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LIMITING STEPS IN PHOTOSYSTEM II AND WATER DECOMPOSITION IN *CHLORELLA* AND SPINACH CHLOROPLASTS*

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SUMMARY

1. By varying the redox potential of a chloroplast suspension, we obtained new evidence for an equilibrium between states S_0 and S_1 in the model of Kok, B., Forbush, B. and McGloin, N. (1970, *Photochem. Photobiol.* 11, 457–475). The mid-point potential of the S_0 to S_1 couple is close to that for the pool of the electron acceptor of System II, A to A^-.

2. The limiting steps between two consecutive photoreactions of System II in *Chlorella* and spinach chloroplasts, have been studied.

(a) The limiting step from S_1 to S_2 (noted $\gamma_1(\Delta t)$) is not exponential. Its temperature coefficient becomes greater as the reaction proceeds. The shape of the kinetics is an intrinsic property of each center. Chloroplasts fixed with 2% glutaraldehyde, show simple first order kinetics.

(b) The limiting step from S_0 to S_1 ($\gamma_0(\Delta t)$) exhibits the same characteristics as $\gamma_1(\Delta t)$.

(c) The limiting step from S_2 to S_3 ($\gamma_2(\Delta t)$) shows sigmoidal kinetics; two reactions are involved. One of the reactions exhibits the same properties as $\gamma_0(\Delta t)$ and $\gamma_1(\Delta t)$.

(d) The limiting step from S_3 to S_0 ($\gamma_3(\Delta t)$) is a first order reaction, two times slower than the other transitions. This reaction is interpretated in terms of oxygen release.

3. We also studied the limiting steps in the presence of low concentrations (50 μM) of hydroxylamine. The results favor the binding of two molecules of hydroxylamine to every photochemical center.

INTRODUCTION

During the last few years, we have learned much about water decomposition and oxygen evolution performed by Photosystem II in green plants. Kok *et al.*[1] have shown that four consecutive photochemical reactions per center are necessary for

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Abbreviation: DCIP, dichlorophenolindophenol.

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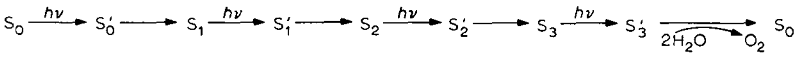
the production of an oxygen molecule. This theory can be summarized by the following linear scheme;



$$S_0 \xrightarrow{h\nu} S_0' \longrightarrow S_1 \xrightarrow{h\nu} S_1' \longrightarrow S_2 \xrightarrow{h\nu} S_2' \longrightarrow S_3 \xrightarrow{h\nu} S_3' \xrightarrow[2H_2O \;\to\; O_2]{} S_0 \qquad (1)$$

States S refer to the general state of a center, which comprises its oxidizing and reducing sides, and differ according to the number of charges stored on the oxidizing side of Photosystem II. During a short flash ($\leq 5\,\mu$s) a photochemically inactive state S_n' is formed. A dark reaction $S_n' \rightarrow S_{n+1}$ leads to the photochemically active state S_{n+1}. Vater *et al.*[2] and Joliot[3] measured the average rate of the dark reactions for the 4 S states (half time between 0.6 and 1 ms, which is slow relative to the duration of the flash). In this way, during a short saturating flash, each photochemical center reacts once and only once.

Only states S_0 and S_1 are stable in the dark. It has been shown[4] that there is an equilibrium between S_0 and S_1 in the dark. In this communication, we will present some new results with regard to this equilibrium.

These results provided us with a useful tool for the study of reactions $S_0' \rightarrow S_1$ and $S_3' \rightarrow S_0$. We also investigated $S_1' \rightarrow S_2$ and $S_2' \rightarrow S_3$, thus extending further the study begun by Kok *et al.*[1] and Bouges[5].

MATERIAL AND METHODS

Chlorella pyrenoidosa are grown on Knop medium with Arnon's trace elements A5 and B6. Before use, cells are suspended in 0.05 M phosphate buffer (pH 6.4) containing 0.1 M KCl. Spinach chloroplasts are prepared according to the method of Avron[6] and are suspended in 0.05 M Tris–HCl buffer (pH 7.8), containing 0.01 M NaCl, 0.1 M KCl, 0.4 M, saccharose, 9 g/l serum albumin, 0.2 mM $NADP^+$, and 0.5 μM ferredoxin.

Oxygen evolved by a flash is measured by the polarographic method described by Joliot *et al.*[7,8]. We used a flash lamp General Radio (Strobotron: duration; 6 μs at 1/3 peak intensity).

Chloroplasts were fixed by glutaraldehyde, according to a method described by Utsumi and Packer[9].

NOTATION

We shall designate Y_n the oxygen yield after the nth flash of a sequence, fired after 6 min dark.

When the dark time between all the flashes of the sequence is 300 ms, (infinite time compared to the limiting step $S_n' \rightarrow S_{n+1}$), we shall write Y_n or Y_n (∞).

When the time Δt between two consecutive flashes of the sequence is varied, we shall write $Y_n(\Delta t)$, indicating in each case the number of the two flashes between which the time is varied. For example, $Y_n(0)$ corresponds to two synchronized flashes in the sequence.

