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ACID-INDUCED DOUBLE HITS IN GLUTARALDEHYDE-TREATED *CHLORELLA*

BRIGITTA MAISON-PETERI

Laboratoire de Photosynthèse, C.N.R.S., 91190 Gif-sur-Yvette (France)

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Summary

Glutaraldehyde-treated *Chlorella* cells show pH-dependent oxygen evolution characteristics.

1. The oxygen yield on the second flash (Y_2) of a sequence of 2 μs flashes is pH-dependent; the rest of the sequence is unchanged.

2. This yield on the second flash increases as the dark-time between sequences is increased.

3. The deactivation times for the S_2 and S_3 states are not significantly changed at different pH values.

4. Using a short flash (0.5 μs) for the first flash, no oxygen was detected on the second flash, indicating that there is no S_2 remaining in the dark.

5. When the intensity of the first flash is varied, a sigmoidal curve is obtained for Y_2 as a function of flash intensity at pH 6.0 but not at pH 7.5. This is indicative of a double hit process.

6. At pH 6.0 the turnover of the Photosystem II centers after the first flash has a fast rise ($t_{1/2} < 25$ μs) followed by a slow phase. This behaviour is not seen at pH 7.5, nor after two flashes.

7. We propose a scheme in which an auxiliary acceptor is in series with Q_1 and in competition with B. The redox potential of the auxiliary acceptor is pH dependent.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Q_1, first primary acceptor; Q_{aux}, second or auxiliary primary acceptor; B, secondary acceptor; Y_n, oxygen yield on the *n*th flash.

Introduction

Over the last few years a number of authors have shown that the reaction center of System II is able to undergo a double turnover during the time of a short flash (2—5 μs) [1—7]. For a double turnover to occur it is necessary to postulate, as did Gläser et al. [1], the presence of an auxiliary primary acceptor. Under normal conditions, the reduction of P^+-680 takes place in approximately 30 ns [8] whilst the reoxidation of Q^- is much slower: $t_{1/2}$ = 500 μs [9—10]. Thus for a second photochemical act to take place during a short flash the reaction is limited by the acceptor side and cannot take place unless there is a second acceptor.

So far, the position of the auxiliary primary acceptor with respect to the electron transfer chain has not been elucidated; it may either be in series, parallel or rapid equilibrium with the principal primary acceptor. Depending on the scheme considered, parallel or series, double hits may take place once or several times during an illumination sequence. As the reoxidation of the auxiliary acceptor is considered to be slow ($t_{1/2}$ = 75 s—4 min [6,11]) a double hit only occurring once seems the more likely. The redox potential of the acceptor has been characterized and seems to vary from —300 to +400 mV under the conditions used by different authors [4,11—13].

Most of the measurements leading to the conclusion that an auxiliary acceptor must be present, as well as those concerning its characterisation, have been done using chloroplasts, or subchloroplast particles [13], often in the presence of ferricyanide or other reagents. Only Van Best and Duysens [2] have provided evidence for an auxiliary acceptor in algal cells, under anaerobic conditions.

Double hits have been detected via measurements of the *P*-680 absorption change [1,7], fluorescence induction kinetics in the presence of DCMU [3,14], the 550 nm absorption change [14] and fast fluorescence and luminescence [2,11,14]. In addition, Diner [6] has shown that in Tris-washed chloroplasts the photo-oxidation of phenylenediamine is greatest on the first flash and he concludes that under these conditions a double hit occurs on the first flash. He is not able to detect the double hit via the oxygen-evolving site in untreated chloroplasts and supposes that the extra charge generated cannot be used for oxygen evolution. In contrast Velthuys and Kok [5] provided evidence for double hits from oxygen evolution experiments using ferricyanide-treated chloroplasts. They obtained a high oxygen yield on the second flash of a series, a yield which is normally low [15]. Furthermore, when they measured the amount of ferricyanide reduced per flash, the yield on the first flash was significantly lower than that on the subsequent flashes, also in favour of a double hit on the first flash.

