EXCITON ANNIHILATION IN THE TWO PHOTOSYSTEMS IN CHLOROPLASTS AT 100°K

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ABSTRACT The fluorescence yield (F) of spinach chloroplasts at 100°K measured at 735 nm (photosystem I fluorescence—F 735) and at 685 nm (photosystem II fluorescence—F 685) has been determined with different modes of laser excitation. The modes of excitation included a single picosecond pulse, sequences of picosecond pulses (4, 22, and 300 pulses spaced 5 ns apart) and a single nonmode-locked 2-μs pulse (MP mode). The F 735/F 685 intensity ratios decrease from 1.62 to 0.61 when a single picosecond pulse (or low-power continuous helium-neon laser) is replaced by excitation with the 300-ps pulse train (PPT mode) or MP mode. In the PPT mode of excitation, the 735-nm fluorescence band is quenched by a factor of 45 as the intensity is increased from 10^{15} to 10^{18} photons/cm^2 per pulse train and the 685-nm fluorescence is quenched by a factor of 10. In the MP mode, the quenching factors are 25 and 7, respectively, in the same intensity range. Fluorescence quantum yield measurements with different picosecond pulse sequences indicate that relatively long-lived quenching species are operative, which survive from one picosecond pulse to another within the pulse train. The excitonic processes possible in the photosynthetic units are discussed in detail. The differences in the quenching factors between the MP and PPT modes of excitation are attributed to singlet-singlet annihilation, possible when picosecond pulses are utilized, but minimized in the MP mode of excitation. The long-lived quenchers are identified as triplets and/or bulk chlorophyll ions formed by singlet-singlet annihilation. The preferential quenching in photosystem I is attributed to triplet excitons. The influence of heating effects, photochemistry, bleaching, and two-photon processes is also considered and is shown to be negligible.

INTRODUCTION

In the photosynthesis of green plants, energy is absorbed by the antennae pigment molecules (chlorophyll and carotenoids) and is transferred to the reaction centers of photosystem I (PS I) and photosystem II (PS II). PS I is associated with the CO_2-reducing side, while PS II is associated with the O_2-evolving side of the photosynthetic apparatus. Some of the energy not utilized for photosynthesis is emitted as fluorescence (quantum yield ~ 1–3%) by chlorophyll *a* molecules (1). At low temperatures (~ 150°K) the fluorescence of chlorophyll associated with either PS I or PS II can be resolved (2–3). According to Butler and Kitajima (4), PS I pigments exhibit a maxi-

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mum in the fluorescence spectrum at 735 nm while chlorophyll *a* associated with light harvesting and PS II pigments display a maximum at 685 nm and a shoulder at 695 nm. At ambient temperature the PS I fluorescence is almost completely absent and only the maximum at 685 nm is observed.

Recently, Mauzerall (5) and Campillo et al. (6) have reported that the fluorescence quantum yield of *Chlorella pyrenoidosa* at room temperature decreases with increasing photon flux per pulse when either a single 7-ns (5) or single 20-ps pulse (6) of laser light is used for excitation. These effects have been attributed to the multiple excitations and subsequent exciton-exciton annihilation in photosynthetic units (6, 7). Such processes have been well characterized in organic crystals and solutions where singlet-singlet, singlet-triplet, and triplet-triplet annihilation have all been observed (8).

The pigment composition of the two photosystems is quite different. The light-harvesting pigment-protein complexes that give rise to the emission at 685 nm (4) contain most of the chlorophyll *b* (chlorophyll *a* to chlorophyll *b* ratio 1:1 [9]). Since energy transfer between these light-harvesting chlorophyll molecules and chlorophyll antenna molecules associated with PS II is believed to occur readily in both directions (4), we shall, for simplicity, refer to the fluorescence band at 685 nm as the PS II emission band. Since the energy levels of chlorophyll *b* are higher than those of chlorophyll *a*, energy transfer from chlorophyll *b* to chlorophyll *a* occurs readily, but does not occur in the reverse direction. Therefore, exciton migration between chlorophyll *a* molecules within the light-harvesting pigments should be inhibited by the presence of chlorophyll *b* molecules, which act as "obstacles" (10). Since this effect tends to reduce the macroscopic diffusion coefficient of the excitons, the bimolecular annihilation rate constant should be reduced as well. These considerations indicate that bimolecular exciton quenching of the PS II emission at 685 nm may be less efficient than quenching within PS I.

In this paper we report our results on the quenching of the fluorescence of spinach chloroplasts at 100°K. Low temperatures were utilized to resolve the PS I and PS II fluorescence bands. Different modes of excitation with sequences of varying lengths of picosecond pulses (single, 4, and 22 pulses in a pulse train) and a nonmode-locked pulse 2 μs long were utilized to differentiate between the different quenching mechanisms. The various excitonic interactions possible in photosynthetic units are discussed in detail. With microsecond excitation pulses, singlet-singlet annihilation processes are expected to be negligible, while the opposite is true when picosecond pulses are utilized. Singlet excitons do not survive from one picosecond pulse to another (spaced 5 ns apart) when pulse sequences are used. Thus, with pulse sequences of different lengths, the fluorescence quantum yield can provide information about the presence of relatively long-lived quenching states that survive from one excitation pulse to another when picosecond pulse sequences are used. These experiments demonstrate that long-lived quenchers are indeed present and that quenching by these long-lived quenchers, most likely triplets, is more efficient in PS I than in PS II.

