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INVESTIGATION OF DOUBLE TURNOVERS IN PHOTOSYSTEM II CHARGE SEPARATION AND OXYGEN EVOLUTION WITH EXCITATION FLASHES OF DIFFERENT DURATION

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Summary

The characteristics of double hitting in Photosystem II charge separation and oxygen evolution in algae and chloroplasts were investigated with saturating excitation flashes of 3 μs, 300 ns and 5 ns duration. Two types of double hitting or advancement in S-states were found to occur in oxygen evolution: a non-photochemical type found even with 5 ns flashes and a photochemical type seen only with microsecond-long flashes, which have extensive tails. The non-photochemical type, occurring with a probability of about 3%, is sensitive to the physiological condition of the sample, and is only present in algae or chloroplast samples that have been freshly prepared. In chloroplasts incubated with ferricyanide, a 3-fold increase in double advancement of S-states is observed with xenon-flash illumination but not with 300 ns or 5 ns laser illumination. However, double turnovers in Photosystem II reaction center charge separation are large with xenon flash or 300 ns laser illumination but not with 5 ns laser illumination. This indicates that quite different kinetic processes are involved in double advancement in S-states for oxygen evolution and double turnovers in charge separation. Various models of the Photosystem II reaction center are discussed. Also, based on experiments with chloroplasts incubated with ferricyanide, an unique solution to the oxygen S-state distribution in the dark suggested by Thibault (Thibault, P. (1978) C.R. Acad. Sci. Paris 287, 725—728) can be rejected.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll.

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Introduction

Measurements of oxygen evolution from chloroplasts and algal cells that have been dark adapted for a few minutes and then illuminated by a series of 10 μs long saturating flashes [1] showed oxygen to be evolved in bursts that vary in magnitude with a damped oscillation of period four. Maximum yields occur on flash 3, 7, 11, etc. Kok et al. [2] proposed a four-step linear mechanism of positive charge accumulation that led to the discharge of molecular oxygen. The damping of the period-of-four oscillation was explained by Forbush et al. [3] by including in the model two photochemical parameters, α and β, which represent 'misses' and 'double hits', respectively. It was suggested [3] that misses occurred at reaction centers that did not receive a quantum and double hits at reaction centers that were able to accept a second quantum during the decay tail of a flash.

Various attempts were made to test this proposal for the origin of double hits or double advancement in S-states of oxygen evolution, but the data are inconclusive. With a 40 ns laser flash, without a tail on the microsecond scale, double advancement in S-states were observed (see Fig. 7 of Ref. 4 and discussion of this point by Weiss et al. [5]). However, these results were deficient since the laser flashes had to be given at low flashing rates (1 flash/15 s) or were of subsaturating intensity. Also, with a 3 μs xenon flash at intensities below saturation, Jursinic [6] observed significant double advancement in S-states. However, Joliot et al. [7], using a 2 μs xenon flash and Govindjee et al. [8] using a 600 ns laser flash, found no double advancement in S-states.

Recently, it has been reported that chemical means can be used to increase double advancements as evidenced by enhanced oxygen yields after flash number two. Low concentration of ferricyanide [9] or ferricyanide with 200 mM $MgCl_2$ [10] were conditions capable of causing increased double advancement in S-states.

In this report, an attempt is made to determine whether double advancements do occur under nanosecond flash illumination by observing the oxygen flash pattern, using either a xenon flash lamp, a 300 ns dye laser or a 5 ns dye laser for excitation. Objections to earlier laser experiments [4,5] were overcome here, since saturating laser flashes at a rate of 2 Hz could be provided. Also, a better understanding of the origin of misses and double advancements was pursued by observing the effects of ferricyanide on the oxygen flash-yield pattern and on chlorophyll *a* fluorescence yield changes.

Materials and Methods

Chloroplasts were isolated from leaves of dwarf peas (*Pisum sativum*) grown in the laboratory and harvested 10—14 days after germination. Isolation procedures were as previously reported [11]. *Chlorella pyrenoidosa* were grown under batch conditions as previously described [6] and were resuspended for experiments in the medium 20 mM Tris-HCl/40 mM NaCl, pH 7.0.

A rate electrode was used for making oxygen flash yield measurements as previously described [11]. Chloroplasts were applied to the electrode at a chlorophyll concentration of 0.5 mg/ml, and *Chlorella* at a chlorophyll concentra-

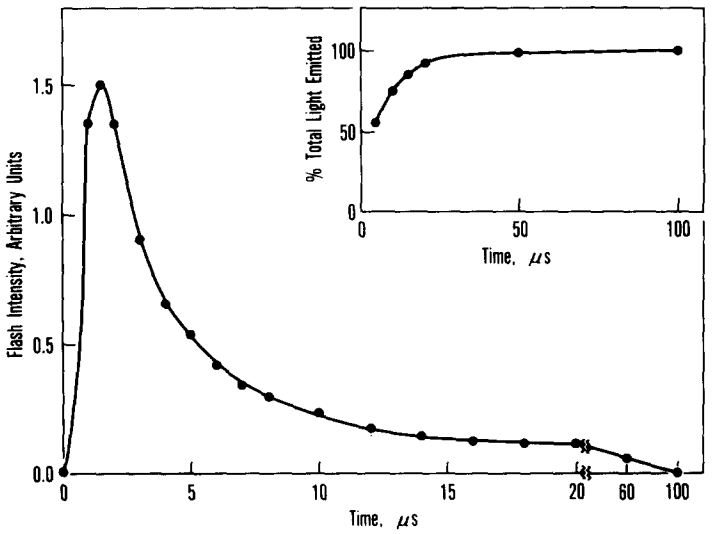


Fig. 1. Plot of the time dependence of the intensity of the xenon flash used for excitation in these experiments. As in the experiments, this measurement was made with no optical filter before the flash lamp. Inset: an integration of the flash time dependence curve showing the portion at any time of the total light emitted per pulse.

tion of 0.2 mg/ml, which corresponded to a single layer of cells on the platinum surface. When comparing oxygen evolution using different excitation flash sources, excitation intensities were adjusted with neutral density filters to be at approximately the same point above saturation on the light curve.