In this paper we present evidence for the occurrence of double hits on the first flash of a series of flashes, in glutaraldehyde-treated algae in the absence of exogenous acceptor. The double hits are detected via the oxygen-evolving side and are shown to be dependent on pH. The same results are obtained for algae washed with 10^{-4} M benzoquinone and for isolated chloroplasts. However, for the latter the amplitude of the effect is variable depending on the state of the material and generally requiring the addition of the System I acceptors NADP

and ferredoxin. We have chosen the glutaraldehyde treatment to vary the internal pH of the algae because of the reproducibility of the results obtained and because of the ease of preparation. We do show some results obtained with chloroplasts to confirm that the effects seen are dependent on pH and are not induced by the glutaraldehyde treatment.

Materials and Methods

The experiments were performed with the alga *Chlorella pyrenoidosa* grown and harvested as previously described [16], or with broken chloroplasts.

Chloroplasts were isolated from spinach, lettuce or peas, according to a procedure previously described [17] and were then resuspended in 0.4 M sorbitol, 10 mM NaCl, 10 mM $MgCl_2$ and 10 mM Tricine, pH 7.8. They were stored in the dark at 0°C at a concentration of 1—3 mg chlorophyll per ml.

Glutaraldehyde fixation is carried out with reactivated glutaraldehyde (50% v/v) stored at —18°C. The glutaraldehyde is added under continuous stirring to a suspension of *Chlorella* cells in their culture medium, 0.1—0.2% glutaraldehyde for a concentration of 50 μg chlorophyll/ml. The cells are incubated for 5 min with the glutaraldehyde before centrifugation and resuspension of the cells in appropriate buffer: 50 mM phosphate buffer, 0.1 M KCl is used for the pH range 6.0—7.5, whilst for pH 5.0 and 5.5 20 mM Mes also containing 0.1 M KCl is used.

The algae are kept in the dark on ice before use.

For oxygen measurements the algae and choroplasts are used at a concentration of 500 μg chlorophyll/ml. The yield of oxygen evolved at each flash of a series was measured with a rate electrode. The length of flash used is defined in the text.

For details of the DCMU-triggered luminescence experiments see Etienne and Lavorel [18].

The Hill reaction is measured using a Cary 14 with saturating lateral actinic illumination of the cuvette. Dichlorophenolindophenol is used at a concentration of $5 \cdot 10^{-4}$ M.

The fluorescence inductions were followed using an apparatus previously described [19]. A sample of suspension flows to a preilluminating chamber where it is submitted to a varying number of 2 μs saturating flashes. During a further flow step the sample is mixed with an equal volume of medium containing 10^{-4} M DCMU. The fluorescence is detected using weak modulated light, provided by 2 green light emitting diodes (Hewlett Packard, modulation rate 1 kHz) filtered through a 4.96 Corning + Wratten filter. A complementary red, 2.64 Corning filter is mounted on the PM window. The modulated fluorescence signal is processed by a PAR lock-in amplifier and the kinetics recorded with an oscilloscope.

Results

Oxygen evolution as a function of pH

The oxygen evolved per flash by untreated algae exposed to a sequence of short (2 μs), saturating flashes shows characteristic damped oscillations of

period four [15], with a low yield on the second flash and a high degree of damping (see Fig. 1a control). Glutaraldehyde treatment modified the form of the oxygen sequences (Fig. 1a): there is a decrease in the damping and a fall off in yield after 20—30 flashes. The sequences resemble those of chloroplasts in the absence of System I acceptors. The fall off in yield is attributed to a slowed reoxidation of the plastoquinone pool because glutaraldehyde inhibits plastocyanin [20]. We have calculated the degree of damping of the oxygen sequences for different pH values using the method of Lavorel [21] and find that the values of σ_1 are similar to those of chloroplasts and independent of pH (glutaraldehyde-treated algae $\sigma_1 = 0.3$—0.4; whole algae $\sigma_1 = 0.6$—0.7; chloroplasts $\sigma_1 = 0.3$—0.4). The first two flashes are omitted from the calculation to avoid the introduction of anomalies because, in addition to the changes described above, glutaraldehyde renders the oxygen yield on the second flash