We shall call $\gamma_n(\Delta t)$ the probability that a center in state S_n' just after a flash will be in state S_{n+1} at time Δt after the flash.

RESULTS AND DISCUSSION

Equilibrium between S_0 and S_1

Chloroplasts or *Chlorella* subjected to a sequence of short saturating flashes, following a 5-min period of dark adaptation show a highly reproducible pattern of oxygen emission[1,8]; there is no oxygen evolved after the first two flashes: the oxygen yield after the third flash is maximal, and is followed by an oscillation of period four (Fig. 1).

This pattern has been interpreted according to Scheme 1. To obtain the damping, additional hypotheses are required, *i.e.* during a short intense flash some centers do not react (probability of misses, α) and some react twice (probability of double hits, β)[1]. In our experiments, double hits are negligible (about 0.01) and will be neglected.

The misses cannot be suppressed even with a highly saturating flash. The origin of the misses is unknown, and we don't know whether the percentage of misses is the same for each of the S states. An analysis of the general case in which the probabilities of misses are not necessarily the same for all states is required.

Let us call $\tilde{S}_0$ the concentration of centers in state S_0 after a dark period, $\tilde{S}_1$ the concentration of centers in state S_1, and α_i the probability of misses in state S_i.

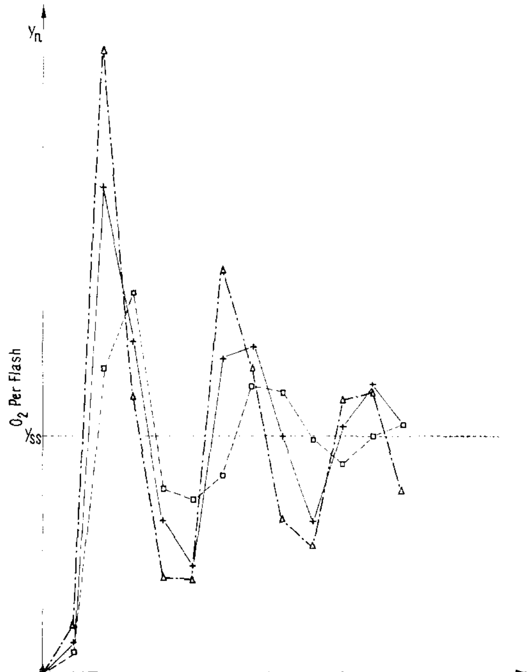


Fig. 1. Oxygen flash yield sequence Y_n, for spinach chloroplasts at 20 °C after 10 min dark. The time between two flashes was 300 ms. +, control; □, 0.1 mM DCIP and 1 mM ascorbate; △,0.1 mM $K_3Fe(CN)_6$.

The oxygen yield after the third and fourth flashes are given by the equations;

$$Y_3 = (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)\tilde{S}_1$$

$$Y_4 = (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)[(1 - \alpha_0)\tilde{S}_0 + (\alpha_1 + \alpha_2 + \alpha_3)\tilde{S}_1]$$

The average value $\bar{\alpha}$ of α_i is, as a first approximation, independent of the distribution of α_i among the different states. Thereby, the damping of the oscillations shall be characterized by $\bar{\alpha}$ which is calculated from the sequence.

With chloroplasts, $\bar{\alpha}$ is 0.11. $\bar{\alpha}$ remains unchanged by the presence of ferricyanide. Thus, the increase in the ratio Y_3/Y_4 must be interpreted as an increase of $\tilde{S}_1$ in the presence of 0.1 mM ferricyanide (Fig. 1). A computer best fit indicates that all centers are in state S_1 after 5 min dark in the presence of ferricyanide and that the chemical change of S_0 to S_1 is negligible in the 300 ms dark between two flashes. This second assumption can also be proved by varying the time between the flashes; the shape of the sequence is the same when the flashes are given every 80 ms or every second.

In the presence of reduced dichlorophenolindophenol (DCIP) (0.1 mM), a partial inhibition of the steady-state yield (not shown in Fig. 1, which presents normalized curves) is also observed. We know[10] that reduced DCIP has no effect on the deactivation of states S_2 and S_3. On the other hand, the midpoint redox potential of the A to A^- couple (acceptor pool for System II) is 0.240 mV (Kok *et al.*[11]): it is 0.217 mV for the oxidised and reduced form of DCIP (Wurmser[12]). So, the partial inhibition of the steady-state yield in the presence of reduced DCIP indicates a reduction of A to A^-. A change in the non-reacting System II centers between two flashes involves an increase of the damping. Actually, in the presence of reduced DCIP (0.1 mM), α is increased ($\bar{\alpha}=0.15$).

It can also be proved by computation, that reduced DCIP enhances S_0 relative to S_1, following dark adaptation. We conclude that in chloroplasts, the ratio $\tilde{S}_1/\tilde{S}_0$ depends upon the redox potential of the solution. This is new evidence for the existence of an equilibrium between S_0 and S_1. The redox potential that corresponds to equal concentrations of S_0 and S_1 is close to the midpoint potential of the A to A^- couple, as A becomes reduced at approximately the same potential as S_1.

