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LIFETIME OF THE LONG-WAVELENGTH CHLOROPHYLL FLUORESCENCE

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SUMMARY

The fluorescence lifetime of chlorophyll *in vivo* and *in vitro* was measured with a phase fluorimeter. The lifetime *in vivo* of the long-wavelength emission (730 mμ) which appears at low temperature was 3.1 mμsec. It is concluded that this emission is fluorescence rather than phosphorescence as had been previously reported by BRODY.

INTRODUCTION

Several reports[1-3] have shown that at low temperature chlorophyll, *in vivo* and in concentrated solution, develops a relatively strong emission band near 730 mμ. The fluorescence-emission spectra from a bean leaf at different temperatures are shown in Fig. 1. The 730-mμ-emission band is of interest because it is emitted by relatively small amounts of a pigment complex absorbing near 705 mμ, denoted C-705 (see ref. 3). The C-705 molecules *in vivo* have been implicated as energy sinks and centers of photochemical activity by virtue of the resonance transfer of excitation energy from chlorophyll *a* to C-705 (see ref. 3). BRODY has reported in several papers[4-6],

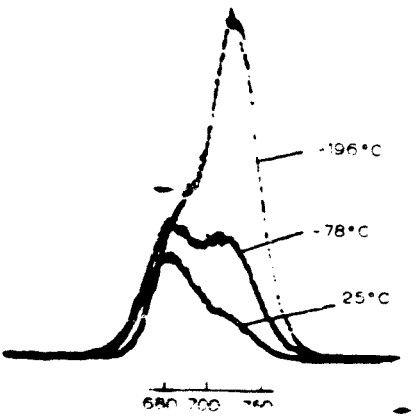

Fig. 1. Fluorescence emission spectra (uncorrected for phototube response, S-20) from a bean leaf at different temperatures

without giving experimental data, that the lifetime of the low-temperature, 730-mμ emission was 10^{-4} sec and has suggested it emanates from a triplet state of a chlorophyll dimer[5]. He has also used this value of the lifetime to calculate some physical parameters of this pigment on the assumption that it is a chlorophyll dimer[6]. The measurements which will be reported in the present paper, however, show that the lifetime of this emission is in the millimicrosecond range which indicates that the emission is from the lowest $\pi - \pi^*$ singlet state of C-705.

METHODS

In the experiments described, fluorescence was excited with blue light and was measured through a cut-off filter which did not transmit the exciting light. The long-wavelength, low-temperature emission was measured with a cut-off filter which transmitted wavelengths longer than 720 mμ (Corning filters 2030 plus 5031). The chlorophyll *a* fluorescence was measured with a filter having a cut-off at 640 mμ (Corning 2030).

The initial attempts to measure the lifetime of the low-temperature emission were made by measuring the decay of emission (displayed on an oscilloscope) following excitation with a repetitive light flash from a condenser discharge. The lifetime of the emission, however, was shorter than 50 mμsec which was the shortest lifetime that could be measured with this equipment. In order to measure shorter lifetimes, a phase fluorimeter was assembled. (Several such instruments have been described in the literature[7-9].) In this measurement, fluorescence is excited by a light beam which is modulated at a radio frequency. The fluorescence emission is modulated at the same frequency but is shifted in phase due to the exponential decay of the fluorescence. The lifetime of fluorescence, τ, is related to the angle of the phase shift, θ, by:

$$\tau = \frac{1}{2\pi f} \tan \theta \qquad (1)$$

where f is the frequency of modulation[6]. In the limit of long lifetimes, the phase shift approaches 90° and the amplitude of modulation goes to zero.

In our phase fluorimeter, shown schematically in Fig. 2, a light beam defined by the slit, S, is modulated in passing through an X-cut quartz crystal, Q, which is vibrating in a radio-frequency field at a natural harmonic frequency of 6.5 megacycles/sec. The standing waves in the quartz crystal act as a diffraction grating which modulates the diffracted light at twice the field frequency, *i.e.*, 13.0 megacycles/sec. The zero order of the diffraction pattern is blocked at B. The diffracted light is split into two beams by the partially silvered beam splitter, M_1. One beam goes directly to the multiplier-type phototube, P_1, which serves as a monitor of the exciting light. The other beam is incident on the sample. The multiplier-type phototube, P_2, (EMI 9558) measures fluorescence or transmission from the sample through the cut-off filter, F_3. Fluorescence is measured when the blue filter, F_1 (Corning 9782), is in the light beam and transmission is measured when F_1 is exchanged for the variable-density filter, F_2. (In the latter case, fluorescence is negligible compared to transmission.) Both phototube circuits are tuned to the modulating frequency. The light signals from P_1 and P_2 are displayed on a high-frequency, dual-beam oscilloscope. A continuously adjustable variable delay line, D, in the phototube circuit for P_1, permits the phase between the

two light signals to be adjusted. The phototube power supplies, V_1 and V_2, are initially adjusted so that the amplitudes of the signals from P_1 and P_2 will be equal when P_2 is measuring fluorescence from the sample. The variable-density filter, F_2, is adjusted so that the amplitude of the signal for transmission is the same as that for fluorescence. These adjustments are made in order that the signals for fluorescence and transmission can be compared with the reference signal from P_1 without altering the gains of the phototubes.

