Biochimica et Biophysica Acta, 305 (1973) 281–291

BBA 46537

ISOLATION OF A FAST DECAY IN SUBMILLISECOND *CHLORELLA* LUMINESCENCE

O. LUMPKIN* and Z. HILLEL**

Department of Physics, The City College of the City University of New York, New York, N.Y. 10031 (U.S.A.)

(Received November 24th, 1972)

SUMMARY

Using a simple He–Ne (632.8-nm) laser phosphoroscope steady-state luminescence from *Chlorella pyrenoidosa* was studied from 50 μs to 1.1 ms between 1 ms long exciting flashes. The following results were obtained: (1) prior freezing or ultraviolet irradiation changed the time course of the luminescence to a rapid decay with a half-time of about 110 μs; (2) 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) suppressed the 110-μs luminescence; (3) spectrally, all observed luminescence was, within possible error, identical to fluorescence; (4) no effect on the luminescence intensity from pulsed magnetic fields up to 30 kgauss was observed; (5) the relative fluorescence yield, measured simultaneously with luminescence, was found to be constant.

Our principal conclusions, supported mainly by experiments with DCMU, are: (1) the 110-μs decay is a distinct component of the total steady-state luminescence; (2) prior freezing or ultraviolet irradiation isolates this component of the luminescence by suppressing all other components; (3) the half-time and intensity of this component are temperature independent in the interval 0–22 °C.

INTRODUCTION

There is much current interest in millisecond and submillisecond luminescence*** from chlorophyll in green plants[1–6] because of its suspected relation to the primary photochemical processes of photosynthesis.

Using a laser phosphoroscope, we have studied *Chlorella* luminescence from 50-μs to 1.1–1-ms long flashes. Briefly, we find evidence for a component in the luminescence, whose half-time, about 110 μs, and intensity are temperature independent. Spectrally, this component is identical, within possible error, to fluorescence. The mechanism behind this luminescence is uncertain, but a non-chemical process on the donor side of System II seems probable.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

* Present address: University of California, San Diego, Physics Department, P.O. Box 109, La Jolla, Calif. 92037, U.S.A.

** Graduate Student at The City University.

*** We will use "luminescence" to denote what is variously called "delayed light", "delayed fluorescence", and "delayed luminescence".

MATERIALS AND METHODS

All experiments were performed with *Chlorella pyrenoidosa*, Strain 251, obtained from the Culture Collection of Algae at Indiana University and grown continuously in shaking flasks immersed in a water bath at a temperature of 16–17 °C. Ne arcs provided continuous illumination and the growth medium (Knop's) was aerated with a CO_2–N_2 (5:95, v/v) gas mixture. To study luminescence, a suspension of cells in about 1 ml of growth medium was transferred into a short length of 2 mm quartz tubing closed at one end. Luminescence was then excited and recorded using a laser phosphoroscope (Fig. 1) whose essential element was an aluminum block with slots for two spinning discs mounted on a common shaft. The shaft was flexibly coupled to a 400 cycles/s (24000 rev./min) synchronous motor and the sample was rigidly mounted in a vertical hole located in the aluminum block between the discs. The discs were made of thin plexiglass with large areas painted in black. One disc

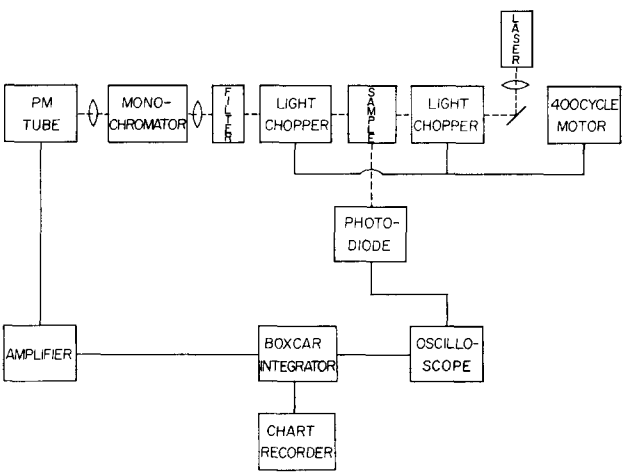


Fig. 1. A schematic diagram of the laser phosphoroscope. The filter indicated is a Corning 2-51 and was simply a precaution against accidental exposure of the photomultiplier tube (PM) to strong laser light. A lens focused the laser on the sample, while other lenses focused the sample on the 2.68-mm entrance slit of the monochromator and also focused the 1.50-mm exit slit of the monochromator on the photocathode of the photomultiplier. Power to drive the 400-cycle motor was obtained by amplifying the output of an audio oscillator.

