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NEW RESULTS ON THE PROPERTIES OF PHOTOSYSTEM II CENTERS BLOCKED BY 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA IN THEIR DIFFERENT PHOTOACTIVE STATES

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SUMMARY

We have studied the 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) action on the different S states by oxygen, fluorescence and luminescence measurements.

We show that no oxygen is evolved during a flash following the addition of DCMU to centers in their S_3 state. This suggests that oxygen inhibition cannot be attributed solely to a blocking between Q and A. For all the photoinactive states, the only remaining pathway for the quencher reoxidation, in the presence of DCMU, appears to proceed through a back reaction. Therefore, the complete quencher regeneration still occurring when the fourth positive charge is formed in the presence of DCMU is also an indication of an action by DCMU at the donor side.

The data well fit the model in which the oscillations of the fluorescence yield and their damping are attributed to a fast equilibrium between two forms of the centers: a photoactive and a photoinactive form, both of which are quenchers. The equilibrium constant depends on the number of positive charges stored and DCMU changes the characteristics of this equilibrium.

INTRODUCTION

A reaction center of Photosystem II can be considered as a permanent association between a primary electron donor Y, a chlorophyll molecule and a primary electron acceptor Q [1]. There exist four photoactive states of the centers S_0, S_1, S_2 and S_3 which differ, at least, by the number of positive charges stabilized on the donor side [2].

After a photochemical charge separation, the centers are for a while in a photoinactive state S'_n ($0 \leqslant n \leqslant 3$). Under normal conditions, the dark transitions $S'_n \rightarrow S_{n+1}$ are fast [3, 4]: Y^+ is reduced by Z (the water-splitting enzyme) and the fast reoxidation of Q^- is mainly achieved by the secondary pool of acceptor A [5]. When four

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

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positive charges have been stabilized on the donor side, oxygen is produced and the donor side is reduced back to its S_0 state within milliseconds [6, 7].

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is a well known inhibitor of oxygen evolution. It blocks the fast Q^- reoxidation by preventing the normal flow of electrons from Q^- to A [8]. Since only S_0 and S_1 are stable in the dark [2], the inhibition of oxygen evolution when DCMU is added to dark-adapted material could simply be due to its action between Q and A: by forbidding more than one photoreaction per center, it would prevent the formation of the state S'_3 precursor of oxygen.

If this assumption is correct, centers blocked by DCMU in their S_3 state should still produce oxygen during a subsequent illumination. This mechanism was tested and by the use of a new technique we were also able to study the effects of DCMU addition on fluorescence and luminescence properties of the different S states.

We are, therefore, able to suggest a possible answer to the following questions: Does DCMU act on the donor side of Photosystem II? Is the blocking by DCMU equivalent to the blocking at low temperature?

MATERIALS AND METHODS

Material

Algae and chloroplasts were used. The algae, *Chlorella pyrenoïdosa*, were cultivated as previously described [7]. They are used at concentrations between 5 and 20 μg total chlorophyll per ml, in their growing medium. The chloroplasts are from market spinach. They are prepared according to a method described by Avron [9] and kept in a darkened vessel at 0 °C until used.

The oxygen measurements were made with a rate electrode (Haxo and Blinks type). Fluorescence and luminescence measurements were made with a laboratory-constructed apparatus (Fig. 1). The biological material is initially contained in a dark vessel and is magnetically stirred, under air pressure (approx. 1.5 atm). Its flow