A photograph of the oscilloscope record obtained from a leaf at −196° is shown in Fig. 3. In the upper curve, the variable-delay line was adjusted so that signals from P_1 and P_2 were in phase when P_2 was measuring transmitted light. In the lower record, P_2 was measuring fluorescence causing the phase of the signal to be shifted to

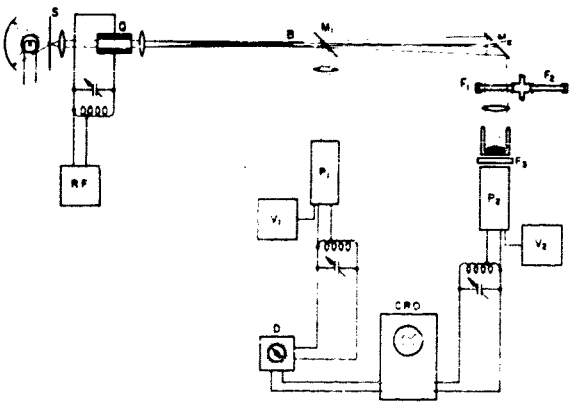

Fig. 2. Schematic diagram of phase fluorimeter. Tungsten or mercury arc lamp illuminates slit, S. Transmitter, RF, with tuned circuit drives quartz crystal, Q. Zero order of diffraction pattern blocked at B. F_1, Corning filter 9782. F_2, variable-density filter (polaroid). F_3, cut-off filter (see text). V_1 and V_2, variable high-voltage power supplies for multiplier phototubes P_1 (RCA 6217) and P_2 (EMI 9558). D, variable-delay line (GR 301-S104). CRO, dual-beam, cathode-ray oscilloscope (HP-150A).

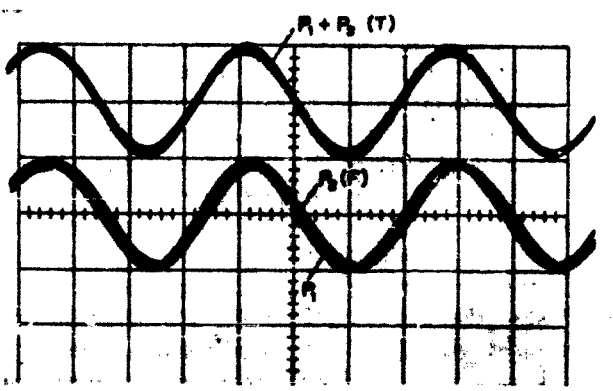

Fig. 3. Modulated photosignals from P_1 and P_2. The sample is a leaf at −196° measured through a 720-mμ cut-off filter. Upper curve: $P_2(T)$ measures transmission. Photosignals superimposed. Lower curves: $P_2(F)$ measures fluorescence. Photosignal from P_2 shifted to right. Time scale: $2 \cdot 10^{-6}$ sec/cm.

longer time. The angle of the phase shift can be estimated directly from the oscillogram. However, a more sensitive measure of the phase shift can be obtained by operating the oscilloscope in a differential mode to measure the difference between the signals from P_1 and P_2. When P_1 and P_2 are equal in amplitude and phase, a null signal is obtained. The variable-delay line is initially adjusted so that the differential signal gives a null when P_2 is measuring transmitted light. The variable-density filter is then exchanged for the blue filter so that P_2 measures fluorescence and the delay line is readjusted to give a null signal. The change of the delay line in the two cases can be converted to the phase angle, $\theta = 2\pi f(\Delta t)$, where Δt is obtained from the calibration of the variable-delay line. The variable-delay line is calibrated against the change of delay obtained by moving the phototube P_1 along an optical bench. The 2-m optical bench permits direct calibration of delay times up to 6.7 mμsec. Knowing θ, the lifetime of the fluorescence can be computed from Eqn. 1.

The measurements of the fluorescence lifetimes are made to a precision of $\pm$ 0.1 mμsec. Measurements *in vivo* were made on leaves freshly picked from 7-day-old, greenhouse-grown bean plants. Measurements *in vitro* were made on chlorophyll *a* which was stored in cold ether solution. Just prior to an experiment, the ether was removed from an aliquot of the chlorophyll solution with a stream of nitrogen and the dry chlorophyll was re-dissolved in absolute ethanol. Oxygen was removed from the ethanolic solution by flushing with nitrogen. Samples of concentrated chlorophyll solutions were made by placing a drop of $8 \cdot 10^{-3}$ M solution between two glass plates. These samples were frozen quickly with liquid nitrogen and the emission spectrum of the sample was measured before and after an experiment to make sure that the long-wavelength emission was present. Measurements were also made on dilute (10^{-5} M) solutions of chlorophyll *a* in ethanol.