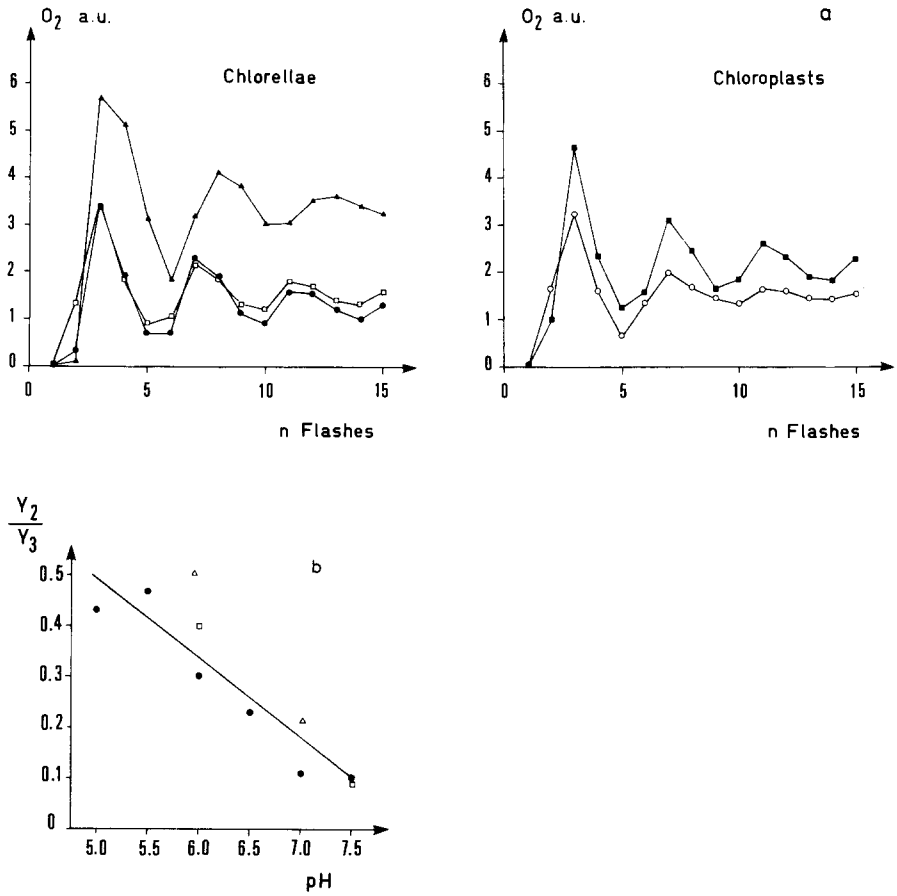

Fig. 1.a. Oxygen yield (non-normalised) as a function of flash number for untreated control Chlorellae, glutaraldehyde-treated algae and untreated chloroplasts at different pH values. Control: ▲; glutaraldehyde-treated algae: ●, pH 7.5; □, pH 6.0; chloroplasts: ■, pH 7.0; ○, pH 5.9. Flash spacing is 300 ms. Chloroplasts in the presence of $2 \cdot 10^{-4}$ M NADP and ferredoxin. b. Ratio of the oxygen yield on the second flash to that on the third flash as a function of pH. The values obtained from the experiment of (a) are also shown: □, glutaraldehyde-treated Chlorellae; △, chloroplasts.

(Y_2) dependent on pH (Figs. 1a and b). Fig. 1a also shows the oxygen sequences obtained for untreated chloroplasts in the presence of NADP and ferredoxin at two pH values, 5.9 and 7.0 As for the glutaraldehyde treated material, Y_2 increases with decreasing pH.

In Fig. 1b we have plotted the ratio of Y_2 to Y_3 as a function of pH. We have used this ratio as we cannot normalise to the steady state because of the fall off in yield, yet we need to take into account the inhibition of oxygen yield which takes place after long times of incubation at low pH.

In the rest of this study we have compared the results obtained with glutaraldehyde treated material at two pH values: 7.5 where we observed little effect on Y_2 and 6.0 where Y_2 has sufficiently increased but where the inhibition of the oxygen signal is not prohibitive. We have verified by Hill reaction measurements that the activities of the treated algae were comparable for these pH values: with water as donor and dichlorophenolindophenol as acceptor we obtain values of 50 ± 10 μmol O_2/mg chlorophyll/h.

