FLUORESCENCE DECAY STUDIES OF CHLOROPHYLL *A IN VIVO*

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ABSTRACT The study of the fluorescence of chlorophyll *a* offers a useful approach toward better understanding of the primary act of photosynthesis. This paper describes new measurements of the decay of chlorophyll *a* fluorescence *in vivo*, made with a considerably improved oscilloscopic-display technique. The main result is the identification of two decay periods both of the order of a few nanoseconds. Possible interpretations of this phenomenon are discussed.

INTRODUCTION

The fluorescence lifetime of chlorophyll *a in vivo* and *in vitro* has been determined in our laboratory first by Brody (1) and later by Tomita (2), using a hydrogen flash lamp for excitation. Their determinations had a serious drawback in that the time constant of the lamp and the detecting system was longer than the decay time of fluorescence. Because of this, the exact shape of the light pulse could not be determined. Assuming that the shape of the light flash was a delta function of time, they treated the data by the method of moments (1) and by the method of "convolutions of the first kind" (2) to calculate the decay time of the fluorescing species. In both procedures, a simple exponential decay had to be postulated. In the present determinations, we were able to considerably reduce the time constant of the lamp and the detecting system so that it became shorter than the decay time of fluorescence. We are now also able to observe the true shape of the light flash.

Terenin, Ermolaev, and Dmitrievsky (3) also measured the decay time of fluorescence of chlorophyll and other pigments with a phase fluorometer postulating an exponential decay.

Since the experiments described in the present paper were made, Butler and Norris (4) measured the decay time of the chlorophyll *a* fluorescence in a bean leaf, using a phase fluorometer, again a simple exponential decay had to be postulated.

EXPERIMENTAL

Fluorescence decay time measurements were made with an instrument similar in principle to that used by Brody (1) and Tomita (2). Light flash from a hydrogen

lamp, pulsed at a repetition frequency of 2 kc was used for excitation. An RCA 7102 photomultiplier tube, with the fastest response was selected from among half a dozen such tubes. The illuminated area of the photocathode was reduced to less than 2 mm in diameter to minimize the spread of electron transit times. The output of the DC operated photomultiplier was displayed on an EG&G traveling wave oscilloscope. Details of the instrument will be published elsewhere.[1]

The response of the lamp and the detecting equipment was determined by photographing the pulse displayed on the cathode ray tube screen. The light pulse had a rise time of 0.6 nanoseconds and a fall time of 0.8 nanoseconds. The width of the pulse measured at 50 per cent amplitude was 6.3 nanoseconds. Fig. 1 is an actual

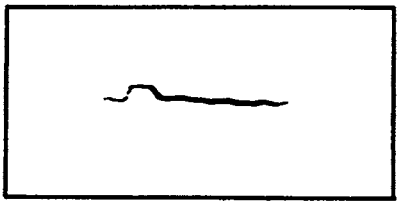

FIGURE 1 Light pulse as viewed on the traveling wave oscilloscope screen. Sweep speed position 5; 20 kv.

photograph of the light pulse and Fig. 2 is a semilogarithmic plot of the same photograph. An algal suspension placed in a cuvette of 1 cm path length was excited by the light flash through a Corning C.S. 4-76 blue filter. For the detection of the chlorophyll *a* fluorescence a sharp cutoff red filter (Corning C.S. 2-64) was used. Fluorescence decay measurements were made either with the light source, the blue

[1] Under pulsed excitation, when a square wave voltage is applied across the electrodes of the gas in the flash lamp, the current flowing through the electrode gap should also be similar in shape. However, the faithfulness with which this can be observed depends upon the response characteristics of the detecting system. In the present instrumentation, we have been able to reduce the time constant of the detecting system to less than one nanosecond; and that is the reason, we believe, we are able to observe the true shape of the light pulse—rectangular in shape—similar to the voltage impressed upon the electrodes. When the intensity of the light flash was attenuated with neutral density filters, no change in its shape was observed. The signal observed on the oscilloscope had an amplitude of less than 0.5v, well within the limits of the measuring range of the oscilloscope. This eliminated the possibility of overloading either the photomultiplier or the oscilloscope amplifier. The light pulse was observed not only on an EG&G traveling wave oscilloscope, Edgerton Germeshausen and Grier, Inc., Boston, Massachusetts, with which most of the measurements reported here were made, but with a Tektronix 519 oscilloscope, kindly loaned to us by the Betatron Laboratory, Urbana, Illinois. In order to convince ourselves that the complex decay curve of the fluorescence of chlorophyll *a in vivo*, reported in this paper, is not due to an experimental artifact, we examined the fluorescence decay of several compounds, such as *p*-terphenyl, 2,5-diphenyloxazole, and fluorescein, whose decay times were determined under similar experimental conditions. A fair agreement was observed with the published data on these compounds. These and other details will be described in a forthcoming publication.

