Reprinted from

Biochimica et Biophysica Acta, 460 (1977) 171–181

BBA 47283

TIME-RESOLVED FLUORESCENCE SPECTROSCOPY OF SPINACH CHLOROPLAST

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(Received July 5th, 1976)

SUMMARY

Picosecond fluorescent kinetics and time-resolved spectra of spinach chloroplast were measured at room temperature and low temperatures. The measurement is conducted with 530 nm excitation at an average intensity of $2 \cdot 10^{14}$ photons/cm^2, pulse and at a pulse separation of 6 ns for the 100 pulses used. The 685 nm fluorescent kinetics was found to decay with two components, a fast component with a 56 ps lifetime, and a slow component with a 220 ps lifetime. The 730 nm fluorescent kinetics at room temperature is a single exponential decay with a 100 ps lifetime. The 730 nm fluorescence lifetime was found to increase by a factor of 6 when the temperature was lowered from room temperature to 90 K, while the 685 and 695 nm fluorescent kinetics were unchanged. The time-resolved spectra data obtained within 10 ps after excitation is consistent with the kinetic data reported here. A two-level fluorescence scheme is proposed to explain the kinetics. The effect of excitation with high light intensity and multiple pulses is discussed.

INTRODUCTION

Although a great deal of progress has been made in the overall understanding of photosynthesis, little is known about the primary energy conversion processes. The primary conversion of light into chemical energy occurs on an ultrafast time scale which has only recently been explored by picosecond laser techniques. The in vivo fluorescent kinetics of 685 nm from chloroplasts and subchloroplast preparations enriched with Photosystems I and II have been previously investigated [1–4, 7, 9, 10]. This paper reports on the fluorescent kinetics at various wavelengths and on the spectra within 10 ps of excitation at ambient and low temperatures. These kinetic data have been interpreted in terms of a two-level fluorescence scheme. Recently, new effects in photosynthetic fluorescence due to high intensity and multiple picosecond pulse excitations have been reported [5, 6]. In this paper, we report and discuss the effect of excitation with high intensity and multiple pulses.

MATERIALS AND METHODS

The fluorescence intensity within a 10 ps interval was measured as a function of time and wavelength by the ultrafast Kerr shutter technique. The experimental setup has been previously reported [3]. In the experiment a train of approx. 100 laser pulses at a wavelength of 530 nm separated by 6 ns was used to excite the sample. Each pulse had an average energy of 0.02 mJ, pulse width of 6 ps, and energy density at the sample of 0.08 mJ/cm^2 ($\approx 2 \cdot 10^{14}$ photons/cm^2 $\pm 30\,\%$)*. The effect of the exciting light intensity and the use of a train of pulses in this experiment will be discussed in a later section. The Kerr gate was activated by the 1060 nm laser pulse, and has a time resolution of approx. 10 ps. Fluorescence at the rear end of the sample cuvette was collected and passed through the Kerr gate. The fluorescence collected at a particular time is from all the exciting pulses in the laser pulse train. The measured lifetime is therefore an averaged result from the 100 pulses in the laser pulse train. The spinach chloroplast sample was prepared by a method previously described [1, 2]. The chlorophyll concentration was measured to be 40 μg/ml for sample in a 1 cm long cuvette, and 1 mg/ml in a 0.05 cm long cuvette.

EXPERIMENTAL RESULTS

In Fig. 1, the fluorescent kinetics at 685$\pm$2.5 and 730$\pm$2.5 nm, at room temperature and 90 K are displayed. At room temperature, the 730 nm fluorescence is observed to decay approximately as a single exponential with a lifetime of 100 ps, whereas the 685 nm fluorescence decays non-exponentially with a longer overall lifetime. The 685 nm fluorescent kinetics can be fitted by a two component exponential decay with lifetimes of 56 and 220 ps, and amplitudes of 1, and 1.2, respectively. The spinach chloroplast fluorescence data at 685 nm, along with the fluorescence collected from a spinach leaf at 685 nm is also shown. During the experiment, the stem of the spinach leaf was immersed in water. It is evident that the leaf and chloroplast data are almost identical.

