Biochimica et Biophysica Acta, 449 (1976) 329–339

BBA 47208

FLUORESCENCE LIFETIMES OF *CHLORELLA PYRENOIDOSA*

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(Received June 21st, 1976)

SUMMARY

The fluorescence decay of *Chlorella pyrenoidosa* has been investigated under a variety of conditions in the picosecond and nanosecond time regions. Most of the fluorescence is accounted for by an expression of the form

$$I(t) = I_0 \exp-(At+Bt^{\frac{1}{2}})$$

though an additional exponential term is required to include a weak component of lifetime 32 ps observable only at the higher pulse intensities. This interpretation reconciles earlier and apparently conflicting data.

The weak 32 ps component may be associated with Photosystem I, although the possibility that it is an artefact of the high intensity pulses used cannot be excluded at present. The main fluorescence, described by the equation above is attributed to the antenna chlorophyll and is of the form which would be expected from a single light harvesting array with trapping at randomly distributed sites.

INTRODUCTION

Fluorescence from the photosynthetic unit provides direct insight into the initial photochemical processes involved in photosynthesis. Consequently, both the time dependence and quantum efficiency of this fluorescence have been investigated by a number of techniques. Recently, the time resolution of these measurements has been extended by utilizing picosecond pulses from mode-locked lasers [1–8]. The fluorescence decay obtained for dark-adapted *Chlorella* and/or chloroplasts, using picosecond pulses, has been reported to contain a short-lived component of lifetime near to or less than 100 ps [1–8]. There are also several reports of a second component of lifetime near 300 ps [2, 6, 7] and one report of an additional long lived component with a lifetime of 4.5 ns [2].

Previous work using nanosecond flash and phase shift methods on dark-adapted *Chlorella* and/or chloroplasts at room temperature has given lifetimes between 0.350 and 2.0 ns [9–17] with more recent work centring about 0.6 ns [10–14] for dark-adapted systems and between 1.5 to 2 ns for systems with the Photosystem II traps closed by using DCMU and pre-illumination [13–16]. At liquid nitrogen

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temperature, lifetimes between 1.4 to 3.1 ns have been reported [15–17]. In the nanosecond work, the fluorescence decay has been assumed to be from one or two exponential components [9–17].

Recently, there has been a report of a decrease in the quantum yield of dark-adapted *Chlorella* within about 10 ns of the pulse exciting fluorescence [3, 18]. The effect, within the intensity range of 10^{13} to 10^{16} photon/cm^2 of exciting light, was observed using both picosecond [3] and nanosecond [18] pulses. The shortness of the fluorescence decay measured using picosecond pulses, compared to the earlier measurements using nanosecond pulses, has been attributed to the higher intensity of the picosecond pulses [3, 18].

Thus, there remains some disagreement concerning the decay of in vivo chlorophyll fluorescence. We have therefore made measurements of *Chlorella* fluorescence decay using both single and multiple picosecond pulses of varying intensity on dark-adapted systems at room and liquid nitrogen temperature and on systems in which the Photosystem II traps were closed by pre-illumination after addition of DCMU. Additionally, the effect of intensity of the exciting pulse on the fluorescence quantum yield within nanoseconds of laser excitation was investigated using single pulse excitation.

EXPERIMENTAL

The experimental arrangement consisted of a mode-locked Nd^{3+} glass oscillator, which produced a train of more than a hundred 1060 nm pulses with a separation of 6.7 ns and a half maximum duration of 6 ps. A temperature tuned caesium dihydrogen arsenate crystal generated the second harmonic pulse train (530 nm) with 10–15 % efficiency. A single pulse was selected from the centre of the train by an electro-optic shutter (Pockels cell). By inserting a half wave plate before the Pockels cell, the full train could also be used for excitation.

At the 1 mm sample cell the photon density was measured to be 10^{14} photon/cm^2 for a single pulse. The fluorescence from the sample passed through a wavelength selection filter on to the slit of an IMACON 600 streak camera, with an S20 photocathode. A vidicon multichannel analyser stored the streak intensity into a 500 channel memory, from which the data were transferred on to punch tape for analysis.

The algae, *Chlorella pyrenoidosa*, were grown in liquid culture [1], gassed with air, and collected during the logarithmic growth phase, by centrifugation at 3000 $\times$ *g* for 1 min. The cells were resuspended in growth medium to give a transmittance of 40 % at 530 nm in a 1 mm cuvette. A fresh sample of *Chlorella* was used after every firing of the laser.