Measure of the kinetics of reaction $S_n' \rightarrow S_{n+1}$

After 5 min dark, with *Chlorella* or chloroplasts, the centers are in state S_0 (concentration $\tilde{S}_0$) or in state S_1 (concentration $\tilde{S}_1$).

Reactions $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$

After a dark period, the algae or chloroplasts are subjected to a flash sequence. The time between the first two flashes, Δt, is variable. The following flashes are given every 300 ms (infinite time compared to the reactions $S_n' \rightarrow S_{n+1}$).

Scheme 1 indicates that Y_3 is proportional to $\tilde{S}_1\gamma_1(\Delta t)$. $\gamma_1(\Delta t)$ is obtained by the equation:

$$\gamma_1(\Delta t) = \frac{Y_3(\Delta t)}{Y_3(\infty)}$$

Neglecting the misses, as a first approximation, Y_4 is proportional to the sum of:

(1) $\tilde{S}_0\gamma_0(\Delta t)$; number of centers initially in state S_0 which performed two photoreactions during the first two flashes.

(2) $\tilde{S}_1\,(1-\gamma_1(\Delta t))$; number of centers, initially in state S_1 which performed only one photochemical reaction during the first two flashes.

Thus, we obtain the equation:

$$\gamma_0(\Delta t) = \frac{Y_4(\Delta t)}{Y_4(\infty)} + \frac{Y_3(\Delta t) - (Y_3(\infty))}{Y_4(\infty)}$$

The greater the number of centers in state S_0 at the beginning of the flash sequence, the more accurate the estimation of $\gamma_0(\Delta t)$. To obtain this condition, the experiments were performed either in the presence of reduced DCIP (0.1 mM) or after three flash preillumination followed by 6 min (or 3 min) dark for chloroplasts (or *Chlorella*, respectively) (*cf.* ref. 4).

For more precise computation, we supposed that the misses did not change for the same state during the reaction $S_0' \rightarrow S_1$. By taking into account the possible differences in the probabilities of misses of states S, it could be demonstrated (see Appendix 1) that $\gamma_0(\Delta t)$ is between

$$\frac{Y_4(\Delta t) - Y_4(0)}{Y_4(\infty) - Y_4(0)} \text{ and } \frac{Y_3(\Delta t)}{Y_3(\infty)}.$$

Reaction $S_2' \rightarrow S_3$

After a dark period, $\gamma_2(\Delta t)$ is observed by varying the time Δt between the second and the third flash. The time between the first two flashes is 300 ms. Scheme 1 indicates that $Y_3(\Delta t)$ is then proportional to $\gamma_2(\Delta t)$.

$$\gamma_2(\Delta t) = \frac{Y_3(\Delta t)}{Y_3(\infty)}$$

Reaction $S_3' \rightarrow S_0$

In a sequence after a dark period, state S_3' is obtained after the third flash (Scheme 1). Varying the time, Δt, between the third and the fourth flash allows us to study $\gamma_3(\Delta t)$. The oxygen evolved after the 7th flash detects the amount of centers in state S_0 formed by the 4th flash.

In the flash sequence, the dark time between two flashes is 300 ms except between the third and the fourth. We shall call $Y_n(\Delta t)$, the oxygen yield of the nth flash, where Δt is the time between the third and the fourth flash.

The greater the difference between $Y_7(0)$ and $Y_7(\infty)$, the more accurate the results. On the other hand, we obtain $Y_7(0) = Y_6(\infty)$.

The more centers in state S_1, the greater the difference between $Y_6(\infty)$ and $Y_7(\infty)$. The optimum is in the presence of ferricyanide where all centers are in state S_1.

It can be demonstrated then (see Appendix 2) that:

$$\gamma_3(\Delta t) = a \cdot \frac{Y_7(\Delta t) - Y_7(0)}{Y_7(\infty) - Y_7(0)} + b\gamma_1(\Delta t) + c\gamma_2(\Delta t)$$

where $a+b+c=1$, $0.75 \leq a \leq 0.86$, $0 \leq b \leq 0.09$, $0.11 \leq c \leq 0.25$.

Analysis of reaction $S_1' \to S_2$

$\gamma_1(\Delta t)$ is plotted for various temperatures with *Chlorella* (Fig. 2) and chloroplasts (Fig. 3).

For the −15 °C curve, the *Chlorella* were suspended in 40% glycerol. (We verified that between 0 and 20 °C, glycerol does not change the kinetics). Below −9 °C, the sensitivity of the detection apparatus becomes limiting because of the slow rate at which oxygen is emitted. So, for the lower temperatures, the algae were left at −15 °C for only the first two flashes and then heated to 0 °C in time shorter than that for deactivation (3 s). The yield of the third flash was measured at the latter temperature.

The kinetics of these curves for both chloroplasts and *Chlorella* were followed to 80% of completion, and we found that they are not exponential.

The shape of the curve $\gamma_1(\Delta t)$ was not changed in an experiment in which the flashes were non-saturating. Because only a third of the centers reacted per flash in this experiment, all hypotheses involving an interaction between the centers are not likely. Thus, the non-exponential shape of curves is a property of the centers.

Chloroplasts fixed with 2% glutaraldehyde exhibit first order kinetics and constant activation energy between 0 and 30 °C (Figs 3b and 3c). Their temperature coefficient is $Q_{10} = 2.6$.