In Fig. 2, the room temperature fluorescence spectrum of spinach chloroplast at a time less than 10 ps after excitation (denoted by $t \approx 0$) is shown. A time-integrated fluorescence spectrum, which was obtained by uncrossing the polarizers in the Kerr gate setup, is also shown in Fig. 2. The spectra were corrected for the spectral responses of the photomultiplier (RCA 7265), spectrometer (Jarrel-Ash 1 meter), filters (Corning 1-75 and 3-67), and polarizers (Polaroid HN 22). In Fig. 2, no correction was made for the self absorption, which has caused a relatively small peak at 685 nm**. The self absorption-corrected spectra are displayed in Fig. 3a. The correc-

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* The previously reported photon flux density at the sample by Yu et al. (1975) (Biochim. Biophys. Acta, 387, 159–164) is in error by a factor of three due to an underestimate of the size of the focussed laser beam spot at the sample cell. Photographs of the beam spot at the sample cell were taken and determined the beam spot at 5 mm instead of 3 mm.

** The fluorescence lifetime can be corrected for self absorption using the equation $\tau_0 = \tau$ (measured) $(1-Aq_F)$ where q_F is the fluorescence quantum efficiency, and A is the absorption coefficient which is always < 1. (See Birks, J. B. (1970) Photophysics of Aromatic Molecules, p. 92, Wiley-Interscience, London). Since q_F is $\cong 2\,\%$ in plant photosynthesis, the correction in the lifetime is less than 2 %. This is consistent with the observation that the fluorescent kinetics from the almost opaque leaf is identical to that from the chloroplast preparation within experimental error.