MATERIALS AND METHODS

Spinach Chloroplasts

The details of the preparation of the spinach chloroplasts from whole spinach leaves is described elsewhere (11). The chloroplasts were suspended in a sucrose (0.4 M)-Tris (20 mM, pH 8.2)—KCl (20 mM) buffer solution. Several drops of this suspension were squeezed between two microscope slide cover slips so that the effective sample thickness was between 0.05 and 0.2 mm. The optical density was kept between 0.15 and 0.20 at the absorption maximum of 680 nm; thus the optical density at the wavelength of the laser (~ 610 nm) was always kept below 0.04.

Apparatus

The fluorescence was excited with a mode-locked dye laser (Electro-Photonics model 33) operated with the dye rhodamine 6 G, while mode-locking was achieved with the dye 3,3'-diethyl oxadicarbocyanine iodide (DODCI). The output of the mode-locked laser consisted of a train of about 300 pulses, 5–10 ps in width and spaced about 5 ns apart. The pulses were best defined in width and in amplitude within the central part of the pulse train and a Pockels cell was utilized to select pulses from this portion of the train. Gate widths of 3, 20, and 100 ns were utilized to select either 1, 4, or 22 pulses in sequence.

The number of the selected pulses, as well as observations of mode-locking in general, was determined by means of a streak camera (model ICC 512, Electro-Photonics, Belfast, Northern Ireland). The individual pulses and pulse sequences were observed directly on the camera screen and recorded on Polaroid film (Polaroid Corp., Cambridge, Mass.).

The fluorescence spectra were recorded by means of an f/3.5 spectrograph-optical multichannel analyzer arrangement. With this device it was possible to record complete fluorescence spectra with either a single-pulse laser shot or any of the pulse sequences utilized. The fluorescence spectra were stored in digital form in the memory of the optical multichannel analyzer (OMA), which allowed for signal averaging as necessary. The fluorescence spectra were read out on an $x - y$ plotter.

The relative fluorescence quantum yield as a function of the excitation energy was determined by dividing the digital values of the fluorescence intensities at 685 and 735 nm (recorded by the OMA) by the laser output energy. The output energy of the laser was measured by using a 45° beam splitter, which reflected a portion of the total energy onto the detector head of an energy meter (model R 3230, Laser Precision Corporation, Yorkville, N.Y.).

The response of the spectrograph-OMA system was determined by substituting a diffuser for the sample. A nonlinearity of as much as 20% was noted; at low intensities in particular, the readout was lower than at intermediate and high intensities. The fluorescence readings were corrected, as necessary, for this nonlinearity.

The sensitivity of the OMA did not extend to the last decade of excitation intensities studied. In this regime of excitation energies (~ 10^{15} photons/cm^2), a Hamamatsu R 712 photomultiplier fitted with neutral density and interference filters was utilized (Hamamatsu Corp., Middlesex, N.J.). This system, using the entire picosecond pulse train, also exhibited small nonlinearities (~ 15%), for which corrections were made as necessary.

Cooling of the samples was achieved by means of a regulated nitrogen gas flow (stability ± 1°C) in a cryostat. The laser beam was focused onto the sample (area $\simeq$ 0.02 cm^2) and the fluorescence was in turn focused onto the entrance slit (0.5 mm) of the spectrograph.

RESULTS

Several modes of excitation of the fluorescence of spinach chloroplasts were used and are listed below:

(*a*) Continuous low-intensity excitation with a 5 mW helium-neon laser (He-Ne), attenuated to an intensity of not more than 2 mW/cm^2 ($\sim 10^{16}$ photons/cm^2 per s).

(*b*) Entire picosecond pulse train (PPT).

(*c*) Nonmode locked laser pulse of $\sim 2\ \mu$s duration (MP). This pulse was obtained by simply removing the mode-locking dye DODCI.

(*d*) Single picosecond pulse selected from the pulse train.

(*e*) Sequence of four consecutive picosecond pulses.

(*f*) Sequence of 22 consecutive picosecond pulses.

The Pockels cell was used for modes of excitation *d* through *f*. It was verified that the contribution of the background pulses (light that passes through the Pockels cell anyway when the gate is off) contributed less than 4% to the signal observed when the gate was switched on.

The He-Ne laser was primarily used to determine the relative intensities of the 685 and 735 nm fluorescence bands (denoted by F 685 and F 735, respectively) under conditions of relatively low continuous excitation intensities. The ratio of the intensities F 735/F 685 varied from sample to sample within the range of 1.3–1.8.

