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KINETIC AND SPECTRAL RESOLUTION OF TWO COMPONENTS OF DELAYED EMISSION FROM *CHLORELLA PYRENOIDOSA*

CELIA BONAVENTURA AND MICHAEL KINDERGAN

Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, Calif. 91109 (U.S.A.)*

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SUMMARY

1. Decay kinetics of delayed emission from *Chlorella pyrenoidosa* have been determined with a high degree of precision. The decay in the msec-to-sec interval after excitation can be represented accurately by the sum of two exponential decays—a "fast component" whose intensity-dependent lifetime ranges between 3 msec and 10 msec and a "slow component" whose lifetime ranges between 170 msec and 215 msec.

2. The emission spectrum of the slow component has a peak at 685 nm but lacks the shoulder between 710 and 730 nm seen in the fluorescence emission spectrum. The slow component can be isolated by monitoring the emission at 685 nm for long times (>30 msec) after high or low intensity excitation, or at any time in the msec to sec interval following low intensity (< 0.9 mW/cm^2) excitation. Saturation of the slow component occurs in the low intensity region where oxygen evolution is linear with light intensity. The temperature sensitivity of the slow component indicates involvement of an enzymatic and/or diffusion-limited process.

3. The emission spectrum of the fast component is identical to the fluorescence emission spectrum of the cells, with a peak at 685 nm and a shoulder between 710 and 730 nm. The fast component can be isolated by monitoring the emission at 740 nm. The intensity of the fast component parallels the reduction of System 2 electron acceptors and reaches a maximum level when photochemistry is light saturated. This fact was established by simultaneous measurements of oxygen evolution and delayed emission.

4. The fast component can be selectively inhibited by 3 (3,4-dichlorophenyl)-1,1-dimethylurea, heat or ultraviolet irradiation. The slow component can be selectively inhibited by hydroxylamine or low temperatures.

5. Possible mechanisms resulting in fast and slow component delayed emission are discussed in view of the experimental results.

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; fluorescence, denotes total emission and does not imply knowledge of an exponential decay.

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INTRODUCTION

Knowledge of the kinetics of decay of an excited state often provides insight into the nature of the state and the mechanisms that govern its chemical and physical relaxation processes. This has certainly been true in the case of the prompt emission from photosynthetic systems whose relationship to the time courses of photosynthetic events has been so central to our understanding of the electron transport mechanisms in these systems.

Since its discovery in 1951[1], delayed light emission has been one of the most intriguing and potentially rewarding subjects of investigation associated with the photosynthetic process. Yet, at the present time, the apparent complexity of the delayed light and the experimental difficulties associated with measuring its extremely low intensity over the required wide time spans have prevented realization of the full potential of this tool for the study of photosynthesis. It is already known from the work of STREHLER AND ARNOLD[1] that the action spectrum for production of delayed light is the same as the action spectrum for photosynthesis. Prior to the present investigation, it was generally believed that the spectrum of the delayed emission is identical to that of fluorescence. ARNOLD AND DAVIDSON[2] and CLAYTON[3] have suggested that variable chlorophyll fluorescence *in vivo* might be merely caused by the presence of a fast component of the delayed emission. The phase-fluorometric measurements of MULLER AND LUMRY[4], however, indicate that the intensity of the delayed emission is weaker than the intensity of the prompt emission by at least a factor of ten.

As their names imply, the lifetimes of the prompt and delayed emission differ greatly. The fluorescence of chlorophyll *in vivo* is known to decay exponentially with a lifetime of 0.35 nsec at low exciting intensity and 1.92 nsec when the exciting light is sufficiently strong to saturate the photochemical reactions of photosynthesis[5]. In the time range 0.001 sec to 1.0 sec, the decay of the delayed light is not a simple exponential but has been described in past work as a superposition of as many as three different exponentials[6] implying more than one source for this emission.

It has been suggested by many workers that the release of stored energy in the form of delayed light is a result of certain back reactions in the photosynthetic process. Certainly, interesting temperature effects would be expected if this hypothesis were true and indeed some have been found[7]. In addition, the effects of added cofactors and inhibitors on the delayed emission can be used to localize the origin of the delayed light, and an increasing amount of research activity in this area is evident.

The purpose of the present paper is to investigate more quantitatively in live cells of *Chlorella pyrenoidosa* the above phenomena—the precise nature of the time course for delayed emission, the temperature dependence, the effect of various cofactors and inhibitors, and the precise nature of the spectrum of the delayed emission. In addition, the dependence of the delayed light on exciting light intensity from very low light levels up to levels that saturate the photochemical apparatus of photosynthesis was of interest to us because of the known relationships between light saturation and the properties of the photosynthetic unit.

MATERIALS AND METHODS

Cells of *Chlorella pyrenoidosa* were grown in test tube culture in Knop's medium[8] at a specific growth rate of 1.8. Temperature during growth of the samples was

maintained at 26.5°. Aeration by 5 % CO_2 in air kept the cultures suspended. Samples for measurement were taken during the logarithmic growth phase and diluted with Knop's medium or centrifuged to the desired concentration.

Measurements of delayed emission and fluorescence were made using the phosphoroscope system shown schematically in Fig. 1. Excitation was provided by a 6500-W Osram Xenon arc combined with a broad-band interference filter (Baird Atomic) with a maximum transmission at 488 nm and Corning glass filters No. 4-96 and No. 4-97. The spectral output of this excitation source is centered at 480 nm and has a half-width of approximately 40 nm. The exciting light completely illuminated a 1-ml cell suspension of between 20 and 30 % transmission contained in a quartz sample holder. A belt-driven rotating cylinder with a choice of speeds and with unappreciable frequency drift served as a phosphoroscope. The light emitted from the sample passed through Corning glass filters No. 2-62, No. 2-63, and No. 2-73 into a 0.25-m Bausch and Lomb high-intensity grating monochromator with a band-pass of 19.2 nm. The monochromator output was detected by an Amperex 56 TVP photomultiplier cooled to dry-ice temperature. The output from the photomultiplier was amplified by a Keithley Multi-Range electrometer, Model 610B, and displayed by a chart recorder, or amplified by a Tektronix oscilloscope No. 585 with a Type 1A7 plug-in unit.

