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PROBING FLUORESCENCE INDUCTION IN CHLOROPLASTS ON A NANOSECOND TIME SCALE UTILIZING PICOSECOND LASER PULSE PAIRS

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The fluorescence induction and other fluorescence properties of spinach chloroplasts at room temperature were probed utilizing two 30-ps wide laser pulses (530 nm) spaced Δt (s) apart in time (Δt = 5–110 ns). The energy of the first pulse (P_1) was varied (10^{12}–10^{16} photons · cm^{-2}), while the energy of the second (probe) pulse (P_2) was held constant ($5 \cdot 10^{13}$ photons · cm^{-2}). A gated (10 ns) optical multichannel analyzer-spectrograph system allowed for the detection of the fluorescence generated either by P_1 alone, or by P_2 alone (preceded by P_1). The dominant effect observed for the fluorescence yield generated by P_1 alone is the usual singlet-singlet exciton annihilation which gives rise to a decrease in the yield at high energies. However, when the fluorescence yield of dark-adapted chloroplasts is measured utilizing P_2 (preceded by pulse P_1) an increase in this yield is observed. The magnitude of this increase depends on Δt, and is characterized by a time constant of 28 ± 4 ns. This rise in the fluorescence yield is attributed to a reduction of the oxidized (by P_1) reaction center P-680$^+$ by a primary donor. At high pulse energies ($P_1 = 4 \cdot 10^{14}$ photons · cm^{-2}) the magnitude of this fluorescence induction is diminished by another quenching effect which is attributed to triplet excited states generated by intense P_1 pulses. Assuming that the P_1 pulse energy dependence of the fluorescence yield rise reflects the closing of the reaction centers, it is estimated that about 3–4 photon hits per reaction center are required to close completely the reaction centers, and that there are 185–210 chlorophyll molecules per Photosystem II reaction center.

Introduction

Many recent studies of the primary processes of photosynthesis have been made possible because of the availability of short and intense laser light flashes. Nanosecond and picosecond laser flashes have been particularly useful in studies of exciton dynamics in intact membranes [1,2], and in the elucidation of the primary charge separation sequences and charge stabilization processes in isolated reaction centers of photosynthetic bacteria. The exciton lifetimes are of the order of a few hundred picoseconds in bacterial photosynthetic membranes [3] and in green plants [2]. The stabilization of the charge on the first quinone acceptor in bacterial reaction centers also occurs on this time scale [4,5]. In view of the similarity between reaction centers of bacteria and of PS II of green plants, it is likely that a similar time interval of a few hundreds of picoseconds is required for an analogous stabilization of the charge on the first quinone acceptor in PS II reaction centers. It is

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Abbreviations: Chl, chlorophyll; PS, photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

therefore evident that upon excitation with a flash of duration of 500 ps or more, the excitons created in the initial portion of the flash will encounter mostly open reaction centers, while those created towards the tail portion of the flash will encounter mostly closed reaction centers. Thus, in many investigations up till now in which such relatively long laser flashes were used, the photochemical state of the reaction centers changed during the excitation, thus complicating the interpretation of the phenomena being observed. In principle, this difficulty can be circumvented by utilizing very low intensity light flashes; however, it is often necessary to work with saturating light flashes. Furthermore, the excitons can range over large domains of molecules encompassing several reaction centers [1]; thus, even at low excitation intensities the problem of the heterogeneity in the photochemical state of the reaction centers may still present itself.

These experimental difficulties can be reduced by utilizing the short pulses (5–50 ps) delivered by readily available and, by now, conventional lasers. However, another type of problem can arise because a relatively high density of excitons is created in a short time interval. At high pulse intensities singlet-singlet exciton annihilation occurs and a severe quenching of the fluorescence is observed [1]. Furthermore, long-lived quenchers, most likely triplet states, are also created by intense picosecond laser pulses; these quenchers reduce the fluorescence yield observed upon excitation with subsequent laser pulses [6,7]. These exciton-exciton annihilation effects have been utilized to study some of the parameters which characterize the migration of singlet excitons in the photosynthetic membranes [3,7–9].

In this study we have utilized pairs of picosecond laser pulses to probe such mechanisms by analyzing the fluorescence properties of spinach chloroplasts. The first pulse was utilized to close the reaction centers, while the second pulse incident at a time Δt later (variable from 5 from 110 ns) was used to probe the fluorescence properties of the chloroplasts in which either part or all of the reaction centers were closed by the first flash. Both dark and light-adapted chloroplasts were used to investigate exciton capture by the reaction centers of PS II, as probed by the well known fluorescence induction phenomenon [10,11]. Using the improved time resolution of these experiments the rate constant of the fluorescence induction is found to be 28 ± 4 ns.

Experimental procedure

The chloroplasts were isolated from spinach leaves by standard techniques and were resuspended at 130 μg Chl/ml in about 300 ml of buffer (400 mM sucrose, 20 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.2). When necessary 13 μM DCMU and 13 mM hydroxylamine were added to the sample. Preillumination of the sample was achieved by using a saturating HeNe laser light beam. Tris-washed chloroplasts were prepared according to Ref. 12. The chloroplast suspension was stored in an ice-cooled reservoir, magnetically stirred, and was continuously circulated by means of a peristaltic pump (Buchler) via plastic tubing connected to a glass capillary tube (1 mm diameter). The flow rate was 0.2 ml/s. The reservoir, all of the tubing, and the capillary (except for a 6 mm portion exposed to the laser beam) were darkened.

