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DOUBLE PHOTOREACTIONS INDUCED BY A LASER FLASH AS MEASURED BY OXYGEN EMISSION

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When dark-adapted spinach chloroplasts are illuminated by an oversaturating laser pulse (total duration less than 150 ns) a double advancement in the S-states ($S_1 \rightarrow S_3$) is observed, as revealed by the relatively high oxygen yield measured after a second flash. The oxygen evolved on the second (Y_2) and the third (Y_3) flashes of a series has been measured as a function of the energy of a first laser pulse; the half-saturating energy for Y_2 is about 7-times higher than that for Y_3. This high energy requirement for Y_2 shows that double photoreactions occur during the course of the first laser pulse. Experiments with double laser pulses show that for a fraction of the centers, the turnover time is limited by a submicrosecond dark step, which could conceivably be the reduction of photo-oxidized chlorophyll by a secondary donor. Under the same experimental conditions, *Chlorella* cells do not undergo double photoreactions upon illumination by a laser pulse. The absence of such efficient double photoreactions has already been reported (Jursinic, P. (1981) Biochim. Biophys. Acta 635, 38–52) using *Chlorella* or pea chloroplasts. In the presence of a saturating concentration of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (20 μM DCMU), chloroplasts are able to evolve oxygen with a maximum on the second flash. The inhibition is 89% for the second and close to 99% for the third flash. These results indicate that two electron-acceptot sites are accessible during the course of a laser pulse. The probability of charge stabilization for the second photoreaction always remains low in chloroplasts and is close to zero for *Chlorella* cells.

Introduction

The oscillations of period 4 in oxygen emission [1] which are observed when photosynthetic material is illuminated by a series of saturating flashes have been interpreted by Kok et al. [2] by assuming five states of oxidation (S-states) for the PS II electron donors. A small amount of oxygen is generally evolved on the second flash of the sequence which indicates that the first flash induced double advancement in the S-states ('double hits' according to the terminology of Kok et al. [2]. The simplest interpretation is to assume that these double hits occur when the total duration of the flash (including the tail) becomes significant when compared to the reoxidation time of the primary PS II electron acceptor Q^- ($t_{1/2} \approx 600$ μs [3,4]). However, this hypothesis does not explain why the amount of oxygen evolved on the second flash varies with different biological material while it does not seem to correlate with a variation of the reoxidation time of Q^-. Maison-Peteri [5] reported that the oxygen yield on the second flash increases when the pH is decreased, which she explains by a drastic decrease in the reactivation time of PS II centers.

Studies of the efficiency of double photoreactions upon illumination of photosynthetic material by saturating flashes have yielded contradictory results. On the basis of fluorescence experiments, we previously reported [6] that, even in the presence of

Abbreviations: PS, photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

DCMU, oversaturating Xenon flashes could induce double photoreactions in PS II centers which might suggest that PS II centers include two electron acceptors Q_1 and Q_2. No significant double advancement in the S-states induced by the first flash of a series was observed either by Diner [7] using chloroplasts or by Delrieu [8] using *Chlorella* cells. Jursinic [9] measured a rather large emission of oxygen on the second flash both on *Chlorella* and fresh pea chloroplasts but excluded the possibility of double photoreactions during the first flash. Finally, we recently reported [10] oxygen emission on the second flash given to freeze-thawed spinach chloroplasts in the presence of saturating concentrations of DCMU. This seems to indicate that, at least for a fraction of the centers, two electron-acceptor sites must be present. These contradictory data led us to reinvestigate this problem using both *Chlorella* cells and chloroplasts illuminated by short laser pulses.

