems prudent in view of the chemically induced dynamic electron polarization (CIDEP) phenomenon recently associated with PSII activity in algae (McIntosh and Bolton, 1976). Flash illumination of algae generated an electron paramagnetic resonance (EPR) emission signal with g values of 2.0025 and 2.0042, corresponding to the radicals $P680^+$ and Q^-, respectively. One interpretation of this CIDEP phenomenon is that, upon receiving a quantum, P680 goes to a singlet excited state, and intersystem crossing occurs, populating the three triplet levels unequally. Charge separation between P680 and Q occurs before spin–lattice relaxation restores Boltzman equilibrium in the triplet states, and the resulting radicals are observed to have initially inverted spin populations. As stated by McIntosh and Bolton (1976) their results do not prove that triplets are intermediates in PSII photochemistry, but further work in this area seems warranted.

Of the three theories, that of charge recombination is consistent with the largest portion of experimental results. Reviews on DLE by Lavorel (1975), Malkin (1977*a*,*b*), and Amesz and Van Gorkom (1978) should be consulted. Here we will be primarily discussing the current literature, stressing results in the μs range.

3.2. DLE Experimental Methods

Since DLE can be observed over an extremely wide time range (ns to many min), under a wide variety of illumination conditions, and pH, temperature, ionic strength, etc., a wide variety of techniques have been used to monitor it (Lavorel, 1975). In order to measure DLE in the μs time range (a) the excitation source must be turned off completely before the measurement can begin since any residual excitation light will generate a large fluorescence artifact that will mask the weak DLE signal, and (b) the photomultiplier must be capable of responding to the weak DLE signals in a few μs after the excitation light has been shut off. Two methods are now commonly used to measure μs DLE, the phosphoroscope method and the single shot method (Lavorel, 1975).

In the phosphoroscopes employed to measure μs DLE (Lavorel, 1971; Haug *et al.*, 1972), focused beams of continuous emission lasers are chopped either by a rapidly rotating slotted disc or an electro-optical shut-

ter. The falling edge of the actinic light pulses, produced with these methods, have drop times of a few μs for the rotating disk and approximately 25 ns for the electro-optical shutter. The phosphoroscope method has a number of disadvantages that must be kept in mind when interpreting the data. The excitation occurs as multiple flashes so the DLE observed is a complex signal having contributions from all the previous flashes and the final flash. Errors involved in interpreting phosphoroscope data have been discussed by Mar *et al.* (1975). Also, complications brought about by pre-illumination in interpreting electron transport inhibitor effects on ms DLE have been described by Ruby (1977). The duration of the actinic flash in the phosphoroscope method is often about 1 ms, and any DLE decay component due to a charge stabilization reaction having a rate much faster than this time is either unobservable or distorted (Lavorel, 1973), since these stabilization steps take place before the actinic flash is terminated.

In the single shot method, individual flashes are given to the sample and the DLE decay is followed after the flash. The actinic flashes are provided either by a xenon flash lamp with its long decay tail cut off by a rotating slotted disk (Zankel, 1971), or by various self-terminating lasers (Lavorel, 1973; Duysens *et al.*, 1975; Jursinic *et al.*, 1976). Normally the photomultiplier is gated off electronically during the flash to avoid excessive anode current and to allow linear response of the photomultiplier at full sensitivity after the flash. A variety of methods have been used for gating photomultipliers (de Martinia and Wacks, 1967; de Marco and Penco, 1969; Jursinic, 1977; Van Best, 1977), which allow DLE to be measured after single saturating flashes within less than a μs (Van Best and Duysens, 1977) or a few μs (Jursinic and Govindjee, 1977*a*). The advantages of the single shot method are the following: stabilization steps occurring a few μs after the flash can be observed, since the excitation lasts only a few ns instead of tens of μs or longer as in the phosphoroscope method; decays after a single excitation are not complicated by the decay of states generated by previous phosphoroscope cycle excitations; and there is freedom in giving a variety of pre-illumination regimes. The major drawback of the method is the extremely high intensity of the excitation light that must be used. In intact, well-coupled chloroplasts, each electron transport chain turns over once every 15 ms, and absorption of one quantum of light every 15 ms per PSU is needed for efficient photosynthesis (Junge, 1977*a*). However, to saturate photosynthesis with a single laser pulse of 15-ns width, six orders of magnitude greater intensity is required than if continuous light is used. The problems involved with using light intensities of this magnitude have been stressed by Mauzerall (1976*a*,*b*).

3.3. DLE in the Microsecond Range

3.3.1. Complex Decay Kinetics

The decay in DLE in the μs range, following single flash illumination or under phosphoroscope conditions, is extremely complex. Certainly this complicated decay of DLE reflects its sensitivity to many on-going processes during photosynthesis. In order to analyze DLE decay curves, most authors plot their data in a semilogarithmic form, apply standard graphical procedures, and present amplitudes and lifetimes of the exponential components. The analysis of DLE decay data as a summation of exponential components can lead one into a certain fixed framework of interpretations, which must be avoided. The mathematically generated exponential components by themselves have no particular significance, but are convenient for comparing data gathered under different conditions. The mechanistic reality of a DLE component can only be demonstrated by comparison with other measurements. A large portion of the following discussion will be devoted to the identification of a particular DLE decay component with PSII reactions proposed in various theories.

3.3.2. DLE Decay in Normal Chloroplasts and Algae

In the μs range following a single flash a variety of DLE decay components have been reported (Table 4): ~1, 6, 35 to 45, and 135 to 290 μs.