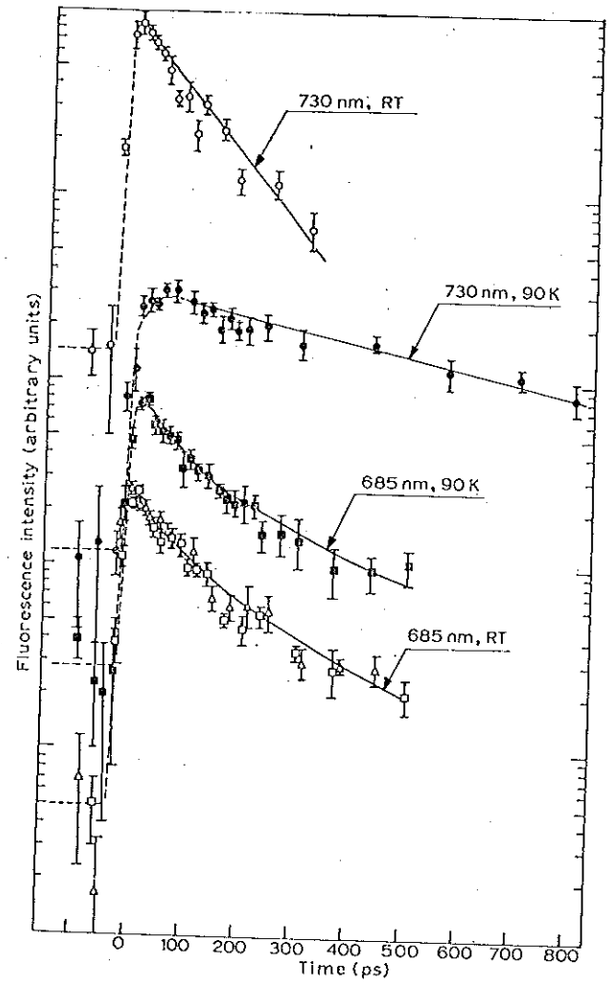

Fig. 1. The time dependence of the fluorescence emission of spinach chloroplast at various wavelengths and temperatures. (○, 730 nm at room temperature; ●, 730 nm at 90 K; □, 685 nm at room temperature; ■, 685 nm at 90 K; △, 685 nm fluorescence of spinach leaf at room temperature.) The solid curves represent $I = 1.5 + 92 \exp(-t/100)$ for ○; $I = 1.2 + 31 \exp(-t/600)$ for ●; $I = 3 + 48 \exp(-t/45) + 48 \exp(-t/200)$ for ■; $I = 0.5 + 12.5 \exp(-t/56) + 14.5 \exp(-t/220)$ for □, △ combined. The dashed lines indicate the background noise level and the rising of the fluorescence. The four curves are displaced vertically for clarity of presentation. The error bar is the standard deviation of the mean of six or more data points.

tion factor for each wavelength due to self absorption was obtained by comparing the time-integrated emission spectra of the sample excited by a weak He-Ne laser with the spectra of the very dilute sample. For the purpose of comparison, the spectra are normalized to the same value at 685 nm. From the curves displayed in Figs. 2 and 3a, one observes that at wavelengths > 700 nm, the fluorescence intensity measured relative to 685 nm is greater in the $t \approx 0$ spectrum than in the time-integrated spec-

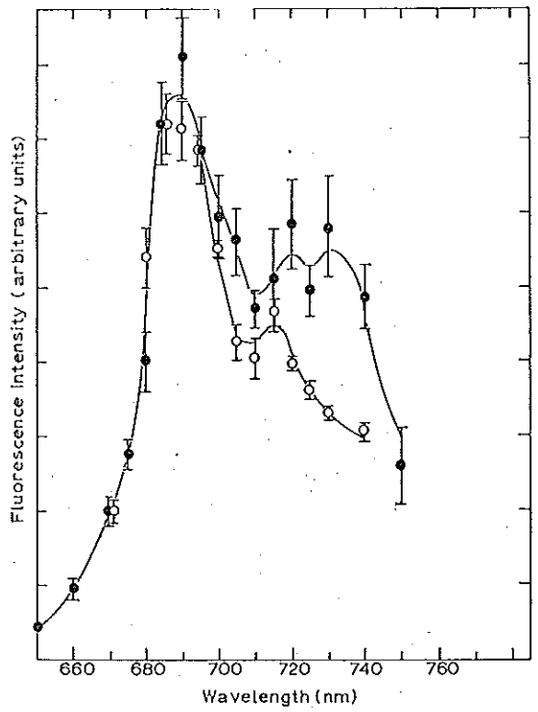

Fig. 2. Fluorescence spectra of spinach chloroplast at room temperature. (●, spectrum at a time less than 10 ps after excitation; ○, time-integrated spectrum.) The $t \approx 0$ and time-integrated spectra are normalized at 685 nm. The spectra are corrected for instrument response, but not for the self absorption of the sample. The error bar is the standard deviation of the mean of six or more data points. The spectral width of the spectrometer is 5 nm.

trum. This indicates that at longer wavelengths the overall lifetime at room temperature is shorter than that at 685 nm, which is consistent with the kinetic measurement.

The fluorescent kinetics at liquid nitrogen temperature at 685 and 730 nm are also shown in Fig. 1. As the temperature is lowered to 90 K, the 730 nm lifetime is observed to increase by a factor of 6, whereas the 685 nm fluorescent kinetics is essentially unchanged. The $t \approx 0$ and time-integrated spectra at 90 K are shown in Fig. 4 and the curves corrected for self absorption are shown in Fig. 3b. Predictably, in this case the long wavelength (> 700 nm) fluorescence intensity measured relative to 685 nm is greater in the time-integrated spectrum than in the $t \approx 0$ spectrum. In addition to the lengthening of the lifetime at 730 nm at 90 K the $t \approx 0$ intensity of the 730 nm fluorescence measured relative to 685 nm is found to increase by a factor of 2.5 from that measured at room temperature. It should be noted that the $t \approx 0$ fluorescence intensity is a measure of the initial population of the excited fluorescent states. The fluorescent kinetics also measure the rise of the relative populations of the excited fluorescent states. For all the kinetic data we have reported here, the risetime of fluorescence at various wavelengths and temperatures was always observed to be short, < 10 ps.

