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THE EMISSION YIELDS OF DELAYED AND PROMPT FLUORESCENCE FROM CHLOROPLASTS

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Summary

1. The decay of delayed fluorescence from chloroplasts blocked with 3-(3,4-dichlorophenyl)-1,1-dimethylurea and uncoupled with gramicidin has been measured in the time range 0.75—45 ms by use of a laser phosphoroscope.

2. The decays have been analysed as the sum of three first-order components of approximate half-lives 0.2, 2.5 and 300 ms by a computer-assisted least-squares fit procedure.

3. The prompt fluorescence yield of the chloroplasts was manipulated by changing the cation concentration of the chloroplast-suspending medium.

4. Analysis of the concentration dependence of the components of the delayed fluorescence decay and of the prompt fluorescence inductions indicates that the emission yield of the intermediate ($\tau \sim 2.5$ ms) component of the decay is equal to the fluorescence yield of a Photosystem II photosynthetic unit with an open trap, and that for the slow ($\tau \sim 300$ ms) component the emission yield is equal to the total Photosystem II prompt fluorescence yield.

5. It is concluded that the delayed fluorescence yield in the time range studied is a complex function of time, which may be due to there being different mechanisms leading to delayed fluorescence production at short and long times after cessation of illumination.

Introduction

The emission of delayed fluorescence from oxygen-evolving photosynthetic organisms is thought to have its origins in a back-reaction between the reduced primary electron acceptor (Q^-) and the oxidised primary electron donor (Y^+) of Photosystem II [1]. In principle, delayed fluorescence is a valuable tool for investigating the initial steps of photosynthetic energy conversion in Photo-

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system II, but several factors act to complicate the emission process. The principal factor is the high-energy state of photophosphorylation, which is thought to influence the rate of the back-reaction by lowering the activation energy barrier [2,3]. Secondly, the intensity of delayed fluorescence is modified by the availability of precursors at the reaction centre. This effect is seen both on the oxidising side [4] and on the reducing side [5] of Photosystem II. These two factors, together with the assumption that the back-reaction is first order [1], can be combined in the expression [6]:

$$J = [Y^{+}\mathrm{Chl}Q^{-}]k'\nu \exp\{-(E_{ac} - \Delta p)/kT\} \quad (1)$$

where J is the rate of chlorophyll excited singlet formation via the back-reaction, $[Y^{+}\mathrm{Chl}Q^{-}]$ is the concentration of the precursor complex, k' is a constant containing entropy terms, ν is a frequency factor, E_{ac} is the activation energy for the back reaction and Δp is the high-energy state of photophosphorylation expressed as Mitchell's proton motive force [7], given by:

$$\Delta p = \Delta\psi + 2.303\frac{RT}{F}\Delta pH \quad (2)$$

where $\Delta\psi$ is the electrical gradient and ΔpH is the pH gradient across the thylakoid membranes, and the other symbols have their usual meanings.

A third factor which modulates the intensity of delayed fluorescence was suggested by Butler [8] and by Lavorel [9]. These authors proposed that since the last stages of delayed fluorescence emission involve the production of chlorophyll excited singlet states in the Photosystem II pigment bed [10], the yield of delayed fluorescence should be subject to the same factors that modify prompt fluorescence yield. Lavorel [9], by analogy with the expression for the intensity of fluorescence, (F) $F = \phi I$, where I is the incident light intensity and ϕ is the fluorescence yield, wrote for the intensity of delayed fluorescence (L), $L = \phi' J$ where ϕ' is the delayed fluorescence yield. Hence, Eqn. 1 becomes:

$$L = \phi'[Y^{+}\mathrm{Chl}Q^{-}]k'\nu \exp\{-(E_{ac} - \Delta p)/kT\} \quad (3)$$

The relationship between ϕ and ϕ' is at present uncertain, but it will depend on the characteristics of the migration of delayed fluorescence excitations through the Photosystem II pigment bed, and on the nature of the photosynthetic unit of Photosystem II [1].