The ~1-μs component has been reported only by Van Best and Duysens (1977) and has its greatest amplitude if the PSII reaction center,

TABLE 4. Microsecond Delayed Light Emission Components[a]

Reference	Sample	Time range studied (μs)	DLE decay component lifetime (μs)
Zankel (1971)	Chloroplasts	65–800	14, 50, 290
Lavorel (1973)	*Chlorella*	6–600	5–6, 36–45, 135
Duysens *et al.* (1975)	*Chlorella*	0.6–>50	0.8, 14–25, 200
Jursinic and Govindjee (1977*a*)	Chloroplasts	6–100	5–7, 30–35
Van Best and Duysens (1977)	*Chlorella*	0.2–25	0.7–1.4, 10–30

[a] DLE decay components found by various investigators using *Chlorella* cells and chloroplast fragments. Decay data is shown for DLE following a single flash after dark adaptation of the samples. In all cases the flash widths were 3 μs or less, but flash intensities were subsaturating for Lavorel, just saturating for Zankel and Jursinic and Govindjee, and oversaturating for Van Best and Duysens.

prior to excitation, is in the P680 Q^- state, which up to this time has been considered a state closed for photochemistry. To explain their results, Van Best and Duysens (1977) propose that an additional acceptor, W, operates when Q is in its reduced state, thus allowing photochemistry to take place (see Section 2.3.3a). DLE under these conditions is then due to the recombination of $P680^+$ with W^-, and the 1-μs lifetime is due to $P680^+$ being reduced by Z at that rate [see Eq. (9)]. There are difficulties with this explanation. The 1-μs $ZP680^+ \rightarrow Z^-P680$ reaction seems to occur only on the first flash after dark adaptation (Duysens *et al.*, 1975) while the 1-μs component of DLE was observed after flashes other than the first (Van Best and Duysens, 1977). Gläser *et al.* (1976) believe that the fast $ZP680^+ \rightarrow Z^-P680$ reaction is photochemically transformed into a slower reaction at flash intensities above saturation; however, the 1-μs DLE component of Van Best and Duysens (1977) is not inhibited at intensities exceeding saturation. Also, the need to hypothesize an auxiliary acceptor W in order to satisfy the charge recombination hypothesis for DLE is awkward, since samples having DCMU present have not yet been shown to generate $P680^+$ after the first flash (Döring *et al.*, 1969; Gläser *et al.*, 1974). With W available, $P680^+$ is expected to be generated on flashes after the first. The generation of DLE in reaction centers that are in a closed form (P680 Q^-), instead of indicating the presence of W (Van Best and Duysens, 1977), may indicate that DLE in this time range does not originate from charge recombination but from triplet fusion or electron–hole recombination!

The 6-μs component was reported by Lavorel (1973) and Jursinic and Govindjee (1977*a*); it may be considered equivalent to Zankel's (1971) 14-μs and Van Best and Duysen's (1977) 10-μs component (Table 4). Both Lavorel (1973) and Jursinic and Govindjee (1977*a*) have suggested that this component reflects the $ZP680^+ \rightarrow Z^-P680$ charge stabilization reaction. A 6-μs time for this reaction is by no means an established fact (see Section 2.3.3). An alternative is to suggest that triplet quenchers, formed due to the high intensities used, decay with this lifetime, increasing the fluorescence as observed and providing delayed fluorescence due to triplet $\rightarrow$ singlet conversion.

A 25- to 50-μs component is observed by all investigators (Table 4), and correlates well with the 50-μs lifetime decay of $P680^+$ of Gläser *et al.* (1974). Zankel (1971) and Lavorel (1973) proposed that this component reflects charge stabilization steps on the oxygen side of the PSII reaction center. Based on results with Tris-washed samples, Jursinic and Govindjee (1977*a*) suggested this component to be related to the movement of charge between Z_1 and Z_2, electron carriers between P680 and the oxygen evolving system (see Sections 2.3.3b and 3.3.4). The concept of a 25- to 50-μs charge stabilization reaction on the oxygen side of PSII, however, is unable to

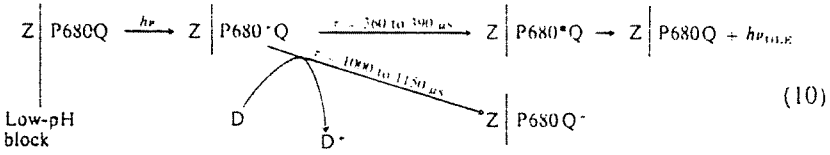
explain the continued existence of a 25- to 50-μs DLE component in samples treated with high concentrations of NH_2OH, a treatment that eliminates normal electron flow from Z to P680$^+$ (see Section 3.3.5 for further discussion of this component).

The 100- to 200-μs lifetime decay component was correlated only with the decay of Q^- by Zankel (1971), presuming that the intrinsic back reaction between P680$^+$ and Q^- must be much slower than this time. Van Gorkom and Donze (1973), however, suggested that this component determined the intrinsic back reaction rate and originated from reaction centers in the Z$^+$P680 state prior to excitation; experiments supporting this theory will be discussed below.

3.3.3. DLE Decay at Low pH

At pH 4.5 the predominant DLE decay had a lifetime of 175 μs in spinach chloroplasts, as measured by the phosphoroscope method (Haveman and Lavorel, 1975). A somewhat slower decay component, having a 290-μs lifetime, was reported by Van Gorkom *et al.* (1976) under identical conditions. The low-pH treatment caused internal acidification of the thylakoid, and thus inhibited oxygen evolution, which depends on the release of protons to the thylakoid interior (Fowler and Kok, 1974; Junge *et al.*, 1977; Junge and Ausländer, 1977; Fowler, 1977; Saphon and Crofts, 1977), and eliminated the more rapid DLE components due to charge stabilization.