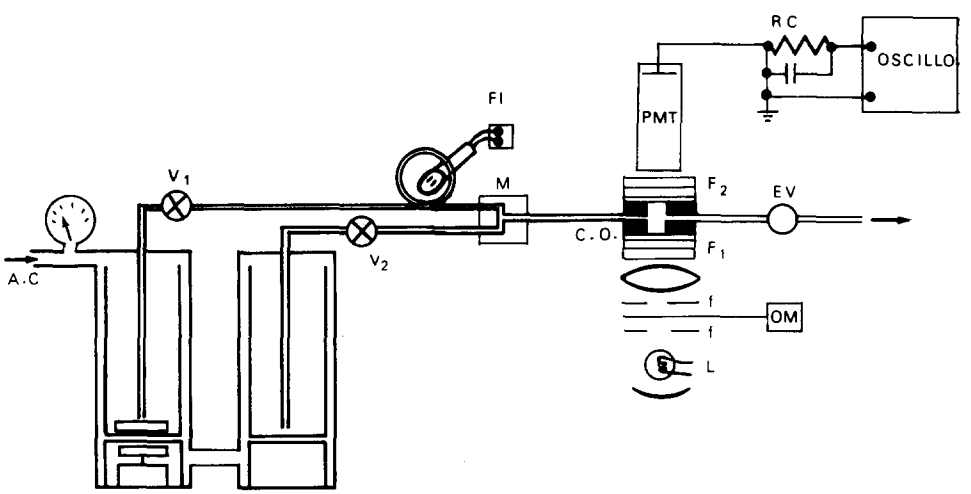

Fig. 1. Stopped-flow apparatus. AC, compressed air; EV, electro valve; V_1, V_2, mechanical valves, Fl, Flash lamp; M, Mixing chamber; f, lenses; F_1, F_2, complementary filters; C. O., observation chamber; OM, electromechanical shutter; PMT, photomultiplier tube; L, Tungsten lamp.

through a pyrex capillary tube to a small-volume observation chamber is controlled by an electrovalve. It can be rapidly mixed with another solution (initially in an identical vessel) in a 4-jet mixing chamber situated before the observation chamber. The minimum time between the mixing and the observation is about 80 ms. The biological material can be illuminated before the mixing in a spiral of capillary tubing surrounding a flash lamp. The preilluminating flashes are obtained with a "Strobotac", their duration is 2 μs at half-peak, and their energy 0.1 J. Blue actinic light selected from a 750-W projection lamp by a series of filters (Corning 4-96 and Calflex) is admitted to the observation chamber by an electromechanical shutter (opening time approx. 1 ms, closing time 10 ms). The light intensity is about $4 \cdot 10^4$ erg $\cdot$ s^{-1} $\cdot$ cm^{-2}. Fluorescence emission is observed in the axis of the detecting beam and is detected by means of an EMI photomultiplier tube equipped with Corning 2-64 and Wratten 70 filters. The voltage through an appropriate resistor is displayed either on a storage oscilloscope (Tektronix) or on a multichannel analyzer (Intertechnique) which allows direct integration of the fluorescence rise curves. For luminescence measurements the anode current is measured by a sensitive D.C. amplifier (Lemouzy) and the amplified signal is fed to the oscilloscope. The experiments are performed at room temperature.

RESULTS

Oxygen evolution in the presence of DCMU

One of the points of this work is to see whether, oxygen can be produced by centers which have reached their S_3 state before the addition of DCMU. The oxygen

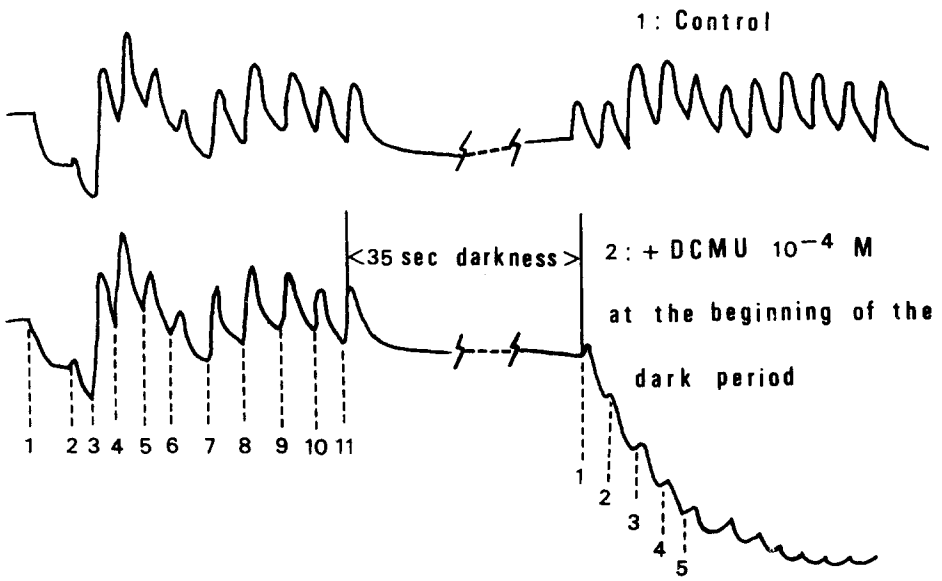

Fig. 2. Recordings of the rate electrode response for chloroplasts illuminated by two series of 11 flashes, 35 s apart. (The time between flashes is 500 ms.) On the ordinate, the relative rate of oxygen emission. On the abscissa, the time. The height of the peaks is an indication of the amount of oxygen evolved per flash. In the control experiment 1 (top curve) no DCMU was added. In the second experiment 2 (bottom curve) 10^{-4} M DCMU was added just after the last flash of the first series.

