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RAPID DELAYED LUMINESCENCE FROM CHLOROPLASTS: KINETIC ANALYSIS OF COMPONENTS; THE RELATIONSHIP TO THE O_2 EVOLVING SYSTEM

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SUMMARY

Delayed luminescence from saturating flashes given to isolated chloroplasts was measured in the time range of 65–800 μsec with the following results:

1. Three distinct components having decay half times of approx. 10, 35 and 200 μsec could be detected.

2. The yields of both the 35- and 200-μsec delayed luminescence components oscillate with a period of four, in phase with oscillations of O_2 yield; no large oscillations of fluorescence paralleling those of luminescence or O_2 were observed.

3. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) abolished the 10- and 200-μsec components and the oscillatory behavior of the 35-μsec component.

4. The 35- and 200-μsec components are not directly influenced by System I.

The DCMU isolated 35-μsec component showed the following properties:

1. The decay is first order and the emission spectrum is essentially identical to that of chloroplast fluorescence;

2. The yield saturates with a total emission of about 10^{-4} quanta/trap.

3. The temperature dependence indicates an activation energy of about 250 mV for the yield and 200 mV for the decay.

4. Maximal emission was obtained when Q, the acceptor of System II, was oxidized prior to the flash.

The results are discussed in terms of possible mechanisms concerning the production and behavior of the luminescence.

INTRODUCTION

Ever since the observation[1] that green plants show chlorophyll luminescence at times longer than the intrinsic fluorescence lifetime, this delayed luminescence* has been studied in the hope that such studies could be used to elucidate some of the processes of photosynthesis.

We studied the yield of delayed luminescence from 65 to 800 μsec in the hope

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

* The term "delayed luminescence" was used rather than "delayed light" or the more precise term "delayed fluorescence" for purposes of readability.

that at these short times, the luminescence might reflect some of the initial steps in the O_2 evolving process. Following the lead of BARBIERI *et al.*[2], who showed that in a sequence of flashes the longer delayed luminescence (> 20 msec) oscillated with a period of four, out of phase with the oscillations of the oxygen yield, we studied the luminescence from a similar sequence of flashes given to dark adapted chloroplasts and algae. We will describe the behavior of three new, short lived ($t_{1/2}$ approx. 10, 35 and 200 μsec), components of the delayed luminescence. Two of these oscillate with a period of four, in phase with oxygen evolution. One component could be isolated and its yield determined.

MATERIALS AND METHODS

Chloroplasts were prepared from greenhouse grown spinach. They were suspended in 0.4 M sucrose, 0.05 M NaCl and 0.05 M Tricine, pH 7.5 at a concentration of about 1 mg chlorophyll per 20 ml total. The algae Chlorella and Scenedesmus were cultured autotrophically at 30° in Roux bottles containing 600 ml of medium described by NORRIS *et al.*[3]. Measurements were made in the culture medium. Mutant No. 8 of Scenedesmus[4], lacking functional System I was grown under the same conditions with yeast extract and dextrose added to the medium. Unless otherwise stated, measurements apply to chloroplasts and were made at pH 7.5 at room temperature.

Delayed luminescence was measured in the apparatus shown in Fig. 1. An actinic flash (EGG FX-101, 4 μF at 900 V, half width approx. 3 μsec) is focussed by lens L_1 on a 1-mm slit adjacent to the chopper disc. The actinic light is filtered by F_1 (Corning filter 3-71). The chopper disc rotates at 3600 rev./min and serves to shut off the tail of the flash. The time from fully open to fully closed is 20 μsec. Lenses L_2 and L_2' then focus the beam, *via* mirror M_1, onto the sample. The sample holder is a 1-cm section of a 2-mm diameter teflon tubing. The sample can be changed automatically by flowing fresh suspension through the tubing. Luminescence from the sample is focussed by lenses L_3 and L_3' *via* mirror M_2 onto a 1-mm slit adjacent to the chopper disc. Here, the chopper prevents the actinic flash, and fluorescence induced by it, from

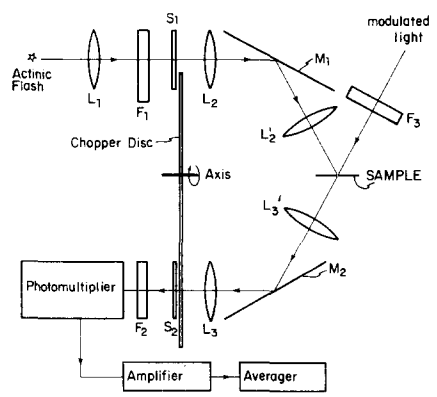


Fig. 1. Schematic diagram of delayed luminescence and fluorescence measuring apparatus. The chopper disc rotates past slits S_1 and S_2. In position shown, light from the flash can go to the sample The chopper is preventing light going through the slit S_2 to the photomultiplier. As the disc rotates, it cuts off the tail of the flash at S_1 and permits light to enter S_2. By using narrow slits and close distances, opening and closing times of 20 μsec each are obtained (see text for further details).