A neodymium YAG mode-locked laser (Quantel YG 402) provided 30 ps (full-width half-maximum) excitation pulses at 530 nm at a rate of 5 Hz. The laser output was divided into two pulses P_1 and P_2 by a beam splitter as shown in Fig. 1. The main pulse P_1 was focussed on the sample in the capillary tube, while the second pulse P_2 entered a variable delay path (5–110 ns). A beam splitter was utilized to render P_2 colinear with P_1. Another beam splitter was utilized to direct light from a 5 mW HeNe laser onto the same sample area which is exposed to pulses P_1 and P_2. The area of all of these beams at the location of the sample was $8 \cdot 10^{-3}$ cm^2. Electromechanical shutters were positioned along the paths of the pulses P_1 and P_2 so that either one alone or both pulses were allowed to reach the sample. Calibrated neutral density filters (f_1 and f_2) were utilized to vary the intensity of the pulses P_1 and P_2, although the energy of the pulse P_2 was generally held constant, while the energy of P_1 was varied. A beam splitter directed a portion of P_1 to an energy meter (RJ 7200, Laser Precision Corp.). This energy meter can be operated in the ratio mode with

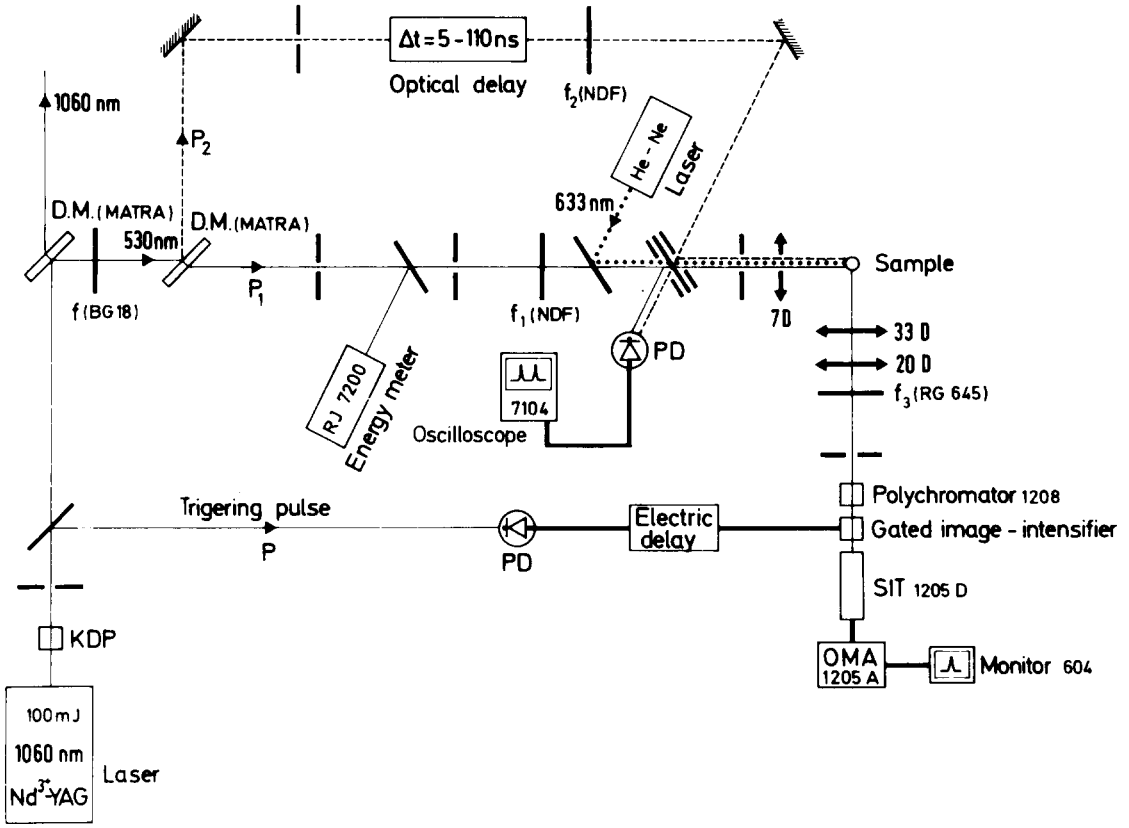

Fig. 1. Schematic diagram of apparatus. D.M., dichroic mirror (Matra); NDF, neutral density filter; BG 18, RG 645, cut-off filter (Schott); 7104, oscilloscope; 604, monitor (Tektronix); PD, fast photodiode; KDP, KH_2PO_4 doubling crystal.

two detector heads, and was also used to calibrate the absolute energies of P_1 and P_2 at the location of the sample. A fast-response photodiode was used to monitor the delay Δt between the pulses P_1 and P_2.

The fluorescence emitted by the sample was focussed onto the entrance slit of a spectrograph protected by an RG645 (Schott) cutoff filter. A gated image intensifier with an opening time of 10 ns was inserted between the spectrograph and an optical multichannel analyzer. By adjusting the opening of the gate in time, it was possible to detect the fluorescence generated either by one or the other of the two closely spaced pulses, and also to discriminate between the fluorescence generated by the pulses P_1 or P_2 and that generated by the continuous He-Ne laser illumination. The opening time of the gate was determined by means of a pulse derived from another fast photodiode and an adjustable electronic delay line. Each data point was obtained by averaging 100 laser shots (the intensity fluctuation from pulse to pulse was $\pm 20\%$) which gave a value for the total integrated fluorescence signal, and another value of the averaged laser intensity (output of the energy meter). The fluorescence yield was obtained by dividing the fluorescence intensity by the actual intensity of the laser pulse incident on the sample.