We have found that the F 735/F 685 ratio, and thus the general aspect of the fluorescence spectrum, varied strongly with the type of excitation utilized and with the intensity of the exciting laser pulses. This effect is demonstrated in Table I for a particular sample and for different pulse sequences. It is evident that the F 735/F 685 ratio is

TABLE I

AVERAGE INTEGRATED QUANTUM YIELD (ϕ, IN RELATIVE UNITS) OF THE PHOTOSYSTEM I (735 nm) AND PHOTOSYSTEM II (685 nm) FLUORESCENCE OF SPINACH CHLOROPLASTS AT 95°K AS A FUNCTION OF THE NUMBER OF SUCCESSIVE PICOSECOND PULSES

Number of pulses	Average energy	ϕ_{685}	ϕ_{735}	ϕ_{735}/ϕ_{685}
	μJ§/pulse			
1*	15 ± 2	740 ± 70	1,170 ± 130	1.62 ± 0.07
4*	19 ± 2	250 ± 35	400 ± 40	1.63 ± 0.10
22*	18 ± 2	170 ± 8	207 ± 15	1.20 ± 0.09
300‡	15 ± 1	980 ± 140‖	588 ± 70‖	0.61 ± 0.02
(He-Ne)	–	–	–	(1.62)

*The results given are averages of 10 separate experiments.

‡Three experiments; the total energy for the entire pulse train was 4.5 ± 0.2 mJ and the figure of 15 ± 1 μJ/pulse was obtained by dividing the total energy by ~ 300.

§10 μJ of energy incident on the sample corresponds to 3.7 × 10^{16} photons/cm^2.

‖When the entire pulse train is utilized, the individual pulses vary in intensity, some with less and some others with more than the average energy indicated. The average quantum yield values thus cannot be easily compared to the other pulse sequences where the pulse energies were fairly uniform.

unchanged when either the He-Ne, a single picosecond pulse, or four successive picosecond pulses are utilized to measure the fluorescence spectrum. However, when the 22-pulse sequence or the entire pulse train (PPT) is used, there is a strong decrease in this ratio. The fluorescence intensity at 735 nm, i.e., the PS I fluorescence, is weakest when the whole train is used for excitation. A similar effect is observed when the MP excitation mode is used.

The preferential quenching of the 735 nm fluorescence band also depends strikingly on the intensity of the excitation. Typical spectra at two different intensities for the PPT and MP excitation modes are shown in Fig. 1 and are compared, in each case, to the spectrum obtained with the He-Ne laser. If the excitation energy is reduced below $\sim 10^{15}$ photons/cm^2 for the entire pulse train or the 2-μs pulse, a fluorescence spectrum identical to the one generated with the He-Ne laser is obtained (not shown in Fig. 1).

These changes in the spectra are attributed to a preferential quenching of the PS I fluorescence at 735 nm. However, strong quenching can also be observed within PS II. This is also illustrated in Table I. The relative quantum yield of fluorescence drops by a factor of three within both PS I and PS II when a single pulse is replaced by the four-pulse sequence. When the 22-pulse sequence is used, there is a still further drop in the quantum yield, but the decrease within PS I is more pronounced. When the entire pulse train is used, the preferential drop in the quantum yield at 735 nm is still further accentuated and the F 735/F 685 ratio decreases to $\sim$ 0.61.

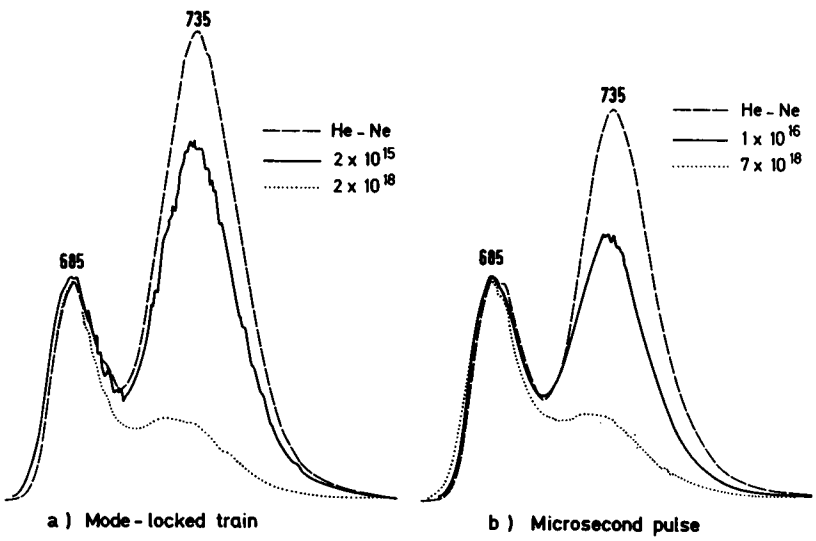

FIGURE 1 (*a*) Fluorescence spectra of spinach chloroplasts at 95°K with either a helium-neon laser for excitation or the entire train of picosecond pulses (PPT) at two different intensities (in units of photons/cm^2 per pulse train incident on the sample). (*b*) Fluorescence spectra of spinach chloroplasts at 100°K with a $\sim$2-μs nonmode locked pulse (MP) for excitation at two different intensities. The helium-neon laser-induced spectrum is also shown for comparison. No corrections for the spectral response of the spectrograph or the OMA detector head (type 1205 D) have been made.

