MEASUREMENT OF THE FLUORESCENCE LIFETIME OF CHLOROPHYLL *a* IN VIVO

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ABSTRACT New measurements have been made of fluorescence lifetime (τ) of chlorophyll *a* in the algae *Chlorella pyrenoidosa*, *Porphyridium cruentum*, *Anacystis nidulans*, and in spinach chloroplast. τ-values of 0.6 and 0.7 nsec were obtained with green plants. *Anacystis* and *Porphyridium* gave a τ of 0.5 nsec. The previously described two stage decay of fluorescence in vivo in these organisms could not be confirmed. This observation could have been caused by a second wave of light emission from the exciting hydrogen lamp (not detected in earlier work). The lifetimes found in this study (calculated, as before, by the method of convolution integrals) were close to those found by other observers for "low" excitation intensities; the value first reported from this laboratory (1.0–1.7 nsec) may have corresponded to "high" excitation intensity.

INTRODUCTION

In recent years, several studies of average fluorescence decay time, τ, of chlorophyll *a* in vivo have been made. In our laboratory, first Brody and Rabinowitch (1) and then Tomita and Rabinowitch (2) used a flash technique to determine the fluorescence decay curves of chlorophyll *a* in vitro and in vivo. The exponential decay constants, reported by them for the green alga, *Chlorella*, the blue green alga, *Anacystis*, and the red alga, *Porphyridium*, ranged between 1.2 and 1.7 nsec. Murty and Rabinowitch (3) using the same method with an improved oscilloscopic display technique reported the existence, in the same algae, of two superimposed exponential fluorescence decays—one was observed between 0.95 and 1.7 nsec, the other, between 4.3 and 5.6 nsec. Nicholson and Fortoul (4) found from time distribution of photons emitted after flash excitation, a much shorter decay period, $\tau = 0.6$ nsec, for chlorophyll *a* in *Chlorella* and in *Porphyridium*.

Using a phase fluorometer—a method first applied by Dimitrievsky et al. (5)—Butler and Norris (6) also found (postulating a simple exponential decay) a τ-value of 0.6 nsec for the fluorescence lifetime of chlorophyll *a* in a bean leaf. Muller and Lumry (7), using a phase fluorometer, concluded that the lifetime of chlorophyll *a*

fluorescence in vivo depends upon the intensity of excitation. The lower limit of the observed lifetime, τ_{min} ($I \rightarrow 0$) for *Chlorella* was as short as 0.35 nsec; the upper limit, τ_{max} ($I \rightarrow \infty$), as high as 1.92 nsec (8). Recently, Merkelo et al.,[1] using a mode locked He-Ne gas laser ($I \approx 10^5$ erg cm^{-2} sec^{-1}) as excitation source, found a τ-value of 1.4 nsec for *Chlorella* with both "flash" and "phase" method.

This paper presents a reinvestigation of the lifetime of fluorescence of algae by the flash method, using an instrument with a faster response than those used earlier in this laboratory (1–3), and which permits use of a very low intensity of exciting light.

EXPERIMENTAL

The green alga, *Chlorella pyrenoidosa* (Emerson's strain 3), the red alga, *Porphyridium cruentum*, and the blue-green alga, *Anacystis nidulans*, were grown in inorganic media (9). *Chlorella* and *Anacystis* were grown in incandescent light at 22°C and at 25°C, respectively; *Porphyridium* in fluorescent light, at 18°C. A mixture of 5% CO_2 and 95% air was bubbled through the cultures during growth; 4–6 day old cultures were used. The cell suspension was

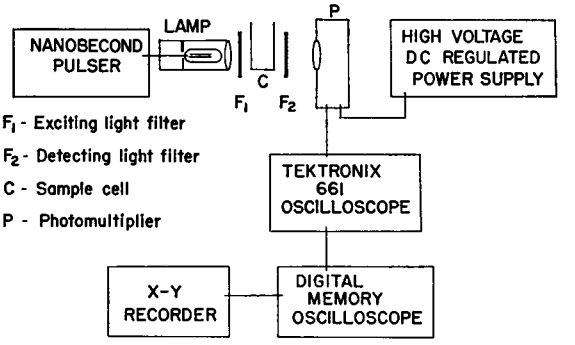

FIGURE 1 Block diagram of the instrument.