Material and Methods

Chloroplasts were isolated from market spinach as follows: 50 g of leaves were cut into small pieces and put into the bowl of a blender (Turmix). 100 ml of chilled homogenizing medium. (0.4 M sucrose, 0.05 M Tris, 0.01 M NaCl, pH 7.5) were added and the homogenizer run for a few seconds at high speed and a few seconds at low speed (total duration less than 15 s). The slurry was squeezed through several layers of gauze and centrifuged at 0–2°C for 7 min at $4000 \times g$. The pellet was resuspended in a small volume of homogenizing medium and stored at −70°C in the presence of 1% serum albumin and 5% dimethyl sulfoxide. Prior to use, chloroplasts were suspended in 0.05 M phosphate buffer, pH 6.5, with 0.1 M sucrose and 0.05 M KCl and maintained in the dark at 0°C. Experiments were performed at room temperature. *Chlorella pyrenoidosa* was grown under white fluorescent light (approx. 3000 lx), on Knop medium containing the trace elements A5 and B6 of Arnon [11]. The cells were used in 0.05 M phosphate buffer, pH 6.5, with 0.05 M KCl.

Amperometric titration of oxygen was performed with an apparatus described in Ref. 12 and modified as follows: the apparatus includes a single membrane and the thickness of the buffer compartment has been reduced to 3 mm. The thickness of the compartment bounded by the platinum electrode and the dialysis membrane has been reduced to 0.06 mm in order to decrease the settling time of the biological material. Chloroplasts were used at a concentration of 800 μg Chl/ml and algae at approx. 600 μg Chl/ml. The material can be placed on the electrode in total darkness. Experiments were performed after 8 min settling on the electrode.

The electrode is illuminated through a Y-shaped fiber optic guide (Schott) of which the common output section is rectangular and of the same size as the electrode. In the common output window, the optical fibers are randomly distributed, which allows homogeneous illumination of the biological material. One of the arms of the light guide is used to illuminate the sample (by Xenon or laser flashes). The amount of oxygen evolved is determined by calculating the integral of the amperometric signal from 4.6 to 16 ms after each flash (integration of 20 digitized samples).

The duration of the xenon flash (General Radio, Stroboslave 1539A) is about 3 μs at half-height; 70% of the energy is emitted in less than 25 μs. The duration of the laser flash (Ruby laser, System 2000, JK Lasers; 694 nm, 300 mJ) is close to 30 ns at half-height; 99% of the energy is emitted in less than 150 ns. The laser pulse can be attenuated by stainless-steel screens (Buckee Mears Co.) and its energy is measured, in relative units, by collecting the light reflected by the electrode and then transmitted to a photocell via the other arm of the Y-shaped optical guide. Double laser pulses can be generated by opening the Q-switch twice during the same pumping flash. A small fraction of the light emitted by the pumping flash is also collected by the light guide and illuminates the biological material. This parasitic illumination, which lasts several hundred microseconds, can induce double photoreactions when superimposed on the laser pulse. The actinic effect of this parasitic light was measured when the pumping flash is fired without opening the Q-switch, which prevents the laser emission. Curve 2 in Fig. 3A and B and curve 2 in Fig. 5A and B have been corrected by subtracting the double hits induced by this parasitic light. This correction, significant only for the highest energies of the laser pulse, never exceeds $0.01 Y_{ss}$.

The energy of the different flashes has been expressed in terms of the average number of absorbed

photons per PS II center (hν/center) calculated on the basis of the single-hit saturation curve. Although this measurement is only approximate, it permits us to compare the energy of flashes of different durations and wavelengths.

Results

Chloroplasts

Oxygen sequences as a function of duration and energy of the first flash. Chloroplasts suspended in buffer were dark-adapted for several hours at 0°C. For each measurement, a new sample was placed on the electrode without any preillumination and allowed to settle for 8 min. Fig. 1 shows the oxygen emission sequence upon illumination of chloroplasts by a series of saturating xenon flashes for different duration and energy of the first flash. Y_n is the amount of oxygen evolved by the nth flash of the series and Y_{ss} is the stationary value of the oxygen emission. The experimental values have been corrected by subtracting the small negative artifact induced by the xenon flash; the validity of this correction will be discussed below. An increase in the energy of the first laser flash (from 9 to 900 photons/center, Fig. 1) induced an increase in Y_2 and a significant decrease in Y_3. A slight advance of phase was also observed for the following flashes of the sequence (see Y_4 and Y_5). This increase in Y_2 and the subsequent decrease in Y_3 strongly favor the hypothesis that a strong laser flash induces double advancement of the S-states. It must also be noted that the sequences measured after a first oversaturating laser flash or after a weaker xenon flash are of the same order of magnitude.