Flash excitation was provided by three different sources. A xenon strobe lamp (General Radio 1538-A), a dye laser (Phase-R model DL-1100) and the

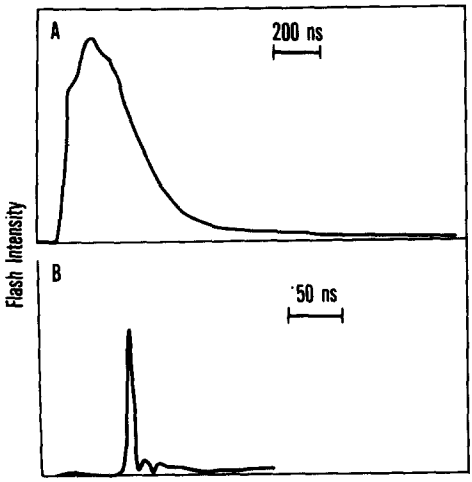


Fig. 2. Traces of the time dependence of the intensity of laser flashes used for excitation in these experiments. The laser pulse designated as 300 ns in the text is shown in (A) and as 5 ns is shown in (B).

same dye laser with a Phase-R model CD 700-4 cavity dumper. The dye laser was operated with Rhodamine-6G in ethanol, which has an output centered at 584 nm. Time profiles of the various flashes shown in Figs. 1 and 2 were measured with a reversed biased United Detector Technology PIN020A photodiode and Tektronix 7623A storage oscilloscope with a 7A18 amplifier and 7B50A time base. The longest duration flash is that of the xenon lamp (Fig. 1), which reaches its peak intensity by 1.5 μs, has a width at 1/3 height of 4.5 μs, and decays to 1/10 peak intensity by 12 μs. The inset of Fig. 1 shows the time dependence of the total light output of the xenon flash. Approx. 80% of the total light output has occurred by 1/10 peak intensity (12 μs).

The 300 ns dye laser flash intensity profile is shown in Fig. 2A and the 5 ns cavity dumped laser flash in Fig. 2B. The actual 5 ns laser rise and fall times, which should be in the order of 2.5 ns, are not directly observable in this figure due to limitations in measuring equipment. The rise time, which is equal to the fall time, of the combined system of photodiode, cable and oscilloscope amplifier is 8 ns. The rise time of Fig. 2B is approx. 8 ns; thus, it is certain that the laser profile has a rise and fall time more rapid than 8 ns. The 5 ns figure for the laser pulse width is expected on theoretical grounds based on the length of the laser optical cavity. The small signal following the 5 ns laser pulse in Fig. 2B is due to electrical noise pick-up by the detection circuit.

Fluorescence yield changes were measured with a conventional fluorimeter with the actinic source at right-angles to the photomultiplier (Hamamatsu R928) shielded with a Corning CS 2-64 glass filter. The actinic source consisted of an incandescent lamp powered by a voltage-regulated direct current supply. The lamp emission was focused by a lens and chopped at the focal point by an electronic shutter (Uniblitz model 23X2A0X5) with an opening time of 0.8 ms. The actinic light was filtered with Corning CS 4-96 and CS 3-71 glass filter. The photomultiplier output was recorded by a Biomation 805 waveform recorder and hard copies of the data were made by output to a chart recorder. Areas above fluorescence rise curves were measured by weighing tracings on an analytic balance. Pre-illumination flashes given before fluorescence measurements were supplied as indicated in the data by one of the above flash sources through a prism. Timing between the pre-illumination flash and shutter opening was controlled by laboratory-built pulse-delay circuits. All experiments were carried out at temperatures of about 20°C.

Calculation method for obtaining theoretical parameters for O_2 flash-yield patterns

The matrix-multiplication technique introduced by Delrieu [12] was used to calculate the miss (α), single (β) and double (γ) advancement in S-state probabilities and dark S-state distribution that correspond to a particular oxygen flash-yield pattern. The transition of the S-states on the n-th flash is given by the following:

$$|S^{(n)}> = K\,|S^{(n-1)}>$$

where $|S^{(n)}>$ is a column vector the elements of which are $S_0^{(n)}$, $S_1^{(n)}$, $S_2^{(n)}$, $S_3^{(n)}$, the S-state distribution after flash n. K is a matrix of which the elements

are the transition probabilities arranged as follows:

$$K = \begin{bmatrix} \alpha & 0 & \gamma & \beta \\ \beta & \alpha & 0 & \gamma \\ \gamma & \beta & \alpha & 0 \\ 0 & \gamma & \beta & \alpha \end{bmatrix}$$

presuming homogeneity of transition probabilities between S states. The yield after any flash n is calculated by:

$$Y_n = \beta S_3^{(n-1)} + \gamma S_2^{(n-1)}$$

and the steady-state yield is

$$Y_{ss} = (\beta + \gamma)(0.25) = (1 - \alpha)(0.25)$$

It is assumed that $S_3^{(0)} = 0$, initial values for α, γ, $S_0^{(0)}$ and $S_1^{(0)}$ are chosen and values for Y_n/Y_{ss} are calculated. The values of α, γ, $S_0^{(0)}$ and $S_1^{(0)}$ are varied by a computer program to minimize the sum of the squared deviations

$$E = \sum_{i=2}^{n} \{ Y_n/Y_{ss} \text{ (calculated)} - Y_n/Y_{ss} \text{ (experimental)} \}^2$$