Dark time between sequences and deactivation

The increase in Y_2 at low pH can have at least two explanations: either the 10 min dark time between sequences is insufficient for complete deactivation of the S_2 state, or a double hit takes place on the first or second flash.

When the dark time between sequences was increased as shown in Fig. 2,

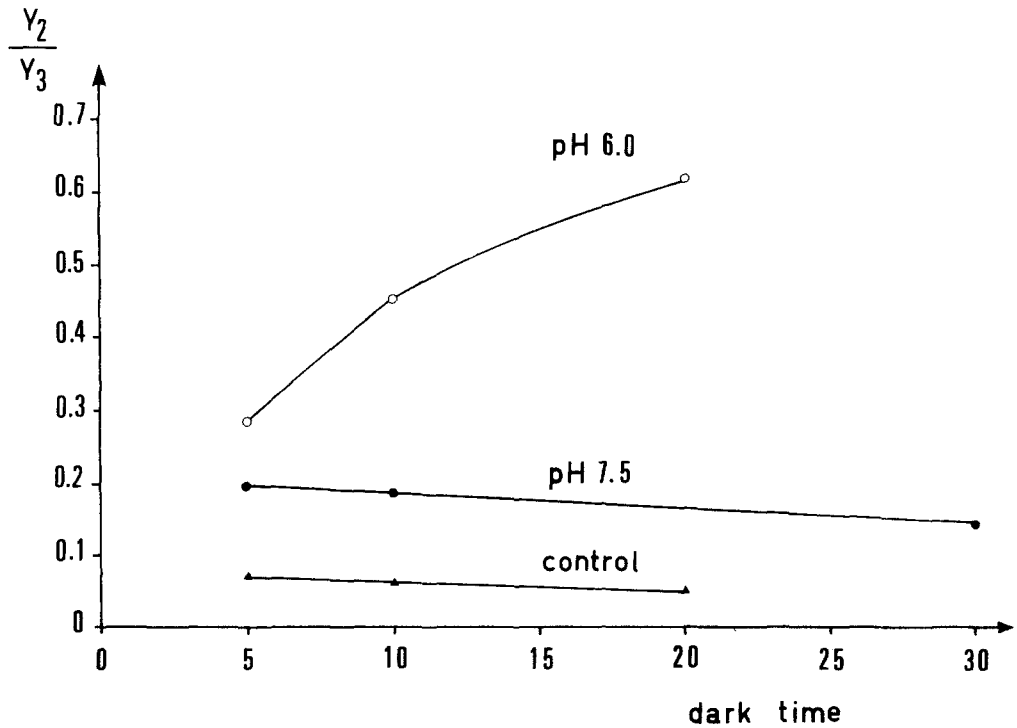

Fig. 2. Ratio of the oxygen yield on the second flash to that on the third flash as a function of dark-time between sequences for untreated control algae and glutaraldehyde-treated algae at pH 6.0 and 7.5.

Y_2 increases as a function of this time. Thus the increase in Y_2 is not due to a simple slowing of the deactivation of S_2 at pH 6.0.

To obtain further information about the behaviour of the S_2 and S_3 states we have followed their dark decay. The rates of deactivation of S_2 and S_3 are determined by giving 1 or 2, 2-μs preilluminating flashes, followed by a variable dark time (0.3—200 s) and measuring the oxygen evolved on the third flash [22].

The dark decay of S_3 (Fig. 3a) is slowed for glutaraldehyde treated algae, as compared with untreated algae, but there is no significant difference between the rates at pH 6.0 and 7.5. The dark decay of S_2 (Fig. 3b) is also slowed compared with untreated algae. The main difference which emerges when comparing the kinetics for pH 6.0 and 7.5 is an increase in the amount of S_2 at pH 6.0. This increase is likely to be partly due to some S_3 which has been formed by double hits and which decays to S_2.