Results

A typical set of data is shown in Fig. 2. The two curves labeled F_1 and F_1(HeNe) represent the fluorescence yield of dark-adapted and light-adapted chloroplasts, respectively, as a function of the intensity of the P_1 pulse (the fluorescence signal is detected during the excitation by P_1). When the reaction centers are closed by the background

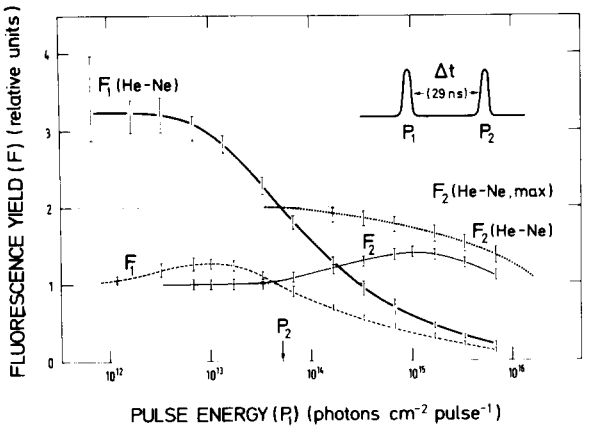

Fig. 2. Dependence of fluorescence yields F on the intensity of the pulse P_1. F_1(HeNe) and F_1, fluorescence measured during excitation by P_1 with and without background illumination, respectively. F_2(HeNe) and F_2, fluorescence yields measured during excitation by pulse P_2 preceded by P_1. P_2, energy of pulses P_2 utilised to measure F_2 and F_2(HeNe). Typical error bars are shown; the reproducibility of the measurements in the F_1 experiments improves as the P_1 energy is increased. In the F_2 measurements the error bars are constant, since the P_2 energy is constant.

illumination with the He-Ne laser, the fluorescence yield is about 3-times larger than for the dark-adapted chloroplasts (in the absence of background illumination). The F_1(HeNe) curve exhibits a decrease with increasing P_1 intensity which sets in at about 10^{13} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}; this decrease in the fluorescence yield can be attributed to singlet-singlet exciton annihilation [8,8a]. A similar effect can be observed with dark-adapted chloroplasts, but F_1 exhibits a slight rise of 10–20% at about 10^{13} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}, before exciton annihilation effects become dominant at higher pulse intensities.

Similar experiments were performed in which the P_1 pulse was succeeded by a P_2 pulse a time interval Δt later. The image intensifier was gated on at the time of arrival of pulse P_2. The curves F_2 and F_2(HeNe) represent the fluorescence yields generated by pulse P_2 for dark-adapted and light-adapted chloroplasts, respectively, as a function of the intensity of P_1. Thus, pulse P_2 probed the fluorescence state of the PS II previously subjected to pulse P_1. The delay between P_1 and P_2 for the data shown in Fig. 2 was 29 ns. The energy of the pulse P_2 was selected as low as possible, but still consistent with a reasonably good signal-to-noise ratio. The energy of the P_2 pulse was held constant at $4 \cdot 10^{13}$ photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}. For this energy, as expected from the F_1(He-Ne) curve, the fluorescence yield in the light-adapted state F_2(He-Ne,max) is about twice the fluorescence yield in the dark-adapted state.

The fluorescence yield F_2(HeNe) exhibits a decrease when the energy of the P_1 pulse exceeds $4 \cdot 10^{14}$ photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}. The yield F_2 is, as expected, lower than F_2(HeNe), and goes through a distinct maximum for P_1 energies of 10^{15} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1} before it declines at still higher energies of P_1.

When the delay time Δt between P_1 and P_2 was varied, results similar to those shown in Fig. 2 were obtained. However, the amplitude of the maximum in F_2 increased when Δt was increased

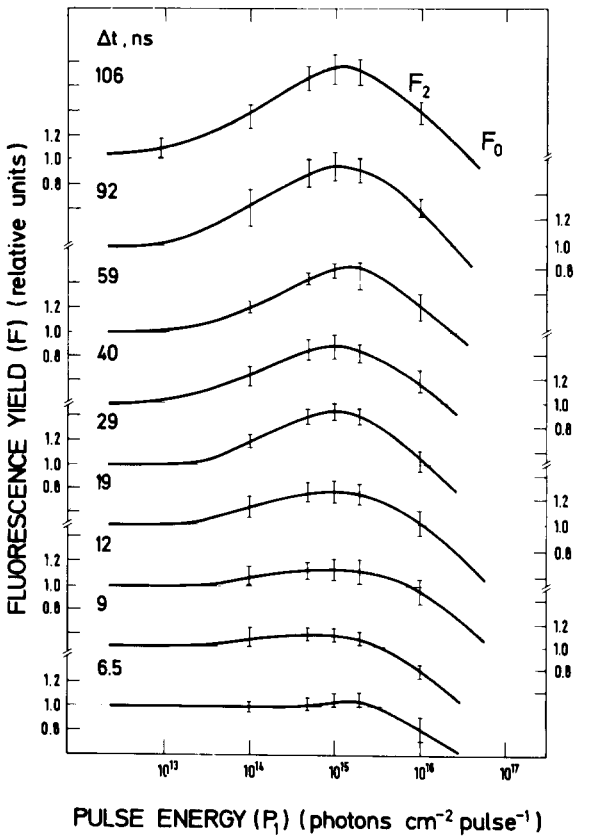

Fig. 3. Dependence of the fluorescence yield F_2 on the delay time Δt between the pulses P_1 and P_2. Most curves are averages of two to three separate experiments and the typical error bars are indicated.

from 5 to 110 ns. The behavior of F_2 for different delay times is summarized in Fig. 3. The error bars shown represent the spread in the data points obtained in different experiments (typically two to three runs).

