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PERIODIC CHANGES IN THE OXIDATION STATE OF MANGANESE IN PHOTOSYNTHETIC OXYGEN EVOLUTION UPON ILLUMINATION WITH FLASHES

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Summary

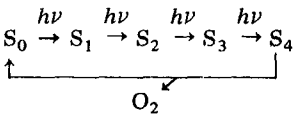
The pattern of manganese released from chloroplast membranes by a rapid temperature shock after various illumination regimes indicates that changes in the oxidation state of bound manganese occur during photosynthesis. Continuous illumination decreases by 35—40% the amount of Mn(II) released in the presence of $K_3Fe(CN)_6$ compared with a dark-adapted control. Following illumination and heat treatment, the addition of the reductant H_2O_2 to the samples causes an increase in the level of electron paramagnetic resonance (EPR)-detectable manganese. The pH dependence of the H_2O_2 reduction indicates that the non-EPR-detectable manganese present in the heated sample after illumination is in the form of higher oxidation state compounds, e.g. MnO_2. The light-induced Mn(II) decrease is reversible in the dark with $t_{1/2}$ approx. 40 s and can be prevented by the presence of the Photosystem II inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethyl urea or fluorocarbonylcyanide phenylhydrazone during the illumination period.

After a series of brief flashes of light the Mn(II) released by heat treatment oscillates over periods of four flashes. The pattern is similar to the O_2 yield flash pattern and suggests that a cycling of manganese oxidation states is involved in the O_2 evolution mechanism. The oscillations in the Mn(II) release are analyzed in terms of the current four-step model for O_2 evolution. The analysis suggests that manganese is successively oxidized in the first two steps, but undergoes a partial reduction on the third step. This result is consistent with the concept that water undergoes a partial oxidation prior to the release of O_2 from the water-splitting complex.

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; FCCP, fluorocarbonylcyanide phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

At present it appears that a manganese-containing complex is involved in the reactions that lead to the production of O_2 during photosynthesis [1]. The exact nature of these reactions, however, remains undetermined. In the current model based on O_2 flash yield kinetics, a chemical intermediate (S) is proposed to cycle through five different states after successive photoreactions before leading to the release of O_2 [1—3].



(1)

Implicit to this model is the assumption that each S_i state (where $i = 1,2,3,4$) differs from the preceding state, S_{i-1}, by the loss of an electron. Upon reaching the most oxidized, S_4, the intermediate then reacts to produce O_2 and the original S_0. Indirect experimental evidence based on proton magnetic resonance relaxation rates and thermoluminescence measurements of chloroplasts has implicated changes in manganese oxidation states in the cycling of the S intermediate [4—7]. In this paper we report additional evidence to support the role of manganese as a redox mediator in photosynthetic oxygen evolution.

Under favorable circumstances paramagnetic Mn(II) may be monitored directly using electron paramagnetic resonance (EPR) techniques. In chloroplasts at room temperature the Mn(II) EPR signal is detectable only after the bound manganese has been released from the membrane [8,9] (for a possible exception see Ref. 10). One treatment which causes the release of bound manganese is a mild temperature shock [11]. Because higher oxidation state complexes of manganese may be released as non-EPR-detectable species it appeared to us that the amount of Mn(II) released from the membrane might reflect oxidation state changes of the bound manganese. We investigated the Mn(II) EPR signal in heat-treated chloroplasts in the presence of oxidants and reductants before and after illumination, either continuous or as a series of brief flashes. The results indicate that bound manganese does undergo photooxidation in connection with Photosystem II reactions. After a series of brief flashes of light the Mn(II) signal shows period four oscillations similar to those observed for the O_2 yield. This result strongly suggests that changes in manganese oxidation states are directly involved in the O_2 mechanism. Analysis of the manganese oscillations in terms of the four-step mechanism of Eqn. 1 suggests that manganese is successively oxidized in the first two steps, but undergoes a partial reduction in the third step.