We observe here for chloroplasts in the absence of glutaraldehyde, as previously for *Chlorella*[5], that the temperature coefficient is time dependent; at time zero, the slopes of the curves (Fig. 3a) show a temperature coefficient $Q_{10} = 1.7$, between 20 and 30 °C. At longer times (*e.g.* $\geqslant 1$ ms at 21 °C), the curves tend to become

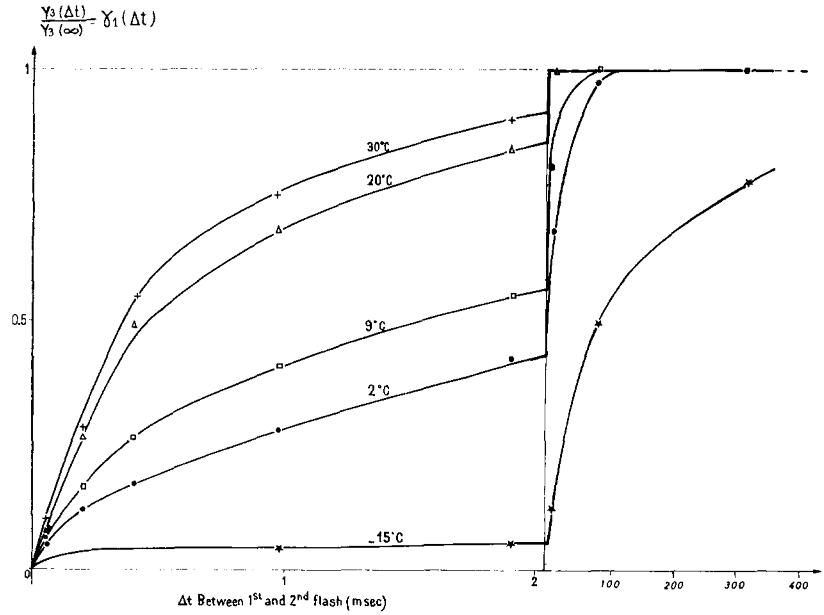


Fig. 2. Y_3 as a function of time Δt between the 1st and 2nd flash for *Chlorella*. The time between 2nd and 3rd flashes was 300 ms. The temperature for each curve is indicated.

