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MEASUREMENT OF THE FLUORESCENT LIFETIMES OF CHLORELLA AND PORPHYRIDIUM IN WEAK LIGHT

W. J. NICHOLSON AND J. I. FORTOUL
IBM Watson Laboratory, Columbia University, New York, N.Y. (U.S.A.)
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SUMMARY

The fluorescent lifetimes of Chlorella and Porphyridium were measured by determining the emission time distribution of fluorescent photons following excitation by a flash lamp. A lifetime of 0.6 ± 0.2 nsec was determined for both species exposed to light of an average intensity less than 1 erg/sec per cm^2. No lifetime greater than 1 nsec with a relative yield greater than 10% was found.

INTRODUCTION

During the past decade, several measurements have been made of the fluorescent lifetimes of various photosynthetic species[1–6]. Two independent methods have been utilized, direct observation of a phototube output pulse following flash excitation and phase fluorimetry. In the older flash work, BRODY AND RABINOWITCH[1], by means of a moment analysis, obtain lifetimes of 1.2, 1.5, and 1.6 nsec for Anacystis, Porphyridium and Chlorella, respectively. Using phase fluorimetry, BUTLER AND NORRIS[4] obtained a value of 0.6 nsec for the fluorescence of bean leaf. In each of these measurements, it was explicitly assumed that the fluorescent species had unique lifetimes. More recently, MURTY AND RABINOWITCH[5] have cast doubt on the validity of this assumption by observing a complex decay spectra of fluorescent species excited by a flash lamp. However, in recent phase fluorimetric measurements[6] at both low and high light intensities, lifetimes of from 0.44 to 1.15 nsec were observed with no indication of significant contributions to the lifetimes by longer lived components. In order to clarify some of these discrepancies we have begun a study of fluorescent lifetime measurements using an approach differing from each of the above studies.

EXPERIMENTAL METHOD

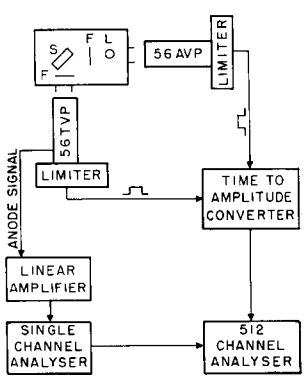
The *Porphyridium cruentum* were cultured in 300-ml erlenmeyer flasks under sterile conditions in an inorganic medium described by BRODY AND EMERSON[7]. The *Chorella pyrenoidosa* were cultured in 250-ml flasks containing the following salts (in g/l): $Ca(NO_3)_2 \cdot 4H_2O$, 0.96; KCl, 0.17; KH_2PO_4, 0.17; $FeCl_3 \cdot 6H_2O$, 0.011; $MgSO_4 \cdot 7H_2O$, 0.34. Air containing 5% CO_2 was bubbled through the flasks which were illuminated by four 20 W white fluorescent rods. The Chlorella were harvested after 3–4 days and the Porphyridium after 1–2 weeks. The fluorescence measure-

ments were made on algae at a concentration such that an absorbance of 0.7 was measured at the chlorophyll *a* absorption peak.

The decay of fluorescence was measured using a method that minimized the effects of electron transit time spread in photomultiplier tubes[8]. This method measured the distribution of time intervals between the absorption of light and the emission of single fluorescent photons. In the case where no more than one fluorescence photon could be detected from a single excitation event the distribution of time intervals between excitation and emission is a direct manifestation of the fluorescence decay curve. Since these time intervals can be measured using the leading edges of phototube pulses, the dispersion introduced by using the entire phototube output is significantly reduced. For example, the time dispersion using leading edges of single photon pulses is approx. 0.4 nsec while the full width at half height of such pulses is approx. 3 to 4 nsec on well designed fast phototubes.

Fig. 1 is a schematic diagram of the experimental system. The electronic components are modified versions of those designed by SIMMS[9] for use with significantly larger signals. A short light pulse was provided by an air spark gap (approx. 0.2 mm) made with 2 tungsten needles. The time spectrum of this light source is shown in Fig. 2. It can be seen that the lamp output has both a fast component decaying with a mean lifetime of 1.1 nsec and a slower component with a lifetime of about 4.5 nsec. The average light intensity delivered to the photosynthetic species was in all cases less than 1 erg/sec per cm^2.