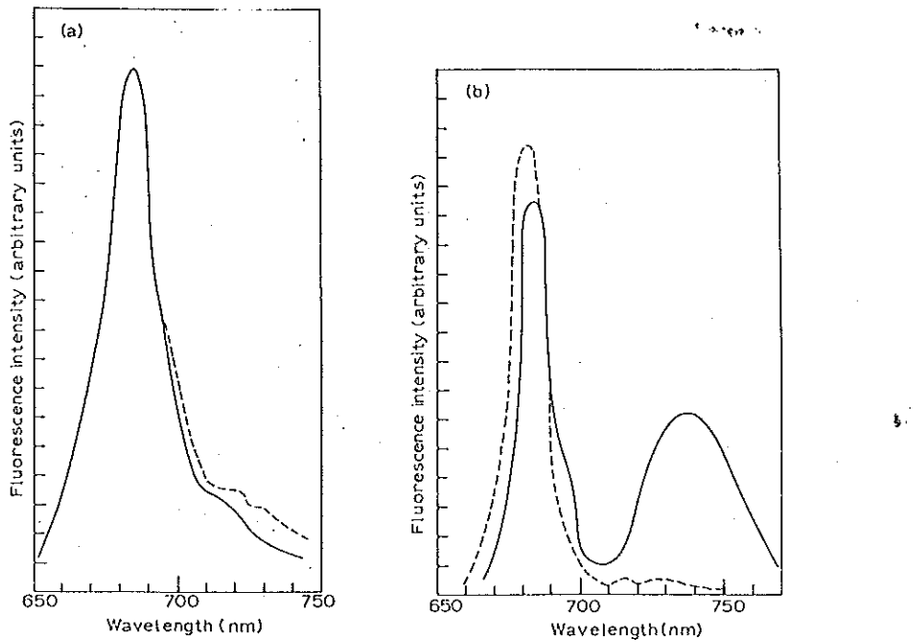

Fig. 3. (a) The fluorescence spectra of spinach chloroplasts at room temperature after correction is made for self absorption. —, time integrated; – – –, $t \approx 0$. (b) The fluorescence spectra of spinach chloroplast at 90 K after correction is made for self absorption. —, time integrated; – – –, $t \approx 0$.

In Table I, a summary of the fluorescence lifetimes and risetimes measured at various wavelengths (685, 695, and 730 nm) and temperatures are tabulated.

The general interpretation of the picosecond fluorescence is that its kinetics describe the energy transfer processes from antenna pigments to the trapping molecules. It is then important to question the state of the traps under a train of exciting pulses at a given intensity.

We have measured the profiles of the exciting laser pulse train and the fluorescence, and observed that the ratio of the intensities between the fluorescence and excitation light remains constant ($\pm 10\,\%$) throughout the pulse train at the given intensity ($\approx 2 \cdot 10^{14}$ photons/cm^2, pulse). This result implies one of the following possibilities: (a) the pulses, separated by 6 ns within the pulse train, at the given intensity, are independent of each other; (b) shortly after the first few exciting pulses of the laser, a long lived state with a rather constant concentration exists in the photosynthetic apparatus, and it quenches the fluorescence in such a manner that the fluorescence yield remains constant throughout the pulse train. Beddard et al. [7] have found the fluorescence lifetime obtained by multiple pulse excitation to remain constant over a decade change in exciting intensity. This result has been confirmed by Campillo et al. [6], who in addition have found that the lifetime attained from single pulse excitation becomes longer when the intensity is lowered. In view of these observations, it is unlikely that at the present exciting intensity ($2 \cdot 10^{14}$ photons/cm^2, pulse) the pulses in the pulse train can be considered as independent. Processes involving the annihilation of the fluorescing singlets by the long lived states or exces-