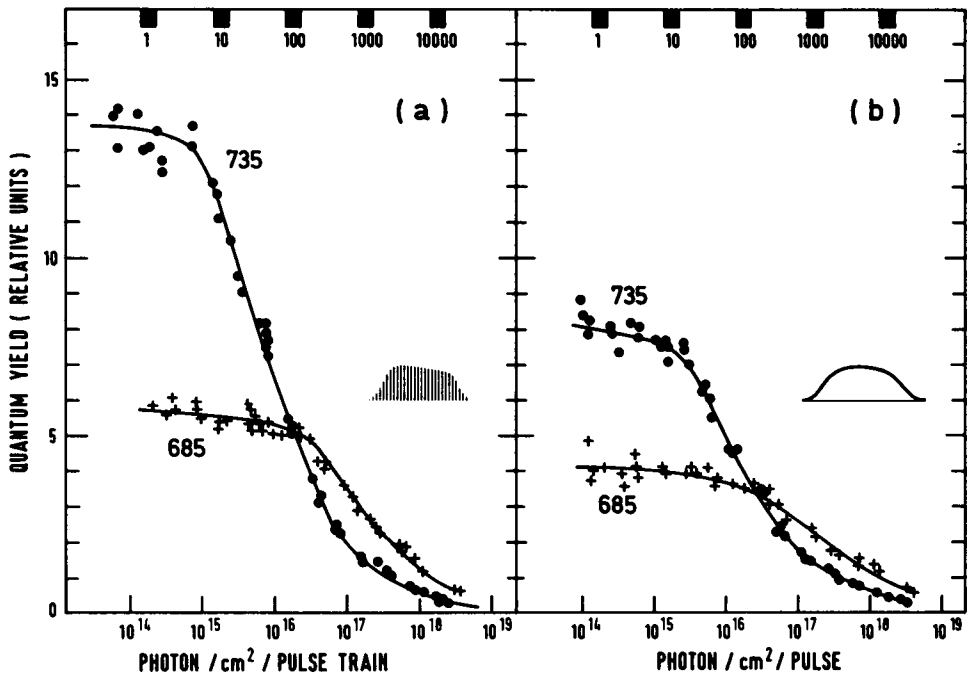

FIGURE 2 Fluorescence quantum yield of spinach chloroplasts (in relative units) as a function of excitation energy. The data in both (*a*) and (*b*) were obtained with the same sample at 100°K. The calculated total numbers of photon hits per photosynthetic unit are given at the top of the figure (see Results for details). (*a*) picosecond pulse train excitation (PPT); (*b*) excitation with a ~ 2-μs pulse (MP). The type of excitation is also indicated schematically in *a* and *b*.

The results shown in Table I indicate that a relatively long-lived quenching intermediate (or intermediates) is produced during each individual picosecond pulse, persists at least until the subsequent pulse, and is able to quench the fluorescence produced by the subsequent pulse. For pulse sequences of 22 or 300 pulses, this effect is particularly pronounced for the PS I fluorescence.

The relative quantum yields of fluorescence measured at 685 and 735 nm as a function of laser pulse energy with the PPT and MP modes of excitation are shown in Fig. 2. In this figure it can be seen that quenching at 735 nm sets in at intensities of 10^{15} photons/cm^2 per pulse train, about 30 times lower than the intensity at which quenching at 680 nm sets in. Similar effects are observed in the MP mode of excitation (Fig. 2 *b*). Thus qualitatively similar results are obtained with the two different modes of excitation in Fig. 2. However, in the PPT mode the quenching factors (quantum yield at the lowest intensity divided by the yield at the highest intensity utilized) are higher than in the MP mode. For the sample of Fig. 2, the quenching factors are 45 and 10 at 735 and 685 nm, respectively, for the PPT pulse train mode, and 25 and 7, respectively, for the MP mode. Analogous results were obtained with other samples.

We have also studied quenching curves with the single pulse excitation mode in the intensity range of 4×10^{14} to 2×10^{16} photons/cm^2 per pulse. Our OMA system at the present time is not sufficiently sensitive to extend these quenching studies to lower intensities. In the excitation energy range studied, the quenching factor is about four for both the 685 and the 735 nm fluorescence. The quenching factor is about four for both the 685 and the 735 nm fluorescence. The quenching factor at 735 nm is at most 10–15% higher than at 685 nm. This is consistent with the results given in Table I, where, within experimental error, the quenching factors are the same for the PS I and PS II fluorescence with single pulse excitation. If the same amount of total energy is supplied in the whole pulse train mode, the energy per individual picosecond pulse is, of course, much smaller. As can be seen in Fig. 2*a*, there is very little quenching at 685 nm, while the quantum yield at 735 nm drops by more than a factor of two in the range of 4×10^{14} to 2×10^{16} photons/cm^2 per pulse train.

