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CORRELATION BETWEEN FLASH-INDUCED OXYGEN EVOLUTION AND FLUORESCENCE YIELD KINETICS IN THE 0 TO 16 μs RANGE IN *CHLORELLA PYRENOIDOSA* DURING INCUBATION WITH HYDROXYLAMINE

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SUMMARY

Following flash excitation, oxygen pulses and fluorescence kinetics in the time range 0–16 μs were studied in the alga *Chlorella pyrenoidosa* during incubation with various concentrations of hydroxylamine. The obtained results could be explained considering four effects of hydroxylamine.

1. Hydroxylamine removes (reduces) oxidizing equivalents, generated in the water-splitting system by flash excitation. This process does not markedly affect the fluorescence yield kinetics between 0 and 16 μs following the ignition of a flash and reaches a constant rate within a few minutes, but possibly within a few seconds, after addition of hydroxylamine. In a sequence of flashes separated by dark time t_d, the steady-state oxygen yield in the flashes is $\exp(-kt_d)$, the yield at $t_d = 0$ being taken equal to 1, where $k = (0.1+\beta[NH_2OH])s^{-1}$, with $[NH_2OH]$ in mM and $\beta = 0.6\ mM^{-1}$, provided $[NH_2OH] \geqslant 0.5$ mM.

2. An inhibition between Z, the physiological donor and the oxidized reaction center pigment P^+ occurs, proceeding as $\exp(-k_i t_i)$ where t_i is the incubation time with hydroxylamine and $k_i = (\alpha[NH_2OH])\ min^{-1}$, with $[NH_2OH]$ in mM and $\alpha = 0.14\ mM^{-1}$. This process not only inhibits oxygen evolution capability, but also decreases the amplitude of the fluorescence yield difference $\Delta\Phi = \Phi(16\ \mu s)-\Phi(2\ \mu s)$ induced by a flash in the steady state. In a fraction of the reaction centers this inhibition occurs "immediately" after the addition of hydroxylamine. These observations, combined with the conclusion of Cheniae and Martin (1971, Plant Physiol. 47, 568–575) that the inhibition of the Hill reaction is related to the extraction of bound manganese, indicate that the reaction between Z and P^+ requires bound manganese.

3. In the inhibited centers a second donor for P^+, D, connected to an entry site for the artificial electron donor hydroxylamine becomes apparent.

4. A flash-induced oxygen uptake signal was observed in the presence of hydroxylamine, which was shown to be caused by a system II reaction.

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

The effects under (1) and (4) were reversed in the dark if hydroxylamine was removed by washing. The effects under (2) and (3) were reversed during illumination of a washed sample.

INTRODUCTION

Hydroxylamine has been known for a long time to affect photosynthesis of green plants [1]. Oxidation of hydroxylamine by chloroplasts has been reported by Vaklinova [2]. Cheniae and Martin [3, 4] proposed different sites of action of hydroxylamine in Photosystem II and concluded that the release of manganese was related to the inhibition of oxygen evolution by hydroxylamine. In algae but not in spinach chloroplasts this inhibition proved to be reversible in the light, if hydroxylamine had been removed by washing. Bennoun and Joliot [5] concluded that the oxidation of hydroxylamine was caused by a system II reaction. Recently the oxidation of hydroxylamine by superoxide in a system I reaction has also been proposed [6]. Several authors ascribed the oxidation of hydroxylamine to the action of hydroxylamine as an artificial electron donor for Photosystem II [3, 5, 7]. An inhibition of oxygen evolution, which could be reversed by two light flashes has been observed at low hydroxylamine concentrations [8, 9]. Measurements of the fluorescence yield of chlorophyll *a* in the presence of hydroxylamine were usually performed during continuous illumination [5, 8, 10]. Recently measurements of fluorescence yield kinetics in algae in the presence of hydroxylamine during saturating xenon flashes have been performed [11]. Flash measurements have the advantage that the influence of hydroxylamine on the fluorescence yield can be studied during a single turnover of the reaction centers. It was found that the inhibition of oxygen evolution by hydroxylamine was accompanied by a diminishing of the light-limited fluorescence yield increase during a saturating flash of 13 μs halfwidth, and that this light-limited fluorescence yield increase was replaced by a 25-μs increase, caused by a dark reaction [11].

The extraction of manganese, which plays an important role in the donor complex of Photosystem II [3], may very well be the cause for these alterations in the behaviour of the fluorescence yield kinetics. In this paper we describe a systematic study of the behaviour of *Chlorella pyrenoidosa* during incubation with hydroxylamine. We were able to explain the experimental results with the four effects mentioned in the summary. Moreover, the results gave information about the donor complex of the reaction center of Photosystem II.

MATERIALS AND METHODS

Algae (*C. pyrenoidosa* Chick, strain Emerson 3) were grown as described previously [12]. Following centrifugation, the algae were suspended in growth medium (pH = 6.0) and diluted to an extinction at 680 nm (with correction for scattering at 750 nm) of 0.3 in a 1mm cuvette. A measuring device, in principle equivalent to the one described by Joliot et al. [13] was used for oxygen measurements. The sus-

