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EFFECTS OF CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE AND HYDROXYLAMINE ON THE PHOTOSYSTEM II ELECTRON EXCHANGE MECHANISM IN 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA TREATED ALGAE AND CHLOROPLASTS

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SUMMARY

The effects of NH_2OH and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated algae and chloroplasts were studied. In the presence of DCMU, the photochemically separated charges can only disappear through a recombination back reaction; both substances induce an irreversible reduction of the donor side and after sufficient illumination their action in the presence of DCMU leads to the formation of a permanent fluorescent state.

In the DCMU+CCCP system, a fast fluorescence induction curve is observed. The fluorescence yield is brought to its maximum by two flashes. The luminescence emission is strongly inhibited and most centers reach their permanent fluorescent state after one flash.

In the DCMU+NH_2OH system, a slow fluorescence rise is observed and several saturating flashes are needed for the fluorescence yield to reach its maximum. The exhaustion of the NH_2OH oxidizing capacity and the complete transformation to a permanent fluorescent state also require a large number of flashes.

The reduction pathway catalyzed by CCCP appears to be a good competitor to the back reaction, while NH_2OH seems to be a relatively inefficient donor.

In addition the action of NH_2OH and CCCP on fluorescence suggests that the donor side influences the quenching properties of Photosystem II centers. A possible mechanism is proposed.

INTRODUCTION

Photosystem II reaction centers can be regarded as an association between a primary electron donor Y, a primary electron acceptor Q and a chlorophyll molecule [1]. In their photoactive states, acting as efficient exciton traps, they quench the fluores-

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

cence emission. After a photochemical charge separation:

$$Y\ Chl\ Q \xrightarrow{h\nu} Y^+\ Chl\ Q^-,$$

they are transitorily in a photoinactive state unable to trap an exciton and hence to quench the fluorescence. Under normal conditions, the dark regeneration of a photoactive state is rapidly achieved with a corresponding fast fluorescence decay [2, 3]. Y^+ is reduced by the water-splitting enzyme Z and Q^- is mainly reoxidized by the secondary acceptor A [4]. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is known to inhibit the electron flow from Q^- to A [5]. Although the fast pathway for the quencher regeneration is then blocked, there remains a slow Q^- dark reoxidation which has been attributed to a recombination of charges [6]. Therefore, even in the presence of DCMU, after a sufficient dark time, the fluorescence yield decays to its low dark-adapted level. This process will be abolished if an irreversible reduction of the oxidized donor occurs without involving Q^-. It can be achieved in two ways: by the action of NH_2OH or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). NH_2OH at a high concentration destroys Z by manganese extraction [7]. It is irreversibly photooxidized by System II in a simple one quantum process [8]. CCCP, at concentrations higher than needed for its uncoupling effect, interacts with the donor side and inhibits oxygen evolution by an acceleration of the deactivation rate constants [9]. It does not seem to be itself reduced [10] but rather it catalyses a reduction pathway distinct from the back reaction, which involves carotenoids [11] or another endogenous electron donor located between Q and $NADPH_2$ [12].

Homann [13], indeed, found a complete inhibition of the fluorescence decay remaining in the presence of DCMU by the action of either CCCP or NH_2OH. Analogous results were found by Bennoun for the NH_2OH treatment [6].

In the present study, CCCP and NH_2OH were both found to modify the fluorescence rise curve which follows the progressive reduction of Q in DCMU-treated material. Whereas the fluorescence rise was slightly speeded up by the CCCP action, it was slowed down by the NH_2OH action.

The slow fluorescence rise in the presence of DCMU and NH_2OH is associated with a failure to bring the fluorescence yield to its maximum during the first short saturating flashes of a series. Since neither CCCP nor NH_2OH are supposed to act on the presumed fluorescence quencher Q, these experimental facts suggest that the donor side influences the fluorescence quenching properties of Photosystem II centers. Several recent experiments led different authors to the same conclusion [14–16]. A thorough study of the effects of NH_2OH and CCCP was undertaken in the aim of investigating the mechanism of such an influence.

MATERIALS AND METHODS

Algae (*Chlorella pyrenoïdosa*, Chick, Emerson strain) were cultivated and harvested as previously described [17]. They are used at an equivalent chlorophyll concentration of 5–50 $\mu g \cdot ml^{-1}$ in their culture medium.