reaching the phototube. The slit opens fully in about 20 μsec, and the time delay between the peak of the actinic flash to the beginning of the measurement could be minimized to 50 μsec. The light enters the photomultiplier (Amperex XP 1002) through filter F_2 (Corning 2-64). The signal is amplified and stored in a signal averager (Biomation 102S). The electronic time response was adjustable; signal storage $\geqslant$ 2 μsec per address.

Fluorescence could be measured in the same apparatus by introducing a modulated measuring beam through filter F_3 ($CuSO_4$ and Corning Filter 4-96). An ultrasonic modulator similar to that described by SPENCER AND WEBER[5] was used to obtain a 14-MHz modulated beam. The remainder of the system remained the same as for delayed luminescence measurements except that a tuned, 14-MHz, phase-sensitive detector was used which eliminated the (d.c.) signal due to delayed luminescence.

Typical luminescence results

Delayed luminescence was measured from a sequence of flashes given to dark adapted chloroplasts. The details of the measurements are given in the legend of Fig. 2, which shows some typical data. From such data, information was gained concerning the oscillations in yield, kinetics, total yield and general behavior. These will be discussed in the following sections.

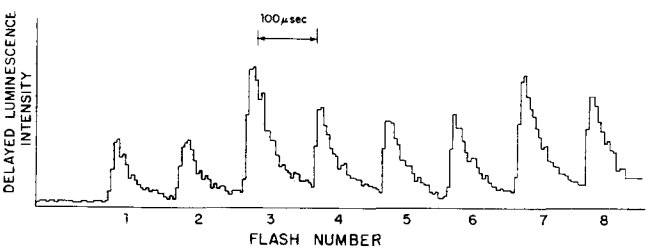


Fig. 2. Delayed luminescence from chloroplasts as a function of time from a series of eight, 3-μsec-long, saturating flashes, spaced 1 sec apart. The curve represents the average of 64 measurements on separate, dark adapted samples. The instrument records the signal from 45 to 160 μsec after each flash in 5-μsec steps or address advances. There is a time of one second between flashes when no measurements are recorded. The peaks are therefore spaced about a second apart and come at 65 μsec, a time determined by the opening of the shutter and the 3-μsec electronic time constant. The sample was dark adapted 20 min prior to the flashes. The first part of the recording is the signal without a flash. Similar data were obtained out to 800 μsec by adjusting the starting and address times of the averager.

Kinetics: Isolation into components

Fig. 3 shows the log of delayed luminescence intensity after the 1st and 3rd flash as a function of time and compares it to the intensity after the 1st flash in the presence of 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU). The decay in the presence of DCMU (Fig. 3C) is clearly first order over a wide (65–250-μsec) range, with a half time at room temperature of about 35 μsec. A predominant 35-μsec component was present in all the measurements we made.

Additional components can be seen in the absence of DCMU. The luminescence decay after the third flash (Fig. 3B) reveals a component which decays more slowly. Data similar to those shown, extended out to 800 μsec, indicate that in chloroplasts this component (subtracting the 35-μsec component) decays in approx. 200 μsec.

However, the data were not taken over a sufficiently long time range to determine whether this decay is first order.

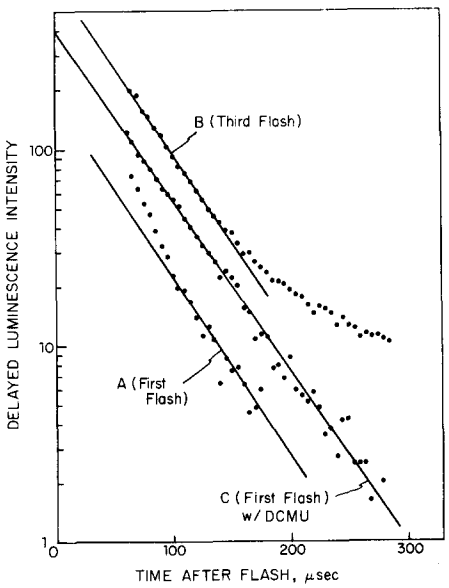


Fig. 3. Log of delayed luminescence from isolated, dark adapted, chloroplasts as a fuuction of time after: A, the first ffash; B, the third, no DCMU was added; C, the first flash, 20 μM DCMU was added. The straight lines are theoretical for a first order decay with a half time of 35 μsec. The luminescences in A and B are both plotted to the same (arbitrary) scale. C has been shifted to prevent overlapping.

