Biochimica et Biophysica Acta, 459 (1977) 329–336

BBA 47248

INTENSITY EFFECTS ON THE FLUORESCENCE OF IN VIVO CHLOROPHYLL

G. PORTER, J. A. SYNOWIEC and C. J. TREDWELL

Davy Faraday Research Laboratory of The Royal Institution, 21 Albemarle Street, London W1X 4BS (U.K.)

(Received August 30th, 1976)

SUMMARY

A technique for measuring relative quantum yields of fluorescence with a picosecond streak camera is described. We show that *Chlorella pyrenoidosa* exhibit an intensity dependent quantum yield when irradiated with single picosecond light pulses. This effect also occurs under conditions that inhibit the activity of the reaction centres, which can therefore be excluded as the cause.

When a pulse train (pulse separation 6.9 ns) was used, the quantum yield was further reduced by the light absorbed from previous pulses, which indicates the formation of a quenching species having a relatively long lifetime.

Absolute quantum yields calculated from the fluorescence decay show that single excitation pulses of $3 \cdot 10^{13}$ photons/cm^2 give results comparable to those obtained by very low intensity methods.

INTRODUCTION

The study of in vivo chlorophyll *a* fluorescence on a picosecond time scale is a powerful method of investigating the efficient energy transfer processes of photosynthesis. However, the very high intensities employed have led to doubts about the validity of such measurements. Until recently intensities in excess of $5 \cdot 10^{14}$ photons/cm^2 per laser pulse have been used, and, under these conditions, initial lifetimes ($\tau_{1/e}$) of between 50 and 100 ps have been reported [1–4]. These are much shorter than the 0.6 ns lifetime determined by phase fluorimetry [5, 6].

It has been shown [7, 8] that, above a threshold of approximately 10^{13} photons/cm^2, the quantum yield of dark-adapted *Chlorella pyrenoidosa* decreases with increasing excitation intensity. Campillo et al. [9] have reported a parallel decrease in the initial lifetime ($\tau_{1/e}$) from 375 ps to 50 ps over the range of 10^{14} to $3 \cdot 10^{15}$ photons/cm^2. This effect has been attributed [7, 8] to the high singlet exciton population generated in each photosynthetic unit by the laser pulse. Both singlet-singlet

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

annihilation [7, 9] and multiple trapping [8] have been suggested as the quenching process.

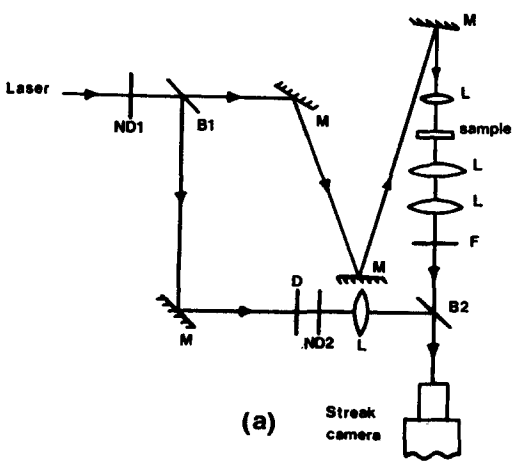
Breton and Geacintov [10] have also observed a cumulative decrease in the quantum yield of spinach chloroplasts over several pulses of a laser pulse train. They attribute this quenching to another species, which has a relatively long lifetime, such as the triplet or ion of chlorophyll *a*.

We have designed a system for simultaneously measuring the relative quantum yield and fluorescence decay with a picosecond streak camera. This has been used to investigate the effect of pulse intensity on the quantum yield and fluorescence decay of *Chlorella pyrenoidosa* under various experimental conditions.

EXPERIMENTAL

A train of 1060 nm pulses was produced by a mode-locked Nd^{3+}: glass laser oscillator. The half maximum duration of pulses from the centre of the train was 6 ps and the pulse separation was 6.9 ns. The second harmonic (530 nm) was generated with 10–15 % efficiency by a temperature tuned caesium dihydrogen arsenate crystal. A Pockels cell electro-optic shutter selected a group of 10 pulses from the centre of the pulse train. After selection the average energy per pulse was 50 μJ but, due to the voltage risetime in the transmission cable, the first pulse was approximately 50 % weaker than subsequent pulses.

