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# LIGHT EMISSION FROM THE *SCENEDESMUS OBLIQUUS* WILD TYPE, MUTANT 8, AND MUTANT 11 STRAINS, MEASURED UNDER STEADY-STATE CONDITIONS BETWEEN 4 NANOSECONDS AND 10 SECONDS

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SUMMARY

1. The entire time course of the delayed light emission, from 4 ns to several seconds, was measured from the wild type $D_3$ strain, from the mutant 8 strain, and from the mutant 11 strain of *Scenedesmus obliquus* algae. The algae were illuminated by 488 nm, 12 mW/cm$^2$ laser light which was modulated on and off by either a rotating slotted disc or by an electro-optic laser light modulator. The kinetics of the light emission from the algae were measured through a 690-nm interference filter during the time intervals between the stimulus laser light flashes.

2. The $D_3$ wild type strain and mutant 8 produced delayed light emission of an intensity which could be measured by our apparatus, while any emission present from mutant 11 was not intense enough to be recorded. This indicated that most, if not all, of the delayed light emission originated from photosystem II.

3. The delayed light emission curve for the wild type and mutant 8 strains decayed in a complex fashion, *i.e.* the delayed light emission decay curves could not be described analytically by a single exponential term.

4. The initial microsecond section of the delayed light emission curve intersected the prompt fluorescence decay curve at virtually a right angle for both the wild type and the mutant 8. The lack of a transition zone between the two curves indicated that the prompt fluorescence was not a fast component of delayed light emission. Also, the lack of a transition zone between the two curves is evidence that delayed light emission occurred concurrently with prompt fluorescence during the periods the algae were exposed to the laser light. Following the instant the stimulus laser light was extinguished, the prompt fluorescence intensity fell to zero and the presence of the less intense delayed light emission was revealed.

5. The intensity of light emission from an algal sample could be measured (a) a few microseconds before the stimulus light was extinguished, and (b) in the first microsecond after the stimulus light was extinguished. Since the delayed light emission decay curve was rather flat throughout the first microsecond following stimulus light extinction, the ratio of prompt fluorescence intensity to delayed light emission intensity could be closely approximated as the ratio of (a)/(b). This

ratio was found to be 165:1 for the wild type algae and 165:0.3 for the mutant 8. The algal samples were always preilluminated by the modulated laser light for 20 min prior to the ratio measurements.

## INTRODUCTION

Approximately 100 years ago, fluorescence was observed in illuminated green plants[1]. Delayed light emission was not discovered until 1951, and various mechanisms have been proposed[2-4] to explain the origin of delayed light. For times shorter than 0.1 s, delayed light seems to be produced by electron-hole recombination in a quasi-crystalline lattice of the photosynthetic unit[4]. Despite numerous experiments of various kinds (kinetics, quantum yields, lifetimes, biochemical alterations, mutants), the origin of delayed light emission and fluorescence, their interrelationships, and their quantitative involvement in photosynthetic processes remain undecided[5-7]. It has been suggested that part of the fluorescence may perhaps represent a fast component of delayed emission[8]. The delayed emission spectrum from higher plants and algae is similar to that of fluorescence[5]. The delayed light intensity, measured at times longer than 50 $\mu$s, is known to decay in a complex fashion.

We present in this article the entire decay curve, from 4 ns to several s, of the light emission from the wild type and from two mutant strains of *Scenedesmus obliquus*. The data support the conclusions that (a) the nanosecond and microsecond delayed light emission originates in photosystem II, similar to the slower components of delayed light emission[9], (b) the delayed light emission decays in a complex fashion, and (c) the delayed light is not a fast component of prompt fluorescence.

## METHODS

Cultures of *S. obliquus*, wild type strain $D_3$, were grown at 22 °C in a synthetic medium[10]. The illumination was provided by white fluorescent tubes. The algal cultures were continuously shaken and flushed with a stream of water-saturated air containing 2 % $CO_2$. The *S. obliquus* mutants 8 and 11 were heterotrophically grown at room temperature in the dark[11-13]. The mutant 8 has a defect in the pyridine nucleotide-reducing Photosystem I, and mutant 11 has a defect in the oxygen-evolving Photosystem II (ref. 11). These mutants have revealed no major difference in the amounts of the main pigments of the photosynthetic apparatus[11].