The fluorescence kinetics of *Chlorella* were studied up to 10 ns after excitation, by selecting progressively slower streak speeds. On the fastest time scale, resolution was better than 10 ps. The monitoring wavelengths were set by the following filters: > 600 nm DB5 CYAN (cut-off), 691 nm BALZER (12 nm bandwidth interference filter), > 715 nm SCHOTT RG715 (cut-off).

The *Chlorella* fluorescence decay was measured for four differing sets of experimental conditions:

(i) Dark-adapted *Chlorella* at room temperature were excited with a pulse train. The pulse exciting the recorded fluorescence had an energy of 10^{15} photon/cm^2

(the laser pulse energy varies by about 20 % from shot to shot).

(ii) Dark-adapted *Chlorella* at room temperature were excited with a pulse train attenuated by a factor of 10 using a neutral density filter or with a single pulse. The pulse in the pulse train and the single pulse exciting the recorded fluorescence both had an energy of 10^{14} photon/cm^2.

(iii) *Chlorella* at room temperature, with the traps of Photosystem II closed, were excited by a single pulse which had an energy of 10^{14} photon/cm^2. The traps of Photosystem II were closed prior to laser excitation by illumination with broad band blue light after addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) to a concentration of $0.5 \cdot 10^{-3}$M in the suspending medium. The fluorescence induction curve, (the change in the photostationary fluorescence intensity when excited by the broad band blue light) was measured by a fibre-optic coupled to a photomultiplier and storage oscilloscope immediately prior to laser excitation.

(iv) Dark-adapted *Chlorella* at 77 K were excited by a single pulse having an energy of 10^{14} photon/cm^2. The samples were frozen in a phosphorescence tube, and maintained at 77 K in a Dewar of liquid nitrogen. At 77 K, the *Chlorella* fluorescence intensity at 715 nm increased dramatically, whereas the fluorescence intensity at 690 nm changed only slightly.

Fluorescence quantum yields, as a function of exciting pulse photon density, were investigated by directing a portion of an exciting single pulse of known photon density on to the streak camera slit approximately 500 ps before the arrival of the fluorescence. The relative quantum yields were determined by dividing the integral over the fluorescence decay from time zero to 2.5 ns by the integral over the exciting pulse.

RESULTS

The fluorescence obtained using experimental conditions (i) was of sufficient intensity to allow the decay to be followed from 10 ps to 1.5 ns through combination of the results obtained using the various time scales of the streak camera. The logarithmic plot of this decay (shown in Fig. 1) shows clearly that the decay is non-exponential. However, as shown in Fig. 2, from approximately 100 ps to 1 ns the logarithm of the intensity of the fluorescence is linear with the square root of time. The fluorescence is described by

$$I(t) = I_0\{\alpha/\exp(t/\tau_1)+(1-\alpha)/\exp(t/\tau_0+At^{\frac{1}{2}})\} \quad (1)$$

within the 10 % experimental accuracy of the intensity measurements. Eqn. 1 contains both an exponential component with lifetime τ_1 and initial intensity αI_0 (where I_0 is the total initial intensity) and a non-exponential component with a square root of time term in the exponent and initial intensity $(1-\alpha)I_0$. The decay is adequately described by only the second term 100 ps after excitation. The exponential and non-exponential terms (components) are subsequently referred to as C1 and C2, respectively. Although the fluorescence decay can alternatively be described as a sum of exponentials, at least four exponentials (eight parameters) must be used to obtain a fit better than that given by eqn. 1.

Since the fluorescence decays obtained under experimental conditions (ii), (iii) and (iv) were not of sufficient intensity to allow measurements on the fastest time