Changes in Chl *a* fluorescence yield (Van Gorkom *et al.*, 1976) and absorbance at 820 nm (Haveman and Mathis, 1976) indicate that the reduction of P680$^+$ at low pH proceeds with a 260–290 μs lifetime. Since the PSII reaction center was only partially closed by the first flash, Van Gorkom *et al.* (1976), and Haveman and Mathis (1976) proposed that the reduction of P680$^+$ proceeds mostly by a back reaction with Q^-, but partly by a reaction with an unidentified donor D. At low pH, the following scheme is believed to operate:



(10)

where all symbols are as previously defined. The combined rates of P680$^+$ reduction by back reaction with Q^- and electron donation by D give the P680$^+$ lifetime of 260 to 290 μs, and a ratio of open (P680Q) to closed (P680Q$^-$) centers of about 75%.

The decay of Q^- under low-pH conditions has not been determined. However, under Tris treatment, which causes changes very similar to low pH (Haveman and Lavorel, 1975; Haveman and Mathis, 1976), Renger and Wolff (1976), by measuring absorption changes at 320 nm, found Q^- to decay with a 145- to 290-μs lifetime. This is somewhat faster than the 360- to 390-μs back-reaction reduction of $P680^+$ predicted in the above scheme, but is of comparable value in view of the different experimental techniques, samples, and errors involved.

DLE in the μs range is suggested to be of the "leakage" type (Lavorel, 1975). In the simplest view of the leakage type DLE, the intensity of DLE (L) is proportional to $[P680^+]$ and $[Q^-]$; thus,

$$\frac{dL}{dt} \propto [P680^-] \frac{d[Q^-]}{dt} + \frac{d[P680^+]}{dt} [Q^-] \qquad (11)$$

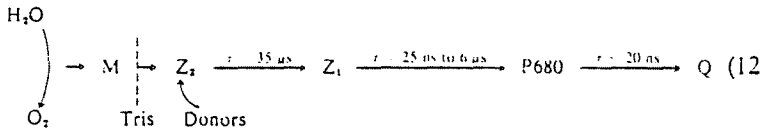
For the rates of $P680^+$ and Q^- decay cited above, this equation gives a DLE decay time of 100 to 165 μs, which agrees fairly well with the 175-μs DLE decay component found by Haveman and Lavorel (1975), but poorly with the 290-μs DLE component found by Van Gorkom *et al.* (1976). Thus, on kinetic grounds there is reason to believe that the 175-μs DLE decay is the intrinsic back reaction time between $P680^+$ and Q^- at low pH. Also, if this is the intrinsic back reaction time in normal samples, and the $ZP680^+ \rightarrow Z^-P680$ has a lifetime of no greater than 6 μs (see Section 2.3.3), then a PSII quantum efficiency of $\geq$ 97% is expected, which is in agreement with observed efficiencies (Sun and Sauer, 1971) of near unity.

3.3.4. DLE Decay in Tris-Washed Samples

If the recombination of charges takes place with a lifetime of 175 μs, then the more rapid components of DLE decay seen in normal samples must reflect charge stabilization reactions such as $ZP680^-Q^- \rightarrow Z^-P680Q^-$. As pointed out by Van Gorkom and Donze (1973), any treatment that would increase the fraction of traps in the Z^-P680Q state will stimulate DLE. Incubation with high concentrations of alkaline Tris buffer (Yamashita and Butler, 1968) inhibits electron flow between the oxygen evolving complex and the PSII reaction center, but does not destroy the reaction center photoactivity in the presence of exogenous electron donors. Using electron spin resonance and polarographic techniques, Babcock and Sauer (1975) and Babcock *et al.* (1976) observed a rapid build up and a slow decay of positive charges on the donor side of PSII in Tris-washed chloroplasts. Thus, Tris washing interrupts the charge stabilization reactions on the donor side of PSII without interfering with PSII charge separation.

Haveman and Lavorel (1975) measured DLE decay in the μs range in Tris-washed chloroplasts using a phosphoroscope. They found that Tris washing enchanced a 175-μs lifetime component and inhibited more rapid decay components. The effect of Tris washing was believed to be similar to low pH, and the 175-μs component to again reflect the intrinsic charge recombination rate.

Parallel measurement of the rise in Chl *a* fluorescence yield (to determine the rate of change of $P680^+$, a quencher of Chl *a* fluorescence, see Section 2.3.2c) and DLE decay in the 6- to 100-μs range, following single saturating 10-ns laser flashes, was carried out by Jursinic and Govindjee (1977*a*) in Tris-washed chloroplasts under various conditions. Tris washing inhibited a 6-μs rise in Chl *a* fluorescence yield (Fig. 8). The effects of Tris were eliminated if PSII electron donors (such as Mn^{2+}, ascorbate, reduced phenylenediamine, and reduced benzidine) were present. Jursinic and Govindjee (1977*a*) also observed that two pre-illumination flashes were needed before the full effect of Tris washing on DLE decay and Chl *a* fluorescence yield rise became apparent. Thus, they suggested that a capacity to hold two charges exists between the site of Tris washing and P680 (see Section 2.3.3b). PSII electron flow reactions could be written as follows:



(12)

where Z_1 and Z_2 are charge carriers, M is the oxygen evolving system, and other symbols are as previously defined. The 25 ns–6 μs- and 35-μs decay components of DLE correspond to the charge stabilization steps of electron movement from Z_1 to $P680^+$ and Z_2 to Z_1^+, respectively. In Tris-washed samples, after a couple of pre-illumination flashes, Z_2 and Z_1 become oxidized, and these charge stabilization steps are inhibited as are the 6- and 35-μs components of DLE decay, and the yield of DLE increases by about 2.5-fold (Jursinic and Govindjee, 1977*a*). (The fast components of DLE decay were not completely inhibited in Tris-washed samples, which was probably due to regeneration of some centers to the Z_2Z_1 P680Q form between flashes.)