pension containing the algae (0.5 ml) was spread on top of a surface area with a diameter of 29 mm, in the center of which there was a platinum electrode with a diameter of 8 mm for oxygen measurements. The suspension was covered with a nuclepore membrane with 4×10^5 pores of 5 μm diameter per cm^2. After the membrane had been fixed, the growth medium was pressed through the nuclepore membrane by pressing the surface, containing the platinum electrode, against the membrane. A thin homogeneous layer of algae remained firmly fixed between nuclepore membrane and platinum electrode. A diaphragm made it possible to illuminate only the algae between membrane and platinum electrode via the light transmitting nuclepore membrane. A flow of growth medium along the membrane offered the possibility of varying the oxygen concentration in the measuring device by changing the flow rate. The steady-oxygen concentration is determined by an equilibrium between oxygen uptake by the algae and oxygen supply by the medium. 0.1 M KCl was added to the growth medium to ensure sufficient conductance in the polarograph vessel. Usually the oxygen pulses increased during the first hour(s) after putting the algae into the measuring device. If the algae were preincubated with 0.1 M KCl before use, the oxygen pulses usually became constant in a much shorter time. The measurements were started when the amplitude of oxygen pulses had become stable. Hydroxylamine incubation was started by exchanging the growth medium without hydroxylamine for medium with the desired hydroxylamine concentration. This was done at a high flow rate, in order to obtain a complete medium exchange within 10 s. The pH of the medium with hydroxylamine was adjusted with KOH to the pH value of the growth medium (pH = 6.0). The platinum electrode was polarized at a voltage of -0.7 V with respect to an Ag/AgCl electrode. The electrical current flowing through the measuring device was, via a current to voltage converter with low input impedance, transformed into a corresponding voltage. After elimination of the D.C. component by compensation or by filtering with a high-pass filter, the signal was registered with a rapid recorder with a time constant of several milliseconds (Siemens, oscillomink). The use of the high-pass filter is convenient, if during the measurements the electrode signal undergoes slow changes (e.g. during the medium exchange). The method for fluorescence measurements during a flash has been described previously [14–16].

Different flash types were used for experimental and technical reasons. Measurements of the fluorescence yield during short flashes (several microseconds) made clear that fluorescence quenching state(s) remain present during the first microseconds during illumination with a short flash [17]. The influence of these short-lived quenching states on the fluorescence yield kinetics becomes more considerable if the energy required for saturation is absorbed in a short flash with a half time comparable to the life time of the quenching states. To reduce this effect flashes of 13 μs halfwidth and low intensity were used. The flashes were saturating with respect to oxygen evolution however. The flashes for fluorescence measurement have to be very reproducible and monochromatic. The latter because the shape of the flash is wavelength dependent, and corrections for the absorption spectrum of the algae and the spectral sensitivity of the light detector would be necessary otherwise. The requirements for fluorescence measurements could be satisfied by using a FT 230 (General Electric) flash tube supplied by a 40 μF capacitor operated at 1500 V. The light was filtered with a filter combination consisting of: Calflex-C, Balzers K_1, Schott BG 18/2 and a neutral density filter with a transmittance of 3.9 %. The trans-

mitted band has a maximum at 414 nm. The flash intensity is plotted as a function of time in Fig. 5a (dashed line). The flash repetition rate during fluorescence measurements was one flash per 2.56 s. The maximum power dissipation of the flash tube sets an upper limit to the flash repetition rate. Therefore, the flash tube was provided with a capacitor of 1 μF, operated at 1000 V for the higher flash repetition rates during some of the oxygen measurements. The light was filtered with a filter combination consisting of: Calflex-C(1 mm), Balzers K_1 and BG 18/2 (Schott). The flash then had a halfwidth of about 1 μs and was able to saturate oxygen evolution.

All experiments were done at room temperature (about 24 °C).

RESULTS

Decay of amplitude of flash-induced oxygen evolution pulse during incubation with several concentrations of hydroxylamine as a function of dark time between the flashes

The effect of various dark times between flashes on the decrease of the amplitude of the flash-induced oxygen evolution pulse in the steady state during incubation with 0.5 mM hydroxylamine is shown in Fig. 1. The results were obtained from a single sample to avoid variations among different samples. The dashed line is obtained by extrapolation to zero dark time between the flashes (see Discussion). The ratio of the amplitudes of the oxygen evolution pulses at a fixed incubation time with e.g. 0.5

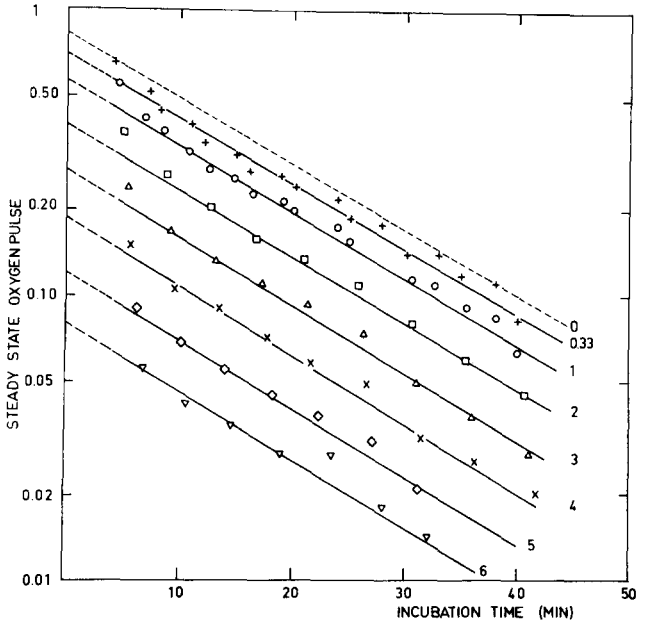

Fig. 1. Amplitude of oxygen pulses in the steady state during a flash series as a function of incubation time with 0.5 mM hydroxylamine at different dark times between the flashes (indicated on right in seconds). The results were obtained from a single sample. The curves are normalized by dividing the amplitude of oxygen pulses at the indicated incubation time by the amplitude of the oxygen pulse without hydroxylamine at the respective dark times between flashes. The dashed line for zero dark time between flashes is obtained by extrapolation.