diluted with its culture medium to an optical density (in the red absorption maximum) between 0.15 and 0.2 for a 1 cm path length. In separate experiments, synchronous *Chlorella* cultures were grown at 30°C by a method similar to that of Pirson, Lorenzen, and Rupel (10), using a regime of 14 hr light and 10 hr dark; algae were harvested at different times in order to get cells in different physiological stages of development. Spinach chloroplasts were prepared by the procedure of Anderson and Boardman (11).

The instrumentation (Fig. 1) was, in principle, the same as previously used in this laboratory (1–3). A PEK 118 series hydrogen flash lamp (PEK Labs, Inc., Sunnyvale, Calif.) was used as light source. It was operated by means of a Huggins Laboratories (Sunnyvale, Calif.) Kilovolt nanosecond pulse generator (60 cycles/sec). The light intensity was of the order of 10^{-2} erg cm^{-2} sec^{-1}. A broad band filter, CS 4–96 (Corning Glass Works, Corning, N. Y.), was used in front of the lamp to isolate the appropriate spectral region for excitation. A sharp cut-off filter, CS 2–64, was used to free fluorescence from admixture of incident light; an

[1] H. Merkelo, S. Hartman, G. S. Singhal, and T. Mar. 1968. Sub-nanosecond radiative lifetime measurements using mode-locked lasers. Paper presented at the International Congress on Electron Devices, Washington, D.C. in October 1968.

RCA photomultiplier type C70045C (anode pulse rise time, 5×10^{-10} sec) (Radio Corp. of America, New York) was used to measure the fluorescence. The lamp, the sample cell, and the photocathode of the photomultiplier were placed in a straight line, and the distances between them made as short as possible to maximize the fluorescence signal. There was practically no contribution of scattered actinic light. This was checked by making measurements with a "scatter control" sample—a suspension of bleached cells in water. The sample cell had a 1 cm square base. Tektronix (type 661) sampling oscilloscope (Tektronix, Inc., Beaverton, Ore.) was used to observe the fluorescence decay curve. This oscilloscope consisted of 5T1A type timing plug-in unit and a 4S1 Duel Trace sampling unit with a 0.35 nsec rise time. As the fluorescence signal was very weak, the signal-to-noise ratio was quite low. A much better ratio could be achieved by feeding the signal into a digital memory oscilloscope (model NS 514, from Northern Scientific Inc., Middleton, Wisc.). A satisfactory clean signal was obtained by averaging the signal on the digital memory oscilloscope for half an hour. Averaging of the fluorescence signal was started after 5 min of illumination to avoid fluorescence transients. The fluorescence decay curve was recorded on a X–Y recorder (Moseley 2D-2; F. L. Moseley Co., Pasadena, Calif.).

RESULTS AND DISCUSSION

When the instrument response time (1 nsec in our case) is comparable to the fluorescence lifetime of the system, the observed decay curves are the combined effect of both. True fluorescence lifetime was calculated in previous work (1, 2) by the method of convolution integrals. The observed decay of fluorescence, $F(t)$, is given,