3.3.5. DLE Decay in Hydroxylamine Treated Samples

As with low pH and Tris, incubation of samples with NH_2OH disrupts oxygen evolution, but does not eliminate primary charge separation in PSII

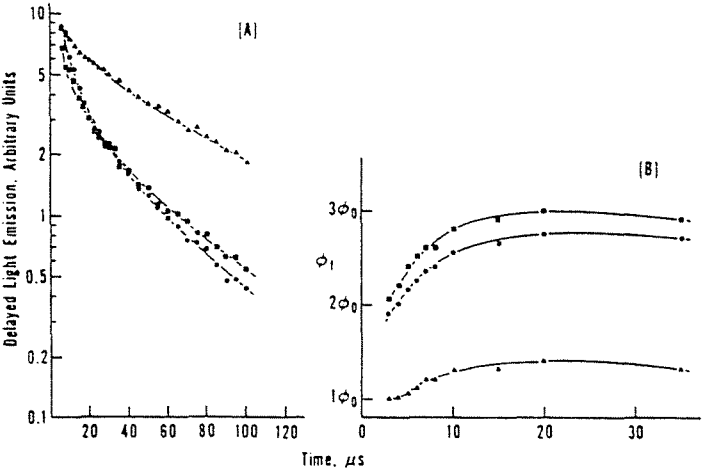


Fig. 8. (A) Logarithmic plot of the decay in delayed light emission after a final flash following a series of pre-illumination flashes: ●——●, untreated control chloroplasts with pre-illumination flashes at a rate of 1 flash/s; ▲——▲, Tris-washed chloroplasts, 1 flash/s; and ◘——■, Tris-washed chloroplasts with 10^{-3} M ascorbate plus 10^{-5} M benzidine, 20 flashes/s. (B) Plot of the rise in Chl *a* fluorescence yield (ϕ_f) in terms of ϕ_0, the level of fluorescence yield prior to excitation. Data from Jursinic and Govindjee (1977*a*).

or PSI (Cheniae and Martin, 1971). Oxygen-evolving capacity was eliminated when two-thirds of the Mn pool was extracted from the thylakoids as a result of incubation with NH_2OH (Cheniae and Martin, 1970). Zankel (1971) observed that NH_2OH treatment caused an enhancement of DLE intensity at 90 μs (indicative of a 50-μs-lifetime decay component) after a single flash excitation. Lavorel (1973) made a thorough study of the effects of NH_2OH on μs DLE following a single flash or multiple flash excitation in *Chlorella* cells. It was found that in 1 mM NH_2OH or greater, a 5- to 10-μs-lifetime DLE component was inhibited, while the 50- to 70-μs and 110- to 300-μs components were enhanced (Fig. 9). The interpretation of this NH_2OH effect (Lavorel, 1973) was that the 5- to 10-μs DLE decay reflected the charge stabilization step, $ZP680^{\cdot} \rightarrow Z^{\cdot}P680$, and NH_2OH inhibited this reaction. This inhibition of charge flow had to be quite close to P680, since unlike Tris (Section 3.3.4) its effect was observed after just one flash (Fig. 9) (see Jursinic, 1977). This NH_2OH effect on charge flow and DLE was supported by experiments of Den Haan *et al.* (1974, 1976) on μs rise kinetics of Chl *a* fluorescence yield (Section 2.3.3c). The enhanced 50- to 70-μs phase was suggested (Lavorel, 1973) to be due to changes on the Q side of PSII. The 50- to 70-μs component could

not be due to electron donation from NH_2OH to $P680^+$, since this component occurred even when NH_2OH was washed out.

When both NH_2OH and DCMU were present and the sample was pre-illuminated the μs DLE did not disappear, but a "new" 5-μs-lifetime component appeared (Lavorel, 1973). This was surprising since illumination of samples with NH_2OH and DCMU present should cause reaction centers to change into a photoinactive state ($P680\ Q^-$). This was the argument used to explain the loss of DLE in the millisecond (Stacy *et al.*, 1971; Mohanty *et al.*, 1971) and second ranges (Bennoun, 1970) in samples having both NH_2OH and DCMU present. Electron flow inhibitors besides DCMU (Ducruet and Lavorel, 1974) also gave rise to this 5-μs-lifetime component even after prolonged illumination. It appears that some centers may not enter the $P680Q^-$ inactive state, or the DLE is from some other source

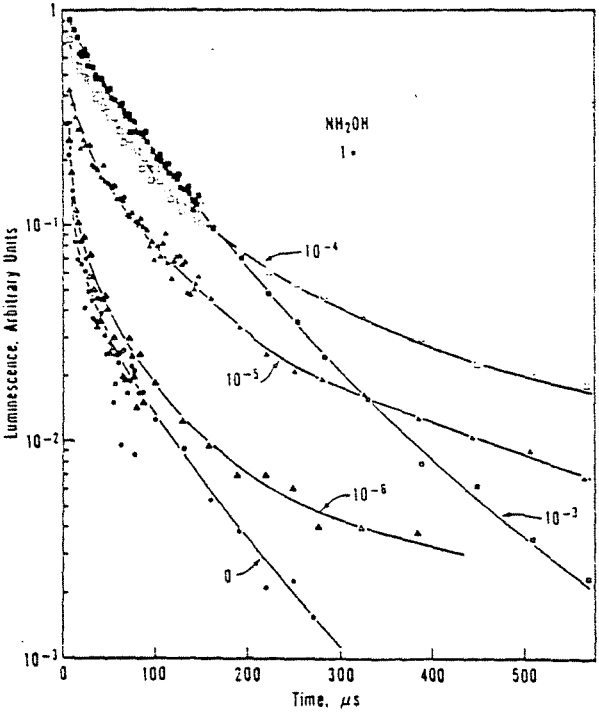


Fig. 9. Effect of NH_2OH on the luminescence decay in *Chlorella*. Molar concentrations of NH_2OH are as indicated. Each sample received one flash (1•) following dark adaptation. Data from Lavorel (1973).

besides charge recombination—perhaps triplet fusion (Stacy *et al.*, 1971) or electron-hole recombination (Arnold, 1976)!