RESULTS

Fig. 4 is an oscilloscope record of the direct flash excitation of the long-wavelength emission from a concentrated solution of chlorophyll *a* ($8 \cdot 10^{-3}$ M) at $-196°$. The same results were obtained with a leaf at $-196°$. The fluorescence lifetime could not be determined in this measurement because the speed of response of the oscilloscope was not rapid enough to follow the brief pulse of light. The measurement of the exciting flash (made without the cut-off filter) showed precisely the same time course as Fig. 4. These measurements are important, however, because they established

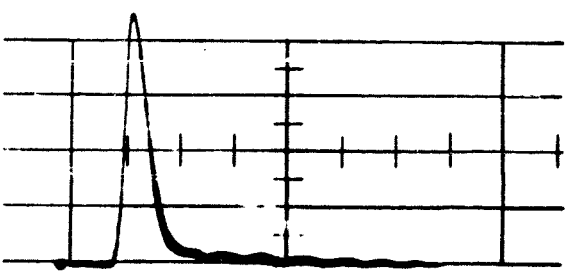

Fig. 4. Oscilloscope record of flash-excited fluorescence (measured through 720-mμ cut-off filter) from $8 \cdot 10^{-3}$ M solution of chlorophyll *a* in ethanol. Repetitive excitation, through a Corning 9782 filter, was from a condenser discharge in air. Time scale: 10^{-7} sec/cm.

that the lifetime of the fluorescence is less than 50 mμsec and that there is no longer-lived emission of detectable intensity. The phase fluorimeter would not detect a long-lived emission if it were present.

The oscilloscope record of the modulated photosignals from the phase fluorimeter are shown in Fig. 3. The fluorescence signal is due to the long-wavelength emission from a bean leaf at −196°. The phase shift shown in Fig. 3 is approx. 15°. With the null method, it was determined that this same phase shift could be produced by moving the phototube P_1 90 ± 3 cm along the optical bench. This corresponds to a Δt of 3.0 ± 0.1 mμsec and a τ of 3.1 ± 0.1 mμsec (for small values of θ, $\tan\theta \simeq \theta$ and $\tau \simeq \Delta t$). Values for the fluorescence lifetimes of a leaf and of chlorophyll *a* solutions are given in Table I. The values obtained with the 720-mμ cut-off filter are for the long-wavelength fluorescence. In dilute solutions of chlorophyll *a* the long-wavelength emission does not appear and the lifetime of the chlorophyll *a* emission is little affected by temperature.

TABLE I

FLUORESCENCE LIFETIMES

Sample	*Measured lifetime* (10⁻⁶ sec)	
	25°	−196°
Leaf	0.7 (640 mμ)*	3.1 (720 mμ)
Chlorophyll *a* in ethanol ($8 \cdot 10^{-3}$ M)	2.5 (640 mμ)	4.4 (720 mμ)
Chlorophyll *a* in ethanol (10^{-5} M)	0.3 (640 mμ)	0.2 (640 mμ)

* Wavelength of the cut-off filter used in the fluorescence measurement is shown in parentheses.

DISCUSSION

The values for the fluorescence lifetime of chlorophyll *a* agree fairly well with previous reports. TERENIN[10], using a phase fluorimeter, obtained values of 0.6 and 1.0 mμsec for the lifetime of chlorophyll *a* fluorescence *in vivo* and BRODY[11], with the direct flash method, obtained values between 1.2 and 1.6 mμsec from different algae. TERENIN[10] reported that the fluorescence lifetime of chlorophyllide *a* in ethanol increased from 5.2 to 7.0 mμsec on cooling to −180°; an effect which we did not find with dilute chlorophyll *a* solutions. It is possible in TERENIN's measurement that the lifetime at room temperature was shortened by quenching processes or that the lifetime at −180° was lengthened by self absorption of fluorescence and re-emission. A longer optical path, due to turbidity in the frozen sample, would increase self absorption. BRODY gives a value of 6.9 mμsec for the fluorescence lifetime of chlorophyll *a* in methanol.

With regards to the lifetime of the low-temperature, long-wavelength emission it is difficult to discuss the discrepancy between our value of 3.1 mμsec and BRODY's value of 10^{-4} sec (see ref. 6) because he has given no experimental data and little experimental detail. BRODY made his measurements with a phosphoroscope which probably could not measure lifetimes much shorter than 10^{-4} sec. (The method he used previously[11] to measure short lifetimes was not employed for the low-temperature

emission.) It is possible that he was measuring a very low-yield phosphorescence or chemi-luminescence which had this order of lifetime, although our direct flash excitation experiments did not detect such an emission.

At $-196°$ the fluorescence yield, ϕ, of C-705 must be quite high (approaching unity) in order to account for the relatively large emission at 730 mμ which results from the rather low absorption at 705 mμ (see ref. 3). The natural lifetime, τ_0, is thus not much greater than the measured lifetime, $\tau_0 = (1/\phi)\tau$. The short natural lifetime indicates that the emission is from a $\pi - \pi^*$ singlet state of C-705.

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