The number of hits per photosynthetic unit, assuming that a unit consists of 200–300 molecules, has been estimated from the photon flux and the absorption of the sample. For optical densities below 0.05, the light is fairly uniformly absorbed throughout the sample and the calculated number of hits per unit is constant as a function of optical density for values less than 0.05. For higher optical densities the light is absorbed nonuniformly and the number of hits per unit calculated for the same flux decreases with increasing optical density. Therefore, in fluorescence-quenching experiments, the optical density of samples must be kept below ~0.05. Otherwise, the inhomogeneous intensity of the light beam within the sample will give rise to fluorescence emanating from photosynthetic units receiving few hits as well as from units in the front portion of the sample, facing the laser, which receive many hits. In such cases the quenching factors will be too low.

Furthermore, even if the absorbance of the sample is kept below 0.05, nonuniform absorption of light within a single chloroplast must be taken into account. Wavelengths of excitation in which the absorption coefficient of chlorophyll and accessory pigments is low are to be preferred. The wavelength of 610 nm, used in this work, corresponds to a relatively small absorption coefficient; using typical absolute absorptivities provided elsewhere (12), we estimate that within a given chloroplast the light intensity gradient is 10% or less at 610 nm. The error thus introduced in the quenching factors is probably negligible as compared to the other errors inherent in such work (estimate of absolute flux densities, area of the sample, and inhomogeneities within the light beam).

The estimated number of hits per photosynthetic unit is indicated at the top of Fig. 2 by black squares. The left-hand side of the square reflects the calculation of the number of hits using a value of 200 molecules/photosynthetic unit, while the right hand side corresponds to a calculation using 300 molecules/unit.

DISCUSSION

The striking difference in the quenching of the fluorescence of chloroplasts viewed at 685 and 735 nm once more confirms that these two bands have different origins. It is

well known that the fluorescence band at 735 nm attributed to PS I decreases in intensity as the temperature is raised (2, 3, 13). Since high-energy densities are utilized in our experiments, possible heating effects and their subsequent influence on the fluorescence spectrum must be considered.

Possible Temperature Effects

We first note that the samples are partially bleached when excitation energies approach the values of 10^{19} photons/cm^2 per pulse train. In such cases, subsequent quantum yield measurements at lower intensities are, of course, no longer reproducible. To verify that no irreversible changes had taken place at the higher excitation energies, quantum yields at lower energies were always remeasured after a given series of experiments.

In should be also noted that in Fig. 2, selective quenching within PS I sets in at relatively modest excitation energies. At energies of 10^{17} photons/cm^2 per pulse, the extent of quenching in PS I approaches 80% of the limiting values in Fig. 2. If we take a typical optical density of 0.05 and a sample thickness of 0.01–0.02 cm, then 10% of the photons are absorbed, which corresponds to an energy density of the order of $\sim$ 0.01–0.1 cal/cm^3 if all of the photon energy is converted to heat. This is clearly not enough energy to cause significant heating effects (estimated to be $< 1°$/pulse train).

We consider next local transient heating effects that may occur due to possible nonuniform absorption of the light and due to the fact that the energy is delivered within a very short time (picoseconds). Rather than calculating thermal equilibration times using a diffusion equation, we shall proceed to a direct experimental demonstration that this type of effect cannot account for the observed preferential quenching of the 735 nm fluorescence band. We have compared fluorescence spectra obtained upon excitation with a single pulse of 4×10^{16} photons/cm^2 and an entire pulse train, containing an equal number of photons, but spread out over the 300 pulses within the train. Transient heating effects should be strongest with the single pulse and weakest with the pulse train, since within the pulse train the average energy per pulse is much lower; therefore in the single pulse mode the F 735/F 685 ratio should be lower than in the PPT mode if local heating effects are important. However, exactly the opposite is observed. In the single pulse mode the F 735/F 685 ratio was 1.6–1.8 while in the PPT mode it was $\sim$ 0.6. We thus conclude that the change in the fluorescence spectra obtained at the higher excitation energies, multiple pulse sequences, and MP mode are not due to thermal effects.

Photochemistry

The effects of the photochemical events in chloroplasts on the fluorescence intensity are well known (1). The fluorescence intensity rises when the samples are illuminated and as the photochemical events at the reaction centers take place. The fluorescence of PS II depends on photochemistry in this manner. Using a strong actinic flash and a much weaker second flash to probe the quantum yield of fluorescence ϕ at variable time intervals (Δt) after the actinic flash, Mauzerall (14) has shown that ϕ rises by a

factor of two for $\Delta t \approx 100$ ns and an additional $\approx 50\%$ for $\Delta t \approx 20\ \mu$s. This latter rise appears to be linked to photochemistry (15). The fast, 100-ns rise time may be linked to a drop in the concentration, on these time scales, of triplet excitons created by the actinic flash.