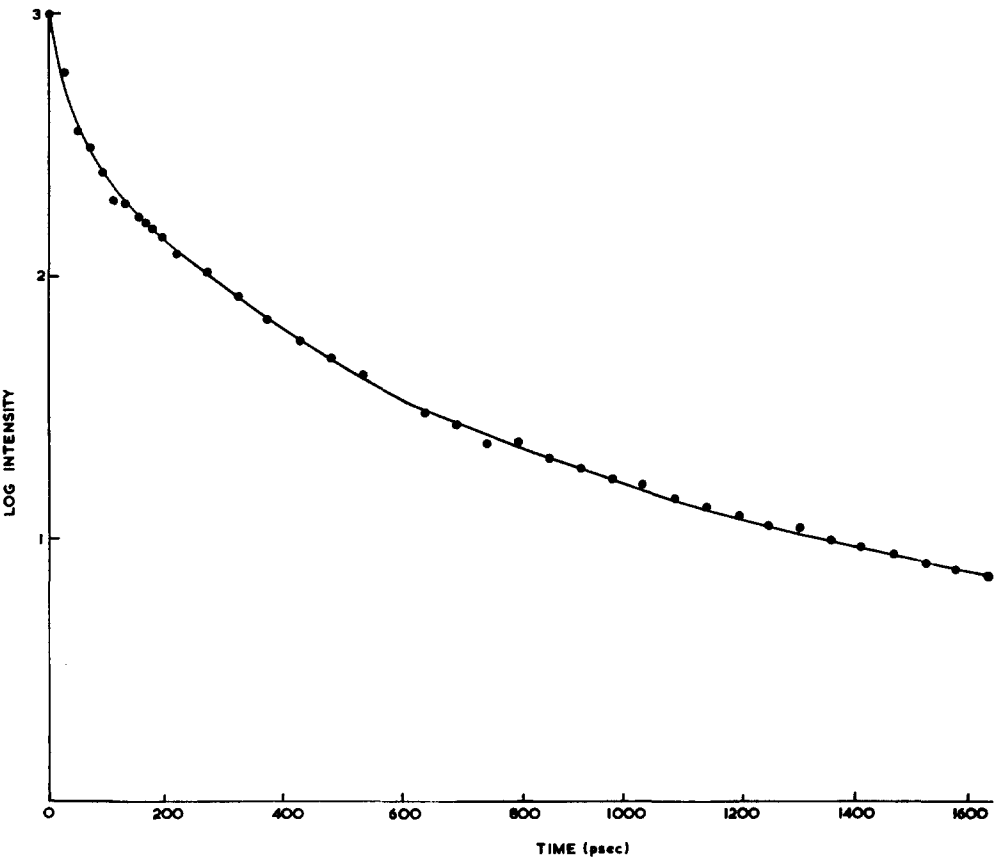

Fig. 1. The log of the intensity of fluorescence versus time (ps) of dark-adapted *Chlorella* at room temperature excited by a pulse train (the exciting pulse had an energy on the order of 10^{15} photon/cm^2).

scales, C1 could not be studied under these conditions. However, the fluorescence decays of (ii), (iii) and (iv), taken 100 ps after excitation, were found to have the same form as C2 in (i). The measured fluorescence decays and the fluorescence decay calculated using the parameters in Table I are shown in Fig. 3. The fit of the measurable fluorescence decay to C2 is not very sensitive to τ_0 since only a small fraction of the total intensity of fluorescence is measured at times approaching τ_0. A value of τ_0 of 5 ± 1 ns was derived for (i) and (iv). For (ii) and (iii), all the parameters could not be simultaneously extracted from the data; I_0 and A were obtained for $\tau_0 = 5$ ns and τ'_0 was subsequently calculated with I_0 and A fixed at these values. In both cases, τ'_0 is near $\tau_0 = 5$ ns. The absolute yields listed in Table I were calculated from the expressions