This fitting procedure was done for $n = 12$ for the data presented here. Even though a set of transition parameters and dark S-state distribution can be found to give a good fit to the flash-yield data, other solutions exist [12,13]. The solutions shown for this data were found to give the minimum value for E.

The above method establishes values for α, β and γ with the assumption that the behavior of the S-state transitions is the same on all flashes. Recently, this assumption has been challenged [14,15] and extraordinarily different values for the transition probabilities have been suggested to occur on the first flash following dark adaptation. The possibility was accommodated in the present calculation method as follows. From the method described earlier, a K and an $|S^{(1)}>$ are found that describe the flash-yield pattern for many flashes. If the first flash transition probabilities are unique, then the following equation can be written:

$$|'S^{(1)}> = K' \, |'S^{(0)}>$$

where K' is a unique matrix for the first flash only, consisting of elements α', β', γ' different to α, β, γ. $|'S^{(0)}>$ represents a unique dark S-state distribution different to $|S^{(0)}>$. Initial guesses are made for α', γ', $'S_0^{(0)}$ and $'S_1^{(0)}$ and it is presumed that $'S_3^{(0)} = 0$. These parameters are then varied by a computer program to minimize the sum of the squared deviations

$$E = \sum_{i=0}^{3} (S_i^{(1)} - {}'S_i^{(1)})^2$$

In this way possible unique sets of transition probabilities (K') and dark S-state distributions ($|'S^{(0)}>$) that give good predictions of the oxygen flash-yield pattern ($|'S^{(1)}> = |S^{(1)}>$) can be found.

For completing the various minimization calculations required, a MODCOMP

II/25 computer was used with a general program for minimizing deviations involving a multi-variable function.

Results

Oxygen flash-yield patterns for Chlorella with different excitation flashes

In order to test the photochemical nature of the oxygen S-state transition parameters α, β, γ, the oxygen flash-yield pattern was observed with excitation from a xenon flash, a 300 ns laser and a 5 ns laser. Fig. 3 shows typical oxygen yield patterns that are observed. The yield on flash two, Y_2, is not zero with any of the excitation sources even after 15 min dark adaptation, which is sufficient time for decay of all S_2-states. These results show that double advancements occur even with a 5 ns excitation flash. Mathematical analysis of *Chlorella* oxygen flash-yield patterns in five separate measurements for each excitation type gave the average parameters shown in Table I, lines 1—3. With 300 ns and 5 ns laser excitation, the oxygen parameters are almost identical, while with the xenon flash, excitation misses are less probable and double advancements are more probable. This change in the miss parameter is puzzling, since the xenon and laser flashes were saturating. An explanation of this phenomenon was not pursued in this work. It is important to note that even under laser excitation, double advancement in S-states did not drop to zero but decreased from 6.6% to 4%.

Oxygen flash-yield patterns for chloroplasts with different excitation flashes

Freshly prepared chloroplasts excited with a xenon flash, 300 ns laser or 5 ns

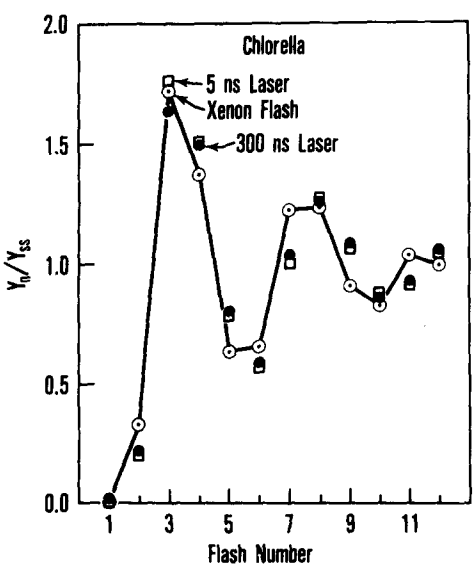


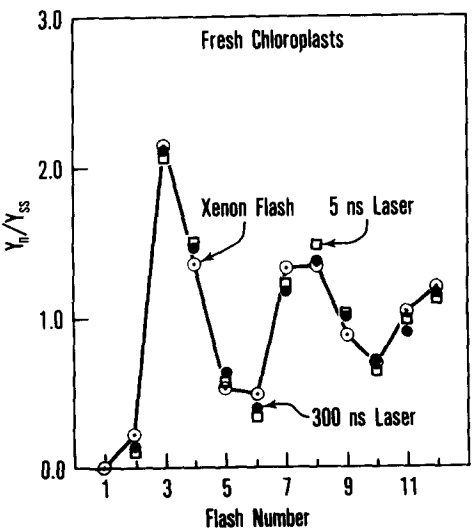
Fig. 3. Oxygen flash-yield as a function of flash number following 5 min dark adaptation. The oxygen yield on any flash, Y_n, is normalized by the steady-state yield, Y_{ss}, reached after many flashes. The sample was *Chlorella*, receiving excitation at a rate of 2 Hz from a xenon flash (○———○), a 300 ns laser (●———●) or a 5 ns laser (□———□).