Chloroplasts were prepared from market lettuce according to a method described by Nelson et al. [18]. They were kept in a darkened vessel at 0 °C until used at

an equivalent chlorophyll concentration of 1 mg/ml in a sucrose–Tris–NaCl buffer at pH 7.8 with 0.1 M KCl added.

Fluorescence measurements were made with an apparatus already described [19]. Fluorescence is either monitored during continuous illumination with blue actinic light (light intensity: 2.10^4 erg $\cdot$ cm^{-2} $\cdot$ s^{-1}) or during brief saturating flashes (duration: 5 μs, flash energy: 0.3 J). The photoelectric signal is displayed on a multichanel analyzer (Intertechnique) which allows direct integration of the curves, or on a storage oscilloscope (Tektronix). Due to input impedance limitations, the fluorescence signal during short flashes was widened and only the maximum was taken into account.

Luminescence at 685 nm was monitored with a laser phosphoroscope built by Lavorel and described elsewhere [20]. The light source is a Helium Neon laser (λ = 632,8 nm), 50-μs flashes at a given frequency are produced by a combination of rotating discs. A 50- to 100-μs flash duration is needed to give more than one quantum per photoactive center (saturating flash). The photomultiplier tube was turned off during the flashes to avoid the artefact resulting from the intense fluorescence signal. The luminescence signal in the form of amplified photoelectron pulses was stored with a 3-μs or 30-μs resolution in a multichannel analyzer (Intertechnique).

The photooxidation of hydroxylamine was detected with the polarographic method described by Joliot and Joliot [21]. All experiments are performed at room temperature (20 °C).

The fluorescence and luminescence measurements are done with flow methods which require a large volume of photosynthetic material for each experiment. This is one of the reasons why the fluorescence and luminescence experiments were done with algae. The main reason, however, is that with algae in the presence of NH_2OH+ DCMU, the fluorescence induction is invariably slower than with DCMU alone [22]. With chloroplasts either "fast" or "slow" induction can be obtained depending on an unknown varying factor (probably linked to the chloroplast membrane integrity). Bennoun and Li [23] published experiments in which a fast fluorescence rise was observed for chloroplasts mixed with DCMU and NH_2OH. The same authors have also obtained slow inductions with different batches of chloroplasts (personal communication). We, therefore, use chloroplasts only for the study of NH_2OH photooxidation since with algae the oxidized NH_2OH remains inside the algae cells and cannot be detected amperometrically .The chloroplasts, we used, displayed a slow fluorescence rise in the presence of NH_2OH+DCMU.

RESULTS

Fluorescence induction under continuous illumination

In presence of DCMU, the fast initial sigmoidal rise is followed by a slow increase of the fluorescence yield which lasts for several seconds (Fig. 1, curve 1). If the algae are preincubated with DCMU ($5 \cdot 10^{-5}$ M) and CCCP (10^{-5} M), the initial part of the curve is identical to the former but a maximum fluorescence yield lower than the former maximum is reached at the end of the fast rise (Fig. 1, curve 2). After a dark incubation of at least 10 min with NH_2OH ($2 \cdot 10^{-3}$ M) and DCMU ($5 \cdot 10^{-5}$ M), the fluorescence induction starts from a higher level than with DCMU alone and, through a non-sigmoidal biphasic rise, it slowly reaches a lower maximum yield (Fig. 2,