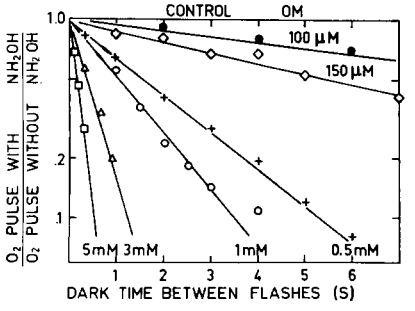

Fig. 2. Ratio of the amplitude of the oxygen pulses (steady state) in the presence of several concentrations of hydroxylamine (at a fixed incubation time $\geq$ 10 min) and of the oxygen pulses in the absence of hydroxylamine as a function of dark time between flashes. The amplitudes of the oxygen pulses in the presence (at the incubation time considered) and in the absence of hydroxylamine were arbitrarily chosen equal to one at $td = 0$.

mM hydroxylamine, and of the oxygen pulses in the absence of hydroxylamine is plotted as a function of dark time between the flashes in Fig. 2 (curve marked 0.5 mM). The amplitudes of the oxygen evolution pulses in the presence and in the absence of hydroxylamine were arbitrarily chosen equal to one at zero dark time between the flashes. The obtained curve represents the deactivation (reduction of oxidizing equivalents) as a function of time of the water-splitting system due to the presence of 0.5 mM hydroxylamine. This deactivation, which can be approximated by a first-order process, has e.g. a half time of $t_{\frac{1}{2}} \approx 1.5$ s at a concentration of 0.5 mM (Fig. 2). The fact that the straight lines in Fig. 1, representing the decay of the amplitude of the oxygen pulses, remain parallel during incubation, indicates that the deactivation rate of the water-splitting system is independent on the incubation time with hydroxylamine, provided this time is longer than about 10 min. The deactivation rate has been determined for various hydroxylamine concentrations

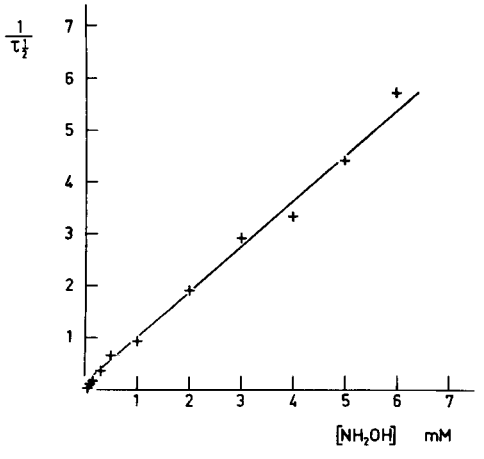

Fig. 3. Rate of deactivation of the oxygen-evolving system due to the presence of hydroxylamine during a flash series as a function of hydroxylamine concentration.

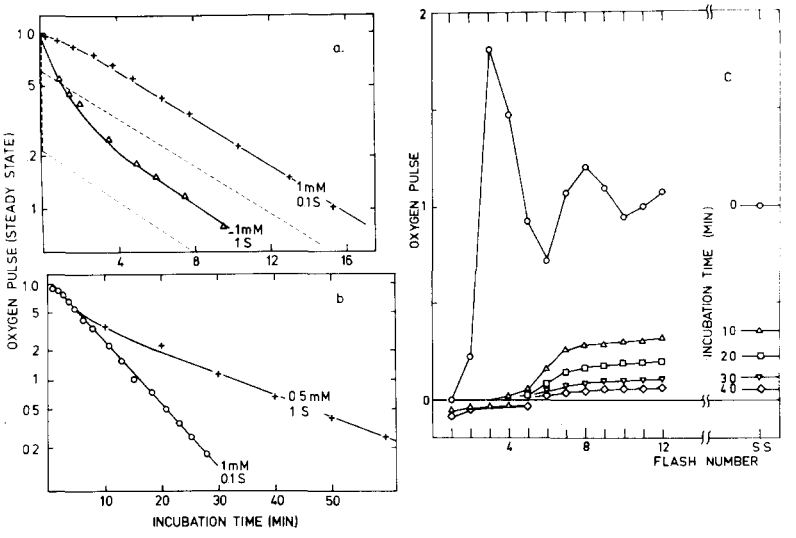

Fig. 4. (a, b) Amplitude of the oxygen pulses (normalized at zero incubation time) in the steady state during a flash series, as a function of incubation time with hydroxylamine. Dark time between flashes and hydroxylamine concentrations as indicated. (c) Oscillation pattern of amplitude of oxygen pulses during a flash series following 10 min of dark adaptation. The measurements were done simultaneously, on the same sample, with the measurement of the decay curve (0.5 mM; 1 s) in b. Notice the oxygen uptake in the presence of hydroxylamine indicated during the first five flashes following dark adaptation (for kinetics of pulses, see Fig. 9).

(Fig. 2). The inverse of the halftime, $t_{\frac{1}{2}}^{-1} \approx 1.4\ k$, is plotted against hydroxylamine concentration in Fig. 3. For hydroxylamine concentrations of 0.5 mM and higher k depends on the hydroxylamine concentration according to $k = (0.1 + \beta[NH_2OH])$ s^{-1} with $\beta = 0.6\ mM^{-1}$ and $[NH_2OH]$ in mM. At lower concentrations deviations occur, and e.g. at a concentration of 50 μM the half time for deactivation is 30–40 s.

The decay of oxygen evolution during the first minutes of incubation are shown in Fig. 4a. The deviations from first-order decay at short incubation times are due to the time interval of about 4 min necessary for the diffusion of hydroxylamine through the nuclepore membrane to the sites of action. Diffusion through the *Chlorella* cells is less likely to be the limiting factor, because Cheniae and Martin [18] found no deviation from first-order decay for oxygen evolution capability during incubation with hydroxylamine at incubation times of several minutes in algae and spinach chloroplasts brought into direct contact with medium containing hydroxylamine. The diffusion process, which delays the action of hydroxylamine on the organisms, appears to be terminated after 4 min of incubation with 1 mM hydroxylamine, since at longer incubation times the decay of the amplitude of the oxygen pulse does not deviate from a first-order decay any more (Fig. 4a). An approximate correction for the diffusion process is obtained by shifting the decay curves in Fig. 4a over a time interval of 4 min. This yields the dashed lines. These lines intercept the ordinate at values lower than the control value, due to the mentioned deactivation process, which requires about 2 s at the concentration considered. Moreover, an additional "immediate" decrease is involved (see Discussion). In the following we consider the decay of oxygen evolution to consist of a slow phase, represented by the

straight lines for incubation times longer than 4 min (Fig. 4a), and a fast bent phase which becomes apparent at low flash repetition rates and lasts for about 4 min (lower curve in Fig. 4a).