Finally, without DCMU, the luminescence decay after the first flash (Fig. 3A) reveals in addition to the 35-μsec component a still faster component. Although the time resolution of the instrument did not allow an accurate measurement of the faster component, the difference was seen consistently in alternate measurements with and without DCMU. When this faster component is subtracted off, the yields after the 1st flash (35-μsec component) seem to be independent of DCMU. Assuming a first order decay for the fast component, its half time is approx. 10 μsec.

Oscillations of luminescence yield

The magnitude of the flash yield of the 35-μsec component of delayed luminescence is fairly well represented for chloroplasts by the total luminescence intensity at 90 μsec, at which time the faster (10-μsec) component has decayed. Similarly the "200-μsec" component is fairly well represented for chloroplasts by the total luminescence at 500 μsec. We therefore plotted the flash yields at these times in Fig. 4 and will use this plot to compare the behavior of the two components.

Fig. 4A shows the flash yields as a function of flash number for dark adapted chloroplasts. An oscillation in amplitude with a period of four similar to that seen in the oxygen yield is apparent for both components. As in the oxygen yield, the luminescence yield is highest after the third flash. The patterns can be described as being composed of a fixed level (dashed line of Fig. 4A), *plus* an amount which is propor-

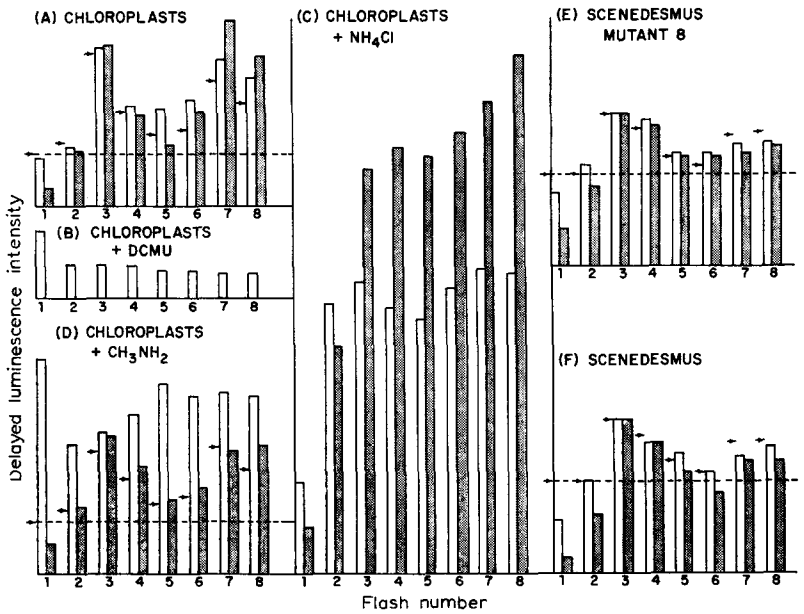


Fig. 4. Delayed luminescence at 90 μsec (open bars) and 500 μsec (shaded bars) after each of a series of eight flashes as a function of flash number. Data taken from curves obtained under the same conditions as Fig. 2. The samples contain the following: (A) chloroplasts, no additions; (B) chloroplasts, 20 μM DCMU added; (C) chloroplasts 50 mM NH_4Cl added; (D) chloroplasts, 90 mM methylamine added; (E) mutant No. 8 of Scenedesmus; (F) wild type Scenedesmus. The luminescence yields at 90 μsec, and at 500 μsec from the chloroplast containing samples are all approximately to the same scale. Normalization was made between the 90-μsec and the 500-μsec data to make them comparable in magnitude. The distances from the arrows to the dashed lines are proportional to the corresponding flash yields of oxygen taken from ref. 6 for chloroplasts and ref. 7 for algae. The luminescence data are shown at 90 μsec to minimize contributions from components other than the one decaying in 35 μsec and shown at 500 μsec to minimize contributions from the 35-μsec component.

tional to the O_2 yield for the flash (arrows). Small deviations at high flash number are to be expected since the O_2 and luminescence yield of these are very much dependent on the quality of the flashes, which differed in the measurements. The difference between the luminescence yields at 90 and 500 μsec after the first flash is significant: The first flash yield for the 35-μsec component is definitely not zero; that for the 200-μsec component is zero to within experimental error.