The purpose of the experiments described here is to investigate the relationship between ϕ and ϕ'; one way in which this may be done is to employ some means of manipulating the Photosystem II chlorophyll fluorescence yield and then to observe its effects on the intensity of delayed fluorescence. This has been achieved by using a phosphoroscope to measure delayed fluorescence from chloroplasts whose fluorescence yield can be modified by means of the 'cation effect' [11,12] whereby the addition of cations to broken chloroplasts suspended in a low-cation medium causes an increase in fluorescence. This effect takes place under experimental conditions chosen to keep other factors in Eqn. 2 constant, i.e., in the presence of uncouplers to suppress the high-energy state, and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to ensure that Q^{-} is reoxidised only via the back-reaction of Photosystem II [5]. In addition, experiments were carried out in the steady state, so that no modification

of delayed fluorescence intensity due to induction effects on the oxidising side of Photosystem II [4] was expected.

Materials and Methods

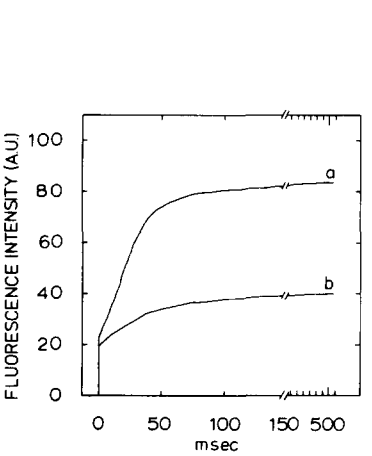
Intact chloroplasts were isolated from peas by the method of Stokes and Walker [13]. The chloroplasts were osmotically shocked and resuspended in a medium containing 0.33 M sucrose/10 mM KCl/20 mM tricine, pH 7.0, together with the required concentration of $MgCl_2$. Gramicidin ($5 \cdot 10^{-7}$ M final concentration) and DCMU ($5 \cdot 10^{-5}$ M final concentration) were added to the chloroplasts prior to experimenting.

Fluorescence measurements were made in an apparatus already described [14]. The fluorescence of the sample (pathlength 5 mm) was excited by broad-band blue light (Corning 4-96 filter) at an intensity of $4 \cdot 10^4$ erg $\cdot s^{-1} \cdot cm^{-2}$. Chlorophyll fluorescence was detected by a photomultiplier after passing through Corning 2-64 and Wratten 70 filters. The output of the photomultiplier was stored in a transient recorder (Datalab model 905) and finally recorded on a chart recorder. The concentration of chlorophyll in the chloroplast suspensions was 10 $\mu g \cdot ml^{-1}$ as estimated by Arnon's method [15].

Delayed fluorescence decays were measured in the time range 0.75—45 ms by means of a laser phosphoroscope described previously [16,17], but with the following modifications. The light source was a 4 W continuous argon-ion laser (Spectra Physics model 164) with 75% of the light energy in lines at 514.5 nm and 488.0 nm. Light pulses of 200 μs duration at a frequency of 20 Hz were obtained by use of an acousto-optic coupler (Spectra Physics model 365) in conjunction with the rotating disc of the phosphoroscope. The energy of each pulse was about 4 mJ. The laser beam (area 4 mm^2) was incident on the chloroplasts at a chlorophyll concentration of 60 $\mu g \cdot ml^{-1}$ contained in a capillary tube (inside diameter, 1.5 mm) and kept at 20°C by a water jacket. 10 000 delayed fluorescence decays were averaged using a multichannel analyser (Didac 800) with a time resolution of 0.25 ms per point. Measurement of delayed fluorescence decays was not started until induction effects in each sample were complete.

Results

The effect of adding cations to broken chloroplasts suspended in a low-cation medium is to increase the Photosystem II chlorophyll fluorescence intensity [11,12]. Fig. 1 illustrates chlorophyll fluorescence inductions from broken chloroplasts blocked with DCMU, uncoupled with gramicidin and suspended in a medium containing 2 mM $MgCl_2$ (a) or no $MgCl_2$ (b). The inductions show the features noted by other authors (see, for example, ref. 18): upon addition of $MgCl_2$ to the suspending medium there is a small increase in the initial level of fluorescence (the f_o level), and a larger increase in the variable fluorescence, f_v (above the f_o level), whilst the shape of the induction changes from being approximately exponential to being markedly sigmoid. Table I summarises data from a series of inductions using a range of $MgCl_2$ concentrations.