evolved is measured during a flash sequence. The photosynthetic material is maintained on the surface of a platinum electrode by a dialysis membrane which separates it from the flowing medium. 30 s are necessary in this experimental set-up for the complete inhibition of oxygen production, when DCMU (10^{-4} M) is added in the medium. In *Chlorella*, the S_3 deactivation half-time is less than 30 s, it is thus impossible to do the experiment with algae (in contrast to the experiments described by Duysens [10]) and chloroplasts, where deactivation processes are slower [11], are used instead. Two series of flashes are given 35 s apart. In the DCMU experiment, an over-saturating DCMU concentration (10^{-4} M) is injected immediately after the last flash of the first series.

The oxygen evolved by the first flash of the second series corresponds to the amount of centers brought to their S_3 state by the first flash series which has not been deactivated during the dark period. The results are shown in Fig. 2 (the amount of oxygen evolved per flash is proportional to the peak height). There is much less oxygen evolved in the presence of DCMU than in the control experiment. It can be explained in two ways: (a) The S_3 stability is decreased by DCMU, most centers are back to the S_2 state when the flash is fired. (b) S_3 states are still present but DCMU blocks the transfer of the fourth positive charge to the water molecule.

In both cases, it implies that DCMU has an action on the donor side.

Intrinsic rate of DCMU action

The fluorescence rise curve in strong light consists of two successive phases: the fast initial photochemical rise $\Phi_0 \rightarrow \Phi_I$ is followed by a slower "thermal" phase $\Phi_I \rightarrow \Phi_p$. These fluorescence variations cannot be attributed solely to the primary electron acceptor Q, the quencher of fluorescence in its oxidized form [8], and Delosme introduced the concept of a non-photochemical quencher R, the destruction of which seems to follow the reduction of the secondary electron acceptor pool A [12]. The effect of DCMU addition prior to illumination is to suppress the thermal phase by raising Φ_I to Φ_p. In the two-quencher hypothesis introduced above, the action of DCMU has two consequences: (1) A blocking of the fast electron transfer from Q^- to A as classically admitted [8]. The threshold for saturating light intensities which corresponds to a photochemical reduction of Q faster than its reoxidation is therefore lowered: for the light intensities used in this work Φ_I and Φ_p of the control are not saturated but in the presence of DCMU, the maximum yield is attained. (2) A disconnection of the quencher R.

Not long after the fast mixing in the dark with DCMU, the fluorescence rise curves are of the following type: a fast initial photochemical rise of greater amplitude than that of untreated algae, followed by a slow rise up to the maximum level (Fig. 3). We determined the time necessary for a "normal" DCMU rise curve to occur and came to the following conclusions: (a) It is concentration dependent; we, therefore, used a high DCMU concentration ($5 \cdot 10^{-4}$ M) to obtain a complete action 400 ms after the mixing. (b) No kinetic heterogeneity between the two DCMU actions was found by this method. We, therefore, cannot exclude a hypothesis in which both actions are attributed to the same mechanism. (c) The presence of chemicals (carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) or NH_2OH) acting on the donor side [13] does not change the rate of action whereas it is lowered by a low concentration of *m*-dinitrobenzene acting on the acceptor side [14].

