KINETICS STUDIES ON THE FLUORESCENCE QUENCHER IN ISOLATED CHLOROPLASTS

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ABSTRACT The chlorophyll fluorescence yield in isolated chloroplasts without an added electron acceptor is increased by actinic illumination. The decline in the fluorescence yield when the actinic illumination is extinguished can be accurately represented by three, independent, exponential decays with half-times of approximately 0.8, 5, and 30 sec. These results have been interpreted using Duysens' theory of fluorescence quenching by a compound (Q) on the reducing side of photosystem II. This theory states that changes in fluorescence yield are indicative of electron flow through Q. The most rapid decay is eliminated by an EDTA washing of the chloroplasts and the half-time is increased by uncoupling with ammonia and by added electron acceptors in suboptimal concentrations. Thus, this decay may represent electron flow from Q to intermediates on the oxidizing side of photosystem I. The decay with a half-time of 5 sec is affected in the same manner as the decay with the shortest half-time by the same procedures. However, electron donors to photosystem II lengthen the half-time of the 5 sec decay while eliminating the most rapid decay. This 5 sec decay can be interpreted as electron flow from Q to intermediates either on the reducing side of photosystem II or on the oxidizing side of photosystem I. The decay with the longest half-time is affected only by pH and electron donors to photosystem II. Therefore, this decay may indicate electron flow from Q to intermediates on the oxidizing side of photosystem II which may be connected to the regeneration of the oxygen burst.

INTRODUCTION

In the absence of added electron acceptors, the fluorescent yield of chlorophyll in chloroplasts is increased with actinic illumination. Duysens (1963) has explained this phenomena by assuming that a compound (Q) which is the primary electron acceptor of the photoact of photosystem II suppresses fluorescence when oxidized and permits fluorescence when reduced. Thus, according to Duysens' hypothesis the increase of the fluorescent yield upon actinic illumination is due to Q being reduced by system II more rapidly than being oxidized by system I. The decline of the high fluorescent yield after the actinic light is extinguished is presumably caused by oxidization of the reduced Q.

Many investigators (Joliot, 1965 *b*; Lavorel, 1966; Murata, Nishimura, and Takamiyo, 1966; Forbush and Kok, 1968; Heber and French, 1968) have noticed that the decay of the fluorescence yield in the dark proceeded with at least biphasic kinetics involving half-times of approximately 1 sec and 1 min. This dark decay, or reoxidization of Q, has been interpreted as electron flow through the oxidizing side of system I resulting in the regeneration of the oxygen burst (Joliot, 1965 *a*, *b*).

From their studies of the fluorescence from chloroplasts, Murata et al. (1966) postulated that a cycle around photosystem II (from Q to oxidized intermediates on the water-splitting side of the photoact) must exist in isolated chloroplasts. Likewise, Homann (1968), and Malkin and Jones (1968) observed a higher fluorescence from chloroplasts with added dithionite than from DCMU-poisoned chloroplasts (where DCMU is 3-[3,4-dichlorophenyl] 1,1-dimethylurea). These results indicate that either DCMU did not block all electron transport into system I or a cycle within system II caused Q to remain slightly oxidized in the presence of DCMU.

This paper will examine the decay of the fluorescence yield when the actinic light is extinguished. The decay curve has been mathematically fitted to three exponential rates. Each rate has been examined in order to ascertain conditions which affect it and to see if this rate could be indicative of a cycle in photosystem II.

METHODS

Chloroplasts were isolated from spinach plants unless otherwise noted by either a sucrose-washed preparation (Hind, Nakatani, and Izawa, 1969) or an EDTA-washed preparation (Izawa, Heath, and Hind, 1969). The fluorescence yield was measured by a fluorometer using a modulated measuring light (270 Hz) described earlier (Heath and Hind, 1969 *a*); however, in these experiments the signal output of the lock-in amplifier was connected to a signal averager (Fabri-Tek Instruments, Inc., Madison, Wis., model 1052H). The averaged fluorescence yield from the signal averager was plotted using an X-Y recorder. The data was then digitized and punched onto cards which were fed into a computer (Control Data Corp., Melville, N. Y., model 6600).

The computer fitted the data points to kinetic curves involving three simultaneous exponential decay processes (equation 1) using a least squares estimate of the nonlinear parameters (Marquardt, 1963, 1964).

$$\phi = \phi_0 + \sum_{j=1}^{3} A_j e^{-k_j t}, \tag{1}$$

where ϕ = data points (fluorescence at time, t) and ϕ_0, A_j, k_j = constraint constants noted as b_j. The computer program minimized the sum of the squares of the difference between the predicted data points and the observed data points by varying the constraint constants. The convergence criterion was that:

$$|\delta_j| / (T + b_j) < 5 \times 10^{-5}, \quad \text{for all } j, \tag{2}$$

where the last increment to b_j is $K\delta_j$, $T = 10^{-3}$ and K = the number of constraint constants, i.e. 7. It was determined that the initial values of the b_j's would not influence the final values found by the computer program if the initial values were not different from the final values by more than an order of magnitude. The sum of the squares of the residuals was normally from 1 to 5 and the standard error of fit was 0.15–0.25 for 50 data points ranging from 20.0 to 100.0. The data points were read to ±0.1.