Oscilloscope output was fed into a Nuclear Data memory unit to separate

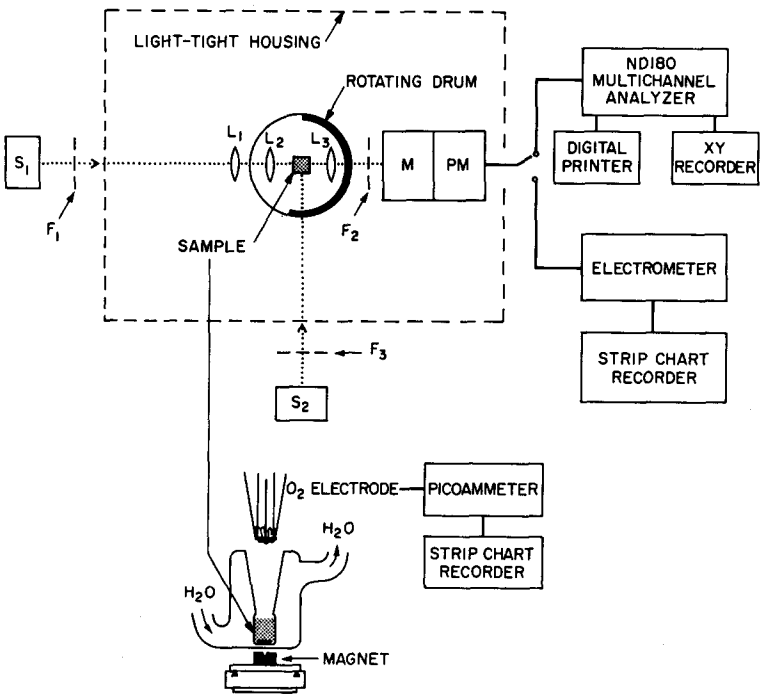


Fig. 1. Schematic representation of sample holder, phosphoroscope, and data collection components. S_1 and S_2, positions of a 6500 W Osram Xenon arc for stimulation of delayed emission (S_1) and fluorescence (S_2); F_1, F_2 and F_3, filters; L_1, L_2 and L_3, quartz lenses; M, Bausch and Lomb monochromator; PM, Amperex 56TVP photomultiplier. The multichannel analyzer was used for measurements of delayed emission and the electrometer for measurements of fluorescence.

signal from noise. The memory unit consists of two parts: integrator and time base (ND 180 ITB), and 512-channel memory unit (ND 180 M). The triggering of the ITB unit is taken from the output of a photodiode, which is synchronized with the rotating cylinder. After averaging for 10- to 100-k counts, the output of the memory unit is printed in digital form by a Hewlett-Packard digital recorder, Model 5050B, or plotted by a Hewlett-Packard X-Y recorder. The data in digital form were subjected to kinetic analysis by an IBM 360-75 computer.

The quartz sample holder was water cooled to maintain the temperature at 26.5° during the measuring interval. Magnetic stirring by a quartz-jacketed stirring bar at 600 rev./min kept the cells in suspension, and a flow of 5 % CO_2 in air over the cells prevented CO_2 limitation from occurring even at high light intensities. Exciting intensities were measured by a calibrated circular Eppley thermopile of 1-cm diameter whose photosensitive element was placed in a position corresponding to the center of the cell suspension. The scattered light contribution to measurements of fluorescence was minimized by side illumination through narrow-band interference filters (Baird Atomic) with appropriate blocking. No scattered light contribution to the delayed emission curves was detectable.

Delayed emission spectra were obtained by taking the digital output of the Hewlett-Packard 5050B recorder and averaging the values of five channels centered at the desired time after excitation and subtracting the average base line from this value. Signal averaging was done at each selected wavelength. The emission spectra were adjusted for differences in spectral sensitivity of the photomultiplier and monochromator. The correction factors were determined by using a calibrated Bureau of Standards tungsten lamp as the source and measuring the photomultiplier output with the Keithley electrometer. The prompt emission spectrum was obtained in a similar manner except for side illumination and electrometer amplification in place of signal averaging.

The ultraviolet treatment involved using only Corning Glass Filter No. 7-54 between the source and the sample. This filter passed most of the ultraviolet emission from the 6500-W Xenon arc. The cells were exposed to 15.5 mW/cm² for 7 min. The heat treatment consisted of exposing the cells to 55° for 85 sec.

It was possible to measure delayed emission and rates of O_2 evolution simultaneously by insertion of a Yellow Springs Instrument Co. oxygen electrode, Model 5531, into the sample compartment. Its output was measured with a Keithley picoammeter, Model 417.

RESULTS

Decay kinetics

The decay kinetics of delayed emission at three different exciting light intensities are illustrated in Fig. 2. The solid lines in the figure are generated by a least-squares fit of the data to the sum of two exponential decays. At 12 mW/cm² exciting light intensity, where O_2 evolution is virtually light saturated, the lifetimes of the two exponential components are 3.31 msec and 173 msec with pre-exponential coefficients of 148 and 8.62, respectively. At 0.9 mW/cm² exciting light intensity, in the region where O_2 evolution is linear with light intensity, the lifetimes are 5.48 msec and 215

msec with pre-exponential coefficients of 3.78 and 4.62, respectively. At 0.09 mW/cm² exciting light intensity, the lifetimes are 9.6 msec and 187 msec with pre-exponential coefficients of 3.13 and 4.70, respectively.

