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INDUCTION PATTERNS OF DELAYED LUMINESCENCE FROM ISOLATED CHLOROPLASTS

I. RESPONSE OF DELAYED LUMINESCENCE TO CHANGES IN THE PROMPT FLUORESCENCE YIELD

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Summary

1. Using a phosphoroscope, delayed luminescence and prompt chlorophyll fluorescence from isolated chloroplasts have been compared during the induction period.

2. Two distinct decay components of delayed luminescence were measured a "fast" component (from ≈1 ms to ≈6 ms) and a "slow" component (at ≈6 ms).

3. The fast luminescence component often did not correlate with the fluorescence changes while the slow component significantly changed its intensity during the induction period in a manner which could usually be linearly correlated with variable portion of the fluorescence yield change.

4. This correlation was evident after preillumination with far-red light or after allowing a considerable time for dark relaxation.

5. The close relationship between the slow luminescence component and variable fluorescence yield was observed with a large range of light intensities and also in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea which considerably changes the fluorescence induction kinetics.

6. Valinomycin and other antibiotics reduced the amplitude of the 6 ms (slow) luminescence without affecting its relation with the fluorescence induction suggesting possibly that a constant electrical gradient exist in the dark or formed very rapidly in the light, which effects the emission intensity.

7. Changes in salt levels of suspending media equally affected the amplitude of both delayed luminescence and variable fluorescence under conditions when the reduction of Q is maximal and constant.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS I, Photosystem I; PS II, Photosystem II.

8. The results are discussed in terms of several models. It is concluded that the model of independent Photosystem II units together with photosynthetic back reaction concept is incompatible with the data. Other alternative models (the "lake" model and photosynthetic back reaction; recombination of charges in the antenna chlorophyll; the "W" hypothesis) were in closer agreement with the results.

Introduction

A survey of the literature reveals quite a few papers which report that delayed luminescence and prompt fluorescence from isolated chloroplasts often vary in a parallel way. To give a few examples, Clayton demonstrated [1] that during a regime of repetitive on-off irradiation, typical of the phosphoroscope technique, the delayed luminescence observed after 250 ms from the on period was modulated by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and $K_3Fe(CN)_6$ in exactly the same way as the prompt fluorescence. Wraight [2] showed using the same technique that the delayed luminescence observed at 1 ms increased during the chopped continuous irradiation, parallel to a similar increase in the prompt fluorescence yield, both tending finally to a steady-state level. For this experiment uncoupled chloroplasts were used to eliminate any influence of pH gradients and membrane potential, which were also claimed [3—7] to modulate the delayed luminescence in addition to the fluorescence yield. Mar et al. [8] reported similar behaviour of delayed luminescence (observed in a range of several milliseconds) and fluorescence from non-treated chloroplasts excited by a continuous series of flashes. However, the faster component of the delayed luminescence behaved in a more complex way. More recently, Barber et al. [9] have also shown a close relationship between yield factors controlling prompt and millisecond-delayed luminescence from illuminated uncoupled chloroplasts treated with DCMU.

In contrast to the above, however, there are also other works which fail to observe a clear-cut correlation between prompt and delayed fluorescence (e.g., ref. 4).

There may be two different reasons why the delayed luminescence should be correlated to the fluorescence yield. Both reasons were put forward by Lavorel [10,11].

(1) When an exciton is formed by the luminescence-generating reaction it would essentially sense the pigment system at which it was formed. Thus the quantum yield of emission from this exciton would be the same as from an exciton formed at the same place by direct absorption. Hence the intensity of delayed luminescence is factored to $\phi_e \cdot J$, where ϕ_e is the emission yield and J is the rate of exciton production. Whether ϕ_e should be taken as equal to ϕ_F, the macroscopic fluorescence yield, depends on the model assumed for the photosynthetic unit.

(2) The recombination hypothesis [11] assumes that delayed luminescence is generated from a back reaction of the primary photoact. If Q^- (the primary acceptor of PS II) is one of the recombination partners and if the second recombinant, on the oxidzing side of PS II, is quickly adjusted to a certain

steady-state in the light, the delayed luminescence should depend directly on Q^-. On the other hand the variable prompt fluorescence yield ($\phi_F - \phi_{F_0}$) is a monotonic function of Q^- [12]. Thus the apparent dependence of delayed luminescence on ϕ_F in the case is indirect, reflecting the dependence of both ϕ_F and delayed luminescence on Q^-.

There are essentially two models of the photosynthetic unit which also must be considered:

(1) The independent-units model predicts that as an exciton is formed by a back-reaction the unit has an open trap and hence the emission yield from this unit would correspond to ϕ_{F_0}. The delayed luminescence intensity will be proportional to $\phi_{F_0} \cdot [Q^-]$, (i.e., $\phi_e = \phi_{F_0}$; $J \propto [Q^-]$). Writing q for the fraction of "closed" reaction centers (i.e., Q^-/Q_{total}) and $\Delta\phi_F$ for the variable fluorescence when normalizing its maximal extent to 1 (i.e., $\Delta\phi_F = (\phi_F - \phi_{F_0})/(\phi_{F_{max}} - \phi_{F_0})$), the relation between the two in the independent-units model is simply $\Delta\phi_F = q$. Hence the relation between the delayed luminescence and the fluorescence should be written:

$$L \propto \phi_{F_0} \cdot q = \phi_{F_0} \cdot \Delta\phi_F \tag{1}$$

(2) The "lake" model, where energy transfer between several photosynthetic units is allowed, results in the following equation which connects between q and the fluorescence yield [13]:

$$\phi_F = \frac{R_p \phi_{F_0}}{R_p - (R_p - 1)q} \tag{2}$$

R_p is a parameter, constant for a given sample, equal to the ratio $\phi_{F_{max}}$: ϕ_{F_0}. From this equation the (normalized) variable fluorescence ϕ_F is given by:

$$\Delta\phi_F = \frac{(1-p)q}{1-pq} \tag{3}$$

where p is a constant defined as $p = 1 - 1/R_p$.