The optical arrangement for relative quantum yield measurements is shown in Fig. 1a. The pulse train was focused to a $5 \cdot 10^{-3}$ cm^2 spot at the 1 mm cuvette, to produce a maximum photon density of $3 \cdot 10^{16}$ photons/cm^2 per pulse. Fluorescence from the sample was passed through a wavelength selection filter (> 665 nm, SCHOTT RG665 and > 715 nm, SCHOTT RG715) on to the slit of an S20 Imacon



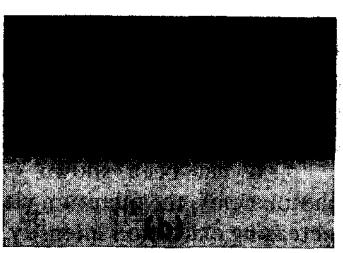


Fig. 1. (a) Optical arrangement for measuring relative quantum yields. M1-4, 100 % reflecting front surface mirrors; B1,2 10 % reflecting beam splitters; L1-4, lenses; D, ground glass diffuser; ND1,2 Neutral absorbance filters; F, Wavelength selection filter. (b) A typical trace obtained by this technique. Each group of two pulses consists of a reference pulse (left) and the fluorescence (right) due to that pulse.

600 (John Hadland (P.I.) Ltd.) streak camera. 10 % of the 530 nm pulse train was split off before the sample and travelled over a shorter path to the camera slit. Each reference pulse arrived approximately 2 ns before the fluorescence from the sample.

The streak camera was optically coupled to a 500 channel optical multichannel analyser (OMA, SSR 1205 B), which stored the streak traces and displayed them on an oscilloscope or chart recorder. An I.T.L. laser calorimeter was used to calibrate the integral of the reference pulse (OMA integrate function) in terms of the energy of the excitation pulse. A slow streak speed was selected so that the first three pulses and the resulting fluorescence decay curves could be recorded. Fig. 1b shows a typical oscilloscope trace where the second, fourth, and sixth pulses are the fluorescence emissions due to excitation by the first, third, and fifth reference pulses respectively. Incident intensities were determined by the value of ND1 and the variation in the laser output from shot to shot. Values of ND1 were selected at random to preclude time dependent artifacts. The relative quantum yields of the three fluorescence decay curves were calculated by dividing the integral of the second pulse by that of the first, the fourth by that of the third, and so on.

The linearity of the streak camera detection system was determined by monitoring the excitation pulse intensity attenuated by a range of calibrated neutral optical density filters. By placing these filters after the beam splitter Bl (Fig. 1a), the reference pulse could be used to calculate the transmittance; response was found to be linear within ± 2 %. A similar determination was performed for the fluorescence emission from rose bengal, a xanthine dye, monitored above 600 nm. Over the range of experimental intensities employed, response was again linear within ± 2 %, and the error in the relative quantum yield was ± 5 %.

Chlorella pyrenoidosa were grown and prepared as previously described [11]. Samples had a measured transmittance of 56 % at 530 nm in a 1 mm cuvette, and a fresh sample from a stock suspension was used for each laser shot. The experimental conditions were:

(i) Dark adapted *Chlorella* at room temperature.

(ii) *Chlorella* at room temperature, with the reaction centres of Photosystem II closed. This was accomplished by adding DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) to a concentration of $5 \cdot 10^{-4}$ M, and irradiating with 633 nm light from a CW helium : neon laser (1.25 mW/cm^2).

(iii) *Chlorella* at 77 °K irradiated with 633 nm light as described in (ii).

The fluorescence emitted above 665 nm was observed in the case of (i) and (ii), and above 715 nm in the case of (iii).

The fluorescence decay traces from (i), (ii), and (iii) were recorded over a wide range of excitation intensities ($3 \cdot 10^{13}$–$8 \cdot 10^{15}$ photons/cm^2) for the first and subsequent pulses in the train. The sensitivity of the streak camera is determined by the slit width and streak speed selected; a relatively high photon input is required if a good signal to noise ratio is to be attained at maximum time resolution (5 ps). Selection of a streak speed with 50 ps time resolution gave the best compromise between sensitivity and time resolution. By increasing the excitation area to $2.8 \cdot 10^{-1}$ cm^2 still lower intensities could be used without decreasing the photon input to the camera. The collection optics shown in Fig. 1a were modified to contend with the increase in beam diameter.