In contrast to the F_2 curves, the shapes and magnitude of the F_2(HeNe) curves were independent of the delay time Δt. Thus, quenchers are generated by pulse P_1 which survive until pulse P_2 arrives. We define a quenching factor q as follows:

$$q = \frac{F_2(\text{HeNe})}{F_2(\text{HeNe,max})} \quad (1)$$

The significance of q is apparent from Fig. 2; it represents the normalized (with respect to F_2(HeNe,max)) fluorescence yield which is reduced as a result of the generation of long-lived quenchers by pulse P_1. In Fig. 4 it is shown that the fraction of the fluorescence quenched, which is equal to $1 - q$, is independent of the delay time Δt. These data (obtained at a constant energy of $P_1 = 7 \cdot 10^{15}$ photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}) demonstrate that there is no apparent change in the concentration of the long-lived quenchers for Δt up to 100 ns. Their lifetime is therefore much longer than the maximum delay between the pulses P_1 and P_2 utilized in our experiments.

Illumination of chloroplasts in the presence of DCMU and hydroxylamine leads to curves F_1(DCMU), F_2(DCMU) and F_2(DCMU,max) which are very similar (data not shown) to the corresponding curves F_1(HeNe), F_2(HeNe) and F_2(HeNe,max) of Fig. 2, respectively, although the fluorescence yields are 20–40% higher in the DCMU-treated than in the control chloroplasts subjected to HeNe illumination. The curve F_2(DCMU) was also found to be independent of Δt; moreover, the values of q are identical (within experimental uncertainty) to the ones obtained for light-adapted chloroplasts.

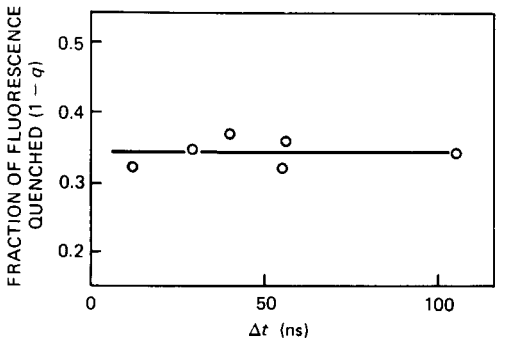

Fig. 4. Fraction of residual fluorescence $(1 - q)$ detected by pulse P_2, as a function of the delay time. The energy of P_1 was held constant at $7 \cdot 10^{15}$ photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}. The parameter q is the fraction of the fluorescence quenched by long-lived quenchers (Eqn. 1).

Using Tris-washed chloroplasts, the fluorescence yield F_1(HeNe,Tris) measured at approx. 10^{13} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1} is about 30% higher than F_1, while this relative increase is about 200% in the control (F_1(HeNe)). Furthermore, for $\Delta t =$ 106 ns the curve F_2(Tris) does not show any significant increase (data not shown), while an approx. 70% increase is observed under normal conditions.

All of the results described were independent of the flow rate in the range 0.05–0.2 ml/s.

Discussion

Before proceeding to an interpretation of the results we first review in some detail the events which occur in reaction centers of green plants and the factors which can influence the fluorescence yields of such systems.

Effects of the state of the reaction centers on fluorescence yields

Since the original hypothesis of Duysens and Sweers [10] relating the increase in the fluorescence yield (induction) observed upon illumination of dark-adapted chloroplasts to the photochemical state of the acceptor side of PS II, the induction of fluorescence has been extensively used to investigate the primary reactions of PS II. Using 10-ns laser flashes, Mauzerall [13] observed a biphasic rise time (25 ns, 3 μs) in the induction curve and concluded that these kinetics were too slow to reflect the primary charge separation. Considering that P-680 as well as P-680$^+$ are fluorescence quenchers, Butler [11] argued that the limiting steps in the fluorescence induction are dark reactions on the donor side of PS II. The observation that in dark-adapted chloroplasts P-680$^+$ decays in 35 ± 10 ns [14] reinforces this interpretation. Furthermore, Sonneveld et al. [15] measured fluorescence yields in *Chlorella* as a function of excitation

energy utilizing 15- and 30-ns laser pulses; they were able to account for the shapes of the fluorescence yield vs. pulse energy curves by assuming (i) that P-680$^+$ and P-680 have the same quenching efficiencies, and (2) that the rate constant of reduction of P-680$^+$ was 20–25 ns.