Experimental

Chloroplast samples were prepared from spinach maintained in growth chambers according to previously described procedures [9]. The standard sucrose buffer consisted of 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.6, 0.4 M sucrose and 0.01 M NaCl. After isolation the chloroplasts were washed once with 10^{-4} M EDTA in sucrose buffer to remove any extraneous loosely bound ions and twice more with sucrose buffer

to remove the EDTA. Chlorophyll concentration was determined by the method of Mackinney [12]. The samples were diluted to 1 or 2 mg Chl · ml^{-1} in sucrose/buffer containing $MgCl_2$ at 0.005 or 0.010 M, respectively. Other additions to the samples are given in the figure legends.

Heat treatment to cause the release of bound manganese was performed directly in the EPR cell. The flat cell (0.25 mm × 10 mm × 60 mm) containing the sample was placed in a hot water bath, 53—55°C, for 2 min, then cooled in a stream of tap water and dried before measurements were made.

The characteristics of the white-light flashes (FWHM approx. 20 μs) or continuous white light for illumination experiments, as well as the polarographic detection of the O_2 flash yield, were described previously [13]. The EPR spectra were measured on a Varian E-3 spectrometer (X Band, 9.5 GHz). The cavity was continuously flushed with dry N_2. All spectra were recorded at room temperature and, unless otherwise noted in the text, the operating conditions were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude 16 G; time constant, 3 s; scan rate, 125 G/min. The flat cell was positioned in the cavity using special clips which provided reproducibility in the signal amplitude of ±3%.

The results were analyzed in terms of the four-step model of Eqn. 1 using a computer program written in collaboration with D.B. Goodin and J.A. Kirby. The fitting algorithm employed a least-squares minimization procedure and is available under the title MINUIT in the Lawrence Berkeley Laboratory computer center program library.

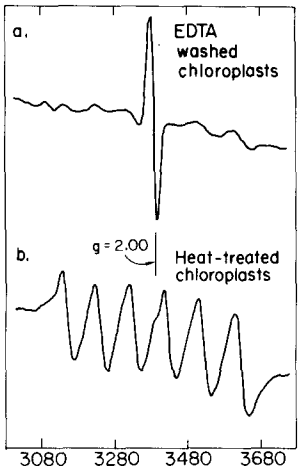
Results

Manganese release measurements

The effect of heat treatment on the chloroplast EPR spectrum is demonstrated in Fig. 1. An untreated control sample (Fig. 1a) shows a large signal at about $g = 2$, the signal II_u and II_s of Babcock and Sauer [13], and several smaller, broadly distorted signals in the region of the manganese transitions. These smaller signals may be due to some extraneous manganese trapped within the thylakoids. When the same sample is heated in the EPR cell for 2 min at 55°C and repositioned in the cavity (Fig. 1b), the signal at $g = 2$ largely disappears, and the six strong hyperfine lines characteristic of the manganese hexa-aquo complex appear, indicating the release of manganese from the membrane [9]. The increase in the manganese EPR signal upon heat treatment is about ten fold.

For convenience, we define the parameter ΔP as the average of the peak-to-trough heights for the manganese lines, given in arbitrary amplitude units. In those cases in which the middle lines become distorted by $g = 2$ signals (e.g. by the formation of signal I in the dark upon addition of $K_3Fe(CN)_6$) only the undistorted lines are used in calculating ΔP. The mean and S.D. for nine determinations of different heated aliquots of one sample preparation was 67 ± 5, indicating a reproducibility within ±10%.

According to Blankenship and Sauer [9] the amplitude of the derivative EPR signal is directly proportional to the concentration of free manganese in the chloroplast suspension. In our samples, containing 1 mg Chl · ml^{-1},



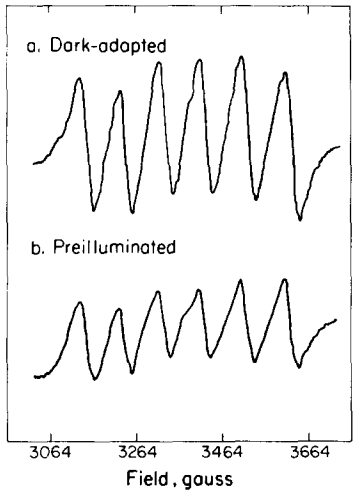


Fig. 1. Room temperature EPR spectra (1st derivative) for (a) EDTA-washed spinach chloroplasts and (b) the same sample heat treated at 55°C for 2 min in the EPR cell. Receiver gain was the same for both spectra. Chlorophyll content was 2 mg Chl · ml^{-1}. The manganese signal amplitude (ΔP) is defined as the average sum of the peak-to-trough heights for the manganese lines.