filter, the red filter, and the detector being in a straight line, or with the source and the detector on the same side of the sample, so as to record the fluorescence from the front surface, and minimize self-absorption. The decay times of chlorophyll *a* in *Chlorella pyrenoidosa* (green alga), *Porphyridium cruentum* (red alga), *Anacystis nidulans, Plectonema boryanum,* and *Phormidium luridum* (blue-green algae), were measured while the cells were in their respective growth media. The suspensions had optical densities between 0.2 and 0.4 in the maximum of the red absorp-

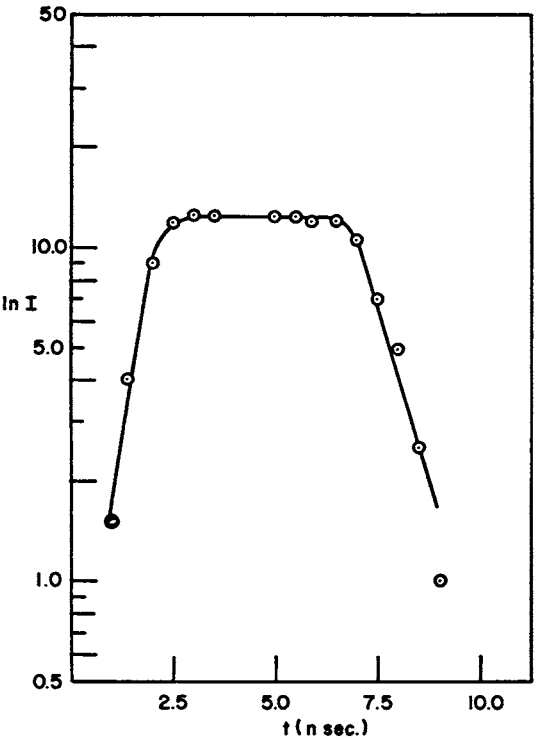

FIGURE 2 A semilogarithmic plot of the photograph shown in Fig. 1.

tion band. Photographs of the light pulses were evaluated on a microscope stage, fitted with 10 turn helipot dials with an accuracy of 5 per cent at low amplitudes of the signal and as low as 1 per cent at high amplitudes.

RESULTS

All the algae examined showed complicated decay curves. Within the limitations of the present technique these curves could be interpreted as superpositions of two exponential curves. The curves in Fig. 3 illustrate this phenomenon for *Chlorella,* (curve 1) and *Phormidium* (curve 2). Table I gives the calculated decay times for all species investigated. τ is the initial decay time, τ_2, the decay time of the "long-

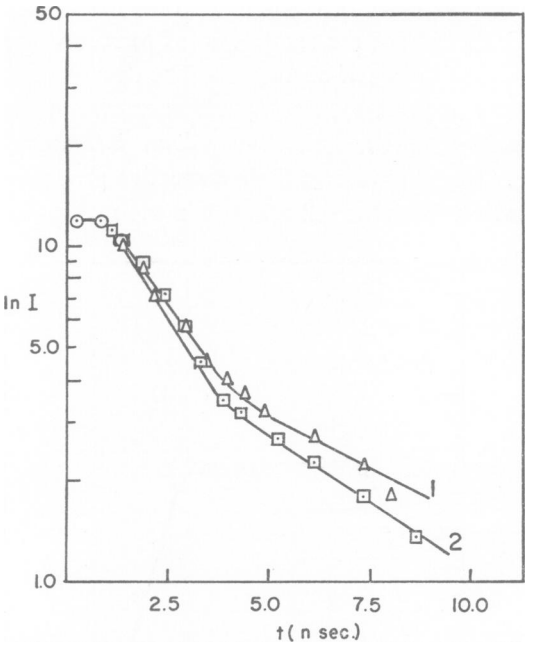

FIGURE 3 A semilogarithmic plot of the fluorescence of (1) *Chlorella* and (2) *Phormidium*.

TABLE I

FLUORESCENCE DECAY TIME OF CHLOROPHYLL *a* IN ALGAE

Alga	τ	τ_1	τ_2
	nsec.	*nsec.*	*nsec.*
Chlorella	2.7	1.7 [1.7 (2), 1.6 (1)]	5.6
Anacystis	2.3	1.4 [1.2 (1, 2)]	5.4
Porphyridium	2.3	0.95 [1.5 (1, 2)]	4.2
Phormidium	2.3	0.93	5.0
Plectonema	2.2		

Numbers in parentheses refer to references.

lived" component, and τ_1, the initial decay time τ_2 corrected for the long-lived component τ_2. We found for *Chlorella* a τ_1 value of 1.7 nanoseconds, identical with that obtained earlier by Brody and Tomita; but our value of τ_1 for *Porphyridium* (0.95 nanoseconds) is considerably shorter than that calculated by Brody (1) and Tomita (2) (1.5 nanoseconds). This may merely mean that our *Prophyridium* cells were 37 per cent less fluorescent than those of Brody (1) and Tomita (2). Butler and Norris's value of 0.6 nanoseconds time of chlorophyll *a* fluorescence in the bean leaf also suggests a relatively low yield of fluorescence (4).