DCMU-triggered luminescence and fluorescence oscillations

Etienne and Lavorel [18] have shown that it is possible to obtain a DMCU-triggered luminescence in dark-adapted material by the recombination of the positive charge on S with the negative charge on Q, the latter being reduced by B after the addition of DMCU.

$$S^+QB^- \xrightarrow{DCMU} S^+Q^-B \rightarrow SQB + h\nu_L$$

Furthermore Lavorel [23] has postulated that the S states can all function as substrates for luminescence, the luminescence intensity being dependent on the number of positive charges stored on the donor side, $S_4 > S_3 > S_2 > S_1$. Thus, if S_2 is present in the dark at pH 6.0 and not at pH 7.5, we would expect a greater emission of DCMU-triggered luminescence at pH 6.0, providing the acceptor side is not limiting. Compared with untreated algae the luminescence emission is very much smaller for the glutaraldehyde-treated material and there

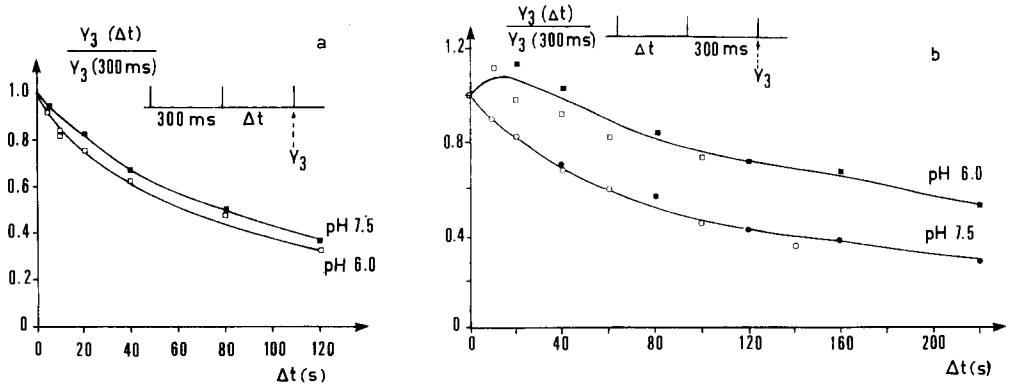

Fig. 3.a. Deactivation of the S_3 state at pH 6.0 and pH 7.5. Two flashes spaced by 300 ms are given, followed by a variable dark-time, Δt, oxygen is detected on the third flash (insert). b. Deactivation of the S_2 state at pH 6.0 and pH 7.5. A preilluminating flash is given followed by a variable dark-time, Δt, the oxygen evolved as a function of dark-time is measured on the third flash (insert). The results obtained from two different experiments are plotted on the same curve to provide more points.

is no difference between pH 6.0 and pH 7.5. It seems unlikely that there is some S_2 remaining in the dark at pH 6.0, but it is necessary to verify that the concentration of negative charges is sufficient.

To check this we have tried to obtain information about the concentration of B^- present in the dark using a protocol similar to that of Wollman [24]. The algae are dark adapted for 10 min and mixed with 10^{-4} M DCMU after a variable number of 2 μs preilluminating flashes. The initial fluorescence level is measured rapidly (100 ms after mixing), before recombination reduces the concentration of Q^-. Pronounced oscillations of period 2 of the fluorescence indicate that in the dark the concentrations of B and B^- are not equal; whereas in the absence of oscillations B and B^- are taken to be present in equal amounts. For glutaraldehyde-treated algae weak oscillations are seen at pH 6.0 and 7.5 which would indicate that there is B^- present in the dark (40—60%). However at pH 6.0 the fluorescence yield without any preilluminating flashes shows a deficit not observed at pH 7.5 (Fig. 4). This implies that at pH 6.0, after addition of DCMU in the dark, the concentration of Q available for combination with an eventual S_2 is low. Hence the absence of DCMU-triggered luminescence is not conclusive for the absence of S_2.

Variations of the length or the intensity of the first flash

To confirm that there is no S_2 stable in the dark and to show that double hits occur on the first flash at pH 6.0, the effect on Y_2 of a 0.5 μs length first flash was compared with that of the regular 2 μs flash. Table I shows that at pH 6.0 Y_2 is dependent on flash length and completely abolished when the 0.5 μs flash is used.