$$\phi = (\tau_1/\tau_0)\phi_0 \quad \text{for C1}$$

and

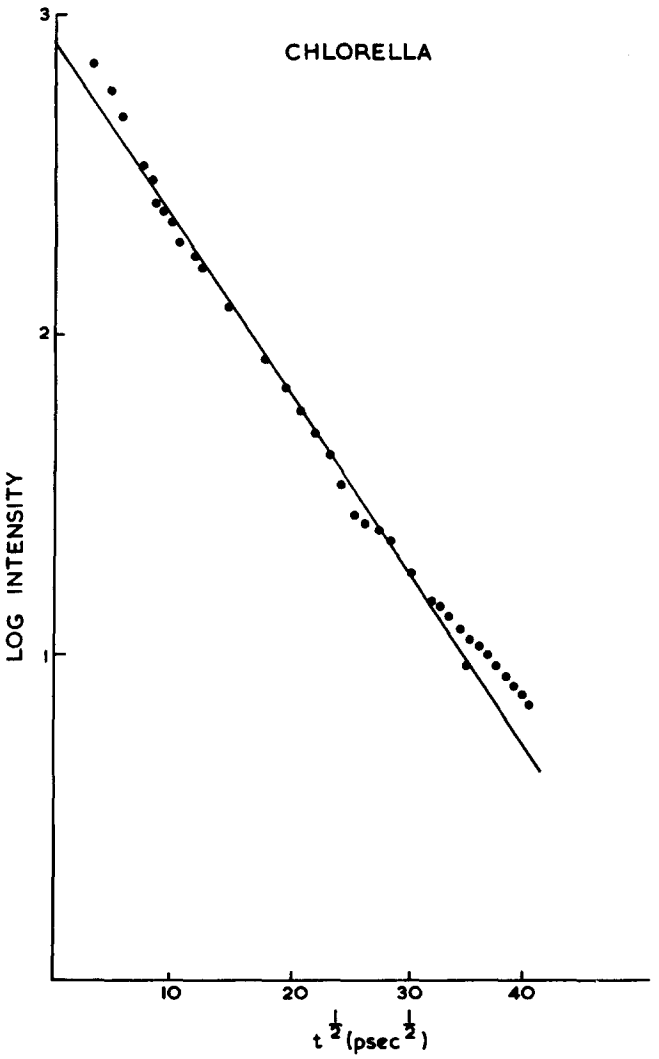

Fig. 2. The log of the intensity of fluorescence versus (time)$^{\frac{1}{2}}$ (ps$^{\frac{1}{2}}$) of dark-adapted *Chlorella* at room temperature excited by a pulse train (the exciting pulse had an energy on the order of 10^{15} photon/cm^2).

$$\phi = \{1-\pi^{\frac{1}{2}}\, q e^{q^2}(1-\mathrm{erf}\, q)\}\phi_0;\; q = \frac{(\tau_0)^{\frac{1}{2}}}{2}\, A \tag{2}$$

for C2, where τ_0 and ϕ_0 were taken as 5 ns and 0.33, respectively, from data on chlorophyll in dilute solution [11, 19]. Eqn. 2 is a previously derived expression [20] for a decay of the same time dependence as C2. τ_M, the mean time of fluorescence, was obtained by numerical integration of the expression

$$\tau_M = \int_0^\infty t\, I(t) / \int_0^\infty I(t)$$

TABLE I

CHARACTERISTICS OF *CHLORELLA* FLUORESCENCE DECAY

Fluorescence decay is described by: $I(t) = I_0\{\alpha/\exp(t/\tau_1)+(1-\alpha)/\exp(t/\tau_0+A(t)\ddagger\}$ for the conditions: (i) dark-adapted, room temperature, excitation by pulse train (approx. 10^{15} photon/cm^2); (ii) dark-adapted, room temperature, excitation by attenuated pulse train or single pulse (approx. 10^{14} photon/cm^2); (iii) pre-illumination after addition of DCMU, room temperature, excitation by attenuated pulse train of single pulse (approx. 10^{14} photon/cm^2); (iv) dark-adapted, low temperature, excitation by single pulse (approx. 10^{14} photon/cm^2). The initial intensities of C1 and C2 are given by αI_0 and $I_0(1-\alpha)$, respectively. The fluorescence decays were normalized to an initial intensity, I_0, of one.

Component	(i)			(ii)	(iii)	(iv)
	C1	C2	Total	C2	C2	C2
Initial intensity	0.436	0.564	1.000	0.780	0.780	0.994
τ_1 (ps)	32.3	—	—	—	—	—
τ_0 (ns)	—	3.9	—	—	—	4.4
τ'_0 (ns)*	—	—	—	5.5	5.1	—
A (ps$^{-\ddagger}$)	—	0.103	—	0.094	0.048	0.025
Quantum yield**	0.00085	0.0057	0.0066	0.0095	0.032	0.076
τ_M (ps)	32.3	451	282	530	1460	2460
$\tau_{1/e}$ (ps)	32.3	90.1	48	109	436	1230

* The other parameters were held fixed at their values for $\tau_0 = 5$ ns while τ_0' was varied.