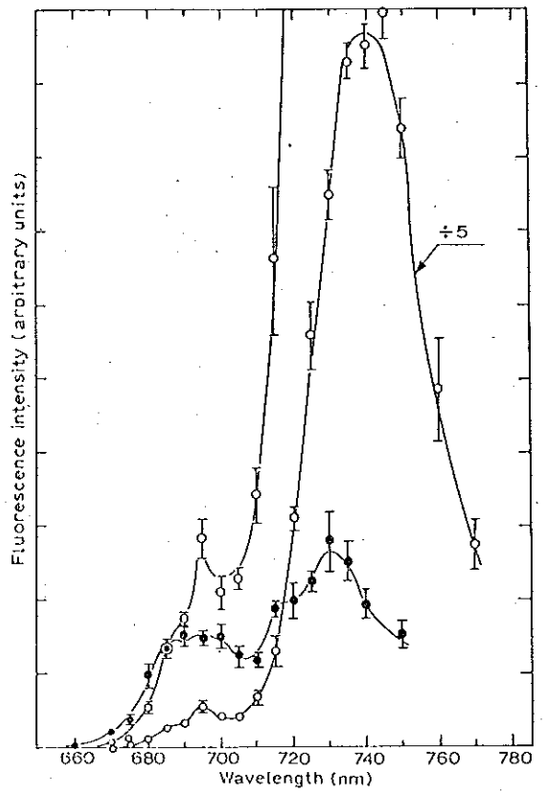

Fig. 4. Fluorescence spectra of spinach chloroplast at 90 K. (●, spectrum at a time less than 10 ps after excitation; ○, time-integrated spectrum). The $t \approx 0$ and time-integrated spectra are normalized at 685 nm. The spectra are corrected for instrument response, but not for the self absorption of the sample. The error bar is the standard deviation of the mean of six or more data points. The spectral width of the spectrometer is 5 nm.

TABLE I

TYPICAL VALUES OF RISETIMES AND LIFETIMES OF CHLOROPLAST FLUORESCENCE

The risetime τ_r is calculated from the equation $\max = \tau[\ln(\tau/\tau_r)]/(\tau/\tau_r - 1)$ where τ is the average decay time, and t_{max} is the time when the fluorescence intensity is a maximum [1, 2]. The lifetime and amplitude follow the relative fluorescence intensity equation $I = A_I \exp(-t/\tau_I) + A_{II} \exp(-t/t_{II})$. Typical error is about 20 %.

Wavelength (nm)	Temperature (K)	Risetime (ps)	Lifetime and amplitude			
			τ_I (ps)	A_I	τ_{II} (ps)	A_{II}
685	280	≈5	56	1	220	1.2
685	90	≈5	45	1	220	1
730	280	≈5	100	1		0
730	90	13±7	600	1		0
695	280	≈5		0	220	1
695	90	≈5		0	220	1

