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EVIDENCE FOR A DOUBLE HIT PROCESS IN PHOTOSYSTEM II BASED ON FLUORESCENCE STUDIES

P. JOLIOT and A. JOLIOT

Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris (France)

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SUMMARY

1. The amplitudes of the fast (0–20 μs) and slow (20 μs–2 ms) fluorescence rise induced by a 2 μs flash have been measured as a function of the energy of the flash in chloroplasts inhibited by 3(3,4-dichlorophenyl)-1,1-dimethylurea. The saturation curve for the slow rise shows a characteristic lag which is not observed for the fast fluorescence rise. This lag indicates that Photosystem II centers undergo a double hit process which implies that (a), each photocenter includes two acceptors Q_1 and Q_2; (b), after the first hit, oxidized chlorophyll Chl^+ is reduced by a secondary acceptor Y in a time short compared to the duration of the flash; (c), after the second hit, Chl^+ is reduced by another secondary donor, D.

2. According to Den Haan et al. ((1974) Biochim. Biophys. Acta 368, 409–421), hydroxylamine destroys the secondary donor responsible for the fast reduction of Chl^+. In the presence of 3 mM hydroxylamine, only the secondary donor D is functional and a flash induces mainly a single hit process.

3. The saturation curves for the fast and the slow rises have been studied in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea for a second actinic flash given 2.5 s after a first saturating one. The large decrease in the half-saturating energy indicates the existence of efficient energy transfer occuring between photosynthetic units.

4. Two alternate hypotheses are discussed (a) in which D is an auxiliary donor and (b) in which D is included in the main electron transfer chain.

INTRODUCTION

Duysens and Sweers [1] established that the fluorescence yield of chlorophyll in vivo is controlled by the redox state of the primary System II electron acceptor Q. It appears now that a large number of other parameters also control the fluorescence yield: (a) According to Okayama and Butler [2] and Butler et al. [3] the oxidized form of the photoactive chlorophyll ($Chla_{II}{}^+$) quenches the fluorescence with an efficiency

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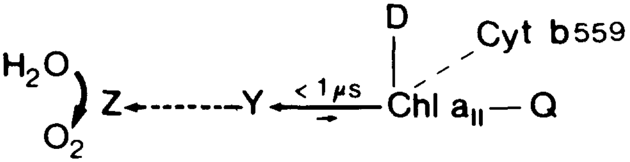
Abbreviation: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

comparable to that of oxidized Q; (b), the number of oxidizing equivalents stored on the donor side of System II centers (Kok's S states [4]) modulates directly or indirectly the fluorescence yield [5–6]; (c), the formation of a light-induced quencher T has been reported upon excitation by a high energy flash in both active (Q-oxidized) or inactive (Q-reduced) System II centers [7–8]. This quencher is ascribed to a carotenoid triplet state which fully relaxes in approx. 15 µs; (d), other factors such as the nature and the concentration of cations [9–10], the proton gradient [11] and the electric field [12] all affect the fluorescence yield.

The following scheme summarizes our present knowledge of Photosystem II centers where $\mathrm{Chla_{II}}^+$ and Q represent respectively the primary donor and acceptor:



Scheme I

Z, the final donor involved in the water-splitting reaction, is able to store four oxidizing equivalents. According to Duysens et al. [13] the transfer of the positive charge from $\mathrm{Chla_{II}}^+$ to the secondary donor here called Y, occurs in less than 1 µs. As shown by Delosme [6], the amplitude of the fast fluorescence rise (< 1 µs) in the absence of DCMU is a function of the number of oxidizing equivalents stored on Z.

Mauzerall [14] measured the fluorescence yield after a 10 ns laser flash and observed a fast increase ($t_{\frac{1}{2}} \simeq 30$ ns) which probably corresponds to the reduction of $\mathrm{Chla_{II}}^+$ by the secondary donor Y. Using 2 µs saturating flashes, Zankel [8] and then A. Joliot [15] observed a slow component in the fluorescence increase. The amplitude of this component increases markedly when the temperature is lowered and at 2 °C in the presence of DCMU, the maximum fluorescence level is reached in about 2 ms [15].

Hydroxylamine is known to inactivate the oxygen evolving system by release of bound manganese [16]. Den Haan et al. [17, 18] showed that in the presence of hydroxylamine, the fluorescence rise measured during a saturating flash is limited by a dark reaction ($t_{\frac{1}{2}} \simeq 25$ µs) ascribed to the reduction of oxidized $\mathrm{Chla_{II}}^+$ by an auxiliary donor D. A. Joliot [19] showed that this electron transfer reaction is polyphasic and is complete in about 2 ms at 2 °C. We stress that the kinetics of this rise are similar to the slow rise observed in the presence of DCMU alone. When the temperature is lowered to below −40 °C, a photooxidation of cytochrome *b*-559 is observed [20]. Den Haan et al. [18] established that the auxiliary donor D cannot be identified with cytochrome *b*-559. Thus, it appears that at least three donors can directly interact with the photoactive chlorophyll.

However, the rather complex model for the System II centers mentioned above (Scheme I) does not explain the non-exponential character of the fluorescence induction curve observed in the presence of inhibitors which prevent the reoxidation of Q^- by the plastoquinone pool: The fluorescence rise then presents a characteristic lag first interpreted by Morin [21] who assumed that Q can accept successively two electrons. A more detailed study of this hypothesis was undertaken by Lavorel [22].

Joliot and Joliot [23] and Joliot et al. [24] proposed an alternate hypothesis in which energy transfer occurs within the photosynthetic units. Measurements of the fluorescence life-time [25] provide additional support for this hypothesis.

The observation that a saturating flash does not destroy all the fluorescence quenchers present led Doschek and Kok [26] to propose that each center includes two acceptor sites. From low temperature (−50 °C) experiments, we reached the same conclusion [27]; nevertheless, unlike Morin [21] and Doschek and Kok [26], we proposed that the sigmoidal shape of the fluorescence induction curve was due to efficient energy transfer between units. The existence of two acceptors has also been recently proposed by Glaser et al. [28]. Based on a theoretical analysis of the fluorescence induction curves, Paillotin [29] concluded first, that energy transfer occurs between the photosynthetic units and second, that System II centers can exist in more than two quenching states. Such would be the case if the centers included two acceptor sites.