** The absolute quantum yield for each component multiplied by the initial intensity of that component.

$\tau_{1/e}$, the time for fluorescence decay to reach 1/eth of its initial intensity, was obtained using the Newton-Raphson method from the parameters listed in Table I for C2. Both τ_M and $\tau_{1/e}$ are listed in Table I.

In (ii), the same results were obtained within experimental error for excitation with either the single pulse or the attenuated pulse train. Thus the increase in quantum yield and τ_M in (ii) relative to (i) are affected by the decrease in the intensity of the exciting pulse rather than by the effect of pre-illumination by pulses preceding the excitation pulse in the pulse train. The intensity effect of the exciting pulse on the quantum yield up to 2.5 ns after the exciting pulse was investigated directly over the exciting pulse intensity range used in these experiments. The relative quantum yields measured, using single pulse excitation, increased by a factor of 1.7 as the exciting pulse power density was reduced from 10^{15} photon/cm^2 to 10^{13} photon/cm^2.

All the room temperature fluorescence decays ((i), (ii) and (iii)), and the relative quantum yield measurements reported were obtained using the 600 nm cut-off filter. Attempts to obtain decay curves using the 691 nm interference filter and 715 cut-off filter were difficult, owing to the lower intensity of the room temperature fluorescence in these wavelength regions. Nevertheless, within the limits imposed by the lower intensity obtained when using the 691 and 715 nm filters, the fluorescence decays were independent of wavelength.

The room temperature *Chlorella* fluorescence spectrum has a peak at 685 nm with a shoulder at 740 nm. However, at low temperature (77 K), the maximum shifts to 718 nm with weak shoulders at 688, 700 and 740 nm for the *Chlorella* used in these experiments. The main portion of the fluorescence intensity results from the 718 nm

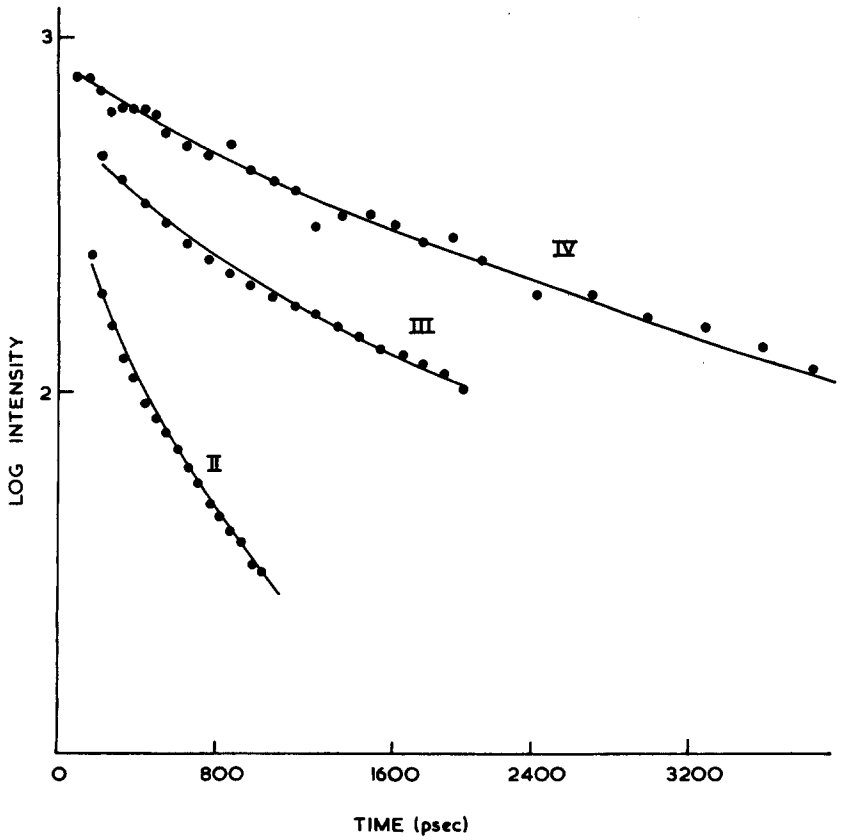

Fig. 3. The log of the intensity of fluorescence versus time (ps) for: (ii) Dark-adapted *Chlorella* at room temperature excited by an attenuated pulse train or a single pulse; (iii) *Chlorella* at room temperature pre-illuminated by broad band blue light after addition of DCMU excited by an attenuated pulse train; (iv) Dark-adapted *Chlorella* at 77 K excited by a single pulse. In each case the exciting pulse had an energy of the order of 10^{14} photon/cm^2.

peak. The higher quantum yield at 77 K results in better decay curves, particularly when using the 619 and 715 nm filters; however, the fluorescence decays were still independent of the filter used.