The effect of 20 μM DCMU on the 35-μsec component is shown in Fig. 4B. As was indicated previously, DCMU abolishes the 200-μsec component. Although DCMU has little effect on the 35-μsec component of the luminescence yield after the 1st flash, the yield of subsequent flashes is diminished, depending on the dark time between flashes. The luminescence yields from the second and subsequent flashes are smaller, the shorter the dark intervals. Recovery of the maximal delayed luminescence required a half time of about 1 sec, qualitatively the same time in which, under these conditions, the fluorescence yield, after being raised by a flash, returns to a low level. This suggests that for luminescence to occur, the acceptor (Q) of System II must be in its active (oxidized) form prior to the flash.

Various types of inhibitors of O_2 known to act on System II were used, and, as

expected, they all altered the oscillatory pattern of the luminescence, *i.e.* changed the phase, magnitude, *etc.* For example a high concentration (50 mM) of NH_4Cl, which is known to inhibit oxygen evolution[8], produced the pattern shown in Fig. 4C. An unexplained result, however, was obtained with 90 mM methylamine (Fig. 4D); the pattern at 90 μsec appears to be advanced in phase by two; that at 500 μsec appears unaffected. Parallel experiments* indicated no change in either the oscillatory pattern or yield of oxygen. Further changes in the luminescence, similar to those seen with NH_4Cl, were seen with methylamine at pH 8.4, probably reflecting the loss of oxygen seen at this higher pH in the presence of methylamine[8]. Similar high values of luminescence were also seen with high concentrations (5 mM) of hydroxylamine.

It was found that in the time range studied the phosphorylation uncoupler NH_4Cl (10 mM) and the System I acceptor methyl viologen (100 μM) had no detectable influence on the luminescence yields from the first six flashes given to dark adapted chloroplasts. In Scenedesmus mutant No. 8 (ref. 4), which lacks a functional System I (Fig. 4E) the 35-μsec emission oscillated in a manner very similar to that observed with spinach chloroplasts (Fig. 4A) and the wild type algae (Fig. 4F). Differences can be accounted for by the faster deactivation time for O_2 in algae. This behavior is very similar to that of the O_2 yield and strongly suggests that the luminescence involves neither System I nor the electron transport chain between the two systems. The observation that DCMU (which presumably blocks electron transport beyond Q, the acceptor for System II) does not alter the 35-μsec component of luminescence after the first flash also indicated no involvement of electron transport components in the chain beyond the primary acceptor Q for this component.

BARBIERI *et al.*[2] found that, at times greater than 20 msec after flashes, oscillations of luminescence from Chlorella had a different phase from what we have shown for chloroplasts at shorter times. They saw the highest yield after the second flash. The difference in phase seen at 20 msec is a result of the time range studied. We found that the phase changed at about 5 msec (Fig. 5).

Rapid fluorescence decay and its relation to delayed luminescence

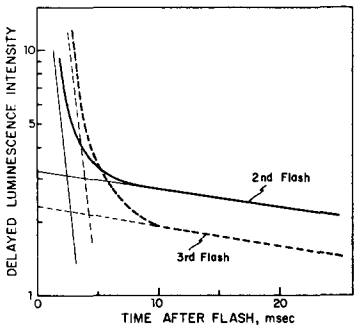
Delayed luminescence represents stored energy emitted by the chloroplasts. LAVOREL[9] has pointed out that the amount emitted depends not only on how much stored energy is transformed to singlet excitation energy of chlorophyll, but also on the probability that the energy is then emitted rather than quenched by internal conversion or retrapped. Thus the delayed luminescence yield should (1) reflect the amount of stored energy transformed to singlet energy, and (2) reflect the state of the traps which is reflected in the yield of the prompt fluorescence ("Lavorel effect"). It is not clear whether this dependence should be on the total fluorescence yield or, as has been suggested by the work of CLAYTON[10], on the variable part of the yield.

We first compared the spectrum of delayed luminescence between 70 and 110 μsec with that of fluorescence and found them to be the same within experimental limits ($\pm$ 20 %).

We then studied the fluorescence yield under the conditions of some of our luminescence measurements in order to find out to what extent the behavior of the shorter lived delayed luminescence corresponds to similar behavior of fluorescence.

* Oxygen measurements quoted in this paper were performed by Marion McGloin Smith using apparatus described in ref. 6.