A fast, high-gain Amperex 56 AVP photomultiplier tube (S-11 response) viewed the lamp directly and provided a zero-time signal at the beginning of the flash-lamp emission. The samples were irradiated with blue light through Corning 5-58 and 5-59 filters. The fluorescence was detected using an Amperex 56 TVP (S-20 response) phototube through a Corning 2-60 sharp cut-off red filter. Neutral density filters were



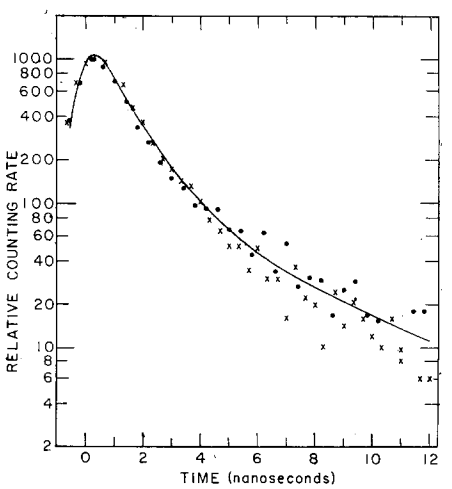


Fig. 1. Schematic diagram of the electronic apparatus used in the measurement of fluorescent lifetimes. (F = filter, L = flash lamp, S = sample cell.)

Fig. 2. Time spectrum of the flash lamp. Data from 2 independent runs are indicated by ● and ×. The solid line represents the fit to the data using as the lamp spectrum the function $I(t) = 10e^{-t/1.1} + e^{-t/4.5}$ and taking into account the electronic response of the apparatus.

also used when necessary to reduce the light output such that a fluorescent photon would be observed in no more than 5 % of the excitation events. In a typical experiment this value was usually 1 %. This low value minimizes to the same degree the distortion of the decay time spectrum by 2 photon events.

The anode signal from each tube was shaped by a limiter circuit into a 25 nsec long, 1 V square pulse. These signals were then fed to a time-to-amplitude converter, the output of which was directly proportional to the time overlap of the two limiter signals. The output of the time-to-amplitude converter was stored in a Nuclear Data 512 Channel Analyser which was gated by single photons of a prescribed energy. This gating assured that all limiter output pulses used for time determinations were of identical amplitude. The system was calibrated and its linearity checked by inserting various known delays after one of the limiters. Fig. 3 shows that the system was linear over a range of 20 nsec.

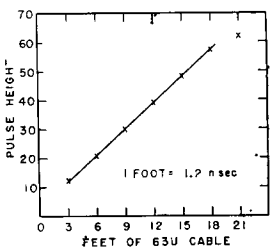


Fig. 3. The calibration of the time-to-amplitude converter over the time range used in this experiment.

The time resolution of the electronic system, measured using large light signals in each phototube, was approx. 0.2 nsec. Unfortunately, we were unable to obtain a light source with a decay time sufficiently short to measure directly the time resolution of the apparatus using single photon pulses in one phototube. In lieu of such short light sources, however, the single photon response of the equipment was determined by calculating the electronic response from the observed response to light from a fast plastic scintillator and from the lamp flash. In this analysis both the lamp and the scintillator were assumed to have a rise time less than 0.2 nsec and an exponential decay. If this assumption concerning the rise times proves invalid, we will have attributed dispersion to the phototube which more properly should have been assigned to the lamp or scintillator. This incorrect attribution, however, will little affect the analysis of the light emitted from fluorescent species.