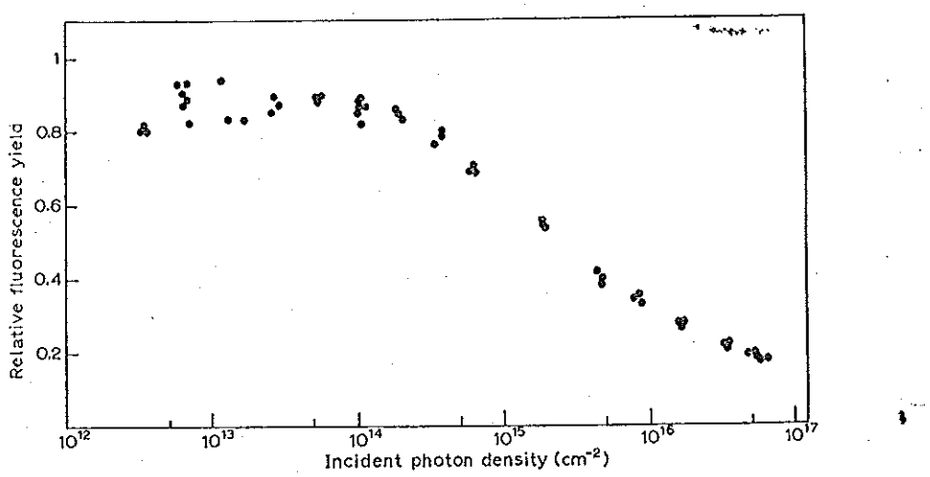

Fig. 5. The 690±6 nm fluorescence yield as a function of excitation intensity per pulse in a train of 6-ps pulses separated by 6 ns,

sive excited singlet states (shortening the fluorescence lifetime), and the closing of the traps (lengthening the fluorescence lifetime) may act concurrently. We have also varied the intensity of exciting light, and observed the fluorescence yield to decrease when the photon flux exceeds about 10^{14} photons/cm^2, pulse as shown in Fig. 5. Similar results were reported by measurements using a 7 ns 377 nm pulse [8], and a 20 ps, 530 nm pulse [5]. It should be noted that the constant yield level in Fig. 5 extends about a factor of 10 towards higher intensity compared to that of the single pulse result [5]. This observation is consistent with the possibility that a steady-state concentration of a fluorescence quenching state exists due to multiple pulse excitation.

As shown in Fig. 5, the present experiment is conducted at an intensity such that the fluorescence yield is just about to decrease. For higher intensities, however, the question of whether the measured fluorescence kinetics are compounded with processes other than that of energy trapping is quite an important one. We have varied the exciting intensity over a few hundred fold to measure its effect on the kinetics. At the exciting intensity of the present experiment ($\approx 2 \cdot 10^{14}$ photons/cm^2, pulse), the fluorescence kinetics can be interpreted by a two component decay (56 and 220 ps lifetimes) with about equal amplitude. We have assigned these two components to the fluorescence from Photosystems I and II, respectively. Increasing the exciting intensity by 100-fold, the relative amplitude of the fast component is observed to increase by a factor of 3, and the lifetime of the slow component is essentially unchanged. Decreasing the exciting intensity by a factor of 3.5, the kinetics are observed to be unchanged. The relatively weak dependence of the relative amplitude of the fast component on the exciting intensity and its asymptotic behavior at low light intensity (less than 10^{14} photons/cm^2, pulse) suggest that the fluorescence at constant yield level would possess a two component character as measured in this experiment.

DISCUSSION

Earlier [3, 4] we reported the fluorescence decay from Photosystems I- and

II-enriched preparations with lifetimes 60 and 200 ps, respectively. In our present measurement a two component decay of the fluorescent emission from chloroplast at 685 nm with lifetimes 56 and 220 ps and amplitudes of 1, and 1.2, respectively, was observed. This result is consistent with the lifetime measurement of the Photosystems I and II preparation. This has enabled us to assign the fast and slow fluorescence components from the chloroplast data to the emission from Photosystems I and II, respectively. Since fluorescence at 730 and 695 nm are mainly emissions from Photosystems I and II, respectively, the measured single exponential decay at these wavelengths is further evidence to this assignment.