At low temperatures, fluorescence induction effects (increase in fluorescence intensity) attributed to photochemistry are also observed (16, 17). The amount of variable fluorescence is larger in PS II than in PS I; furthermore, the variable fluorescence observed at 735 nm is attributed to a spillover of singlet excitons from PS II to PS I (18).

In our quenching experiments, particularly in the PPT and MP excitation modes where the excitation times range up to 2 μs, the effects of photochemical events on the fluorescence yield cannot be eliminated a priori. We note, however, the following facts, which indicate that photochemical effects, if any, do not play any decisive role in the experiments described here:

(*a*) In our experiments the reaction centers were always in the closed state, since the sample was excited many times, with the individual experiments being spaced 10–20-s apart. Thus the fluorescence was at its maximal level (16–17), since not enough time was allowed between experiments to allow the chloroplasts to return to their dark, adapted state.

(*b*) Photochemical events tend to increase the fluorescence, while we observe strong decreases by factors of up to 45. Thus, photochemical events might only affect somewhat the magnitudes of the quenching factors.

(*c*) The effects of photochemical events on fluorescence yields are much less important in PS I than in PS II. According to Kitajima and Butler (18), while the PS II fluorescence increases by a factor of four, the PS I fluorescence increases by only $\sim 30\%$ during the induction.

Possible Influence of Two-Photon Absorption and Bleaching Effects

Quenching due to photon-exciton interactions and subsequent ionization can be also discounted at the excitation energies used in these experiments (7). Two-photon ionization is also possible at high energy densities, which can again lead to a loss of fluorescence. Such nonlinear effects should manifest themselves most strongly with the single picosecond pulse excitation mode.

To estimate the possible contributions of these effects to fluorescence quenching, we studied the relative quantum yield of a 20 mMchlorophyll *a* solution in diethyl ether at room temperature using single picosecond pulse excitation. Within the excitation range of (5–200) $\times 10^{14}$ photons/cm^2 per pulse, there was no change in the fluorescence quantum yield within experimental error ($\pm$ 5%). This shows that two-photon absorption and exciton-photon interactions can be discounted as important fluorescence-quenching mechanisms.

However, when the 22-pulse train sequence is used to excite the fluorescence of this particular chlorophyll *a* solution, there is a decrease by a factor of 1.8 in the quantum yield, as the integrated energy of the pulse train is increased from 5×10^{14} to 5×10^{17} photons/cm^2 per pulse train. This quenching factor is much smaller than

observed in whole chloroplasts over the same energy range. The observed quenching by a factor of 1.8 in the solution cannot be due to bimolecular annihilation by excited states of chlorophyll. This can be easily shown by refering to the Stern-Volmer equation, $F_o/F = 1 + K\tau_o Q$, where τ_o ($\sim$ 5 ns) is the lifetime of the singlets in the absence of the quencher and F_o is the corresponding fluorescence yield. Q is the concentration of quencher and F is the fluorescence yield in the presence of Q. Typical values of K, the bimolecular quenching constant, are of the order of 10^{10} M^{-1} s^{-1} for diffusion-controlled processes (19). If we take an even larger value, $K = 10^{11}$ M^{-1} s^{-1}, and use $\tau_o = 5$ ns and $F_o/F \sim 2$, we find that $Q \approx 2 \times 10$ mM, 10 times higher than the actual chlorophyll concentration of the solution used in our experiments.

We attribute the small observed drop in the fluorescence quantum yield of the chlorophyll *a* solution to a bleaching of the ground state as molecules are promoted to their triplet excited states. In air-saturated solutions, the chlorophyll triplet lifetime is controlled by dissolved molecular oxygen. Quenching constants for the radiationless deactivation of chlorophyll triplets by oxygen are expected to be of the order of 10^9 M^{-1} s^{-1} (19). Thus the triplet lifetime in air-saturated solution is expected to be of the order of several hundred nanoseconds (19). Using a 100-ns pulse train, we can thus excite a significant proportion of chlorophyll molecules to their triplet excited states. At the highest intensities we used with the 22-pulse train, four photons per molecule were absorbed (if no bleaching is assumed). The bleaching mechanism could thus in principle be important in accounting for the drop in the fluorescence of chloroplasts under conditions of high intensity when pulse trains are used for excitation. However, the quenching factors observed ($\sim$ 10–40) appear too large to be accounted for in these terms.

Exciton Interactions and their Occurrence When Different Modes of Excitation Are Employed

Annihilative bimolecular exciton interactions are efficient processes for quenching singlet excitons in organic media (8). Singlet excitons (S_1) are quenched by any of the following excitonic processes, in which the quenchers themselves are excited species created by the excitation:

$$\text{singlet-singlet: } S_1 + S_1 \xrightarrow{\gamma_{SS}} S_o + S_n; \tag{1}$$

$$\text{singlet-triplet: } S_1 + T_1 \xrightarrow{\gamma_{ST}} S_o + T_n; \tag{2}$$

$$\text{singlet-ion: } S_1 + N(\pm) \xrightarrow{\gamma_{Si}} S_o + N(\pm). \tag{3}$$

S_o denotes the ground state, T_1 a triplet state, while the subscript n denotes excited states higher than the first excited singlet (S_1) or triplet (T_1) states. $N(\pm)$ denotes either a positive or negative ion (of chlorophyll in our case), and the bimolecular rate constants are denoted by γ. The rate constants γ_{SS}, γ_{ST}, and γ_{Si} depend on the diffusion constant of the singlet excitons and on other factors (20). Rahman and Knox (20) have shown that quenching of chlorophyll *a* singlets by triplet excitons is

allowed by the Förster energy transfer mechanism. The rate constants γ_{ST} and γ_{SS} are thus probably not too different in value.