TABLE I

OXYGEN EVOLUTION TRANSITION PARAMETERS FOR *CHLORELLA* AND FRESH AND FROZEN CHLOROPLASTS

Homogenous transition parameters for oxygen evolution S-states, calculated as described in the text from oxygen flash-yield patterns. The corresponding dark adapted S-state distribution is also shown. In all cases, $S_2^{(0)}$ was found to equal zero. These values are averages of at least five separate determinations of the oxygen flash-yield patterns as in Figs. 3—5, and standard deviations are indicated.

Line	Sample conditions	$\overline{\alpha}$	$\overline{\beta}$	$\overline{\gamma}$	$S_0^{(0)}$	$S_1^{(0)}$
1	*Chlorella* (xenon)	0.139 ± 0.029	0.795 ± 0.20	0.066 ± 0.012	0.33 ± 0.02	0.67 ± 0.02
2	*Chlorella* (300 ns laser)	0.197 ± 0.015	0.773 ± 0.024	0.040 ± 0.020	0.29 ± 0.03	0.71 ± 0.03
3	*Chlorella* (5 ns laser)	0.191 ± 0.018	0.769 ± 0.026	0.040 ± 0.013	0.30 ± 0.03	0.70 ± 0.03
4	Fresh chloroplasts (xenon)	0.136 ± 0.019	0.826 ± 0.010	0.038 ± 0.002	0.23 ± 0.02	0.77 ± 0.02
5	Fresh chloroplasts (300 ns laser)	0.162 ± 0.014	0.816 ± 0.018	0.022 ± 0.005	0.22 ± 0.01	0.78 ± 0.01
6	Fresh chloroplasts (5 ns laser)	0.150 ± 0.015	0.837 ± 0.017	0.017 ± 0.003	0.20 ± 0.02	0.80 ± 0.02
7	Frozen chloroplasts (xenon)	0.122 ± 0.016	0.812 ± 0.020	0.066 ± 0.003	0.27 ± 0.03	0.73 ± 0.03
8	Frozen chloroplasts (300 ns laser)	0.179 ± 0.018	0.817 ± 0.019	0.004 ± 0.003	0.25 ± 0.03	0.75 ± 0.03
9	Frozen chloroplasts (5 ns laser)	0.170 ± 0.020	0.826 ± 0.021	0.004 ± 0.003	0.27 ± 0.03	0.73 ± 0.03



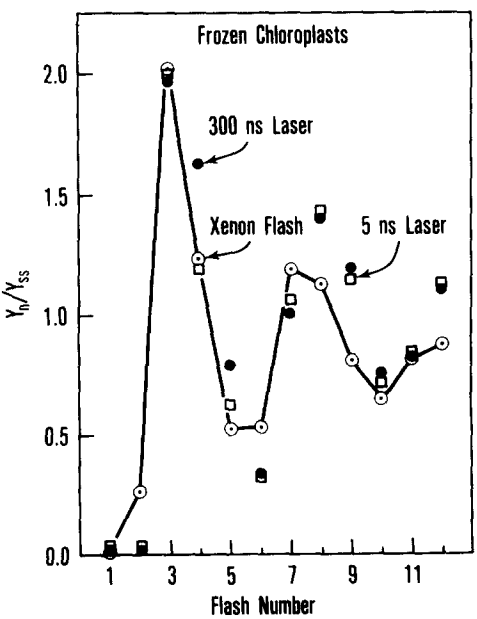


Fig. 4. Oxygen flash-yield as a function of flash number for freshly prepared chloroplasts. All other information is the same as for Fig. 3.

Fig. 5. Oxygen flash-yield as a function of flash number for chloroplasts that had been quick frozen in liquid nitrogen, stored for 10 days and rapidly thawed prior to the measurement. All other information is the same as for Fig. 3.

laser give oxygen flash-yield patterns as shown in Fig. 4. The 300 ns and 5 ns laser excitations give very similar patterns with non-zero yields on flash two. The yield on flash two is somewhat greater with xenon flash excitation. The average oxygen evolution transition parameters calculated from five separate measurements for each condition are listed on lines 4—6 in Table I. Compared to the xenon flash excitation, the laser excitation results in a reduced probability for double advancement in S-states and an increased probability for misses.

When the same type of experiment was done with chloroplasts that had been kept frozen in liquid nitrogen, freshly prepared chloroplasts that had been kept on ice for 5 h or longer, or chloroplasts prepared from market spinach, different results were observed. With these types of sample, excitation with 300 ns or 5 ns laser flashes gives yields on flash two of zero, while the xenon flash still gives non-zero yields on flash two. Freezing of chloroplasts or aging on ice did not seem to damage significantly the oxygen evolution apparatus, since steady-state yields were observed to be reduced by 10% or less. Typical oxygen flash-yield patterns are shown in Fig. 5. This is seen in the oxygen evolution transition parameters on lines 7, 8 and 9 of Table I as a 6.6% probability for double advancement in S-states with xenon flash excitation and essentially zero probability with laser excitation.

Ferricyanide effects on oxygen flash-yield patterns of chloroplasts with different excitation flashes

It has been previously reported [16,9] that ferricyanide could increase the oxygen yield on flash two and this has been interpreted [10] as an increase in double advancement in S-states only following the first flash. The effects of different excitation sources on the ferricyanide-induced double hitting were investigated.