We observed a significant increase in the amount of oxygen evolved on the second flash as a function of the time of dark adaptation at 0°C: for chloroplasts used in the experiment of Fig. 1, Y_2/Y_{ss} underwent a 30% increase when the dark-adaptation time at 0°C was varied from 30 min to 7 h, while Y_{ss} remained constant.

We must stress that we observed much larger values of Y_2/Y_{ss} than those measured by Jursinic [9] on frozen pea chloroplasts. This author observed practically no oxygen emission on the second flash when the chloroplasts were illuminated by a 5 or 300 ns laser flash. We thus conclude that, depending upon the type of material or its pretreatment, the oxygen

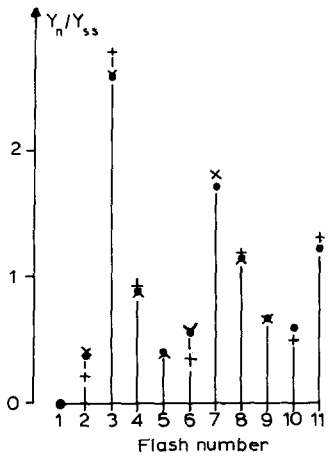


Fig. 1. Oxygen evolved by chloroplasts upon illumination by a series of saturating flashes 400 ms apart. Yn, oxygen evolved on the nth flash; Y_{ss}, stationary level of oxygen emission. The first flash of different energies and durations is followed by a series of xenon flashes (9 photons/center). First flash: (•) laser flash, 900 photons/center; (+) laser flash, 13 photons/center and (x) xenon flash, 9 photons/center. After thawing and diluting, chloroplasts were dark adapted at 0°C for about 160 min.

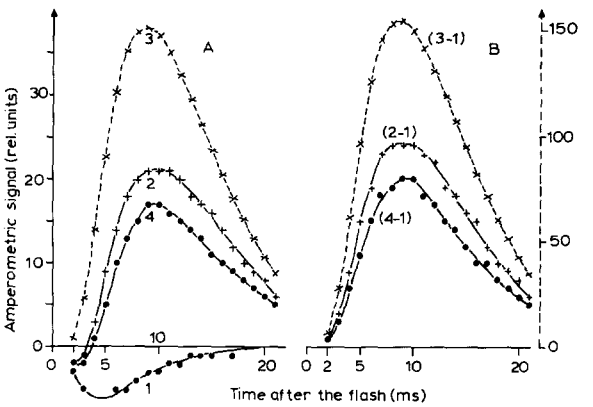


Fig. 2. (A) Time course of the amperometric signal after illumination of chloroplasts by a saturating xenon flash. Curves 1, 2 and 3 give the amperometric signals after the first, second and third flashes, respectively, of a series 400 ms apart. Curve 4: amperometric signal after a xenon flash given to chloroplasts preilluminated by a laser pulse (about 340 photons/center). (B) Time course of the amperometric signal due to oxygen, calculated after subtraction of the photoelectric artifact (A, curve 1) from curves 2–4 (A). Note that dashed curves 3 and (3-1) refer to the right-hand ordinate.

production on the second flash varies widely.

Fig. 2A shows the amperometric signal as a function of the time after a saturing xenon flash under different experimental conditions (see figure legend). When the flash is given to dark-adapted material (curve 1), the signal is principally due to a photoelectric artifact caused by the illumination of the electrode. Fig. 2B shows the amperometric signal when this photoelectric artifact has been subtracted. One of the parameters which determines the wave form is the rate of the limiting step which leads to oxygen formation from the primary photoproducts. After correction, the same wave form is observed for the three curves in Fig. 2B, which means that the same limiting step on oxygen formation is involved, independent of the number or type of preilluminating flashes. The fact that the wave forms shown in Fig. 2B are identical proves that the amplitude of the photoelectric artifact does not vary appreciably during the sequence. This result justifies a posteriori the method used to correct the sequences for the photoelectric artifact in the experiment of Fig. 1.