In all measurements of fluorescence yields involving microsecond or sub-microsecond pulse excitation, the role of other fluorescence quenchers must be considered. Among these quenchers is a carotenoid triplet state which was first described by Duysens et al. [16], and which has a lifetime of several microseconds. Quenching by this carotenoid triplet could explain the slow phase (approx. 3 μs) in the fluorescence induction curve observed by Mauzerall [13]. The close parallelism between the kinetics of quenching of the fluorescence and of the buildup and decay of carotenoid triplets has been investigated [9,17]. Furthermore, it has been demonstrated that the yield of these long-lived quenchers was much higher for laser flashes of nanosecond or microsecond duration than in the case of single picosecond laser flashes [6,9]. While the carotenoid triplets can be created directly within the antenna [6,9], it is also possible that excitation of chloroplasts by light pulses of longer duration than the time required for the stabilization of the primary charges (and which leads to multiple excitation of the reaction center) can create triplet states within the reaction center itself.

According to the present state of knowledge [18], the reaction center of PS II consists of a primary chlorophyll donor species (P-680) in close proximity to a pheophytin (Phe) molecule which acts as an intermediate acceptor. A quinone (Q) molecule acts as the first stable acceptor and is believed to be located in close proximity to pheophytin.

A singlet exciton S_1 is captured by an open reaction center complex and gives rise to an excited P-680* molecule which decays by a charge separation, and ultimately results in the stabilization of the charge on Q:

$$S_1 + \text{P-680-Phe-Q} \rightarrow \text{P-680*-Phe-Q} \rightarrow \text{P-680}^+\text{-Phe}^-\text{-Q} \rightarrow$$

$$\rightarrow \text{P-680}^+\text{-Phe-Q}^-$$

The initial charge separation occurs on picosecond time scales, while the transfer of an electron from pheophytin to Q probably occurs within several hundred picoseconds. An unidentified donor (D) reduces P-680$^+$ on a time scale of 35 ± 10 ns [14], thus regenerating P-680:

$$D + \text{P-680}^+\text{-Phe-Q}^- \rightarrow D^+ + \text{P-680-Phe-Q}^-$$

When all of the reaction centers of PS II are in the open state (P-680-Phe-Q), the fluorescence yield is low, since S_1 excitons are readily quenched by the open reaction centers. However, when the reaction centers are closed (state P-680-Phe-Q$^-$) either by illumination or by chemical reduction they can still capture an exciton according to:

$$S_1 + \text{P-680-Phe-Q}^- \rightarrow \text{P-680*-Phe-Q}^- \rightarrow \text{P-680}^+\text{-Phe}^-\text{Q}^-$$

The back-reaction can regenerate P-680* and gives rise to a delayed luminescence [19] which is believed to account for the fluorescence induction phenomenon.

Single-pulse fluorescence yields (F_l and F_l(HeNe))

The relative values of F_1 and F_1(HeNe) observed at low P_1 pulse intensities (Fig. 2) can be accounted for in terms of fluorescence induction. We note that the curves F_1 and F_1(HeNe) presented here are very similar to those recently reported by Hirsch et al. [20], although these authors used maize leaves and the inhibitor DCMU rather than background illumination to close the reation centers. The slight increase in the yield F_1 which is observed at P_1 pulse intensities of about 10^{13} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1} cannot be attributed to the usual fluorescence induction phenomenon. Only a negligible fraction of the reaction centers can be reduced to the state D$^+$-P-680-Phe-Q$^-$ during the short lifetimes [2] of the singlet excitons (less than 500 ps) created by the P_1 pulse, while the reduction of P-680$^+$ by D occurs on time scales of tens of nanoseconds [14]. This increase in F_1, which was also noted by Hirsch et al. [20], is discussed below in the section comparing the trapping efficiencies of P-680 and P-680$^+$. A quantitative analysis of these data in terms of absorption cross-section, singlet-singlet annihilation coefficients, etc., using the newly developed master equation theory of Paillotin et al. [21] will be presented in a subsequent publication.

In the light-adapted state only the prominent

decline in the yield due to singlet-singlet annihilation is evident (curve F_1(HeNe) in Fig. 2). A similar decline is also evident in the dark-adapted state (curve F_1, Fig. 2), but it sets in at higher pulse energies. This difference in pulse energies at which exciton-exciton annihilation events become noticeable reflects the competition between capture of S_1 excitons by reaction centers and $S_1 + S_1$ annihilation. When the reaction centers are open, the mean exciton lifetime is lower than in the case of closed reaction centers [2], and thus a higher exciton density is required in order to observe the effects of exciton-exciton annihilation on the fluorescence yield. At the highest pulse energies the F_1 and F_1(HeNe) curves approach one another, indicating that exciton annihilation dominates over reaction center capture processes.

Fluorescence induction – double-pulse experiments (F_2)

The curve labeled F_2(HeNe) shown in Fig. 2 is a measure of the influence of the pulse P_1 on the fluorescence of light-adapted chloroplasts measured a time Δt later. Above a P_1 energy of approx. $4 \cdot 10^{14}$ photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1} a long-lived quencher which persists for tens of nanoseconds, or more, manifests itself by causing a decrease in the fluorescence yield measured by P_2. It should be noted that the decrease in F_2(HeNe) at P_1 energies of 10^{16} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1} is only approx. 40% (as compared to the low-intensity regime), while F_1(HeNe) is decreased by a factor of approx. 15 at the same P_1 energies. This comparison indicates that relatively few long-lived quenchers which can survive until pulse P_2 arrives are generated by the first pulse P_1. The large quenching observed in F_1(HeNe) is due to the mutual annihilation of short-lived singlet excitons which cannot survive until the probe pulse P_2 arrives at the sample. A similar conclusion concerning the relatively low efficiency of generation of long-lived quenchers by single picosecond laser pulses was reached earlier by utilizing sequences of picosecond pulses spaced 5 ns apart [6,22].