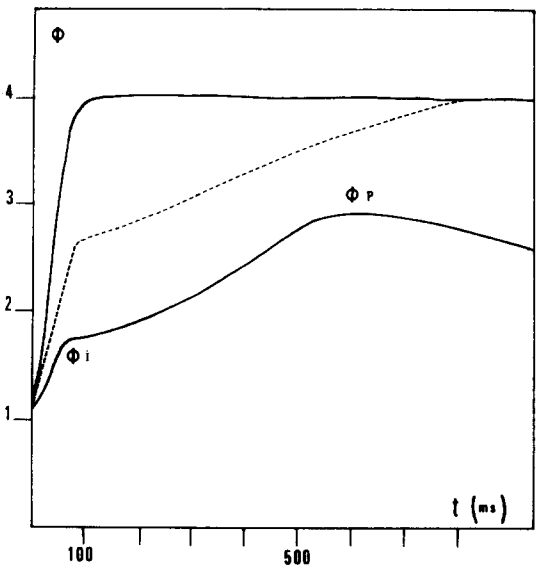

Fig. 3. Fluorescence rises for *Chlorellae*. Bottom curve: in the absence of DCMU. Φ_1: end of the fast rise. Φ_P: maximum of the thermal rise. Medium curve (dotted line): 200 ms after mixing with 10^{-4} M DCMU. Top curve: in the presence of 10^{-4} M DCMU. Ordinate: Fluorescence yield (arbitrary units). Abscissa: Time of illumination (ms).

Fluorescence rise curves in the presence of DCMU and evaluation of the concentration of the photochemical quencher Q

In order to vary the distribution of the S states, the algae are preilluminated by a number of short flashes. The algae are then mixed with DCMU after a dark period sufficient to allow the relaxations $S'_n \rightarrow S_{n+1}$ but much shorter than the Z^{n+} lifetimes [10]. After complete action of DCMU, a continuous illumination reveals the fluorescence rise curves which correspond to the transitions $S_n \xrightarrow{h\nu} S'_n$ occurring in the presence of DCMU.

The experimental procedure is the following: the sample in the preilluminating chamber is first renewed by a flow period. It is then illuminated by flashes 300 ms apart. A suitable flow time is used to transfer this sample through the mixing chamber (where it is mixed with DCMU) to the observation chamber where light is admitted 400 ms after the mixing.

The fluorescence rise curves depend on the number of preilluminating flashes. The maximum difference is between the fluorescence rise of control algae (dark adapted) and the fluorescence of algae preilluminated by 2 flashes (Fig. 4). The first one corresponds mainly to the transition: $S_1 \xrightarrow{h\nu} S'_1$, the second one corresponding mainly to $S_3 \xrightarrow{h\nu} S'_3$. The first consists of a single fast rise up to a maximum level (when the DCMU concentration is lower, a small slow phase can be detected as reported elsewhere [15]). In the second one, the fast initial rise is followed by a slow phase which finally reaches a maximum fluorescence yield lower than that of the control algae. The initial fluorescence yield (Φ_0) and the fluorescence yield at the end of the fast rise (Φ_1) oscillate with the number of preilluminating flashes. The amplitude of the fast rise also shows an oscillatory pattern (Fig. 4 insert).