The observation of several kinetic components in the fluorescence induction has led to the introduction of a third concept: The heterogeneity of the antenna [24] or of the photocenters [30]. Melis and Homann [30] thus proposed the existence of two types of centers having different photochemical efficiency.

Based on new fluorescence results in chloroplasts, we attempt to present here a coherent model which integrates most of the concepts mentioned above.

MATERIALS AND METHODS

Fluorescence experiments were performed using an apparatus previously described [27]. Chloroplasts were illuminated by a brief actinic Xenon flash (Verre and Quartz Co. model VQ X CAD 22, 1 μs at half-height; 90 % of the energy is emitted in less than 5 μs). For the saturation curves, the energy of this actinic flash is attenuated by calibrated stainless steel screens (Buckee Mears Co.). As in ref. 15, the fluorescence yield is measured by weak detecting flashes (General Radio Stroboslave, 1539A) given at various dark times following the actinic flash. 20 μs was chosen as the shortest time of analysis to minimize the contribution of the quencher T. In experiments where Q is reduced (chloroplasts preilluminated in the presence of 20 μM DCMU plus 10 mM hydroxylamine), the fluorescence yield 20 μs after an unattenuated actinic flash is 96 % of the maximum yield, indicating a minimal contribution of quencher T; where Q is oxidized, this contribution is lower due to the competition between Photoreaction II and the formation of T.

Chloroplasts were isolated from market spinach according to the method of Avron [31] and stored at −70 °C in the presence of 5 % dimethylsulfoxide. Prior to use, the chloroplasts were suspended at a concentration of approximately 90 μg chlorophyll/ml in 0.05 M phosphate buffer (pH 6.4) with 0.4 M sucrose, 0.1 M KCl, 0.1 μM gramicidin and 7 % ficoll. All the experiments were performed at 2 °C, in the presence of 20 μM DCMU added to dark-adapted chloroplasts.

Experiments in the presence of ferricyanide were performed according to the following procedure: Chloroplasts (1400 μg chlorophyll/ml) were treated in the dark by 1.75 mM ferricyanide for 10 ms at room temperature. They were then diluted to 88 μg chlorophyll/ml with the above mentioned buffer plus 20 μM DCMU, which leads to a final concentration of 0.1 mM ferricyanide.

Hydroxylamine (pH 6.4) was added to dark-adapted chloroplasts prior to the addition of DCMU. The incubation time (about 15 min) was always sufficient to totally inactivate the water-splitting system.

RESULTS AND DISCUSSION

Saturation curves in the presence of DCMU: Double-hit process

Effect of ferricyanide. In Fig. 1 is depicted the fluorescence yield at 20 µs and 2 ms after a single actinic flash as a function of the energy of the flash. The 0–20 µs fluorescence increase gives essentially a measurement of the fast rise (< 1 µs) and the difference (2 ms–20 µs) gives a relative measurement of the amplitude of the slow rise [15]. The part of the slow rise which occurs in the 0–20 µs time range is difficult to evaluate due to the contribution of the quencher T in the same time range.

The existence of a double-hit process is demonstrated by the observation of a well-developed lag in the saturation curve of the slow phase. By integrating a large number of experiments, we checked that no significant slow rise is observed for energies of the actinic flash less than 3 %. If, as proposed by Melis and Homann [30],

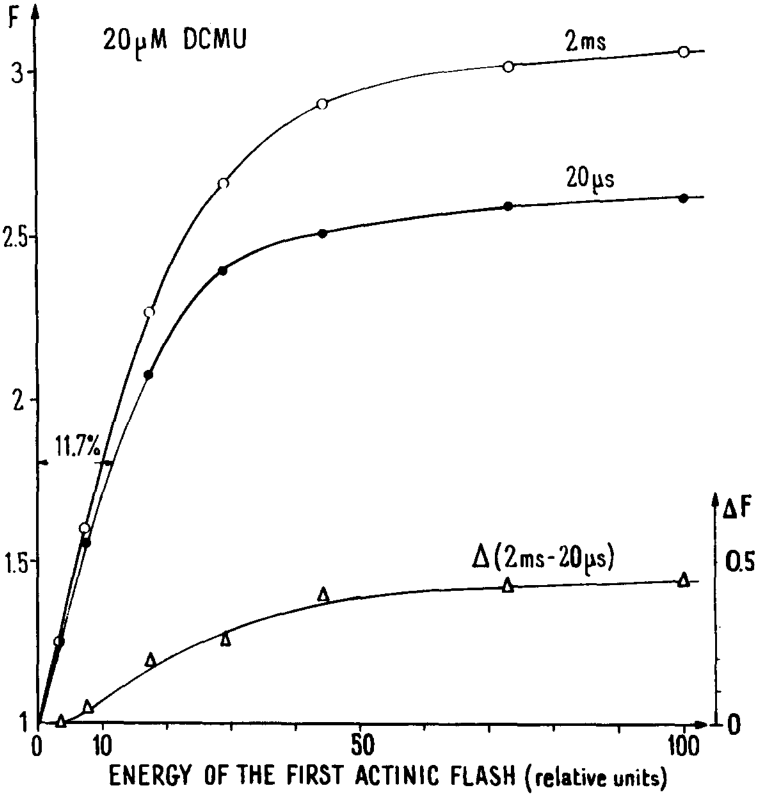
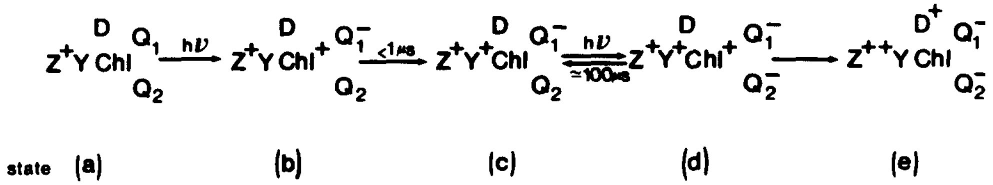


Fig.1. Left ordinate, fluorescence yield in chloroplasts in the presence of 20 µM DCMU 20 µs (●) and 2 ms (○) after a 2 µs actinic flash of variable energy. The initial fluorescence level is equal to 1. Right ordinate, difference ΔF (Δ) between the two preceeding curves.

there were two types of centers with different photochemical efficiency, the initial slope of the saturation curve for the less efficient quencher would not be zero. A double-hit process implies the presence of two electron acceptors, Q_1 and Q_2. As the rate of reduction of $\mathrm{Chla_{II}}^+$ by Y ($< 1\ \mu s$) is short compared to the duration of our flash, there is a high probability that each center will undergo a second charge separation during a single flash. The sequence would be as follows starting from state S_1 [4]:



Scheme II

The appearance of a slow increasing phase in the fluorescence yield is easily explained if one assumes that state (d) shows a higher quenching efficiency than state (e). This is in agreement with the quenching property of Chl^+.