The behavior of the fluorescence yield curve F_2 indicates that the effect of the rise in the fluorescence yield due to the closing of reaction centers by P_1, and the partial reduction of P-680$^+$, is diminished by the long-lived quencher which is also generated by P_1. In order to characterize further the long-lived quencher and the fluorescence rise, both F_2 and F_2(HeNe) were measured as a function of the delay time Δt. The fluorescence rise is, within experimental error, absent in F_2(HeNe), and only the long-lived quencher manifests itself. It is shown in Fig. 4 that the effect of the long-lived quencher on the fluorescence yield curve F_2(HeNe) is independent of the delay time Δt in the range 5–110 ns. The nature of this quencher has not been investigated in this work. However, as mentioned above, carotenoid triplets are known to be created by a variety of high-energy laser flashes, including picosecond pulse trains [1]. Since these carotenoid triplets have lifetimes of several microseconds, it is likely that the long-lived quenchers which are observed in this work are indeed carotenoid triplets.

The effect of the long-lived quenchers on the yield F_2(HeNe) is independent of the delay time Δt (Fig. 4) and is the same for control or DCMU-treated chloroplasts. This suggests that the curve F_2 can be adjusted by subtracting the effects of these long-lived quenchers. In this manner, the actual effect of the energy of the P_1 pulse on the fluorescence increase at different values of Δt can be estimated. This correction is made by assuming that the effects of the quenchers and of the partially closed reaction centers on the fluorescence yield are additive. It is thus assumed that the long-lived quenchers proportionally have the same effect on the fluorescence yield whether the reaction centers are completely open, completely closed, or only partially closed. With this assumption, if we denote by F_2' the fluorescence yield after correction for the effect of the long-lived quencher, we can write:

$$F_2 = F_2' q \tag{2}$$

where q is the factor by which the overall fluorescence is assumed to be reduced by the long-lived quenchers (Eqn. 1). The quantity F_2' thus represents the fluorescence yield which would be observed if long-lived quenchers were entirely absent. Thus, by utilizing the correction of Eqn. 2 to adjust each one of the F_2 curves shown for different delay times in Fig. 3, the effect of P_1 energy on the fluorescence yield F_2 detected with a constant-energy probe pulse P_2 can be obtained. Examples

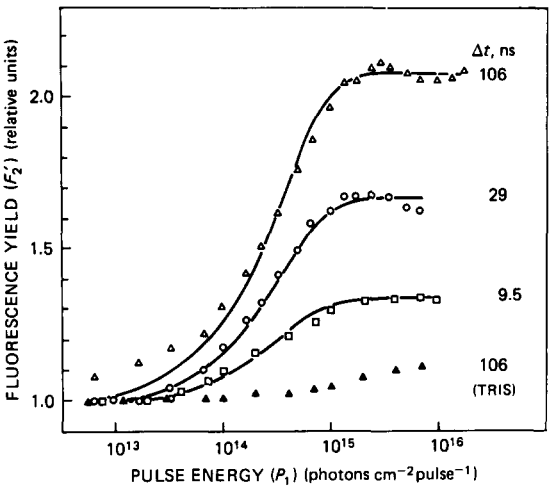

Fig. 5. Fluorescence yield F_2' (corrected for quenching) (see Eqn. 2), as a function of the energy of P_1 for different delay times. The curve labeled 106 (TRIS) was obtained with Tris-washed chloroplasts. The data points were obtained from the curves in Fig. 3, while the solid lines are plots of Eqn. 3 adjusted to the experimental data as discussed in the text.

of F_2' curves as a function of the P_1 pulse energy are shown for three different delay times in Fig. 5.

In Fig. 5 we have also plotted an F'_2 curve for Tris-washed chloroplasts at $\Delta t = 106$ ns. The very modest induction which is obtained under these conditions is consistent with the observation that Tris treatment slows down considerably the reduction of P-680$^+$ [12].

The observation that the maximum value of F_2' at the longest delay time of 106 ns is identical (within the precision of our measurements) to F_2(HeNe,max) indicates that all of the fluorescence induction exhibited by dark-adapted chloroplasts is complete within this time interval. This correlates well with the observation [14] that under similar conditions all of the P-680$^+$ states are also reduced with a time constant of 35 ± 10 ns.

Analysis of fluorescence induction curves

For every one of the delay times shown in Fig. 5 the corrected F_2' curves reach a saturation value at similar values of the P_1 pulse energy (approx. 10^{15} photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}), suggesting that all of the reaction centers are closed at this level of excitation energy. The saturation levels in F_2' become higher the larger the value of Δt.

Mauzerall [23], utilizing 7 ns laser excitation pulses followed by weak intensity probe pulses 30 μs later to probe the fluorescence state of *Chlorella*, obtained results similar to those shown in Fig. 5.