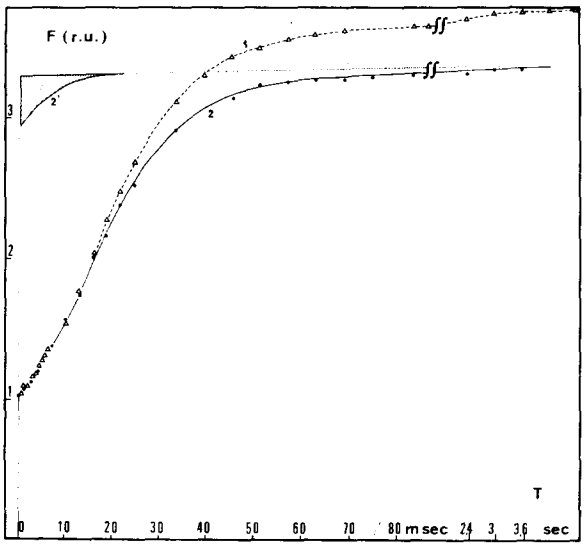


Fig. 1. Effect of CCCP on the fluorescence induction curve of DCMU-treated *Chlorella*. Algae concentration : 5 μg chlorophyll total per ml in growing medium. Light intensity $\approx 10^4$ erg · cm^{-2} · s^{-1}. Abscissae: time of illumination. Ordinate: fluorescence yield (relative units). Curve 1, 50 μM DCMU added in the dark; Curve 2, 50 μM DCMU and 10 μM CCCP added in the dark; Curve 2', after one preilluminating flash 50 μM DCMU and 10 μM CCCP added in the dark. Darkened area: evaluation of the quencher concentration.

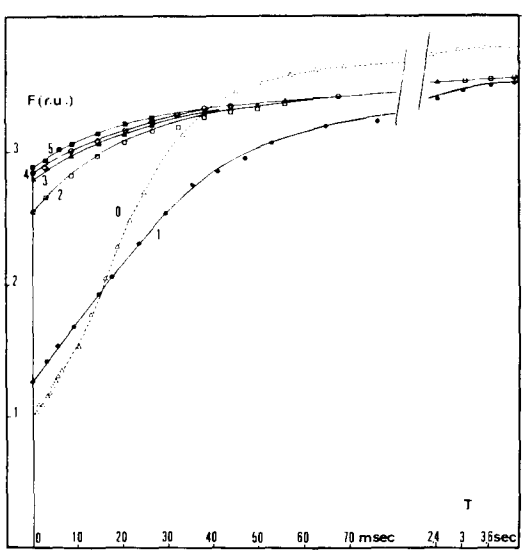


Fig. 2. Effect of NH_2OH on the fluorescence induction curve of DCMU-treated *Chlorella*. Curve 0, 50 μM DCMU added in the dark; Curve 1, 50 μM DCMU and 2 mM NH_2OH added in the dark (20 min of incubation); Curve 2, 50 μM DCMU and 2 mM NH_2OH preillumination by one flash; Curve 3, 50 μM DCMU and 2 mM NH_2OH preillumination by two flashes; Curve 4, 50 μM DCMU and 2 mM NH_2OH preillumination by three flashes. Other conditions same as in Fig. 1.

curve 1). If CCCP is added to the NH_2OH+DCMU-treated algae, it suppresses the second slow phase without affecting the initial part of the rise.

Without DCMU, the fast initial photochemical rise is followed by a large "thermal phase" attributed to the destruction of a non-photochemical quencher R [24]. The slow phases observed in DCMU or in NH_2OH+DCMU could result from the same mechanism: the destruction through a slow thermochemical reaction of a non-photochemical quencher. If it were the case, the fluorescence induction curves would not depend only on the number of photons absorbed. The kinetics of fluorescence in the presence of NH_2OH+DCMU were, therefore, recorded with two light intensities differing by a factor of 100. The identity of the two curves when the number of incident photons (product of the light intensity by the time) is plotted on the abscissae (Fig. 3) shows that the slow phases observed have no correlation with the normal "thermal phase" [24] and are indeed photochemically limited.

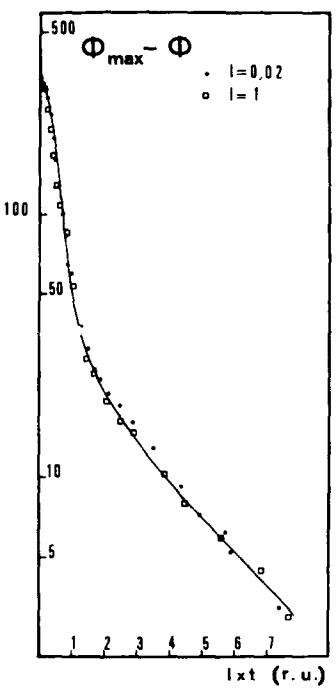
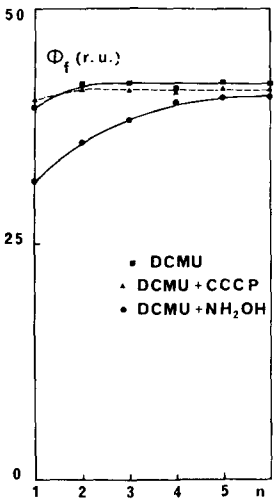


Fig. 3. Semi-logarithmic plot of the difference between the maximum fluorescence and the fluorescence at a given time versus the number of photons absorbed per unit time (relative units): $I \cdot t$. Algae 5 μg Chlorophyll total ml^{-1} in presence of 50 μM DCMU and 2 mM NH_2OH. ●, intensity of illumination: 1 (relative units); □, intensity of illumination: 0.02.