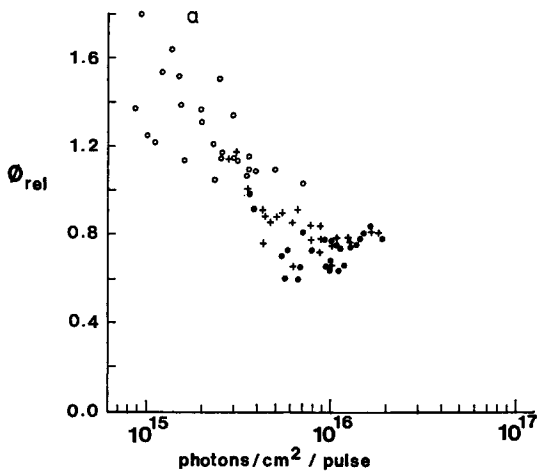


Fig. 2. See opposite page for legend.

RESULTS

The relative quantum yields (ϕ_{rel}) of fluorescence for (i), (ii), and (iii) were found to decrease as the intensity of the first pulse was increased. Subsequent pulses in the train showed a similar effect, and also a further decrease in ϕ_{rel} compared with that of the first pulse. At low excitation intensities, ϕ_{rel} decreased from pulse to pulse in the train, even though the excitation pulse intensity remained constant. At high excitation intensities ($> 3 \cdot 10^{15}$ photons/cm^2), ϕ_{rel} reached a constant minimum value after the third pulse in the train. The quantum yield changes are apparent when ϕ_{rel} is plotted as a function of intensity for each of the first three pulses. Figs. 2a, 2b, and 2c show the curves obtained from (i), (ii), and (iii) respectively. The values of ϕ_{rel} are not normalised, and only Figs. 2a and 2b have a comparable scale.

The closure of the Photosystem II reaction centres for (ii) is confirmed by the twofold increase in ϕ_{rel} from figure 2a to 2b. The > 715 nm emission monitored in (iii) is attributed to fluorescence from Photosystem I [12]. This also shows a marked increase in ϕ_{rel} compared with room temperature measurements, but, due to the change of monitoring wavelength, Fig. 2c cannot be compared with Fig. 2a.

Fluorescence decay traces obtained by single pulse excitation at various intensities under conditions (i), (ii), and (iii) are shown in Figs. 3, 4, and 5 respectively. The time calibration for Figs. 3 and 4 is 470 ps per major division (50 ps time resolution), and that for Fig. 5 is 780 ps per major division (78 ps time resolution). An analysis of these curves was performed using the numerical printout from the OMA memory. Over the time scale spanned by these traces, most of the curves were satisfactorily described by the equation [13]:

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

where I_0 is the initial intensity, $I(t)$ is the intensity at time t, and A is a constant. Whilst this equation is very similar to that for the Förster mechanism of energy transfer at short times, the actual kinetics of these processes are far more complex. It is