Two extreme hypotheses with regard to the emission yield of the delayed luminescence exciton can be assumed: (a) The luminescence exciton escapes easily the trap formed after recombination. Its emission yield is essentially equal to ϕ_F. Assuming again that $J \propto (Q^-)$. The intensity of delayed luminescence given by $L \propto \phi_F \cdot q$ will result after simple algebraic manipulation of Eqn. 2 and 3 in the following equation:

$$L \propto \phi_F \cdot q = \phi_{F_{max}} \cdot \Delta\phi_F \tag{4}$$

(b) The luminescence exciton is in the vicinity of an open trap, and most probably will be quenched by it. In this case, the situation is similar to the independent units model, with some modification. Writing in this case that $\phi_e \approx \phi_{F_0}$ and $J \propto Q^-$:

$$L \propto \phi_{F_0} \cdot q = \phi_{F_0} \cdot \frac{\Delta\phi_F}{(1-p) + p\Delta\phi_F} \tag{5}$$

This equation predicts a monotonous but not a linear relation between L and $\Delta\phi_F$, with a coefficient which is independent of the absolute value of $\phi_{F_{max}}$. This point is actually very similar to the case predicted by Eqn. 1.

The three models give thus 3 different relations of L with respect to the fluorescence yield. The first two predict linear relation of L vs. $\Delta\phi_F$ but differ in that they predict a relation to either ϕ_{F_0} or $\phi_{F_{max}}$. These predictions can be checked by the experiment.

This work is an extension of the preliminary work reported elsewhere [14]. The main aim was to establish the experimental relations between the fluorescence and the delayed luminescence and to decide what actually controls the delayed luminescence: ϕ_F, Q^-, or both in accordance with the various models.

Materials and Methods

Apparatus. The basic apparatus was a conventional phosphoroscope, in which delayed luminescence could be detected by a photomultiplier during the off-period of the chopped exciting light. A second photomultiplier was optically connected by a light-guide from the sample to monitor the prompt fluorescence during the on-period. The outputs of the two photomultipliers were fed into two oscilloscopes having matched time scales. Both oscilloscopes had memory screens and the traces were photographed on Polaroid film. Both oscilloscopes were triggered at the same time by the opening of an actinic light shutter. The phosphoroscope motor speed could be adjusted continously, however, one speed was chosen after some initial experimentation. This speed gave 3 ms for the illumination period and ≈6 ms for the off-period with a small interval of time (≈0.8 ms) between the beginning of the off-period and the commencement of the delayed luminescence measurement.

The exciting light and preillumination were provided from a 25 V quartz-iodine lamp with focusing arrangement. The light was filtered either by a broad band filter (Schott glasses BG18, BG38 and GG475) transmitting roughly between 470—620 nm (limits of 10% of the peak transmission), or by a narrow band interference filter peaked at 550 nm (Balzer B40, half band width ≈10 nm). In some experiments the exciting light was not filtered at all, to achieve maximum intensity (in this case the fluorescence could not be measured, but only the delayed luminescence). Far-red preillumination was supplied from the same light source and was isolated through an interference filter (725 nm, Balzer B40). In some experiments very brief (≈10 μs) flashes were given for preillumination. For this, a xenon flash lamp was installed in the apparatus (Applied Photophysics). The delayed emission was filtered through a cut-off filter (Schott glass RG 660) and the fluorescence was filtered through an interference filter (685 nm, Balzer B40). Some of the experiments were carried out in a flash phosphoroscope. In this case, the illumination source was a flash lamp delivering brief (≈10 μs), nearly saturating flashes. Again, rotating discs were provided to screen the photomultiplier. In this set-up the signal was processed in such a way so that only a certain chosen portion in time of the decaying delayed luminescence signal was selected.

Procedure of the experiment. About 2—4 μl of stock chloroplast suspension (containing 4—6 mg chlorophyll per ml) were diluted in 2 ml of reaction mixture and placed in a regular 4-sided 1-cm spectrophotometric cell. In most of the experiments far-red illumination was given for 1 min, then the shutter

was closed and the far-red filter was replaced by one of the actinic light filters. The two oscilloscopes were triggered together to start their sweeps and the shutter was opened as quickly as possible (≈2 ms). When the effect of various dark intervals (elapsing from a previous exposure) was checked, actinic light was given for 10 s followed by the appropriate dark period.

Materials. For most experiments whole chloroplasts were prepared from market spinach, as described previously [15], following essentially the method of Stokes and Walker [16]. The chloroplasts were either diluted directly in any chosen reaction medium, or first osmotically shocked by diluting into distilled water, and then a reaction mixture was added. No essential difference was found between these two methods. With the flash phosphoroscope class B chloroplasts were prepared following the method of Schwartz [17].

Results and Discussion

General characteristics of the delayed luminescence trace

Fig. 1A shows fluorescence and delayed luminescence of two phosphoroscope cycles recorded on the same oscilloscope (double beam mode). It shows the time relationships of the on-period (at which fluorescence was recorded), the off-period and the time interval (most of the off-period) at which delayed luminescence was recorded. This figure was taken at the steady-state. However, the essential form of the delayed luminescence trace is not much different if measured at other regions of time elapsing from the start of the exposure. It is composed of relatively fast decaying component ($t_{1/2}$ = 2—3 ms) followed by a slow component ($t_{1/2} \approx 50$ ms). The slow component cannot be defined from the phosphoroscope traces, since the measurement period was relatively short. It could be isolated after the actinic light was terminated by closing the shutter (Fig. 1B). This general profile of the delayed luminescence in this time range was reported previously, for example, by Itoh and Murata [18].

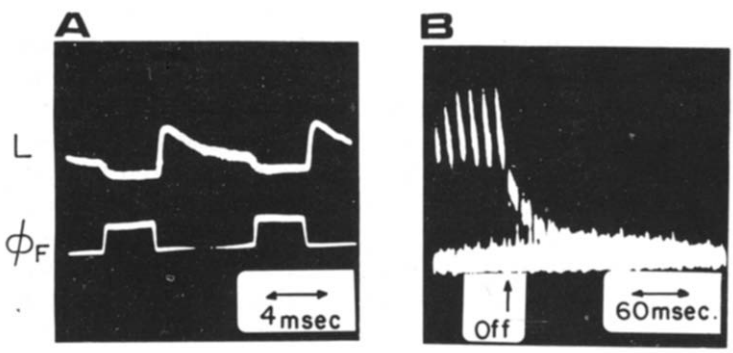

Fig. 1. Delayed luminescence decay pattern. (A) Simultaneous measurement of prompt and delayed luminescence. Lower trace-fluorescence (showing also the on-period of the phosphoroscope). Upper trace-delayed luminescence. Actinic light-broad filter ($2 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} average incident intensity); steady-state conditions. (B) Isolation of the "slow" component of the delayed luminescence decay. The "fast" component is recorded as almost vertical lines, the top of which is out of scale. At the arrow the shutter was closed. Before the arrow about 20 phosphoroscope cycles were given. After the arrow one observed the decay of the slow component. Conditions as in (A).