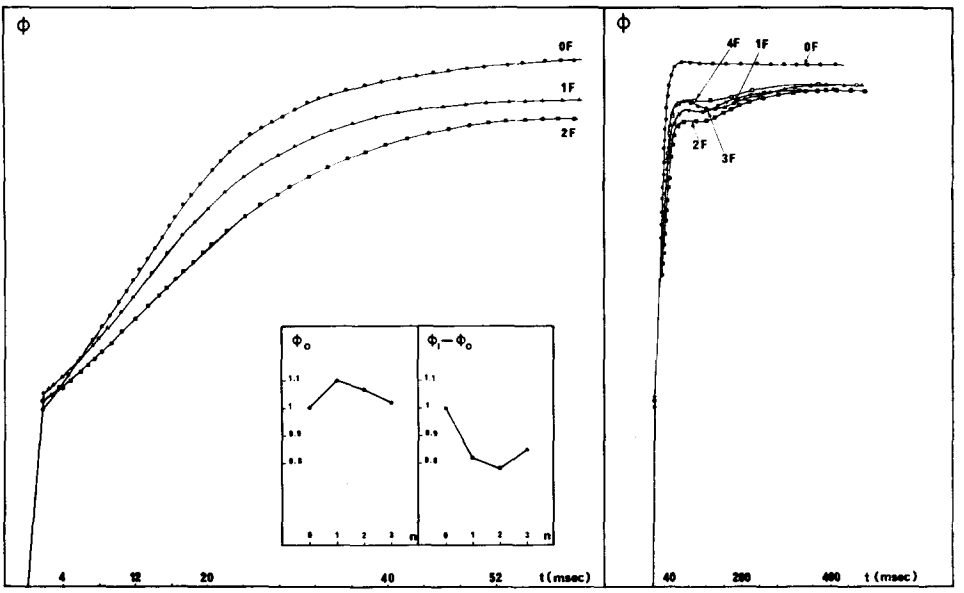

Fig. 4. Fluorescence rise curves for *Chlorellae* after 0 to 4 flashes and mixing with $5 \cdot 10^{-4}$ M DCMU. ●–●, 0 flash; ▽–▽, 3 flashes; ▲–▲, 1 flash; □–□, 4 flashes; ■–■, 2 flashes. Ordinate: two different time scales (in ms). Abscissa: fluorescence yield (arbitrary units). Insert: Oscillations of the Φ_0 level (arbitrary units) and of the photochemical phase, Φ_1–Φ_0, (arbitrary units) (revealed by a continuous illumination) with the number of preilluminating flashes. (Algae mixed, after preillumination with $5 \cdot 10^{-4}$ M DCMU.)

It is very important to know whether or not the same amount of quencher is destroyed during the different fast rises. Since no linear relationship exists between the fluorescence yield and the quencher concentration, this concentration has to be evaluated by other means [16]. Bennoun and Li recently proved that a linear relationship exists, for DCMU-treated chloroplasts, between variable fluorescence and photochemical rate [17]. This validates the evaluation of the quencher concentration by the complementary area of the fluorescence curves when the same maximum yield is attained. When the maximum yield varies the significance of the complementary area is not straightforward.

If, in only case, a reduced quencher Q corresponds to a fluorescent state, variations of the maximum fluorescence yield can be attributed either to a change in the amount of non-photochemical quenching, the amount of photochemical quencher being constant, or to a change in the amount of photochemical centers (some centers are transformed into permanent quenchers). In the first case, inasmuch as the non-photochemical quenching does not vary during a given fluorescence rise, Lavergne showed that the complementary area normalized to the maximum fluorescence yield gives an absolute evaluation of the Q concentration [18]. In the second case, Lavorel has tested, on the "îlot model" [19] that the blocking of a number of centers in a permanent quenching state did not result in an appreciable decrease of the normalized area. Experimentally by ultraviolet irradiation [20] or DNB treatment [21], a large amount of centers can be blocked in a permanent quenching state and no appre-

ciable change of the normalized area is found. One has thus to admit that the active light absorption [21] decreases with a decreasing number of photoactive centers.

With the accuracy used in obtaining the actual measurements, the normalized area was found to be invariant. The half-time of the rise is equivalent for all the curves of Fig. 4. The Φ_I variations can, therefore, be due to some centers in a non-photochemical quenching state.

Fluorescence decay and regeneration of the quencher in the dark

Although DCMU blocks the fast reoxidation of Q^- by A, for dark-adapted material there remains a slow dark decay of the fluorescence yield from the light-induced value [22, 23]. This fluorescence decay exists also for algae mixed with DCMU after any number of preilluminating flashes. The fluorescence yield, restored after a variable dark time (Δt), was revealed by a second illumination. The time course of this decay depends on the number of preilluminating flashes. The fastest decay is observed for algae preilluminated by one flash, the slowest for control algae. These decays correspond to a regeneration of photoactive states where the oxidized acceptor Q is a quencher of fluorescence. Fig. 5 shows the time courses of quencher restoration. The fastest corresponds to algae preilluminated by one flash. Even after two flashes, which bring the centers mainly to an S_3 state, mixing with DCMU and illumination during which a photochemical charge separation occurs, the fluorescence decay and quencher restoration are relatively fast and completed within seconds. This fact will be discussed in detail later.

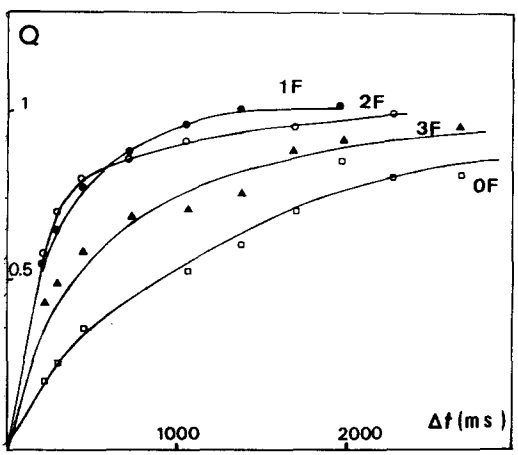

Fig. 5. Dark restoration of the quencher Q after a continuous illumination in presence of $5 \cdot 10^{-4}$ M DCMU. □–□, unpreilluminated algae; ●–●, after 1 preilluminating flash; ○–○, after 2 preilluminating flashes; ▲–▲, after 3 preilluminating flashes.