DISCUSSION

One may suggest that the complex decay curves could be the result of self-absorption and secondary emission. To evaluate this effect both "front wall" emission and "back wall" emission were compared and no significant difference was found, despite the much longer path of the primary fluorescence in the second case. Barring experimental artifacts, the complex decay curves suggest the participation of more than one fluorescing species, or the existence of two mechanisms by which one and the same species may be brought to fluoresce.

The average concentration of chlorophyll *a* in the pigment containing grana *in vivo* is of the order of 0.01 to 0.1 M. In solution at such high concentrations of chlorophyll *a* self-quenching of fluorescence occurs. Concentration quenching can be due (*a*) to non-fluorescent dimers, formed by two non-excited pigment molecules (*b*) to exciting energy dissipation by encounters between excited and non-excited pigment molecules and (*c*) to the formation of "eximers" (5)—dimers formed by resonance interaction of one excited and one non-excited molecule. Of these three quenching mechanisms, the first reduces the yield of fluorescence, but does not affect its lifetime, the second reduces both yield and lifetime, while the third can have either the same effect as the second (if the eximer dissociates into two ground state monomers); or it can fluoresce with a different lifetime either emanating from the eximer itself, or from the excited monomer formed by dissociation of the eximer. Pertinent to this discussion are the results obtained by Butler and Norris (4). They obtained a value of 6.3 nanoseconds for the lifetime of chlorophyll *a* fluorescence in ethanol solutions which was shortened as the concentration was increased from 10^{-5} to 8×10^{-3} M. They found that temperature had no noticeable effect on the fluorescence from diluted solutions of chlorophyll *a*. However, concentrated solutions when cooled to −196° C. exhibited a new emission component beyond 720 mμ having a value of 4.4 nanoseconds. It should be pointed out that Brody (6) originally obtained evidence for the existence of such long wavelength fluorescing species from the fluorescence spectra of concentrated chlorophyll *a in vitro*. A bean leaf cooled to −196°C. also gave rise to a long wavelength fluorescing band with a τ of 3.1 nanoseconds. Their report does not indicate the effect of temperature on the short wavelength component of fluorescence either in concentrated solutions or in the bean leaf. From these results one may infer that mechanism (*c*) operates perhaps in addition to mechanism (*a*). Had mechanism (*b*) been the only operative mechanism a shortening of τ might have been explained but not the long wavelength fluorescing species.

Brody and Brody (6, 8) observed a long wavelength fluorescing species in chloroplast preparations when cooled to −196°C. From the rather insufficient data they attribute the emission as to an aggregate of chlorophyll rather than due to an eximer fluorescence. They suggested that in view of the "long lifetime" this species—this value agrees neither with that obtained by Butler and Norris (4)

nor with our value for the second component—should be attributed to triplet emission. It is interesting to note, that in the same report they remarked that the τ_0 (the natural lifetime) as evaluated from the absorption spectra corresponded to the singlet transition and so the possibility of an n-π transition may be considered.

Evidence for the long wavelength emission as derived from fluorescence excitation spectra has been a subject of controversy. Tollin, Fujimori, and Calvin (9) as well as French and Young (10) believe that this could be due to the effect of reabsorption while Brody and Brody (7, 8) believe it to be real. Duysens (12) observed a fluorescence band at 720 mμ in *Porphyridium* at room temperature. He attributed it to an "unknown" pigment, presumably chlorophyll *d*. However, chlorophyll *d* is shown not to exist in this alga (8). This band corresponds to the 710 mμ emission component reported by Butler and Brody and Brody. The reasons for the occurrence of a long wavelength emission especially at low temperature will be examined in a later publication.

The two different decay times may also be interpreted in terms of the two pigment system hypothesis. The two components correspond either to different kinds of chlorophyll or to the same chlorophyll in different proportions. The fast emission component may be associated with the pigment system having a higher concentration of chlorophyll *a*. In this excitation energy can be rapidly transferred to the "reaction site." The slow emission component may be associated with the pigment system containing less of chlorophyll *a*, in which the transfer rate for the excitation energy will be slow compared to the fast emission component system.

In conclusion, it should be pointed out that the fluorescence studies of chlorophyll *a* either in solution or *in vivo* suggest that there is more than one species fluorescing. Brody and Brody (7) and Govindjee and Yang (11) using a matrix method for the analysis of fluorescence and of the fluorescence excitation spectra, of chlorophyll *a in vivo* find evidence for two or more species fluorescing at room temperature and perhaps more at lower temperatures. Their speculation as to the existence of several fluorescing species is consistent with the observation of several species present in the decay time curves for the chlorophyll *a* fluorescence *in vivo*.

This paper was presented at the Eighth Annual Biophysical Society Meeting; Chicago, Illinois, February 1964.

This work is supported by grants from the National Science Foundation and by the Office of Naval Research Equipment loan contract.

Received for publication, August 10, 1964.

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