This experiment provides direct proof for the absence of S_2 in the dark, otherwise we would obtain oxygen on the second flash even with a very short flash. Thus the high oxygen signal observed on the second flash at low pH is probably due to a double hit process.

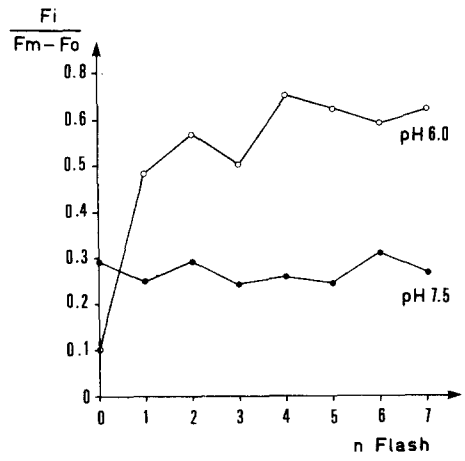

Fig. 4. Initial fluorescence yield (F_i) after addition of DCMU as a function of the number of preilluminating flashes at pH 6.0 and 7.5. F_i is normalised to the variable fluorescence ($F_{maximum} - F_o$, fluorescence after no flashes).

TABLE I

OXYGEN YIELD ON THE SECOND FLASH AS A FUNCTION OF FLASH LENGTH

First flash	Y_2/Y_3	
	pH 6.0	pH 7.5
0.5 μs laser	0	0
One 2 μs flash	0.15	—
Two simultaneous 2 μs flashes *	0.28	0.12

* The effective time during which the flash is saturating is increased with the simultaneous flashes. The remainder of the sequence is measured using 2 μs flashes; note that in all cases the first flash is saturating.

Velthuys and Kok [5] detected double hits in ferricyanide-treated chloroplasts by varying the intensity of the first flash of a sequence of flashes. They followed the oxygen yield on the second (Y_2) or third (Y_3) flash as a function of the intensity of the first flash. With S_2 present Y_2 and Y_3 plotted as a function of flash intensity should follow the same curve. If a double hit occurs on the first flash, this double hit will disappear at low flash intensity and Y_2 as a function of flash intensity will show a lag which is not seen for Y_3.

We have used the same technique and found a lag for Y_2 as a function of the intensity of the first flash, but not for Y_3. This lag was only observed at pH 6.0 and not at pH 7.5. This confirms that a double hit occurs on the first flash, and leads us to measure the turnover times for the system II centers.