Absence of the S_2-state in dark-adapted material. Table I shows the oxygen evolved for the first and the second flash of a series of xenon flashes for three different energies of these flashes. For nonsaturating xenon flashes, Y_2/Y_{ss} (which is proportional to the concentration of S_3 formed by the first flash) decreased by a factor of 5 when the flash energy is halved. If the small amount of oxygen evolved by the second flash were linked to the presence of S_2 in the dark-adapted material, we would expect a decrease in Y_2/Y_{ss} proportional to the decrease in flash energy. The quadratic decrease in Y_2/Y_{ss} as a function of flash energy indicates that the concentration of S_2 in the dark is negligible. On the other hand, a simplified mathematical analysis of the sequences reported in Table I indicates that a large majority of the centers are in the S_1-state. This conclusion agrees with the pattern of sequences (Fig. 1) in which the value of Y_4 is particularly low.

TABLE I

Oxygen evolved by dark-adapted chloroplasts after illumination by the second (Y_2/Y_{ss}) or third (Y_3/Y_{ss}) flash of a series of xenon flashes of different energies 400 ms apart

Energy of the flashes (photons/center)	Y_2/Y_{ss}	Y_3/Y_{ss}
9	0.390	2.5
0.4–0.5	0.020	0.471
0.2	0.004	0.121

Saturation curves. In Fig. 3, dark-adapted chloroplasts were illuminated by a series of flashes 400 ms apart. The first flash (ruby laser) was of different energies and was followed by a series of nonsaturating xenon flashes (about 0.4 photons/center). Contrary to the experiment of Fig. 1, relatively weak xenon flashes were used to minimize the probability of the double hits they could induce. Actually, the values of Y_2/Y_{ss} observed here are lower than those obtained in the experiment of Fig. 1 for the same energy of the laser flash. Y_3/Y_{ss} (curve 1) is proportional to the number of centers initially in the S_1-state which have stabilized one charge after the laser flash (single-hit saturation curve). For energies higher than 5–10 photons/center, Y_3/Y_{ss} becomes independent of the flash energy. Y_2/Y_{ss} (curve 2) displays a biphasic increase as a function of the laser flash energy. For the first phase of curve 2, the half-saturating energy is about 7-times higher than that for curve 1. This high

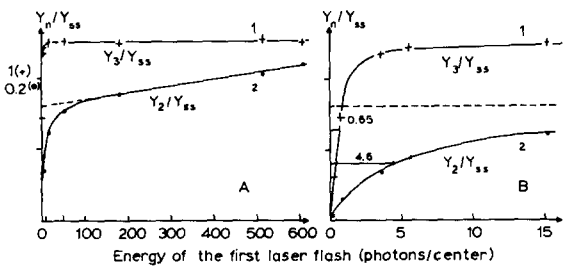


Fig. 3. Oxygen evolved on the second (Y_2/Y_{ss}, 2) or third (Y_3/Y_{ss}, 1) flash of a series of nonsaturating green xenon flashes given 400 ms apart to chloroplasts, as a function of the energy of a first laser flash. Xenon flashes were filtered through Wratten filter No. 58. To take into account the progressive increase in Y_2 as a function of the dark incubation time at 0°C, the plotted value for each point is the average of two measurements performed in two independent experiments with two batches from the same chloroplast preparation. The first experiment has been performed from the high to the low values of the laser flash energy, and in the opposite way in the second experiment. The average incubation time for each point is about 170 min. The dashed line gives the extrapolation to zero energy of the linear increasing phase of Y_2/Y_{ss} observed in the high energy range. The half-saturating energies for Y_3/Y_{ss} and Y_2/Y_{ss} are 0.65 and 4.6 photons/center, respectively.