The algae were harvested three days after inoculation and diluted with growth medium to an absorbance of 0.1 to 0.4 at 690 nm, and placed into rectangular quartz cuvettes with 1 cm optical path length. The cuvettes were maintained during the experiments at 22 °C and the algal suspension of 2 ml was stirred by bubbling with an air stream saturated with water vapor and containing 2 % $CO_2$. The sample was illuminated with modulated blue light (488 nm) from an argon laser (Model 54A, Coherent Radiation, Palo Alto, Calif.). Light intensities of 12 mW/cm$^2$, measured at the sample position, were employed. Prior to collecting emission data, the algae were preilluminated with blue laser light of 12 mW/cm$^2$ for 20 min. The light emitted

from the algae first passed through a Schott double interference filter (filter peak at 690 nm) and struck the cathode of a dry ice-cooled photomultiplier (Amperex 56CVP).

In the time interval from 4 ns to about 2 ms, the decay pattern of the emitted light was recorded with photon counting[14] or by sampling techniques[15]. The excitation laser beam was chopped periodically with a frequency of 50 Hz by an electro-optical shutter[15]. The shutter could switch the laser beam off within about 25 ns and keep the beam off for approx. 6 ms. During the entire period the shutter remained closed, the decay curve of delayed light could be measured from the algal sample. The contrast ratio for the shutter was defined as the intensity of laser light at the sample during the time interval the shutter was open compared to the intensity of laser light at the sample during the time interval the shutter was closed. Contrast ratios better than 20000:1 were routinely obtained. These contrast ratios were measured in the absence of an interference filter, with the laser beam being scattered by a suspension of inert latex particles placed at the usual sample position. Numerous tests have been carried out to assure that the electro-optical shutter had a sufficient contrast ratio for delayed light emission experiments. An insufficient contrast ratio would result in a low level of fluorescence of the algal sample during the time intervals in which the delayed light emission was to be measured.

In addition to experiments with the electro-optical shutter, a mechanical rotating slotted disc shutter was employed. The rotating disc extinguished the focused laser beam in 250 ns and produced a laser light modulation pattern of 1 ms duration light flashes with a 500 Hz repetition rate.

Both the electro-optical shutter and the rotating disc shutter gave identical results for the time course of delayed emission, as measured between 0.3 $\mu$s and several milliseconds. For time ranges or measurement shorter than 300 ns, only the electro-optical shutter was fast enough to be used. Nevertheless, upon extrapolating the data obtained with the mechanical shutter to times shorter than 300 ns, the values agree with the data from electro-optical modulator experiments.

For decay times longer than 1 ms, the delayed light emission was measured with a phosphoroscope[16].

## RESULTS

The light emission from the algae was recorded as a function of time after the 488 nm laser light had been switched off. The reference point, $t = 0$, was defined to be the onset of the closure of the electro-optical shutter, and is designated by an arrow in Fig. 1. This figure includes the interval of time in which the electro-optical shutter switched the laser light off at the sample. The broken line in Fig. 1 represents the change in relative intensity of the laser light striking the sample during the 100 ns time interval described by Fig. 1. This "closure" curve was recorded by first removing the algae sample and the 690 nm interference filter and then scattering a small amount of the modulated laser light from the electro-optical shutter onto the photomultiplier cathode.

The algal emission curve and the light scatter "closure" curve of Fig. 1 were recorded at the same absolute light intensity level by the photomultiplier. This was accomplished by adjusting an iris in front of the photomultiplier such that the photomultiplier output was the same number of c/s while recording both curves.

The numerical data forming the closure curve and the algal emission curve were then normalized to the same value in the region $t < 0$ of Fig. 1 in order to allow direct comparison of the two curves. From a mathematical analysis[15] of the time course of the decay of these two curves, it can be determined that the algal prompt fluorescence can be best approximated by a first order decay having a lifetime of 8.5 ns at 77 °K and 2 ns at 295 °K.