A detailed master equation approach for calculating the excitation energy dependence for the transformation of reaction centers (open → closed) was developed by Paillotin et al. [21]. The fraction of reaction centers (ϕ_{RC}) which become closed as a result of excitation with a light pulse of total number of incident photons I (photons $\cdot$ cm^{-2} $\cdot$ pulse^{-1}) depends on the number (m) of reaction centers per photosynthetic unit and the parameter $R = K_o/K_m$, and the quantum yield χ for the transformation:

open reaction center → closed reaction center

when an exciton interacts with a reaction center. The rate constants K_m and K_o denote the exciton decay constants when all m reaction centers are in the closed and open states, respectively. In this theory the finiteness of the photosynthetic units is taken into account; thus, the $m = 1$ case usually refers to the 'puddle' model, while the $m = \infty$ case refers to the 'lake' model of the photosynthetic unit.

In general, for arbitrary values of R and m, ϕ_{RC} is represented by a series of exponential terms; however, when R is close to unity, the energy dependence of ϕ_{RC} reduces to the following, single-exponential expression [21]:

$$\phi_{RC} \approx 1 - \exp\left[-\left(\frac{n}{m}\right)\chi_o \sigma I\right] \qquad (3)$$

where σ (cm^2) is the absorption cross-section (per chlorophyll molecule) at the excitation wavelength, and n the number of chlorophyll antenna molecules per domain. Since, in the derivation of Eqn. 3, it is assumed that there are m reaction centers per domain, the ration n/m is the number of chlorophyll molecules per reaction center.

Mauzerall [24] has previously derived the same expression for ϕ_{RC} based on a different, statistical model; the exponential dependence of the quantum yield is termed the 'Poisson saturation curve'. The argument of the exponent is X/t, the number of hits X per trap, where t is the number of traps per PSU in his notation (m in eq. 3). Mauzerall makes the point that the exponential dependence

of ϕ_{RC} is independent of the number of reaction centers per PSU. However, the master equation theory of Paillotin et al. [21] shows that this is generally not true and that the exponential dependence (Eqn. 3) is obtained for all values of m only if $R \approx 1.0$. The use of Eqn. 3 in our work is thus appropriate, since there is no change in the fluorescence yield within at least several nanoseconds after P_1, when the excitons created by the first flash convert the reaction centers from the open to the closed state. The usual higher values of R are obtained only after 100 ns, and thus Eqn. 3 is no longer valid only for excitation flashes whose duration is of the order of 10–100 ns, or longer. Making the additional assumption that $q \propto F_2'$, the dependence of ϕ_{RC} on I according to Eqn. 3 is compared to the experimental data in Fig. 5; a fit to the data was obtained by setting the arguments of the exponential to unity and matching the calculated curves to the experimental F_2' data at 63% of the maximum values. The fits are reasonable except at the low-energy values of the $\Delta t = 106$ ns data. In Mauzerall's experiments of fluorescence induction measured 30 μs after a 7 ns laser flash, a good fit of Eqn. 3 to the data was obtained as well [23,24]. For each of the three curves in Fig. 5 the 63% valule of ϕ_{RC} is obtained when $I = (3.2 \pm 0.2) \cdot 10^{14}$ photons $\cdot$ cm$^{-2} \cdot$ pulse^{-1}; at this excitation energy:

$$\left(\frac{n}{m}\right)\chi_o \sigma I = 1.0 \qquad (4)$$

and the number of chlorophyll molecules per reaction center can be estimated.

At the excitation wavelength of 530 nm, the absorption cross-section is $\sigma = 1.6 \cdot 10^{-17}$ cm$^2 \cdot$ molecule^{-1} [25]. Assuming that $\chi_o \approx 1$, the number of chlorophyll molecules per reaction center is estimated to be $n/m \approx 185$–210.

Recently, Ley and Mauzerall [26,27] have estimated from oxygen yield measurements in *Chlorella vulgaris* that this ratio is 130–260, depending on the level of irradiance during the growth of the algae. This value is thus comparable to our ratio $n/m = 185$–210 for spinach chloroplasts.

The limiting energy necessary to close all the reaction centers is about 10^{15} photons $\cdot$ cm$^{-2} \cdot$ pulse^{-1} (Fig. 5) which corresponds to 3–4 hits per reaction center.

It is noteworthy that the closing, or oxidation of the PS II reaction centers as measured by the corrected fluorescence induction curves F_2', appears to be just as efficient when single 30 ps picosecond laser pulses or 5–7 ns excitation pulses [27] are utilized. In our picosecond pulse experiments, the excitation energy dependence of F_2', and thus the closing of the reaction centers, is fairly well approximated by Eqn. 3, even though singlet-singlet exciton annihilation is occurring in the same pulse energy range of 10^{14}–10^{15} photons $\cdot$ cm^{-2}. In deriving Eqn. 3, exciton-exciton annihilation was completely neglected. The reasonable fit of this equation to the data suggests that exciton capture by reaction centers may be much more efficient than singlet-singlet annihilation. This subject will be analyzed in more detail elsewhere.

Utilizing laser flashes of 450–750 ns duration, a decrease in the oxygen yield was noted at the highest pulse energies (approx. 10^{16} photon $\cdot$ cm$^{-2} \cdot$ pulse^{-1}) by Ley and Mauzerall [27]. Under these conditions [9], carotenoid triplets may well start to compete effectively with the reaction centers for the excitons; however, this explanation is not favored by Ley and Mauzerall [27].