During the decay of the oxygen evolution pulses following the addition of hydroxylamine (Fig. 4b, curve marked 0.5 mM; 1 s), the oscillation patterns of the amplitude of oxygen evolution pulses (each measurement was done after 10 min dark adaptation) were registered (Fig. 4c). The oscillation became gradually (not shown in this figure) strongly damped during the establishment of the fast phase of oxygen evolution decay, whereas the damping remained unaltered during the slow phase, after completion of the fast phase (Fig. 4c).

Correlation between the decay of the fast fluorescence yield increase and of the amplitude of the oxygen evolution pulse during incubation with hydroxylamine

In Fig. 5 the fluorescence yield kinetics in the steady state during a saturating flash (dashed line) are plotted for various incubation times with 0.5 mM hydroxylamine. The fluorescence yield increase during the flash is light limited in the absence of hydroxylamine [11]. We notice that the difference of the fluorescence yields between 16 and 2 μs, $\Delta\Phi = \Phi(16\ \mu s) - \Phi(2\ \mu s)$ diminishes with increasing incubation time with hydroxylamine, whereas the shape of the fluorescence induction curves remains unaltered, at least at short incubation times. During incubation with hydroxylamine the dark fluorescence yield increases (Fig. 5). During this increase the contribution of the light-induced fluorescence quenching state T, which occurs in the increased fluorescence, becomes gradually more pronounced. At longer incubation times (33 min) the light-limited fluorescence yield increase presumably would have the same shape, if the formation of the quenching state T [14] would not decrease the increased dark fluorescence yield during the flash.

In Fig. 6 the fluorescence yield difference $\Delta\Phi$ (see Fig. 5) and the amplitude of the oxygen evolution pulse are plotted against the incubation time with hydroxylamine for concentrations as indicated and a dark time of 2.56 s between the flashes.

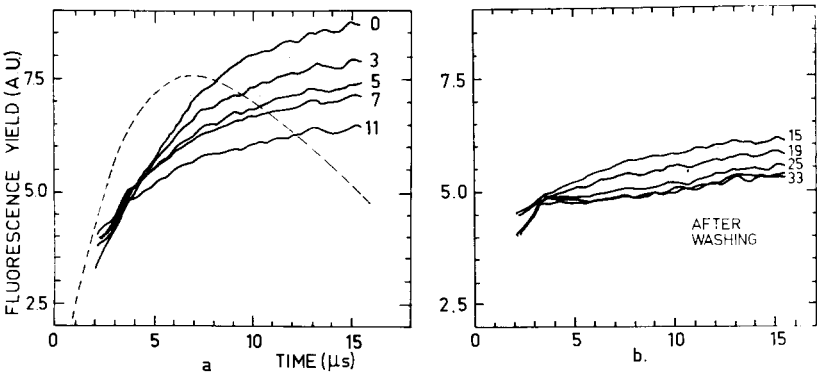

Fig. 5. (a, b) Fluorescence induction curves (solid lines) during 13-μs flashes (dashed lines) in the steady state during a flash series at increasing incubation times from 0 to 33 min with 0.5 mM hydroxylamine (indicated in minutes on the right), and in the lower curve of b: after having washed the sample following 33 min incubation. Flash repetition rate, one flash per 2.56 s.

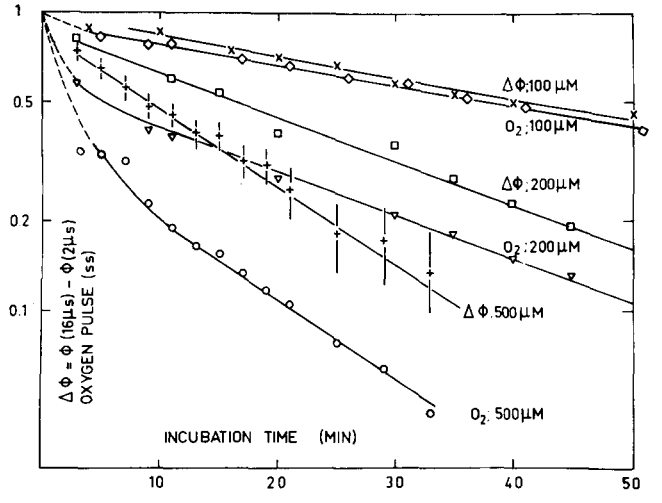

Fig. 6. Amplitude of fluorescence yield difference $\Delta\Phi = \Phi(16\,\mu s) - \Phi(2\,\mu s)$ (see Fig. 5) and of oxygen evolution pulse (log scale) during and following a saturating xenon flash, respectively, as a function of incubation time with hydroxylamine. Concentrations as indicated. Curves are normalized at zero incubation time, by dividing the amplitudes of the oxygen pulses by the amplitude of the oxygen pulse of the control, before addition of hydroxylamine. The points on the curves were obtained from measurements in the steady state, after disappearance of the oscillations which occur during the first flashes following dark adaptation (see text). Flash repetition rate, one flash per 2.56 s.

$\Delta\Phi$, and the amplitude of the oxygen pulse were measured on the same sample in the steady state after the disappearance of oscillations which occur in $\Delta\Phi$ [19] and the amplitude of the oxygen pulse [20] during the first flashes following dark adaptation.

