CHARACTERISTICS OF PROMPT AND DELAYED FLUORESCENCE FROM SPINACH CHLOROPLASTS

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ABSTRACT Effects of ferricyanide, dichlorophenyldimethylurea (DCMU), and uncouplers of phosphorylation on the prompt and delayed fluorescences from spinach chloroplasts are described. Any factor that affects the yield of prompt fluorescence will similarly influence the intensity of delayed fluorescence. This idea, recently investigated by Lavorel, should be expressed in terms of a "live" component of fluorescence; that is, the component from chlorophyll associated with the photochemical traps of System II. Some of the effects of ferricyanide and DCMU on delayed fluorescence can then be explained in terms of effects on the yield of prompt fluorescence. From the internal consistency of the explanation, applied to various observations, a judgment can be made that most of the prompt fluorescence observed initially when dark-adapted chloroplasts are first illuminated is "dead," coming from chlorophyll not associated with trap II. The live fluorescence is represented almost entirely by the time-varying component that develops during illumination. The observed intensity of delayed fluorescence can be divided by the yield of live prompt fluorescence to give an intrinsic delayed fluorescence. This intrinsic delayed fluorescence is proportional to the square root of exciting light intensity (as long as the excitation is not saturating) and decays with second order kinetics. This behavior may reflect the photochemical formation and second order dissipation of an oxidized product of Photosystem II.

INTRODUCTION

In green plant tissues the intensity of chlorophyll (Chl) fluorescence shows variations during constant illumination, attributable in part to changes in the states of the photochemical traps serving the oxygen-evolving System II. The traps for System II remain hypothetical, in the sense that they have not been identified chemically. However, an extensive phenomenology (1–3) indicates that they are sites of photochemical oxidoreduction, and that they become closed (nonfunctional) when the primary electron acceptor has been converted to its reduced form. One can write the hypothetical reaction center as a complex between photoactive Chl and an

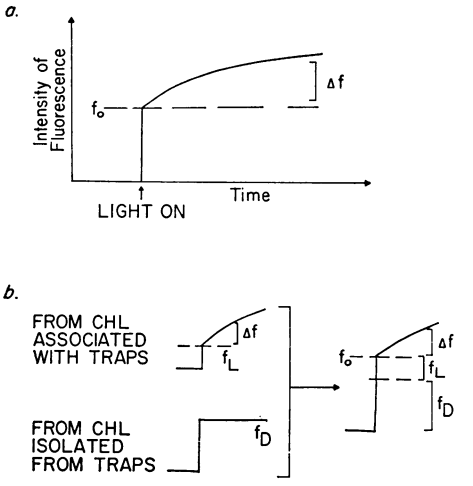

FIGURE 1 (*a*) Typical appearance of the prompt fluorescence from spinach chloroplasts vs. time during constant illumination. (*b*) This fluorescence can be partitioned into hypothetical live (f_L) and dead (f_D) components, from chlorophyll that is or is not associated with photochemical traps. The time-varying part Δf is all "live."

electron acceptor E (in the terminology of Joliot), and formalize the photochemical cycle as follows:

Excitation:	$Chl \cdot E$	$\rightarrow$	$Chl^* \cdot E$	(1)
Photochemistry:	$Chl^* \cdot E$	$\rightarrow$	$Chl^+ \cdot E^-$	(2)
Rapid removal of oxidizing equivalent:	$Chl^+ \cdot E^-$	$\rightarrow$	$Chl \cdot E^-$	(3)
Slower removal of reducing equivalent:	$Chl \cdot E^-$	$\rightarrow$	$Chl \cdot E$	(4)

An open (functional) trap is $Chl \cdot E$; a closed one is typically $Chl \cdot E^-$. This is indicated by the facts that reducing agents tend to close the traps whereas oxidizing agents tend to keep them open. Reaction 4, in which a closed trap becomes open once more, involves transfer of an electron to the carriers leading to System I, or to a Hill oxidant such as ferricyanide. This step is thought to be inhibited by DCMU.[1] The hypothetical photoactive Chl, associated with E in the traps, should be distinguished from the larger component of light-harvesting Chl that absorbs energy and delivers excitation quanta to the photoactive Chl.

The appearance of a typical record of the fluorescence from spinach chloroplasts during constant illumination is sketched in Fig. 1 *a*. An initial level f_0 is displayed at the onset of illumination; this rises to a higher level $f_0 + \Delta f$ in the illuminated steady state. The rise occurs because traps become closed as the illumination progresses. The fact that a dark-adapted preparation emits some fluorescence (f_0) shows that the trapping is not perfectly efficient even when the traps are maximally open. This

[1] The following abbreviations are used: DCMU, (3(3,4-dichlorophenyl))-1,1-dimethylurea. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

can be expected if a quantum, absorbed in an aggregate of Chl associated with traps, has a certain probability of encountering a trap and a residual probability of being dissipated either thermally or as fluorescence. The rise from f_0 to $f_0 + \Delta f$ then reflects the shifting of these relative probabilities as the density of open traps decreases. But the initial fluorescence might also be due, in whole or in part, to a component of "dead" Chl; pigment that is not associated with traps and that returns a certain yield of fluorescence regardless of the state of the traps. We shall therefore distinguish between "live" and "dead" fluorescence; the former is from Chl that can transfer energy to the System II traps, and the latter is from Chl that is isolated from these traps. The trace shown in Fig. 1 *a* can be resolved into these components as shown in Fig. 1 *b*: the time-varying part Δf is all live; the initial part f_0 might be composed of live (f_L) and dead (f_D) contributions.