A comparison of other picosecond fluorescence studies reflects the complication inherent in measuring fluorescence lifetimes when the exciting source is an intense pulse or multiple pulses. Paschenko et al. [9] measuring the fluorescence from one pulse in a train of exciting pulses at $\leq 3 \cdot 10^{14}$ photons/cm^2, pulse have reported the observation of a three component decay with lifetimes of 80 ($\lambda \geq 730$ nm), 300 ($\lambda \geq 650$ nm), and 4500 ps. These components were attributed to the emissions from Photosystems I, II, and chlorophyll pigment not involved in photosynthesis, respectively. A similar experiment by Beddard et al. [7], at an intensity of approx. $5 \cdot 10^{14}$ photons/pulse, reported a short lifetime of about 130 ps ($\lambda \geq 580$ nm). Our results of 100 ps ($\lambda = 730 \pm 2.5$ nm) and of 220 ps ($\lambda = 685 \pm 2.5$ nm) are in reasonable agreement with those of Paschenko et al.* The difference between our results and that of Beddard et al. [7] may be due to the difference in the wavelength ranges investigated and the difference in excitation intensities used. This may also have been the problem of the earlier reports [1,2] of a fast fluorescence decay (≥ 10 ps)**. Kollman et al. [10] have reported a short lifetime of 75 ps ($\lambda \geq 640$ nm) for *Anacystis* and 41 ps ($\lambda \geq 640$ nm) for *Chlorella pyrenoidosa*, also measuring the fluorescence from one pulse in a train of exciting pulses of high intensity. Recently they have used a single pulse to measure fluorescence lifetimes, and have obtained $1/e$ point lifetimes at $\lambda = 700$ nm of 175 ps measured at $3 \cdot 10^{14}$ photons/cm^2, and 375 ps measured at 10^{14} photons/cm^2 in *C. pyrenoidosa* [6]. Traditional methods have placed the fluorescence lifetime within the range of 0.35–0.8 ns [11–14], which is approximately the same result estimated by Campillo et al. [6]. It should be noted here that comparison of our data with those from other picosecond studies (except for those of Paschenko et al. [9]) is further complicated by the difference in the spectral regions investigated. It is well established that Photosystems I and II fluoresce with characteristic wavelengths at 730 and 685 nm, respectively [15]. Furthermore, we and Paschenko et al. have observed different lifetimes for these components, although the effect of high intensity and multiple pulse excitation on the photosystems can be different. It is apparent that in order to determine the true energy trapping rate in photosynthesis an accurate, single pulse measurement at low light intensity ($< 10^{13}$ photons/cm^2) and various wavelengths is needed.

The observed kinetics and the low temperature behavior of the long wave-

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* The long risetime data at 530 nm excitation by Paschenko et al. is not in agreement with ours, and those of ref. 10. The difference in collecting geometry (transmitted versus right angle), and stimulated emission, in our opinion, are unlikely the cause for the discrepancy.

** The dip and rise of the fluorescence temporal profile reported in references 1 and 2 can also be caused by an insufficient amount of data points and the then large shot to shot statistical fluctuation in the laser output.

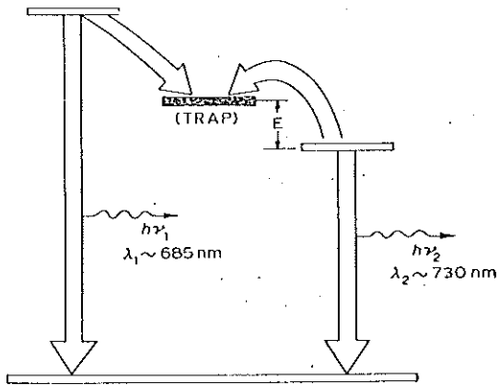

Fig. 6. Two-level fluorescence scheme of primary energy conversion in chloroplast photosynthesis. The excited states of the chlorophyll pigment (denoted by white bars) transfer the excitation energy to the trap(denoted by a black bar), for initiating photosynthetic reactions. The energy level of the trap is E unit above the long wavelength component, which can be determined by measuring the fluorescence decay rate at various temperatures.