The decay of $\Delta\Phi$ and the decay of the amplitude of the oxygen evolution pulse as a function of incubation time with hydroxylamine can both be approximated by a first-order process with the same rate constant for incubation times longer than 10 min. The rate constant is proportional to the hydroxylamine concentration:

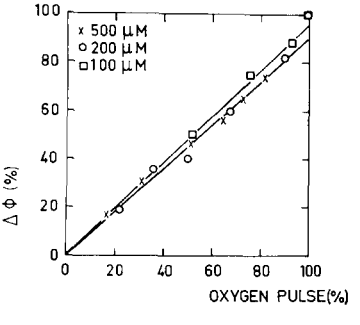

Fig. 7. $\Delta\Phi$ from Fig. 6 plotted against the amplitude of only the slowly decaying phase of oxygen evolution (see Fig. 6) during incubation with hydroxylamine at concentrations as indicated. The amplitudes of the oxygen pulses at the respective incubation times were obtained from straight lines, intercepting the ordinate at value 1, and parallel to the straight lines representing the first-order decay kinetics of oxygen pulses at longer incubation times (see Fig. 6). One flash per 2.56 s.

$k_i = \alpha[NH_2OH]\ min^{-1}$ where $\alpha = 0.14\ mM^{-1}$ and $[NH_2OH]$ in mM (compare the slopes of the decays of Fig. 6). The initial level of the fluorescence yield Φ_0 ($\Phi_0 \approx \Phi$ (2 μs)) increases during incubation with hydroxylamine at a comparable rate (Fig. 5).

In Fig. 7 the slowly decaying component of oxygen evolution (see Figure legend) is plotted against $\Delta\Phi$. A linear dependence exists, except for a discontinuity due to an "immediate" decrease in $\Delta\Phi$ following addition of hydroxylamine (see Discussion).

Properties of fluorescence yield kinetics in algae in which oxygen evolution capability has completely disappeared due to incubation with hydroxylamine

If after a sufficient incubation time, the fast light-limited fluorescence yield increase and oxygen evolution capability have disappeared, a relatively slow fluorescence yield increase induced by a flash occurs due to a dark reaction [11]. This fluorescence yield increase with a half time of about 25 μs is probably due to a reduction of the oxidized primary donor P^+ by a secondary electron donor which we call D[11]; the other secondary donor Z associated with water oxidation presumably reduced P^+ in 1 μs or less [17].

From Fig. 8a we conclude that a fluorescence yield increase (due to the 25-μs component) was still present if hydroxylamine, after having inhibited the larger part of oxygen-evolving capability, had been removed from the sample by washing (Fig. 8a). Oxygen measurements (Fig. 8b) done simultaneously with the fluorescence yield measurements of Fig. 8a, show a periodicity with the flash number comparable to the control measurements but with much smaller amplitude. Tightly bound hydroxylamine which remains in the algae after washing had been released from the centers by a series of preilluminating flashes. If residual hydroxylamine would still have been present a delay by two flashes should have occurred [8]. The normal periodicity in the small fraction of remaining oxygen-evolving centers indicates that hydroxylamine had been removed successfully.

The 25-μs fluorescence yield increase in the centers inhibited by hydroxylamine has some interesting features. After removal of hydroxylamine by washing an appre-

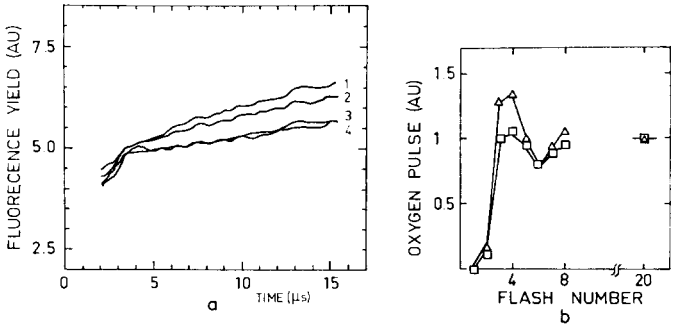

Fig. 8. (a) Fluorescence induction curves during the first four flashes (flash numbers indicated on the right) after dark adaptation, following 33 min of incubation with 0.5 mM hydroxylamine, washing and removal of bound hydroxylamine by several preilluminating flashes. (b) Oscillations of the amplitudes of oxygen pulses, simultaneously recorded with the fluorescence yield measurements of a (□), and a control measurement, performed before incubation with hydroxylamine (△). Steady-state values of the oxygen pulse are normalized under both conditions.

TABLE I

Dark time (t_d) between flashes necessary to observe the 25 μs fluorescence yield increase during the flashes in the steady state with an amplitude equal to half the maximum amplitude, observed after several minutes dark adaptation. (Amplitude of the fluorescence yield increase was measured in the time interval from 0 to 16 μs following the ignition of the flash).

Hydroxylamine concentration	t_d
0 M (after washing)	30–45 s
0.5 mM	3.2 s
1 mM	1.2 s
2.5 mM	0.8 s
5 mM	0.6 s

ciable 25 μs fluorescence increase occurred twice, induced by two consecutive flashes given 2.56 s after each other. This fluorescence increase was much smaller during the following flashes (Fig. 8a). Apparently Photosystem II is still active during the first two flashes after dark adaptation whereas the activity becomes less in the following flashes. If the flash repetition rate is low, the 25-μs fluorescence rise is observed throughout the flash series. A dark interval of 30–45 s between the flashes was necessary to observe the slow fluorescence yield increase with an amplitude of half of the maximum obtainable amplitude, which was observed following a dark adaptation of several minutes. In the presence of hydroxylamine the required dark time between the flashes became much shorter, dependent on the hydroxylamine concentration used (Table I).

The 25-μs fluorescence yield increase will only considerably contribute to the fluorescence yield difference $\Delta\Phi = \Phi(16\ \mu s) - \Phi(2\ \mu s)$ in the steady state during flash series with low flash repetition rates and if high concentrations of hydroxylamine are present. Hence $\Delta\Phi$ only represents the light-limited fluorescence yield increase at a flash repetition rate of one flash per 2.56 s and low hydroxylamine concentrations. At a hydroxylamine concentration of 0.5 mM, however, the 25-μs fluorescence yield increase becomes able to contribute to $\Delta\Phi$, therefore the values for $\Delta\Phi$ are slightly higher than would be expected on the basis of first-order decay kinetics (Fig. 6).