Fluorescence changes induced by short saturating flashes in series

The fluorescence yield reached during successive flashes varies in a way similar to the "slow" and "fast" fluorescence induction described above. Several flashes are needed in the DCMU+NH_2OH system to bring the fluorescence yield to its maximum whereas the maximum yield is reached during the second flash (and the following ones) in the DCMU+CCCP-treated algae (Fig. 4, curves 2 and 3).



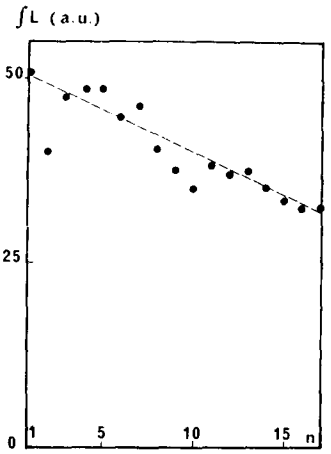


Fig. 4. Effect of DCMU, DCMU+CCCP, DCMU+NH_2OH on the fluorescence yield reached during 5-μs flashes 500 ms apart. Algae in their growing medium at an equivalent chlorophyll concentration of 15 $\mu g \cdot ml^{-1}$. Curve 1, with 50 μM DCMU; Curve 2, with 50 μM DCMU and 10 μM CCCP; Curve 3, with 50 μM DCMU and 2 mM NH_2OH.

Fig. 5. Effect of 20 μM DCMU and 3 mM NH_2OH on the luminescence decay in *Chlorella*. Each sample is illuminated by 50-μs flashes, 40 ms apart. The integral of the luminescence intensity over 1.274 ms after each flash is plotted as a function of the number of flashes received by each sample. The recording is the average of 200 runs.

TABLE I

CONCENTRATION OF Q RESTORED AFTER 1–4 FLASHES

In each case, the maximum area, corresponding to dark-adapted algae, is normalized to 1.

	One flash	Two flashes	Three flashes	Four flashes
In the presence of DCMU+CCCP	0.09	0.08	0.06	
In the presence of DCMU+NH_2OH	0.3	0.22	0.15	0.14

In DCMU alone, where no YChl Q^- is formed, a maximum yield is also reached during the flashes (Fig. 4, curve 1). A maximum fluorescent yield can correspond either to a permanent photoinactive state YChl Q^- or to a Y^+ChlQ^- state. An additional experiment is necessary to know the amount of $YChlQ^-$ formed per flash.

Evaluation of the concentration of photoactive states regenerated after 1–4 successive flashes

For both DCMU+NH_2OH and DCMU+CCCP systems, the number of centers converted by a flash to a permanent photoinactive state YChl Q^- depend on the competition between the back reaction and the alternative pathway for the Y^+ reduction. This number is complementary to the number of regenerated photoactive states: YChl Q-titrated by the area bound between the fluorescence induction curve

and its asymptote [25] (Fig. 1, curve 2′ and Fig. 2, curves 2, 3, 4, 5). The results are shown in Table I: A majority of centers are blocked after the first flash in the presence of DCMU+CCCP. On the contrary in the DCMU+NH_2OH system a complete blocking requires several flashes.

Luminescence decays

The results depicted in Fig. 5 have been kindly provided by Dr Lavorel from an unpublished experiment.

The competition between the back reaction and the irreversible reduction of Y^+ should also have an influence on the luminescence originating from the back reaction. In the presence of DCMU, the back reaction is the only pathway for the Q^- reoxidation, therefore, the luminescence intensity will be proportional to the reoxidation rate: $-dQ^-/dt$ and its integral over the whole decay proportional to the amount of regenerated centers [26].