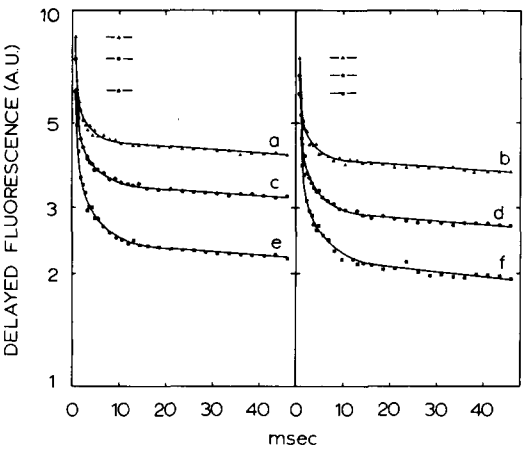


Fig. 1. The induction of Photosystem II chlorophyll fluorescence from pea chloroplasts blocked with $5 \cdot 10^{-5}$ M DCMU and uncoupled with $5 \cdot 10^{-7}$ M gramicidin. The chloroplasts were suspended in a medium containing 0.33 M sucrose/10 mM KCl/20 mM tricine, pH 7.0, in the presence (a) or absence (b) of 2 mM $MgCl_2$.

Fig. 2. The decay of delayed fluorescence from chloroplasts blocked with DCMU, uncoupled with gramicidin and suspended in media containing different concentrations of $MgCl_2$. The solid lines represent the result of applying a least-squares procedure to fit a curve comprising three first-order phases to the experimental points. Suspending medium as in legend to Fig. 1 except that the concentration of $MgCl_2$ added (in mM) was a, 2.0; b, 0.5; c, 0.3; d, 0.2; e, 0.1; f, 0. For clarity, the short horizontal lines at the left of each half of the figure indicate the initial values of each decay (at 0.75 ms).

In a parallel series of experiments the decay of delayed fluorescence from chloroplasts was measured as a function of $MgCl_2$ concentration. Fig. 2 shows the decay of delayed fluorescence in the time range 0.75—45 ms from DCMU-blocked chloroplasts uncoupled with gramicidin and suspended in media containing different concentrations of $MgCl_2$. It can be seen that the intensity of

TABLE I

THE EFFECT OF $MgCl_2$ ON THE PROMPT AND DELAYED FLUORESCENCE OF CHLOROPLASTS

Experimental conditions as in the legend to Fig. 1.

$MgCl_2$ (mM)	Prompt fluorescence *			Delayed fluorescence **			
				Intermediate phase		Slow phase	
	f_0	f_{vmax}	f_{tmax}	τ (ms)	Intensity	τ (ms)	Intensity
0	20.3	19.9	40.2	3.06	1395 ± 227	272	2159 ± 123
0.1	28.0	25.7	53.7	2.58	1670 ± 125	349	2412 ± 46
0.2	28.3	37.3	65.6	2.08	1788 ± 151	312	2930 ± 51
0.3	25.2	46.7	71.9	2.47	1582 ± 139	369	3464 ± 57
0.5	24.2	54.3	78.5	1.63	1680 ± 217	371	4026 ± 47
2.0	23.0	58.5	81.5	1.62	1708 ± 237	340	4515 ± 47

* Fluorescence intensity is measured in arbitrary units. f_0 is the level of fluorescence attained on first illuminating the chloroplasts; f_{vmax} is the maximum fluorescence above the f_0 level. $f_{tmax} = f_0 + f_{vmax}$. Results are the average of three determinations.

** Delayed fluorescence intensities are measured in arbitrary units and represent the extent of the phases determined by the least square-fit extrapolated to zero time. The errors on the intensities are the standard errors of the least-squares fit.