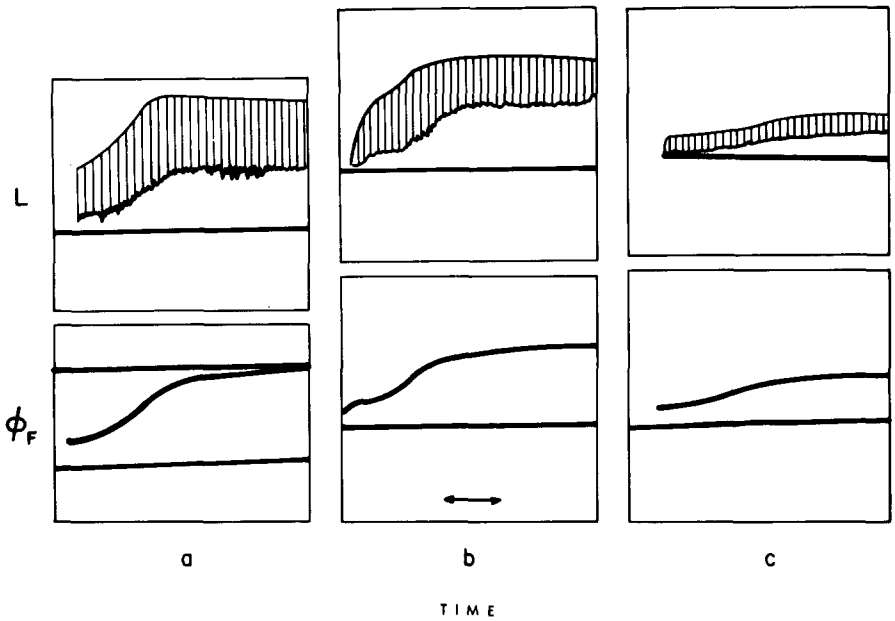

Fig. 2. Copies from oscilloscopic traces, examplifying a correlation between delayed luminescence and prompt fluorescence during the induction period. Upper traces, delayed luminescence. Lower traces, fluorescence. Chloroplasts broken by osmotic shock. (a), suspension in 0.3 M sucrose, light intensity 100%. (b) suspension in 0.1 M KCl, light intensity 51%. (c) as in (b), light intensity 24%. Chlorophyll concentration ≈ 5 μg/ml. Far-red preillumination was given (725 nm, 1.8 neinsteins · $cm^{-2} \cdot s^{-1}$) and an actinic illumination from a broad band filter ($\approx$480—620 nm) was used. 100% intensity $\equiv 2 \cdot 10^5$ ergs · $cm^{-2} \cdot s^{-1}$ ($\approx$10 neinsteins · $cm^{-2} \cdot s^{-1}$).

To observe slower induction phenomena, the brightness of the electron beam of the oscilloscope was reduced, so that only the signals from the delayed luminescence proper were registered and the off and on (almost vertical) signals were not. At very low sweep rates, the individually delayed luminescence traces superimposed on the screen and only the top and bottom envelope were discriminated (Fig. 2). The final point of each delayed luminescence trace (i.e. the bottom of the envelope) represents approximately the slow component of delayed luminescence. This approximation was checked by switching off the illumination at any induction time (e.g., Fig. 1B), obtaining a slow decay trace which indeed extrapolated to the bottom of the envelope. The difference between the top and bottom of the delayed luminescence envelope was taken as the contribution of the "fast" component.

Correlation of delayed luminescence and prompt fluorescence during the induction period

After far-red preillumination, an exposure to continuous actinic light causes simultaneous rise both in the delayed luminescence and the prompt fluorescence. The rise in the prompt fluorescence was previously interpreted in terms of the reduction of Q, which quenches the fluorescence in its oxidized state [12]. Concomitantly with the reduction of Q, a large pool of electron carriers is also reduced, very probably by electron transfer from Q^-, requiring relatively

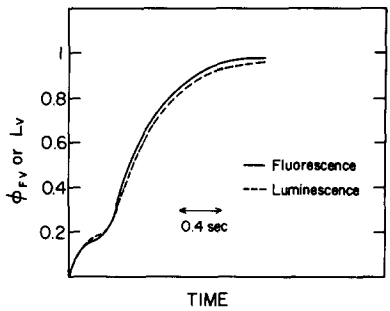

Fig. 3. Graphical comparison of the variable fluorescence and variable luminescence transients. In this particular experiment pea chloroplasts were used, suspended in 0.1 M KCl. Preillumination: far-red (1 min). Actinic illumination, broad-band filter; intensity ≈ 10 neinsteins $\cdot$ cm^{-2} $\cdot$ s^{-1}.

large input of quanta (10—20 per reaction center). This is reflected by the relatively slow rise kinetics of the prompt fluorescence [19].

We observed that the delayed luminescence increased as the fluorescence yield increased. However there was a striking difference between the two decay components. A clear correlation to the fluorescence rise was seen only for the bottom of the delayed luminescence envelope ("slow" component). The "fast" component, however, did not follow the fluorescence changes, especially at high actinic light intensities (see Fig. 5). In this paper we have only concentrated on the properties of the slow component especially since, as we discovered later, some of the fast component included a constant luminescence artifact from the cuvette. However, a thorough study of the fast component has now been made and will be reported elsewhere [20].

The rise in the "slow" delayed luminescence shows all the characteristics of the fluorescence rise, noticeably the typical marked inflection at the beginning of the induction. If only the variable parts are compared, after normalization to the same amplitude, there is an almost linear relation between delayed luminescence and prompt fluorescence. Fig. 2 shows a few traces of the original recordings. Fig. 3 compares graphically the normalized changes of a selected example, and Table I brings the ratio of the variable delayed luminescence and the variable prompt fluorescence, obtained at different times for several experiments. This ratio remains essentially constant as required by a linear correlation *.