This model is formally identical to the one proposed by Glaser et al. [28]. From spectrophotometric studies, these authors concluded that the long-lived state of $\mathrm{Chla_{II}}^+$ (approx. 35 μs) exists only after a double-hit process generated by a long saturating flash. When the water-splitting enzyme is destroyed by Tris treatment, the formation of the long-lived state of $\mathrm{Chla_{II}}^+$ only requires an efficient single-hit process. Surprisingly, no lag appears in the curve of Glaser et al. for the double-hit process. We shall come back to this point later.

In Fig. 1, a fraction (approx. 20 %) of the quenchers are inactivated simply by the addition of DCMU in the dark [27]. This inactivation is now easily interpreted if it is assumed that, in dark-adapted chloroplasts, a fraction of the secondary acceptor B [32], or R [33], is in the B^- form. According to Velthuys and Amesz [33], the centers in the QB^- form are transformed to Q^-B upon addition of DCMU, probably due to a change in the potential of B. Wollman [34] reported recently that B^- can be oxidized by quinone in *Chlorella*. Ikegami and Katoh [35] showed that the addition of ferricyanide prior to DCMU slows down the fluorescence rise measured in continuous light. The simplest interpretation is that ferricyanide, as quinone, is able to oxidize B. In order to confirm this hypothesis, we measured with continuous light the half-times ($t_{\frac{1}{2}}$) of the fluorescence induction curves in dark-adapted chloroplasts at −50 °C. The values of $t_{\frac{1}{2}}$ obtained under various conditions were: (a) No addition, 160 ms; (b), 20 μM DCMU, 120 ms; (c), ferricyanide, 170 ms; (d), ferricyanide+DCMU, 170 ms. At −50 °C, the electron transfer from Q to B is blocked either in the presence or in the absence of DCMU. The half-time of the fluorescence induction is a rough indication of the number of available quenchers. As we reported previously [27], the addition of DCMU accelerates the fluorescence rise, i.e., decreases the number of active quenchers. On the contrary, neither ferricyanide (c) nor ferricyanide plus DCMU (d) induces any significant change in the half-time rise. Thus, we propose that, when ferricyanide is added prior to DCMU, all the quenchers are in their oxidized form.

In Fig. 2 are depicted the saturation curves in the presence of 0.1 mM ferricyanide added to chloroplasts before DCMU. Compared to the control Fig. 1, the

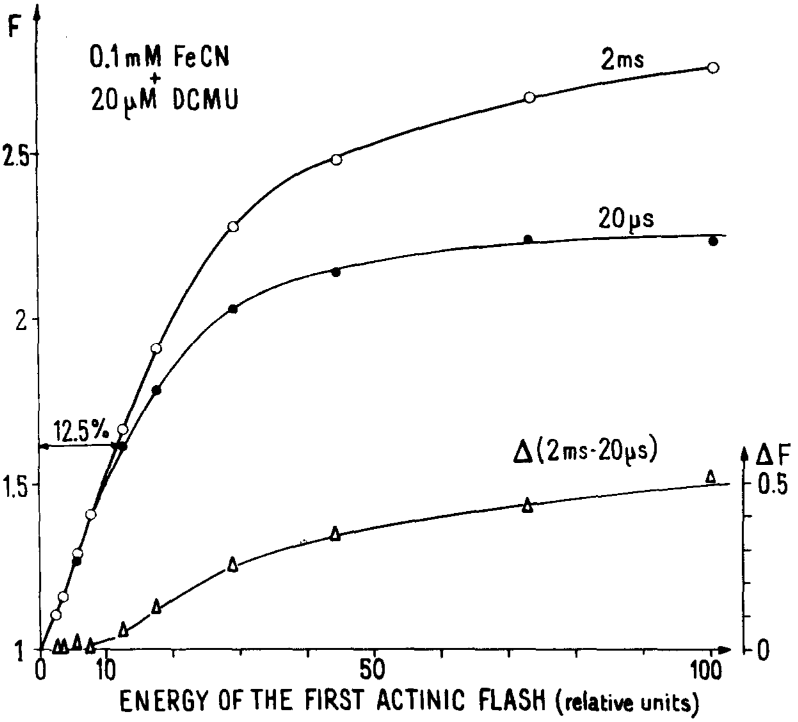


Fig. 2. Same as Fig. 1 except that the chloroplasts are treated with 0.1 mM ferricyanide prior to the addition of 20 μM DCMU (see Materials and Methods). The initial fluorescence level was 7 % lower than that observed in the presence of DCMU alone. The maximum amplitude of the (2 ms–20 μs) rise upon excitation by a flash of energy 166 % is 0.6.

maximum amplitude of the fast rise is decreased while the amplitude of the slow phase is slightly increased. In the absence of ferricyanide, a fraction of the centers has at least one of the two acceptors in the reduced form. For these centers, only a single-hit process occurs and the quenching state (d) (Scheme II) is not formed. After action of ferricyanide, most of the centers include two oxidized acceptors. Immediately after a saturating flash, all the centers are in state (d), which explains the decrease in

TABLE I

FLUORESCENCE YIELD MEASURED 1 ms AFTER EACH FLASH OF A SERIES OF SHORT SATURATING FLASHES GIVEN 200 ms APART

	Dark-adapted level	1st Flash	2nd Flash	3rd Flash	Stationary level
20 μM DCMU	1.07	3.31	3.47	3.48	3.56
0.1 mM FeCN +20 μM DCMU	1.00*	2.88	3.58	3.63	3.73

* The dark-adapted level in the presence of ferricyanide plus DCMU is arbitrarily taken to be equal to 1.

amplitude of the fast phase. As expected, a correlated increase in the slow phase is observed. We checked that when ferricyanide is added after DCMU, the saturation curves show only minor changes compared to those of Fig. 1: One observes an approximately 10 % increase of the slow phase, which can be interpreted assuming that Q_2, the less efficient quencher, is partially reduced in the dark and can be slowly oxidized by ferricyanide even in the presence of DCMU.