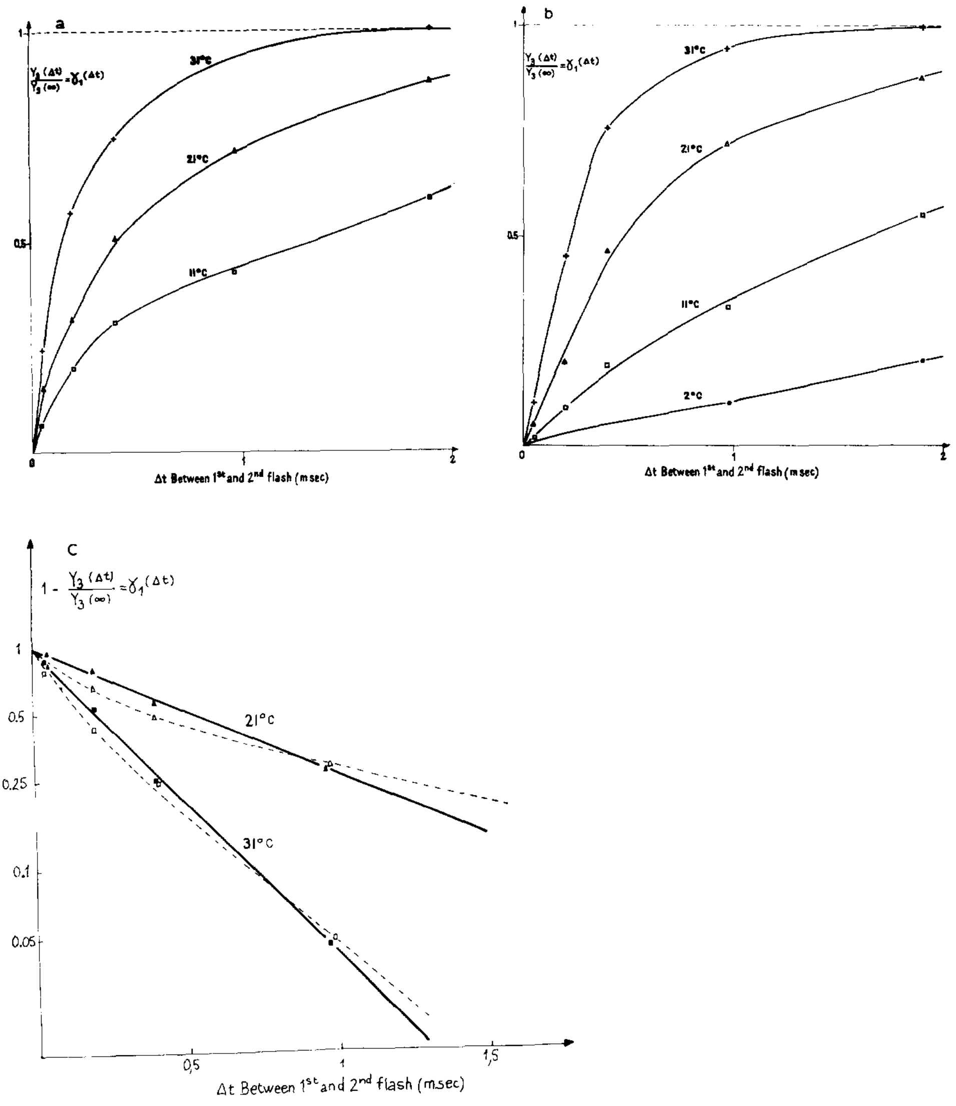


Fig. 3. Same as Fig. 2 for spinach chloroplasts. (a) Control. (b) Chloroplasts fixed with 2% glutaraldehyde. (c) Semi-log plot. △, control at 31 °C; ▲, 2% glutaraldehyde at 31 °C; □, control at 21 °C; ■, 2% glutaraldehyde at 21 °C.

exponential (Fig. 3c). The rate constant shows a temperature coefficient $Q_{10} = 2.6$ between 20 and 30 °C.

The temperature coefficient becomes greater as the reaction takes place, and at long times, goes to the temperature coefficient of the same chloroplasts fixed with glutaraldehyde.

The time dependence of the temperature coefficient is observed with *Chlorella* (Fig. 2).

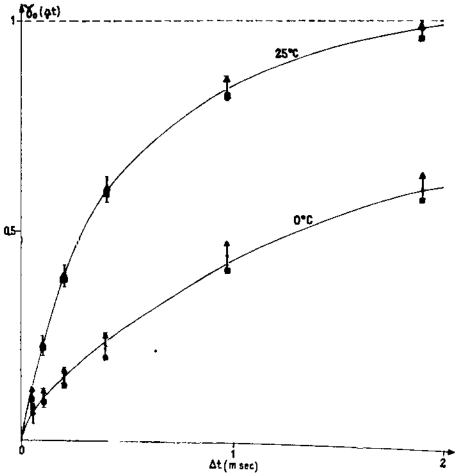
If $\gamma_1(\Delta t)$ consisted of only two first order reactions with two appreciably different temperature coefficients (as was supposed in ref. 5), we could separate these two reactions by lowering the temperature. We exclude this hypothesis because at −15 °C, the curve exhibits no exponential phase. In addition, the smaller the variations of the temperature coefficient with time, the more the curves $\gamma_1(\Delta t)$ tend to be exponential.

All those results favor a single reaction for the transition $S_1 \xrightarrow{} S_2$. This reaction could be the recovery of chlorophyll a_{II} put forward by Döring *et al.*[13], which shows the same half time and a time-dependent temperature coefficient (Döring, unpublished). It could also be the reoxidation of the acceptor Q measured by Kok *et al.*[11] and Joliot *et al.*[1].

The variation of the temperature coefficient could then be interpreted by two kinds of models.

(1) In each center, the activation energy varies during the reaction. After a flash, a state with low activation energy would be reached: for example, two molecules may become closer, or the state of the membrane may change. This lowering of activation energy would take place within less than 50 μs and disappear within a few milliseconds. Glutaraldehyde would block this mechanism probably in fixing a protein of the photochemical center. This is a dynamic model.

(2) Each center presents a constant activation energy during the reaction, but different centers have different activation energies. To account for the temperature effects, it must also be assumed that the number of centers in a given activation energy depends on the temperature. Glutaraldehyde would fix all centers at the same activation energy. This is a static model.



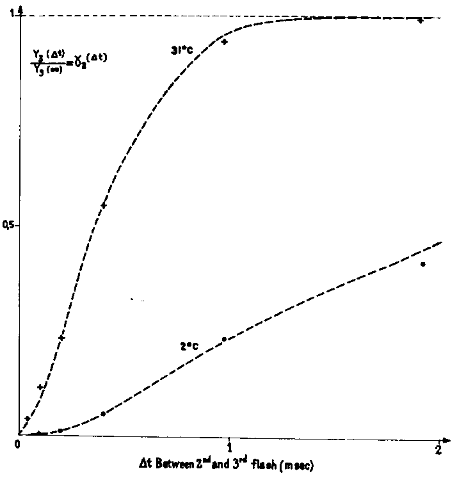


Fig. 4. Calculated curve (—) for $\gamma_0(\Delta t)$ in *Chlorella*. Δt is the time between the first two flashes of the series. The temperatures are indicated. ■, $[Y_3(\Delta t)]/[Y_3(\infty)]$; ▲, $[Y_4(\Delta t) - Y_4(0)]/[Y_4(\infty) - Y_4(0)]$.

Fig. 5. Y_3 as a function of time Δt between the 2nd and 3rd flash. Chloroplasts were fixed with glutaraldehyde. We used the same batch as for Fig. 3. The time between the 1st and 2nd flash was 300 ms. The temperature for each curve is indicated. The points are experimental and the dashed curves are theoretical.