Thus, it is either Eqns. 1 or 4 which are obeyed. Eqn. 5 is approximated to a linear relation if p is small enough; if the accuracy of our measurements is within 10%, Eqn. 5 will agree with the results for $p \leqslant 0.2$, as checked by a numerical comparison of Eqn. 5 for various values of p and $\Delta\phi_F$. This, however, is in contradiction with the value of p obtained from its definition (i.e., $p = 1 - 1/R_p = 1 - \phi_{F_0}/\phi_{F_{max}}$ (cf. Introduction)), which in our case was around $p = 0.7$, as well as to the value of p frequently cited in the literature, which is around 0.5. Later we shall drop Eqn. 5 for still another reason (the relation to ϕ_{F_0}).

* At short induction time the ratio seems to vary; however, this is mainly due to the comparison of small extents which are difficult to estimate accurately from the recording and are subject to very large errors.

TABLE I

NUMERICAL COMPARISONS BETWEEN FLUORESCENCE AND LUMINESCENCE TRANSIENTS
Time scale: for Sample I ≈0.5 s, for Sample II ≈3 s, for Sample III ≈0.5 s.

Time * (arbitrary scale)	Ratio of extent of variable fluorescence : variable luminescence **		
	Sample I ***	Sample II †	Sample III ††
0	—	—	—
0.1	1.0	1.4	—
0.2	1.6	1.3	0.98
0.3	1.4	1.2	0.98
0.4	1.25	1.1	1.0
0.5	1.09	1.0	1.0
0.6	1.14	1.0	
0.7	1.16	1.0	1.0
1.0	1.25		1.0

* For each experiment the absolute time scale is different due to different induction times. The absolute time scale is given for each sample below. At time zero the induction transient starts with a value zero, at time 1 the extent of the transient is about 90—95%, and at time 0.5 it is about 40—60%.

** In this table there was no attempt to normalize the extents and the ratios are therefore arbitrary (incidentally very close to 1). The significant point is that they remain approximately constant throughout the induction time.

*** Chloroplasts at high light density (≈10 neinsteins · cm^{-2} · s^{-1}).

† Low light intensity ≈2 neinsteins · cm^{-2} · s^{-1}.

†† With DCMU $5 \cdot 10^{-6}$ M, low light intensity ≈1.4.

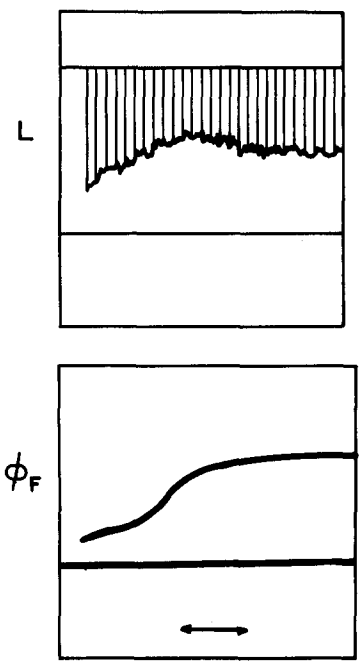

Fig. 4. Fluorescence and delayed-luminescence transients at the highest intensity attainable. Whole chloroplasts in the grindling medium. Light intensity ≈20 neinsteins · cm^{-2} · s^{-1} (wide-band filter).

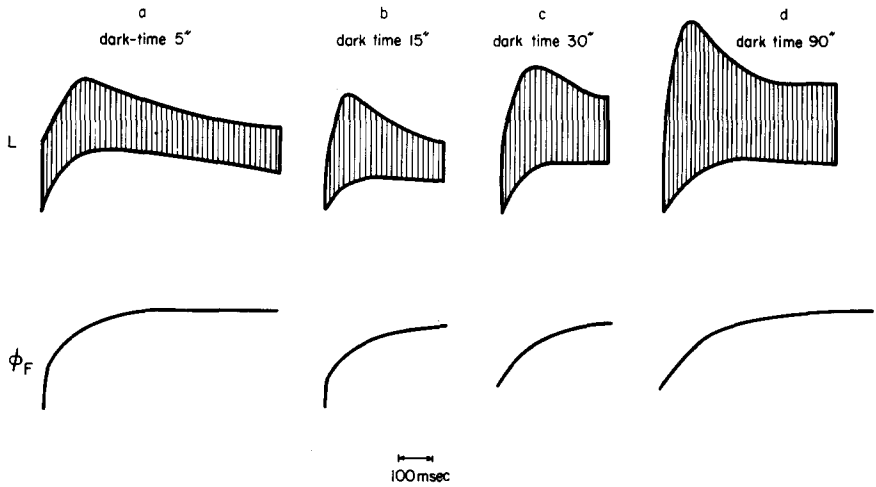

Fig. 5. Effect of dark intervals between a previous exposure to the experiment. (a) 5 s; (b) 15 s; (c) 30 s; (d) 90 s. Osmotically shocked chloroplasts susupended in 0.1 M KCl. Light intensity ≈10 neinsteins · $cm^{-2} \cdot s^{-1}$.

Conditions at which the correlation of delayed luminescence and prompt fluorescence is observed

The correlation observed between the fluorescence rise and the delayed luminescence was obtained in a wide range of light intensities, approx. between $2 \cdot 10^4$ erg · $cm^{-2} \cdot s^{-1}$ * (averaged in time) down to 70 erg · $cm^{-2} \cdot s^{-1}$ (where the measurement terminated because of increased noise : signal ratio). At very high intensities this correlation became gradually less obvious and other factors probably exert more influence (Fig. 4).

The correlation to the fluorescence rise was particularly lost when the induction was measured, not after far-red preillumination, but after a certain dark period elapsing from a previous actinic exposure. Fig. 5 shows a series of delayed luminescence and fluorescence rise curves at various dark-times. Both signals increase during illumination, but their kinetics do not match: the fluorescence rise is now composed of a fast and slow phase while that of the "slow" delayed luminescence is smoother and does not correspond to any one of the fluorescence phases. Furthermore, the "slow" delayed luminescence decreases slightly, following the initial increase, towards a steady-state level. However, as the dark-time increases the correlation between delayed luminescence and fluorescence gradually reappears.