A typical experiment consisted of suspending chloroplasts (40 μg/2.0 ml) in a solution of 0.1 M sucrose, 0.03 M tricine-NaOH at pH 8.0 and 0.005 M $MgSO_4$. The measuring light (432 ± 10 nm) had an intensity of 20 erg cm^{-2} sec^{-1} and the actinic light (470–540 nm) had an intensity of 5 kerg cm^{-2} sec^{-1}. The temperature was 12°C. The time schedule of the actinic light for signal-averaging experiment, unless otherwise noted, consisted of: 15 sec on, 15 sec off (for conditioning), and 4 or 5 cycles of 15 sec on followed by 60 sec off.

RESULTS

The intense actinic light produces noise in the measured yield which can be markedly reduced by the use of signal averaging techniques (cf. Methods). The yield kinetics are reproducible if the chloroplasts are conditioned by a flash of actinic light (10–15 sec) prior to the averaging. The fluorescence yield before the conditioning flash is slightly below that prior to the subsequent flashes; after the first flash the yield requires several minutes to reach the low initial yield. Fig. 1 A shows a recording of the increase in yield upon illumination after averaging. The rise of the yield initially is linear with time. Only after the yield has increased to approximately 40% of the final steady-state value does this rate become nonlinear. Fig. 1 B shows the full kinetics of the decay of the yield when the actinic light is extinguished. This decay is not a simple exponential, but a complex one.

Decay Kinetics of Fluorescence Yield: Components

In Fig. 1 B the decay curve shows a rapid initial drop of the yield followed by a much slower decline. As found through curve fitting, the decay curve can be accurately represented by a sum of three independent, simultaneous exponential processes with time constants differing by orders of magnitude, as given in equation 1 *a*.

$$\phi = \phi_F - \phi_0 = A_\alpha e^{-k_\alpha t} + A_\beta e^{-k_\beta t} + A_\gamma e^{-k_\gamma t}, \qquad (1\,a)$$

where ϕ_F is the observed fluorescence at time (t) and ϕ_0 is the "nonquenchable" fluorescence induced by the measuring beam. A sum of one or two exponentials does not fit the data points as well as the sum of three exponentials, judged by the standard error of the parameters and of the fit and by the least squares residuals. In addition, the sum of three exponentials requires less iterations to fit the data points.

The decay curve was digitized, and the resulting series of points is represented by crosses in Fig. 2. These experimental points were then fitted by a computer to the sum of three exponential curves, as shown by the solid line in the figure. The three separate decay curves are also shown. They have been denoted as the α mode (for that with the longest half-time), β mode, and the γ mode (for that with the shortest

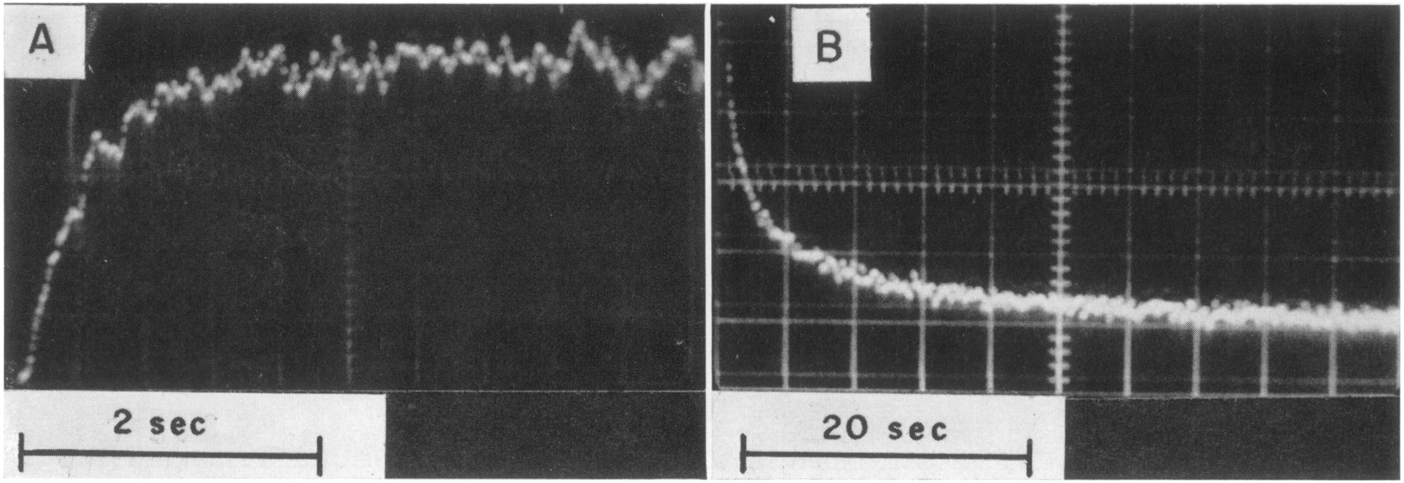

FIGURE 1 Kinetics of fluorescent yield induced by actinic illumination. Pea chloroplasts suspended as in Methods at pH 8.2 with NaCl (10 mM). Curve A: full scale = 5.1 sec. Actinic light on. Curve B: full scale = 51.2 sec. Actinic light off.