Van Best and Duysens (1977) observed a 1-μs-lifetime DLE decay component in *Chlorella*, believed by them to be due to the $ZP680^+Q^- \rightarrow Z^-P680Q^-$ charge stabilization reaction; this component was inhibited by incubation with NH_2OH, but a 20- to 30-μs component was enhanced. Since these experiments were carried out under conditions where Q was in its reduced form prior to excitation, continued photoactivity was suggested to be due to another acceptor, W (Section 2.3.3).

3.3.6. Effects of Membrane Potential on DLE

DLE originates from the repopulation of singlet excited states of Chl *a* from stored energy. The large scale loss of energy by DLE is avoided presumably by charge separation and stabilization into states having large activation energy barriers for back reaction (Arnold and Azzi, 1968). Any experimental change that might alter these activation barriers would alter DLE.

Modifications of the "high energy state" by uncouplers of phosphorylation (Mayne, 1967), and by the production of transmembrane pH and salt gradients, strongly affect the intensity of DLE in the millisecond and seconds range (Mayne, 1968; Miles and Jagendorf, 1969; Barber and Kraan, 1970; Wraight and Crofts, 1971; Barber and Varley, 1972; Fleischman and Mayne, 1973). During illumination, DLE shows induction kinetics controlled by electron transport and the development of light-induced proton uptake and membrane potential (Kraan *et al.*, 1970; Kraan, 1971; Wraight and Crofts, 1971; Wraight *et al.*, 1971). These effects were ascribed to a modulation of the effective activation energy for DLE by the electrical and chemical gradients of the proton motive force of the chemiosmotic coupling hypothesis (Fleischman, 1971; Crofts *et al.*, 1971).

Lavorel (1975) has reviewed the membrane potential effects on millisecond and second range DLE. Thus, this section will deal with changes in microsecond DLE attributed to thylakoid membrane potential and externally applied electric fields.

DLE decay components† with lifetimes of 50 μs (Zankel, 1971) and 20

† Changes in Chl *a* fluorescence yield with flash number have also been reported by Joliot *et al.* (1971) 1–2 s after *n* strong flashes, and by Delosme (1972) during 2-μs saturating flashes. The quantum yield of fluorescence in the former experiment oscillated in parallel with the $[S_2 + S_3]$, whereas in the latter the yield was complementary to it. The relationship of the changes to the DLE under discussion here remain to be examined. The oscillations in 2-μs fluorescence yield suggest that electron flow from Z_1 to $P680^+$ may be faster when S_2 or S_3 is present than when S_0 or S_1 is present, if we accept the hypothesis that fluorescence yield in this time scale reflects reduction of the quencher $P680^+$.

μs (Duysens *et al.*, 1975) have been observed to vary in intensity with the number of light flashes given to dark adapted samples. (For changes in ms DLE with the number of light flashes, see Barbieri *et al.*, 1970.) One interpretation of these changes is that a positive charge builds up in the oxygen evolving system with flash excitation, resulting in an electric field near the reaction center, which decreases the DLE activation energy. If this interpretation is correct, then the variation with flash number is the first demonstration of an electric field effect or μs DLE.

Jursinic *et al.* (1978), in their studies on the effects of light-induced and salt-jump-induced thylakoid membrane potential on μs and ms DLE from chloroplasts, following a single laser flash, discovered that DLE in the first 100 μs after a flash was independent of the thylakoid membrane potential. Millisecond DLE following a single flash showed sensitivity to thylakoid membrane potential *only if* a proton gradient was present. Earlier studies of ms DLE did not reveal this proton gradient requirement, since they were conducted with the phosphoroscope, not the single excitation flash method, and, thus, always had a proton gradient established. From the lack of an effect of membrane potential on μs DLE, but its presence on ms DLE (when a proton gradient was available), it was concluded that the primary charge separation does not span the entire thylakoid membrane but occurs in ≤ 5 Å, while the secondary charges that give rise to ms DLE are approximately 11 Å apart (Fig. 10). Also, by measuring changes in ms DLE caused by simultaneous injection of KCl and sodium benzoate, which creates a proton gradient, the light-induced potential generated across the thylakoid membrane by a single flash was calibrated and found to be 128 ± 10 mV (Jursinic *et al.*, 1978). This determination by DLE of membrane potential generated by a single flash agrees well with the recent measurements of Zickler *et al.*, (1976) based on voltage dependent ionophores.