It is important to distinguish between live and dead fluorescence, and it is not easy to do so experimentally. One extreme (no dead component) has been adopted in evaluating the fluorescence from photosynthetic bacteria with reference to the state of the trapping pigment P870, because the data then agree closely with a simple mathematical model (4, 5). In the case of Chl fluorescence from green plant tissues, the other extreme has usually been adopted: all of the initial f_0 has been ignored, as befits a foreign contribution (2, 6). Live and dead components should have distinct lifetimes; this difficult question has not been completely settled (7, 8). One aspect of the importance of this distinction can be seen in the ensuing discussion of delayed fluorescence.

The delayed fluorescence of Chl in green plants (9) is a sign that light energy has been stored, and then excitation quanta have been regenerated from this stored energy. The mechanism depends on the functioning of System II (10). It is likely, therefore, that the regenerated quanta are formed in the component of Chl that communicates with the System II traps. The spectra of delayed and prompt fluorescence are similar, indicating that the regenerated excited state of Chl is the same as the singlet state formed initially during light absorption (11).

Since prompt and delayed fluorescence both reflect the presence of singlet excitation quanta in the Chl associated with System II, any factor (such as the state of the traps) that modifies the yield of the prompt fluorescence should have a corresponding effect on the yield of the delayed emission. This has been recognized by Butler (12) and by Lavorel (13), who suggested that in any formulation of the delayed fluorescence, the yield as derived from the prompt fluorescence should be written explicitly as a separate factor:

$$\text{Delayed fluorescence intensity} = (\text{prompt fluorescence yield}) \times (\text{other factors}). \quad (5)$$

The correctness of this general idea can be seen in many instances where the time-varying patterns of delayed and prompt fluorescence (corresponding to Δf in Fig. 1)

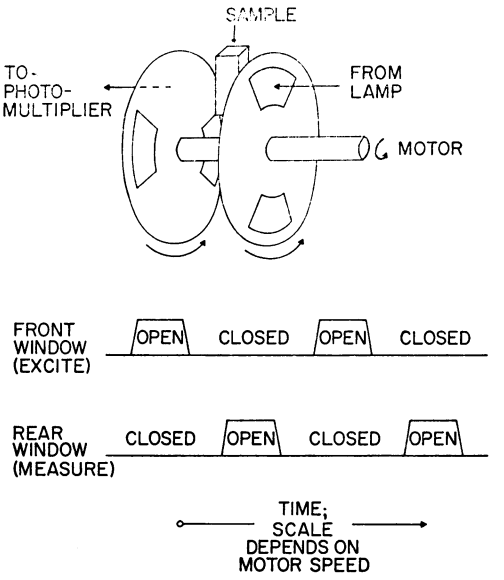

FIGURE 2 Outline of a Becquerel phosphoroscope.

are similar[2] (14). However, a consistent feature of these patterns is that the variations of delayed fluorescence are more conspicuous, forming a larger fraction of the whole, than the corresponding variations of prompt fluorescence. This feature has a simple explanation. The excitation quanta that give delayed fluorescence have been formed by a mechanism involving the System II traps, and have therefore been deposited only in the live Chl that communicates with these traps. The variations in delayed light intensity therefore reflect variations in the yield of the live fluorescence, $f_L + \Delta f$. These variations are relatively much larger than the variations in the total fluorescence, $f_0 + \Delta f$.

The foregoing argument and comparison between variations of prompt and delayed fluorescence can be used in order to decide what fraction of the prompt fluorescence is live and what fraction is dead. The validity of this procedure can be assessed by the degree to which self-consistent results are obtained when the variations are induced in different ways. Once the partition between live and dead fluorescence has been made, the formulation suggested by Lavorel can be made to read

$$\text{Delayed fluorescence intensity} = (\text{yield of live prompt fluorescence}) \times (\text{other factors}). \quad (6)$$

When the fluorescence yield has thus been properly segregated in the expression for delayed fluorescence, the significance of other factors can be evaluated properly.

[2] In reference 14 the basis of the kinetic similarity between prompt and delayed fluorescence was given a less simple and direct interpretation.