As shown in Table I, the fluorescence quantum yield increases and τ_M of C2 lengthens after pre-illumination and the addition of DCMU (experimental conditions (iii)). The increase in photostationary quantum yield was calculated as 300% from the fluorescence induction curve obtained on illumination, after addition of DCMU and immediately prior to laser excitation.

DISCUSSION

The parameters given in Table I for fluorescence decay are compatible with all previous nanosecond and picosecond work. Most previous picosecond fluorescence decays [1–8] were obtained using excitation conditions similar to those used in (i). Under these conditions, the fluorescence decay recorded for 200 ps, following exci-

tation, is determined primarily by τ_1 (32 ps) and $\tau_{1/e}$ for C2 (90 ps) and is thus compatible with the previously reported lifetimes of 100 ps or less. τ_M for the total fluorescence decay (282 ps) is compatible with reports of a second component of fluorescence decay of lifetime near 300 ps [2, 6, 7]. τ_0, calculated as near 5 ns, is compatible with the report of a long-lived component with a lifetime of 4.5 ns [2], τ_M of C2 (450 ps) is nearer to the lifetime of 0.6 ns obtained [10, 14] for dark-adapted systems, using the phase shift method. The phase shift method presumably measures τ_M for a non-exponential decay. The calculated total quantum yield of 0.66 % is in good agreement with a recent experimentally determined value of 0.7 % [21].

The calculated quantum yield increase in (ii) relative to (i) is consistent with the recent report of an increase in quantum yield with a decrease in pulse intensity for times of the order of 10 ns following the exciting pulse [3, 18] and with our measurements, which show that the effect occurs within 2.5 ns of the exciting pulse. τ_M of C2 only increases by a factor of 1.2 in (ii) relative to (i) and is thus still near to 0.6 ns. In general, τ_M for decay of the form of C2 is not linearly proportional to quantum yield, although for low values of q it is more nearly so.

The quantum yield change for (iii) relative to (ii) is 340 % (the exciting pulse used in (iii) was of comparable intensity to that used in (ii)) which is in good agreement with the measured 300 % change in photostationary fluorescence prior to laser excitation. The change in photostationary fluorescence is thus directly related to change in fluorescence lifetime, reflecting the closing of the traps of Photosystem II. τ_M of C2 (1.5 ns) is also in good agreement with previous estimates of between 1.5 to 2.0 ns [13–16].

The quantum yield at low temperature is also increased relative to (ii). τ_M (2.5 ns) agrees reasonably well with the previously reported lifetime of 3.1 ns [17] and the reported long wavelength component of Mar et al. [16], 2.3 ns. As the fluorescence decay obtained here does not show wavelength dependence, no direct comparison can be made with the short wavelength component of Mar et al. (1.4 ns); however this value is close to the calculated value of $t_{1/e}$ (1.2 ns).

The use of a non-exponential component of the form of C2 thus results in compatibility between the results obtained on both picosecond and nanosecond time scales for a wide range of experimental conditions. Four or more exponential components produce as good a fit as C1 and C2 but, since the fluorescence decay did not vary with wavelength under our experimental conditions, there is no direct evidence of physically separate components exhibiting exponential fluorescence.

There are several mechanisms which have been shown to lead to fluorescence decay of the form of C2 (Eqn. (1)) in vitro. The square root of time term in C2 has been shown to arise from N exponential components, resulting from quenching by N molecules in random distribution, whose rate of quenching varies with the sixth root of the distance between the excited molecule and the quenchers [20]. Decay of the form of C2 can also occur in systems which have molecular diffusion-controlled kinetics under certain conditions [22, 23]. In the photosynthetic unit, a process analogous to molecular diffusion, excitation energy migration among the antenna chlorophyl via Förster, and/or some other process of exciton energy transfer, could occur before trapping [24, 25]. Thus, excitation energy-migration controlled kinetics in the photosynthetic unit could also give rise to decay of the form C2 [23]. In this instance, one can take τ_0 in C2 to represent a parameter reduced in value from the

dilute solution τ_0 in proportion to quencher concentration times the coefficient of energy migration [23–25].