A comparison of DLE decay 10 ms or longer after termination of

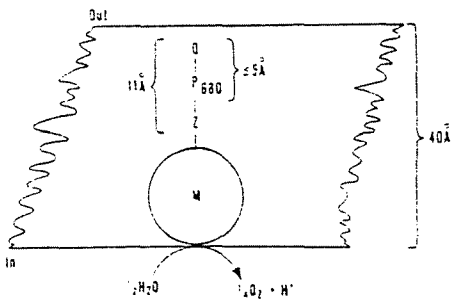


Fig. 10. A working model for the possible arrangement of photosystem II components in the thylakoid membrane based partly on interpretations of DLE membrane potential data (see Section 3.3.6). P680 is the reaction center Chl *a* and the primary electron donor, Q is the "primary" electron acceptor, Z is the first secondary electron donor, and M is the chemical species which accumulates four positive charge equivalents before reacting with water to evolve oxygen. From Jursinic *et al.* (1978).

illumination with the 520 nm absorption change (indicative of thylakoid membrane potential) in *Chlorella* by Joliot and Joliot (1975) showed a close correlation between the enhancement of DLE amplitude at 22 ms after illumination and 520 nm absorption change. However, decay in the stimulation of DLE was found to be more rapid than the decay in 520 nm absorption change. To explain this apparent inconsistency, it was suggested that due to some structural heterogeneity the back reaction in various centers was affected differently by thylakoid membrane potential. For unequal sensitivity of the centers to the electric field, see Diner and Joliot (1976).

Enhancement of DLE by the application of external AC fields was first shown by Arnold and Azzi (1971*a*,*b*). The kinetics of DLE enhancement by pulsed DC fields have been elucidated by Ellenson and Sauer (1976). The enhancement of ms DLE had a risetime of a few tens of microseconds and was a function of the applied field strength, and it decayed as a fast ($\tau_{1/2}$ = 15 μs) and a slow ($\tau_{1/2}$ = 38 μs) component. Experiments using different applied field strengths, photosynthesis inhibitors, ionophores, and electron donors and acceptors affect these fast and slow components differently, suggesting that they have different origins, which are unidentified at this time. Also, intact thylakoid membranes from isolated chloroplasts were required, since this external field enhancement of DLE was not observed in thylakoids with disrupted membranes or in whole cells.

The enhancement of DLE by external AC fields was observed only in a special structure called a "bleb" (Arnold and Azzi, 1977), which is a spherically distended chloroplast formed by suspending chloroplasts in a medium of low osmotic pressure. The "bleb" behaves as a thin electric insultating shell (thylakoid membranes) containing a conductor (ion containing medium), and this intensifies the apparent local field by about 100-fold (Ellenson and Sauer, 1976; Arnold and Azzi, 1977). For intact cells, such as *Chlorella*, the field intensification must occur at an outer membrane where the DLE process does not take place, and no external field enhancement of DLE is observed.

Ellenson and Sauer (1976) did observe an external field enhancement of DLE 100 ms after a single excitation flash. Jursinic *et al.* (1978), however, did not see an enhancement of DLE, observed 1 to 4 ms following a single excitation flash, nor a decrease when the membrane potential was abolished by adding gramicidin or K^+ and valinomycin, unless a proton gradient was present. The reason for the difference in these results is not known at this time, but wide differences in experimental conditions exist: externally applied field versus light or salt-jump-produced membrane potential, "bleb" chloroplasts versus isolated chloroplasts in isotonic medium, and DLE observed at 100 ms versus 1 to 4 ms after single-flash excitation.

Arnold and Azzi (1977) found that if the external electric field was applied during illumination, then a field of the same polarity applied at a

later time stimulated DLE to a lesser extent than a field of opposite polarity. This polarization of the external field stimulation lasted for a few seconds. Ellenson and Sauer (1976) also reported a polarization phenomenon; however, it lasted only 20 ms, affected their fast and slow components differently, and was complicated by the manner in which the field pulses were given. The field polarization phenomenon is believed to be due to the antiparallel orientation of the thylakoid membranes of opposite faces of the "bleb" (Ellenson and Sauer, 1976), and the recovery is due to compensation by ions in the medium and chloroplast rotation.

3.3.7. Effects of Temperature on DLE

DLE is of extremely low intensity presumably because the redox energy of the photoproducts is below that required to reach the first excited state of Chl. In essence, there is an activation energy requirement that may be altered by an electric field, as discussed in the previous section, or by thermal activation, as will be discussed here. The sensitivity of DLE to temperature has been studied by the slow heating of pre-illuminated samples, temperature-jumps, and kinetic studies at various temperatures.

DLE upon slow heating of pre-illuminated samples (glow curve) was first observed by Arnold and Sherwood (1957) on dried chloroplasts. (For glow peaks in photosynthetic bacteria, see Govindjee *et al.*, 1977*c*.) Glow curves from algae, leaf disks, and chloroplasts led Arnold and Azzi (1968) to suggest that the photosynthetic reaction centers behave like semiconductors by analogy with glow curve theory in solid state physics. Slow heating of chloroplasts, pre-illuminated while being cooled at 77°K, produced four glow peaks (Arnold and Azzi, 1968); Shuvalov and Litvin (1969) observed five peaks in plant leaves. According to Arnold and Azzi (1968) these glow peaks are due to thermal lifting of electrons and holes from traps in the photosynthetic pigment solid state system.

Desai *et al.* (1975) observed six peaks at a very slow rate of warming of pre-illuminated and frozen samples: peak Z at 118°K, peak I at 236°K, peak II at 261°K, peak III at 283°K, peak IV at 298°K, and peak V at 321°K. The Z peak is believed to be unrelated to normal photosynthesis (Arnold and Azzi, 1968; Desai *et al.*, 1975; Sane *et al.*, 1977), as it is formed when other peaks and photosynthesis are saturated; we consider it likely that it is due to triplet → singlet transition in aggregated forms of Chl *a* emitting at 740 nm (also see Sane *et al.*, 1974, 1977). Peak II, which is prominent in leaves but almost absent in chloroplasts (Sane *et al.*, 1977), was shown to be directly related to the loss of Q^- (Desai *et al.*, 1975), presumably by back reaction with oxidized donor(s) to give this glow peak. By using subchloroplast fragments enriched in PSI or PSII pigments, Sane *et*

al. (1977) have identified peaks I and II with PSII, peak IV with both PSI and PSII, and peak V with PSI. Inoue (1976) and Inoue *et al.* (1977) have suggested the relationship of glow peaks in isolated chloroplasts to manganese and the oxygen evolution activity. [For the effects of various electron transport inhibitors on glow peaks, see Lurie and Bertsch (1974) and Sane *et al.* (1977), and for the effect of intermittent illumination on the greening of wheat leaves and on the glow peaks, see Ichikawa *et al.* (1975) and Inoue *et al.* (1976*a*,*b*).]