Fig. 2. The effect of illumination on the manganese release from chloroplasts. Spectrum (a) was obtained after heat treatment from a dark-adapted sample while spectrum (b) was obtained from a sample which was illuminated for 60 s in continuous white light before heat treatment. The two spectra represent two aliquots from the same sample preparation. Receiver gain was the same for both spectra. Other conditions are given in Fig. 1.

the amount of manganese released from different preparations was in the range 0.02—0.05 μmol · ml^{-1}, a variation observed previously in spinach [9] and in peas [14]. This corresponds to a ratio of 3—8 manganese atoms/400 Chl molecules.

Effect of continuous illumination on manganese release

Fig. 2 shows the effect of illumination with continuous white light on the manganese release. Spectrum (a) was taken from a dark-adapted aliquot while spectrum (b) was taken from an aliquot which was illuminated for 60 s with continuous white light before heat treatment. The ΔP for the illuminated sample is 43% less than that for the dark-adapted sample. The two spectra are nearly identical in linewidth and hyperfine coupling, indicating that the light has changed only the amount of EPR-detectable manganese released from the membrane.

The extent of the light-induced decrease in ΔP, however, was variable for different sample preparations. This variability apparently arises from a rapid aging effect. Table I shows that for one chloroplast preparation the light-induced decrease was no longer present 3 h after homogenization. But the aging effect was overcome in another aliquot of the same sample when 2 mM $K_3Fe(CN)_6$ was added to the suspension medium. Under these conditions a large light-induced decrease could be maintained during 6 h after isolation. It is interesting to note that the reversal of the aging effect by $K_3Fe(CN)_6$ occurred only when the $K_3Fe(CN)_6$ was added before the illumination period

TABLE I

THE EFFECT OF AGING ON THE LIGHT-INDUCED DECREASE IN MANGANESE RELEASE AFTER HEAT TREATMENT IN SPINACH CHLOROPLASTS

Chloroplast samples were stored on ice in the dark at a concentration of 2 mg Chl · ml^{-1}. Conditions for EPR measurements are given in Fig. 2.

Time after homogenization (h)	Experimental procedure	Mn EPR signal (ΔP)	Percent change
2	Dark, heat	59	
	Light, heat	47	−20
	+ 2 mM $K_3Fe(CN)_6$, dark, heat	43	
	+ 2 mM $K_3Fe(CN)_6$, light heat	28	−34
3—4	Dark, heat	56	
	Light, heat	50	−9
	+ 2 mM $K_3Fe(CN)_6$, dark, heat	44	
	+ 2 mM $K_3Fe(CN)_6$, light, heat	29	−33
6—7	Dark, heat	58	
	Light, heat	59	0
	+ 2 mM $K_3Fe(CN)_6$, dark, heat	47	
	+ 2 mM $K_3Fe(CN)_6$, light, heat	36	−38
	Light, heat, + 2 mM $K_3Fe(CN)_6$	47	0

and heat treatment. The last line in Table I shows that when the $K_3Fe(CN)_6$ was added after heat treatment, no light-induced change in ΔP was observed. This effect of $K_3Fe(CN)_6$ may be due to its role as a Hill oxidant which allows complete turnover of Photosystem II in aged chloroplasts, or to its influence on the redox environment of the sample.