It was interesting to observe that the correlation between the fluorescence and delayed luminescence persisted even in the presence of DCMU (Fig. 6). In this case the rise time of the fluorescence and delayed luminescence was smaller by a factor of 10 approximately, due to the inhibition of the electron transport between Q^- and the rest of the electron carrier pool [21]. With

* This number corresponds roughly to 10 neinsteins · $cm^{-2} \cdot s^{-1}$ of incident light (taking as an average wavelength λ = 550 nm), roughly equal to 3 neinsteins · $cm^{-2} \cdot s^{-1}$ of absorbed light in a sample containing 5 μg chlorophyll/ml. From the fluorescence induction time in presence of DCMU, this light intensity corresponds approx. to 20 quanta/reaction center s.

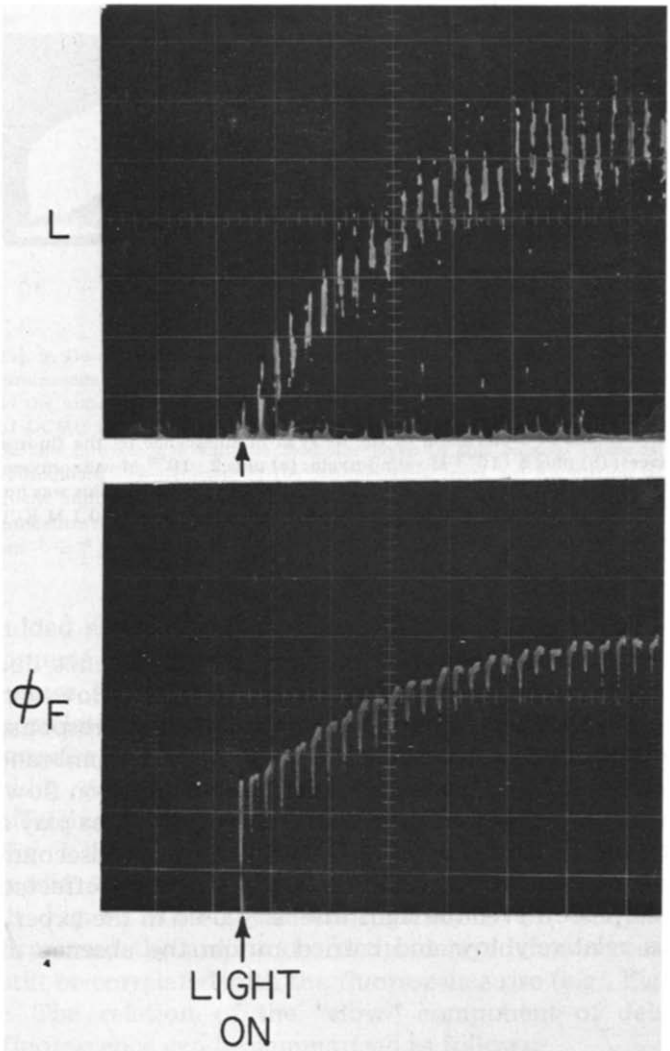

Fig. 6. Induction of delayed luminescence and fluorescence in the presence of DCMU (5×10^{-6} M). Lettuce chloroplasts in 0.1 M KCl and 8 mM Tris, pH 9. Illumination was provided from a narrow band filter (590 nm) of intensity 2.5 neinsteins · cm^{-2} · s^{-1}. 50 ms/div.

DCMU, preillumination with far-red light did not make any difference, and the complete rise transient was restored within 10 s. We noticed a secondary effect of DCMU on the steady-state level of the delayed luminescence attained at the end of the rise, which in some cases slowly dropped to very low level (in a time range between a few seconds to minutes). This decay was seen equally with dark or light incubation with DCMU. This effect cannot be related immediately to the direct inhibition by DCMU of the electron transport chain, which is observed at the moment of its addition. (cf. Fig. 9).

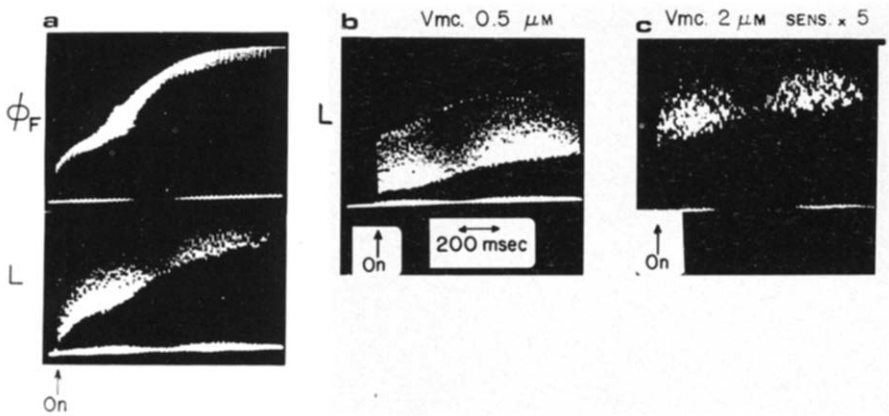

Fig. 7. Effect of valinomycin on the extent of modulation of the delayed luminescence by the fluorescence yield. (a) control (bottom trace); (b) plus $5 \cdot 10^{-7}$ M valinomycin; (c) plus $2 \cdot 10^{-6}$ M valinomycin. The sensitivity of trace (c) is 5 times higher than that of (a) or (b). (a) top trace-fluorescence; this was not affected by the addition of valinomycin. (Osmotically shocked) chloroplasts suspended in 0.1 M KCl. Light intensity $\approx$10 neinsteins $\cdot$ cm^{-2} $\cdot$ s^{-1}.

Effect of uncouplers

The correlation shown above indicates a rise of delayed luminescence due either to the rise in the emission yield or in the concentration of Q^-. However, it has also been reported [3—7] that delayed luminescence increases in response to the development of a "high energy state" (pH gradient and membrane potential across the thylakoids) brought about by light-induced electron flow. It seems that in the above experiments the "high energy state" does not play a role for the following reasons. Firstly, only the slower phase of millisecond-delayed luminescence has been considered, which is not significantly effected by the high energy state [22]. Moreover the light intensity used in the experiments reported above was relatively low and carried out in the absence of

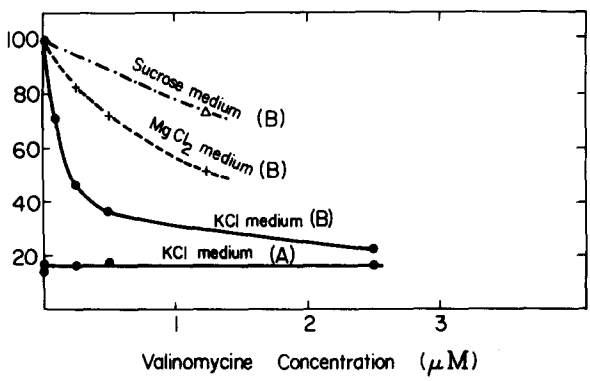

Fig. 8. Concentration dependence of the valinomycin effect in different media. KCl medium $\equiv$ 0.1 M KCl, 5 mM Tris, pH 7.6. $MgCl_2$ medium $\equiv$ 2.5 mM $MgCl_2$, 25 mM Tricine, pH 7.6, 0.1 M sucrose. Sucrose medium $\equiv$ 0.25 mM sucrose. A and B designated the parameters of the delayed luminescence transient. B is the amplitude of the transient and A is the initial value (Eqn. 5).