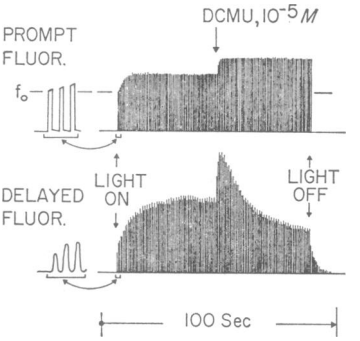

FIGURE 3 Traces of prompt and delayed fluorescence from spinach chloroplasts, obtained with a Becquerel phosphoroscope (see Methods). A spike of prompt fluorescence was recorded whenever the front (excitation) window was open, and a spike of delayed fluorescence when the rear window was open. Exciting light intensity at the sample, with the front window open, was 0.082 mw/cm². Motor speed was 1 rev/sec, giving a delay of 250 msec between excitation and measurement of delayed fluorescence. During the measurement DCMU was injected to give a final concentration of 10^{-5} M. The density of chloroplasts in this and the other measurements corresponded to 5 μg/ml of Chl *a*. The delayed fluorescence was actually about two orders of magnitude weaker than the prompt fluorescence. This should be borne in mind when examining Figs. 3–9.

We shall see that this gives new insight into the effects of some chemicals on the delayed fluorescence, and into the quantitative relations between delayed fluorescence intensity, time, and exciting light intensity.

METHODS

Market spinach was disrupted in an aqueous medium containing 0.6 M sucrose, 0.01 M KCl, and 0.02 M Tricine (pH 7.8) by means of a Waring Blender (Waring Products Co., Winsted, Conn.). The disrupted mixture was filtered through Miracloth (Chicopee Mills, Inc., Milltown, N. J.), centrifuged briefly (about 1 min) at about 1000 *g* to remove larger particles, and then centrifuged at 10,000 *g* for 10 min to sediment the chloroplast fraction. The pellet was redispersed in the medium used for disruption. Concentrations of Chl *a* and Chl *b* were determined as described by Arnon (15). For individual measurements the chilled stock suspension was diluted to a concentration of 5 μg/ml Chl *a* in a medium like that used for disruption but without sucrose.

Additions of chemicals during measurement of light emission were made by injecting, as vigorously as possible for good mixing, 0.15 ml of reagent into 3.0 ml of chloroplast suspension held in a cuvette. Injections of CCCP or DCMU were made by first diluting an ethanolic solution of the reagent 10-fold with water and then injecting the diluted solution into the cuvette. Such experiments were accompanied by controls in which 10% ethanol was injected. All experiments were attended by controls in which water was injected; dilution of the chloroplast suspension caused small changes in the fluorescence signals that were discounted in evaluating the effects of reagents.

Delayed fluorescence was measured with a Becquerel phosphoroscope, the principle of which is shown in Fig. 2. The motor speed determined the time that elapsed between an exciting flash and the next measuring interval; this time could be varied conveniently between

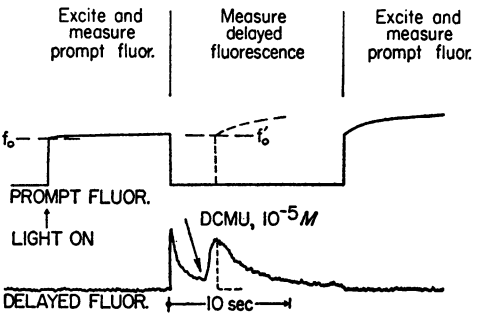

FIGURE 4 Prompt and delayed fluorescence from spinach chloroplasts. In this measurement the motor shaft of the phosphoroscope was given an abrupt quarter-turn in order to change from excitation (and measurement of prompt fluorescence) to measurement of delayed fluorescence, or vice versa. Note that injection of DCMU (10^{-5} M final concentration) caused an increase in the intensity of delayed emission. The dashed line indicates how the delayed fluorescence measurement could be interrupted at any time in order to evaluate the prompt fluorescence. Exciting light intensity 0.036 mw/cm².

about 5 and 500 msec. By leaving the motor off and giving the shaft a quarter turn by hand, it was also possible to make an abrupt transition (in about 50 msec) from a period of excitation to a period of measurement.

Prompt fluorescence could be measured with the same instrument through another window, permanently open, at right angles to the exciting beam. Thus a record of prompt fluorescence was obtained whenever the front (exciting) window was open, and delayed fluorescence when the rear window was open. The detector for prompt fluorescence was shielded from scattered exciting light by a red Corning No. 2-64 filter (Corning Glass Works, Corning, N. Y.); the exciting light was passed through a blue (Corning No. 4-97 plus $CuSO_4$) filter combination.

Both detectors were EMI 9558 photomultipliers (Whittaker Corporation, Plainview, N. Y.) (S-20 spectral response); the photocurrents were amplified and displayed on two channels of a Texas Oscillo-Riter Recorder (Texas Instruments, Inc., Houston, Tex.). Exciting light intensity was measured in units of milliwatts per cm² with a Yellow Springs Instruments Radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The exciting lamp was a 500 w tungsten-iodine lamp operated from a variable transformer. Excitation could also be delivered in short (about 0.2 msec) pulses by means of a xenon flash lamp; the sample then received about 0.1 joules per cm² in a single flash.

Typical records of prompt and delayed fluorescence are shown in Figs. 3 and 4, the former with the motor running and the latter with the shaft given an abrupt quarter turn. These are reproductions of actual recorder traces, chosen to illustrate the effects of DCMU injected during a measurement.