Oxygen uptake

In the presence of hydroxylamine the oxygen evolution in *C. pyrenoidosa*

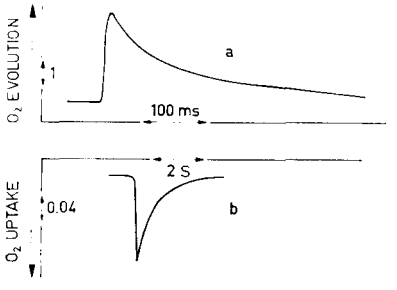

Fig. 9. a, Kinetics of oxygen evolution pulse following a flash, and b, idem for oxygen uptake pulse in the presence of 2 mM hydroxylamine.

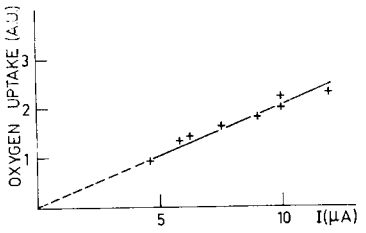

Fig. 10. Amplitude of oxygen uptake pulse in *C. pyrenoidosa* in the presence of 2 mM hydroxylamine during a flash series (steady state) as a function of electrical current (which is a measure for the oxygen concentration in the measuring device) through the electrode system.

gradually disappears and a polarographic signal of a polarity opposite to the polarity of the oxygen evolution signal and much slower kinetics appears (compare Figs. 9a and 9b). The new signal reaches a steady state after a few flashes (Fig. 4c). The signal is thought to be due to oxygen uptake because the amplitude of the signal depends strongly on the oxygen concentration in the measuring device (Fig. 10). Extrapolation of the curve in Fig. 10, suggests that the signal disappears if the oxygen concentration in the measuring device approaches zero. In order to have an indication whether oxygen uptake was driven by a system II or a system I light reaction, the amplitudes of oxygen uptake and oxygen evolution pulses were measured in the linear region of the saturation curves with flashes at wavelengths of 656 and 702 nm, respectively. Flash intensities were chosen such as to have a ratio of the amplitudes of oxygen evolution pulses in the absence of hydroxylamine equal to

$$\frac{O_2 \text{ evolution at } 702 \text{ nm}}{O_2 \text{ evolution at } 656 \text{ nm}} = 0.94.$$

The ratio of the amplitudes of oxygen uptake pulses in the presence of hydroxylamine was

$$\frac{O_2 \text{ uptake } 702 \text{ nm}}{O_2 \text{ uptake } 656 \text{ nm}} = 1.08 \text{ at the same flash intensities.}$$

Oxygen uptake was, like oxygen evolution, inhibited by 10^{-5} M 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The more or less similar ratios for the amplitudes of oxygen evolution and of oxygen uptake pulses at 702 and 656 nm, respectively, and the DCMU sensitivity indicate that oxygen uptake is driven by the same light reaction as oxygen evolution (system II). Slight deviations in the ratios for O_2 uptake and evolution may be caused by a difference in light saturation properties at the different wavelengths, due to geometrical factors in the measuring device, which will be discussed elsewhere (in preparation).

Fig. 11 gives the amplitude of the oxygen uptake pulse (steady state) as a function of hydroxylamine concentration. The curve reaches a maximum at a concentration of 1–2 mM and decays again at higher concentrations. The results were obtained from a single sample, treated with increasing concentrations of hydroxylamine. At a fixed concentration the amplitude of the oxygen uptake pulse remained constant.

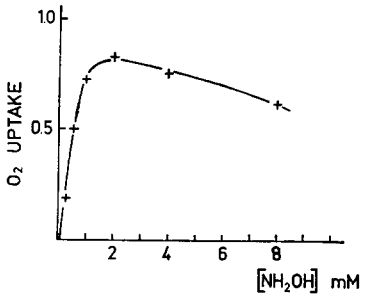

Fig. 11. Amplitude of oxygen uptake pulse (steady state) as a function of hydroxylamine concentration at a fixed oxygen concentration in the measuring device.

A dark interval is required to restore the ability for oxygen uptake following a flash. For hydroxylamine concentrations in the range of 2–6 mM the amplitude of the steady-state oxygen uptake pulse was half the amplitude of the first oxygen uptake pulse following 6 min of dark incubation if the dark time between the flashes was about 0.5 s.

Reversibility of processes after elimination of hydroxylamine from the algae by washing

(1) The fast phase of oxygen evolution decay (see e.g. Fig. 4a) and the increased damping in the oscillation pattern of oxygen pulses during a flash series (Fig. 4c) were reversed completely during the removal of hydroxylamine by washing. For this reversal no light was necessary.

(2) The slow phase of oxygen evolution decay (see also ref. 21) and the decrease of the light-limited fluorescence yield increase in a flash during incubation with hydroxylamine could, at least partially, be reversed in the light after hydroxylamine had been removed from the algae by washing.

(3) The 25 μs fluorescence yield increase was also present in algae treated with hydroxylamine after hydroxylamine had been removed by washing (Fig. 8a), but this increase was gradually replaced by the light-limited fluorescence yield increase, if the washed sample was illuminated (see also under 2).

(4) The oxygen uptake (Fig. 9b) which is observed in the presence of hydroxylamine disappears if hydroxylamine is removed from the sample. During the first flashes after washing the oxygen uptake is still present, however, which is likely due to residual bound hydroxylamine.