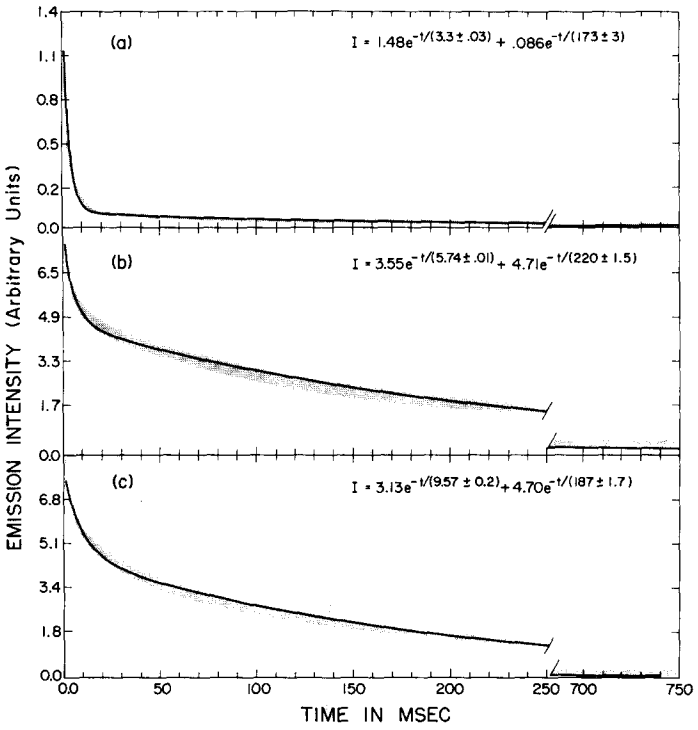


Fig. 2. Decay kinetics of delayed emission from *Chlorella pyrenoidosa* excited by (a) 12 mW/cm², (b) 0.9 mW/cm², and (c) 0.1 mW/cm² of 488 nm light. The solid lines are plots of the indicated functions that were fit to the data by the method of least squares. The shaded region is an envelope of the data points recorded by the 5050B recorder.

This kinetic analysis and the earlier observations of ARTHUR AND STREHLER suggest the existence of two components in the time range studied here. Delayed emission in the 1–5 msec interval after high intensity excitation is predominantly due to the rapidly decaying "fast component", while at low exciting intensity or at times greater than 20 msec, the fast component contributes little to the emission. To understand better the underlying causes of the delayed emission and its relationship to the various photochemical events in photosynthesis, we have made a study of the physical characteristics of the delayed emission under conditions where it will be dominated by one or the other component. Table I summarizes the results obtained.

Emission spectra of prompt and delayed emission

Fig. 3 compares the emission spectrum of fluorescence with that of delayed emission measured 2 msec after high intensity (12 mW/cm²) excitation. Both spectra have a peak at 690 nm and a shoulder between 710–730 nm. The shoulder of the fluorescence emission appears to be more distinct than that of the fast component of

TABLE I

COMPARISON OF FAST AND SLOW COMPONENTS OF DELAYED EMISSION

	Fast component	*Slow component*
Relative intensity 685 nm/730 nm	2.8	38
Lifetimes (msec)		
0.09 mW/cm²*	9.6	187
0.9 mW/cm²*	5.7	210
12.0 mW/cm²*	3.2	178
Relative initial intensity at 690 nm		
0.09 mW/cm²*	0.67	1
0.9 mW/cm²*	0.75	1
12.0 mW/cm²*	17	1
Saturation intensity (mW/cm²)*	20.0	0.6
Effect of treatment		
DCMU	Strongly suppressed	Stimulated
Ultraviolet	Strongly suppressed	Suppressed, faster decay
55°	Strongly suppressed	Suppressed
5°	Stimulated	Strongly suppressed
Hydroxylamine	Suppressed	Strongly suppressed

* Intensity of 488-nm excitation.

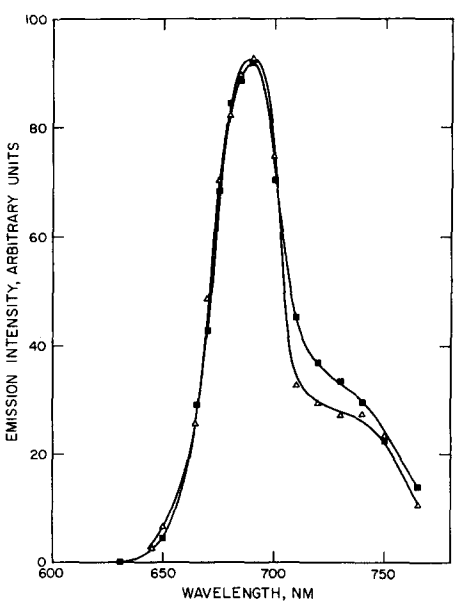


Fig. 3. The emission spectrum of fluorescence (△) and of the fast component of delayed emission (■). The delayed emission was measured 2 msec after 12 mW/cm² of 488-nm excitation. Fluorescence was measured during illumination by 10 mW/cm² of 488 nm light.

delayed emission. We believe that this is due, at least in part, to small scattering differences arising from side illumination for fluorescence excitation and front illumination for excitation of delayed emission. This confirms recent work by AZZI[10], which clearly shows the similarity between the spectrum of the fast component of delayed emission and the emission spectrum of fluorescence.