TABLE II

OXYGEN EVOLUTION TRANSITION PARAMETERS FOR FRESH CHLOROPLASTS WITH AND WITHOUT FERRICYANIDE PRESENT

Homogeneous transition parameters for oxygen evolution S-states, calculated as described in the text from oxygen flash-yield patterns. The corresponding dark adapted S-state distribution is also shown. In all cases, $S_2^{(0)}$ was found to equal zero. These values are averages of at least five separate determinations of the flash-yield patterns as in Fig. 6 A—C, and standard deviations are indicated. All other conditions are as in Fig. 6.

Line	Sample conditions	$\overline{\alpha}$	$\overline{\beta}$	$\overline{\gamma}$	$S_0^{(0)}$	$S_1^{(0)}$
1	No $Fe(CN)_6^{3-}$ (xenon)	0.097 ± 0.017	0.850 ± 0.020	0.053 ± 0.003	0.26 ± 0.04	0.74 ± 0.04
2	+$Fe(CN)_6^{3-}$ (xenon)	0.102 ± 0.007	0.848 ± 0.010	0.050 ± 0.004	0.24 ± 0.03	0.76 ± 0.03
3	No $Fe(CN)_6^{3-}$ (300 ns laser)	0.145 ± 0.012	0.833 ± 0.015	0.022 ± 0.006	0.26 ± 0.03	0.74 ± 0.03
4	+$Fe(CN)_6^{3-}$ (300 ns laser)	0.146 ± 0.006	0.833 ± 0.010	0.021 ± 0.004	0.23 ± 0.02	0.77 ± 0.02
5	No $Fe(CN)_6^{3-}$ (5 ns laser)	0.140 ± 0.014	0.840 ± 0.011	0.020 ± 0.004	0.25 ± 0.03	0.75 ± 0.03
6	+$Fe(CN)_6^{3-}$ (5 ns laser)	0.136 ± 0.017	0.850 ± 0.015	0.014 ± 0.005	0.23 ± 0.03	0.77 ± 0.03

Fig. 6 A—C shows oxygen flash-yield patterns for chloroplasts incubated with 1.5 mM ferricyanide and excited with a xenon flash, 300 ns laser or 5 ns laser. Only with xenon flash excitation, not laser excitation, is there any significant increase in the oxygen yield on flash two. Mathematical treatment of these data gave the transition parameters shown in Table II. The mathematical fitting procedure actually gives transition parameters that reflect what occurs on the average for flashes 1—12. The lack of a ferricyanide effect on the data in Table II means that there is no significant ferricyanide effect on the transitions of the oxygen states after the first flash. Thus, the effects of ferricyanide seen