We measured the fluorescence yield 1 ms after each flash of a series of short saturating flashes given 200 ms apart, in the presence or in the absence of ferricyanide (Table I). The stationary level is nearly the same for the two experimental conditions, which proved the absence of a direct quenching effect of 0.1 mM ferricyanide. As pointed out by Doschek and Kok [26], a single saturating flash is not able to destroy all the quenchers, i.e., only a fraction of the centers has stabilized two positive and two negative charges. As the reduction of Chl^+ by Y is very fast compared to the rate of the back reaction between Chl^+ and Q^-, one would expect that the charges separated during the first photoact are all stabilized. However, after the second hit, a competition should occur between the rate of reduction of Chl^+ by D and the back reaction between Chl^+ and Q^- (approx. 100 μs [36, 37]). The fraction of the centers which have stabilized two positive charges after the first flash is difficult to estimate because the exact quenching efficiency of state (c) is unknown. In the presence of ferricyanide, the second flash destroyes about 80 % of the remaining variable fluorescence, which indicates that the rate of reduction of Chl^+ by D is considerably greater than the rate of the back reaction. This efficiency drastically decreases from the third flash on. This

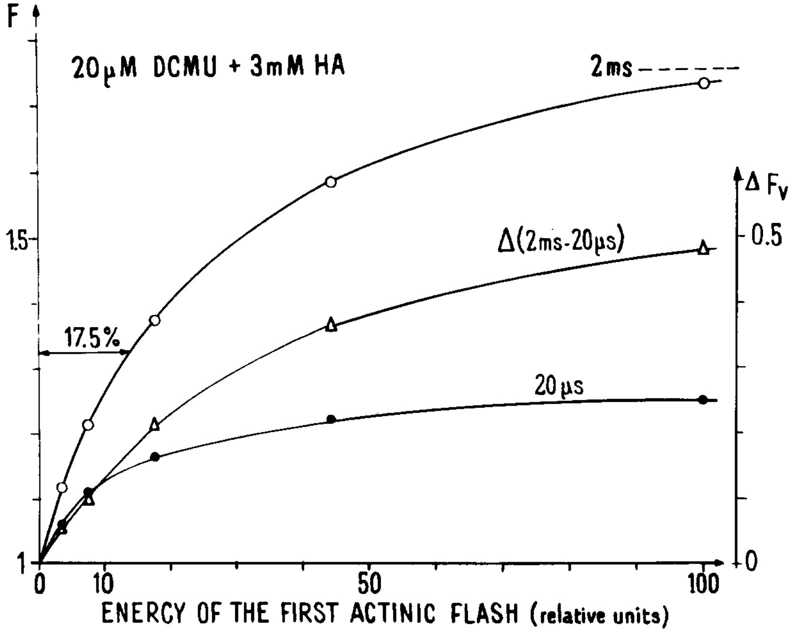


Fig. 3. Same as Fig. 1 except that chloroplasts are treated with 20 μM DCMU plus 3 mM hydroxylamine. Dashed line, maximum fluorescence yield reached after an actinic flash of energy 166 %. The stationary fluorescence level reached after a series of saturating flashes is 3.16.

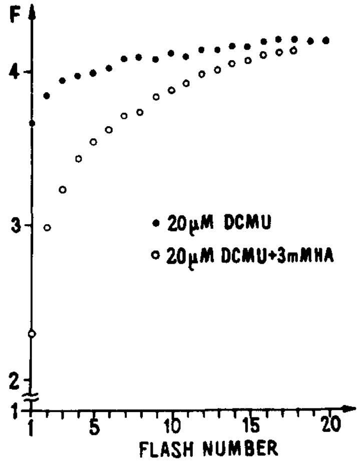


Fig. 4. Fluorescence yield 2 ms after each flash of a series of short saturating flashes given 300 ms apart, in the presence of 20 μM DCMU (●) or 20 μM DCMU plus 3 mM hydroxylamine (○).

suggests a large variability in the rate of reduction of Chl^+ by D among the centers. This variability explains why the slow fluorescence rise is multiphasic [15, 19].

Effect of hydroxylamine

Den Haan et al. [17] and A. Joliot [19] interpreted the effect of hydroxylamine on the fluorescence rise in assuming that the donor Y is destroyed and that Chl^+ is slowly reduced by an auxiliary donor D. Then, one would predict that the probability of double hits would be very low for the duration of the flashes we used. The saturation curves obtained in the presence of hydroxylamine (Fig. 3) which do not present

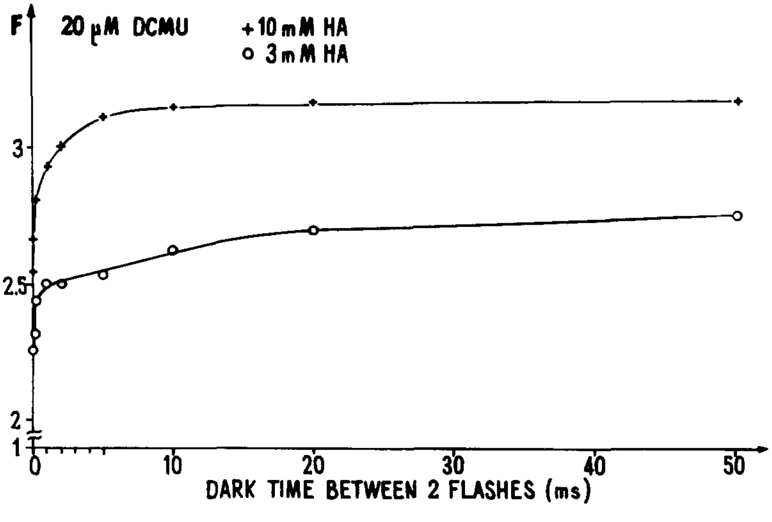
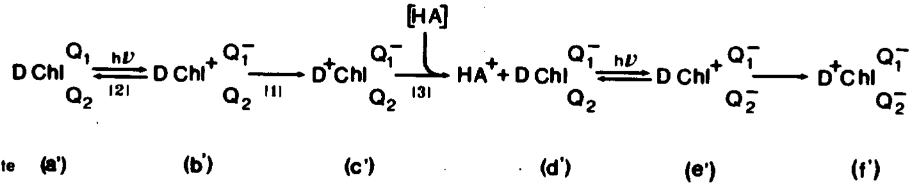