A representative result of such studies is shown in Fig. 6. Such studies (using faster and slower recording times) indicated that variations of fluorescence yield with flash number are less than 25 % of the variable part of the fluorescence and less than 15 % of the total. The variations seen were both too small and in the wrong direction to account for the oscillations of either the 35-μsec or 200-μsec component of delayed luminescence.



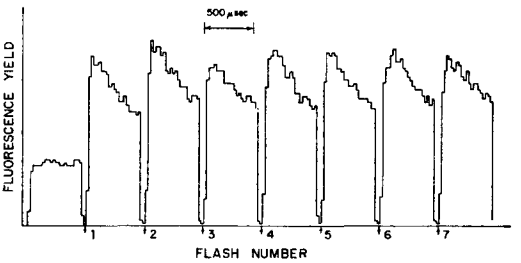


Fig. 5. Delayed luminescence from dark adapted Chlorella as a function of time after the second flash, solid lines (A); after the third flash, dashed lines (B). Flashes were spaced 0.25 sec apart. Each of the luminescence decays can be represented by the corresponding two first order decays (straight lines) shown in the figure.

Fig. 6. Fluorescence produced by a weak modulated measuring beam as a function of time after a series of actinic flashes. Same conditions as Fig. 2. Flashes are spaced one second apart, and measurements made at 25 μsec per address from 0 to 550 μsec after each flash. The peaks occur at about 80 μsec after the flash, a time determined by the opening time of the shutter to the photomultiplier and the 10-μsec time-constant of the electronics. The initial 550 μsec is the fluorescence observed in the absence of any flash. Sample contained chloroplasts and 100 μM methyl viologen. Arrows indicate time of flash. The curves represent the average of 128 measurements on separate dark adapted samples.

Also the decay of the 35-μsec luminescence component cannot be explained by a Lavorel affect. This luminescence component is observed in the presence of DCMU: No rapid decay of fluorescence could be seen in the presence of that poison. Also, we did not observe a 35-μsec fluorescence decay in non poisoned chloroplasts.

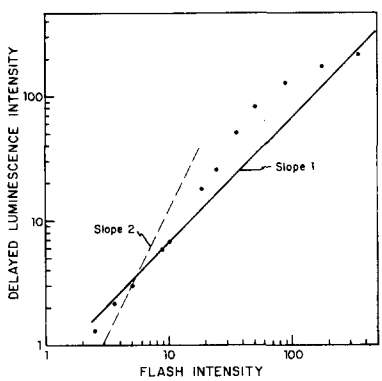
The relationships between fluorescence and luminescence is not completely clear for the 200-μsec component. The fluorescence decay can be described as a first order 200-μsec decay, followed by some slower decay. (This does not rule out a more complex decay that is not first order.) Both the fluorescence decay and the 200-μsec luminescence are eliminated by DCMU. This suggests a correlation between the 200-μsec luminescence and fluorescence decays. The fractional change of fluorescence in 200 μsec is small (< 30 % of the variable and 20 % of the total fluorescence) indicating that the correlation is not due to a Lavorell effect.

Light saturation, absolute yield and temperature dependence of the 35-μsec component in the presence of DCMU

To get some better understanding about possible mechanisms for the formation of the 35-μsec component, we determined the magnitude, temperature dependence and intensity dependence of its yield. The intensity of delayed luminescence from the first flash in the presence of DCMU is plotted as a function of flash intensity on a log–

log scale in Fig. 7. It is seen that at low light intensities, where there should be few neighboring closed traps, the luminescence appears to be proportional to flash intensity, indicating a one quantum process. At higher intensities the luminescence rises in a manner suggesting a response to an increase of fluorescence yield due to closed neighboring traps. At even higher intensities, the luminescence saturates.

Since the 35-μsec emission decays first order and saturates, it is possible to estimate the energy emitted per trap. This was done by comparing it with the fluorescence yield, assuming it takes one quantum per trap to raise the fluorescence in the presence of DCMU. The procedure is outlined in the legend of Fig. 8.