In unheated chloroplasts 2 mM $K_3Fe(CN)_6$ eliminates any measurable manganese EPR signal, while in heated chloroplasts the magnitude of the signal is significantly decreased compared with samples without $K_3Fe(CN)_6$ (Table I). The attenuation in the manganese EPR signal could be due to a chemical oxidation. However, ferricyanide is too weak an oxidant to oxidize Mn(II) appreciably in a neutral aqueous environment [15]. A more likely explanation is the formation of a complex between manganese and ferricyanide. Iron and copper complexes of ferricyanide are known [15], and similar complexes with manganese may have no EPR-detectable signal. In heat-treated chloroplasts, as opposed to Tris-washed chloroplasts [9], the released manganese appears to be freely permeable through the membrane. A considerable fraction of the EPR-detectable manganese can be washed away after heat treatment (data not shown). Thus, the released manganese and ferricyanide are accessible to each other to allow complex formation.

In subsequent experiments, except as noted, 2 mM $K_3Fe(CN)_6$ was included in the suspension medium to insure a consistent light effect. Because of the effect on the manganese EPR signal, light-induced changes are always compared with dark controls containing the same amount of $K_3Fe(CN)_6$.

The continuous light effect on ΔP in chloroplasts is reversible. Fig. 3 shows a semi-logarithmic plot of the dark-minus-light difference in ΔP versus the dark time between the end of a 60 s illumination period and heat treatment. The first point in the curve was taken 30 s after the end of the illumination period. (Although chloroplasts were routinely heat treated for 2 min, under our condi-

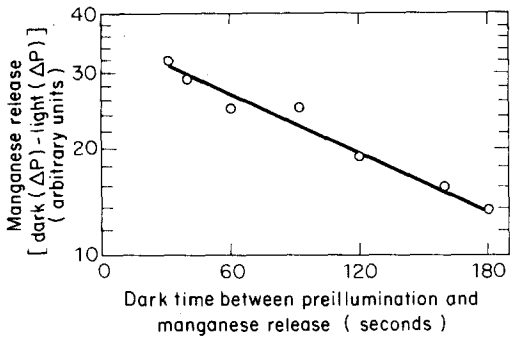


Fig. 3. Dark decay of the light-induced decrease in manganese release from spinach chloroplasts. The log of the dark-minus-light difference in ΔP is plotted as a function of dark time between the end of a 60 s preillumination period and heat treatment. The suspension medium contained 2 mM $K_3Fe(CN)_6$. Other conditions are given in Fig. 1.

tions most of the manganese was released from the membrane within the first 20—30 s). The curve appears to be linear, with $t_{1/2}$ approx. 40 s.

Effects of various chemical treatments on the light-induced change in manganese release

Photosystem II inhibitors. Two commonly used inhibitors of Photosystem II activity are DCMU and FCCP. DCMU blocks electron flow through Photosystem II at the level of the primary acceptor, while FCCP at high concentrations (greater than 100 μM) accelerates the deactivation reactions of the higher S states [16]. As shown in Table II the presence of these reagents during the illumination period eliminates any light-induced change in the manganese release. These results implicate the involvement of Photosystem II reactions in the light effect on manganese release.

Glutaraldehyde fixation. The manganese EPR signal under the conditions used in our experiments reflects only the +2 oxidation state. An important question is whether the light-induced decrease in ΔP arises from some conformational change which decreases the amount of Mn(II) released from the membrane or from the creation of higher oxidation state complexes of manganese that are present in a non-EPR-detectable form after heat treatment. Glutaraldehyde fixation is known to prevent macroconformation changes in the membrane [17]. In Table II the light effect is shown to be still present in the manganese release from glutaraldehyde-fixed chloroplasts. Macroconformation changes, therefore, seem unlikey as an explanation for the light effect, although contributions from microconformation changes cannot be excluded.