broke the continuous output beam of a 1.5-mW He–Ne gas laser (632.8 nm) into rectangular flashes 1 ms long which illuminated the algae after passing through a small hole. The second disc shielded the photomultiplier during laser flashes but between flashes passed luminescence exiting through a small window in front of the sample. This window was completely exposed about 50 μs after each laser flash and was covered about 1.1 ms. later. Before the next flash a dark interval about 400 μs long was used to record the zero level of luminescence intensity.

Luminescence was detected by an RCA C31034 photomultiplier tube operated at 1500 V and cooled by cold gas evaporated from a liquid N_2 storage dewar. The photomultiplier signal was amplified and then fed into a Princeton Applied Research

Model CW-1 Boxcar integrator synchronized with the flashing laser light. Synchronization was accomplished by placing a small photodiode adjacent to the sample cell where it could detect the laser flashes.

A high-intensity Bausch and Lomb grating monochromator with a bandpass of 9.6 nm preceded the photomultiplier tube, and to record spectrally unresolved luminescence its grating could be replaced by a mirror. To measure spectra we calibrated the monochromator–photomultiplier with a standard lamp obtained from the Eppley Laboratories.

Fluorescence and fluorescence yield could be measured by illuminating the sample through a transverse hole in the aluminum casing of the phosphoroscope. Also, through another set of transverse holes, cold N_2 evaporated from a storage dewar could be blown over the quartz sample tube for temperature studies. Temperature was measured by means of a copper–constantan thermocouple inserted into the suspension of algae.

Incident laser light intensities were measured with a Spectra Physics 401 C power meter.

RESULTS

Typical results

Fig. 2 shows some typical recordings of the luminescence at room temperature made with our phosphoroscope. Details of the measurements are given in the caption. The lower part of the figure, shows the inhibition of the decay in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

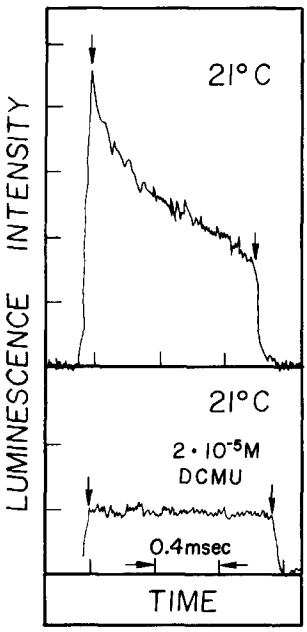
The following remarks apply to all the work reported here: (1) the *Chlorella* suspension was not dark adapted before placing it in the phosphoroscope; (2) typically the decay was recorded starting 3–5 min after turning on the exciting flashes. This decay, we verified, was identical to that recorded starting 10–15 min after turning on the exciting flashes; (3) the actual exciting light intensity may be less than the measured incident powers due to scattering and absorption in the suspension; (4) with only growth medium in the sample cell, no luminescence was detected; and (5) the concentration of algae in the suspension was not measured.

Effect of freezing on the luminescence

To explore the mechanism of the luminescence, we decided first to study luminescence at sub-freezing temperatures. We observed, however, that the luminescence abruptly disappeared as soon as the suspension of algae froze and only reappeared when the suspension melted. Immediately after melting* though, and particularly in the temperature range 0–4 °C, the luminescence exhibits an initial fast decay with a half-time of about 110 μs. Fig. 3 shows a recording of such luminescence and Fig. 4 a semi-log plot of the decay and for comparison data recorded at 21 °C before freezing.

Varying the freezing temperature also has an effect on the luminescence decay, and in Fig. 5 we have plotted the log of the luminescence recorded at 21 °C for

* Tollin *et al.*[7] observed luminescence after single short flashes at 77 °K, but Arnold and Azzi[8] report that no luminescence is observed at 77 °K between repeated flashes. In our experiment luminescence (excited by repeated flashes) always disappeared at the freezing point.