Fig. 5. Fluorescence yield reached 2 ms after the second flash as a function of the dark interval between two short saturating flashes in the presence of 20 μM DCMU plus 3 mM hydroxylamine (○) or plus 10 mM hydroxylamine (+).

any lag favor this interpretation. Nevertheless, the relative amplitude of the 0–20 μs rise to the total rise is higher for a weak flash than for a saturating flash.

The efficiency of quencher destruction by a series of saturating flashes (Fig. 4) is lower in the presence of 3 mM hydroxylamine than in its absence, as already pointed out by Etienne [38]. We interpreted this low efficiency as due partly to the competition between the charge stabilization on D and the back reaction, and partly to the fact that mainly single-hit process occurs in the presence of hydroxylamine. In this case, the reduction of the two electron acceptors would require at least two successive flashes; in order to check this hypothesis, we measured the turnover time of Photosystem II centers in the presence of DCMU plus hydroxylamine. In Fig. 5 is depicted the fluorescence yield reached 2 ms after the second flash as a function of the dark interval between two flashes. The turnover curve is strongly biphasic: A first phase terminated in 1 ms is clearly related to the slow phase in the fluorescence rise, i.e., the reduction of Chl^+. The time course of the second phase is strongly dependent on hydroxylamine concentration contrary to the first phase. This experiment can be interpreted by the following sequence of reactions:



Scheme III

When the second flash is fired, states (a′), (c′) and (d′) undergo a second photoreaction, but only states (a′) and (d′) can stabilize their charges. The fast component of the turnover which does not depend upon the hydroxylamine concentration is due to the reaction [2] in competition with reaction [1]. The slow component (hydroxylamine concentration dependent) is due to reaction [3]. This slow component in the turnover is difficult to interpret if the centers have only one acceptor.

The two-acceptor model also explains the results obtained at high hydroxylamine concentration (0.1 M [19]). At this concentration, a fast rise ($t_{\frac{1}{2}} \simeq$ 5–10 μs) due to a direct reduction of Chl^+ by a prefixed hydroxylamine is followed by a slow rise similar to that observed at lower hydroxylamine concentration. As the duration of this fast reduction is of the same order of magnitude as the duration of the flash, the probability of double hits again becomes significant. For the second hit, Chl^+ is reduced by D, which explains the existence of the slow phase. The fraction of the centers for which the photoactive chlorophyll has fixed a hydroxylamine molecule is certainly not negligible at lower concentration (10 mM). This might be responsible for the difference in the fluorescence increase measured at 10 mM and 3 mM. Even in the presence of 3 mM hydroxylamine, it is likely that for a fraction of the centers, a hydroxylamine molecule has been fixed on the photoactive chlorophyll. These centers undergo a low efficiency double-hit process which might explain the ‘tail’ in the 2 ms saturation curve (Fig. 3).

Energy transfer

Fig. 6 shows the saturation curve observed when a second flash of variable

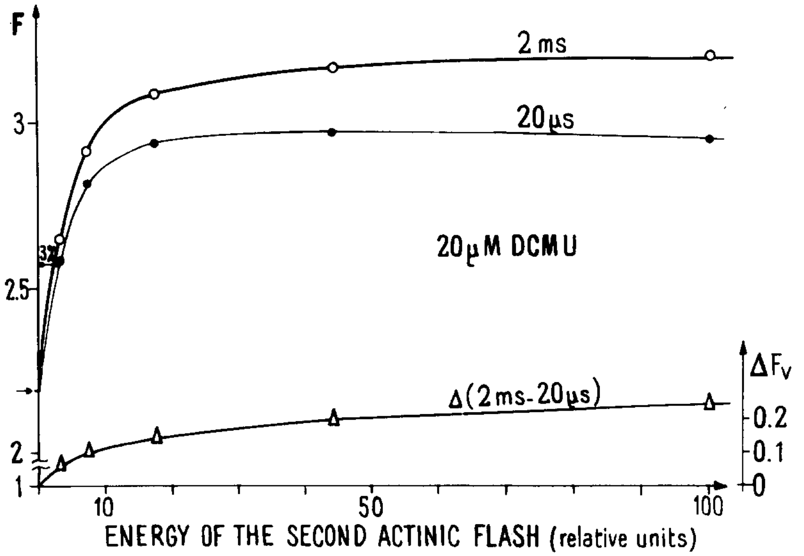


Fig. 6. Left ordinate, fluorescence yield reached 20 μs (●) or 2 ms (○) after a second flash of variable energy given 2.5 s after a first saturating actinic flash. Chloroplasts plus 20 μM DCMU. Right ordinate ΔF, difference between the two preceeding curves.

energy is given 2.5 s after a single flash. Compared to Fig. 1, two major differences appear: First, the half-saturating energy for the 20 μs curve is about 3.5 times lower than that observed for the first flash; this result demonstrates the existence of very efficient energy transfer which increases the cross section of a center when the number of active quenchers decreases. Second, the saturation curve for the slow phase does not show the characteristic lag. This will be discussed later. Similar results have been