RESULTS AND DISCUSSION

Effects of Ferricyanide and DCMU

The effects of ferricyanide and DCMU on the prompt fluorescence of Chl in chloroplasts are well known and easily interpreted (3). Ferricyanide, acting as an electron acceptor in a Hill reaction, promotes the removal of electrons from the System II

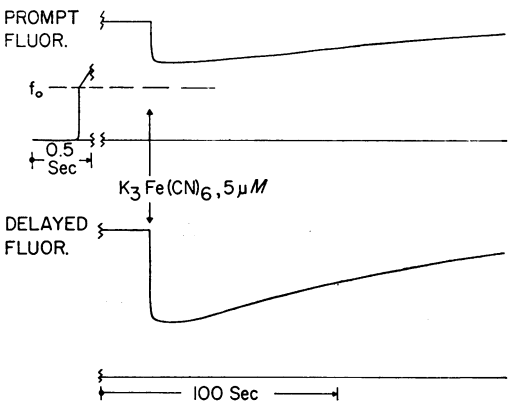

FIGURE 5 Prompt and delayed fluorescence from spinach chloroplasts showing the effects of injecting ferricyanide (5×10^{-6} M final concentration). The measurements were made as in Fig. 3. The curves represent the envelope of the spikes, which are not shown individually. Exciting light intensity 3.1 mw/cm². Time between excitation and measurement of delayed fluorescence 500 msec.

reaction centers (see Equation 4 above);

$$\begin{array}{ccc} Chl \cdot E^- & & Fe^{+++} \\ \downarrow & \cdots\cdots & \downarrow \\ Chl \cdot E & & Fe^{++} \end{array}$$

DCMU, according to traditional interpretation, blocks the first step of this electron transfer sequence. Ferricyanide thus promotes turnover of the photochemical system and keeps the traps in the "open" form Chl·E, whereas DCMU inhibits turnover and allows light to drive the traps into the "closed" form Chl·E⁻. With exciting light strong enough to establish a steady state in which most of the traps are closed, addition of ferricyanide causes an immediate drop in the yield of fluorescence, signifying that the steady state has been shifted to one in which most of the traps are open. Just the opposite effect is induced by DCMU; here the effect is most striking if the initial steady state is one in which most of the traps are open (weak exciting light).

DCMU, 10^{-5} M, blocks completely the transfer of electrons from Chl·E⁻ to ferricyanide. This can be shown by adding ferricyanide to chloroplasts after DCMU (for example, during the second half of the record shown in Fig. 3), and noting that the ferricyanide then causes no change in the intensity of fluorescence. In such an experiment the ferricyanide also has no effect on the delayed fluorescence. This shows that any reduced substances, on the pathway from Chl·E⁻ to ferricyanide but beyond the DCMU block and hence susceptible to attack by ferricyanide, are *not* involved in a rate limiting way in the mechanism for producing delayed fluorescence.

Fig. 3 shows that there is a progressive decline in the intensity of delayed fluorescence after addition of 10^{-5} M DCMU, under the conditions specified in the legend.

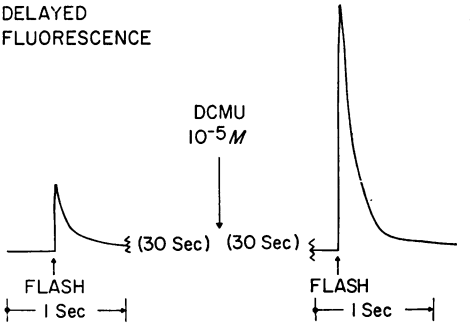

FIGURE 6 Delayed fluorescence from spinach chloroplasts induced by a xenon flash, before and after addition of 10^{-5} M DCMU. Duration of the flash was about 0.2 msec; intensity at the sample was about 0.1 joules/cm^2. The phosphoroscope motor was running at 50 rev/sec and the flash was introduced through the entrance window while it was open.

If the illumination had been continued the delayed fluorescence would eventually have declined to a small fraction of its initial intensity. This decline reflects the progressive dissipation of some precursor of the delayed fluorescence, after the photochemical turnover has been inhibited by adding DCMU. The decaying precursor that governs the intensity of delayed fluorescence is not $Chl \cdot E^-$, which remains present at a high level as shown by the continued high yield of prompt fluorescence.[3] These observations therefore suggest that an oxidized product of Photosystem II is a precursor of the delayed fluorescence. This might be a hole in the Chl aggregate, as suggested by Arnold (16), or it might be any oxidized entity on the pathway from Chl^+ to oxygen.

Whereas DCMU causes a decline in the intensity of delayed fluorescence, ferricyanide causes a stimulation, probably owing to increased photochemical turnover. This effect is striking only under strong exciting light, such that the turnover is limited (and can be influenced) by chemical factors and not by light intensity.

Inhibition by DCMU and stimulation by ferricyanide have been associated, in the published literature, with fast decaying components of delayed fluorescence that are observed during the first few milliseconds after a flash of exciting light (17). Exactly the opposite effects have been reported (18, 19) for the "slow" delayed light measured 100 msec or more after excitation. This has given the impression that the fast and slow components of delayed fluorescence involve different mechanisms. The present experiments will confirm most of the published observations, but will show that the associations of certain effects with fast or slow components of delayed fluorescence are accidental or trivial, and do not require more than one mechanism. It will be shown that the second pair of effects, inhibition by ferricyanide and augmentation by DCMU, can be attributed entirely to changes in the yield of the live component of prompt fluorescence.