It has also been shown, in an analytic treatment with several approximations, that the simultaneous occurrence of both one-step Förster energy transfer and energy migration give a decay of the form of C2 [26]. In this treatment, τ_0 again refers to the chlorophyll dilute-solution lifetime while the exponent of the "square root" of t term is reduced from the value of 0.5, valid for the situation in which there is no energy migration. Recent, direct ab initio calculations on concentration quenching in chlorophyll solution, incorporating Förster energy-transfer migration, have used Monte Carlo methods to obtain the time dependence of fluorescence decay. These calculations also indicate that a large energy migration contribution to energy transfer reduces the exponent of the "square root" of t term below 0.5 [27].

The precision of our data does not allow discrmination between one-step Förster transfer, energy migration, or a mixture of the two. However, since each of these processes results from transfer to a random distribution of traps, the kinetics of the fluorescence decay are compatible with a model of the photosystem unit having the Photosystem I and Photosystem II traps randomly dispersed in bulk antenna chlorophyll molecules.

Using the parameters obtained from the fluorescence decay with the exponent of the "square root" of t term set exactly to 0.5 and $\tau_0 = 5 \pm 1$ ns, it is possible to calculate the concentration of traps using an expression appropriate to one-step transfer in a homogeneous environment

$$C_q = C_0\, q;\; C_0 = \frac{3000}{2\sqrt{\pi^3\, N R_0{}^3}}$$

where C_q is the concentration of the traps, N is Avogadro's number and R_0 is set to 70 Å, a value appropriate for $\tau_0 = 5$ ns with complete spectral overlap of donor emission and trap absorption [20, 24]. Complete spectral overlap should approach the situation in the photosytem unit since the trap absorption is adequately red-shifted with respect to the donor emission [24]. The calculated concentration of traps is only an upper bound since energy diffusion in the heterogeneous photosystem unit environment would lower the number of traps necessary to produce a given amount of quenching. The values given by this simple calculation are 4.8, 4.0, 2.0 and 0.95 % of total chlorophyll molecules for systems (i) to (iv), respectively, assuming a chlorophyll concentration of 0.1M.

The contribution of C2 to the decrease of (ii) relative to (i) as shown by the calculation of quenchers, C_q, is related to lowering the number of intensity-induced quenchers. The concentration of these intensity-induced quenchers is related to the absorbed photon density. Changing the absorbance of the sample by lowering the *Chlorella* concentration, without change in the incident photon density, results in an altered fluorescence decay. However, since at constant absorbed photon density lowering the sample absorbance does not alter the fluorescence decay, the effect of reabsorption due to *Chlorella* concentration appears negligible.

Part of the increased quantum yield of (ii) relative to (i) is due to the decreased initial intensity of C1. This suggests that C1 may arise from the high intensity of the exciting pulse; although the exponential form of C1 is not consistent with attributing its origin to single-singlet annihilation, as had previously been suggested [3]. Since it

has not, at present, been possible to show experimentally that C1 is not present at lower intensities, it remains a possibility that C1 pertains, at least in part, to Photosystem I fluorescence the lifetime of which has recently been estimated as $\geqq 30$ ps [28].

Thus, whereas C1 may be present below the threshold of the observed decrease of quantum yield with intensity, most, if not all, of the in vivo fluorescence decay is describable by a single non-exponential component, C2. C2 reflects the state of the traps. When the traps of Phostosystem II are closed as in (iii), the quantum yield of C2 increases. When the traps of Photosystem I and Photosystem II, and/or energy transfer to these traps, are rendered less effective by low temperature as in (iv), the fluorescence yield of C2 is further increased.

Thus we may conclude that a single non-exponential component containing a square-root of time term adequately describes most, if not all, of the in vivo fluorescence decay of the Photosystem unit at low intensities of the exciting pulse. The form of this single non-exponential component is consistent with traps, randomly dispersed in bulk attena chlorophyll molecules, through which excitation energy diffuses to the traps via excitation energy transfer. Further work on the individual particles of Photosystem I and Photosystem II is now in progress.

ACKNOWLEDGEMENTS

We wish to thank the United States Army for award of the Secretary of the Army Fellowship to L. H., the Science Research Council for the award of a Studentship to J.A.S., and the Ministry of Defence for an award of a Fellowship to C.J.T.

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