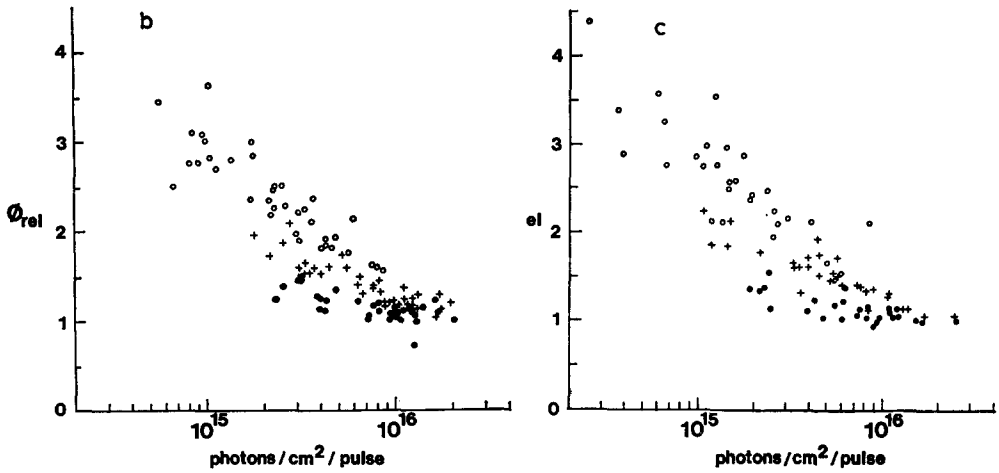


Fig. 2. ϕ_{rel} plotted as a function of pulse intensity for the first (○), second (+), and third (●) pulses in the train. (a) Dark-adapted *Chlorella* at room temperature. (b) Pre-illuminated *Chlorella* with DCMU at room temperature. (c) Pre-illuminated *Chlorella* at 77 °K.

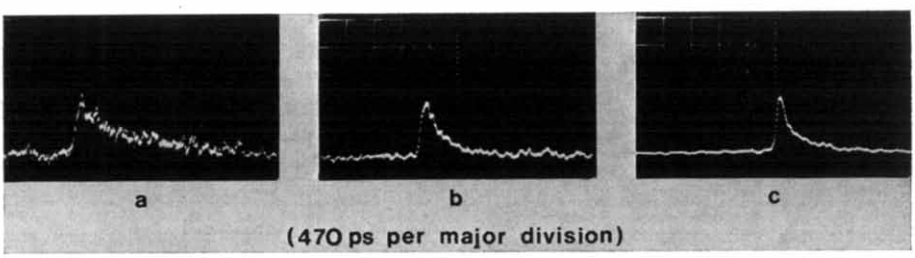


Fig. 3. Fluorescence decay traces for dark-adapted *Chlorella* at excitation intensities of: (a) $3 \cdot 10^{13}$, (b) $5 \cdot 10^{14}$, (c) $8 \cdot 10^{15}$ photons/cm^2.

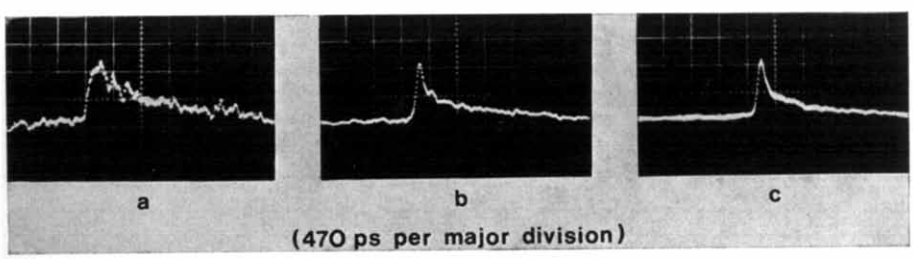


Fig. 4. Fluorescence decay traces for pre-illuminated *Chlorella* with DCMU, at excitation intensities of (a) $3 \cdot 10^{13}$, (b) $5 \cdot 10^{14}$, (c) $8 \cdot 10^{15}$ photons/cm^2.

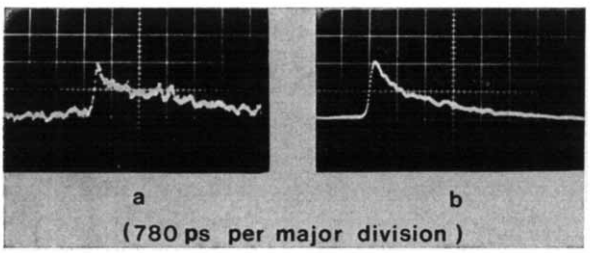


Fig. 5. Fluorescence decay traces for pre-illuminated *Chlorella* at 77 °K, at excitation intensities of (a) $3 \cdot 10^{13}$, and (b) $8 \cdot 10^{15}$ photons/cm^2.

only used here as a means of obtaining a value for the absolute quantum yield of fluorescence. Under conditions (ii) and (iii) at low excitation intensities, the decay curves followed a predominantly exponential decay law of the form:

$$I(t) = I_0 \exp(-\mathrm{kt})$$

where k is a constant.