In the presence of NH_2OH (10^{-3} M) Bertsch et al. [27] have previously shown that the luminescence emission is enhanced during a short period and inhibited thereafter. In the presence of DCMU+NH_2OH, the luminescence integration is done over

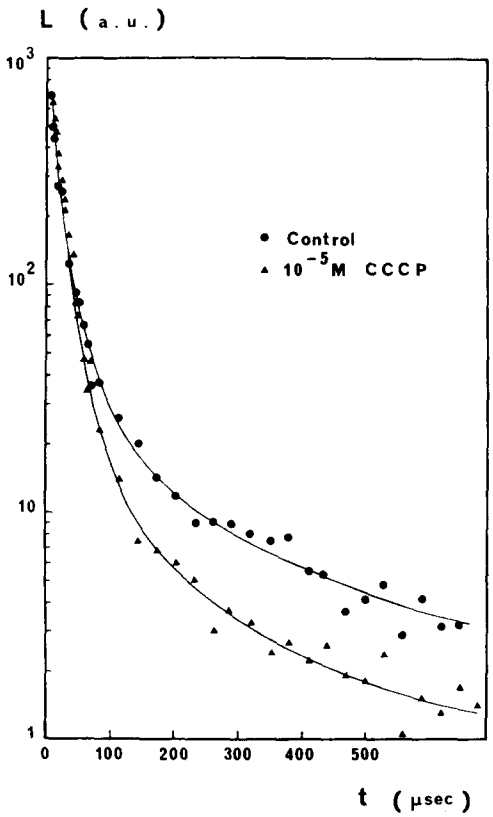


Fig. 6. Effect of 10 μM CCCP on luminescence decay in *Chlorella*. Each sample receives 200 flashes. The recording is the average of 10.000 decays and starts at $t = 3$ μs. Semi-log plot. Other conditions as in Fig. 5.

the first millisecond since for longer times the luminescence intensity becomes negligible. The slow decrease of the luminescence integral during a sequence of flashes shows that the inhibition of the back reaction is very progressive (Fig. 5). NH_2OH appears to be a poor competitor to Q^- for the Y^+ reduction.

The effect of CCCP 10^{-5} M on the fast luminescence decay after a flash is shown in Fig. 6. An inhibition occurs after the first microseconds and rapidly increases with time. The back reaction is strongly inhibited in the DCMU+CCCP system: The luminescence integral over 1 ms reaches a minimum constant value from the second flash, onwards (Fig. 7).

Photooxidation of hydroxylamine

High concentrations of NH_2OH inhibit oxygen evolution in algae and chloroplasts [7]. It then serves as a substitute donor to Photosystem II in both cases and it is photooxidized in a one-quantum process [8]. Whereas the photooxidized product of hydroxylamine is easily detected amperometrically when chloroplasts are used, no amperometric signal is seen with the algae. Chloroplasts were, therefore, used in the following experiments and the results will be regarded as qualitative information keeping in mind the reservations made on p. 499.

Photooxidation under continuous illumination. In the NH_2OH+DCMU system, the rate of photooxidation will give an indication how the permanent photoinactive state, YChl Q^-, is formed. The time course of the rate shows a slow component, a complete inhibition would require several seconds of illumination (Fig. 8, curve 1).

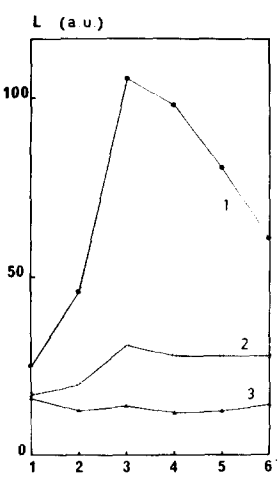


Fig. 7. Effect of CCCP and DCMU on the luminescence emission during a flash sequence. Curve 1, control; Curve 2, +10 μM CCCP; Curve 3: +10 μM CCCP+20 μM DCMU. Other conditions as in Fig. 5.