half-time). The half-times of each decay are approximately 30 sec (α mode), 5 sec (β mode), and 0.8 sec (γ mode). Since the fluorescent yield depends upon the pH (Heath and Hind, 1969 *a*), it is natural to expect hydrogen ion involvement in the decay pathways (Table I). The decay constants (k and A, see equation 1 *a*) for each

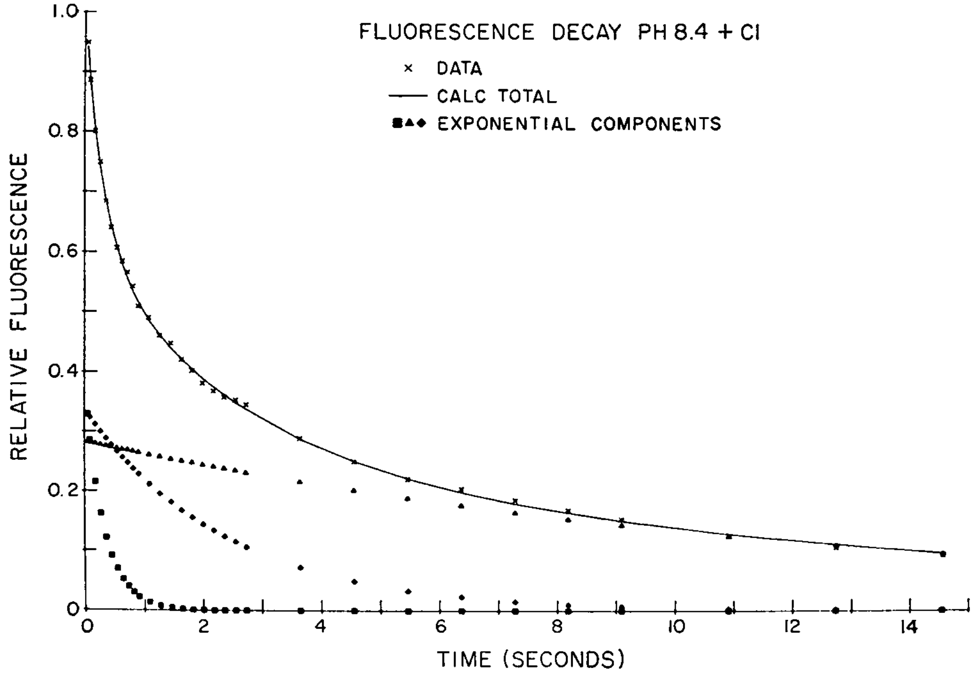

FIGURE 2 Resolution of decay kinetics of fluorescence yield. Chloroplasts suspended as in Methods, with NaCl (10 mM).

TABLE I

EFFECT OF pH UPON THE RATE CONSTANTS OF THE DECAY OF THE FLUORESCENCE YIELD

Mode*	pH 7.4	pH 8.5
	k (*sec*$^{-1}$)	
α	0.028 ± 0.005	0.037 ± 0.002
β	0.14 ± 0.02	0.22 ± 0.03
γ	1.5 ± 0.2	1.9 ± 0.2
	A (*units of* ϕ_0)	
α	0.49 ± 0.09	0.83 ± 0.03
β	1.25 ± 0.09	1.05 ± 0.05
γ	1.13 ± 0.11	0.68 ± 0.04

* Chloroplasts (sucrose washed) suspended as in Methods in NaCl (10 mM) at indicated pH. Average of 20 trials (±SE), k, A, and ϕ_0 defined as in equation 1 *a*.

TABLE II

EFFECT OF OXYGEN TENSION UPON THE RATE CONSTANTS OF THE DECAY OF THE FLUORESCENCE YIELD

Mode*	−Air	+Air
	k (sec^{-1})	
α	0.016 ± 0.003	0.019 ± 0.002
β	0.09 ± 0.01	0.13 ± 0.01
γ	0.6 ± 0.1	1.2 ± 0.2
	A (*units of* ϕ_0)	
α	0.20 ± 0.01	0.24 ± 0.01
β	0.61 ± 0.04	0.55 ± 0.02
γ	0.22 ± 0.02	0.21 ± 0.02

* Chloroplasts (sucrose washed) were suspended as in Methods in NaCl (10 mM) and at pH 8.2. Light intensity = 2×10^3 erg/cm^2 per sec. Average of 6 trials (±SE). k, A, and ϕ_0 as defined in equation 1 *a*.

mode show changes with pH. The k constants for all three modes are decreased some 30–60% by a higher hydrogen ion concentration. The A constants show more diverse changes; at higher pH the A for the γ mode is decreased markedly while the A for the α mode is increased. There seems to be only a slight decrease for the β mode. Also, as the pH increases, there is a small decrease of the total steady-state fluorescence, as previously noted (Heath and Hind, 1969 *a*).