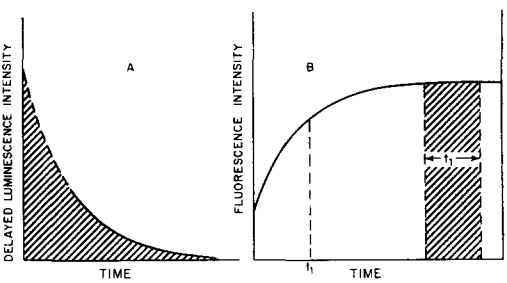


Fig. 7. Log of the intensity of delayed luminescence (arbitrary scale) as a function of the log of flash intensity (arbitrary scale) for dark adapted chloroplasts in the presence of 20 μM DCMU. Solid line (Slope 1) is theoretical for luminescence proportional to the actinic flash intensity; dashed line (Slope 2) is theoretical for luminescence proportional to the square of the actinic flash intensity. The flash intensity was varied with neutral density filters.

Fig. 8. Illustration of how quantum yield for luminescence was determined. Curves are hypothetical. (A) Delayed luminescence in the presence of DCMU as a function of time. Dashed portion is extrapolated from data assuming an exponential decay starting at time of flash, $t = 0$. Area of shaded portion is proportional to total emitted light. (B) Fluorescence in the presence of DCMU as a function of time after measuring light is turned on. Measuring light also serves as actinic light and brings the fluorescence yield to saturation. The time, t_1, to bring the variable part of fluorescence to about 70% of its maximum is taken as the time for absorption of 1 quantum per trap. After reaching saturation the fluorescence yield is about 5%. Therefore, during the time t_1, the area of the shaded portion represents 0.05 quantum emitted. 20 times the area represents 1 quantum emitted. The detection system for A and B remained the same. Assuming the area in A comes from 1 quantum, the ratio of the shaded area of A to 20 times the shaded area of B gives the approximate quantum yield of luminescence.

We computed that the yield of the 35-μsec luminescence for dark adapted chloroplasts in the presence of DCMU is about 10^{-4} quanta emitted per quantum reaching a System II trap. By comparison, if we assume a first order decay for the 200-μsec component, the total yield from the third flash is also on the order of 10^{-4} quanta/trap.

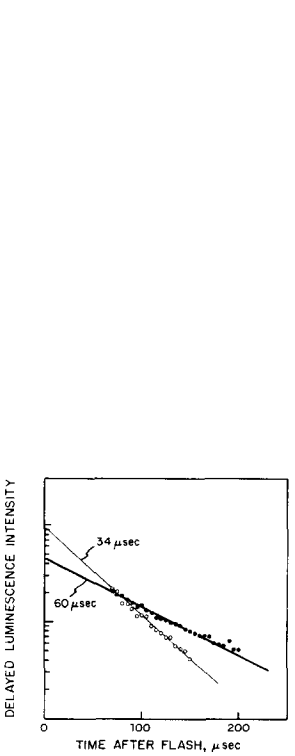
The temperature dependence of the delayed luminescence was measured in the presence of DCMU. As can be seen in Fig. 9, the maximum rate of emission (obtained by extrapolating to zero time) decreased by a factor of almost two in going from 25 to 4°. The decay time also proved to be temperature dependent; the same decrease in temperature increased the half time from 35 to 60 μsec. These results will be discussed later.

DISCUSSION

35-μsec component of delayed luminescence

There are a number of steps in the electron transport chains from System II traps to both oxygen and System I, and the rate of each step could influence the delayed luminescence and produce a luminescence "component". It was hoped that since the faster components are closer in time to the initial photoact and are influenced by fewer events, (*i.e.* are not influenced by later ones) they would be simpler in nature. We found this to be true in some ways for the 35-μsec luminescence component. It occurs after a single brief flash, decays first order, saturates and can be isolated with DCMU. This allowed us to determine its total yield.

It would be tempting, therefore, to speculate that the 35-μsec component comes from a direct recombination of positive and negative equivalent on the main path of photosynthesis formed by a single quantum. We can evaluate this hypothesis by calculating and comparing the total energy of such a pair from two different measurements: from the total luminescence yield at room temperature and from the temperature dependence of the initial intensity. If the hypothesis is correct, the two determinations must agree.