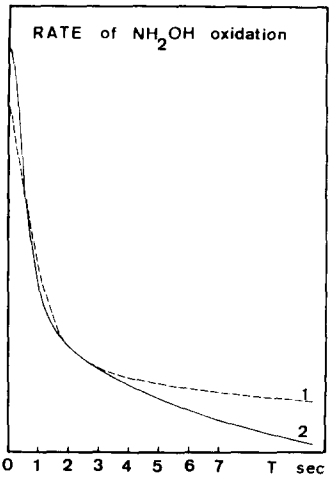


Fig. 8. Rate of NH_2OH photooxidation by lettuce chloroplasts under a weak illumination. Chloroplasts are at an equivalent chlorophyll concentration of 1 mg · ml^{-1}. The platinum electrode is polarized at −0.7 V. The rate is plotted as a function of the illumination time. Curve 1, 50 μM DCMU, 2 mM NH_2OH; Curve 2, same as 1 with 10 μM CCCP added.

Because of the linear relationship between the photochemical rate and the variable fluorescence [23] this behavior is expected from the fluorescence data. If CCCP is added to the NH_2OH+DCMU system, the decay is hastened (Fig. 8, curve 2).

Photooxidation under flashing light. As could be expected, a large number of flashes is necessary in the presence of DCMU and NH_2OH to exhaust the NH_2OH oxidizing capacity. If CCCP is added, fewer flashes are required for a complete inhibition (Fig. 9, curves 1, 2). The amount of NH_2OH oxidized per flash follows a geometrical recurrence law (Fig. 9, insert).

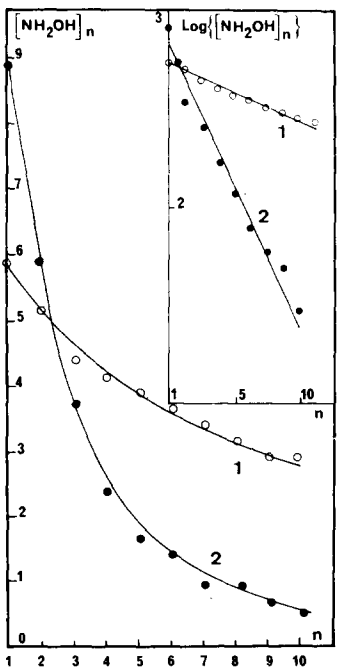


Fig. 9. Photooxidation of hydroxylamine illuminated by 5-μs flashes, 500 ms apart. Curve 1, 50 μM DCMU and 4 mM NH_2OH. Curve 2, same as 1 with 10 μM CCCP added. The amount of NH_2OH oxidized per flash is plotted as a function of the flash number. Other conditions same as in Fig. 8. Insert: semi-log plot.

DISCUSSION

Because of its effect on the fast luminescence decays, NH_2OH was already considered to be moderately efficient as a donor for the System II of algae [20]. This inefficient electron donation can partly explain the results of the present report. According to the following scheme: (where H stands for hydroxylamine):

$$HY\ Chl\ Q \Big|_{DCMU} \underset{k_{-0}}{\overset{k^*1}{\rightleftharpoons}} HY^+\ ChlQ^- \Big| \xrightarrow[DCMU]{k_1} H^+Y\ Chl\ Q^- \Big|_{DCMU} \qquad (1)$$

if k_1 is reduced when NH_2OH is the secondary donor, the back reaction (k_{-0}) will be promoted. Depending on the relative values of k_1, k_{-0} a fraction of centers

will be regenerated through a back reaction after each flash (until the permanent fluorescent state H^+ YChl Q^- is attained for each center). This explains the enhanced fast luminescence and the slow kinetics of NH_2OH photooxidation. In this scheme a maximum fluorescence yield should be reached during each flash. The experimental findings do not agree with this and their explanation requires an additional hypothesis.

To explain a decrease in the rate constant of the photochemical reduction of Q and the failure for a single flash to bring the fluorescence yield to its maximum, one might think of a fast equilibrium between two forms of the centers: a photoactive form T and a photoinactive form T', both quenchers (although T' might be a less efficient quencher) with $K = T'/T$. Only the T form leads to the reduction of Q. Under continuous illumination, as long as the light intensity is low enough for the photochemical rate to be smaller than the rates of the equilibrium between the T and T' forms, the fluorescence rise will be photochemically limited with a rate constant reduced by a factor $1/1+K$. At higher light intensities, the kinetics would be limited by the thermal reactions of equilibrium. During a flash sequence each flash will yield an additional amount of $(Q^-)_n$ such as:

$$(Q^-)_n = \left[1 - \Sigma_{k=1}^{k=(n-1)} (Q^-)_k\right] \frac{1}{1+K} \tag{2}$$

This finally leads to a geometrical recurrence law for the formation of Q^-:

$$(Q^-)_n = \frac{K^{n-1}}{(K+1)^n}$$ (experimentally found in Fig. 9 for the NH_2OH oxidation).