In organic media, singlet-singlet annihilation (21, 22) can also produce ionized charge-carrier pairs, and the possibility that $S_1 + T_1$ interaction also produces ions within the antenna pigment molecules cannot be excluded either. These two processes, denoted by

$$S_1 + S_1 \rightarrow N(+) + N(-) \text{ and} \tag{4}$$

$$S_1 + T_1 \rightarrow N(+) + N(-), \tag{5}$$

should be energetically possible. If we take the energy of the singlets as 1.8 eV and the energy of triplets in vivo as ~ 1.3 eV (23, 24), then the pooled energy is ~ 3.6 eV in process 4 and 3.1 eV in 5. The ionization threshold in chloroplast antenna pigment systems (not at the level of the reaction centers) is not known. However, in nonpolar crystals, such as anthracene and tetracene, the values are 3.7 and 3.0 eV, respectively. It is thus entirely possible that the ionization threshold in chloroplasts is of the order of or less than 3.0 eV, which would energetically permit processes 4 and 5.

Triplets can be formed by the usual intersystem crossing mechanism. If this rate constant is k_{IS}, and the average singlet lifetime is τ_S, then the quantum yield of triplet formation is $k_{IS}\tau_S$. Effectively this means that fewer triplets will be produced under conditions of strong singlet exciton quenching, an effect observed by Mathis (25). However, under these conditions, there may be another source of triplet excitons. If ion pairs are formed according to process 4 by $S_1 + S_1$ annihilation, these ions can recombine to form either triplet or singlet excitons:

$$N(+) + N(-) \rightarrow T_1 \text{ or } S_1. \tag{6}$$

The ion pairs formed initially according to process 4 have overall singlet character. However, if the recombination time is of the order of nanoseconds or more, the unpaired spins may be located in different magnetic environments due to anisotropic g-factors or hyperfine interactions. This will give rise to a time-dependent mixing of the $m_s = 0$ triplet sublevels with the singlet. Under conditions of very high excitation intensities, when singlet-singlet annihilation can occur, ion recombination may thus be an important source of triplets, while intersystem crossover may be unimportant due to the short singlet lifetimes.

To summarize, a consideration of excitonic quenching mechanisms indicates that there are two types of quenchers of singlets: the short-lived ($\lesssim$ 1.5 ns [26]) singlets, and longer-lived quenchers, which may be triplets or ions. With different modes of excitation, it is possible to obtain information about these short-lived and longer-lived quenching species.

Single Picosecond Pulse Excitation

When a single picosecond pulse is used to excite the fluorescence, the influence of long-lived quenchers should be minimal (7) and singlet-singlet annihilation (6, 7, 27) is the dominant quenching channel. Campillo et al. (27) have found that the drop in the

quantum yield of fluorescence is accompanied by a decrease in the singlet lifetime, as expected, when single picosecond pulses are used; thus, fewer triplets should be formed as the intensity of the pulses is increased. On the other hand, more ions may be formed per pulse according to process 4, and triplets may be formed by the recombination of these ions according to process 6.

Excitation with Picosecond Pulse Trains

At 100°K, the fluorescence (singlet) decay times (under conditions of low exciting intensities) have been measured by Hervo et al. (26). The PS I fluorescence at 685 nm exhibits a decay time of 0.9 ns. Thus, in sequences of picosecond pulses in which the pulses are 5 ns apart, singlets do not survive from one pulse to the next. Ions or triplets, on the other hand, may survive from pulse to pulse and thus give rise to additional quenching channels. The fluorescence quantum yield with multiple pulse sequences will depend on the ion recombination times, the triplet lifetimes, the quenching efficiencies, etc. Since these quantities are not yet known, we will limit ourselves here to a qualitative, rather than quantitative, discussion of the results.

The relative fluorescence yields at 685 and 735 nm with different pulse sequences are summarized in Table I. These relative yields represent an average obtained by summing the total fluorescence signal and dividing it by the total energy of the pulse train. The rather precipitous drop (by a factor of three) from a single to four-pulse excitation is noteworthy, particularly since the subsequent drop when the excitation is changed from 4 to 22 pulses is relatively small. The preferential quenching in PS I does not appear either in the single or four-pulse excitation mode, since the F 735/F 685 peak ratios, and thus the spectra, remain unchanged even during strong quenching. The preferential quenching in PS I appears in the 22-pulse and even more strongly when the entire pulse train is used, which leads to the dramatic change in the emission spectrum (Fig. 1).