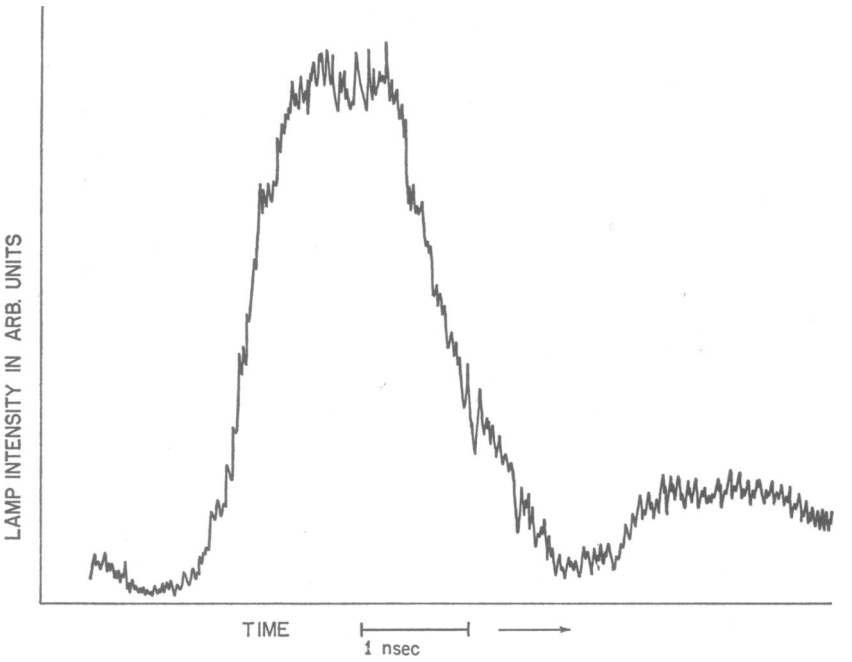

FIGURE 2 Time curve of lamp emission.

in this theory, by

$$F(t) = \int_0^t f(x)I(t - x)\, dx,$$

where $f(x)$ and $I(t-x)$ are, respectively, the true fluorescence decay and the time course of the response of the instrument. Full details of this analysis are given in reference 2. A number of curves were constructed for different assumed lifetimes of fluorescence. The curve which fitted best the observed curve was taken as corresponding to the true decay curve of fluorescence. The time resolution of this procedure was 0.2 nsec.

Fig. 2 shows the decay curves of the lamp emission. The tail of the lamp decay curve is seen to be complex, involving a "second wave" of emission. The intensity of the latter varied from lamp to lamp. A lamp in which this distortion was weakest was selected for measurement. Fig. 3 gives the observed fluorescence decay curve obtained with this lamp for *Chlorella*. A comparison of Figs. 2 and 3 gives no suggestion of a double decay of fluorescence, as reported by Murty and Rabinowitch (3). The measurements were repeated several times with the same result. (Only once during the whole study did we observe a decay curve with an indication of the double decay, but the same shape was observed in this case also with fluorescein, which is not known to show any double decay. The lamp decay curve was again recorded after this finding; and its characteristics were found to have changed in a way which could explain the results obtained with *Chlorella* and fluorescein.)

If the lamp does not have a simple exponential decay, a simple plot of observed

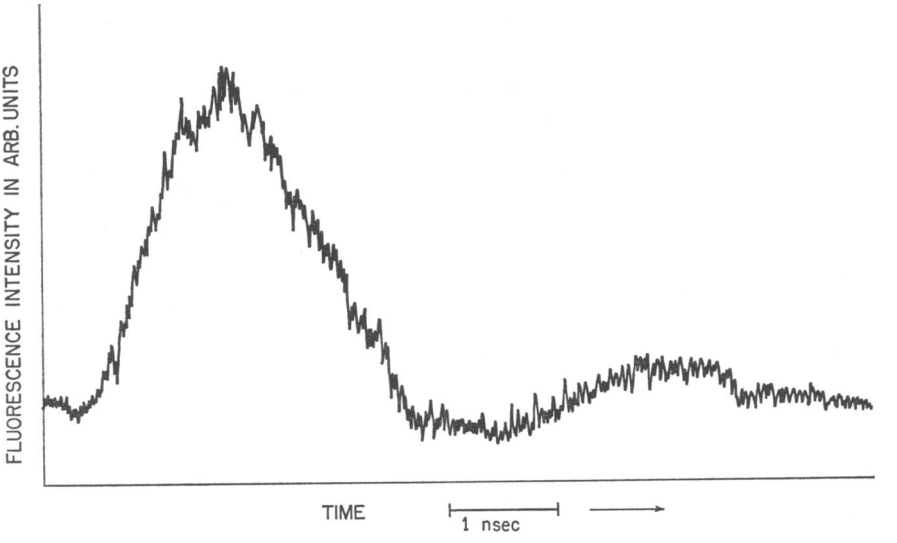

FIGURE 3 Time curve of fluorescence of chlorophyll *a* in *Chlorella*.

fluorescence intensity as a function of time on a semilog scale cannot be expected to give a simple straight line and may suggest a double decay of fluorescence, caused, however, by lamp characteristics rather than by the fluorescing system.