Table II shows the effect of low oxygen tension upon the decay constants. The atmospheres used were air and vacuum (in which reduced indophenol dye is not observed to be oxidized). There is no effect of oxygen pressure upon the k of the α mode, while the k constants of the β and γ modes are nearly doubled. Only the A of the α mode shows a small change.

Effect of System I Activity

It is possible that O_2 does not interact with the reduced Q but is merely acting as an electron acceptor in the Mehler reaction (1951), thus increasing the electron flow through system I. Heber and French (1968) have found that the amount of fluorescence quenching and the Mehler reaction rate are linearly related at different oxygen concentrations.

In order to test further the effects of photosystem I activity on the decay, a small amount of methyl viologen (MV) was added to the chloroplasts. Fig. 3 shows that this amount of MV alters the light intensity dependency of the steady-state fluorescence. The presence of MV has the same effect as the absence of Cl^- (Heath and Hind, 1969 *a*). It causes a decrease in the intercept of the ϕ axis and in the intercept with the ϕ/I axis. A large amount of MV (> 10 μM) causes the fluorescence yield to decrease to near ϕ_0.

Table III shows the effect of MV on the decay constants. MV causes no change in k_α but, much like O_2, causes a nearly twofold increase in k_β and k_γ. Both A_β and A_α are lower in the presence of MV while A_γ is increased. If the total steady-state yield for +MV is normalized to that for −MV, it is then seen that MV causes

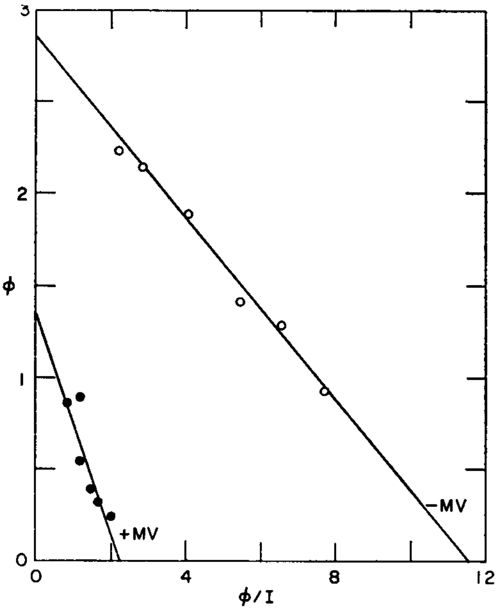

Figure 3 Dependency of steady-state fluorescence yield upon system I activity. Pea chloroplasts suspended as in Methods in tricine (0.015 M) and $MgSO_4$ (5 mM), NaCl (10 mM) at pH 8.4 and, where indicated, MV (0.2 μM).

TABLE III

EFFECT OF SYSTEM I ACTIVITY UPON THE RATE CONSTANTS OF THE DECAY OF THE FLUORESCENCE YIELD

Mode*	−MV	+MV	+MV
	k (*sec*$^{-1}$)		
α	0.025 ± 0.002	0.029 ± 0.002	
β	0.15 ± 0.01	0.29 ± 0.01	
γ	0.90 ± 0.09	2.0 ± 0.4	
	A (*units of* ϕ_0)		*A* (*normalized*)‡
α	0.68 ± 0.06	0.46 ± 0.06	0.68
β	0.94 ± 0.06	0.36 ± 0.06	0.54
γ	0.36 ± 0.01	0.52 ± 0.06	0.78

* Chloroplasts (sucrose washed) were suspended as in Methods at pH 8.4 with NaCl (10 mM) and where indicated MV (0.2 μM). Average of five trials (±SE). *k*, *A*, and ϕ_0 defined in equation 1 *a*.

‡ The total steady-state yield of all components for the +MV case was set equal to the total steady-state yield for the −MV case. From this ratio the individual normalized *A* for each component of the +MV case was calculated.

A_γ to be greatly increased and A_β to undergo a sizeable decline. No change is observed for A_α.

Since the k constants of the β and γ modes (but not the α mode) are increased by conditions that would increase the system I activity, these could be tentatively identified as reduced Q reacting with compounds on the oxidizing side of system I.

Effect of System II Activity

When system II activity is inhibited by the absence of Cl^-, as in Table IV, the A and k constants of the α mode are unchanged. On the other hand, the k constants of the two shorter modes are increased by nearly a factor of two, similar to the case for increased system I activity. However, the A constants are both decreased. If the total steady-state yield for $-Cl$ is normalized to that for $+Cl$, it is then seen that $-Cl$ causes A_α to increase by about 30%, A_β to remain the same, and A_γ to decrease about 20%.

Addition of 3-(3,4-dichlorophenyl) 1,1-dimethylurea (DCMU) to chloroplasts blocks the electron pathway from Q to photosystem I (Butler, 1966). As shown in Fig. 4, DCMU causes a pronounced change in the observed fluorescence kinetic curve. The weak measuring beam causes the fluorescence yield to rise slowly to a value higher than that of the control (compare Fig. 4 A and B). Once the fluorescence induced by the measuring beam reaches steady-state, actinic illumination produces only a small increase in fluorescence yield. When the actinic light is extinguished, the fluorescence yield rapidly falls to the high initial yield. The three distinct decay processes are not observed.