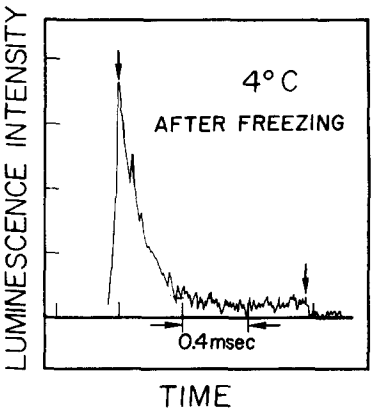


Fig. 2. Upper. Luminescence decay from non-dark-adapted *Chlorella* excited by repeated 1-ms-long, 632.8-nm, 400-mW/cm^2 (incident power) laser flashes spaced 1.5 ms apart. The decay is recorded from about 50 μs (position of first arrow) to about 1.1 ms (position of second arrow) after each flash. The boxcar integrator's time constant was 1 ms and its gate 20 μs wide. The scan of the luminescence began about 3 min after turning on the laser and approx. 24000 light flashes were incident on the algae during the scan. The monochromator grating was replaced by a mirror during this measurement. Lower. Same as above but in the presence of 20 μM of DCMU added to the suspension about 2 h before. The constant intensity of this luminescence is about 1/3 the peak intensity observed without DCMU.

Fig. 3. Luminescence decay recorded at 4 °C immediately after freezing to −55 °C. Other experimental parameters are the same as in Fig. 2.

different samples of algae previously frozen to −9 °C, −50 °C, and −196 °C, respectively. These data show clearly that lower freeze temperatures produce faster overall decays immediately after freezing.

Effect of ultraviolet light

Exposure to ultraviolet light affects the luminescence decay very much like freezing does. This is shown in Fig. 6. Prolonged irradiation suppresses all luminescence, but does not affect the initial decay rate of the altered luminescence as Fig. 7 shows. This suggests that the decay may be caused by a non-chemical mechanism.

Effect of DCMU

Fig. 2 shows that $2 \cdot 10^{-5}$ M DCMU eliminates the luminescence decay. With only $5 \cdot 10^{-6}$ M DCMU, however, a fast initial decay (in the first 200 μs) is still evident at 21 °C and freezing (or irradiating with ultraviolet light) will make this fast decay even more evident. Fig. 8 shows this for a sample frozen to −9 °C. One can also

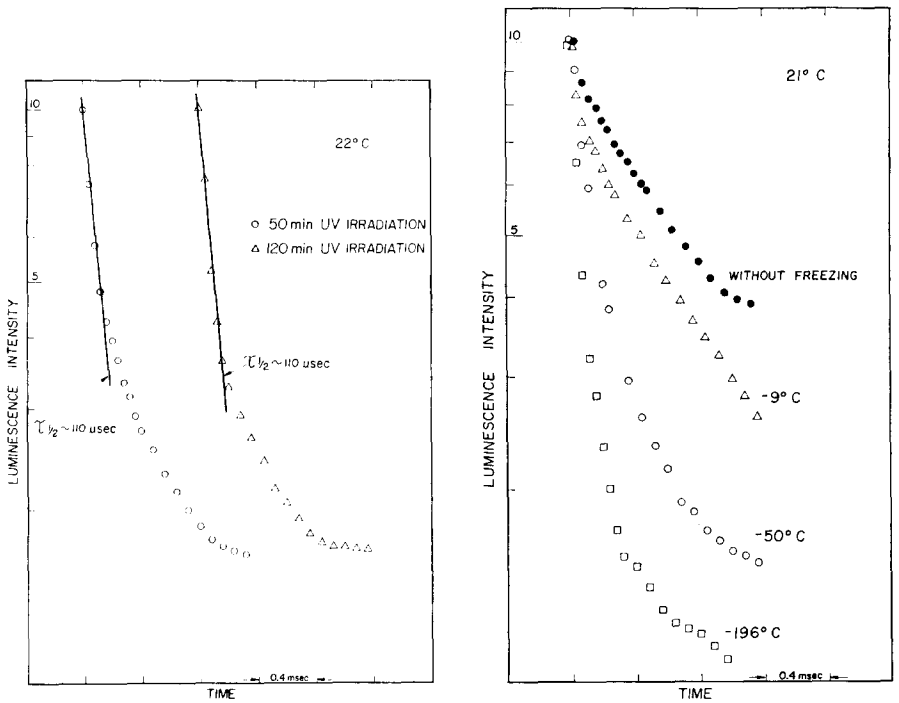


Fig. 4. Log plots of luminescence intensity at 4 °C after freezing to −55 °C (+), and of the decay at 21 °C before freezing (●). Intensities have been normalized to the same initial value.