Even though various peaks have been correlated with PSI and PSII activity, further experiments with flashing light and with partial reactions are needed before a better understanding of the origins of glow peaks and their relation to various DLE components will become available. An excellent beginning in this direction has been made by Inoue and Shibata (1977).

Another temperature effect on DLE is observed upon rapid heating (temperature-jump) of pre-illuminated samples, which was first observed by Mar and Govindjee (1971) to cause a burst of DLE. This phenomenon was studied in greater detail by Jursinic and Govindjee (1972) and Malkin and Hardt (1973); this method of stimulating DLE was similar to salt and acid–base jump triggering methods (Lavorel, 1975) and was believed to allow recombination of normally stabilized charged photochemical products. Correlation of T-jump DLE with the T-jump electric field, and the release of protons, recently observed by Shimizu and Nishimura (1977) and Takahama *et al.* (1976), respectively, awaits further experimentation.

The amplitude and decay kinetics of delayed light emission are temperature dependent in the time range of seconds (Strehler and Arnold, 1951; Tollin *et al.*, 1958; Sweetser *et al.*, 1961; Jursinic and Govindjee, 1972; Laine-Böszörmenyl *et al.*, 1972; Malkin and Hardt, 1973), milliseconds (Tollin *et al.*, 1958; Sweetser *et al.*, 1961; Lavorel, 1969), and microseconds (Zankel, 1971; Jursinic and Govindjee, 1977*b*). [For a recent study on the temperature dependence of DLE in *Chlorella*, see Drissler *et al.* (1977).] Both the amplitude and decay rate of DLE decay components in the second and millisecond ranges are sensitive to temperature and increase with increasing temperature. Activation energy, calculated from Arrhenius plots of decay rates, is found to be about 0.68 eV for components in the seconds range (Malkin and Hardt, 1973), but it varies in the different segments of the millisecond range (Sweetser *et al.*, 1961). Jursinic and Govindjee (1972) observed a region of no slope around 10–15°C in the Arrhenius plot of the DLE in the seconds range; this implies a region of no activation energy! Furthermore, in the temperature-jump experiments, the activation energies varied depending upon the initial temperature of the sample. The interpretation of these observations requires further experiments.

The temperature effects on a DLE decay component having a 50-μs

lifetime at room temperature were studied by Zankel (1971). The amplitude and kinetics of this component were found to be temperature sensitive, having activation energies of 0.25 and 0.20 eV, respectively. Based on these temperature data and measurements of DLE quantum yield, various models for the DLE reaction scheme were discussed by Zankel (1971), and the possibility that the 50-μs component arose from direct recombination of positive and negative charges was rejected.

Jursinic and Govindjee (1977*b*) extended the temperature experiments to the early microsecond range and found that a 6-μs DLE decay component was temperature insensitive as was a 6-μs rise component in Chl *a* fluorescence yield. These authors suggested that these components reflected the $ZP680^+ - Z^-P680$ charge stabilization reaction. The origin of this component, however, remains to be explored and established.

The temperature-sensitive 50-μs component was studied under a variety of conditions in chloroplasts isolated from several plant species. It was found that Arrhenius plots of the exponential decay constants are: (a) linear for lettuce and pea, but discontinuous for bush bean (12–17°C) and spinach (12–20°C) chloroplasts (Fig. 11), and (b) unaffected by DCMU (that changes rate of electron flow), gramicidin D (that abolishes membrane potential), and glutaraldehyde fixation (that abolishes gross structural

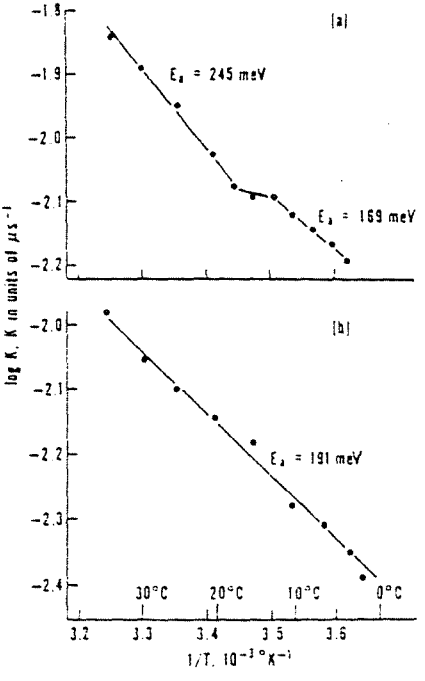


Fig. 11. Arrhenius plots of the exponential decay constant, k, obtained from semilogarithmic plots of delayed light emission decay in the 120–340 μs range in (a) bush bean chloroplasts and (b) lettuce chloroplasts. Both samples had 0.1 μM DCMU present and were at a Chl concentration of 5 μg/ml. The activation energy, E_a, calculated from Arrhenius plot slope is given in millielectron volts (meV). From Jursinic and Govindjee (1977*b*).

changes). The discontinuities were correlated with abrupt changes in (a) the thylakoid membrane lipid fluidity (monitored by ESR spectra of 12-nitroxide stearate, 12NS) and (b) the fluidity of extracted lipids (monitored by differential scanning calorimetry and ESR spectra of 12NS). Thus, it is clear that the temperature sensitivity of μs DLE is not dependent upon intersystem electron transport rate, thylakoid membrane potential, or gross structural changes. Instead, the DLE temperature sensitivity is correlated with changes in thylakoid membrane lipid fluidity, possibly involving a charge stabilization step on the primary acceptor side of PSII, which probably involves lipophilic quinone molecules (Jursinic and Govindjee, 1977*b*).