Analysis of reaction $S_0' \rightarrow S_1$

When Δt is the time between the first and the second flash,

$$\frac{Y_4(\Delta t) - Y_4(0)}{Y_4(\infty) - Y_4(0)}$$

is within 10% of

$$\frac{Y_3(\Delta t)}{Y_3(\infty)}.$$

So $\gamma_0(\Delta t)$, which is between the two, is very close to

$$\frac{Y_3(\Delta t)}{Y_3(\infty)} \ (e.g.\ \gamma_1(\Delta t))\ (\text{Fig. } 4).$$

The results for γ_0 are the same with DCIP or three flash preillumination.

The similar shape, half time and temperature dependence of $\gamma_0(\Delta t)$ and $\gamma_1(\Delta t)$ suggest that the mechanisms of the limiting step in reactions $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$ are the same.

Analysis of reaction $S_2' \rightarrow S_3$

For $\gamma_2(\Delta t)$, the curves are sigmoidal, even with glutaraldehyde-fixed chloroplasts (ref. 5 and Fig. 5). This shape shows that there are two reactions with close half times. With glutaraldehyde-fixed chloroplasts, the two reactions are first order (dashed curves represent theoretical curves in Fig. 5). Computation does not allow us to determine whether the two reactions are consecutive:

$$S_2' \xrightarrow{k_1} S_2'' \xrightarrow{k_2} S_3 \tag{2}$$

or independent:

$$\begin{tikzcd}
 & S''_{2,1} \arrow[dr, "k_2"] & \\
S'_2 \arrow[ur, "k_1"] \arrow[dr, "k_2"'] & & S_3 \\
 & S''_{2,2} \arrow[ur, "k_1"'] &
\end{tikzcd} \tag{3}$$

Thus, dashed curves represent as well

Scheme 1. Then the half times of the two reactions could be:
at 31 °C ($t_1 = 72\,\mu$s, $t_2 = 290\,\mu$s) ($t_1 = 160\,\mu$s, $t_2 = 160\,\mu$s)
($t_1 = 290\,\mu$s, $t_2 = 72\,\mu$s)
at 2 °C ($t_1 = 200\,\mu$s, $t_2 = 2$ ms) ($t_1 = 600\,\mu$s, $t_2 = 1.2$ ms)
($t_1 = 1.2$ ms, $t_2 = 600\,\mu$s) ($t_1 = 2$ ms, $t_2 = 200\,\mu$s)

Scheme 2. Then the half times of the reactions could be:
at 31 °C ($t_1 = 105\,\mu$s, $t_2 = 420\,\mu$s) ($t_1 = 230\,\mu$s, $t_2 = 230\,\mu$s)
at 2 °C ($t_1 = 280\,\mu$s, $t_2 = 2.8$ ms) ($t_1 = 780\,\mu$s, $t_2 = 1.56$ ms)

To obtain complete conversion of S_2 to S_3, the second step of the first model and both steps of the second must be irreversible.

A direct measure of the intermediary state S_2'' would be necessary to distinguish between those reaction schemes. According to Zankel (personal communication), the decay of the fluorescence yield after two flashes is sigmoidal, and correlated to the formation of S_3. A spectroscopic method could permit observation of an intermediate state.

At least, one of the two reactions implied in transition $S_2' \rightarrow S_3$ displays properties similar to those of $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$; non-exponential with variable temperature coefficient on chloroplasts and *Chlorella*: becoming exponential with chloroplasts fixed with 2% glutaraldehyde.

As for fluorescence[14,15], there is a difference between the properties of S_0 and S_1 on the one hand, and S_2 on the other (for S_3, as will be seen later, some additional reactions happen so that oxygen is evolved).

Analysis of reaction $S_3' \rightarrow S_0$

$\gamma_3(\Delta t)$ is exponential (Fig. 6). The half time is 1.2 ms at 20 °C. The reaction $S_3' \rightarrow S_0$ is roughly two times slower than the reactions $S_0' \rightarrow S_1$, $S_1' \rightarrow S_2$ and $S_2' \rightarrow S_3$.

Zankel (personal communication) observed the same sigmoidal decay of the fluorescence yield after two and three flashes but not after one and five, indicating that the sigmoidal decay of fluorescence yield is a property of S_2' and S_3'. After two flashes, the reaction exhibited by the fluorescence decay was the limiting step $S_2' \rightarrow S_3$. After three flashes, a similar reaction is observed for the fluorescence decay, but not for the limiting step $S_3' \rightarrow S_0$ measured in this article. An additional slower reaction is probably involved in transition $S_3' \rightarrow S_0$. A likely candidate for this slow step is the release of oxygen measured by Joliot *et al.*[16] (half time, 0.9 ms).

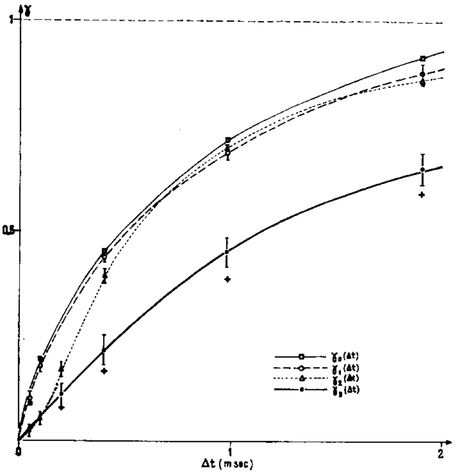
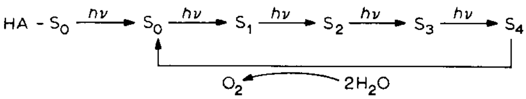