The spectrum of the fast component is compared in Fig. 4 with that of delayed emission measured at 30 msec after low intensity (0.9 mW/cm^2) excitation. Under these conditions, the delayed emission is almost entirely due to the slow component of delayed emission. This spectrum has a peak at 685 nm, but no shoulder at longer wavelengths. The apparent blue shift of the peak of the slow component emission relative to the peak of the fast component may be explained by the fact that the bandwidth necessary to measure the signals was wide enough to include some of the light in the 710–720-nm region even when the monochromator was centered at 690–700 nm. Hence, due to the method of measurement, the asymmetric spectrum would be expected to have its peak shifted to longer wavelengths than a symmetric one. The true peak, in both cases, is probably 685 nm.

The spectra show that isolation of the fast component from the slow component may be accomplished by measuring the emission at 740 nm using high intensity exciting light and by making measurements 2 msec after cutoff of the exciting light. Under these conditions, virtually all of the delayed emission in the time interval of 1–1000 msec may be described by a single exponential with a lifetime of 3.2 msec. The fact that the observed lifetimes of the fast component at the 685 nm peak and at the 710–730-nm shoulder are virtually identical indicates that these emissions are

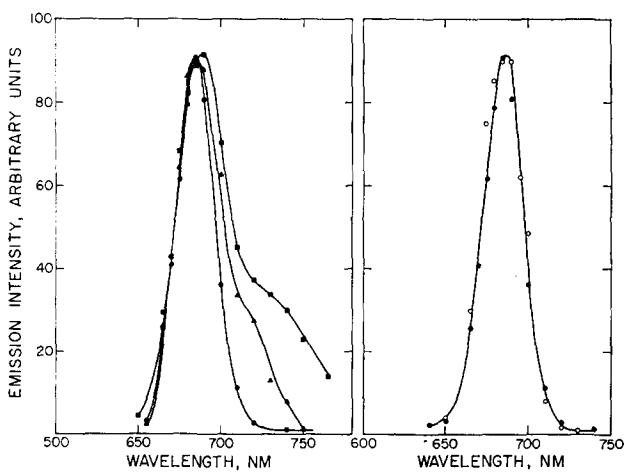


Fig. 4. The emission spectrum of the fast component of delayed emission (■), of the slow component of delayed emission (●), and a spectrum obtained when both fast and slow components contribute to the emission (▲). Measurements of the fast component (■) were made 2 msec after 12 mW/cm^2 of 488 nm excitation. After 10 msec both fast and slow components contribute (▲). Measurements of the slow component (●) were made 30 msec after 0.9 mW/cm^2 of 488-nm excitation.

Fig. 5. The emission spectrum of the slow component, observed 20 msec after 12 mW/cm^2 excitation (○) and 30 msec after 0.9 mW/cm^2 excitation (●).

either the result of a single mechanism or of different mechanisms having almost identical lifetimes.

At long times after the cutoff of exciting light, the contribution of the fast component should become negligible even when using high intensity excitation. Fig. 4 shows that at 10 msec after excitation, the emission spectrum has a much less pronounced shoulder than at 2 msec after excitation. Fig. 5 shows that at 20 msec after excitation, the shoulder is not present and the spectrum is identical to that of the slow component. Hence, the differences in the spectra are a result of the relative contribution of each component to the intensity of delayed light emission, which is a function of both exciting light light intensity and time after excitation, and not due to changes in the components themselves.

Treatments resulting in isolation of the components of delayed emission

The decay kinetics of algae exposed to $3 \cdot 10^{-5}$ M twice-recrystallized DCMU are similar to those of the slow component of untreated algae. We observed that DCMU decreases the intensity of the fast component drastically and increases the intensity of the slow component, as previously reported by SWEETSER *et al.*[11]. Consistent with the observations is the fact that the spectrum of delayed emission from the inhibited cells (at 13 mW/cm^2, 2 msec after excitation) is identical to that of the slow component.

STREHLER AND ARNOLD[1] reported that ultraviolet light decreases the luminescence and oxygen evolution at a comparable rate. We found that cells subjected to ultraviolet irradiation have a spectrum of delayed emission (at 12 mW/cm^2, 2 msec after excitation) identical to that of the slow component. The kinetics of decay indicate that a small portion of the normal fast component, with a lifetime of 3.96 msec, is present. However, the slow component, with a lifetime of 73.0 msec, is dominant. Their respective pre-exponential coefficients are 6.02 and 15.3. The decay of the slow component is three times faster as a result of the ultraviolet treatment.

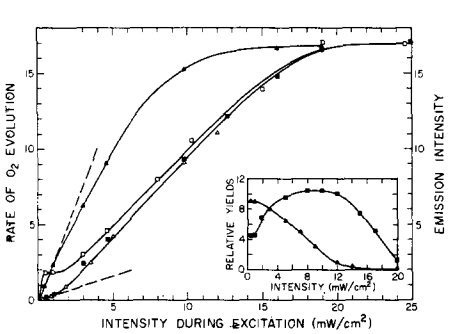
Heat treatment of cells is also effective in isolating the slow component. After 85 sec at 55°, the delayed emission spectrum (at 13.5 mW/cm^2, 2 msec after excitation) is identical to that of the slow component. Upon prolonged exposure of the cells to ultraviolet light or heat, the slow component is also eliminated.