Luminescence decays

Luminescence was observed after the first illumination. The decay depends on the flash number (Fig. 6).

Luminescence originates from a back reaction, Q^- being one of the necessary substrates. Following Lavorel's formulation [24], we can, therefore, consider that

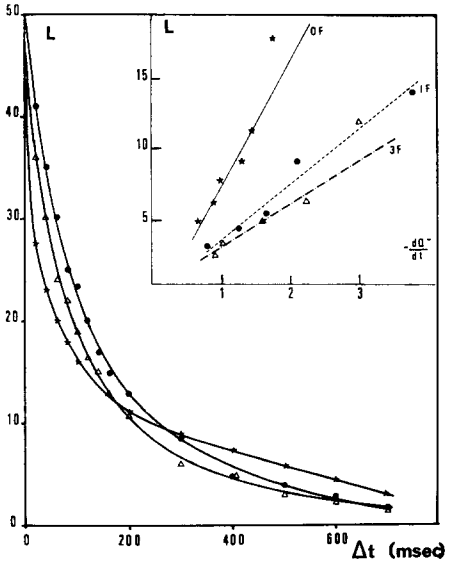

Fig. 6. Luminescence decay after mixing with $5 \cdot 10^{-4}$ M DCMU and a 200-ms continuous illumination. ★–★, no preilluminating flash; ●–●, 1 flash (decay after 2F is equivalent); △–△, 3 flashes. Ordinate: luminescence intensity (arbitrary units). Abscissa: dark time (ms). Insert: Luminescence intensity versus the rate of the quencher regeneration. In presence of $5 \cdot 10^{-4}$ M DCMU. ★–★, no preilluminating flash; ●- - -●, 1 preilluminating flash; △- - -△, 3 preilluminating flashes.

the luminescence intensity (L) is proportional to the rate of Q^- decay by recombination: $L\alpha - [dQ^-/dt]_L$. It is, in general, part of the total Q^- decay:

$$-\left[\frac{dQ^-}{dt}\right] = -\left[\frac{dQ^-}{dt}\right]_L + \sum_K -\left[\frac{dQ^-}{dt}\right]_K$$

where the summation is in competition with the recombination. In the DCMU-blocked system, the back reaction might be the only remaining cause of the Q^- decay and in this case there should exist a linear relationship between the luminescence intensity L and the Q^- decay rate. Such a linear relationship has been established (Fig. 6, insert). However, the accuracy is relatively poor because of experimental error and of a restricted time period.

Effects of CCCP and NH_2OH on fluorescence and luminescence

CCCP is one of the chemicals which accelerates the kinetics of S deactivation [25]. It catalyzes an irreversible reduction in competition with the back reaction. If the algae are preincubated with 10^{-5} M CCCP and submitted to the same experimental procedure as previously described, the fluorescence rise curves during the first illumination still start from different Φ_0 levels (Φ_0 increasing with the flash number) but they all reach the same maximum yield at the end of a monotonous fast rise. The dark fluorescence decay is considerably slowed down (Fig. 7), and the luminescence is inhibited.

The same sort of results are obtained with NH_2OH which reduces irreversibly

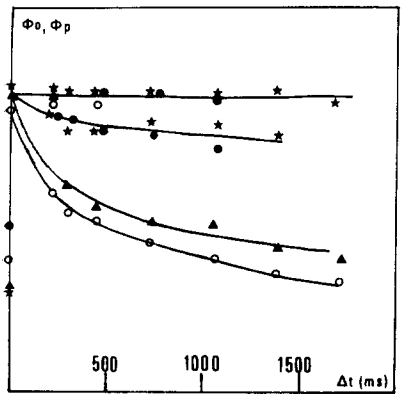

Fig. 7. Dark fluorescence decay in the presence of $5 \cdot 10^{-4}$ M DCMU and 10^{-5} M CCCP after the first 150 ms illumination. The Φ_0 (initial fluorescence yield indicative of the dark decay) and Φ_p (fluorescence yield attained after 150 ms of a second illumination) are both plotted. Control experiment: ▲–▲, no preilluminating flash before the mixing with $5 \cdot 10^{-4}$ M DCMU; ○–○, 1 preilluminating flash. Chlorellae preincubated with 10^{-5} M CCCP: ★–★, no preilluminating flash before the mixing with DCMU; ●–●, 1 preilluminating flash. Abscissa: dark time between the two illuminations (ms). Ordinate: fluorescence yield (arbitrary units).

the oxidized donor [26]. The blocking of the Q^- reoxidation remaining in the presence of DCMU by two chemicals inducing an irreversible reduction of the oxidized donor side also favors a hypothesis which attributes the quencher regeneration to a back reaction.