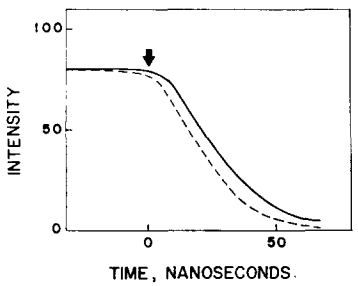


Fig. 1. Light emission from wild type *S. obliquus*. The algal emission (——) was measured at 77 °K through a 690-nm interference filter, utilizing the electro-optical laser light modulator for modulation of the laser beam. The incident light intensity at the algal sample was 12 mW/cm$^2$ from the 488 nm line of the argon laser. - - - -, the intensity of the laser light, scattered by aluminum foil at the sample site, as the laser light is switched off by the electro-optical laser modulator. This "closure" curve was recorded in the absence of the 690-nm interference filter in front of the photomultiplier cathode. The arrow at the onset of the closure curve designates the reference point for zero time. The mathematical analysis referred to in the text must be applied to these two curves in order to determine the actual 8.5-ns fluorescence lifetime of the algal emission at 77 °K. The fluorescence from algae held at room temperature forms a curve which coincides with the closure curve. The mathematical analysis shows that the room temperature fluorescence actually has a lifetime of 2 ns.

In the $t < 0$ time region, the steady-state fluorescence intensities of the wild type strain, the mutant 8 strain, and the mutant 11 strain were tested under identical conditions, *i.e.* with the same exciting light intensity, sample geometry, sample volume and cell density, and with the same preillumination period.

Figs 2, 3 and 4 illustrate the wild type strain's delayed emission at room temperature during the first 1 $\mu$s, the first 8 $\mu$s, and the first 80 $\mu$s, respectively. These curves were obtained from experiments utilizing the rotating disc shutter for the laser light modulation. Very similar curves were measured with the mutant 8 strain, while the mutant 11 strain did not produce enough delayed light emission to be detectable with our apparatus. The delayed emission curves for the wild type and the mutant 8 are compared in Fig. 5 over the first millisecond time interval after the excitation laser light had been switched off.

For the wild type strain, the emission curve beyond 1 ms was measured with a phosphoroscope. The first part of this curve (1 ms $\leqslant t \leqslant$ 3 ms) matched the curve that was recorded with the electro-optical shutter modulation system. The emission curves for times longer than 1 ms were not plotted in Fig. 5. The delayed emission curve for times longer than 1 ms formed a smooth line with a small slope for both the wild type strain and the mutant 8. For the wildtype strain, if the delayed light intensity at 1 ms be normalized to 1.0, the intensity of delayed light at 0.1 s is $1.4 \times 10^{-1}$; at 1 s, the intensity is $1.4 \times 10^{-2}$; and at 9 s, the intensity is $2.6 \times 10^{-3}$.

No measurable differences could be detected in the relative emission curves

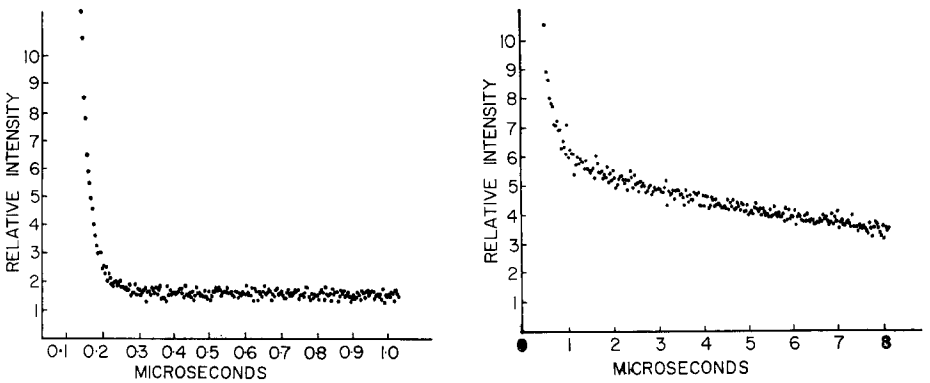


Fig. 2. The first microsecond of delayed light emission from wild type *S. obliquus*. The figure shows the intersection of the (flat) delayed light emission curve with the (vertical) trailing edge of the prompt fluorescence curve. The light emission was measured at room temperature through a 690-nm interference filter, utilizing the high speed rotating disc shutter for laser light modulation. The steady-state algal fluorescence immediately prior to the $t = 0$ point of this figure was 168 times more intense than the delayed light emission shown in this figure at the 1-$\mu$s point.