Hydrogen peroxide. To test whether manganese is produced in a higher oxidation state after a light period, we determined the effect of H_2O_2 on the light-induced decrease in ΔP. H_2O_2 reduces MnO_2 to Mn(II) in a reaction that occurs readily at pH 6.0, but not at pH 7.5 or above [15]. The last set of results in Table II show that the light-induced decrease in ΔP is eliminated when H_2O_2 is added after heat treatment at pH 6.0, but not at pH 7.6. H_2O_2 has little apparent effect on the Mn(II) EPR signal in dark-adapted chloroplasts, regardless of whether it is added before or after heat treatment. Thus, after a

TABLE II

THE EFFECTS OF PHOTOSYSTEM II INHIBITORS, GLUTARALDEHYDE FIXATION, AND H_2O_2 ON THE LIGHT-INDUCED DECREASE IN MANGANESE RELEASE AFTER HEAT TREATMENT IN SPINACH CHLOROPLASTS

Glutaraldehyde fixation was after Zilinskas and Govindjee [18]. DCMU refers to 3-(3,4-dichlorophenyl)-1,1-dimethyl urea and FCCP to fluorocarbonylcyanide phenylhydrazone. The suspension medium contained 2 mM $K_3Fe(CN)_6$. Each ΔP is an average of three determinations. Other conditions are given in Table I.

Experimental procedure	Mn EPR signal (ΔP)	Percent change
Photosystem II inhibitors		
100 μM DCMU, dark, heat	83	
100 μM DCMU, light, heat	83	0
100 μM FCCP, dark, heat	54	
100 μM FCCP, light, heat	51	−5
Glutaraldehyde-fixed chloroplasts		
Dark, heat	67	
Light, heat	36	−46
H_2O_2-treated chloroplasts		
pH 6.0		
Dark, unheated	10	
Dark, unheated, + 0.6% H_2O_2	13	—
Dark, heat	78	
Dark, heat, + 0.6% H_2O_2	76	—
Dark, heat	62	
Light, heat	40	−35
Light, heat, + 0.6% H_2O_2	61	−2
pH 7.5		
Dark, heat	47	
Light, heat	31	−34
Light, heat, + 0.6% H_2O_2	28	−39

light period a portion of the manganese is released in a non-EPR detectable form that can be reduced to Mn(II) with H_2O_2.

Effects of flash illumination on manganese release

To relate the manganese release changes to the O_2 mechanism the ΔP was measured following a series of brief flashes of light. Fig. 4a shows the manganese release flash pattern for control chloroplasts which did not contain $K_3Fe(CN)_6$. Each point represents the average ΔP for 5—11 determinations from several different sample preparations. Results from different sample preparations are normalized to the same dark value. After the first two flashes the ΔP decreases relative to the dark value. After the 3rd flash it increases and then exhibits a periodicity of four after further flashes.

For comparison the O_2 flash yield pattern of the control sample is shown in Fig. 4b. This is a typical pattern for the O_2 yield, except that the yield after the 2nd flash is high and the oscillations are not so deep as is usually observed (the minimum occurs after the 5th flash rather than the 6th flash). This is probably due to the long pulse width of the flash lamp that we used (FWHM approx. 20 μs), which introduces a large number of double hits.

In the presence of DCMU there are no coherent changes in either the

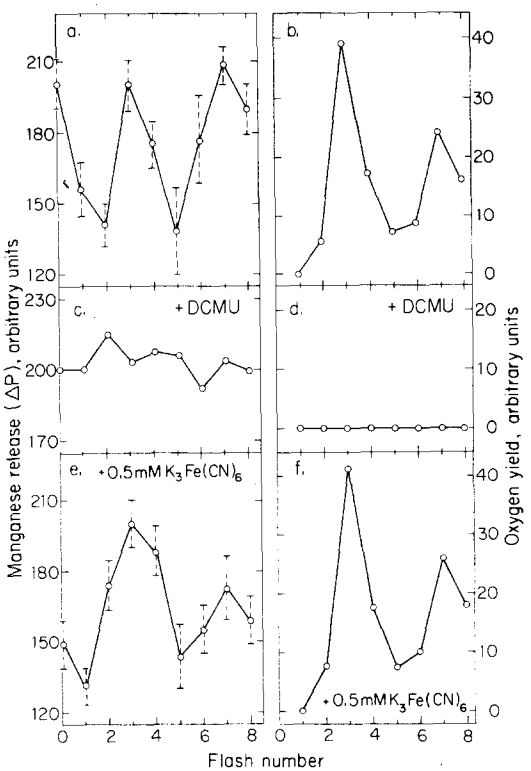


Fig. 4. Manganese release (ΔP) and O_2 yield measured as a function of flash number for control chloroplasts (a and b), chloroplasts containing 100 μM DCMU (c and d) and chloroplasts containing 0.5 mM $K_3Fe(CN)_6$ (e and f). The error bars represent the S.D. for 5—11 measurements. Results from different chloroplast preparations were normalized to the same dark value. Flashes were spaced 4 s apart. Other conditions are given in Fig. 1.