Fig. 5. Log plots of luminescence intensity at 21 °C immediately after being frozen to −196, −50 and −9 °C. For comparison data recorded at 21 °C, and before freezing is also plotted. Intensities have been normalized to the same initial value. In obtaining these data, we verified that the differences in decay shown were mainly due to the specific freeze temperature and not to different durations of freezing.

determine from data like that at 7 °C in Fig. 8 that with DCMU, like ultraviolet, the half-time is also about 110 μs. One interpretation is that, (1) the fast decaying luminescence represents a distinct component of the total luminescence and the formation of this component is blocked by DCMU, (2) ultraviolet irradiation or prior freezing by suppressing other components in the luminescence, isolates this component. Assuming this interpretation, Fig. 8 shows that the intensity and half-time of the fast decay is the same at 7 and 21 °C. This can be seen by subtracting the luminescence intensity at 1.2 ms (position of second arrow) from the intensity at 50 μs (first arrow). In addition, the intensity at 1.2 ms, being about 3 times larger at 21 °C than at 7 °C, implies that the fast decaying luminescence and the constant luminescence are not in competition for excitation energy and perhaps reflect, therefore, separate sources of energy.

With concentrations of DCMU greater than 10^{-4} M no fast decaying luminescence is detectable after freezing or ultraviolet is used to suppress other luminescence.

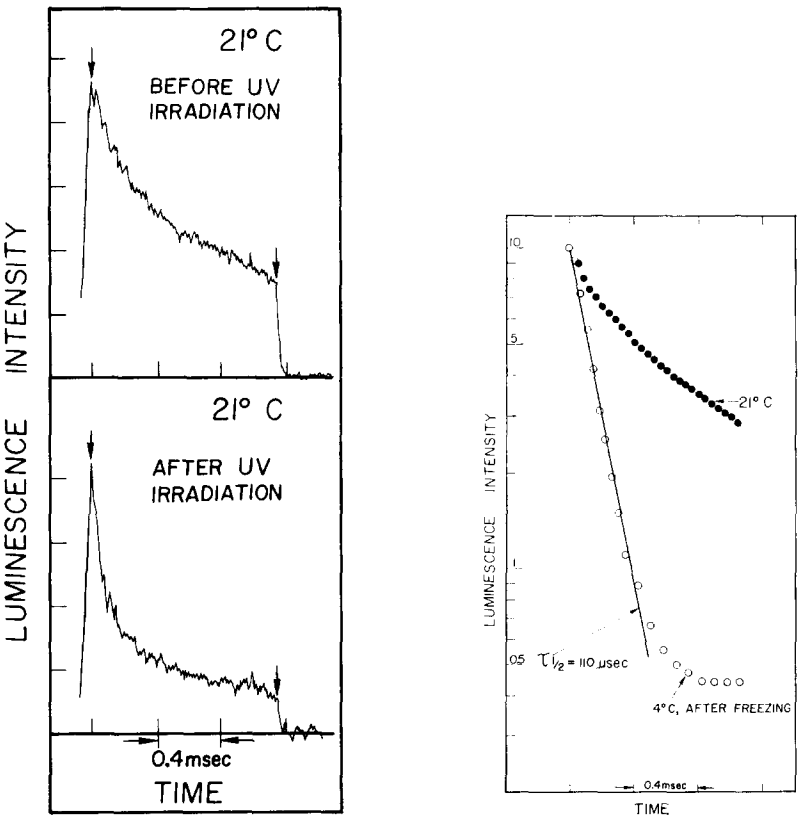


Fig. 6. Upper. Luminescence decay recorded at 21 °C before ultraviolet (UV) irradiation. Other experimental parameters are the same as in Fig. 2. Lower. Luminescence decay recorded immediately after 50 min of irradiation by 20 mW/cm² (incident power), 360 ± 50-nm ultraviolet light from a Spectroline B-100 lamp. Other experimental parameters are the same as in Fig. 2. The peak intensity of the luminescence in this recording is about $^1/_2$ the intensity before irradiation.

Fig. 7. Log of luminescence decay at 22 °C immediately after two different durations of irradiations by 20 mW/cm² (incident power), 360 ± 50 nm ultraviolet. Intensities have been normalized to the same initial value. The peak intensity after 120 min of irradiation is about 1/5 the intensity before irradiation.