The data in Table I indicate that two types of quenchers may be operative. One of these appears to quench the fluorescence strongly and with equal efficiency in both PS I and PS II, and its concentration is already high after only four pulses, while the other one builds up more slowly in time and acts preferentially in PS I. It is entirely possible that the "rapid buildup" quenchers are ions and/or triplets produced by ion recombination, since it is less likely that a significant number of triplets can be formed by the intersystem crossing mechanism in the single or four-pulse excitation mode. The "slow buildup" quenchers, on the other hand, may be triplets. It may be possible to distinguish between these two types of quenchers by monitoring as a function of time either variations in triplet densities by triplet-triplet absorption (25, 28) or by monitoring the ion concentration via the chlorophyll *a* cation absorption band at 820 nm (29). Some of these experiments are presently being planned in this laboratory.

The Microsecond Pulse (MP) Excitation Mode

In this type of excitation it can be shown that singlet-singlet annihilation is not an important mode of quenching, at least for light intensities below 10^{17} photons/cm^2 per pulse. With a singlet lifetime of ~ 1 ns and a pulse duration of 2,000 ns, the probabil-

ity of finding two singlets *at the same time* within a given photosynthetic unit is negligible for less than a total of ~ 4,000 total hits/unit per pulse.

Since $S_1 + S_1$ collisions can be neglected, formation of ions by process 4 can also be discounted. Thus, quenching is due only to long-lived quenching species with this type of excitation. These quenchers are either triplets or ions or both, formed by $S_1 + T_1$ interaction.

The similarity of the fluorescence spectra obtained in the MP and PPT modes of excitation (Fig. 1) indicates that the slow buildup quencher, which acts in PS I preferentially, does not necessarily originate via ion formation due to singlet-singlet exciton collisions.

The general aspect of the quenching curves obtained with MP and PPT types of excitation are also similar. The higher quenching factors obtained in the PPT mode is due to $S_1 + S_1$ and $S_1 + N(\pm)$ annihilation. These processes appear to operate with similar efficiencies in both photosystems, as the data in Table I indicate.

Preferential Quenching in PS I

Quenching within PS I sets in at intensities corresponding to about 10 hits/unit distributed over the entire pulse train in the PPT mode or 2-μs pulse in the MP mode. In the PPT mode this is considerably less than 1 hit/unit per single picosecond pulse, and $S_1 + S_1$ processes are not important at these intensities. The onset of the quenching at the relatively low intensity of ~ 10^{15} photons/cm^2 per pulse train is thus also consistent with the existence of a long-lived quenching intermediate.

The onset of the quenching within PS II takes place at intensities at least 30 times higher than in PS I.

The overall instantaneous rate of decay of singlets can be described by the equation

$$\frac{dn_S}{dt} = -k_S n_S - [\gamma_{SS} n_S + \gamma_{ST} n_T + \gamma_{Si} N(\pm)] n_S, \tag{7}$$

where n_S and n_T represent the densities of singlet and triplet excitons, while $N(\pm)$ represents the densities of ions. k_S is the unimolecular decay constant.

The onset of the quenching occurs approximately when

$$\frac{1}{\tau_S} = k_S = \gamma_{ST} n_T + \gamma_{SS} n_S + \gamma_{Si} N(\pm). \tag{8}$$

The number of hits per unit are sufficiently low that the $\gamma_{SS} n_S$ term can be dropped. Furthermore, the ions are formed only as a result of $T_1 + S_1$ interactions under these conditions. Thus, at the onset of the quenching, $\gamma_{ST} n_T > \gamma_{Si} N(\pm)$, and we conclude that triplets are operative as quenchers. Near the onset of the quenching the condition $\tau_S^{-1} \sim \gamma_{ST} n_T$ thus prevails in both the PPT and MP modes of excitation.

This quenching is considerably more efficient in photosystem I than in photosystem II. The differences may be due to either

$$\gamma_{ST}^{I} \gg \gamma_{ST}^{II} \tag{9}$$

or

$$n_T^{\mathrm{I}} \gg n_T^{\mathrm{II}}, \tag{10}$$

where the superscripts pertain to the two photosystems. Condition 9 would be consistent with the hypothesis of Swenberg et al. (10). However, the possibility that the triplet excitons have different lifetimes in the two photosystems, thus giving rise to condition 10, cannot be excluded. Furthermore, rapid energy transfer from chlorophyll *a* triplets to carotenoids (25), coupled with different efficiencies of this process in the two photosystems (perhaps due to different carotenoid concentrations or distributions), may give rise to different types of triplets in the two photosystems. While chlorophyll *a* – chlorophyll *a* singlet-triplet annihilation is efficient (20), this may not be the case when the triplet excitation resides on a carotenoid molecule.

At the present time, it is not possible to distinguish between the two possibilities in conditions 9 and 10. A knowledge of relative triplet lifetimes and triplet densities in the two photosystems would help to resolve this problem. Experiments to resolve this question are under way.

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