energy requirement for Y_2 suggests that double photoreactions occur during the laser flash. Two charges can be stabilized on the S-state system during the dark period which follows the laser flash for a fraction of the centers (about 10%). These double photoreactions imply that two sites which can accept electrons and two sites which can donate electrons are available during the duration of the flash (30 ns at half-height). The high energy required to observe efficient double photoreactions (Fig. 3, curve 2) suggests that a dark limiting step of which the half-time is close to the duration of the flash takes place between the two photoreactions. An increase in the effective duration of the pulse due to the 'tail' of the flash corresponds to an increase in the energy of the laser flash. This increase in the duration of the flash compared to the time constant of a limiting step could explain the high energy requirement observed for Y_2. We compared the values of Y_2 after illumination by a single laser flash and after two laser flashes 1 μs apart, a negligible interval when compared to the reoxidation time of Q^- ($t_{1/2} \approx 600$ μs). The sum of the energies of these two laser flashes is adjusted to equal the energy of the single laser flash (about 5 photons/center). This energy is just sufficient to reach the saturation of the single-hit process. The values of Y_2/Y_{ss} for the single and the double laser flashes are 0.094 and 0.192, respectively: this large stimulation (more than a factor of 2) indicates that there is a limiting step in the submicrosecond range. This limiting step could be the reduction of the photooxidized chlorophyll of which the half-time is about 30 ns [13,14] on dark-adapted material. It remains difficult to understand why the Y_2/Y_{ss} curve (Fig. 3) does not display a lag for the low flash energies, which would indicates an I^2 dependency on the energy of the flash. In this low energy range, we are too close to the limit of resolution of our techniques to draw any definite conclusion regarding this point.

The slow increasing phase observed for energy of the laser flash higher than 100 photons/center, which does not seem to saturate, remains difficult to interpret. As already discussed in Material and Methods, we can exclude the possibility that this phase is due to the light emitted during the long pumping flash. We cannot exclude the possibility of a double oxidation of the photoactive chlorophyll (or the oxidation of a second chlorophyll close to the center), the probability of this second photoxidation being extremely low.

Oxygen formation in the presence of DCMU. Diner [15] has studied the production of oxygen by chloroplasts illuminated by a series of saturating xenon flashes, in the presence of non saturating concentrations of DCMU. In the presence of 1 μM DCMU, Diner reported abnormally high values for Y_2/Y_{ss}, which suggests that efficient double hits occur on the first flash. We reported recently [10] that chloroplasts were able to emit oxygen even in the presence of an oversaturating concentration of DCMU (20 μM). This ability depends upon the type and state of the chloroplasts studied.

Fig. 4 shows the oxygen emitted along a series of saturating xenon flashes in the absence or presence of DCMU. Sequence 2, observed in the presence of a nonsaturating concentration of DCMU (0.8 μM), dis-

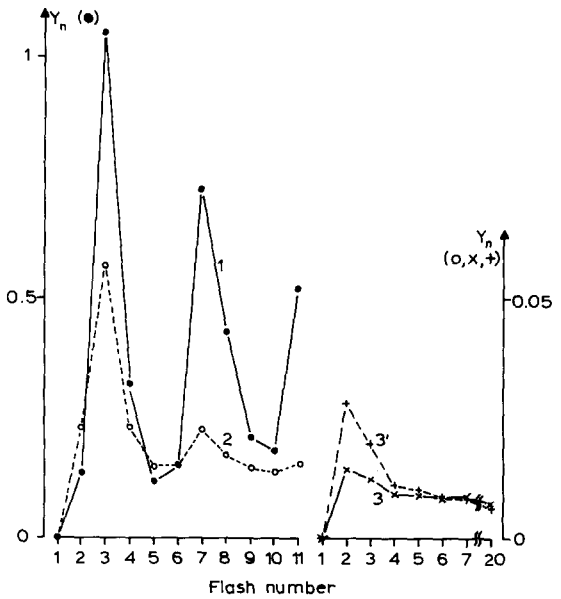


Fig. 4. Oxygen evolved by a series of saturating xenon flashes (6 photons/center) 400 ms apart in the absence (left ordinate) or presence (right ordinate) of DCMU. 1, control (●———●); 2, 0.8 μM DCMU (o- - - - -o); 3, 20 μM DCMU, dark-adapted chloroplasts (x———x); 3' 20 μM DCMU, 23 min dark adaptation after sequence (+- - - - -+). For sequences 2, 3 and 3', the signals, measured using chloroplasts of which the S-state system has been inactivated by treatment with hydroxylamine (see text), have been subtracted from the signals measured using chloroplasts of which the donors have been left intact.