Temperature dependence of luminescence

Evidence for more than one luminescence component may also be seen by studying the luminescence decay as the algae are cooled from room temperature towards freezing. A typical set of data is shown in Fig. 9. The fast decay in the first 200 μs is very evident at both 4 and 1.3 °C as is also the decreasing slope at the end of the decay. Overall, we interpret these data as showing that both the decay rate and intensity of some luminescence components are suppressed by low temperatures (as the freezing experiments first suggested) making the fast decaying luminescence more evident.

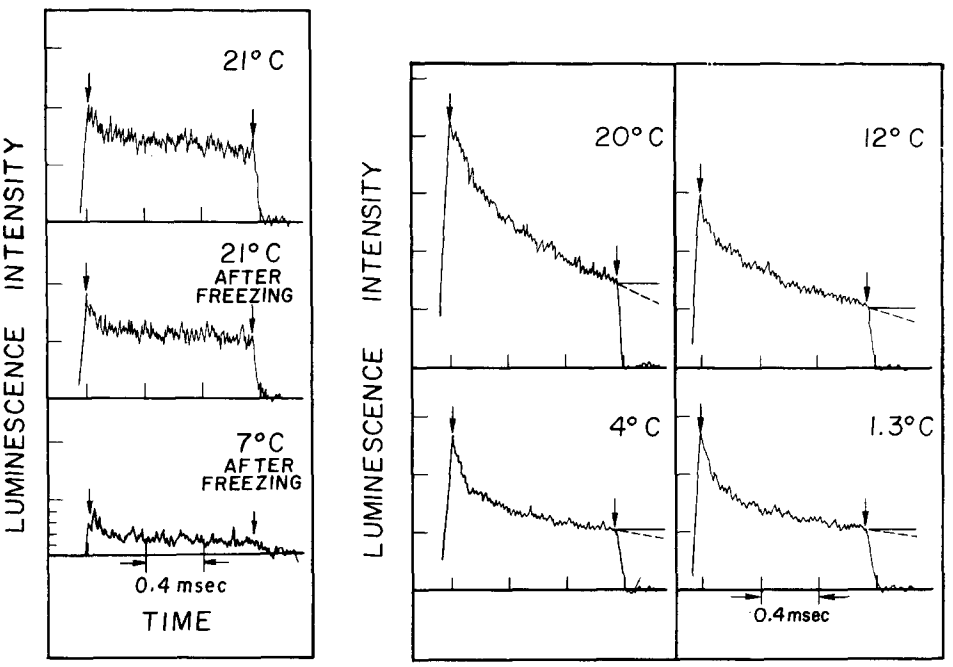


Fig. 8. Upper. Luminescence decay at 21 °C in the presence of $5 \cdot 10^{-6}$ M DCMU added to growth medium 2 h earlier. Other experimental parameters are the same as in Fig. 2. Middle. Same as above except recorded immediately after freezing to −9 °C. Lower. Same as above except recorded at 7 °C immediately after freezing to −9 °C. Correct relative intensities are as given in the figure.

Fig. 9. Luminescence decay recorded at successive temperatures while cooling towards freezing. Correct relative intensities are as given in the figure. Other experimental parameters are the same as in Fig. 2.

Recovery of normal luminescence after freezing and ultraviolet irradiation

Neither the alteration of the luminescence caused by freezing nor by exposure to ultraviolet light is permanent. Cells frozen at −50 °C (see Fig. 3), for instance, exhibit a normal luminescence after about 20 h at 21 °C, while cells exposed 1 h to ultraviolet (see Fig. 7) recover after only 3 h at 21 °C. We have not studied these effects in detail, but the difference in recovery times may indicate different damage sites or may simply indicate that the freeze damage is more extensive. The sensitivity of photosynthetic electron transport to freezing has been suggested by other studies[9], while a deleterious effect of ultraviolet on luminescence (and O_2 evolution) was first reported by Strehler and Arnold[10].