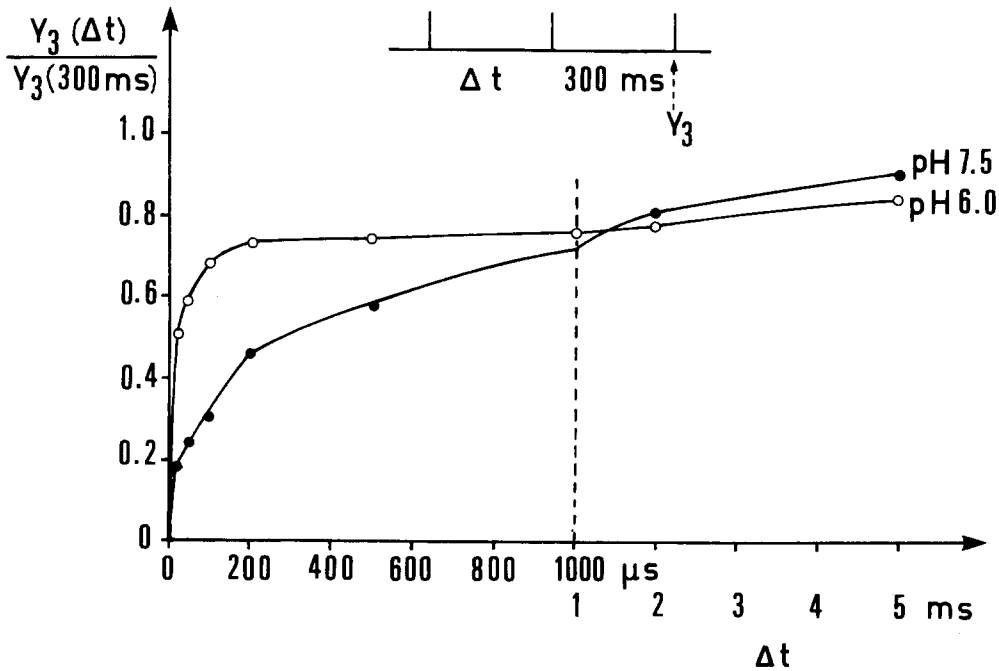

Fig. 5. Turnover of S_2 at pH 6.0 and 7.5. A preilluminating flash is followed by a short variable dark-time, Δt, and oxygen is detected on the third flash (insert). Note the two time-scales.

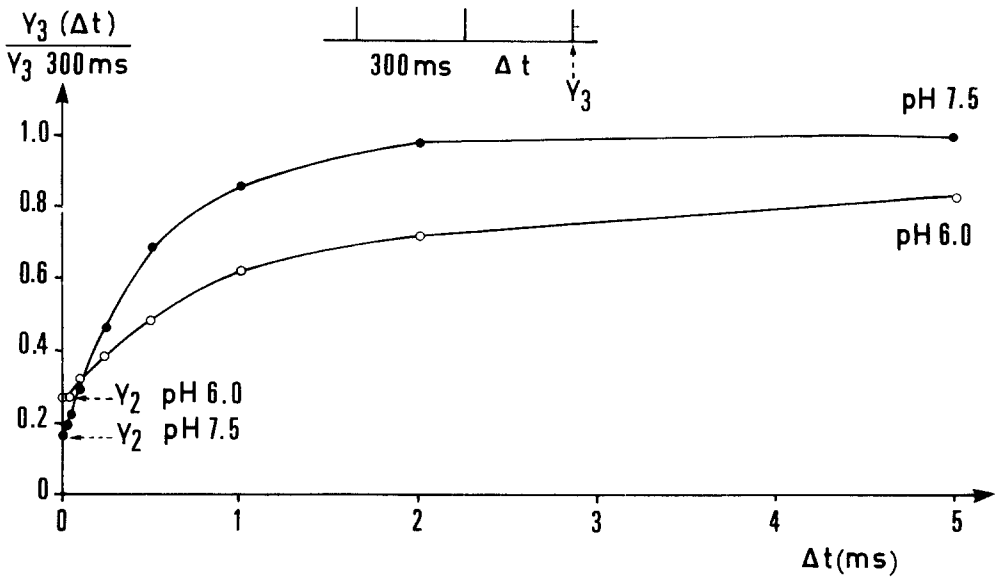

Fig. 6. Turnover of S_3 at pH 6.0 and 7.5. Two preilluminating flashes are given followed by a short variable dark time, Δt, and oxygen is detected on the third flash (insert). For $\Delta t = 0$, the value of Y_2 is obtained, as shown.

Turnover measurements

The turnover is measured by giving 1 or 2, 2 μs preilluminating flashes followed by a short variable dark time (20 μs—300 ms) and a train of flashes spaced by 300 ms. The oxygen yield is measured on the third flash. This allows us to follow the reopening of the Photosystem II centers, i.e. to follow what proportion of centers is able to detect the two closely spaced flashes as two photochemical acts. Figs. 5 and 6 show the curves for the turnover after 1 or 2 flashes, respectively. As Fig. 5 shows, after one flash the turnover at pH 6.0 has a rapid rise followed by a slow phase. It must be noted that the preilluminating flash is a 2 μs flash and thus has already induced some double hits. This implies that the fast phase of the turnover is probably under-estimated. After one flash 70% of the centers are able to reopen with $t_{1/2} < 25$ μs; then during a 2 μs flash a number of centers are able to undergo a double hit. At pH 7.5 the oxygen yield increases regularly with time between the first and second flash $t_{1/2} \simeq$ 300 μs which is of the same order of magnitude as for untreated algae, and corresponds to that detected via the 320 nm absorption change [25]. At pH 7.5 it is the acceptor side which is limiting. After 2 flashes (Fig. 6) the two curves are similar, with halftimes of 300 μs for pH 7.5 and 700 μs for pH 6.0. These values are also in accordance with those obtained from the decay of the 320 nm absorption change. This confirms our hypothesis of a double hit occurring only on the first flash.