Hydroxylamine, at a concentration of 10^{-3} M, appears to suppress the slow component as BERTSCH *et al.* have reported[12]. Kinetic analysis of the decay of delayed light emission of hydroxylamine-treated algae shows a single exponential with a lifetime of 2.8 msec. In addition to this effect on the decay kinetics, it was found that the spectrum of delayed emission after inhibition with hydroxylamine (at 12.5 mW/cm^2, 2 msec after excitation) is similar to that of the normal fast component. The irreversible effect of high light intensities on the delayed emission of hydroxylamine-treated cells, which will be discussed later, caused difficulty in obtaining a precise spectrum. Lowering the temperature to 5° also selectively suppresses the slow component. At 5° both the decay kinetics and the emission spectrum are those of the fast component.

Intensity dependence of delayed emission

We have shown that at high excitation intensities the intensity of the fast component of delayed emission is increased relative to that of the slow component.

Details of the intensity dependence of delayed emission are shown in Figs. 6 and 7. Due to the spectral differences in fast and slow component emission, the intensity dependence observed is dependent on the monitoring wavelength. The delayed emission monitored at 730 nm and measured 2 msec after excitation is predominantly due to the fast component. As shown by the closed squares of Fig. 6, the intesity dependence of the fast component has a complex character, but one that can be easily explained. At excitation intensities below 12 mW/cm², the intensity dependence of the fast component is quite similar to the intensity dependence of fluorescence (open triangles, Fig. 6). This indicates a dependence on the concentration of reduced products of System 2 that determine the fluorescence yield. The complementarity of the yields of the fast component and of oxygen evolution at low intensities (see insert, Fig. 6) is a consequence of this dependence. The rate of oxygen evolution is light saturated at an exciting intensity of 20 mW/cm². The intensity of the fast component, measured simultaneously, is also light saturated at this intensity. The insert of Fig. 6 shows the decrease in yield of the fast component that accompanies light saturation of the turnover rate of System, 2, which is monitored by the rate of oxygen evolution.



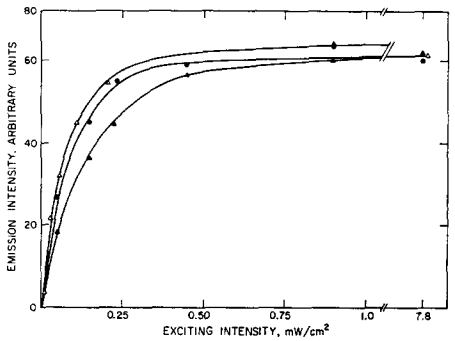


Fig. 6. Steady-state rates of O_2 evolution (▲), fluorescence (△), and delayed emission (□, ■) *vs.* intensity of 488-nm excitation. Delayed emission 2 msec after excitation was monitored at 690 nm (□) or 730 nm (■). The insert shows the intensity dependence of the yields of O_2 evolution (▲) and 730 nm delayed emission (■).

Fig. 7. Intensity dependence of delayed emission at 690 nm: slow component, measured 30 msec after excitation (●); cells with $3 \cdot 10^{-5}$ M DCMU, measured 2 msec after excitation (△); cells at 38°, measured 30 msec after excitation (▲).

If delayed light monitored at 690 nm is measured 2 msec after excitation, one observes the behavior of the fast component at high exciting light intensities and the slow component at low exciting light intensities. As shown in Fig. 6, the transition from slow component character to fast component character takes place when the exciting light intensity is between 0.9 mW/cm² and 3.0 mW/cm².

The dependence of the slow component emission intensity upon excitation intensity is shown by the filled circles in Fig. 7 on an expanded scale. These measurements were made 30 msec after excitation where, even after high intensity excitation, the emission spectrum lacks the 710–730 nm shoulder typical of the fast component. The intensity of emission rises rapidly at very low exciting intensities. JONES[13] and CLAYTON[14] have reported that the increase in intensity of delayed emission is propor-

tional to the square of the exciting light intensity at very low exciting light intensities where only the slow component is present. Our experiments (Fig. 7) did not consider the very low intensity region in sufficient detail to reveal the I^2 dependence. Saturation of the slow component occurs at an exciting intensity of 0.6 mW/cm^2, somewhat below the intensity shown in Fig. 6 to be associated with increased yields of fluorescence and the fast component of delayed emission.

Inhibition of photosynthesis by $3 \cdot 10^{-5}$ M DCMU drastically reduces the contribution of the fast component to the delayed emission. As shown by the open triangles of Fig. 7, the intensity dependence of the delayed emission after DCMU treatment is like that of the slow component, even when measured 2 msec after excitation. Similarly, cells at 38° exhibit little fast component emission and, as shown by the closed triangles, the resulting dependence on excitation intensity is like that of the slow component.

We observe that the delayed emission intensity of cells treated with 10^{-3} M hydroxylamine increases sharply with exciting intensity up to 8 mW/cm^2. At higher intensities there is an irreversible decrease in emission intensity. This photoinhibition is characteristically time-dependent, *i.e.* continued excitation by 8 mW/cm^2 results in a decrease in delayed emission intensity with time. Hence, while the slow component is eliminated by hydroxylamine addition at any exciting intensity, the fast component is also eliminated after sufficient exposure to high light intensity. We observed that photoinhibition of the fast component also occurs when cells kept at 5° are exposed to high intensity excitation.

Temperature dependence of delayed emission

Other workers have investigated the temperature dependence of delayed emission[7]. In view of the many differences between the fast and slow components of delayed emission we have repeated the earlier work under conditions specific for measurement of the fast component and the slow component. The temperature dependence of the fast component (monitored at 730 nm, at 13.5 mW/cm^2, 2 msec after excitation) and the slow component (monitored at 690 nm, at 0.9 mW/cm^2, 30 msec after excitation) is shown in Fig. 8. The slow component is very temperature sensitive. Its intensity is almost negligible at 5° and increases by a factor of 20 between 20° and 40°, while at 50°, its intensity is irreversibly decreased by a factor of 10. The emission spectrum in Fig. 9, taken at 38° 30 msec after excitation, reveals a slight shoulder not seen in the slow component spectrum taken at room temperature. Kinetic analysis of delayed light produced at 38° and excited by 0.9 mW/cm^2 indicates the presence of two components with lifetimes of 9.35 msec and 198 msec. This suggests that the shoulder is due to some fast component emission induced by the elevated temperature.