Fig. 6. Turnover times in spinach chloroplasts at 20 °C, in presence of 0.1 mM $K_3Fe(CN)_6$. ●, $[Y_3(\Delta t)]/[Y_3(\infty)]$ as function of time Δt between the 1st and 2nd flash; ▲, $[Y_3(\Delta t)]/[Y_3(\infty)]$ as function of time Δt between the 2nd and 3rd flash; +, $[Y_7(\Delta) - Y_7(0)]/[Y_7(\infty) - Y_7(0)]$ as function of time Δt between the 3rd and 4th flash; ●, calculated curve for $\gamma_3(\Delta t)$; □, calculated curve for $\gamma_0(\Delta t)$ in absence of $K_3Fe(CN)_6$ as the measurement is very difficult in presence of $K_3Fe(CN)_6$.

Hydroxylamine at low concentration

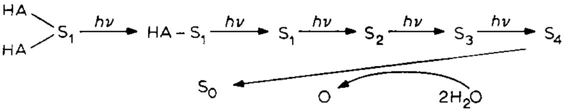
For a series of short flashes given in the presence of 50 μM hydroxylamine, the maximum oxygen yield is shifted from the 3rd to the 5th flash. There is no oxygen evolved after the first four flashes[17]. After a dark period, in the absence of hydroxylamine, most of the centers are in state S_1. Two interpretations are proposed for the delay;

(1) If S_1 gets reduced to S_0 by hydroxylamine (HA), one must assume in addition that one molecule of hydroxylamine is bound to every photochemical center in the dark. The reactions are summarized in Scheme 4;



(4)

(2) If hydroxylamine does not reduce S_1 to S_0, two molecules of hydroxylamine are bound to every center. Scheme 5 summarizes the reactions in the course of a series of short flashes.



(5)

$Y_5(\Delta t)$ is studied as a function of the time Δt between the 2nd and the 3rd flash. According to the first hypothesis, we would observe reaction $S'_0 \rightarrow S_1$ (Scheme 4).

$\gamma_1(\Delta t)$ in the presence of hydroxylamine is detected by $Y_5(\Delta t)$, Δt being then the time between the 3rd and the 4th flash. According to Fig. 7 (Curves b and c), hydroxylamine does not appreciably change the kinetics of $\gamma_1(\Delta t)$.

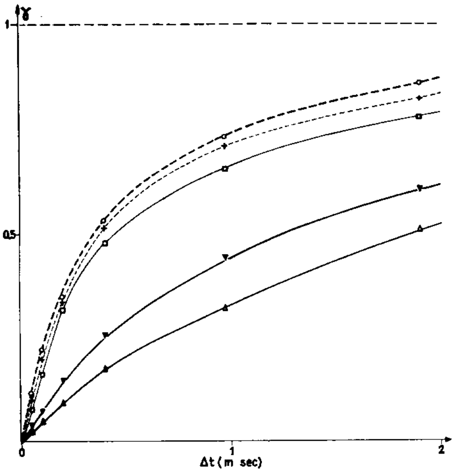


Fig. 7. Turnover times in presence (Curves c, d and e) and in absence (Curves a and b) of 50 μM NH_2OH, for *Chlorella* at 20 °C. Curve a (○), calculated curve for $\gamma_0(\Delta t)$; Curve b (+), $[Y_3(\Delta t)]/[Y_3(\infty)]$ as function of time Δt between the 1st and 2nd flash; Curve c (□), $[Y_5(\Delta t)]/[Y_5(\infty)]$ as function of time Δt between the 3rd and 4th flash; Curve d (▼), $[Y_5(\Delta)]/[Y_5(\infty)]$ as function of time Δt between the 2nd and 3rd flash; Curve e (▲), $[Y_5(\Delta t)]/[Y_5(\infty)]$ as function of time Δt between the 1st and 2nd flash.

Fig. 7 (Curves a and d) shows that the kinetics followed by $Y_5(\Delta t)$ as a function of Δt, time between the 2nd and the 3rd flash, are 3 or 4 times slower than $\gamma_0(\Delta t)$ in the absence of hydroxylamine. This result favors the second hypothesis, *i.e.* two molecules of hydroxylamine bound to every center. The reaction with the first bound molecule (Curve c) would be slower than the reaction with the second one (Curve d). It was shown[18] that if there are two molecules of hydroxylamine bound to every center, the two binding sites are cooperative; the release of the first bound hydroxylamine decreases the affinity of the center for the second one.

Oxidation of the first hydroxylamine molecule (Curve d) takes place in a time close to the time for oxygen release. This is an additional argument for fixation of hydroxylamine at low concentrations, directly on the water site. This could indicate two cooperative sites for water fixation on every photochemical center in System II.

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APPENDIX 1

Let us call Δt the time between the first and the second flash. (The time between the other flashes is infinite with respect to the reactions $S_n' \rightarrow S_{n+1}$.)

$\tilde{S}_0$ and $\tilde{S}_1$ are the concentrations of states S_0 and S_1 at the beginning of the flash sequence.