Fig. 3. The first eight microseconds of delayed light emission from wild type *S. obliquus*. The figure shows the intersection of the (vertical) trailing edge of the prompt fluorescence curve with the leading edge of the delayed light emission curve. The algal emission was measured at room temperature through a 690-nm interference filter, utilizing the high speed rotating disc shutter for laser light modulation.

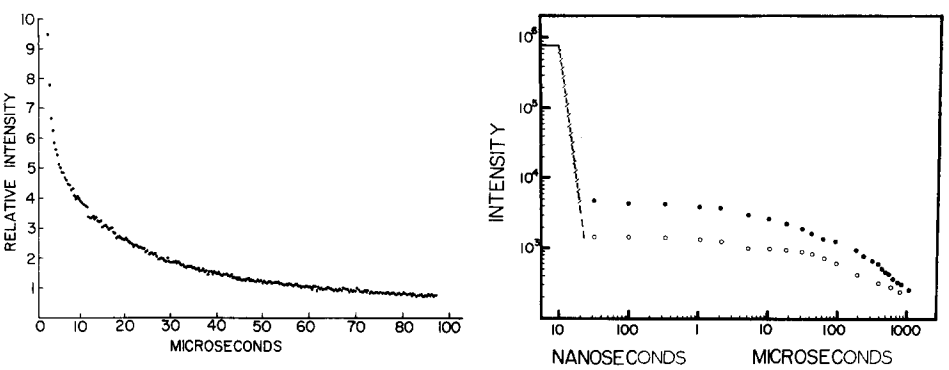


Fig. 4. The first eighty microseconds of delayed light emission from wild type *S. obliquus*. In contrast to Figs 2 and 3, the time axis of this figure is too long to resolve the intersection point of the trailing edge of the prompt fluorescence with the leading edge of the delayed light emission curve. The algal emission was measured at room temperature through a 690-nm interference filter, utilizing the high speed rotating disc shutter for laser light modulation.

Fig. 5. A comparison of the light emission from *S. obliquus* wild type and mutant 8 strains. The algae were excited at room temperature with 488 nm laser light of 12 mW/cm$^2$ intensity. After 20 min of preillumination with the modulated laser light, the emission from the algae was measured through a 690-nm interference filter. The light emission during "closure" is represented by (////) for the wild type, and by (- - - -) for mutant 8. The delayed light emission is represented by (●●●) for the wild type, and by (○○○) for mutant 8. The curve for mutant 8 has been normalized so that the steady-state mutant 8 fluorescence plateau on the left side of the figure will be at the same level as the wild type fluorescence plateau. The text explains that normalization for comparison purposes is reasonable since both wild type and mutant 8 show about the same steady-state fluorescence levels when tested under identical conditions.

from the algal samples when the curves were recorded from algal cell suspensions with absorbance of 0.1 or of 0.4 at the main absorption peak. Some experiments have been performed with a 721 nm interference filter instead of the 690 nm interference filter. No difference could be seen in the relative emission curves recorded through the two different filters.

No attempt was made to measure the delayed light emission from an algal sample held at 77 °K. Our instrumentation could, however, detect a difference in the prompt fluorescence decay curve for algae held at 77 °K as compared to algae held at 295 °K. At 77 °K the sample was a cracked matrix. Fig. 1 illustrates the algal emission curve at 77 °K and also the stimulus light extinction (closure) curve. When the normalized emission curve from algae held at 295 °K was compared to the closure curve, the two curves seemed to coincide. However, the deconvolution analysis indicated a lifetime of 2 ns for the algal prompt fluorescence at 295 °K.

## DISCUSSION

According to our experiments, the steady-state fluorescence emitted from the alga *Scenedesmus* has an exponential decay with a lifetime of the order of 2 ns at room temperature. We found no difference in the lifetime for the emission of the wild type strain, or for the mutants 8 and 11. The 2-ns value agrees with the lifetimes measured *in vivo*, having assumed an exponential decay[17,18]. For chlorophyll *a* in alcohol, the lifetime has been reported to be 5.5 ns[19]. It seems reasonable to designate this type of emission as fluorescence because the measured lifetimes are in accord with the lifetimes of strongly allowed singlet emissions in molecular systems. The lifetime of the fluorescence measured from algae held at 77 °K, which we report to be 8.5 ns, is larger than the values reported in the literature[18,20], probably resulting from self-absorption in the cracked matrix of the sample.