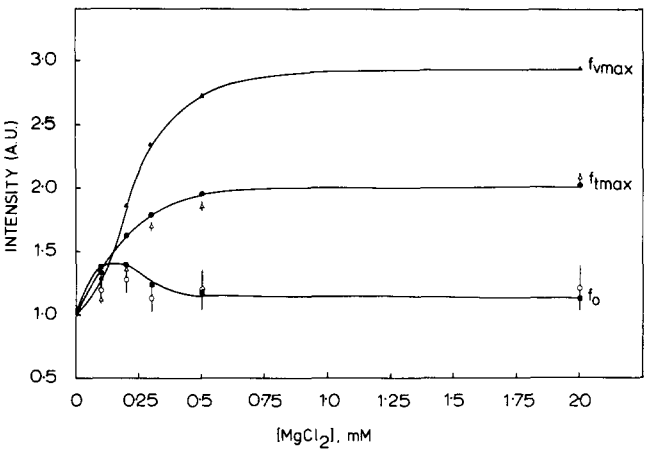


Fig. 3. The dependence of the intensity of prompt and delayed fluorescence on the concentration of $MgCl_2$ in the chloroplast-suspending medium. Closed triangles, maximum variable fluorescence, f_{vmax}; closed circles, maximum total fluorescence, f_{tmax}; closed squares, initial fluorescence, f_o; open triangles, slow phase of delayed fluorescence; open circles, intermediate phase of delayed fluorescence. The error bars on the delayed fluorescence points indicate the standard error of the lest-square fit estimates.

delayed fluorescence increases with increasing $MgCl_2$ concentration.

In order to quantitatively relate the intensity of prompt and delayed fluorescence as a function of $MgCl_2$ concentration the delayed fluorescence decays were decomposed into a series of first-order phases. A computer-assisted least-squares fitting process indicated that in the time range studied all the decays could be described by the sum of three first-order phases of approximate half-times 0.2, 2.5 and 300 ms. Whilst it is unusual to be able to deduce a half-time faster than the characteristic time of the actinic pulse and multichannel analyser, attempts to fit the data to the sum of two first-order phases (omitting the fastest phase) resulted in a much larger sum of squared residuals compared to the three-phase fit. Moreover, the three-phase description is in agreement with previous measurements in this time range using DCMU-blocked algae [17]. However, due to the fast decay of the 0.2 ms phase, it was not possible to determine the intensity of this first phase accurately. Data for the other two phases of the decays shown in Fig. 2 are given in Table I.

Fig. 3 illustrates the dependence of prompt and delayed fluorescence parameters on $MgCl_2$ concentration. It can be seen that the intensity of the slow phase of the delayed fluorescence decay depends on the concentration of $MgCl_2$ in a similar way to the maximum total prompt fluorescence, f_{tmax} ($=f_o + f_{vmax}$). In contrast, the intensity of the intermediate phase has a similar concentration dependence to the f_o level of fluorescence.

Discussion

The mechanism by which Photosystem II chlorophyll fluorescence is stimulated by the 'cation effect' has not yet been completely established (see ref. 19 for review). There are strong indications that the majority of the increase in fluorescence intensity reflects an increase in fluorescence yield due

to a cation-induced change in the rate constant for excitation energy exchange ('spillover') between Photosystem II and Photosystem I [12,18,20,21]. However, it has been proposed that in addition there is a small cation-induced change in the initial distribution of absorbed quanta between the two Photosystems [21].

All the following discussion assumes that, under the experimental conditions employed here, there was no change in J, the rate of delivery of delayed fluorescence singlet states to the chlorophyll pigment bed, but that any changes in delayed fluorescence intensity L were due to changes in ϕ' which were themselves dependent upon a cation-induced change in fluorescence yield, ϕ. On a longer time scale (100 ms—5 s) it has been shown that the addition of $MgCl_2$ to the suspending medium did not change the rate of reoxidation of Q^- in DCMU-blocked broken chloroplasts as measured by the time course of the restoration of the area over the fluorescence induction curve (Hipkins, M.F., unpublished). This observation suggested that J is unaffected by the addition of $MgCl_2$ to the chloroplast-suspending medium.