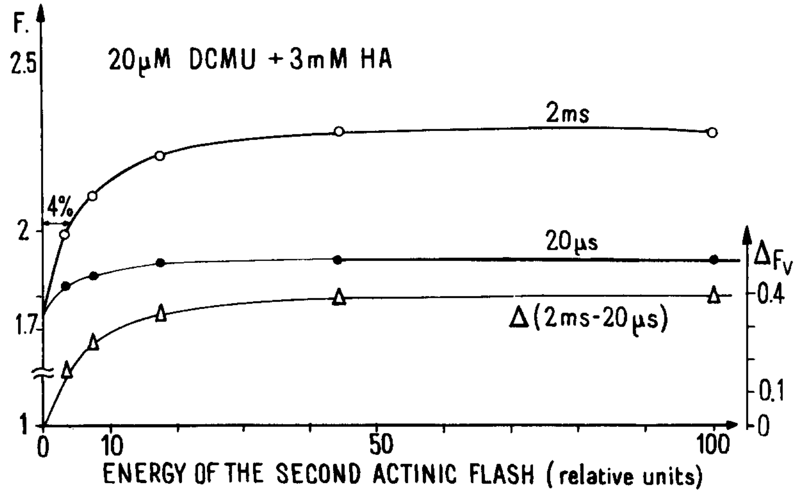


Fig. 7. Same as Fig. 6 except that the chloroplasts are in the presence of 20 μM DCMU plus 3 mM hydroxylamine.

obtained in the presence of ferricyanide (data not shown). The existence of efficient energy transfer is also in agreement with the non-exponential character of the 0–20 μs saturation curves in Figs. 1 and 2. As a significant rise is observed during the flash [6, 17], there is consequently a corresponding increase in the cross-section of the centers. On the contrary, the saturation curves measured in the presence of hydroxylamine (Fig. 3) are closer to an exponential function, which is in agreement with the absence of a fluorescence increase during the flash in this condition [17, 19].

The saturation curves for a second flash fired 2.5 s after a first saturating flash were also studied in the presence of DCMU plus hydroxylamine (Fig. 7). As in the control (Fig. 6), the half-saturating energy is lower for the second flash than for the first one.

Back reactions

The fluorescence decay has been studied in the presence of DCMU after one or six saturating flashes, or after a weak flash (Figs. 8 and 9). The presence of 0.1 μM gramicidin and 0.1 M KCl in the medium eliminates a possible effect of the transmembrane electric field on the back reactions. After a weak flash, i.e., when only single-hit process occurs, the decay is close to an exponential function ($t_{\frac{1}{2}} \simeq 15$ s). The same kinetic behaviour is followed for any flash of lower energy (data not shown). The deviation from a first-order process can be explained if one assumes that the rate constant of the back reaction varies slightly among the centers. After one or six saturating flashes, the decay is more biphasic, as already pointed out by Melis and Homann [30]. This biphasic behaviour is partly due to the non-linear relationship between the concentration of active centers and the fluorescence yield [23].

Photosystem II donors

1. Donors Y and Z. As shown in Fig. 6, an appreciable slow phase is still observed after a second flash given in the presence of DCMU while the corresponding saturation curve does not display any lag. This result is reasonable if one assumes that the equilibrium constant between Z and Y is close to 1. In this case, when the second flash is fired, a fraction of the centers includes the secondary donor Y in the oxidized form Y^+. For these centers, only a single-hit process then occurs and the oxidized chlorophyll is reduced by D through a slow process. Assuming that the value of the equilibrium constant between Z and Y depends upon the number of positive charges stored on Z permits an easy interpretation of the oscillations period 4 of the fast fluorescence rise (< 1 μs) reported by Delosme [6].

Glaser et al. [28] do not observe a lag in the flash saturation curve for the formation of the long-lived $\text{Chla}_{\text{II}}{}^+$ in untreated chloroplasts. This difference with our results may arise from their use of a repetitive flash technique in the absence of DCMU. In these conditions, an appreciable fraction of the centers would include a secondary donor Y in its oxidized form.

2. Donor D. On the basis of kinetic arguments, we argued that the reduction of chlorophyll by the auxiliary donor D is responsible for the slow fluorescence rise either in the presence or in the absence of hydroxylamine. Additional arguments favor this assumption: Den Haan et al. [18] showed that the auxiliary donor functioning in the presence of hydroxylamine is not cytochrome *b*-559. As the slow fluorescence rise is not decreased by addition of ferricyanide (Fig. 2) which is known to oxidize this

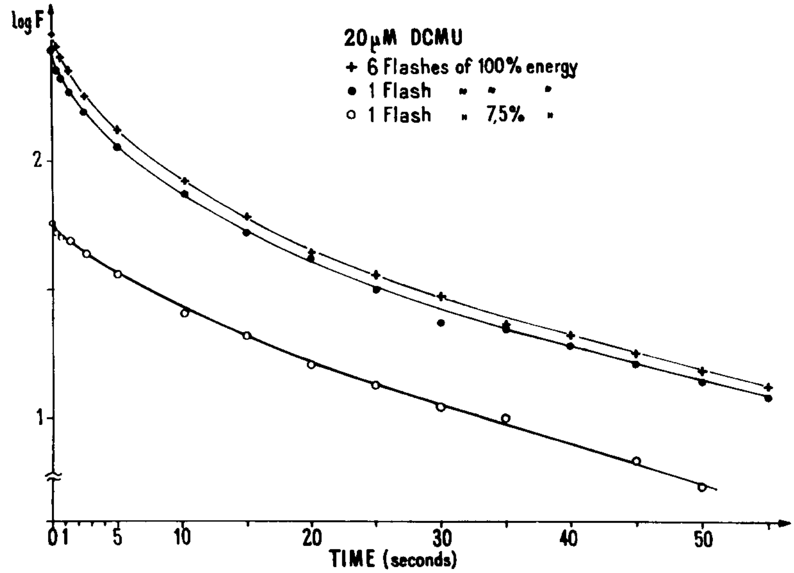


Fig. 8. Fluorescence decay (in log) in the presence of 20 μM DCMU after six saturating flashes given 1 s apart (+), one saturating flash (●) or one weak (7.5 %) actinic flash (○).