The determination of the single photon response can be done in a manner similar to that used by KOECHLIN[10]. Let $G(t)$ be the single electron distribution function, *i.e.*, the response of the phototube and electronics to a series of single photons arriving at the photocathode at a precisely determined time. The output of the system to a lamp with a delta function output would simply be $G(t)$; the output to a lamp with a finite emission time would be proportional to $\int_{-\infty}^{\infty} G(t-t')F(t')\mathrm{d}t'$, where $F(t')$ is the photon emission probability of the light source. Let $Y(t)$ represent the observed response to a light source which decreases exponentially in the early portions of its decay.

$$Y(t) = \int_{-\infty}^{\infty} \lambda F(t')G(t-t')\mathrm{d}t' = \int_{-\infty}^{\infty} \lambda \mathrm{e}^{-\lambda t'} G(t-t')\mathrm{d}t'$$

Integrating by parts and substituting τ for $1/\lambda$ we obtain:

$$Y(t) = G(t) - \tau \frac{dY}{dt}$$

from which $G(t)$ can be obtained directly. Using the above equation $G(t)$ was found to be reasonably represented by a Gaussian, $e^{-(t-t_0)^2/2\sigma^2}$ where $\sigma = 0.4 \pm 0.1$ nsec (see Fig. 4). Because of the agreement between $G(t)$ determined from the lamp data and from the plastic scintillator, it appears that the assumption that the lamp had an extremely fast rise time was justified.

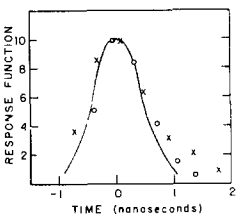


Fig. 4. The experimentally determined phototube response to single photons. The solid line represents the Gaussian $e^{-(t-t_0)^2/2(0.4)^2}$. Flash lamp data are indicated by crosses and data from a plastic scintillator by circles.

Using this response function the best fit to the lamp output was found to be $I(t) = 10\,e^{-t/1.1} + e^{-t/4.5}$ where t is measured in nsec. The accuracy on each decay constant is estimated to be 10 % and on the normalization of the long lived component, 30 %. It should be noted that although the long component of the lamp emission is not manifest until the lamp intensity has fallen to less than 10 % of its initial value, approx. 30 % of the lamp output is in the long lived component.

RESULTS

Fig. 5 shows data obtained for the *Chlorella pyrenoidosa* along with the previously described lamp spectrum. Since the Chlorella fluorescence had no observable lifetime longer than the lamp, it was not possible to obtain the Chlorella decay constant directly. However, the lifetime could be determined by the delay in time of fluorescence light relative to that of the exciting lamp. Representing the lamp output by

$$I(t) = 10\,e^{-t/1.1} + e^{-t/4.5}$$

the expected Chlorella fluorescence for a single component decay having a mean life, τ_F, is calculated to be

$$F(t) = \frac{11}{1.1 - \tau_F} e^{-t/1.1} + \frac{4.5}{4.5 - \tau_F} e^{-t/4.5} - \left[\frac{11}{1.1 - \tau_F} + \frac{4.5}{4.5 - \tau_F}\right] e^{-t/\tau_F}$$

where t is again in nsec. Modified by the phototube response function this gives the solid line in Fig. 5 for which a value of 0.6 nsec was used for τ_F. The data are sufficient for an accuracy of ± 0.2 nsec on the value of τ_F. Because of the close correspondence between the calculated curve and the data at long times, we can say that there is no

long lived component of fluorescence present to an extent such that the integrated output of that component would exceed 10% of the total fluorescence.

As with the lamp, the fluorescence was detected at counting rates which varied by a factor of 5. No change was observed in the decay spectrum. Moreover, at these same counting rates, the fluorescence of uranine was measured and its lifetime found to be 4.2 ± 0.2 nsec (see Fig. 5). These two checks indicated that the measured lifetimes were not affected by distortions introduced from events in which two photons entered the counter from one light flash.

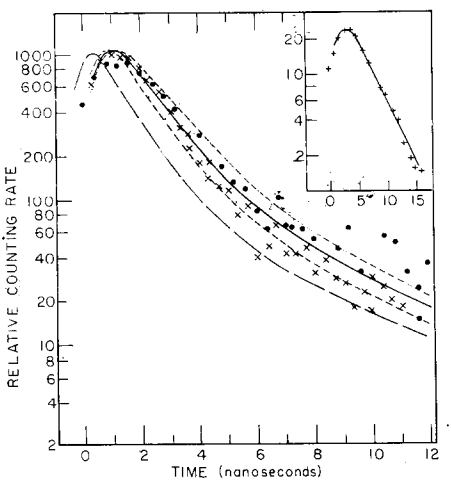


Fig. 5. The time spectrum of Chorella fluorescence. Two independent runs are indicated separately and correspond to the lamp runs indicated by ● and × in Fig. 2. The solid line represents the fluorescence calculated using a lifetime $\tau_F = 0.6$ nsec, the short dashed lines lifetimes of 0.4 and 0.8 nsec. The average lamp excitation spectrum is indicated by the long dashed line. Also shown in the top of the diagram are data obtained with uranine showing a lifetime of 4.2 ± 0.2 nsec.