Discussion

Evidence is presented in this paper for the occurrence of double hits in glutaraldehyde-treated algae at pH 6.0.

It is shown that the high oxygen signal on the second flash observed at pH 6.0 is not due to the presence of S_2 in the dark. Unequivocal proof for the absence of S_2 and the occurrence of double hits on the first flash is provided by the use of a 0.5 μs flash. In this case no oxygen is detected on the second flash.

For a double hit to take place it is necessary to postulate two acceptors, if we assume that there is no cooperation between centers for charge accumulation on the oxygen side. This leads us to examine the possible relations between these two acceptors and *P*-680.

The limiting step is not on the donor side because, as previously shown [8], the reduction of P^+ by the first secondary donor takes place with a halftime of 30 ns. Our results indicate that a 0.5 μs flash is not sufficiently long to induce double hits. Thus a scheme where the two acceptors are connected in parallel to the same primary donor is not possible.

$$D \rightarrow P \begin{cases} \nearrow Q_1 \\ \searrow Q_{aux} \end{cases}$$

At pH 6.0 we observed that the majority of centers have a turnover after one preilluminating flash with a half-time less than 25 μs. These results are in apparent contradiction with those obtained by Diner and Joliot [26] for chloroplasts. They see a slowing of the turnover after one preilluminating flash as the pH is lowered. However, they also used long Xenon flashes, able to induce double hits, so it is probable that they only detected the slow phase of the turnover. Our turnover results lead us to propose the following scheme where Q_1 is oxidized by Q_{aux} (rate constant $3 \cdot 10^4\ s^{-1}$) in competition with B:

$$D - P \rightarrow Q_1 \begin{cases} \rightarrow B \\ \searrow Q_{aux} \end{cases}$$

This scheme can also take into account the results obtained from the measurements of the initial fluorescence level (F_i) after DCMU addition (Fig. 4). At pH 7.5, as in whole *Chlorella*, the oscillations of F_i with flash number are weak, indicative of equal concentrations of B and B^-. At pH 6.0 the F_i level with no preilluminating flashes is very low and may be explained by the absence of B^- in the dark. The disappearance of B^- is a consequence of the presence of Q_{aux} leading to the following equilibria:

$$B^-Q_1Q_{aux} \rightleftharpoons BQ_1^-Q_{aux} \rightleftharpoons BQ_1Q_{aux}^- \overset{\text{slow}}{\rightleftharpoons} BQ_1Q_{aux}$$

The dark incubation of the algae before the measurements is sufficiently long (10 min) for Q_{aux} to be reoxidised [6,11]. Thus after 1 flash Q_{aux} and B are in competition for the charges on Q_1 and this explains why, at pH 6.0, the F_i level is lower after 1 than after 2 flashes. Usually a high F_i yield is seen on the first flash, under conditions where the dark concentrations of B is high, followed by sustained oscillations [24].

From the pH dependency of the double hits it can be inferred that the relative potential of Q_{aux} compared with Q_1 and B has increased, i.e. becomes

more positive, as the pH is lowered. In this case the Q_{aux} detected here at pH 6.0 is similar to, or even the same as the second acceptor postulated by Bowes et al. [11] and Kok and Velthuys [4]. Its potential at pH 7.5 will be such that there is almost no competition with B for accepting electrons from the Photosystem II center, and thus must be lower than that of Q_1.

To detect the auxiliary acceptor in isolated chloroplasts it is necessary to add oxidants such as ferricyanide, which is not necessary in algae. Thus it is probable that the internal environment of *Chlorella* is more oxidising than that of chloroplasts. Furthermore the proportion of double hits in untreated algae is low and in glutaraldehyde-treated algae only seen at $pH < 7.0$. This favours the internal pH of *Chlorella* in the dark as being $\geqslant 7.0$.

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