Effect of exciting light intensity

The initial intensity of luminescence was proportional to exciting light intensity from 0.4 mW/cm^2 to 400 mW/cm^2, the lowest and highest intensities available in our experiment. Adding 20 μM DCMU did not alter these results. The linear dependence of the initial intensity on exciting power doesn't necessarily indicate, however, that a single quantum process creates the luminescence. Suppose, for example, that

the luminescence intensity is proportional to the amount of some species, X (ref. 6), created by the exciting light. If X is created by a single quantum process and if its decay during the flash is first order, then

$$\frac{dX}{dt} = AI_0 - kX \tag{1}$$

where I_0 is the exciting light intensity and A and k are constants. In the steady state dX/dt equals zero and the value of X at the beginning of the dark interval is

$$X_0 = \frac{AI_0}{k} \tag{2}$$

The luminescence intensity, being proportional to X, will thus be proportional to I_0. If, on the other hand, X is created by a two quantum process and decays during the flash with second order kinetics, then

$$\frac{dX}{dt} = AI_0^2 - kX^2 \tag{3}$$

and the same argument as above shows that in the steady state the luminescence intensity is again proportional to I_0.

Finally, we note that the lowest exciting intensity practical in this experiment was still too high to show the square law dependence previously reported[2].

Spectrum of the luminescence

We have measured the spectrum of the luminescence and compared it with that of the fluorescence. In Fig. 10 we plot the fluorescence spectrum and the luminescence spectrum measured between 50 and 100 μs at 21–22 °C. The difference between these spectra on the long wavelength side of the peak is generally larger

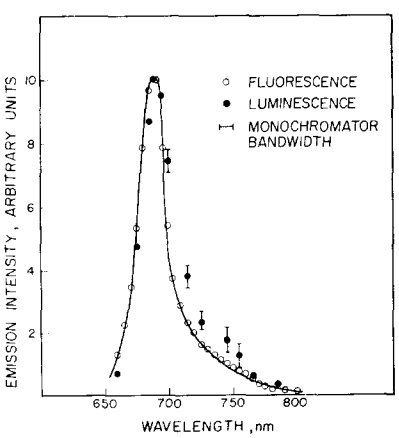


Fig. 10. The spectrum of the fluorescence was measured during illumination by 50 mW/cm^2 (incident power) of 632.8 nm laser light. The luminescence was measured between 50 and 100 μs after repeated 632.8-nm laser flashes of 400 mW/cm^2 (incident power). For both measurements the monochromator bandpass was 9.6 nm.

than the estimated uncertainty of the luminescence measurements but we are not certain that differences in reabsorption are not responsible. We have observed, for example, that increasing the concentration of algae will broaden both the fluorescence and luminescence spectra and thus a generally higher concentration of algae during the luminescence measurements (though we endeavored to avoid this) could account for the difference, as also could the different geometries used to excite luminescence and fluorescence.

We have also compared the wavelength of the peak in the luminescence spectrum in samples with 20 μM DCMU and in samples after exposure to ultraviolet radiation or sub-freezing temperatures. Within ± 10 nm, we found the same peak wavelength as in untreated control samples.

Time dependence of the relative fluorescence yield between flashes

By applying a weak modulated (50 kHz beam of 632.8 nm) light, we could measure, simultaneously with the luminescence, the relative fluorescence yield between flashes. We found a constant time-independent yield. This result was actually anticipated from the experiment of Kok *et al.*[11] who showed that the steady state under repeated closely spaced flashes corresponds to completely reduced primary (Q) and secondary acceptor (A) pools. The fluorescence yield, reflecting the redox state of Q (ref. 12), would then be time independent.

Our result indicates then that the time dependence of the luminescence observed in these experiments is not related to changes in redox state on the acceptor side of System II.

Effect of pulsed magnetic fields

By placing Helmholtz coils around the 2-mm-diameter sample tube, pulsed magnetic fields up to 30 kgauss were applied between exciting light flashes. No effect on the intensity of luminescence was observed. Up to 10 kgauss, intensity changes as small as $\pm 5\%$ could have been detected, but from 10 to 30 kgauss, sample heating reduced our sensitivity to about $\pm 15\%$.

DISCUSSION

The simplest overall interpretation of these experiments, supported primarily by the experiments with DCMU, is that the luminescence decaying with a 110-μs half-time, is a distinct component in the total luminescence. Prior freezing or ultraviolet irradiation isolates this component by temporarily disrupting the mechanism(s) generating other luminescence. The insensitivity of the 110-μs half-time to DCMU, ultraviolet irradiation, and temperature suggests a non-chemical decay mechanism. One possibility is emission from an excited singlet state that is populated by thermal activation from some lower lying metastable state. The decay rate would then reflect the metastable state's life-time. The intensity of the emission, however, would be temperature dependent between 0 and 22 °C, contrary to observation (see results obtained with DCMU) unless the energy gap between the singlet and metastable states were unreasonably small. Another possibility, which can produce a temperature independent intensity is the fusion of two triplets to produce an excited singlet[2]. The triplet life-time, which can be calculated from the 110-μs half-time

would be about 300 μs. This is not an unreasonable chlorophyll *a* triplet life-time[13] and, assuming a temperature-independent formation rate for triplets, the temperature independence of the luminescence intensity simply follows from the temperature independence of the triplet–triplet annihilation rate[2]. Failure to observe a magnetic field dependence in the luminescence intensity, though, casts doubt on this mechanism. Stacy *et al.*[2] argue, however, that this effect can be too small for the best sensitivity (5%) in this experiment.