DISCUSSION

Removal of oxidizing equivalents from the water-splitting system in the presence of hydroxylamine

From Fig. 2 we conclude that the deactivation rate of the water-splitting system increases with increasing hydroxylamine concentration. This indicates that in the presence of hydroxylamine, oxidizing equivalents (precursors of water oxidation) generated during illumination are removed from the water-splitting system. Apparently the reduction of oxidizing equivalents by hydroxylamine is responsible for this deactivation. The deactivation results in a fast decay of the amplitude of the

oxygen evolution pulse during the first minutes following addition of hydroxylamine (see e.g. Fig. 4a, curve marked 1 mM, 1 s). If the flash repetition rate is increased to one flash per 0.1 s the fast decay of the oxygen evolution pulse due to the deactivation of the water-splitting system is not observable any more (Fig. 4a), although the results in Fig. 2 indicate that an inhibition of about 5 % should be expected. The time necessary for the diffusion of hydroxylamine to the sites of action masks this effect. The fast decay is supposed to have a more "immediate" character without the limitation of diffusion of hydroxylamine through the nuclepore membrane. If we correct for the diffusion process, however, an inhibition much larger than the expected 5 % becomes apparent. This will be discussed below. Bouges-Bocquet [22] concluded that at a hydroxylamine concentration of 50 μM, two hydroxylamine molecules became bound to the reaction centers with binding rates of one per 50 s and one per 80 s, respectively. The bound molecules can be oxidized and released from the centers by flash illumination, after which the centers behave like normal unpoisoned centers in oxygen evolution. We measured the deactivation rate of oxygen evolving centers in the steady state at various hydroxylamine concentrations during a flash series (Fig. 3); The half time for deactivation of 30–40 s at a concentration of 50 μM may well be due to the binding rate of hydroxylamine to the centers. Hence the deactivation of the water-splitting system is probably caused by the process described by Bennoun and Bouges [8]. This would suggest that only if hydroxylamine is bound to the centers oxidizing equivalents are lost. A direct electron donation by free hydroxylamine to the oxidizing equivalents cannot be excluded, however. Even at high hydroxylamine concentration the deactivation rate of the water-splitting system remains relatively low ($k = 3.8\ s^{-1}$ at 6 mM).

The oscillation pattern of oxygen pulses becomes strongly damped (Fig. 4c, compare results at 0 and 10 min incubation, respectively) during the establishment of the fast decay component (Fig. 4b). This indicates that the removal of oxidizing equivalents is not restricted to a fixed fraction of reaction centers, but that this process affects all centers able to evolve oxygen in a random process. The damping of the oscillation pattern becomes less if at the same hydroxylamine concentration, higher flash repetition rates are used. Of course the delay by two flashes as described by Bennoun and Bouges [8] cannot be eliminated as the dark adaptation time, necessary for the deactivation of the S states, is sufficient to allow the binding of hydroxylamine molecules to the centers. The fluorescence yield kinetics during a flash (Fig. 5) were not dependent on the dark time between the flashes. We conclude that the removal of oxidizing equivalents causes no concomitant changes in the fluorescence yield increase during a flash in the steady state. The oscillation in the fluorescence yield observed in dark adapted organisms [11, 19] is not observed in the presence of hydroxylamine however [11]. Oscillation in the fluorescence yield increase in *C. pyrenoidosa* was also suppressed in the presence of 10^{-5} M CCCP (carbonylcyanide *m*-chlorophenylhydrazone), which is known, like hydroxylamine, to remove oxidizing equivalents on the water side of Photosystem II [23, 24]. Addition of 10^{-5} M CCCP to *Chlorella* cells did not markedly influence the fluorescence yield increase during a flash in the steady state.

Decoupling of the water-splitting system from the reaction center of photosystem II

The similar decay kinetics of oxygen evolution capability (slow component)

and fast fluorescence yield increase during the incubation with hydroxylamine, both with the same time constant (Fig. 6), strongly support the earlier proposed hypothesis that these phenomena are caused by the same process, more specifically the decoupling of the water-splitting enzyme system from the reaction center of Photosystem II [11]. From Fig. 1 we conclude that for each decrease of one second in dark time between the flashes the straight line, representing the decay of oxygen pulses as a function of incubation time with hydroxylamine, shifts upwards over the same distance. Extrapolation to zero dark time between the flashes yields the dashed line which intercepts the ordinate at a value about 15 % lower than the control. This suggests the presence of an "immediate" inhibition, which has also been reported by Cheniae and Martin [18]. This "immediate" inhibition was also observed in $\Delta\Phi$ (Fig. 6). The 90 % value of $\Delta\Phi$ which corresponds with an amplitude of the oxygen pulse of 100 % in Fig. 7, therefore seems to be due to the way in which the slow phase was constructed from Fig. 6. No account was made for the "immediate" inhibition of the amplitude of the oxygen pulse (see figure legend of Fig. 7). The "immediate" inhibition is probably due to a fraction of the reaction centers which are more easily inhibited by hydroxylamine.

We conclude that the amplitude of the fast fluorescence yield increase in *C. pyrenoidosa* (Figs. 5a and 5b), which is light limited during flashes of 13 μs halfwidth, is an indicator for the amount of system II centers still active in oxygen evolution during incubation with hydroxylamine. This includes the "immediate" inhibition. The inhibition, represented by the slow phase of decay of oxygen evolution capability, is, as reported by Cheniae and Martin [18], correlated with the extraction of bound manganese. The clear correlation, found by us, between the inhibition of the oxygen pulse and the inhibition of the reaction $ZP^+ \rightarrow Z^+P$ (represented by the decrease of $\Delta\Phi$ [11]) then suggests that bound manganese is required for electron transport from Z, the physiological donor to the oxidized reaction center pigment P^+.

During the progressive inhibition between P and Z no further change in the oscillation pattern of oxygen pulses, caused by a flash series, occurs. Only the amplitude of the pulses diminishes (Fig. 4c). This means that an increasing well-defined fraction of oxygen-evolving centers is blocked during this type of inhibition. The linear relationship between $\Delta\Phi = \Phi(16\ \mu s) - \Phi(2\ \mu s)$ and the amplitude of the slowly decaying component of oxygen evolution during incubation of hydroxylamine (Fig. 7) has another interesting implication. If a reaction center in which state P^+Q^- is present is supposed to be a fluorescence quencher [25, 26] with an equal quenching efficiency as a reaction center in the state PQ, the curve of Fig. 7 should be expected to deviate from a straight line if energy transfer occurs [27] from the intact units with the reaction center in the state PQ^- to the inhibited units with the reaction center in the state P^+Q^-. Hence energy transfer from closed intact centers to centers inhibited by hydroxylamine in the state P^+Q^- seems unlikely.