According to Scheme 1, we obtain:

$$Y_3(\Delta t) = (1-\alpha_1)(1-\alpha_2)(1-\alpha_3)\gamma_1(\Delta t)\tilde{S}_1$$

$$Y_4(\Delta t) = (1-\alpha_1)(1-\alpha_2)(1-\alpha_3)[(1-\alpha_0)\gamma_0(\Delta t)\tilde{S}_0 - (1-\alpha_1-\alpha_2-\alpha_3)\gamma_1(\Delta t)\tilde{S}_1 + \tilde{S}_1]$$

$$Y_3(\infty) = (1-\alpha_1)(1-\alpha_2)(1-\alpha_3)\tilde{S}_1$$

$$Y_4(\infty) = (1-\alpha_1)(1-\alpha_2)(1-\alpha_3)[(1-\alpha_0)\tilde{S}_0 + (\alpha_1+\alpha_2+\alpha_3)\tilde{S}_1]$$

We find that γ_0 is a linear combination:

$$\gamma_0(\Delta t) = a\,\frac{Y_4(\Delta t) - Y_4(0)}{Y_4(\infty) - Y_4(0)} + b\,\frac{Y_3(\Delta t)}{Y_3(\infty)}$$

where

$$a = 1 - \frac{(1-\alpha_1-\alpha_2-\alpha_3)\tilde{S}_1}{(1-\alpha_0)\tilde{S}_0} \text{ and } b = \frac{(1-\alpha_1-\alpha_2-\alpha_3)\tilde{S}_1}{(1-\alpha_0)\tilde{S}_0}\,;\ a + b = 1.$$

a presents the same sign as $Y_4(\infty) - Y_3(\infty)$. It will be positive in all our experiments in presence of DCIP or after 3 flashes preillumination. As the average value a calculated from the damping, is smaller than 0.33, b is also positive.

These three properties of a and b indicate that $\gamma_0(\Delta t)$ is between

$$\frac{Y_4(\Delta t) - Y_4(0)}{Y_4(\infty) - Y_4(0)} \text{ and } b\,\frac{Y_3(\Delta t)}{Y_3(\infty)}.$$

APPENDIX 2

Let us call Δt, the time between the 3rd and the 4th flash.

In the presence of 0.1 mM ferricyanide, we assume all the centers to be in state S_1 at the beginning of the flash sequence.

According to Scheme 1, we obtain:

$$
\begin{aligned}
&Y_7(\Delta t) - Y_7(0) = (1 - \alpha_0)(1 - \alpha_1)^2(1 - \alpha_2)^2(1 - \alpha_3)^2\gamma_3(\Delta t) - \\
&(1 - \alpha_1)(1 - \alpha_2)^2(1 - \alpha_3)(\alpha_2^2 + \alpha_2\alpha_3 + \alpha_3^2)(\alpha_1 + \alpha_2)\gamma_2(\Delta t) + \\
&\alpha_1^2(1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)[\alpha_2^2 + \alpha_2\alpha_3 + \alpha_3^2 - (1 - \alpha_1)(\alpha_1 + \alpha_3 + \alpha_3)]\gamma_1(\Delta t)
\end{aligned}
$$

The flash yields in a sequence are given by the equations:

$$
\begin{aligned}
Y_3(\infty) &= (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3) \\
Y_4(\infty) &= (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)(\alpha_1 + \alpha_2 + \alpha_3) \\
Y_5(\infty) &= (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)(\alpha_1^2 + \alpha_2^2 + \alpha_3^2 + \alpha_1\alpha_2 + \alpha_2\alpha_3 + \alpha_3\alpha_1) \\
Y_6(\infty) &= (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)(\alpha_1^3 + \alpha_2^3 + \alpha_3^3 + \alpha_1^2\alpha_2 + \alpha_2^2\alpha_3 + \alpha_3^2\alpha_1 + \\
&\qquad \alpha_1\alpha_2^2 + \alpha_2\alpha_3^2 + \alpha_3\alpha_1^2 + \alpha_1\alpha_2\alpha_3)
\end{aligned}
$$

Thus, we can write:

$$
\gamma_3(\Delta t) = a\,\frac{\mathrm{Y}_7(\Delta t) - \mathrm{Y}_7(0)}{\mathrm{Y}_7(\infty) - \mathrm{Y}_7(0)} + b\gamma_1(\Delta t) + c\gamma_2(\Delta t)
$$

$$
\begin{aligned}
a &= k(Y_7(\infty) - Y_6(\infty)) \\
b &= k(Y_4(\infty) - Y_5(\infty))\alpha_1^2 \\
c &= k(Y_6(\infty) - \alpha_1^2 Y_4(\infty))(1 - \alpha_2)
\end{aligned}
$$

where k is calculated so that

$$a + b + c = 1.$$

From the damping of the oscillations, we calculate $\bar{\alpha} = 0.11$, which involves the following inequalities:

$$
0 \leqq \alpha_1^2 \leqq 0.2 \text{ and } 0.56 \leqq 1 - \alpha_2 \leqq 1 \rightarrow \quad
\begin{array}{c}
0.75 \leqq a \leqq 0.86 \\
0 \leqq b \leqq 0.09 \\
0.11 \leqq c \leqq 0.25
\end{array}
$$

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