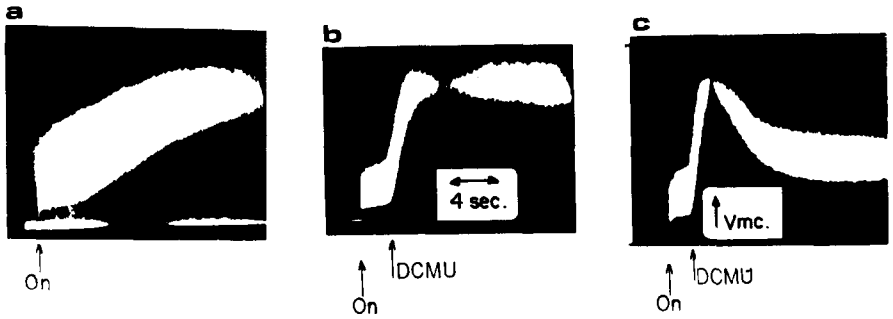

Fig. 9. Demonstration of the effect of DCMU, and valinomycin in the presence of DCMU, on the delayed luminescence. (a) Delayed luminescence transient of control chloroplasts (in 0.1 M KCl). (b) Repetition of the same experiment with addition of DCMU at the point indicated. It is seen that the primary effect of DCMU is to immediately increase the delayed fluorescence level (as expected on the basis of its effect on the fluorescence and the evidence given in this paper). There is, however, a secondary effect of a subsequent slow decrease of the luminescence level. (c) Repetition of the experiment of (b) with subsequent addition of valinomycin. The additions were made by rapid injections from 10-μl syringes. Final concentration of DCMU $5 \cdot 10^{-6}$ M and valinomycin $1 \cdot 10^{-6}$ M. Light intensity ≈ 1.4 neinsteins $\cdot$ $cm^{-2} \cdot s^{-1}$.

added electron acceptor such that it was unlikely that sufficient electron flow occurred to create a ΔpH across the thylakoid membrane. A support to this comes from the fact that DCMU had no immediate effect on the level or the correlation of the prompt and delayed emissions (cf. above). Thus it was a surprise to observe that ionophores, such as valinomycin, nigericin and gramicidin decreased the amplitude of the delayed luminescence rise without affecting fluorescence. The effect of valinomycin on the signal is shown in Fig. 7, while Fig. 8 gives the concentration dependence in various reaction media. The effect of valinomycin in the presence of DCMU is demonstrated in Fig. 9. With those concentrations of ionophore which only partially decreased the amplitude of the delayed luminescence rise, the kinetics could still be correlated with the fluorescence rise (e.g., Fig. 7).

The relation of the "slow" component of delayed luminescence to the fluorescence can be summarized as follows:

$$L = A + B\,\Delta\phi_F(t) \tag{6}$$

A and B are coefficients which are constant during the induction period. The dependence of L on the time t is reflected through the variable fluorescence yield $\Delta\phi_F$, which is a function of time t. The term A may include some artifact luminescence from the cuvette. $\Delta\phi_F$ is defined such that its total maximal extent is 1. Therefore, A is the initial, and $A + B$ the final values of the delayed luminescence. In most experiments the ratio $(A + B) : A$ was in the order of 20 and the increase of delayed luminescence was indeed very striking.

The effect of antibiotics, according to this formulation, is to reduce the amplitude B, without affecting $\Delta\phi_F$ or A. Thus, the ratio $(A + B) : A$ decreases with the addition until no rise is observed and A becomes the dominant factor. Although no satisfactory explanation to the effect of the ionophores is provided it is suggested that B reflects the existence of a constant electrical gradient [23—25], either existing in the dark (resting potential) or very rapidly

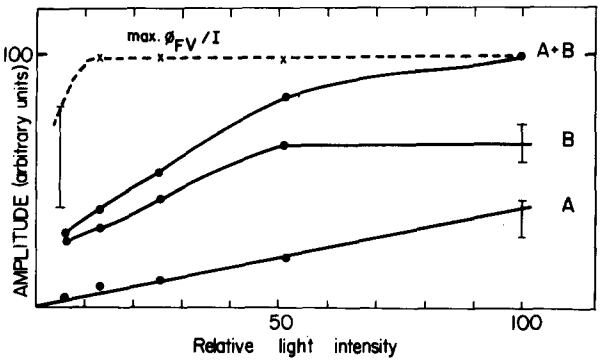

Fig. 10. The effect of actinic light intensity on the parameters of delayed luminescence compared to its effect on the fluorescence yield. Chloroplasts suspended in 0.1 M KCl. 100% intensity corresponds to approx. 10 neinsteins · cm^{-2} · s^{-1}.

induced in the light [4], immediately at the onset of the induction period. This potential collapses by the antibiotics.

Dependence of B on the light intensity

The amplitude *B* depends considerably on the light intensity, even in a range of intensities where the final steady-state fluorescence yield remains constant *. This is shown in Fig. 10 and indicates the effect of other factors besides ϕ_F (or Q^-) on delayed luminescence. These factors could be the steady concentration of the recombining species (besides Q^-), e.g., on the donor side of PS II, or could involve the membrane effects rapidly induced in the light (cf. above).