Fluorescence induction rate constants

To determine the time constant characterizing the induction of the fluorescence we have utilized the data shown in Fig. 3 by calculating F_2' values at the same energies of the pulse P_1, but for different delay times Δt; a series of fluorescence induction curves as a function of the time interval Δt can be constructed for each energy of P_1 (Fig. 6). Assuming that this fluorescence rise is exponential in nature we have fitted the data in Fig. 6 with the equation:

$$F_2' = F_2'(\max)\{1 - \exp(-kt)\} \qquad (5)$$

Satisfactory fits are obtained with values of $k = (28$ ns$)^{-1}$. The error in k, due to the dispersion of the experimental data, is estimated as approx. $\pm(4$ ns$)^{-1}$.

Comparison of the trapping efficiency of P-680 and P-680$^+$

It has been generally assumed [11,15] that P-680 and P-680$^+$ have the same trapping efficiency.

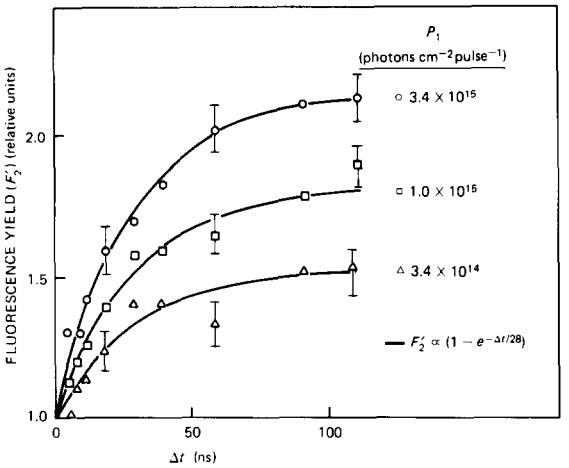

Fig. 6. Fluorescence induction F_2' (corrected for quenching according to Eqn. 2) as a function of delay time measured at three different P_1 pulse energies; the data points were taken from Fig. 3 while the solid lines represent plots of Eqn. 5 with $k = (28\ \text{ns})^{-1}$.

However, Hirsch et al. [20] have interpreted the slight increase of the fluorescence yield when dark-adapted chloroplasts are excited by picosecond pulses of approx. 10^{13} photons · cm^{-2} · pulse^{-1} (see also Fig. 2, curve F_1) by assuming that the state P-680$^+$ is 3-times less efficient as a fluorescence quencher than the state P-680. Our double-pulse experiments do not seem to support this interpretation. In the range of energy of 10^{12}–$3 \cdot 10^{13}$ photons · cm^{-2} · pulse^{-1}, the state of the trap changes as seen by curve F_1 (Fig. 2). However, the yield of fluorescence measured for F_2 in the same energy range is not affected. This is also valid for the curve obtained at $\Delta t = 6$ ns (Fig. 3) and indicates that even after a short delay (6 ns) the fluorescence yield returns to its original F_0 value. Provided that the use of a probe pulse P_2, which has a somewhat high energy, does not affect our data, these observations point towards an identical quenching efficiency of the states P-680-Phe-Q and P-680$^+$-Phe-Q$^-$. The slight increase in the fluorescence yield observed for F_1 at 10^{13} photons · cm^{-2} · pulse^{-1} more probably reflects a lower quenching efficiency of a more primary state (P-680*-Phe-Q and/or P-680$^+$-Phe$^-$-Q). We note that in the case of Sonneveld et al. [15], it was not necessary to take such states into account in the analysis of their data; the instantaneous concentration of these transient species was probably low in their experiments, since the duration of the pulses was relatively long (15 or 30 ns). An investigation of these processes utilizing a lower energy of the probe pulse P_2 and shorter delay times Δt is presently in progress in our laboratory.

Conclusions

The two-pulse picosecond laser excitation experiments described in this work allow for an accurate time resolution for measuring fluorescence yields on nanosecond time scales. The PS II is prepared in a given photochemical state by the first picosecond pulse, while the second pulse is utilized to probe the fluorescence yield of the system in this photochemical state. The fluorescence induction time of 28 ± 4 ns is correlated with the reduction time of P-680$^+$ by a donor D as observed by flash absorption spectroscopy methods. These results further imply that the fluorescence quenching efficiencies of the two different photochemical states of PS II which contain either P-680 or P-680$^+$ are similar. The energy dependence of the fluorescence yield of dark-adapted chloroplasts suggests that a short-lived (1 ns) photochemical intermediate state exists which has a higher fluorescence yield than the reaction centers in either the dark-adapted state, or in the state P-680$^+$.

A complicating feature in the analysis of these two-pulse experiments is that long-lived quenching states, probably carotenoid triplets, are created by the first picosecond laser pulse and survive for 100 ns or more, until the second probe pulse arrives. This effect results in a reduced fluorescence yield detected by the probe pulse. Fortunately, this effect seems to be additive and thus can be subtracted from the overall observed fluorescence generated by the probe pulse. In this manner the increase in the variable fluorescence yield as a function of the delay time between the two pulses can be estimated.

Because of the utilization of intense picosecond laser pulses a large number of excitons are created within the photosynthetic units within a short time interval. Singlet-singlet annihilation, as evidenced by a decrease of the fluorescence yield with increasing pulse energy, is occurring. In principle, a

competition between annihilation and exciton capture by reaction centers can occur. However, the latter process seems to be much more efficient that singlet-singlet exciton annihilation, as evidenced by the fact that the energy dependence of the closing of the PS II reaction centers displays an exponential type of energy dependence and saturation at about 3–4 hits per reaction center.

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