[3] This argument is based on the assumption that the traditional view of the site of action of DCMU is correct.

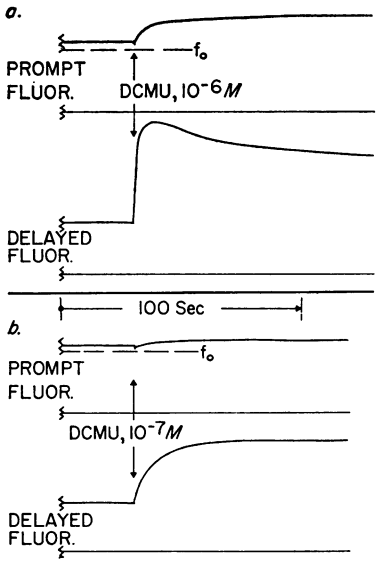

FIGURE 7 Representation as in Fig. 5, showing the effects of 10^{-6} and 10^{-7} M DCMU. Excitation intensity 0.036 mw/cm²; time between excitation and measurement of delayed fluorescence 250 msec.

The action of ferricyanide on prompt fluorescence and on "slow" delayed fluorescence is shown in Fig. 5. This record was obtained with strong exciting light (3.1 mw/cm²) and with the phosphoroscope motor running slowly so that 500 msec elapsed between each flash of exciting light and the subsequent measurement of delayed fluorescence. The representation of Fig. 5 is like that of Fig. 3 except that the individual spikes have been omitted and the envelope of their height is shown instead. Note that the injection of ferricyanide caused an immediate inhibition of both the prompt and the delayed fluorescence. When all of the ferricyanide had become reduced in a Hill reaction the intensities of the fluorescences returned approximately to their original levels. Many such measurements were made, with ferricyanide concentrations ranging from 10^{-6} to 10^{-4} M, and in every case the changes in delayed fluorescence intensity could be correlated with and attributed to changes in the yield of the prompt fluorescence. A quantitative treatment (see later) showed that almost all of the initial fluorescence (f_0 in Fig. 1) should be regarded as dead.

With the motor turning rapidly, so that only 5 msec elapsed between excitation and measurement, ferricyanide caused an initial inhibition of delayed fluorescence followed (within about a second) by a stimulation that was more than enough to offset the inhibition. This stimulation is plausibly the result of a faster photochemical turnover resulting in a higher level of one or more precursors of the delayed fluorescence. The failure to observe stimulation of the "slow" delayed light (slow motor) has a simple kinetic explanation that will be introduced later.

The stimulation of delayed fluorescence by DCMU, like the inhibition by ferricyanide, can be attributed to an effect on the yield of the live component of prompt fluorescence. This stimulation is conspicuous whenever DCMU is added to a sample

in which the traps are predominantly open. If the traps are mostly closed, as under strong exciting light, the addition of DCMU does relatively little toward closing them further. There is then little change in the yield of prompt fluorescence, and correspondingly little stimulation of the delayed fluorescence. Under such conditions the stimulating action of DCMU is usually submerged by the inhibitory action. The general tendency of investigators to use strong exciting light in order to generate a large delayed fluorescence signal is probably responsible for the past failures to notice or report DCMU-induced stimulation of the "fast" delayed fluorescence. Actually the stimulation acts on the fast components as strongly as on the slower components. For example, the record of Fig. 3 was obtained with a low motor speed (250 msec between excitation and measurement of delayed fluorescence), but similar traces were obtained with a high motor speed (5 msec delay) provided that the other conditions of the measurement were not altered. Furthermore the delayed fluorescence elicited by a submillisecond flash, measured within 5 msec after the flash, was magnified more than 3-fold by the prior addition of DCMU (see Fig. 6).

With suitably weak exciting light and low DCMU concentration the inhibiting action of DCMU is negligible in comparison with the stimulating action, and the latter can be studied without the complication of the former (Fig. 7). It then becomes clear that the stimulation of the delayed fluorescence is related to the increase in the prompt fluorescence. As with Fig. 5 this relation will be treated quantitatively later. For the moment it can be said that many records of the sort shown in Fig. 7 were made, and all showed a close relationship between the increases of delayed and prompt fluorescence induced by DCMU.