Correlation of the fluorescence yield increase and delayed luminescence in flash phosphoroscope

In the previous experiments the light was given continuously. Essentially the same results were obtained when saturating, short (3 μs) flashes were delivered to the sample, one flash in each phosphoroscope cycle. In this case, there is one turn-over of the reaction center per phosphoroscope cycle. The conditions are similar to the experiment of Forbush and Kok [28] where it was shown that following a flash, Q is reduced completely and is oxidised very quickly (≈0.6 ms) by transfer of electrons to the secondary pool. The pool and Q equilibrate in electrons and the fluorescence yield rises gradually and reaches maximum in about 20 flashes. Fig. 11A shows the induction kinetics of luminescence at two decay times (20 and 50 ms) and Fig. 11B compares the fluorescence and the luminescence inductions obtained under the same conditions. Both emissions are again nicely correlated. Note, like fluorescence, the delayed luminescence shows a characteristic inflection, which is particularly marked in this kind of experiment.

* At sufficient low intensities, the steady-state fluorescence yield decreases, paralleling a decreased level of reduced Q(Q^-) [26,27].

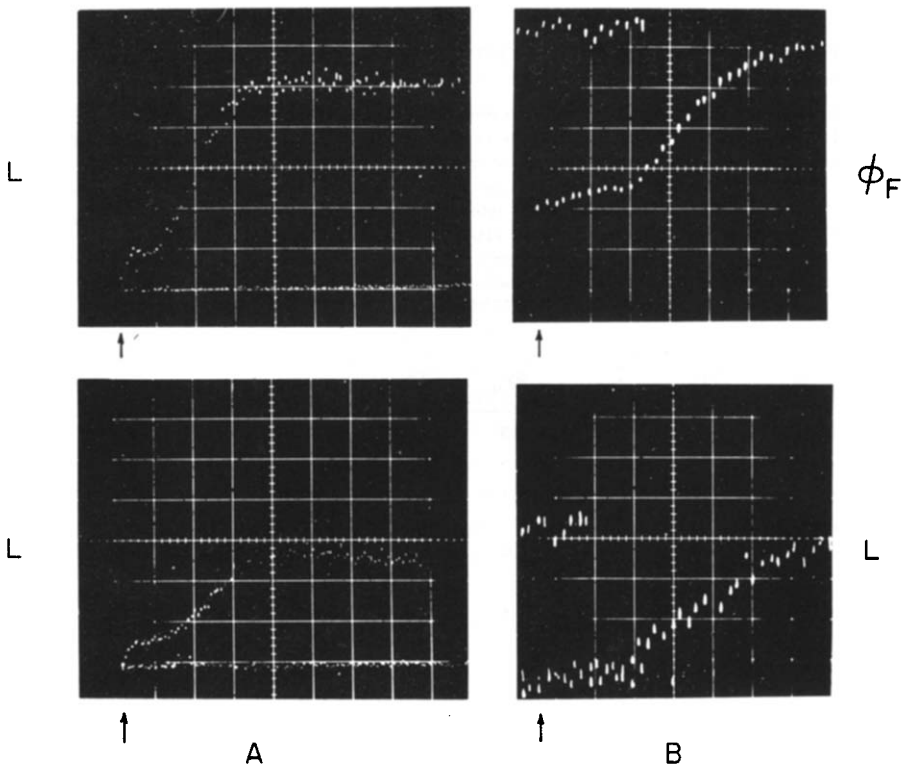

Fig. 11. Observations with the flash phosphoroscope (in the Martin-Marietta Laboratory. The basic construction is described in [29]). (A) Basic observations: each dot corresponds to delayed luminescence at specific time-point of the decay: (Top, at 20—21 ms, Bottom at 50—55 ms) excited by each flash. The base level was achieved by screening the sample from the flashes. The rate of flashing was 20 flashes/s (this rate was checked for saturation with regard to the maximal extent). The top was reached in about 30 flashes (which were checked for saturation). The time scale, 0.5 s/div. (B) Correlation of delayed luminescence and fluorescence transients. The fluorescence was monitored in a separate experiment by weak continuous measuring beam and was changed by the action of the flashes (top). The fast fluorescence transient due to each flash, which occurs within 1 ms [32], were not recorded, and only a small time interval at 50 ms from the flash was monitored. The delayed luminescence at 50 ms was measured in a separate experiment (bottom) done under identical conditions (except for the elimination of the measuring beam). Note here that delayed luminescence starts at level zero, in contrast to the fluorescence starting level. Spinach chloroplasts in 0.1 M KCl and Tris buffer 0.01 M, pH 7.5 (approx. 10 μg chlorophyll/ml).

The effect of the reaction medium

The basic question whether the luminescence rise follows the fluorescence rise or rather the induction of Q or both (cf. Introduction) was approached by testing the effect of environmental factors which affect the fluorescence yield without affecting Q [29]. We have tested the effect of three reaction media which change the extent of the maximal fluorescence, $\phi_{F_{max}}$. The amplitude B of the delayed luminescence rise changed in nearly a parallel way (Table II). Similar results were reported by Barber et al. [9] and by Hipkins [30]. In the present case, the best fit to the data is to write:

$$B \propto (\phi_{F_{max}} - \phi_{F_0}) \tag{7}$$

In agreement with the data of Hipkins [30]. The data of Barber et al. [9] obtained for 1 ms luminescence, in presence of uncouplers and DCMU,

TABLE II

VARIATION OF FLUORESCENCE AND DELAYED LUMINESCENCE PARAMETERS AT VARIOUS REACTION MEDIA

Numbers are in arbitrary units, but comparable for each experiment. Each experiment is with a different chloroplast batch. The main results of this table is the similar ratios for $L_V/\phi F_V$ inspite of considerable changes in the individual values at various reaction media. This shows that L_V follows the variable fluorescence not only when Q is reduced, but also for the same level of Q^- at different reaction media, i.e., $L_V \propto \phi F_{max} \cdot \Delta\phi_F$ rather than $L_V \propto \Delta\phi_F$, only the later expression being equivalent to $L_V \propto (Q^-)$. On the reasons why different media effect the fluorescence yield consult ref. 33.