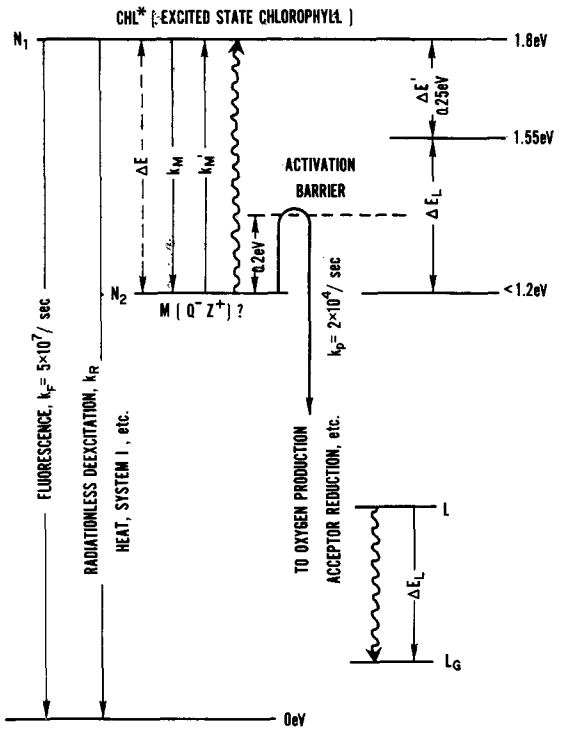


Fig. 9. Log of delayed luminescence from dark adapted chloroplasts in the presence of DCMU as a function of time after a brief saturating flash. ○, at 25°; ●, at 4°. Straight lines are theoretical for first order decay with half times of 34 sec and 60 μsec.

Fig. 10. Energy level diagram describing possible mechanisms for delayed luminescence.

Consider the energy level diagram of Fig. 10. Light is absorbed by the light harvesting chlorophyll which is brought to the singlet excited state, Chl*. The system can then return to the ground state by fluorescence with rate $k_F = 5 \cdot 10^7$/sec (determined by the intrinsic lifetime of chlorophyll) or by radiationless deexcitation with rate k_R; or the energy can go to form a charge–hole pair, represented by the state M (for example, primary acceptor reduced, primary donor oxidized). M is transformed into a metastable configuration M' over an activation barrier (0.2,V see later) with a half time of 35 μsec ($k_p = 2 \cdot 10^4$/sec); for example, either the acceptor or donor transfers an equivalent to a secondary reactant. Luminescence comes about by recombination of the electron–hole pair M, with rate k_{-M}. The recombination rate is related to the trapping rate by the expression

$$\frac{k_{-M}}{k_M} = \frac{N_1}{N_2} e^{-\Delta E/kT}$$

where N_1 is the number of available states per trap in the upper level (about 200, the number of chlorophyll molecules per trap) and N_2 the number of states in the lower level M (which we will take to be one per trap), k is Boltzmann's constant. ΔE is the energy difference between states and T the absolute temperature. If we make the assumption that M represents a step in the normal photosynthetic process and that photosynthesis is efficient, we have sufficiently defined the system so that we can independently calculate ΔE from the total luminescence yield and from the temperature dependence of the initial yield.

From the total luminescence yield of 10^{-4} quanta/trap, we calculate that ΔE is approx. 575 mV. However, a calculation based on the temperature dependence fo the initial yield gives a much lower value: ΔE approx. 250 mV. These calculations are independent of the nature of M and independent of intermediate steps. An investigation of the approximations, the assumptions concerning N_1/N_2 and the probable errors of measurement does little to reconcile the difference between the two determinations of ΔE (575 *vs.* 250 mV). We must therefore conclude that the simple model is wrong — either we deal with a side reaction or we must consider another mechanism.

The elimination by DCMU of the 35-μsec component, after flashes other than the first, indicates that any side reaction leading to the luminescence probably involves the traps. The oscillations of the luminescence can be due to an indirect influence of S.

When we consider alternatives on the main pathway, we must not only consider the measured yields and temperature dependence, but also the apparent one quantum nature of the luminescence. We will present the simplest model that we can think of that incorporates these features. Although such a model is perhaps premature, it illustrates some possibilities which may have some validity, particularly how relatively small (about 30 mV) perturbations due to S, can result in relatively large oscillations. This model consists of a one quantum process on the main pathway assisted by extra energy which is stored prior to the flash. This extra energy might be identical or related to the energy which is presumably stored at the oxygen evolving site: Flash yields of oxygen suggest such stored energy since it takes only three flashes, not four to evolve the first oxygen molecule; *i.e.* an energetic state S_1 is presumed to be stable in the dark[6,7].

This model is illustrated in Fig. 10. We assume that energy stored in some state L can be released with a decay to state L_G in a cooperative process (wavy lines), in which M returns to Chl*. The energy of Chl* is 1.8 eV. Assuming that the energy difference, ΔE, between Chl* and M is great enough ($> 575 + 60 = 635$ mV) so that the direct back reaction accounts for less than 10 % of the luminescence, the energy stored in M is less than 1.15 eV. This energy *plus* the 0.25 eV obtained from heat is at least 0.4 eV below that required to form a quantum at the energy of Chl* (1.8 eV). Therefore, the energy, ΔE_L, obtained from the decay of L must be at least 0.4 eV, or allowing for errors in measurement, 0.35 eV. If we assume that the level of L varies with the state of the oxygen precursor (S), we can account for the oscillation of the 35-μsec luminescence, which we found to be proportional to oxygen yield: A shift in the level of L by only 30 mV would give a 3-fold change in luminescence. Obviously, the influence of the S states need not be direct.