Effects of DCMU on the deactivation kinetics

If the quencher regeneration is indeed due to a back reaction, its kinetics can be compared to the S_2, S_3 deactivation kinetics (Table I). DCMU considerably decreases the stability of the positive charges. However, it is important to note that this effect is observed when the last positive charge is produced in the presence of DCMU. The totality of Q^- is, therefore, involved in the deactivation reaction whereas in the normal case its concentration is rapidly brought to a minimum (Q^- being reoxidized by A). This rapid deactivation is to be compared with the one occurring when A is mainly in a reduced state [27].

TABLE I

DEACTIVATION HALF-TIMES

	S_3	S_2
Normal algae (from Joliot et al. [11])	12 s	30 s
With $5 \cdot 10^{-4}$ M DCMU present during the formation of the last positive charge	200 ms	900 ms

These results cannot give an insight in the stability of S_2 and S_3 when DCMU is added after the Q^- reoxidation. However, in that case one would predict that DCMU would stabilize the positive charges if it was only acting between Q and A when A^- is supposed to be the main substrate for deactivation [28].

DISCUSSION

It has been recently shown that a low concentration of DCMU destabilizes the S_2 and S_3 states in chloroplasts whereas in algae only the S_2 deactivation is accelerated while the stability of S_3 is increased [29, 30]. By extrapolation these differences would forbid a direct comparison between the results on oxygen in chloroplasts and those on fluorescence and luminescence in algae, they would also provide an explanation for a discrepancy between results previously obtained on chloroplasts and algae [10, 31]. Now, let us assume that in algae the S_3 state is indeed stabilized when DCMU is added. In that case after 2 preilluminating flashes and mixing with DCMU, most centers are in their S_3 state and the following fluorescence induction gives evidence for the fourth positive charge formation. If the positive charges were then reduced by water within milliseconds, the centers should be blocked in a permanent fluorescent state $ZYChlQ^-$ since the Q^- reoxidation seems to proceed, in all cases, through a back reaction. This is not experimentally found and the reoxidation of Q^- is completed in some seconds (Fig. 5 curve: "2F"). These facts suggest that DCMU at a high concentration has an inhibitory effect on the donor side in addition to the blocking between Q and A.

The same conclusion can be reached from a comparison between the effects of DCMU and of a low temperature [32]. At -55 °C the fluorescence rise for the S_0, S_1 states is fast, most quenchers can be destroyed by a single flash, on the contrary the fluorescence rise for the S_2, S_3 states is slow, a single flash destroys a small part of the variable fluoresence. The slow fluorescence rise can be correlated in part with the photooxidation of cytochrome b_{559} [33] which is not involved in the oxygen production [34], however, the fluorescence properties of the different S states at -55 °C closely resemble those observed at normal temperature [32]. A slow fluorescence rise photochemically limited can be due to a fast equilibrium between two forms of the centers (as we suggested elsewhere [15]): A photochemical quenching form T and a non-photochemical less quenching form T'. If the rate of photochemical trapping is slower than the rates of the equilibrium, the fluorescence rise will be photochemical with the rate attenuated by a factor $1/(1+K(n)^{-1})$ with $K(n) = T/T'$ the equilibrium constant depending on the state number (high for S_0, S_1, low for S_2, S_3). The existence of two forms of the centers with only one leading to photochemical activity would also provide a possible explanation for the "misses" responsible for the damping of the oxygen and fluorescence oscillations [2, 35]. In this case the misses would be mostly on the S_2 and S_3 states.

In the presence of DCMU, in spite of the fluorescence oscillations, the fluorescence rise time is equivalent for all states. The effect of DCMU on the donor side might be interpreted as a change in the rates of the equilibrium which would then become much slower than the photochemical rate constant. It can occur at concentrations higher than those needed for the blocking between Q and A and this would explain the contradiction between Duysens's conclusions [10] and ours.

An interaction between the donor side and DCMU is plausible in view of the similarities between its chemical formula and those of various agents interacting with the donor side [36].

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