Fig. 4 shows that DCMU added to chloroplasts in the dark caused a stimulation of the delayed fluorescence. Injection of 10% ethanol caused no such stimulation. Under the conditions of Fig. 4 a 4–5-fold increase in the delayed light intensity was induced by DCMU added 3 or more sec after the end of the excitation period. The amplification was less when the injection was made earlier. Since it was likely that this effect could be related to changes in the prompt fluorescence yield, the prompt fluorescence was monitored by interrupting the delayed light measurement, returning the motor shaft to the "excite" position as indicated by the dashed lines in Fig. 4. The initial value f_0' measured in this way indicated the yield of prompt fluorescence at any time during the experiment. Companion measurements were made with and without injections of DCMU, or with 10% ethanol or water injected as controls. The results of one set of measurements are shown in Table I. It can be seen that the amplifying effect of DCMU became larger as the prompt fluorescence yield subsided from the higher "light steady-state" level to the lower levels attained in the dark. It can also be seen that DCMU could cause, in the dark, some closing of the traps leading to an immediate increase in the yield of prompt fluorescence. This rather surprising result suggests that DCMU can cause a shift in the equilibrium between $Chl \cdot E^-$ and $Chl \cdot E$ in the direction of the former (closed trap) without the help of

TABLE I

DATA FOR AN EXPERIMENT OF THE KIND SHOWN IN FIG. 4

Time in dark after excitation	Factor by which the delayed fluorescence was amplified by adding DCMU at the time indicated	Intensity of prompt fluorescence, f_0', in arbitrary units, at time indicated (no injection)	Fluorescence f_0' at the time indicated; H_2O or 10% ethanol added 1 sec earlier	Fluorescence f_0' at the time indicated; DCMU injected 1 sec earlier
sec				
½	1.9	21.0		
1	2.5	20.7		
2	3.5	20.4		
3	4.0	20.2		
5	4.5	19.8		
6		19.6	19.4	21.8
10	4.8	19.4		
20	4.5	19.2		
30	5.8	19.2	19.1	20.6
90		19.0 = f_0		

The exciting light intensity was 0.036 mw/cm²; the final concentration of DCMU was 10^{-5} M.

light. This might be a consequence of disconnecting the system (Chl·E⁻, Chl·E) from the next redox couple in the chain of carriers between System II and System I.

The increase in the yield of prompt fluorescence, caused by adding DCMU and recorded in Table I, is sufficient to account for the amplification of the delayed fluorescence intensity. Assume for the moment that the live fluorescence is represented *only* by the time-varying part: all of the 19.0 units of f_0 (Table I) are dead. At 6 sec after the end of excitation, with nothing injected, the prompt fluorescence had the value $f_0' = 19.6$; we assume now that 19.0 of this is dead and 0.6 is live. Injection of DCMU at 5 sec gave, at 6 sec, a value of $f_0' = 21.8$. When corrected for a dilution effect (see the result of injecting water or ethanol) this value became 22.0. If 19.0 of this is dead, 3.0 is live and the injection of DCMU has raised the yield of live fluorescence 5-fold, from 0.6 to 3.0. The observed 4.5-fold amplification of the delayed fluorescence can thus be accounted for within the precision of these measurements (the precision can be judged by looking at Fig. 4).

When first observed, this effect of DCMU was taken to be an unexpected case of chemiluminescence analogous to the luminescences induced by acid-base transition or reducing agents. Given a trivial explanation in terms of the yield of prompt fluorescence, one might ask whether the other chemiluminescences can be explained in this way. The answer is yes for the "chemiluminescence" induced by hydrosulfite; this agent causes a large increase in the yield of prompt fluorescence. But the lumines-

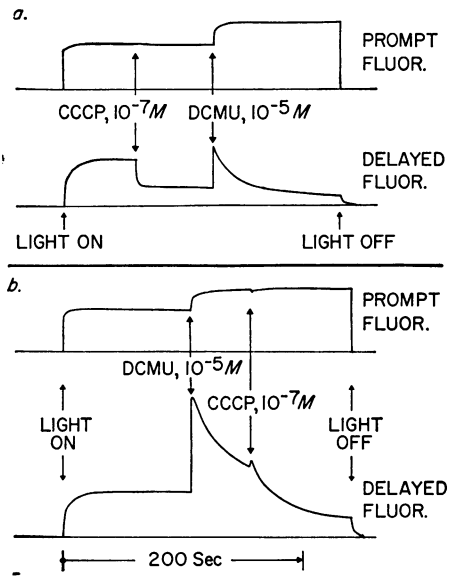

FIGURE 8 Same representation and conditions as in Fig. 7, showing the effects of DCMU (10^{-5} M) and CCCP (10^{-7} M).

cences induced by acid-base transition or by cations[4] require other explanations; measurements showed that these agents did not raise the yield of prompt fluorescence under conditions that led to conspicuous outbursts of light emission.

Effects of CCCP and NH_4Cl

Addition of the uncoupler CCCP to chloroplasts caused an immediate reduction in the intensity of delayed fluorescence with no concomitant change in the prompt fluorescence. The residual delayed fluorescence responded in the usual way to DCMU or ferricyanide, as did the prompt fluorescence. Thus (Fig. 8; compare *a* and *b*) the addition of DCMU after CCCP produced simply a miniaturized "normal" pattern of delayed fluorescence vs. time, and a normal pattern of prompt fluorescence.

If CCCP was added after DCMU, the action of the uncoupler was not expressed immediately but developed more gradually (Fig. 8 *b*). This result suggested that the action of CCCP on the delayed fluorescence required electron flow of the kind that is inhibited by DCMU.