The absolute quantum yields (ϕ_{calc}) were calculated from the expression:

$$\phi_{\mathrm{calc}} = \frac{\phi_0}{\tau_0 I_0} \int_0^\infty I(t)\mathrm{d}t$$

where ϕ_0 and τ_0 are the quantum yield (0.33) and fluorescence lifetime (5700 ps) of in vitro chlorophyll *a* respectively. ϕ_{calc} is the quantum efficiency of the fluorescent part of the chlorophyll *a* attenna system. Table I lists the values of ϕ_{calc}, A or k, and the initial lifetime ($\tau_{1/e}$) of the traces shown in Figs. 3–5. The initial lifetime of (i) at high excitation intensities was within the camera resolution function, and was therefore measured on a faster streak speed (the time resolution 6 ps).

TABLE I

THE EFFECT OF EXCITATION INTENSITY UPON THE FLUORESCENCE EMISSION FROM *CHLORELLA PYRENOIDOSA*

Condition (i) Dark-adapted *Chlorella* at room temperature, (ii) *Chlorella* with DCMU and pre-illumination at room temperature, (iii) *Chlorella* at 77 °K (pre-illuminated).

Experimental conditions	Intensity (photons/cm^2 ± 10 %)	$\tau_{1/e}$ (ps)	A (ps$^{-\frac{1}{2}}$)	k (ps^{-1})	ϕ_{calc}
(i)	$3 \cdot 10^{13}$	450	0.047	—	0.052
(i)	$5 \cdot 10^{14}$	280	0.060	—	0.032
(i)	$8 \cdot 10^{15}$	50*	0.093	—	0.013
(ii)	$3 \cdot 10^{13}$	1800	—	0.00056	0.104
(ii)	$5 \cdot 10^{14}$	660	0.039	—	0.076
(ii)	$8 \cdot 10^{15}$	220	0.068	—	0.025
(iii)	$3 \cdot 10^{13}$	2800	—	0.00036	0.162
(iii)	$8 \cdot 10^{15}$	430	0.048	—	0.050

* Measured with a time resolution of 6 ps.

DISCUSSION

Previous work [7, 8] on the intensity dependence of the relative quantum yield of dark adapted *Chlorella* has shown that the effect has a threshold in the region of 10^{13} photons/cm^2 and reaches a lower limit at 10^{16} photons/cm^2. Over this range ϕ_{rel} decreases by 80 ± 5 %. There is evidence [14] that the lower limit is dependent on the homogeneity of excitation, and is therefore determined by the absorbance of the sample. Since our experimental conditions correspond to those used in previous determinations of this effect [7, 8], the resulting quantum yield curve will be the same. ϕ_{calc} for dark adapted Chlorella (experimental condition (i), Table I), over the range of $3 \cdot 10^{13}$ to $8 \cdot 10^{15}$ photons/cm^2, decreases by 75 %, which is close to the value reported [7, 8.] The calculated quantum yields (ϕ_{calc}) for (i), (ii), and (iii), at $3 \cdot 10^{13}$ photons/cm^2, are all in good agreement with those calculated by the phase shift technique [15]. The initial lifetimes ($\tau_{1/e}$) measured at this intensity also correspond to those obtained by other techniques [6, 15–18] and the mean lifetimes (τ_M) reported by Porter et al [13]. We therefore conclude that, under our experimental conditions, excitation intensities of $3 \cdot 10^{13}$ photons/cm^2 or less are required to obtain decay curves that correspond to singly excited photosynthetic units.

Singlet-singlet annihilation [7, 9] and multiple trapping [8] have both been proposed as possible quenching mechanisms. Simple singlet-singlet annihilation would lead to second order kinetics, which, we have found, do not account for the observed form of the initial decay in (i), (ii), or (iii). It has been suggested (Geacintov, N. E., personal communication) that true second order kinetics are distorted by the distribution of singlet exciton populations amongst the photosynthetic units of high absorbance sample. At present our streak camera does not have the sensitivity to investigate this effect.