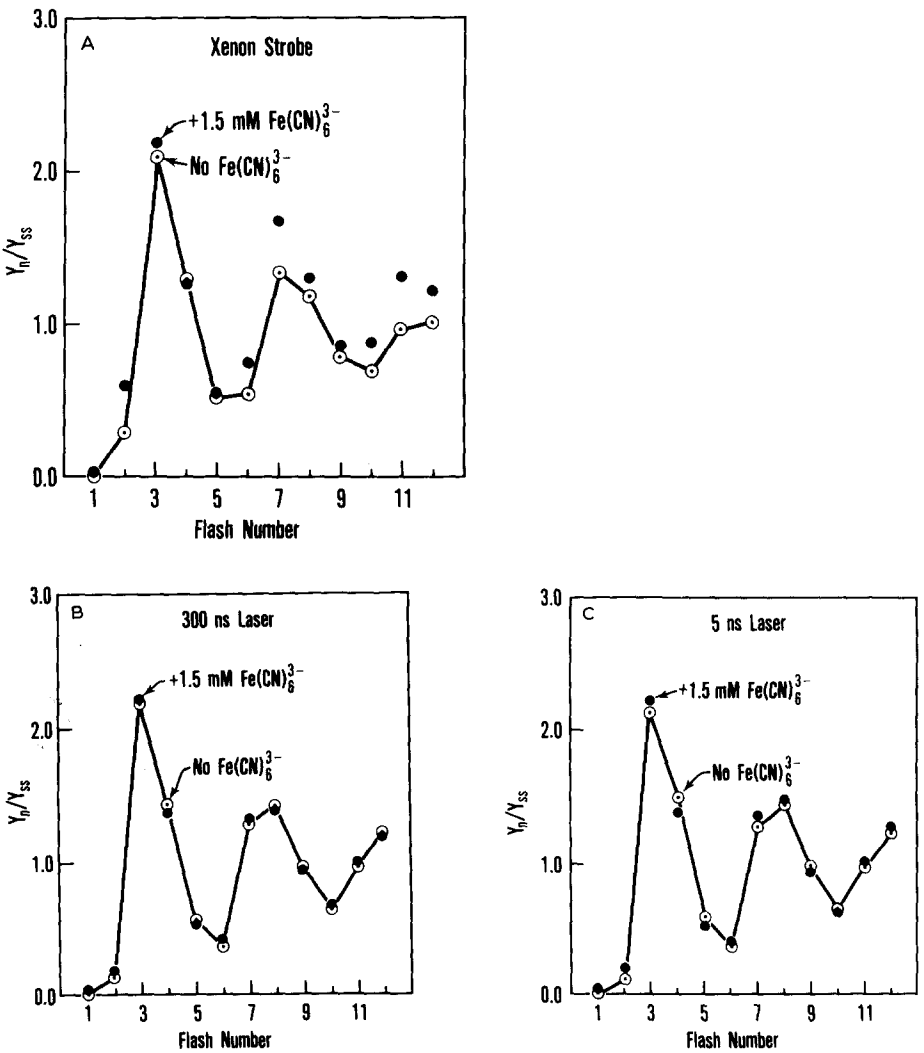


Fig. 6. Oxygen flash-yield as a function of flash number in fresh chloroplasts without additions (○——○) or incubated on ice at a concentration of 5 mg Chl/ml for 10 min with 1.5 mM $Fe(CN)_6^{3-}$ (●——●). The chloroplasts were diluted 10-fold prior to placement on the electrode. The oxygen yield on any flash, Y_n, is normalized by the steady-state yield, Y_{ss}, reached after many flashes. Excitation was given at a rate of 2 Hz in (A) by a xenon flash, in (B) by a 300 ns laser and in (C) by a 5 ns laser.

in Fig. 6A must be due to alterations in the dark S-state distribution or changes in the transition parameters only on the first flash [10].

The transition parameters on flash one and the dark S-state distribution can be described in matrix form as K_1 (K for the first flash) and $|S^{(0)}>$. As described earlier, a computer program was written that allowed K and $|S^{(0)}>$ to vary until a best fit to the entire oxygen flash yield pattern was obtained. On flashes other than the first, K consisted of the appropriate values from Table II. Two solutions for K_1 and $|S^{(0)}>$ that gave equally good fits to the data were found and are shown in Table III. Solution No. 1 has transitions for the first flash and a dark S-state distribution similar to those that have long been accepted [17]. Ferricyanide causes an increase in double advancement in S-states of almost 3-fold. The S-state distribution in the dark remains the same, which is in agreement with Velthuys and Kok [10]. That is, for this solution ferricyanide does not alter the dark-adapted ratio of S_1/S_0. Solution No. 2 is markedly different from solution No. 1, and ferricyanide increases the double advancement in S-states by only a small amount and does alter the S-state distribution in the dark somewhat. Two quite different mathematical solutions for K_1 and $|S^{(0)}>$ have been reported before [15] for normal chloroplasts and algae, but with no way of determining which solution was correct. The data in Table III indicate a test protocol, since only for solution No. 1 does double advancement in S-states greatly increase with ferricyanide treatment. An independent method of looking at the ferricyanide effect on Photosystem II double hitting is described in the next section.

The effect of ferricyanide and different types of pre-illumination flash on the fluorescence rise with DCMU present

Upon continuous illumination of chloroplasts that have been kept in the dark for 5 min or longer, the chlorophyll *a* fluorescence increases from a low to a high level [18]. This rise in fluorescence has been interpreted as due to a reduction by Photosystem II of a fluorescence quencher Q to a non-quenching form, Q^- [19]. For chloroplasts with DCMU present, a linear relationship exists [20] between variable fluorescence and photochemical rate. Thus, if the same

TABLE III

OXYGEN EVOLUTION PARAMETERS CALCULATED SPECIFICALLY FOR THE FIRST FLASH IN CHLOROPLASTS WITH AND WITHOUT FERRICYANIDE PRESENT

Solution No. 1 and solution No. 2 refer to equally good mathematical fits (see text for details) for the oxygen flash-yield data of Fig. 6A. α_1, β_1 and γ_1 are transition probabilities that occur specifically on the first flash following dark adaptation. $S_0^{(0)}$—$S_3^{(0)}$ are the probabilities for the population of each S-state in the dark. All other conditions are as in Fig. 6.

Sample conditions	α_1	β_1	γ_1	$S_0^{(0)}$	$S_1^{(0)}$	$S_2^{(0)}$	$S_3^{(0)}$
Solution No. 1							
No $Fe(CN)_6^{3-}$	0.097	0.850	0.053	0.250	0.750	0	0
+$Fe(CN)_6^{3-}$	0.109	0.751	0.140	0.250	0.750	0	0
Solution No. 2							
No $Fe(CN)_6^{3-}$	0.066	0.314	0.620	0.940	0.060	0	0
+$Fe(CN)_6^{3-}$	0.072	0.272	0.656	0.850	0.150	0	0

maximum fluorescence level is attained under different conditions [21], the areas above the fluorescence-rise curve is proportional to the quencher (electron acceptor) concentration.

Ikegami and Katoh [22] have demonstrated that the normalized area above the fluorescence curve increased 100% upon incubation with ferricyanide prior to the addition of DCMU. It was suggested [22] that an acceptor with a redox potential of 360 mV at pH 7.8 was oxidized in the dark by ferricyanide. The effects on the fluorescence rise have been reported for xenon flashes and 20 ns laser flashes [23] and picosecond laser flashes [24]. In the experiments done here, pre-illumination flashes from a xenon lamp, 300 ns laser or 5 ns laser were given, and the area above the fluorescence rise curve was measured. In this way, the amount of reduction of the acceptor during the flash was estimated and related to a single or multiple turnover of the Photosystem II reaction center.