The possible ways in which the delayed fluorescence yield ϕ' may depend on the fluorescence yield ϕ have been discussed by Lavorel [1]. Essentially, there are two extreme cases which differ in the degree of mobility assumed for the delayed fluorescence excitations. On the one hand, if excitation migration is restricted so that the excitation cannot leave the photosynthetic unit in which it is created, then ϕ' will be equal to the fluorescence yield of a photosynthetic unit with an open trap, ϕ_o *. If, on the other hand, excitations are able to migrate over the whole Photosystem II pigment bed, then the delayed fluorescence yield should be equal to ϕ_{tmax}, the maximum total fluorescence yield of Photosystem II. This is because in the time scale investigated here (0.75—45 ms) few Photosystem II traps reopen during the decay [5].

The data shown in Fig. 2 indicate that in the time range studied the delayed fluorescence decays may be described by the sum of three first-order phases. (There may, however, be additional more slowly decaying phases present, but these will be difficult to detect in the limited time range studied.) The data presented in Table I and Fig. 3 show that the intermediate ($\tau \sim 2.5$ ms) and slow ($\tau \sim 300$ ms) phases of the delayed fluorescence decay have different dependences on fluorescence yield. The intensity of the intermediate phase has a similar concentration dependence for the cation effect as the f_o level of fluorescence, suggesting restricted excitation migration. The intensity of the slow phase, however, increases in line with the maximum total fluorescence, f_{tmax}, suggesting more extensive excitation migration. The different behaviour exhibited by these two phases may be accounted for by assuming that each phase of delayed fluorescence is produced by a distinct mechanism. This could be because the Photosystem II reaction centres are of two types. Alternatively, with homogeneous reaction centres, the mechanism leading to delayed fluorescence production might change during the decay: delayed fluorescence at shorter times being produced by one mechanism and at longer times by

* The concept that the f_o level of fluorescence represents the fluorescence of a Photosystem II photosynthetic unit with an open trap has recently received both experimental [22] and theoretical [23] support. However, there is evidence to suggest that a part of the f_o level of fluorescence arises from outside Photosystem II (see, for example, ref. 24).

another. For example, the intermediate phase might result from a Photosystem II back-reaction with produces a singlet excited state of chlorophyll; this excited state may then be retrapped by the open reaction centre leading to an emission yield equal to ϕ_o. The slow phase might arise from a mechanism involving the production of triplet states in the back-reaction followed by triplet migration and triplet-triplet annihilation. Consequently, singlet states would be produced remote from the photosynthetic unit where the back-reaction occurred and the emission yield would be equal to ϕ_{tmax}. Such a triplet mechanism has been suggested by several authors [25—27], but has yet to find rigorous experimental support [28].

Overall, the results reported here indicate that the delayed fluorescence yield ϕ' is a complex function, and in particular that it changes as a function of time elapsed after illumination has ended. However, since the intermediate phase ($\tau \approx 2.5$ ms) of delayed fluorescence decays relatively rapidly, at times greater than approx. 1 ms after cessation of illumination the slow phase will become dominant and ϕ' will be equal to ϕ_{tmax}. This conclusion is supported by recent experiments performed by Barber et al. [29] where it was found that the envelope of 1 ms-delayed fluorescence from DCMU-blocked uncoupled chloroplasts had an emission yield equal to ϕ_{tmax}. At longer times after the end of illumination this relationship would be expected to break down because a larger proportion of the Photosystem II traps would reopen, leading to the complications of the 'islet effect', which have been discussed by Lavorel [1].

The conclusions presented here contrast with those of Wraight [30] who suggested that delayed fluorescence yield was not influenced by prompt fluorescence yield. It has also been suggested by Clayton [31], and more recently by Malkin [32], that the delayed fluorescence yield is equal to ϕ_v. The experiments of Wraight [30] and of Malkin [32] are however difficult to interpret since they deal with the induction of prompt and delayed fluorescence on first illuminating dark-adapted chloroplasts where, since the Photosystem II traps are not all closed, the islet effect will come into play. Similarly, the experiments of Clayton [31] present problems of interpretation since the variable factors in Eqn. 2, particularly the high-energy state, were not controlled.

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