TABLE IV

EFFECT OF CHLORIDE ION UPON THE RATE CONSTANTS OF THE DECAY OF THE FLUORESCENCE YIELD

Mode*	+Cl	−Cl	−Cl
	k (*sec*$^{-1}$)		
α	0.030 ± 0.002	0.033 ± 0.002	
β	0.18 ± 0.02	0.29 ± 0.04	
γ	1.3 ± 0.1	2.5 ± 0.4	
	A (*units of* ϕ_0)		*A* (*normalized*)‡
α	0.58 ± 0.04	0.60 ± 0.05	0.78
β	0.65 ± 0.03	0.45 ± 0.04	0.59
γ	0.60 ± 0.04	0.35 ± 0.08	0.46

* Chloroplasts (sucrose washed) were incubated as in Methods at pH 8.3. Average of 14 trials (±SE). k, A, and ϕ defined in equation 1 *a*.

‡ The total steady-state yield of all components for the $-Cl^-$ case was set equal to the total steady-state yield for the $+Cl^-$ case. From this ratio the individual normalized A for each component was calculated.

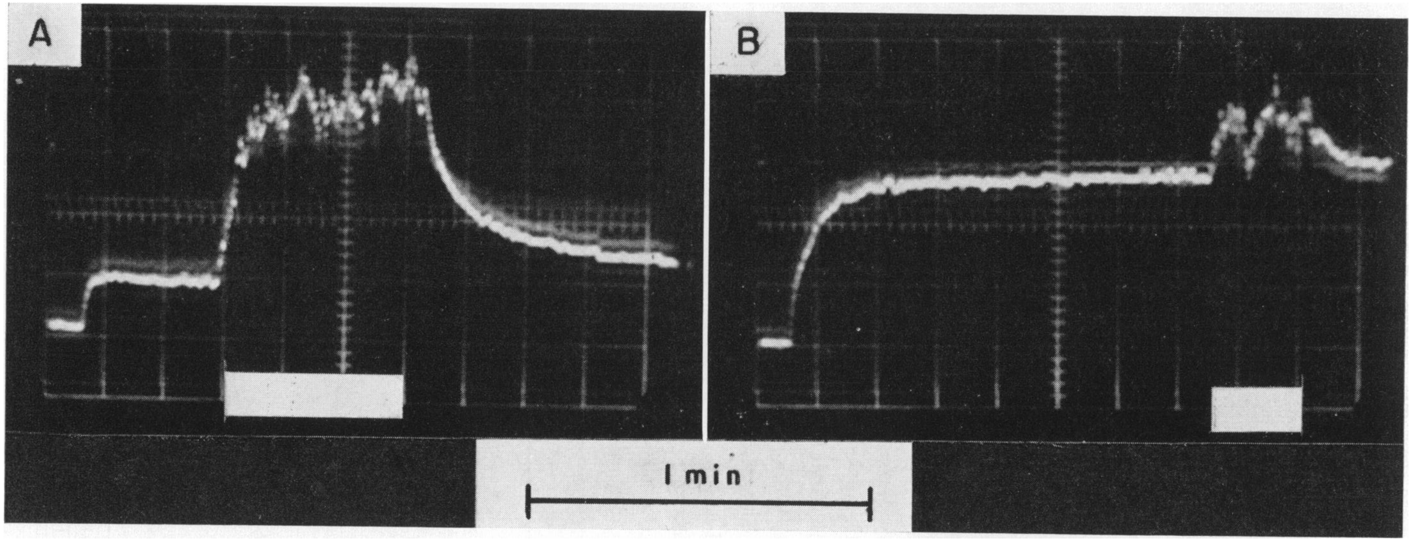

FIGURE 4 Kinetics of fluorescence yield. Chloroplasts suspended as in Methods at pH 8.2 and NaCl (10 mM) and, in Fig. 4 B, DCMU (10 μM). White bar indicates actinic light on.

This rapid decay process, observed when the actinic light is extinguished in the presence of DCMU, is solely the γ mode (Table V A). The observed kinetics can be fit to only one exponential process (as also observed by Heber and French, 1968). In the presence of Cl^-, added DCMU does not alter k_γ or A_γ. In the absence of Cl^-, however, added DCMU causes the high k_γ (observed for $-Cl^-$) to be lowered (to that of +Cl). The increased decay rate due to $-Cl^-$ is eliminated by added DCMU. The effect of *o*-phenanthroline, although not shown here, is exactly the same as that of DCMU.