In the following discussion, we define as delayed light all that light emitted after the decay of the prompt fluorescence. We believe that the 2-ns emission is fluorescence and not a fast component of delayed light[8] because (a) the delayed emission curve intersects the closure curve at an intensity level more than 100-fold smaller than the level of the steady-state fluorescence, (b) there is no indication of a smooth transition between the fluorescence curve and the delayed emission curve at the point of intersection, (c) the true exponential decay of 2 ns for the fluorescence, and (d) the measured fluorescence lifetime of chlorophyll *in vivo* is that expected from quantum yield experiments[19].

The profile of the delayed light emission curve, as measured between about 20 ns and 10 s, clearly demonstrates that delayed light does not decay by a single exponential factor. The intensity of delayed emission at various times after the extinction of the laser stimulus light can be contrasted to the intensity of the steady-state fluorescence which occurs while the algae are being illuminated by the stimulus laser light. For the wild type, the intensity of the steady-state fluorescence relative to that of the delayed light emission was 168:1 at 1 $\mu$s, 256:1 at 10 $\mu$s, and 2900:1 at 1 ms. As can be seen from Fig. 5, between 1 and 10 $\mu$s the intensity of the delayed light emission curve for mutant strain 8 is one-third that of the wild type strain. At the 1-ms point, however, the intensity of the delayed emission from the wild type is greater than that of the mutant 8 strain by a factor of $^4/_3$.

The ratio of the intensity of delayed emission at the $t = 0$ point to the intensity of the steady-state fluorescence immediately preceding the $t = 0$ point is 1:165. When the same ratio is formed from the data of mutant 8, the ratio is 1:500. Mutant 11 data gave a ratio larger than 1:5000. The ratios quoted above were measured from algae which had been preilluminated for 20 min by the modulated laser light. It has been our observation that the delayed light emission is markedly dependent upon the length of time the algae had been preilluminated by the laser light. This is especially true for mutant 8.

The delayed light emission from mutant 8 and from the wild type has been measured over the time interval between 1 ms and about 100 s after stimulus light extinction and was found to originate from Photosystem II[21,22]. Regarding this slow component of delayed light emission, the hypothesis has been presented that the emission is a result of a chemical backreaction between an oxidized donor and a reduced acceptor of Photosystem II, where perhaps a triplet exciton plays a role in an intermediary step[23].

Concerning the origin of the nanosecond and micro-second portion of the delayed light emission, our experiments show that practically all the delayed light emission from the wild type and from mutant 8 also originates from energy storage in Photosystem II. In that time interval, the delayed light intensity of mutant 11 was at the limit of detectability for our instrumentation, *i.e.* the delayed light intensity of mutant 11 was at least a factor of 5000 below the steady-state fluorescence intensity of mutant 11. It is possible that there was no delayed emission at all from mutant 11. Our observations are consistent with the conclusion of Goedheer[24,25] that the oxygen-evolving Photoreaction II[26] is responsible for the production of delayed light emission. In mutant 8, where Photosystem II is intact and where Photoreaction I is non-functional, the light energy stored in Photosystem II cannot be efficiently transferred to Photosystem I because the latter is unable to act as electron acceptor.

The origin of delayed light emission is unclear. At various times during delayed light emission different mechanisms for the production of delayed light are probably operative[4]. For times shorter than 0.1 s, delayed light probably originates from the recombination of electrons and holes. One may tentatively assume that for times shorter than 1 $\mu$s delayed light is generated by exciton processes and charge carrier recombination in the bulk of the quasi-crystalline lattice of the chlorophyll photoreceptor. For metal-free phthalocyanine crystals, effective life-times of $10 < \tau < 100$ ns for the photoinduced charge carriers can be expected[27]. For times longer than 1 $\mu$s, delayed emission probably reflects the stored energy at a particular time at a particular component of the photosynthetic electron transport chain. Enzymatic backreactions can be expected in such a time range because it has been demonstrated that the electron transport system can exhibit absorption changes within a few microseconds after the application of a light pulse[28,29]. It should be kept in mind, however, that *a priori* it is not known whether information obtained with pulsed high intensity light sources is comparable to that determined under steady-state conditions with stimulus light intensities of the same order of magnitude as biologically occurring light levels.

Experiments are under way to elucidate further the mechanism of delayed light emission.

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