It has been found [10] that spinach chloroplasts at 100 °K and at room temperature show a cumulative decrease in ϕ_{rel} over several pulses of a laser pulse train. Fig. 2b and 2c indicate that a similar effect is operative in *Chlorella*. The quenching species must have a relatively long lifetime to survive from pulse to pulse. Singlet-triplet and/or singlet-ion quenching have been proposed [10] as the most probable cause. From Figs. 2b and 2c, and the values of ϕ_{calc}, we find that, at an intensity of $5 \cdot 10^{15}$ photons/cm^2 per pulse, the observed effect accounts for approximately 7 % of the total quenching for each preceding pulse. After 6.9 ns the singlet exciton population is far too low to cause such a marked effect. Similarly, intersystem crossing to the triplet state would not produce enough of a triplet population to explain the observed quenching. There are other pathways to the triplet state which are overall spin allowed and favourable on energy transfer grounds, [19] such as

$$S^* + S^* \dashrightarrow T + T$$

and possibly:

$$S^* + T \dashrightarrow T + T$$

which would form a significant triplet population in the photosynthetic unit, and a quenching process of the form:

$$S^* + T \dashrightarrow S + T$$

would maintain the quencher population. S* is the excited singlet state, S is the singlet ground state, and T is the triplet state of chlorophyll *a*. These processes would be operative during the first excitation pulse, and could also add to the complexity of the kinetics at high excitation intensities.

The fluorescence traces obtained from subsequent pulses in the train (conditions (i), (ii), and (iii) were not significantly different from those of the first pulse. This might have been expected, owing to the predominance of the singlet-singlet annihilation process over the first few pulses. The use of a full pulse train would build up a high triplet population, and singlet-triplet annihilation would then predominate over a wide range of excitation intensities. This explains a previous report that decay rates were unaffected by a ten times attenuation of the excitation pulse train [1].

We have shown that, with careful design of the experimental system, picosecond techniques are capable of studying energy transfer in the photosynthetic unit, without generating spurious quenchers of the kind discussed above.

ACKNOWLEDGEMENTS

We wish to thank 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. We would also like to thank J. Breton and N. E. Geacintov for pre-publication copies of their papers, and L. E. Harris, J. Barber, G. S. Beddard, and N. E. Geacintov for stimulating discussions.

REFERENCES

1 Beddard, G. S., Porter, G., Tredwell, C. J. and Barber, J. (1975) Nature 258, 166–168
2 Shapiro, S. L., Kollman, V. H. and Campillo, A. J. (1975) FEBS Lett. 54, 358–362
3 Kollman, V. H., Shapiro, S. L. and Campillo, A. J. (1975) Biochem. Biophys. Res. Commun. 63, 917–923
4 Paschenko, V. Z., Protasov, S. P., Rubin, A. B., Timofeev, K. N., Zamazova, L. M. and Rubin, L. B. (1975) Biochim. Biophys. Acta 408, 143–153
5 Nicholson, W. J. and Fortoul, J. I. (1967) Biochim. Biophys. Acta 143, 577–582
6 Muller, A., Lumry, R. and Walker, M. S. (1969) Photochem. Photobiol. 9, 113–126
7 Campillo, A. J., Shapiro, S. L., Kollman, V. H., Winn, K. R. and Hyer, R. C. (1976) Biophys. J. 16, 93–97
8 Mauzerall, D. (1976) Biophys. J. 16, 87–91
9 Campillo, A. J., Kollman, V. H. and Shapiro, S. L. (1976) Science 193, 227–229
10 Breton, J. and Geacintov, N. E. (1976) FEBS Lett., in the press
11 Barber, J. (1968) Biochim. Biophys. Acta 150, 618–625
12 Cho, F. and Govindjee (1970) Biochim. Biophys. Acta 205, 371–378
13 Harris, L. E., Porter, G., Synowiec, J. A., Tredwell, C. J. and Barber, J. (1976) Biochim. Biophys. Acta, in the press
14 Geacintov, N. E. and Breton, J., Biophys. J. (1976) in the press
15 Mar, T., Govindjee, Singhal, G. S. and Merkelo, H. (1972) Biophys. J. 12, 797–808
16 Butler, W. L. and Norris, K. H. (1963) Biochim. Biophys. Acta 66, 72–77
17 Singhal, G. S. and Rabinowitch, E. (1969) Biophys. J. 9, 586–791
18 Briantais, J. M., Merkelo, H. and Govindjee (1972) Photosynthetica 6, 133–141
19 Porter, G. and Wright, M. R. (1959) Disc. Faraday Soc. 27, 18