The effects of incubation with ferricyanide and pre-illumination flashes on the area above the fluorescence rise curve are shown in Table IV for freshly prepared chloroplasts. Incubation with ferricyanide causes the area above the fluorescence curve to increase by 1.8-fold, which is in good agreement with previous reports [22,23]. This indicates that an additional acceptor is oxidized by ferricyanide in the dark. A single pre-illumination with a xenon flash or 300 ns laser pulse eliminates the area above the curve due to the primary acceptor and up to 30% of the increase in area (additional area) due to incubation with ferricyanide (lines 3 and 6, Table IV). A single pre-illumination with a 5 ns laser does not eliminate any of the additional area (line 8, Table IV). For all of the flash types, two or more flashes eliminate approx. 90% of the additional area. For fresh chloroplasts, the xenon and 300 ns laser flashes apparently cause multiple turnovers of the Photosystem II reaction center on the first flash, and

TABLE IV

AREA ABOVE FLUORESCENCE RISE CURVES OF FRESHLY PREPARED CHLOROPLASTS WITH DCMU PRESENT, INCUBATED WITH FERRICYANIDE AND PRE-ILLUMINATED

The freshly prepared chloroplasts at a concentration of 10 μg Chl/ml were incubated at room temperature with 0.2 mM $Fe(CN)_6^{3-}$ for 4 min in the dark prior to addition of 10^{-5} M DCMU. 1 min after the addition of DCMU the measurement was started. The additional area is the increase in area due to ferricyanide incubation. Pre-illumination flashes were given at a rate of 2 Hz and the final flash of a series (or the only flash if a single pre-illumination) was given 75 ms prior to the start of continuous illumination. The typical range in the amount of additional area eliminated is indicated on line 3.

Line	Sample conditions	Area/ΔF	% Additional area eliminated
1	No $Fe(CN)_6^{3-}$ (F_0 = 31, F_{max} = 72)	1.80	—
2	+ $Fe(CN)_6^{3-}$ (F_0 = 25, F_{max} = 61)	3.20	—
3	+ $Fe(CN)_6^{3-}$, 1 (xenon)	1.05	25 ± 5
4	+ $Fe(CN)_6^{3-}$, 2 (xenon)	0.53	62
5	+ $Fe(CN)_6^{3-}$, 3 or more (xenon)	0.18	87
6	+ $Fe(CN)_6^{3-}$, 1 (300 ns laser)	0.96	31
7	+ $Fe(CN)_6^{3-}$, 2 (300 ns laser)	0.28	80
8	+ $Fe(CN)_6^{3-}$, 1 (5 ns laser)	1.68	0
9	+ $Fe(CN)_6^{3-}$, 2 (5 ns laser)	0.22	84

this is observed as a decrease in the additional area. The 5 ns laser flash, on the other hand, induces only a single turnover.

Discussion

The data presented here demonstrate that in algae and chloroplasts double advancement in S-states in oxygen evolution does occur when 300 ns and 5 ns laser flashes are used for excitation. It seems that double advancement in S-states can be split into two categories: (1) that occurring even with a 5 ns flash, which will be called the non-photochemical type and (2) the additional double advancement that occurs with excitation flashes having extensive tails, such as xenon flashes, which will be called the photochemical type. Both types of double hitting can occur in algae and chloroplasts, but the non-photochemical double advancement is found to be sensitive to the physiological condition of the chloroplasts. It cannot be observed in frozen chloroplasts, freshly prepared chloroplasts from market spinach, nor in freshly prepared chloroplasts that have been allowed to sit on ice for 5 h or more. The use of non-optimum samples may be the reason why earlier reports [7,8] indicated that double advancement in S-states did not occur when excitation flashes without tails were used.

It was originally suggested [3] that double advancement in S-states of oxygen evolution was due to some reaction centers recovering rapidly enough from an initial quantum absorption to be able to absorb another quantum, during the flash tail. This is felt to be an adequate explanation for photochemical double advancement. It is suggested here that non-photochemical double advancement is an intrinsic property of the Photosystem II system and depends on sample integrity and not the type of excitation flash. A possible mechanism for non-photochemical double advancement would be the involvement of a side carrier 'C' [25] which can exchange charge with the oxygen evolution S-states. The behavior of C would not depend on the type of flash given and can be diagrammed as follows:

$$S_iC \xrightarrow{h\nu} S_{i+1}C \underset{k_1}{\overset{k_\alpha}{\rightleftharpoons}} S_iC^+$$

or

$$S_iC^+ \xrightarrow{h\nu} S_{i+1}C^+ \underset{k_2}{\overset{k_\gamma}{\rightleftharpoons}} S_{i+2}C$$

where S_i is any S-state, k_γ is the rate constant for C^+ accepting an electron, k_α is the rate constant for C donating an electron, and k_1 and k_2 are the rate constants for no change in the charged state of C following an excitation. k_γ and k_α would be related to the tendency for non-photochemical double advances and misses, respectively. A non-optimum sample would have $k_\gamma << k_\alpha$, which would be observed as γ approaching zero and α increasing by a small but significant amount (Table I, lines 5, 6, 8 and 9). The identity or normal biological role of C is not known at this time.

The misses in oxygen evolution occur with much higher probability than

double hits (Table I). In terms of the above model, this will cause C^+ to build up and will require the complication of a reduction reaction to maintain adequate concentrations of C. Another possibility is that only a portion of misses are due to the above side-carrier mechanism. The remainder of misses have a different origin; perhaps extremely slow recovery of some reaction centers, following a flash. This possibility is now being investigated.

Of the two solutions for the effect of ferricyanide on the oxygen evolution transition parameters, only solution No. 1 showed an increase in the double advancement parameter. This solution is believed to be the correct one, since measurement of reduction in area above fluorescence rise curves indicates that the double turnover in Photosystem II charge separation is enhanced by ferricyanide incubation. So, solution No. 2 shown here and suggested earlier by Thibault [15] is of mathematical interest, but is not consistent with all experimental results.