If the photochemical trapping (i.e. the Q reduction) requires a close association of the chlorophyll center with a reduced primary donor and an oxidized acceptor (as seems to be the case in the bacterial reaction centers), the T' form can correspond to centers not associated to a donor or to centers with an oxidized primary donor. After the hydroxylamine treatment one might imagine that the T' form corresponds to a binding between Y and the chlorophyll center loosened by NH_2OH. Schematically if H stands for NH_2OH, the mechanism proposed is the following:

$$\begin{array}{ccccc} \text{HYChlQ} & \underset{k_{-0}}{\overset{k^*\text{I}}{\rightleftharpoons}} & \text{HY}^+\ \text{ChlQ}^- & \xrightarrow{k_3} & \text{H}^+\ \text{YChlQ}^- \\ \Big\downarrow\!\Big\uparrow{\scriptstyle k_1}\ {\scriptstyle k_{-1}} & & & & \\ \text{HY}\text{---}\text{ChlQ} & & & & \end{array} \tag{3}$$

HYChlQ: T form, photochemical quencher, fluorescence yield $\varphi = 0$; HY- -ChlQ: T' form photoinactive, quencher, fluorescence yield $0 < \varphi < 1$; HY^+ $ChlQ^-$: photoinactive state, non-quencher, fluorescence yield $\varphi = 1$; H^+Y $ChlQ^-$: permanent fluorescent state, $\varphi = 1$; k^*I: photochemical rate constant, k_{-0} back reaction rate constant, k_3: NH_2OH oxidation rate constant, k_1, k_{-1} rate constants of the equilibrium T, T'. It is assumed that $k_{-0} \approx k_3$ and that under continuous illumination $k^*I \ll k_1, k_{-1}$, during the flashes $k^*I \gg k_1, k_{-1}$.

When CCCP is interacting with the donor side in the presence of DCMU, the strong inhibition of luminescence, the fast attainment of a maximum fluorescence

yield and the maximum concentration of the $YChlQ^-$ state suggest that the irreversible reduction of Y^+ catalyzed by CCCP is a good competitor to the back reaction. CCCP is also known to suppress the high potential form of cytochrome b_{559} which may serve as a substitute primary donor to Photosystem II [29]. Whenever the photoreduction of Q is associated with the photooxidation of cytochrome b_{559}, the corresponding fluorescence rise is slow and several flashes are needed to obtain a fully oxidized cytochrome [15, 30, 31]. An equilibrium between photoactive and photoinactive forms would therefore exist for the centers in which cytochrome b_{559} is the primary donor. If cytochrome b_{559} participates to a small extent in the electron donation to System II when DCMU or DCMU+NH_2OH are present, it provides an explanation for the slower parts of the fluorescence rises as well as for their suppression by CCCP.

In conclusion, it is inferred that the influence of the donor side on the Photosystem II fluorescence properties results from an indirect effect rather than from a direct quenching by the donor. The fluorescence induction in the DCMU+CCCP case and in the DCMU+NH_2OH system resemble, respectively, the fluorescence induction of the S_0S_1 states and the S_2S_3 states of the centers at -55 °C [15].

The model proposed above, therefore, gives a possibility to reconcile some of the recent results on fluorescence [14–16] with the former hypothesis of a direct correlation between the fluorescence yield and the oxido-reduction level of the acceptor Q [5].

NOTE ADDED IN PROOF (Received February 4th, 1974)

If Y is identical to Chl and Chl^+Q^- is less fluorescent than ChQ^- (as prepared by Butler [30]), all the data can be explained by Eqn (1): By slowing down the K_1 reaction, NH_2OH prevents the attainment of the $ChlQ^-$ state during a 5 μs flash. Under continuous illumination, if $K^*I \gg k_{-0}$, k_1, the fluorescence induction will be photochemically limited with a rate constant attenuated by a factor: $K_1/(K_{-0}+K_1)$.

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