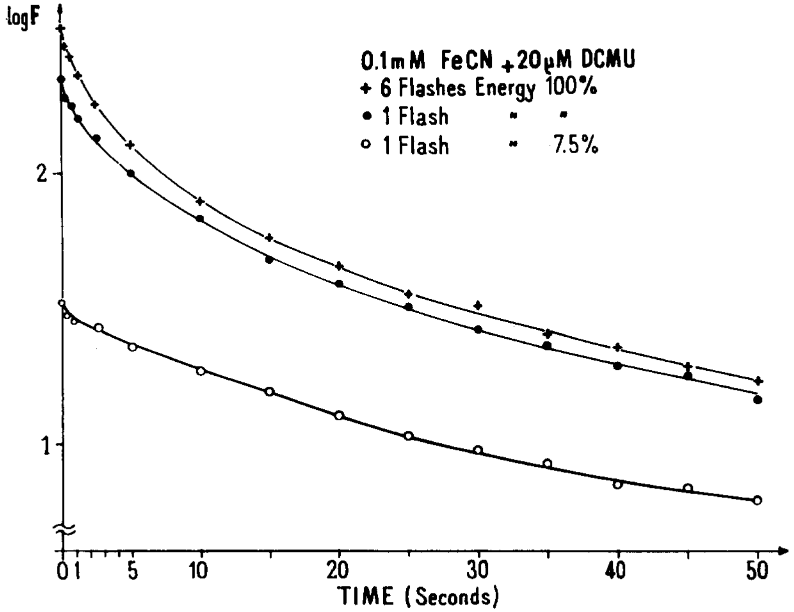
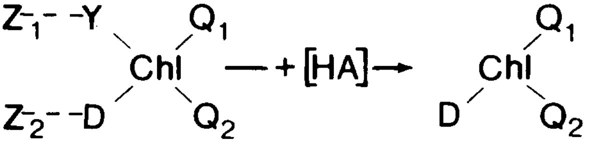


Fig. 9. Same as Fig. 8 except that the chloroplasts are treated with 0.1 mM ferricyanide prior to the addition of 20 μM DCMU.

cytochrome, the same conclusion is reached for the auxiliary donor involved in the absence of hydroxylamine. Thus, the potential of this auxiliary donor is certainly higher than 500 mV.

Two hypothesis can be proposed for the possible function of D : (a) D is an auxiliary donor which is not related to the oxygen-evolving system. Depending on the relative potentials of the transitions $D \rightleftharpoons D^{+} + e^{-}$ and $Z^{n+} \rightleftharpoons Z^{(n+1)+} + e^{-}$, one might expect that charge exchange would occur between Z^{n+} and D via the photoactive chlorophyll. Lavorel and Lemasson [39] proposed a similar hypothesis to interpret the damping of the oxygen oscillations, assuming that a side carrier can reversibly exchange charges with the S states. After six flashes, i.e., when the donors are mainly in the D^{+} and Z^{2+} forms, the back reaction kinetics do not show components of widely different life-times. This suggests that the potentials of the transitions $D \rightleftharpoons D^{+} + e^{-}$ and $Z^{+} \rightleftharpoons Z^{2+} + e^{-}$ are comparable.

(b) D is a part of the main electron transfer chain. The comparison of the rates of the back reactions measured in the presence of DCMU in untreated or hydroxylamine-treated chloroplasts favors this hypothesis: In chloroplasts treated with hydroxylamine and washed afterwards, the life-time of the species D^{+}Chl Q^{-} induced by a single saturating flash is about 100 ms [19]. In untreated chloroplasts subjected to a saturating flash, such a fast phase is not observed in the fluorescence decay (Fig. 8). The D^{+} species thus seems to be 50–100 times more stable when the water-splitting enzyme is intact. In untreated chloroplasts, D might be connected to a carrier Z_2 able to store two charges, the two other positive charges having accumulated on Z_1 connected to Y. The action of hydroxylamine would be as follows:



Scheme IV

In untreated chloroplasts, the positive charge is rapidly transferred from D^{+} to Z_2, which explains the high stability of this state. D could thus be responsible for both EPR signals SIIvf [40] and SIIf [41]. In untreated chloroplasts, Blankenship et al. [42] recently reported a rise time of approx. 20 μs at 20 °C which is coherent with the slow phase in the fluorescence increase. The rate of the charge transfer between D^{+} and Z_2 determines the life time of signal SIIvf (approx. 200 μs). In Tris-treated chloroplasts, D^{+} cannot transfer its charge to Z_2 and one would expect the appearance of a long-lived EPR signal (Signal SIIf). The experiments of Weiss and Sauer [43], which show that the pattern of oxygen evolution stimulated by a 20–40 ns laser flash or by a 10 μs xenon flash are similar, are difficult to reconcile with the presence of D in the main chain. Nevertheless, this hypothesis cannot be ruled out if first, the centers are entirely in the S_0 form in dark-adapted material and second, that efficient double hits occur even with a 20–40 ns laser pulse.

A choice between the two proposed hypotheses requires a refined analysis of the oxygen evolution upon flash and continuous light illumination.

Photosystem II acceptors

If the two proposed acceptors Q_1 and Q_2 were both connected to B, one would expect that in untreated chloroplasts more than one electron would be transferred to B on the first flashes of a series. Bouges-Bocquet (see Fig. 1 in ref. 32) observed that the number of electrons transferred from System II to System I oscillates with a period 2 during a series of flashes. These oscillations are in phase with the ones reported by Velthuys and Amesz [33] in the presence of hydroxylamine, i.e., when mainly single-hit process occurs. Then, it is likely that only one of the two acceptors (Q_1) is connected to B.

We observed that in the presence of DCMU, dithionite is able to reduce neither Q_1 nor Q_2 (data not shown). On the other hand, the fluorescence decay is not accelerated in ferricyanide-treated chloroplasts (Figs. 8 and 9), which means that Q_2^- cannot be appreciably reoxidized by ferricyanide in the 0–60 s time range. These results show that Q_2 is well protected from the external medium. Nevertheless, as pointed out before, it is possible that Q_2^- might be slowly reoxidized by ferricyanide for time of action longer than 60 s.

Quenching properties of the different states of photocenters (Scheme II)

(A). $\mathrm{Chl}^+\left\langle\begin{matrix}Q_1^-\\Q_2\end{matrix}\right.$ (state b): This state appears after a single flash in the presence of hydroxylamine. The absence of a fluorescence increase during the flash [17] shows that its quenching efficiency is identical to that of state (a) $\mathrm{Chl}\left\langle\begin{matrix}Q_1\\Q_2\end{matrix}\right.$, which agrees with the hypothesis of Okayama and Butler [2] and Butler et al. [3].