The above calculations are based on the assumption that both ΔE_L and the interaction described by the wavy lines of Fig. 10 are independent of temperature. Since measurements were only made at two temperatures over a small (20°) range, such an assumption lacks experimental verification. The values given must therefore be taken as being only illustrative.

Whatever the process leading to luminescence, lowering the temperature 20° almost doubles the decay half time (to 60 μsec) indicating an activation barrier for the decay. From the Boltzman equation we calculate this barrier to be 200 mV.

The 35-μsec component was present under all conditions studied for which Q was oxidized prior to the flash. These conditions included the presence of 5 mM hydroxylamine. Since equivalents are probably not transferred to S in the presence of 5 mM hydroxylamine, the 35-μsec luminescence probably does not require such transfer. Assuming that DCMU blocks transfer of an equivalent from Q, the 35-μsec luminescence decay can either reflect a reaction preceding Q reduction, a reaction on the donor side of System II, or a decay in a side reaction.

The 10-μsec component of delayed luminescence

The limitations of our instrument permit us to say little about the 10-μsec luminescence except that a fast component exists which does not oscillate in the same manner as the slower components: It is not readily observable after the third flash. This fast component is eliminated by DCMU, indicating that the formation of the state leading to this luminescence is blocked by DCMU. If, instead, DCMU blocked the decay of this state, the state and consequently the luminescence would be long lived instead of eliminated.

The 200-μsec delayed luminescence component

It seems reasonable to relate the 200-μsec decays in fluorescence and luminescence. The decay in fluorescence may indicate the time for the acceptor Q to transfer its charge and at the same time its ability to participate in luminescence. After a first flash, Q must transfer its electron before a second flash can be effective. KOK *et al.*[6] concluded from the flash yields of O_2, that such activation between the first and second flashes occurred in a second order manner with a first half time of about 200 μsec. Our fluorescence measurements indicate a 200-μsec decay, consistent with this interpretation.

It is obvious that there is no evidence that the fluorescence decaying in these short times is an indication of the redox state of the acceptor Q, rather than the donor Z. The arguments we gave could just as easily have been inverted, putting the 200-μsec decay on the donor side or from a "sensitizer", in which case it may be related to Chl a_{II}[10], which has a similar decay time. In either case, the absence of this luminescence component in the presence of DCMU, indicates that the formation of the luminescent state is blocked by that poison (see discussion of the 10-μsec component). The absence of the luminescence component after the first flash indicates the necessity of two sequential photoacts for the formation of the luminescent state.

Rate limitation in oxidizing side of System II

In the model of KOK *et al.*[6], each flash puts an additional positive equivalent on the precursor, S: The first flash advances S_1 to S_2, the second S_2 to S_3 and the third causes the production of O_2, with a return to S_0. The question arises as to whether S has been advanced by the flash at the time of the luminescence measurements or whether the equivalent has not yet been transferred to S. If S changes rapidly, the system would, after the third flash, be primarily in the S_0 state at the time of our luminescence measurements; if S changes slowly, it would be in the S_3 state. The time for advancement of S must be less than or equal to the time delay between excitation and O_2 evolution. The delay time for O_2 evolution has been measured for Chlorella with the modulated oxygen electrode. The results obtained in two laboratories were contradictory, JOLIOT *et al.*[12] obtained a half time of about 800 μsec while more recently SINCLAIR[13] found that O_2 responded in less than 200 μsec.

The apparent change in phase for the luminescence flash yields of Chlorella at about 5 msec suggests the advance of S. Although our data do not warrant a determination of the type of kinetics involved, the decays between 2.5 and 25 msec can be represented by two first order components, one decaying with a half time of 700 $\pm$ 200 μsec and the other much more slowly (Fig. 5). The yield after the third flash is higher in the faster component; that after the second is higher in the slower. This suggests that at both the shorter and longer times the main influence on luminescence comes from the S_3 state and that S_3 is produced from S_2 in about 700 μsec, in agreement with the time for O_2 response measured by JOLIOT *et al.*[12]. It might suggest that the rate limiting step on the oxidizing side of System II occurs in the transfer to S.

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