Ammonium chloride, 10^{-3} M, produced an effect similar to that caused by 10^{-7} M CCCP with a chloroplast suspension containing 5 μg/ml of Chl *a*.

Resolution of the Prompt Fluorescence into Live and Dead Components

It was shown from the data of Table I that the amplification of delayed fluorescence by DCMU could be explained in terms of an effect on the yield of the live prompt

[4] C. D. Miles, in A. T. Jagendorf's laboratory, has observed luminescence of chloroplasts induced by injecting K^+ and other cations (personal communication).

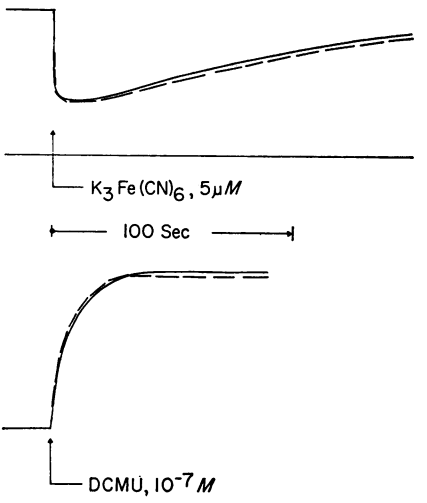

FIGURE 9 The traces of Fig. 5 and Fig. 7 *b* have been redrawn, with the time-varying part of the prompt fluorescence abstracted: the initial fluorescence f_0 was subtracted and the remainder was normalized to the magnitude of the delayed fluorescence before injection of ferricyanide or DCMU. A small dilution effect that attended the injection of a reagent was taken into account. Solid curve, delayed fluorescence; dashed curve, time-varying part of prompt fluorescence.

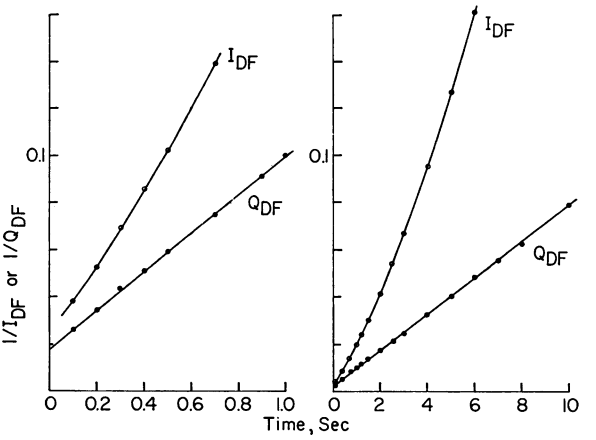

FIGURE 10 Reciprocal of delayed fluorescence intensity I_{DF} and of the intrinsic delayed fluorescence Q_{DF} vs. time after the end of excitation. Q_{DF} equals I_{DF} divided by the yield of the live component of prompt fluorescence; see text. Measurement was made as in Fig. 4; the exciting light intensity was 0.8 mw/cm². I_{DF} and Q_{DF} are in arbitrary units.

fluorescence. It was only necessary to assume that the initial fluorescence, f_0, of dark-adapted chloroplasts was almost entirely of the dead kind. A similar treatment of data obtained with other batches of chloroplasts on other days gave values of f_L never more than 5% of f_0. To a first approximation it appeared that the initial (dark-adapted) fluorescence was all dead and only the time-varying part, Δf, was live.

This conclusion is consistent with the data shown in Figs. 5 and 7. If the time-varying component of prompt fluorescence in either of these figures is abstracted and normalized to the delayed fluorescence the traces are nearly superimposable, as

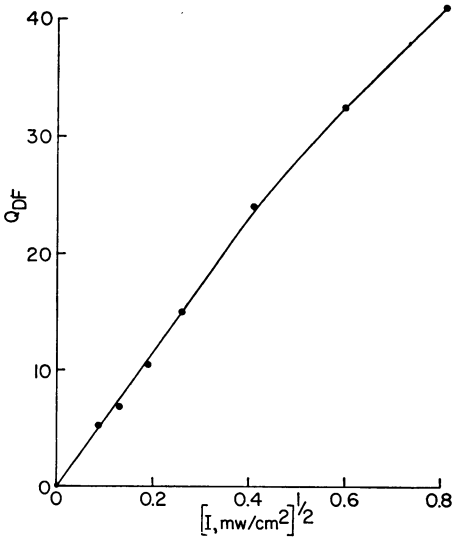

FIGURE 11 Intrinsic delayed fluorescence (see text) vs. the square root of exciting light intensity. The excitation intensity was the time average with the motor running; this was one-fifth the value that prevailed when the front window was open. The time from excitation to measurement of delayed fluorescence was 5 msec (motor 50 rev/sec). The arbitrary units of Q_{DF} are not the same as those in Fig. 10.

shown in Fig. 9. This correspondence was found whenever the inhibitory action of DCMU or the stimulating action of ferricyanide was avoided.

The general internal consistency of these observations provides strong support for the following assertions: all of the time-varying prompt fluorescence, but only a small fraction of the initial part f_0, is live. The stimulating action of DCMU and the inhibitory action of ferricyanide on the delayed fluorescence stem simply from changes in the yield of the live prompt fluorescence.