plays oscillations of period 4, although the inhibition reaches 95%. This sequence is similar to that published by Diner [7]. One must note particularly the high value of Y_2/Y_{ss}. The large damping of the oscillations is most likely due to the life time of the noninhibited chains (1–2 s) which is a function of the turnover time of DCMU. The turnover of DCMU also explains the relatively low value of Y_{ss} observed in the presence of a nonsaturating concentration of DCMU. We would like to stress that the phase of the oscillations remains practically identical both in the presence and absence of DCMU. This result suggests that, for the small fraction of noninhibited electron-transfer chains, a normal rate of double hits is induced by the first flash. It is very likely that the oxygen evolved on the second flash arises from the already inhibited chains. This hypothesis is favored by the sequences observed in the presence of saturating concentration of DCMU (sequences 3 and 3'): sequence 3 does not display any oscillations of period 4 and maximum oxygen production occurred on the second flash, findings we have already described in Ref. 10. A maximum on the second flash was observed for energies of the xenon flashes higher than 3 photons/center. The large difference between sequences 3 and 2 shows that the oxygen emission observed in the presence of a saturating concentration of DCMU cannot be explained by the presence of a few DCMU-insensitive electron-transfer chains. Sequence 2 in Fig. 4 can be described as the sum of an oscillating sequence linked to the noninhibited centers and a nonoscillating sequence (as sequence 3, Fig. 4) associated with the inhibited centers.

We verified that the oxygen emission in sequence 3 is actually associated with the S-state system: the same chloroplasts were incubated for 30 min in the dark in the presence of 4 mM hydroxylamine, in order to inactivate totally the donor chain involved in the water-splitting process. The chloroplasts were then placed on the electrode and the hydroxylamine was completely washed out by circulating a buffer containing 20 μM DCMU. Upon illumination by a series of xenon flashes, these chloroplasts did not evolve any oxygen. The negative signal observed is essentially due to the photoelectric artifact and a small part of its is due to a slight PS I-sensitized oxygen absorption. Sequences 2, 3 and 3' in Fig. 4 have been corrected for this negative signal. We also verified that after this correction, the wave form is the same in the presence of DCMU as in its absence. Sequence 3', measured after a 23 min dark period following sequence 3, shows that the chloroplasts' ability to evolve oxygen in the presence of DCMU is not irreversibly destroyed after a first illumination. We even observed a significant increase in the oxygen evolved on the second and third flashes of the sequence. We also observed that, as in the experiment of Fig. 1, the value of Y_2 measured after a short oversaturating laser flash is of the same order of magnitude as that after a xenon flash (data not shown). It is thus very likely that the same type of double-hit process occurs either in the absence of presence of DCMU.

The fact that chloroplasts which are in the S_1-state are able to evolve oxygen on the second flash even in the presence of DCMU implies that, at least for a fraction of the centers, three electrons can be stabilized on the acceptor side of PS II during the two first flashes of the series. A tentative explanation is proposed below.