It may not be that DCMU eliminates the α and β decay modes but merely that the high yield induced by the measuring beam obscures them. The only possible method that can be used to see if the other modes are operating is to turn off the measuring beam for a given amount of time. This experiment is shown in Fig. 5; the fluorescence yield observed immediately after the measuring beam is turned on is given as a function of the time that the chloroplasts were in the dark. It is clear that there are still two distinct exponential decays — a long and short one. Table V B compares the decay constants observed by the above method for chloroplasts with added DCMU with those for chloroplasts without added DCMU. Both the α and β decays are present. Although, the experimental scatter is high, apparently the k for the β mode is nearly unchanged, while the k of the α mode may be decreased upon adding DCMU. Also with DCMU, the A of the α mode is only slightly increased while the A of the β mode is decreased by nearly 40%. Hence, the addition of DCMU does not eliminate any decay mode. This means either that none of the

TABLE V
EFFECT OF DCMU UPON THE RATE CONSTANTS OF THE FLUORESCENCE DECAY

Condition*		
A. γ mode only†		
	A_γ *(relative)*	k_γ *(sec^{-1})*
+Cl	0.18 ± 0.02	0.87 ± 0.08
+DCMU	0.20 ± 0.01	0.75 ± 0.03
−Cl	0.23 ± 0.02	2.2 ± 0.2
+DCMU	0.28 ± 0.01	0.59 ± 0.04

B. α and β modes§

	k *(sec^{-1})*		A (ϕ_0)	
Mode§	−DCMU	+DCMU	−DCMU	+DCMU
α	0.026	0.008	1.1	1.4
β	0.36	0.46	1.0	0.7

* Pea chloroplasts (sucrose washed) suspended as in Methods at pH 8.4 where indicated DCMU (10 μM) and NaCl (10 mM).

† Average of six trials (+Cl) or three trials (−Cl). See Fig. 5 and text.

§ As in *, data from Fig. 6.

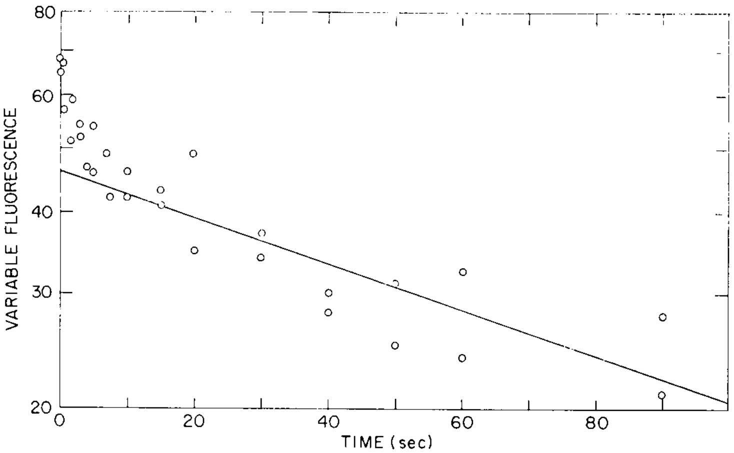

FIGURE 5 Decay of the fluorescence yield in the dark in the presence of DCMU. Pea chloroplasts were incubated as in Methods as pH 8.4 and NaCl (10 mM) and DCMU (10 μM). Variable fluorescence = fluorescence observed immediately upon the onset of illumination after a dark period given by the horizontal axis *minus* fluorescence observed immediately upon the onset of illumination after an extended dark period (2–3 min).

decay mode pathways are through the DCMU inhibition site or the block by DCMU is not complete with low rates of electron transport.

Effect of Uncoupling Treatments

The rates of electron flow in EDTA-washed chloroplasts compared to those in sucrose-washed chloroplasts are much higher because of the elimination of the phosphorylation step (Jagendorf and Smith, 1962). Table VI shows that the k constants for the α and β modes are unchanged by the method of preparation. However the γ mode is totally absent for EDTA-washed chloroplasts. In addition, EDTA-washed chloroplasts also have a lower total, steady-state yield. This type of increased photosystem I activity eliminates the γ decay mode as well as lowering the total yield. The A constants show only a small increase in the α mode and no increase in the β mode. If the γ decay mode is dependent upon the phosphorylation step in system I, then an uncoupler such as ammonia should either increase or eliminate k_γ. Table VII shows that NH_4^+ at concentration sufficient to uncouple the chloroplasts (Hind et al., 1969) nearly doubles the k_γ without affecting the k_β or k_α. In addition, there is no change in the A constants.

Electron Donation to Photosystem II

If one or more of the decay processes is a reaction of reduced Q with the oxidized intermediates on the water-splitting side of photosystem II (a cycle within photosystem II), then a compound which donates electrons to photosystem II should

TABLE VI

COMPARISON OF DECAY CONSTANTS OF THE YIELD BETWEEN SUCROSE- AND EDTA-WASHED CHLOROPLASTS

Mode*	Sucrose washed‡	EDTA washed‡
	k (sec^{-1})	
α	0.050 ± 0.003	0.050 ± 0.005
β	0.29 ± 0.03	0.22 ± 0.02
γ	2.1 ± 0.1	—§
	A (*units of* ϕ_0)‖	
α	0.92 ± 0.06	1.36 ± 0.12
β	1.02 ± 0.04	1.11 ± 0.12
γ	1.25 ± 0.05	—§

* Cf. Table II at pH 8.4, with NaCl (10 mM).

‡ Sucrose-washed chloroplasts were prepared as the EDTA-washed chloroplasts (cf. Izawa et al., 1969) with the replacement of sucrose (0.2 M), N-tris(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid-NaOH (5 mM at pH 7.4) for the EDTA-wash solution.