That DCMU suppresses the fast decaying luminescence probably indicates that it is formed at a site connected to the energy trap of the reaction center by a DCMU-sensitive path. Also, the time independence of the fluorescent yield suggests that this site is on the donor side of the reaction center. In addition, since short periods of ultraviolet irradiation or prior freezing do not suppress the fast decaying luminescence, it may be that this emission originates either in the reaction center itself or at a site connected to the donor side of the reaction center by an unusual, probably short range, mechanism for the transfer of excitation energy. Oxidative-reductive electron transfer can probably be excluded by the evidence linking freeze damage to its interruption[9]. Any other mechanism, such as quantum mechanical tunneling, would seem to require that this emission originates either at or very near the primary donor. One possibility is that the 110-μs luminescence may be related to chlorophyll a_{II} (ref. 14) which has a comparable decay time and is also thought to be on the donor side of System II.

Another possibility is that the 110-μs component is generated in a recombination of the primary photoreactants of System II. The energy to drive this back reaction could come from the high energy state linking electron transport to phosphorylation. Mayne[15] has shown that millisecond luminescence is dependent on this state and has also suggested that DCMU suppresses luminescence by preventing the formation of the high energy state. Structural damage due to freezing or ultraviolet irradiation could cause an accelerated decay of the high energy state and thereby produce the observed 110-μs decay.

Zankel[3] used short (3-μs) saturating flashes to study luminescence from dark adapted chloroplasts. He observed three distinct submillisecond components and one component having a half-time of 200 μs was suppressed by DCMU. It is possible that this component could be related to the 110-μs component we observe, but it is also likely that the short exciting light pulses used in the experiment of Zankel could not appreciably excite the metastable state responsible for the luminescence we observed, possibly because that state is located too far from the reaction center.

ACKNOWLEDGEMENTS

We would like to thank Professor W. Bertsch for drawing our attention to the RCA C31034 photomultiplier tube and also the Research Division of the International Business Machine Corporation for a generous loan of equipment. Dr W. Yu and Mr Michael Silverman provided invaluable technical assistance.

REFERENCES

1 Lavorel, J. (1971) *Photochem. Photobiol.* 14, 261–275
2 Stacy, W. T., Mar, T., Swenberg, C. E. and Govindjee (1971) *Photochem. Photobiol.* 14, 197–219

3 Zankel, K. L. (1971) *Biochim. Biophys. Acta* 245, 373–385
4 Bonaventura, C. and Kindergan, M. (1971) *Biochim. Biophys. Acta* 234, 249–264
5 Ruby, R. H. (1969) *Photochem. Photobiol.* 8, 299–308
6 Clayton, R. K. (1969) *Biophys. J.* 9, 60–76
7 Tollin, G., Fujimori, E. and Calvin, M. (1958) *Proc. Natl. Acad. Sci. U.S.* 44, 1035–1047
8 Arnold, W. and Azzi, J. (1971) *Photochem. Photobiol.* 14, 233–240
9 Heber, U. and Santarius, K. (1964) *Plant Physiol.* 39, 712–719
10 Strehler, B. L. and Arnold, W. (1951) *J. Gen. Physiol.* 34, 809–820
11 Kok, B., Malkin, S., Owens, O. and Forbush, N. (1967) *Brookhaven Symp. Biol.* 19, 446–459
12 Duysens, L. N. M. (1964) *Progr. Biophys. Mol. Biol.* 14, 1–104
13 Livingston, R. (1955) *J. Am. Chem. Soc.* 77, 2179–2182
14 Doring, G., Renger, G., Vater, J. and Witt, H. T. (1969) *Z. Naturforsch.* 24B, 1139–1143
15 Mayne, B. C. (1967) *Photochem. Photobiol.* 6, 189–197