Upon illumination with continuous light or a phosphoroscope, DLE of a photosynthetic system undergoes slow transient changes in intensity (Lavorel, 1975) similar to the transient in Chl *a* fluorescence (Govindjee and Papageorgiou, 1971). The rate at which this induction phenomena becomes established is a discontinuous function of temperature (Itoh and Murata, 1974; Itoh, 1977) and was suggested to be due to the change in the rate of electron transport linked to the Mehler reaction. The DLE transient goes through various phases or changes in intensity, which show discontinuous changes with temperature (Ono and Murata, 1977). These changes with temperature have also been attributed to changes in membrane lipid fluidity, internal pH, and electron transport in the plastoquinone pool. The relationship between these temperature effects on DLE and photosynthesis requires further investigation.

3.3.8. Conclusions

The study of DLE in the microsecond range has not yet disclosed the underlying mechanism(s) for its generation. The charge recombination theory is still adequate for explaining most of the data; however, more data are needed to decide upon the adequacy of proposed revisions required to explain the μs DLE from photocenters in the $P680Q^-$ form, which is normally considered inactive. The possibility of contributions of the triplet–triplet fusion and electron–hole recombination mechanisms to μs DLE also seem worthy of further consideration. Hopefully, in the next few years the DLE mechanism will be understood well enough that it will really assist in the understanding of other aspects of the photosynthetic process.

4. SUMMARY AND CONCLUDING REMARKS

Photosynthesis converts solar energy into chemical energy; it is this process that winds the clocks of our lives. Its understanding may help us

evolve systems to solve the energy crisis threatening all mankind. The discovery of the synergestic and antagonist effects of light of two selected wavelengths on O_2 evolution and on the redox states of several electron carriers, respectively, led to the present-day concept of two pigment systems and two light reactions driving electron flow from H_2O to pyridine nucleotide. The water oxidizing system (PSII) evolves O_2 and reduces plastoquinones (PQ). The other system (PSI) oxidizes PQ and reduces the pyridine nucleotide nicotinamide adenine dinucleotide phosphate. In this review, PSII has been discussed with special emphasis on the information obtained in the subnanosecond to millisecond range by using Chl *a* fluorescence and delayed light emission (DLE) as intrinsic probes of reactions *in situ*. Section I provides a summary of PSII components and reactions; PSI is described only very briefly.

The analysis of Chl *a* fluorescence changes (sub-ns to ms) and of delayed light emission (sub-μs to ms) has led to the following general picture for PSII. The primary electron donor (P680, a Chl *a* dimer) and the electron acceptor (Q, a quinone) do not span the entire thylakoid membrane. An electron acceptor other than Q must exist (whether it is a pheophytin or not remains to be proven). A secondary electron acceptor (R, also a quinone), which is a two-electron acceptor, is an intermediate between Q and PQ. There are two electron donors (labeled Z_1 and Z_2) between M (the charge accumulator) and P680; additionally, an electron donor D can donate electrons to $P680^+$.

The primary photochemical reaction (the charge separation: $P680\ Q \rightarrow P680^+Q^-$) must occur in the sub-ns range as the lifetime of fluorescence (the main competing reaction) is of that order of magnitude as confirmed recently by fluorescence decay measurements after excitation with low-intensity, single ps flash. The primary back reaction appears to have a halftime of 100–200 μs and, the electron donation by Z to $P680^+$ seems to have a lifetime of 50 ns to few μs (depending on the conditions); this is the major charge stabilization reaction of PSII.

There are several components in the rise of Chl *a* fluorescence yield (Table 3) and in the decay of DLE (Table 4) after a single flash. Several components have also been reported for the decay of $P680^+$ to P680 by the absorption technique. Possible interpretations and relationships were discussed. It is concluded that in order to obtain definite interpretations, parallel measurements on absorption changes due to P680, and due to Q, Chl *a* fluorescence yield, delayed light emission changes in the chloroplast (or algal) suspensions, under identical preparative and instrumental conditions, are required.

Although the hypothesis that $P680^+$ is a quencher of Chl *a* fluorescence has a lot of experimental support, we believe it requires experimental proof. Similarly, most of the experimental data appear to be consistent with the

idea that delayed light emission is due to a back reaction of the primary charge separation; yet, there are some experiments which could equally easily be explained by the electron–hole or the triplet theory. Thus, crucial experiments must be planned to test these ideas. The decrease in lifetime and quantum yield of fluorescence in multiple ps flashes are explained to be due to singlet–triplet annihilations; here, direct measurements on the formation and decay of triplets are required to prove the theory. In the same way, Chl *a* fluorescence yield decreases at high light intensities have been explained by the formation of triplet quenchers. Here, again, parallel measurements on triplets are necessary before this theory can be established. The list is endless, and the authors are convinced that Chl *a* fluorescence and delayed light emission, properly used as probes of photosynthetic reactions, have great potentials, since these are very sensitive, nondestructive *in vivo* probes and, depending upon the time scale of measurement, they can monitor specific reactions. Thus, a large number of different reactions can be probed simply by using different time scales of measurement. However, before this stage is reached, parallel measurements on emission and absorption changes are required for the identification of reactions being monitored.

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