(B). $\mathrm{Chl}\left\langle\begin{matrix}Q_1^-\\Q_2\end{matrix}\right.$ (state c): 2 ms after a single saturating flash in the presence of hydroxylamine, the major fraction of the centers is in state (c), the remaining fraction is in state (a) due to the back reaction. $F = 1.75$ (Fig. 3, dashed line), which represents about 0.33 of the variable fluorescence, is thus an underestimate of the fluorescence level corresponding to state (c).

(C). $\mathrm{Chl}^+\left\langle\begin{matrix}Q_1^-\\Q_2^-\end{matrix}\right.$ (state d): Immediately after a saturating flash in the presence of ferricyanide, most of the centers are in state (d). Its corresponding fluorescence level is $F \simeq 2.25$ as shown in Fig. 2. Then, state (d) appears to be a much less efficient quencher that state (b).

A theoretical analysis of this model is in progress. A good fit with most of the experimental data available, particularly the fluorescence induction curves in the presence of DCMU, is obtained if one assumes a probability, p, of energy transfer between units equal to 0.7–0.8 [23] and an efficiency for the photoreaction of state (c) 3–4 times lower than for the first hit. This model explains the main divergences with the simplified model for energy transfer proposed in ref. 23, i.e., the final slow increasing phase in the fluorescence induction curves. As pointed out by Lavorel [22], a model of Photosystem II centers with two acceptors also explains the accelerated fluorescence rise observed after a partial back reaction in the presence of DCMU [44, 26, 24].

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REFERENCES

1 Duysens, L. N. M. and Sweers, H. E. (1963) Studies on Microalgae and Photosynthetic Bacteria, pp. 353–372, University of Tokyo Press, Tokyo
2 Okayama, S. and Butler, W. L. (1972) Biochim. Biophys. Acta 267, 523–527
3 Butler, W. L., Visser, J. and Simons, H. L. (1973) Biochim. Biophys. Acta 292, 140–151
4 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
5 Joliot, P., Joliot, A., Bouges, B. and Barbieri, B. (1971) Photochem. Photobiol. 14, 287–305
6 Delosme, R. (1971) in Proc. 2nd Int. Congr. Photosynth., Stresa 1971 (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 1, pp. 187–195, Dr. W. Junk N.V. Publishers, The Hague
7 Duysens, L. N. M., van der Schatte Olivier, T. E. and den Haan, G. A. (1972) Abstr. VI Int. Congr. Photobiol. Bochum 1972, p. 277
8 Zankel, K. L. (1973) Biochim. Biophys. Acta 325, 138–148
9 Homann, P. (1969) Plant. Physiol. 44, 932–934
10 Murata, N. (1969) Biochim. Biophys. Acta 189, 171–181
11 Wraight, C. A. and Crofts, A. R. (1970) Eur. J. Biochem. 17, 319–327
12 Diner, B. and Joliot, P. (1976) Biochim. Biophys. Acta 423, 479–498
13 Duysens, L. N. M., den Haan, G. A. and van Best, J. A. (1975) in Proc. 3rd Int. Congr. Photosynth., Rehovot 1974 (Avron, M., ed.), Vol. 1, pp. 1–12, Elsevier Scientific Publishing Company, Amsterdam
14 Mauzerall, D. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1358–1362
15 Joliot, A. (1975) in Proc. 3rd Int. Congr. Photosynth., Rehovot 1974 (Avron, M., ed.), Vol. 1, pp. 315–322, Elsevier Scientific Publishing Company, Amsterdam
16 Cheniae, G. M. and Martin, I. F. (1970) Biochim. Biophys. Acta 197, 219–239
17 Den Haan, G. A., Duysens, L. N. M. and Egberts, D. J. N. (1974) Biochim. Biophys. Acta 368, 409–421
18 Den Haan, G. A., Gorter de Vries, H. and Duysens, L. N. M. (1976) Biochim. Biophys. Acta 430, 265–281
19 Joliot, A. (1977) Biochim. Biophys. Acta 460, 142–151
20 Knaff, D. B. and Arnon, D. I. (1969) Proc. Natl. Acad. Sci. U.S. 63, 956–962
21 Morin, P. (1964) J. Chim. Phys. 61, 674–680
22 Lavorel, J. (1972) C. R. Acad. Sci. Paris 274, 2909–2912
23 Joliot, A. and Joliot, P. (1964) C. R. Acad. Sci. Paris 258, 4622–4625
24 Joliot, P., Bennoun, P. and Joliot, A. (1973) Biochim. Biophys. Acta 305, 317–328
25 Tumerman, L. A. and Sorokin, E. (1976) Mol. Biol. 1, 628–638
26 Doschek, W. W. and Kok, B. (1972) Biophys. J. 12, 832–838
27 Joliot, P. and Joliot, A. (1973) Biochim. Biophys. Acta 305, 302–316
28 Glaser, M., Wolff, C. and Renger, G. (1976) Z. Naturforsch. 31c, 712–721
29 Paillotin, G. (1976) J. Theor. Biol. 58, 237–252
30 Melis, A. and Homann, P. (1976) Photochem. Photobiol. 23, 343–350
31 Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272
32 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256
33 Velthuys, B. R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94
34 Wollman, F. A. (1977) Thèse de Troisième Cycle, Paris
35 Ikegami, I. and Katoh, S. (1973) Plant and Cell Physiol. 14, 829–836
36 Lavorel, J. (1973) Biochim. Biophys. Acta 325, 213–229
37 Haveman, J. and Mathis, P. (1976) Biochim. Biophys. Acta 440, 346–355
38 Etienne, A. L. (1974) Biochim. Biophys. Acta 333, 497–508

39 Lavorel, J. and Lemasson, C. (1976) Biochim. Biophys. Acta 430, 501–516
40 Babcock, G. T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328
41 Blankenship, R. E., Babcock, G. T., Warden, J. T. and Sauer, K. (1975) FEBS. Lett. 51, 287–293
42 Blankenship, R. E., McGuire, A. and Sauer, K. (1977) Biochim. Biophys. Acta 459, 617–619
43 Weiss, C. and Sauer, K. (1970) Photochem. Photobiol. 11, 495–501
44 Bennoun, P. (1971) Thèse de Doctorat, Paris