One striking aspect of the data presented here is that the kinetic processes are different for double advancement in S-states of oxygen evolution and double turnovers in Photosystem II reaction center charge separation. It is found that increases in double advancement of S-states caused by ferricyanide can be observed only with a xenon flash not a 300 ns laser flash (Fig. 6 A—C and Table III). This suggests a rate-limiting step of approx. 10 μs (see Fig. 1, inset). A rate-limiting step of 50 μs had been approximated earlier [10] but the inclusion of 200 mM $MgCl_2$ in the chloroplast preparation was undoubtedly a complicating factor. Double turnovers in Photosystem II charge separation are observed with xenon and with 300 ns laser flashes but not with a 5 ns laser flash (Table IV). In view of recent data by Bowes et al. [23,24], the rate-limiting step for double turnover in Photosystem II charge separation is between 5 and 20 ns.

Two models of the Photosystem II reaction center that can account for this behavior are presented. The first model, called the series model, is similar to that of Bowes et al. [23]:

$$\begin{array}{l} \overset{\text{D}}{\text{MZ}_2\text{Z}_1\ \text{P}_{680}\text{Q}_2\text{Q}_1} \xrightarrow[1]{h\nu} \overset{\text{D}}{\text{MZ}_2\text{Z}_1\ \text{P}^+_{680}\text{Q}_2^-\text{Q}_1} \xrightarrow{2} \overset{\text{D}}{\text{MZ}_2\text{Z}_1\ \text{P}^+_{680}\text{Q}_2\text{Q}_1^-} \xrightarrow{3} \overset{\text{D}}{\text{MZ}_2\text{Z}_1^+\ \text{P}_{680}\text{Q}_2\text{Q}_1^-} \\ \xrightarrow[4]{} \overset{\text{D}}{\text{MZ}_2\text{Z}_1^+\ \text{P}^+_{680}\text{Q}_2^-\text{Q}_1^-} \xrightarrow{5} \overset{\text{D}^+}{\text{MZ}_2\text{Z}_1^+\ \text{P}_{680}\text{Q}_2^-\text{Q}_1^-} \end{array}$$

where P_{680} is the reaction center chlorophyll, M is the oxygen evolving system, Z_1 and Z_2 are charge carriers between P_{680} and M, Q_1 and Q_2 are charge acceptors, and D is a secondary charge donor. Reactions 1 and 4 are light reactions, and reaction 4 is the second hit reaction for Photosystem II charge separation that can occur due to quanta in the tail of a flash (indicated by the broken line in the diagram above). Either reaction 2 or reaction 3 must be limiting on the first flash following dark adaptation. As stated previously, this step must take place between 5 and 20 ns. Based on 820 nm absorption changes, van Best and Mathis [26] concluded that upon the first flash, P^+_{680} was reduced with a half-time of 25 to 45 ns. Thus, it is felt that reaction 3, not reaction 2, is the rate limiting step. In this scheme, Q_1 would represent the Photosystem II acceptor

that is reoxidized with $\tau_{1/2} \approx 500$ μs. A complication in this model is that without ferricyanide pretreatment Q_2 will remain reduced, and under these conditions Q_1 must be able to accept electrons directly from P_{680}.

D is a secondary donor which reduces P_{680}^+ (reaction 5) with a half-time of 25 μs [27,28]. In order for reaction 5 to take place, the $Z_2Z_1^+ \rightarrow Z_2^+Z_1$ reaction must take longer than 25 μs and the duration of the flash [29]. To explain the divergence in the ferricyanide increase of double hitting in oxygen evolution and Photosystem II charge separation, the donor D must be unconnected to the oxygen evolving system. This conclusion is in agreement with previously reported results [30]. Also, with 5 ns laser excitation, double turnovers in Photosystem II charge separation do not occur (Table IV, line 8) and D^+ is not generated, and yet double advancement in S-states of oxygen evolution still occur (Table II, line 6). So it seems unlikely that C and D are one-and-the-same, as suggested previously [28].

The second model, called the parallel model, is similar to that of Gläser et al. [31] and later Joliot and Joliot [28]:

$$\begin{array}{lll} & D & Q_1 \\ MZ_2Z_1 & P_{680} & \\ & & Q_2 \end{array}$$

where all symbols are as previously defined. In this case, either Q_1 or Q_2 can accept electrons from P_{680} upon illumination, so the $Z_1P_{680}^+ \rightarrow Z_1^+P_{680}$ step will be the limiting factor for no double hitting in Photosystem II charge separation with 5 ns illumination.

It should be kept in mind that in the models discussed above the secondary acceptor, Q_2, has a midpoint potential of +320 mV and is oxidized only when preincubation with ferricyanide has occurred [22,23]. Earlier investigations [28,30,32,33] performed at lower redox potentials have also suggested multiple acceptors for Photosystem II that are distinct from Q_2 discussed above. These secondary acceptors do not appear to be involved in photochemical double-advancement in S-states. This is suggested by data presented here (Table I and Figs; 3—5) where only non-photochemical double advancement in S-states occurs with a 300 ns flash. A 300 ns flash with oxidized secondary acceptors should cause multiple Photosystem II turnovers and photochemical double advancement in S-states. However, the photochemical double advancement in S-states is not observed. This conclusion is in agreement with recent results of Eckert and Renger [33] published during the preparation of this work.

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