We also searched for a longer lived fluorescence component using the full phototube output and a sampling oscilloscope. To improve the intensity resolution, the sampling oscilloscope output was stored and integrated in a multi-channel analyser. We did not detect any long lived component. However, at the high voltages necessary to measure the fluorescence, we found distortions introduced into the spectra from secondary electron emission by the phototube dynodes.

We also measured the fluorescence lifetime of *Porphyridium cruentum*. Data identical to that shown for Chlorella were obtained.

DISCUSSION

Our value of 0.6 ± 0.2 nsec for Chlorella and Porphyridium lifetimes agrees within experimental error with previous phase fluorimetry measurements[2,4,6]. However, it is shorter than previous flash measurements. This may be due to our use of lower intensities of light (no intensity data of previous flash work were available) or to the improved resolution possible from the timing measurements used in this work.

One of the problems of fluorescence measurements has been the lack of agreement between observed lifetimes and lifetimes calculated from ratios of fluorescent

yields. Recent measurements of MURTY, CEDERSTRAND AND RABINOWITCH[11] gave a value of 5.9 for the fluorescence yield of chlorophyll *a* in ether relative to that of Chlorella at an incident light intensity of 600 erg/sec per cm^2. Using a value of 0.34 (see ref. 3) for the fluorescent yield of chlorophyll *a* in ether, the above ratio suggests a fluorescence lifetime of 0.9 nsec for Chlorella. This value is in reasonable agreement with our measured value for τ of Chlorella considering the different light intensities used and possible variation of environmental conditions for the Chlorella. It does not agree well with the two component τ data of MURTY AND RABINOWITCH[5] in which greater than 50 % of the fluorescence appeared to have a lifetime of 5.6 nsec.

In conclusion, it is felt that the data presented here indicate only a single Chlorella or Porphyridium lifetime. No lifetime greater than 1 nsec with a relative yield greater than 10 % of the total is present. We cannot, however, rule out the existence of 2 fluorescent species having lifetimes differing by 0.2 or 0.3 nsec. Hence, these results cannot be used to rule out the existence of 2 fluorescent species or 2 types of photosynthetic units.

NOTE ADDED IN PROOF (Received September 20th, 1967)

GOVINDJEE (private communication), repeating the work of MURTY *et al.*[11], reports that he has been unable to obtain fluorescent yields in Chlorella greater than 3 %. This gives an estimated lifetime closer to our measured value and that determined by phase fluorimetry.

REFERENCES

1 S. S. BRODY AND E. RABINOWITCH, *Science*, 125 (1957) 555.
2 O. DIMITRIEVSKY, V. ERMOLAEV AND A. TERENIN, *Dokl. Akad. Nauk S.S.S.R.*, 114 (1957) 75.
3 G. TOMITA AND E. RABINOWITCH, *Biophys. J.*, 2 (1962) 483.
4 W. L. BUTLER AND K. H. NORRIS, *Biochim. Biophys. Acta*, 66 (1963) 72.
5 N. R. MURTY AND E. RABINOWITCH, *Biophys. J.*, 5 (1965) 655.
6 A. MULLER AND R. LUMRY, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1479.
7 M. BRODY AND R. EMERSON, *Am. J. Botany*, 46 (1959) 433.
8 Y. KOECHLIN, *Compt. Rend.*, 252 (1961) 391.
9 P. C. SIMMS, *Rev. Sci. Instr.*, 32 (1961) 894.
10 Y. KOECHLIN, *Trans. N.Y. Acad. Sci., Ser. II*, 27 (1964) 227.
11 N. R. MURTY, C. N. CEDERSTRAND AND E. RABINOWITCH, *Photochem. Phytobiol.*, 4 (1965) 917.