The fast component temperature dependence is complex. Between 20° and 30° the intensity is temperature independent. As the temperature is decreased from 20° to 10° there is a sharp increase in emission, most pronounced when the exciting light is not of sufficiently high intensity to saturate the photochemical reactions at room temperature. A similar increase, though at a somewhat lower temperature, was observed by TOLLIN *et al.*[7] who measured the integrated delayed emission intensity from 1 to 10 msec. Between 10° and 5° there is a 25 % decline in intensity. The decrease observed between 10° and 5° may be ascribed to the onset of photoinhibition, which we have observed to occur when cells are exposed to high intensities at 5°.

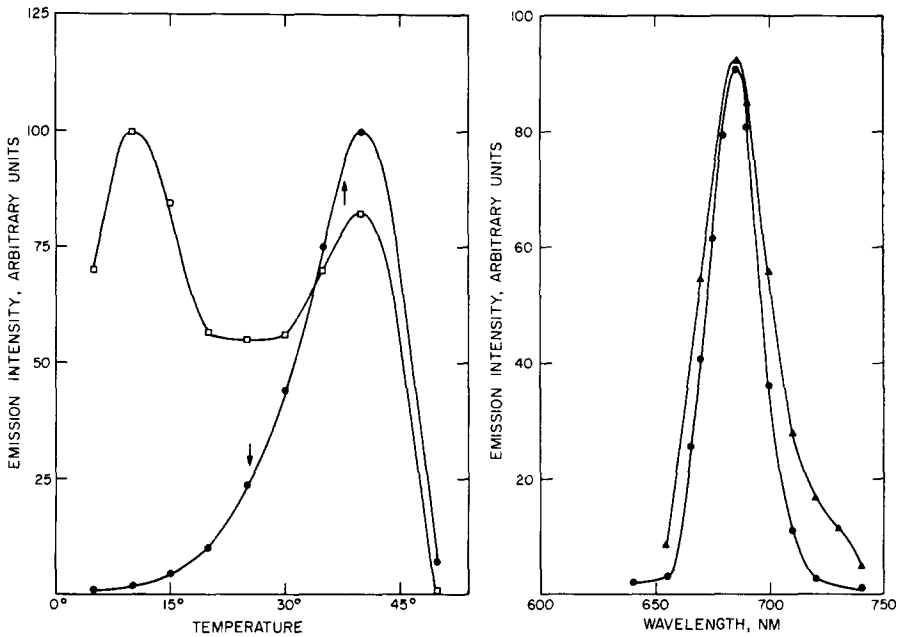


Fig. 8. Temperature dependence of 730 nm delayed emission, measured 2 msec after 12.7 mW/cm² of 488-nm excitation (□) and of 690-nm delayed emission, measured 30 msec after 0.9 mW/cm² of 488 nm excitation (●). Arrows indicate temperatures at which the spectra of Fig. 9 were obtained.

Fig. 9. Temperature dependence of spectrum of delayed emission, measured 30 msec after 0.9 mW/cm² of 488-nm excitation: 26.5°, (●); 38° (▲).

Above 30°, there is a sharp increase in intensity of emission but then at 50° the fast component is completely eliminated.

As one might expect after examination of Fig. 8, only the fast component is observed at 5°. The spectrum at this temperature is the same as that obtained for the fast component of algae at room temperature, and kinetic analysis indicates a single exponential decay with a lifetime of 2.6 msec.

Effect of System 1 excitation on delayed emission

Since there is evidence that System 1 is the main contributor to 710–730-nm emission at low temperatures[15–19], and at least a partial contributor at room temperatures[20,21], we tried to increase the relative intensity of the shoulder emission by exciting the cells using a narrow bandwidth (10 nm) interference filter centered at 715 nm. At this wavelength, approximately 4 times as much light is absorbed by System 1 as by System 2. At the low exciting light intensities (2 mW/cm²) available using the filtered light, the emission spectrum taken at 2 msec was identical to that of the slow component; that is, there was no pronounced shoulder in the 710–730-nm region.

Further comparisons of prompt and delayed emission

As previously reported[1,3,22], when dark-adapted cells are exposed to light both fluorescence and delayed emission exhibit a transitory period of several minutes

duration characterized by an elevated emission intensity. The decrease in emission intensity with time is complementary to an increase in the rate of photosynthesis. The relative changes during this induction period are much more pronounced for delayed emission than for fluorescence. At an exciting intensity of 10 mW/cm^2, we observe a peak-to-steady-state ratio of 10:1 for the fast component of delayed emission (measured 1 to 16 msec after excitation) and of 2.5:1 for prompt emission. The difference in peak-to-steady-state ratios indicates a greater sensitivity of the fast component of delayed emission to the state of the electron transport intermediates. CLAYTON[14] has discussed this phenomenon in terms of "live" and "dead" components of the prompt emission.

We have observed that DCMU inhibition increases the intensity of the prompt emission and of the slow component of delayed emission. At an actinic intensity of 1 mW/cm^2, the fluorescence yield from inhibited cells due to a weak measuring beam ($\ll$ 1 mW/cm^2) is maximized, indicating that the primary electron acceptor of System 2 is largely reduced and unable to act as a quencher of fluorescence. The slow component of delayed emission from inhibited cells is also maximized at this exciting intensity.