Chlorella cells

We performed on *Chlorella* the same experiments as that shown in Fig. 3 using chloroplasts. In Fig. 5, *Chlorella* cells were preilluminated by a first laser flash of variable energy followed by a series of nonsaturating xenon flashes (0.6 photons/center) 300 ms apart. In order to increase the initial ratio S_1/S_0 [2,16], the algae were preilluminated by a series of flashes, dark adapted for 90 s, illuminated by a single flash and then dark adapted for 5 min. As in Fig. 3, Y_3/Y_{ss} (curve 1) becomes independent of the energy

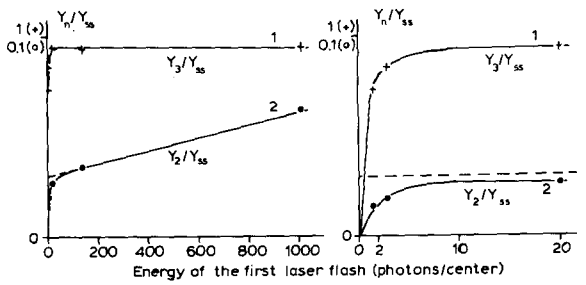


Fig. 5. Oxygen evolved on the second (Y_2/Y_{ss}, 2) or third (Y_3/Y_{ss}, 1) flash of a series of nonsaturating green xenon flashes given 300 ms apart to *Chlorella* cells, as a function of the energy of a first laser flash. Xenon flashes are filtered through a Wratten filter No. 58.

of the laser flash for energies higher than 10 photons/center, and Y_2/Y_{ss} (curve 2) displays a biphasic increase. However, two main differences in the behaviour of chloroplasts and algae in the low energy range must be noted:

(1) the values of Y_2/Y_{ss} are 4–5-times lower in algae than in chloroplasts and are also lower than those reported by Jursinic [9] for *Chlorella* cells illuminated by a 5 or 300 ns laser flash;

(2) in the low energy range, the dependence of Y_2/Y_{ss} and Y_3/Y_{ss} on the energy of the flash is quite similar. On the other hand, the values of Y_2 measured after a single laser flash or after a group of two laser flashes 1 μs apart are not significantly different. These two results show that in *Chlorella*, unlike in chloroplasts the oxygen formation on the second flash depends upon a single-hit process and does not involve a limiting turnover step. Jursinic [9] concluded similarly, since he obtained similar values for Y_2/Y_{ss} after a 5 or 300 ns laser pulse. To interpret his data, Jursinic postulates the existence of a side carrier C (already proposed by Lavorel and Lemasson [17]) which could exchange charges with the S-states. To interpret both our data and those of Jursinic, one must moreover assume that this side carrier could exchange charges between different electron-transfer chains. Another possible interpretation is to assume the presence of a small amount of S_2 in the dark-adapted *Chlorella*. In any case, the low values of Y_2, close to the limit of resolution of our techniques, do not permit us to draw a final conclusion.

The slow increasing phase observed in the high energy range of the laser flash appears to be similar to that observed in chloroplasts.

Discussion

From our experiments on freeze-thawed chloroplasts we conclude that, following a short flash (less than 100 ns), two positive charges can be stabilized on the PS II donor side. The fraction of the PS II centers able to store these two charges depends upon the type of material studied, which explains the failure of several authors [7,8] to observe this type of double-hit process. The amount of oxygen evolved on the second flash depends (1) upon the fraction a of the centers which undergo a double photoreaction during the flash and (2) on the probability b of these centers stabilizing a second positive charge on the S-state system. Experimentally, we can only measure the product $a \times b$, which is proportional to Y_2. The probability a of the centers undergoing double photoreactions depends upon a dark limiting step which is most likely the reduction of the photooxidized chlorophyll by the secondary donor Y, and on the proportion of centers which include two sites able to accept the electrons during the course of the flash. We will discuss what might be the nature of this second acceptor, among those proposed in the literature, in addition to the main electron acceptor Q.

(1) We can exclude the secondary acceptor B [18] or R [19]: the electron transfer from Q^- to B is slow ($t_{1/2} \approx 600$ μs) and we did observe double hits in the presence of DCMU which blocks the $Q \rightarrow B$ reaction.

(2) Under our experimental conditions, the acceptor Q_{400} [20] or C_{400} [21,22] does not seem to be a good candidate: actually, our experiments were performed in the absence of any added oxidant and the chloroplasts settled on a negatively polarized platinum electrode. These conditions exclude the possibility of the presence of the oxidized form of a component the potentiel of which is about 450 mV at pH 6.5. Moreover, in the presence of DCMU, Q_{400} cannot be reoxidized even in the presence of ferricyanide: the sequence 3′ in Fig. 4 shows that the second acceptor site is reoxidized in less than 10 min. Finally, Jursinic [9] showed that the electron-transfer time from Q^- to Q_{400} is much longer than 300 ns.