The values of lifetime found in this study for different fluorescing systems are listed in Table I. The τ-value for chlorophyll *a* in ether (4.9 ± 2 nsec) is in fair agreement with those found in our previous investigations; while those given for chlorophyll *a* in vivo are smaller by a factor of 2–3.

The lifetime of synchronous cells was slightly lower (see Table I) than that of normally grown cells. The values now found for *Chlorella* and *Porphyridium* are in good agreement with those reported in references 4 and 7 for low light intensities. Since there is no data available for the light intensities used by earlier investigators in our laboratory, the longer lifetimes reported previously may have been those corresponding to higher light intensities.

The discrepancy between the fluorescence yield, ϕ_{calc} (about 4–4.7%) calculated from the new τ-values, and the directly measured yield, ϕ_{meas} (about 3% in weak light) is reduced by the newer measurement; but the difference is still compatible with the hypothesis that a considerable fraction (perhaps up to one-half) of chlorophyll *a* (such as Chl_I) is practically nonfluorescent.

The difference between the decay periods in weak and in strong light, if it is confirmed, leaves open the possibility that at certain intensity of illumination, a superposition of two decay periods may be observed. However, the longer "limiting" period, suggested by Muller and Lumry (7) still is much shorter than that observed by Murty and Rabinowitch (3) (1.9 vs. about 5 nsec).

While our present data do not confirm the existence of a longer decay period (about 5 nsec), they do not rule out the possibility of the existence of two decay periods of comparable magnitude.

Systematic studies of fluorescence decay as a function of exciting light intensity remain necessary. Precise measurements of the time course of decay under different

TABLE I

LIFETIMES OF CHLOROPHYLL *a* (NEW VALUES, IN WEAK LIGHT)

Sample	τ
	nsec
Chlorella pyrenoidosa (ordinary culture)	0.7 ± .2
Chlorella pyrenoidosa (synchronous culture, 1–6 hr old cells)	0.6 ± .2
Anacystis nidulans	0.5 ± .2
Porphyridium cruentum	0.5 ± .2
Spinach chloroplasts	0.7 ± .2
Chlorophyll *a* in ether	4.9 ± .2

conditions will be needed to decide whether under certain conditions of excitation, two decay periods may be superimposed on each other.

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REFERENCES

1. BRODY, S. S., and E. RABINOWITCH. 1957. *Science.* **125**:555.
2. TOMITA, G., and E. RABINOWITCH. 1962. *Biophys. J.* **2**:483.
3. MURTY, N. R., and E. RABINOWITCH. 1965. *Biophys. J.* **5**:655.
4. NICHOLSON, W. J., and J. I. FORTOUL. 1967. *Biochim. Biophys. Acta.* **143**:577.
5. DIMITRIEVSKY, O., V. ERMOLAEV, and A. TERENIN. 1957. *Dokl. Akad. Nauk. SSSR.* **114**:75.
6. BUTLER, W. L., and K. H. NORRIS. 1963. *Biochim. Biophys. Acta.* **66**:72.
7. MULLER, A., and R. LUMRY. 1965. *Proc. Nat. Acad. Sci. U.S.A.* **54**:1479.
8. MULLER, A., R. LUMRY, and M. S. WALKER. Report No. 28. Light-intensity dependence of the *in vivo* fluorescence lifetime of chlorophyll. Dept. of Chemistry, Univ. of Minnesota, Minneapolis, Minn.
9. GOVINDJEE and E. RABINOWITCH. 1960. *Biophys. J.* **1**:73.
10. PIRSON, A., H. LORENZEN, and H. G. RUPPEL. 1963. *In* Studies on Microalgae and Photosynthetic Bacteria. Edited by Japanese Society of Plant Physiologists. Univ. of Tokyo Press, Tokyo, Japan. 127.
11. ANDERSON, J. M., and N. K. BOARDMAN. 1966. *Biochim. Biophys. Acta.* **112**:430.