Delayed emission from the inhibited cells can be represented by a single exponential with a lifetime of 120 msec. The signal observed after 1 mW/cm^2 excitation with the weak measuring beam turned on was a composite of the delayed emission resulting from the 1 mW/cm^2 excitation and the prompt emission stimulated by the weak measuring beam. The delayed emission signal was subtracted from the total, and the resulting time-varying component of the prompt emission was subjected to kinetic analysis. The lifetime of the decay of the fluorescence yield obtained in this way is 139 msec. The fluorescence yield decrease after excitation is attributable to reoxidation of the System 2 electron acceptor by a slow back reaction of System 2[23,24]. The similarity of the decay of delayed emission and the decay of the fluorescence yield indicates a mutual dependence on the rate of this back reaction.

A further indication that prompt and delayed emission are both sensitive to the concentration of reduced electron transport intermediates comes from observations of emission changes due to CO_2 limitation. Removal of the ultimate electron acceptor CO_2 results in an increase in concentration of reduced intermediates. Both fluorescence and delayed emission increase due to CO_2 limitation, except at saturating light intensities where CO_2 limitation would be expected to have little effect.

DISCUSSION

The most important new observation uncovered by this investigation is the existence of a slow component of delayed emission that lacks the 710–730-nm emission shoulder typically seen in chlorophyll emission spectra. The striking difference in the 710–730 nm-region of the emission spectra of the fast and slow components of delayed emission is conclusive evidence of heterogeneity in the end points of the two reexcitation mechanisms. The maximum of the slow component emission spectrum lies at the maximum of emission from chlorophyll *a* ($\pm$ 2 nm) but absence of emission in the region usually associated with a vibronic transition[25] indicates that it is either due to a chlorophyll form in a hitherto unknown state or to a pigment different from chlorophyll *a*.

The observed differences in the emission spectra of fast and slow components of delayed emission are relevant to our understanding of the construction and operation of the photosynthetic apparatus. We must conclude that two different mechanisms exist for release of stored energy. One mechanism results in chlorophyll excitation analogous to that accomplished by direct stimulation. The other releases stored energy to emitting molecules that are physically or chemically isolated to the extent that appreciable energy tranfser to the bulk chlorophyll does not occur.

All previous reports of differences between prompt and delayed emission spectra from photosynthetic materials have been understood to be the result of contributions from accessory pigments to the prompt emission spectra[3,10]. There are, however, two instances where transients of *in vivo* fluorescence emission have spectral qualities similar to those observed for the slow component of delayed emission. In the first case, LAVOREL[20] reported that fluorescence emission, observed at the peak of the fluorescence induction period, has a much less pronounced shoulder between 710 nm and 730 nm than does steady-state emission. Second, difference spectra obtained by VREDENBERG AND DUYSENS[21] showed the relative emission at 685 nm to be twice as high following excitation of System 2 than after excitation of System 1. It is possible that the emissions with elevated 685 nm to 730 nm ratios detected in these experiments have the same origin as the slow component of delayed emission and seem to be sensitized by System 2 excitation.

Since two different mechanisms appear to be involved in the production of the fast and slow components of delayed emission, we will discuss the characteristics of the two components separately. The experiments here do not allow a precise delineation of the underlying reactions but do impose restrictions on possible descriptive models. It must be noted that this report deals only with delayed emission in the msec–sec interval after excitation and that the properties of much slower emission may differ from those we observe for our slow component.

Nature of the fast component

The prompt emission spectrum results primarily from direct excitation of the chlorophylls in System 2 by an external light source. The chlorophylls responsible for the fast component of delayed emission could conceivably be either excited at the expense of energy stored in a System 2 photoproduct, or independently excited at the expense of a System 2 photoproduct and System 1 photoproduct at the same rate. The latter is unlikely since (1) Scenedesmus mutant No. 11 with functional System 1 and nonfunctional System 2 provides very little delayed emission[26], (2) cells inhibited by DCMU show very little fast component delayed emission[11]. Thus, it appears that the metastable state whose decay results in fast component delayed emission is the result of System 2 activity.

Fig. 6 shows the similarity between the dependence of prompt emission intensity and fast component emission intensity on exciting intensity. The insert of Fig. 6 clarifies the relationship between the yields of photochemistry (O_2 evolution) and the yield of the fast component. It appears that the fluorescence yield of the reexcited chlorophylls, whose emission is the fast component of delayed emission, reflects the concentration of the reduced primary electron acceptor of System 2. This is true until the concentration of the metastable state reaches an upper limit when the turnover rate of the primary acceptor, as measured by rate of oxygen evolution, is light saturated.

We observe no effect on emission intensity due to removal of the ultimate electron acceptor CO_2 at saturating light intensities. Our interpretation of the fast component of delayed emission is that some reduced intermediate of the electron transport chain may act as the energy source. The DCMU sensitivity indicates that the site of energy storage is beyond the DCMU block, generally assumed to occur between the primary and secondary electron acceptors of System 2. The relative temperature independence of the fast component indicates that the energy source is not very far removed energetically from System 2 chlorophylls. However, the light saturation behavior eliminates the possibility of any purely physical mechanism operative within the bulk chlorophyll models, such as triplet–triplet annihilation, as responsible for the fast component of the delayed emission.

The pool of secondary electron acceptors denoted as A by FORBUSH AND KOK[23] is a possible candidate for the site of energy storage responsible for the fast component of delayed emission. If this hypothesis is correct, then the fact that the fast component of delayed emission exhibits more pronounced induction effects than does prompt emission yield simply reflects the fact that some prompt emission occurs from the bulk chlorophyll molecules even when most of the System 2 electron acceptors are in their open (oxidized) state, while the delayed emission is totally dependent upon the oxidation state of the intermediates.