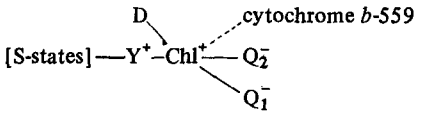
(3) We cannot exclude the possibility that Q, which is a quinone, can store two negative charges (Q^{2-} form). However, this hypothesis is most unlikely as we showed that a fraction of the second acceptor can be reoxidized in less than 400 ms even in the presence of DCMU.

(4) Klimov et al. [23] identified the pheophytin as an intermediary electron acceptor between the photoactive chlorophyll and Q. The very fast back reaction between the photooxidized chlorophyll and the reduced pheophytin ($t_{1/2} \approx 3$ ns [24]) excludes the possibility of stabilization of a negative charge on this component.

(5) Several independent experiments suggest the existence of an acceptor Q_2 [10,25,26] or X_a [27, 28]. This acceptor differs from the main acceptor Q

in its spectral properties (no C-550 signal [10,26], no 320 nm signal [28]) and in its localization on the inner face of the membrane [10,28]. We recently showed [10] that Q_2 is associated with a secondary acceptor, possibly cytochrome *b*-563, and that its reoxidation is DCMU insensitive. Q_1 and Q_2 could be connected to the photoactive chlorophyll either in parallel or in series. In the last case, the electron-transfer time between Q_2 and Q_1 should be much shorter than the duration of the laser pulse. Recent data of Lavergne and Etienne [29] also suggest the existence of two types of acceptors, only one of which would be connected to the secondary acceptor B.

One way of explaining the low probability of double advancement in the S-states is to assume that only a fraction of the centers include both Q_1 and Q_2 in a functional state. A second explanation, which does not exclude the first, is that the probability *b* of charge stabilization on the S-states is low. The following scheme shows the state of the centers which have undergone a double photoreaction:



Y is the secondary donor which transfers its electron to the photoactive chlorophyll (Chl) in less than 30 ns and is connected to the water-splitting system; D [14,30–32] and cytochrome *b*-559 [33] are secondary donors possibly connected in parallel with the photoactive chlorophyll. When Y is in its oxidized form, the probability of stabilizing a second charge on the S-state system will depend upon the relative values of the rate of reduction of Y^+ and of the rates of the reactions which lead to the reduction of photo-oxidized chlorophyll (i.e., electron transfer from D, cytochrome *b*-559 and back reactions between Chl^+ and Q_2^- or Q_1^-).

The presence of a low concentration of reducing substance (precursor of signal II_s [34], substance T [8]) able to compete with water to donate electrons could also decrease the oxygen yielded by the second flash. The increase in Y_2 we observed as a function of dark-adaptation time could correspond to a loss of a reducing substrate responsible for signal II_s.

Comparison of our results with those of Jursinic [9] shows that the probability of double advancement on the first flash is extremely variable depending upon the state and type of biological material studied. Among the parameters which could explain this variability are the relative rates of the dark steps involved in the charge stabilization process, the concentration of reducing substrates able to compete with water, and the fraction of centers including two acceptors.

Oxygen emission in the presence of DCMU. During the first flash, for a fraction of the centers, both Q_1 and Q_2 are reduced which leads to the formation of state S_3. During the dark interval between the first and second flash (400 ms), Q_2 is reoxidized by the secondary acceptor (cytochrome *b*-563?). Then, upon illumination by the second flash, a third charge can be transfered, which allows the formation of state S_4 and oxygen emission. In the presence of 20 μM DCMU, we observed a 90% inhibition for Y_2. This result suggests, as already proposed in Ref. 10, that the charge stabilization process is about 40-times less efficient for the reaction involving Q_2 than that involving Q_1.

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