length component (730 nm) are of significant importance. Since the discovery of longer wavelength fluorescence ($\approx$ 730 nm) in 1958 [16], the origin of this component has not been identified. Various observations support the contention that the 730 nm fluorescence is not merely a vibrational sideband of the chlorophyll *a* fluorescence but a component distinct from 685 nm [15, 17, 18]. One of these observations is that the 730 nm fluorescence is mainly the emission from Photosystem I particles [15]. Since there is no overwhelming evidence to support the preferential appearance of a vibrational sideband fluorescence from Photosystem I alone, the 730 nm fluorescence is likely a distinct component. Furthermore, virescence studies of the etiolated leaves [17, 18] have indicated that the photosynthetic activity is intimately related with the appearance of the 730 nm fluorescence band. Therefore, the 730 nm fluorescence probably emanates from some special aggregate of chlorophyll molecules which appear in the later stages of the greening process. The observed prominence of 730 nm fluorescence at low temperatures measured with low light intensity with conventional methods [15] is consistent with our result of the increased lifetime and amplitude of the fluorescence at 90 K. The temperature independence of the 685–695 nm fluorescence lifetime is also consistent with the lifetime measurement using photon counting techniques [14]. It is therefore reasonable to assume that the data obtained in this experiment can be used to qualitatively describe the energy trapping process.

To explain the observed kinetic data we propose the kinetic scheme shown in Fig. 6. We assume that there exist in the photosynthetic apparatus two types of chlorophyll fluorescence components namely those emitting fluorescence at about 685 nm (including 695 nm) and 730 nm. The energy transfer processes to the trap, which is supposed to be located somewhere between 685 and 730 nm (analogous to Kok's *P*-700 [19] or Butler's *P*-705 [18] absorption components for Photosystem I), are quite different for the two levels. The 730 nm component must overcome a potential barrier to reach the trap. As the temperature is lowered, the energy transfer

rate from the 730 nm level is reduced by an exponential factor, $\exp(-E/kT)$. This accounts for the lengthening of the 730 nm lifetime. If the measured value of 6 for the ratio of lifetime at 90 K to that at room temperature is set to be the ratio for the true lifetimes, the potential energy above the 730 nm level is estimated to be $E \approx 164$ cm^{-1}. For the fast and slow components of the 685 nm and the 695 nm fluorescence, the downhill transfer in both photosystems is not affected by the temperature change. The temperature independence of the 685 and 695 nm fluorescence suggests that the traps in Photosystems I and II are likely to be located between 685–730 nm, and 695–730 nm, respectively. Since the 730 nm fluorescence is predominantly emission from the Photosystem I and the experimental results do not allow us to unequivocally assert that the 730 nm fluorescent kinetics from Photosystem II is identical to that from Photosystem I, the above model likely describes the kinetics and the location of the trap in Photosystem I more closely than those in Photosystem II.

The observed 2-fold increase in peak intensity ($t \approx 0$) at 730 nm (relative to 685 nm) infers that the 730 nm component must have increased its relative population at low temperature. Whether this increase is due to a thermal coupling between the levels or the photosystems, or due to a change of the fluorescing property of the long wavelength component at lower temperatures is speculative at this time.

In conclusion, studies of the effects of temperature on the fluorescence kinetics and spectra appear to be useful in obtaining information on the primary processes in photosynthesis. Picosecond laser excitation not only provides 10^{-11} s time resolution to kinetic studies of photosynthesis, but also with single pulse at low intensity yields direct measurement of energy trapping time, and with multiple or single pulse at various intensity can provide valuable information on the dynamics of excitons (singlet, triplet or other long lived states) in the photosynthetic apparatus.

NOTE ADDED IN PROOF (Received February 3rd, 1977)

It should be noted that the relative fluorescence ratio F_{730}/F_{685} in the time integrated fluorescence spectra at room temperature and 90 K (Figs. 3a and 3b) is much smaller than that obtained from low light excitation. This observation is in agreement with the results of recent publications by Breton and Geacintov (Breton, J. and Geacintov, N.E. (1976) FEBS. Lett. 69, 86–89; Geacintov, N. E. and Breton, J. (1977) Biophys. J., in the press) who have studied the quenching of fluorescence spectra using various numbers of picosecond exciting pulses at high intensity. The quenching was attributed to the existence of long-lived quenching species. They also concluded that this quenching is more effective in Photosystem I.

ACKNOWLEDGEMENTS

This work was supported in part by N.S.F., C.U.N.Y., and Research Corporation grants. R.R.A. is an Alfred P. Sloan Fellow.

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