Medium	Sucrose (0.2 M)			Sucrose (0.2 M) KCl (0.02 M)			Sucrose (0.2 M) KCl (0.02 M) $MgCl_2$ (0.006 M)		
	ϕF_o	ϕF_v	L_V	ϕF_o	ϕF_v	L_V	ϕF_o	ϕF_v	L_V
Expt. 1	1.6	3.9	5.2	1.5	1.9	2.3	1.8	3.2	3.5
Ratio $L_V : \phi F_V$			1.3			1.2			1.1
Expt. 2	1.6	4.3	5.0	1.7	2.2	2.4	1.7	3.5	3.3
Ratio $L_V : \phi F_V$			1.16			1.09			1.09
Expt. 3	1.5	3.5	2.7	2.0	2.2	1.85	1.6	3.8	2.6
Ratio $L_V : \phi F_V$			0.77			0.84			0.68
Expt. 4	2.0	4.0	3.0	2.2	3.8	3.0	2.0	6.0	4.0
Ratio $L_V : \phi F_V$			0.75			0.75			0.66
Expt 5	—	—	—	1.6	2.0	1.4	1.7	3.0	2.2
Ratio $L_V : \phi F_V$						0.7			0.73

supports the relationship

$$B \propto \phi_{F_{max}} \tag{8}$$

The difference between the results may not be serious, especially if ϕ_{F_0} in the last case was small compared to $\phi_{F_{max}}$. Qualitatively, at least they agree in the direction of the effect.

The implication of the last result is considerable: first it eliminates the model in which recombination and independent units are combined together, since this model predicted that B should change only as ϕ_{F_0} (cf. Introduction), which is not the case. It nearly fits with the "lake" model assuming the recombination hypothesis with the additional assumption that the delayed luminescence exciton senses the macroscopic fluorescence yield. The fit would be perfect if B was proportional to $\phi_{F_{max}}$ as in [9] rather than to $(\phi_{F_{max}} - \phi_{F_0})$. May be the term ϕ_{F_0} (or most of it) should be subtracted, even in the "lake" model, if it represents foreign contribution ("dead" fluorescence), as sometimes may be the case [31]. This, however, contradicts with Paillotin [32].

One can interpret the results in terms of other models. For example, one can venture to drop the idea that the slow delayed luminescence is generated by a back-reaction of the photosynthetic primary charge separation. The alternative assumption is that a parasitic side reaction with very low yield generates the luminescence. For example, an exciton could cause charge separation in the pigment system itself ($Chl^*\ Chl \rightarrow Chl^+\ Chl^-$) which are stabilized for some time on some impurity traps and give rise after recombination to lumines-

cence *. This luminescence will sense the actual macroscopic fluorescence yield and hence in this case $L \propto \phi_F$, i.e., $L \propto \phi_{F_{max}} \cdot \Delta\phi_F$. The exact model of the photosynthetic units pigment system is irrelevant for this case. Since the initial delayed luminescence is very small (compared to the final level) in comparison with the initial fluorescence, this model must assume that most of ϕ_{F_0} should be substracted as irrelevant "dead" fluorescence, not originating in PS II. This arguement had been raised before by Clayton [1].

An alternative hypothesis, although yet speculative, stems from the "w" hypothesis of van Best and Duysens [33]. They observed that if they convert Q to Q^- (by dark reduction under anaerobic conditions of whole algal cells) the luminescence in the μs range increases despite the apparent fact that the reaction centers were "closed" and would not support photosynthetic charge separation. Again, this could be explained by non-photosynthetic luminescent reaction, as explained above. However they liked to consider a different explanation in that Q is not the primary acceptor, but rather a secondary one. They introduced a primary acceptor W. Its reduced form, W^-, is very transitory and transfers its electron very rapidly to Q. Because of electrostatic interactions, Q^- inhibits the primary charge separation and hence the fluorescence yield associated with Q^- is high. The primary charge separation of a unit with Q^- can still take place but with a low yield. The following scheme summarizes this hypothesis:

$$PWQ \xrightarrow{h\nu} P^+W^-Q \rightarrow P^+WQ^- \rightarrow PWQ^- \text{ (secondary } e^- \text{ transport from } P^+) \quad \text{(i)}$$

$$PWQ^- \xrightarrow{h\nu} P^+W^-Q^- \rightarrow PWQ^- \text{ (+ delayed luminescence)} \quad \text{(ii)}$$

Reaction i starts with a unit PWQ (P is the primary donor). This unit has low fluorescence yield but also a low yield of delayed luminescence. At the end of the photoreaction the unit is stabilized in the form PWQ^-. Reaction ii starts with PWQ^- having a low yield and stabilizes again in the form PWQ^- but it has relatively high yield of luminescence (recombination of P^+ and W^-).

Originally this scheme was applied to very fast luminescence events (P^+W^- recombination occurring in the μs range). However, it is possible to extend this scheme to much longer times by adding consecutive electron transfer steps on the donor side, e.g., writing a modified Eqn. ii as follows:

$$Z\,PWQ^- \xrightarrow{h\nu} ZP^+W^-Q^- \rightarrow Z^+PW^-Q^- \quad \text{(iii)}$$

while the species $ZP^+W^-Q^-$ contributes to fast delayed luminescence the species $Z^+PW^-Q^-$ contributes to slow delayed luminescence. This argument shows that even "slow" luminescence can be generated from units having intrinsic high fluorescence yield (with Q^-). The emission yield from such units correspond to $\phi_{F_{max}}$. Assuming also the independent units model in this case, the relation between the luminescence and the fluorescence should be written as:

$$L_v(t) \propto \phi_{F_{max}} \cdot [Q^-](t) = \phi_{F_{max}} \cdot \Delta\phi_F(t) \text{ (ignoring the constant term } A)$$

* The evidence that luminescence is affected by photosynthetic parameters (e.g. *S*-states, fluorescence, etc.) and hence must originate from the reversal of the charge recombination is not necessarily true. All the effects could be indirect, e.g. electrostatic field effects can modulate the yield of any reaction taking place in the photosynthetic units.

This equation is similar to the equation obtained previously for the "lake" model assuming the recombination mechanism which again nearly agrees with the experimental results.

In conclusion, our results seem to clarify the type of models with which they nearly agree, and drop others. Still several models survived and further experiments must be conducted to help and choose between them. The following models were found to be inconsistent: (a) independent units plus photosynthetic recombination as the source for delayed luminescence. (b) the "lake" model plus photosynthetic recombination generating an exciton which mainly senses an "open" reaction center. The following models are consistent: (c) the "lake" model, plus photosynthetic recombination generating an exciton which rapidly escapes the open reaction center and senses the macroscopic fluorescence yield, (d) independent units plus photosynthetic recombination in the framework of the "W" hypothesis [33], (e) non-photosynthetic charge separation and recombination.

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