A secondary electron donor associated with an entry site for the artificial electron donor hydroxylamine

In the photosynthetic units in which the water-splitting enzyme system is decoupled from the reaction center, a secondary donor D, different from the normal secondary donor Z or a modification of Z, is able to reduce P^+ with a half time of 25 μs [11]. The results mentioned in this report suggest that only two electrons can

be donated quickly after each other by the modified donor system D. This donation is reflected by the 25-μs fluorescence yield increase during the first two flashes following dark adaptation (Fig. 8a). After the donation of two electrons 30–45 s are required for the rereduction of donor D, by presumably an endogenous electron donor. In the presence of hydroxylamine the rereduction of donor D requires a much shorter time (Table I), which can be explained by an irreversible electron donation to D^+ by hydroxylamine. The rate of electron donation to D^+ is concentration dependent and even at high hydroxylamine concentration this rate remains relatively low (Table I). This limiting step in the electron flow through system II, if hydroxylamine is used as an artificial electron donor, explains the low electron-donating efficiency of hydroxylamine. The dark time required to restore the capability for system II driven oxygen uptake is presumably caused by the same limiting step. More than two electrons can be donated rapidly after each other during the first flash series following long dark incubation with hydroxylamine. The capability for electron donation then degrades progressively during the flash series (see ref. 11). A rapid replacement of electrons by hydroxylamine tightly bound to donor D during the incubation period may explain this phenomenon (see Fig. 12). These bound molecules are not replaced by hydroxylamine molecules after they have been oxidized during illumination. If after oxidation of donor D no other donor is present, the fluorescence yield remains low, because following flash excitation the fluorescence quenching state P^+Q^- returns to the fluorescence quenching state PQ in a back reaction. This back reaction is expected to give rise to an increased luminescence intensity. During a flash series variations of luminescence intensity in the presence of hydroxylamine have been reported [17]. If donor D was exhausted the luminescence intensity, following excitation with a laser pulse, in the time range 1–100 μs was indeed higher than when D was able to donate electrons to P^+ (van Best, J. A., personal communication). No clear correlation, like reported for spinach chloroplasts at low pH in the time range of several hundreds of microseconds [28], between the decay time of the luminescence intensity and the half time for fluorescence yield increase following a flash could be established. The back reaction $P^+Q^- \rightarrow PQ$ is estimated from fluorescence measurements to have a half time of 45–100 μs [11]. Therefore after exhaustion of donor D, the luminescence intensity, if proportional to the concentration of state P^+Q^-, should have the same decay time. The larger part of the luminescence decay had a much shorter decay time after exhaustion of donor D, however (van Best, J. A., personal communication). It may be that the luminescence decay reflects a system II activity which cannot be observed in the fluorescence kinetics.

The identity of D is still an open question. Attempts to measure absorbance changes related to cytochrome *b*-559, in the presence of 0.5 mM hydroxylamine after about 30 min of incubation, failed. Hence the suggestion that cytochrome *b*-559 is identical to D is not supported by these measurements. That cytochrome *b*-559 is oxidized by D^+ [17] in algae not treated with hydroxylamine cannot be excluded, however. If donor D is supposed to contribute in a side path to electron donation also in algae under physiological conditions (e.g. not treated with hydroxylamine), it is likely that only during the first flashes following dark incubation its contribution to electron donation is considerable because of the long rereduction time in the absence of hydroxylamine. The features of donor D suggest that this donor may be related to the donor activity reflected by the EPR signal II which is related to an entry

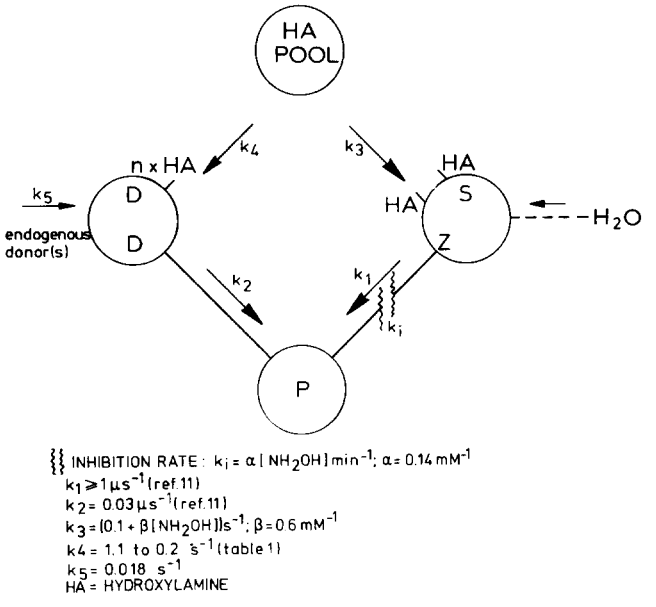

Fig. 12.

site for artificial electron donors in organisms in which the electron transport between the Photosystem II reaction center and oxygen-evolving system is interrupted [29]. The interactions of hydroxylamine with Photosystem II are schematically summarized in Fig. 12. k_i is the rate of the decoupling of the water-splitting systems from the reaction centers at a site located between P and Z. k_n ($n = 1, 2 \ldots 5$) determines the rate of electron transfer between the respective reaction partners. Besides from the two hydroxylamine molecules bound to the donor complex associated with water oxidation [8] a number of hydroxylamine molecules can be bound to donor D. These bound molecules are not replaced by new ones, after they have been oxidized in the light. k_4 and k_3 represent the rate constants for the binding of hydroxylamine to the respective donors or the rate constants for electron transfer from (free) hydroxylamine to these donors. Oxygen uptake, which is presumably due to a reaction of oxygen with a reaction product of hydroxylamine, is not indicated in this figure.

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