Finally, it has been reported by MANTAI *et al.*[27] that ultraviolet irradiation results in a disruption of lamellar structure and of System 2 activity. Similarly, membrane structures have been shown to be disrupted over a small temperature range between 45° and 50°[28]. Indeed, WITT[29] has shown that similar heat treatment prevents the transfer of electrons to the quinones. Such disruptions of the lamellar structure could account for the fact that under these conditions no energy is transferred to the metastable state responsible for the occurrence of the fast component.

Nature of the slow component

As in the case of the fast component of delayed emission, the occurrence of the slow component seems dependent upon the products of System 2 activity. The temperature dependence shown in Fig. 8 of the slow component intensity is characteristic of an enzymatic and/or diffusion-limited process. Extreme heat or ultraviolet irradiation could alter either membrane permeability or the efficiency of an enzyme reaction. It is unlikely though that enzyme damage due to ultraviolet irradiation could increase the rate of the limiting reaction and result in a faster decay. An increase in membrane permeability and the rate of diffusion-limited processes, however, would be an expected result of ultraviolet irradiation since this has been reported[27] to result in membrane damage. Involvement of chloroplast membranes in delayed light production is also indicated by observations of emission from preilluminated chloroplasts stimulated by salt addition[30] or acid-base shifts[31] that establish a potential gradient across the chloroplast membranes. The near identity of the variation of slow component delayed emission intensity with exciting light intensity and the variation of hydrogen ion diffusion across the membrane with exciting light intensity strongly suggests that the two processes are closely related[32].

The fact that DCMU does not prevent occurrence of the slow component of delayed emission indicates that the underlying mechanism does not involve an intermediate on the electron transport chain past the primary acceptor of System 2. An

increase in the concentration of reduced System 2 photoproduct induced by blocking the electron transport chain by DCMU addition or CO_2 limitation increases the intensity of the slow emission, implying an increase in formation of the precursor of the slow component.

We have found that delayed emission from DCMU-treated cells shows the same rate of decay as the fluorescence yield (monitored by a weak measuring beam). This indicates that the decline of the intensity of the slow component of delayed emission reflects in part the decline of the concentration of the primary acceptor of System 2. This result is consistent with the "reverse enhancement" effects reported by GOEDHEER[22]. That the intensity is also influenced by the concentration of System 2 electron donors is shown by inhibition of the slow component by hydroxylamine. The mechanisms by which hydroxylamine inhibits O_2 evolution are thought to be: (1) reduction of the oxidant normally leading to oxidation of water, and (2) extraction of O_2 yielding catalysts like Mn[33]. If the slow component of delayed emission is the result of a recombination involving this oxidant, then the first mechanism would result in its elimination. The second mechanism, which is light stimulated[34], could account for the slow, irreversible inhibition of the fast component.

The concept of delayed emission suggested by LAVOREL[35] and extended by CLAYTON[36] is consistent with our observations and is in accord with reports of I^2 dependence of delayed emission at low exciting intensities[13,14]. The mechanism they suggested for production of delayed emission involves a recombination of charge across a membrane, essentially the reversal of System 2 photoactivation.

During preparation of this manuscript for publication, a report by BENNOUN[24] appeared that lends support to the proposal that the slow component results from a back reaction between the reduced product of System 2 and the primary oxidant of System 2. BENNOUN[24] reported that hydroxylamine blocks one pathway for reoxidation of the reduced product of System 2 and permits reoxidation by another pathway to proceed with unchanged velocity. In the context of the results presented here, the two pathways of reoxidation are linked to production of the slow and fast components of delayed emission.

Of the other models proposed to account for delayed emission, we find that the electron-hole recombination proposed by ARNOLD AND AZZI[37] can account for our observations if a temperature-sensitive step is built into it. The physical model of triplet–triplet annihilation can be eliminated as a possibility due to the temperature sensitivity and the light saturation behavior of the slow emission.

Concluding remarks

Failure to recognize the existence of two delayed emission components whose relative intensities depend upon excitation intensity, CO_2 supply, temperature, culture conditions, and presence of inhibitors has resulted in many contradictory reports concerning delayed emission. Unless these conditions are rigorously controlled during the course of measurement, scatter in the data may obscure the intensity dependence of the kinetics and necessitate use of complex kinetic schemes to fit the data. This is a possible explanation of the fact that RUBY[6] did not observe a change in decay kinetics when the exciting intensity was increased tenfold, and he had to use three exponential components to describe his decay curves.

Contradictory reports concerning light saturation of delayed emission[1,38] and

the effect of DCMU on delayed emission[14] may be resolved by realizing that the first few milliseconds after excitation will be dominated by the fast component, which is suppressed by DCMU and saturates only in the intensity range where O_2 evolution saturates, while longer times (100 msec) are dominated by the slow component, which is stimulated by DCMU and saturates in the intensity range where O_2 evolution is linear.

We find that the behavior of intact cells of Chlorella differs significantly from that of isolated chloroplasts. The intact cells produce a delayed emission whose intensity increases when photochemistry is arrested by CO_2 limitation, except under high intensity excitation where the electron transport intermediates are already in a reduced state and CO_2 limitation has no effect. There have been reports[39,40] that the absence of electron acceptors results in low levels of delayed emission in isolated chloroplasts under high intensity excitation. We suggest that the difference between chloroplast and whole cell behavior may lie in the increased interaction of the electron transport intermediates with O_2 and electron carriers in the suspension medium following isolation of chloroplasts.

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