§ Only two exponential modes could be fit to the decay curve for the EDTA-washed chloroplasts (γ was missing).

‖ k, A, and ϕ_0 as defined in equation 1 a. ϕ_0 (sucrose washed) = 1.27 as to 1.0 for EDTA washed.

TABLE VII

EFFECT OF AMMONIA UNCOUPLING UPON THE DECAY CONSTANTS OF THE FLUORESCENCE YIELD

Mode*	Normal	Ammonia uncoupled
	k (sec^{-1})	
α	0.028 ± 0.004	0.025 ± 0.005
β	0.18 ± 0.1	0.18 ± 0.1
γ	1.7 ± 0.4	2.9 ± 0.4
	A (*units of* ϕ_0)	
α	0.78 ± 0.05	0.78 ± 0.07
β	1.18 ± 0.04	1.31 ± 0.10
γ	0.92 ± 0.10	1.11 ± 0.36

* Pea chloroplasts (sucrose washed) suspended as in Methods at pH 7.7 and with NaCl (10 mM), where indicated NH_4^+ (as $SO_4^=$, 5 mM). Actinic light intensity was 20 kergs/cm² per sec. Average of five trials. k, A, and ϕ_0 defined as in equation 1 a.

alter the decay parameters. Table VIII shows the effect of such a donor — hydrazine. Hydrazine, like hydroxylamine, at high concentrations acts as an electron donor to photosystem II (Izawa et al., 1969; footnote 1).

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[1] Heath, R. L., and G. Hind. Manuscript submitted.

TABLE VIII

EFFECTS OF ELECTRON DONORS TO SYSTEM II ON DECAY OF FLUORESCENCE YIELD

Conditions*	Control	+Cl	$+NH_2NH_2$	+Cl $+NH_2NH_2$
A. α mode†				
A (units of ϕ_0*)*	0.8 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	1.0 ± 0.1
k *(sec*$^{-1}$*)*	0.036 ± 0.007	0.033 ± 0.006	0.017 ± 0.007	0.021 ± 0.008
B. β mode†				
A (units of ϕ_0*)*	0.83 ± 0.08	1.2 ± 0.1	1.6 ± 0.2	1.6 ± 0.1
k *(sec*$^{-1}$*)*	0.22 ± 0.01	0.17 ± 0.01	0.07 ± 0.01	0.11 ± 0.01

* EDTA-washed spinach chloroplasts were suspended as in Methods (pH 8.0) and 25 mM Na_2SO_4 (control) and where indicated NaCl (10 mM), NH_2NH_2 (50 mM). (Average of four trials.)

† Cf. Table II. No γ decay mode was observed.

The k for the α mode declines upon addition of hydrazine with and without added Cl^-. The A for the α mode in the presence of Cl^- shows no change with a donor; while in the absence of Cl^-, the donors cause a marked increase in A_α. Electron donation by hydrazine with and without Cl^- also causes a pronounced decrease in the k_β. In particular, the high k_β caused by $-Cl^-$ is lowered. The A_β is increased by the presence of hydrazine with and without added Cl^-. There is no γ mode shown in Table VIII since EDTA-washed chloroplasts have been used. Hydroxylamine at a high concentration produces the same results as obtained with hydrazine. Hydroxylamine with sucrose-prepared chloroplasts causes a loss of the γ mode, much like EDTA washing.

DISCUSSION

The decay of the fluorescence yield in the dark from the steady-state level induced by actinic illumination has been noted previously by many investigators. Each has found kinetics with two characteristic times (usually half-times) differing by at least one order of magnitude. Joliot (1965 *b*) first described the fluorescence decay by two processes with half-times of 0.5 and 30 sec, and interpreted them to be indicative of the reactivation step for the oxygen burst. Both the 0.5 and the 30 sec times agree well with the half-times for the γ and the α decay modes. Forbush and Kok (1968) also found that the decay from the steady-state level proceeds by two types of reactions; however, one had a half-time of 50 sec and the other appeared to be less than 10 sec. Similarily Malkin and Kok (1966) investigating the restoration of the initial fluorescence level by varying periods of darkness after the fluorescence reached a steady state found half-restoration required about 20 sec while full restoration required 2–4 min. The 20 sec time was confirmed by Murata et al. (1966) and, in addition, was found to be altered by DCMU, much like the α decay mode as seen in Table V. Heber and French (1968) using DCMU-poisoned whole leaves found that the fluorescence decay proceeded by only one first-order reaction with a half-time of about 2 sec, again similar to the γ decay mode (cf. Fig. 4).

Brief flashes of actinic light affect the fluorescence decay in a different manner. Lavorel (1966) found that when the chloroplasts were illuminated by a flash, the high fluorescence yield declines with two pseudo–first-order reactions with half-times of 0.02 and 0.2 sec.

From the previously mentioned investigations, the rates of the dark decay of the fluorescence yield seems to be relatively constant. Unfortunately, it is difficult to quantify these phenomena and, thus, the use of three exponential processes here to describe the decay curves is merely for convenience and does not, at this point, represent adherence to any particular molecular mechanism.