Exciting Light Dependence and Decay Characteristics of the Delayed Fluorescence

The foregoing considerations have shown how the influence of prompt (live) fluorescence yield should be abstracted when the intensity of delayed fluorescence is evaluated. Paraphrasing Equation 6, let Q_{DF} be the concentration of excitation quanta that have been regenerated (by the mechanism for delayed fluorescence) in the live component of Chl, and ϕ_f be the yield of live prompt fluorescence. Then the observed intensity of delayed fluorescence, I_{DF}, is given by

$$I_{DF} = \phi_f Q_{DF} \qquad (7)$$

in arbitrary units. Q_{DF} can be called the intrinsic delayed fluorescence.

To determine ϕ_f in relative units it was sufficient to measure f_0 and Δf as indicated by the prompt fluorescence traces of Figs. 3 and 4. It was found that f_0 varied linearly with exciting light intensity over the entire range used in these experiments, so that f_0 could itself be used as a measure of exciting intensity. Then with the assumption that only the time-varying part of the fluorescence, Δf, is live, the relative yield of this live fluorescence is given by $\Delta f/f_0$.

The decay of delayed fluorescence, after an excitation program of 15 sec at 0.8 mw/cm^2, and the relative values of ϕ_f during this decay, were measured in the manner suggested by Fig. 4. The period of reliable measurement (precision $\pm 5\%$) ranged from about 0.1 to 10 sec after the end of excitation. The decay kinetics for I_{DF} and Q_{DF} are shown in Fig. 10, tested as second order plots (reciprocal of I_{DF} or Q_{DF} vs. time). Semilogarithmic plots showed no sign of any linear sections that would have suggested first order decay. It can be seen that the decay of I_{DF} exhibits no simple pattern, but the decay of Q_{DF} is convincingly second order.[5] Whether this would be true in the millisecond time range remains to be determined. The present instrumentation did not afford measurement of ϕ_f after dark periods less than 100 msec.

The dependence of delayed fluorescence on exciting light intensity was measured with the phosphoroscope operated at a high motor speed so that 5 msec elapsed between excitation and measurement. The average exciting light intensity with the motor running was one-fifth the intensity that prevailed with the motor stopped and the entrance window open. Results of these measurements are shown in Fig. 11. Except for a slight tendency toward saturation at the highest excitation intensities, the intrinsic delayed fluorescence varied as the square root of exciting light intensity.

These results are consistent with a simple formalism: the intrinsic delayed fluorescence reflects the amount X of a certain substance. This substance is formed at a rate proportional (below light saturation) to the exciting light intensity, and decays with second order kinetics:

$$\frac{dX}{dt} = I - kX^2, \tag{8}$$

where I is the exciting light intensity and k is a decay constant. In the steady state, dX/dt is zero and the steady-state value of X is

$$X_0 = (I/k)^{1/2}. \tag{9}$$

If a light steady state is terminated suddenly, the decay in the dark follows the relations

$$\frac{dX}{dt} = -kX^2 \tag{10}$$

and

$$\frac{1}{X} = \frac{1}{X_0} + kt, \tag{11}$$

where X_0 is given by Equation 9. This is the second order behavior shown in Fig. 10.

[5] Lavorel (13) reported second order decay for the quotient, delayed fluorescence intensity divided by total fluorescence yield. His data extended from about 10 to 170 msec.

If kt is much smaller than $1/X_0$, or

$$kt \ll (k/I)^{1/2}, \qquad (12)$$

X is approximately equal to the initial value X_0. Thus the intrinsic delayed fluorescence measured a sufficiently short time after excitation should be proportional (see Equation 9) to the square root of the exciting light intensity, as was observed (Fig. 11).

In Fig. 10 the intercept of the ordinate gives the initial value corresponding to $1/X_0$ in Equation 11. This value is doubled at a time of 0.2 sec; this is the time for which $1/X_0$ equals kt. With an exciting light intensity in the neighborhood of 1 mw/cm^2, then, the approximation given by Equation 12 is valid for times less than about 20 msec. The time used in the measurements of Fig. 11, 5 msec, satisfies this requirement. The reverse approximation, kt much greater than $(k/I)^{1/2}$, can be expected to prevail at times much longer than 0.2 sec. At these longer times X is given approximately by kt and is independent of its initial value X_0. Any factor that influences the light steady state has, therefore, a strong effect on the delayed fluorescence as measured a short time after excitation, when X is approximately equal to X_0, but relatively little effect at long times such that X is fairly independent of X_0. This is enough to explain why the stimulating action of ferricyanide and the inhibiting action of DCMU seem to be associated with the "fast" components of delayed fluorescence.

If we speculate that the parameter X should be identified as an oxidizing entity produced by Photosystem II, for which there is some support (see earlier), the second order decay of X could reflect a point at which two univalent oxidants interact with a "two-electron" transport agent. Such a point of convergence would be needed somewhere between a "one-electron" photochemical act and the formation of an atom of oxygen.

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