The factors which influence the decay rates are summarized in Table IX. The γ decay is eliminated by EDTA washing, which uncouples the phosphorylation step from electron flow and also increases greatly the electron flow from water oxidation to artificial electron acceptors (Jagendorf and Smith, 1962). EDTA washing also lowers the total steady-state fluorescence yield (Table VI), presumably, by increasing the flow of electrons from Q into the oxidizing side of system I with the elimination of the uncoupling step. Both the increased rate constant of the γ mode and the lower steady-state fluorescence are observed when the rate of electron flow from water to O_2 is increased (added MV, Table VI; increased O_2 concentration, Table II; uncoupling by NH_3, Table VII). Increased electron flow, either by uncoupling or more rapid system I turnover would effectively increased the amount of oxidized intermediates between Q and P_{700} (Malkin and Kok, 1966; Kok, Owens, and Forbush, 1967).

TABLE IX

SUMMARY OF EFFECTS UPON FLUORESCENCE YIELD DECAY CONSTANTS

Condition*	$k(sec^{-1})$			$A\ (\phi_0)$			Table
	α	β	γ	α	β	γ	
Increased system I							
$+O_2$	0	+?	+	+?	0	0	III
+MV	0	+	+	−	−	+	IV
EDTA washed	0	−?	*V*	+	0	*V*	VII
$+NH_3$	0	0	+	0	0	0	VIII
Decreased system II							
$-H^+$	+	+	+?	+	−?	−	II
$-Cl^-$	0	+	+	0	−	−	V
+DCMU	−	+?	0	+	−	0	VI
Donors							
$+Cl^-$	−	−	*V*	−	+	*V*	IX
$-Cl^-$	−	−		+	+		IX

* +, −: these represent respectively an increase or a decrease of constant. *V*: this mode does not appear to be present under these conditions. Blank: not measured.

The absence of Cl^- is known to slow the flow of electrons from water into system II; this keeps the fluorescence yield low and Q partially oxidized (Heath and Hind, 1969 *a*; Izawa, Heath, and Hind, 1969). This slower rate of electron flow again allows more oxidized intermediates in the cytochrome chain to build up. Thus, the slowing of electron flow from system II would have nearly the same effect upon the rate constant of any mode of decay as increased electron flow into system I.

The same arguments can be used for the β mode of decay. The largest discrepancy between the β and γ modes is that electron donors to system II (Table VIII) cause a decrease in the rate constant of the β mode and cause the γ mode to vanish. However, these donors may also interact somewhere between the DCMU block and the photoact of system I (Yamashita and Butler, 1968). This interaction would cause a decrease in the amount of oxidized intermediates; also decreasing the rate constant. Therefore, the only conclusion which can be made is that both the β and γ decays are a dark interaction of reduced Q with the intermediates connected to either system I or II.

The α mode is least dependent upon the experimental conditions (Table VII), indicating it is somehow internally limited. The data indicate that the α mode is sensitive only to electron donation to system II and to pH; thus, it may be a system II-linked reaction.

The rate of photosynthetic oxygen liberation and the initial fluorescence intensity have been found to be inversely correlated (Joliot, 1965 *b*; Bannister and Rice, 1968; Bonaventura and Myers, 1969). In addition, Cheniae and Martin (1970) and Heath and Hind (1969 *b*) have found that the intermediates of system II between water oxidation and the photoact are functionally expressed as two separate managanese-linked compounds. This suggests that in system II at least two intermediates (Z and M) exist on the oxidation side of system II and, thus, two decay pathways could exist. These intermediates may be pools, and in this regard, all intermediates must be nearly exhausted of electrons before oxygen can be evolved by water splitting. This hypothesis has been expressed in more detail by Joliot (1968).

Joliot (1965 *a*) has measured a rate constant for the regeneration of the oxygen burst of 0.03 sec^{-1} (reactivation of his E), agreeing very closely with the k_α (Table II). In addition, Fork (1963) has found at least two time constants for the regeneration of the oxygen burst. This may indicate that both Z and M must be reduced for the oxygen burst to reach its maximum. After light has oxidized Z and M in order to repeat the initial kinetics of oxygen liberation, electrons must flow into M and Z during a dark period. No doubt some of these electrons come from water. However, if M is only partially oxidized, it may no longer be able to split water and the electrons then must come from other sources. This would identify the α mode of fluorescence decay with the pathway of electron flow from Q to M (possibly through Z). Chemicals which donate electrons to the oxidizing side of system II would be expected to reduce Z and M and thereby to lower the rate constants for the fluorescence decay. Both α and β modes are so affected.

In summary, the decay pathways may be visualized as:

$$\begin{tikzcd}
H_2O \arrow[r] & M \arrow[r] & Z \arrow[l, dashed, bend right, "\alpha"'] \arrow[r, dash] & \text{photoact II} \arrow[r, dash] & Q \arrow[ll, dashed, bend right, "\beta"'] \arrow[r] \arrow[r, dashed, bend left, "\gamma"] & \text{cytochrome chain} \arrow[r] & \text{photoact I.} \\
 & & & h\nu \arrow[u] & & & h\nu \arrow[u]
\end{tikzcd}$$

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