manganese release or the O_2 yield flash pattern, as shown in Fig. 4c and d.

Fig. 4e and f show the manganese release and O_2 flash pattern, respectively, for samples containing 0.5 mM $K_3Fe(CN)_6$. The oscillations in ΔP show a periodicity similar to the control pattern. However, the initial dark level is low compared to the ΔP after the 1st flash. There also is a downward trend in the ΔP values with increasing flash number. The corresponding O_2 yield pattern shows a small increase in the yield after the 2nd flash and a small decrease in the yield after the 4th flash compared with the control.

Discussion

Manganese EPR measurements as a monitor of changes in the bound oxidation states

As shown in Fig. 1 a sizeable fraction of the chloroplast manganese becomes EPR detectable after a mild temperature shock (55°C for 2 min). A small manganese signal is observable before heat treatment (Fig. 1a), even though the membranes were washed thoroughly with an EDTA solution. After heat treatment the intensity of the manganese EPR signal increases 8—10 fold over that of the unheated sample (Fig. 1b). Similarly to the effect of incubation in

alkaline Tris buffer or high concentrations of NH_2OH, heating causes the large pool of chloroplast manganese associated with O_2 evolution [8] to be released into an apparently aqueous environment [9]. But unlike the other treatments, heating can be used to cause a rapid release of manganese from the membrane directly in the EPR cell. For this reason we chose heating as a convenient method for manganese release in our experiments.

It has been suggested from proton relaxation measurements that a mixture of manganese oxidation states exists in chloroplast membranes [4–6]. The amount of EPR-detectable manganese released from the membrane should then reflect the relative proportion of Mn(II) in the bound oxidation states. Mn(II) is stable in water as the hexa-aquo complex, so that all Mn(II) released from the membrane will be EPR detectable. Mn(III), on the other hand, is highly reactive in an aqueous environment and may disproportionate as follows: 2 Mn(III) → Mn(II) + Mn(IV), where Mn(IV) will precipitate as MnO_2. Thus, half of the bound Mn(III) can be expected to be lost to EPR detection. Likewise, none of the Mn(IV) produced in the membrane would be EPR detectable. Alternatively, the higher oxidation states of the bound manganese could react with endogenous reductants and be partially or completely reduced to Mn(II) upon release from the membrane.

Fig. 2 and the data in Tables I and II show that less Mn(II) is EPR detectable after heat treatment when the samples are illuminated with continuous white light, even in the presence of $K_3Fe(CN)_6$. The light-induced decrease in the Mn(II) EPR signal is apparently not due to major conformational changes in the membrane, because the light effect is still present in glutaraldehyde-fixed chloroplasts (Table II). However, the light effect can be reversed by the subsequent addition of H_2O_2 to the heat-treated sample (Table II). Thus, after an illumination period some of the manganese is released in a non-EPR-detectable form, presumably in higher oxidation state species that can be reduced by H_2O_2. The pH dependence of the H_2O_2 reaction suggests that the non-EPR-detectable form of the manganese is MnO_2. The light-induced change in the manganese release is consistent with a net photooxidation of the bound manganese.

Manganese contribution to the S states

The inhibition of the light effect on manganese release by Photosystem II inhibitors (Table II) and the oscillatory behavior of the EPR signal in brief flashes of light (Fig. 4) suggests that the bound manganese is related to the O_2 evolution mechanism. A possible explanation for these results is that the amount of EPR-detectable manganese released after a flash measures the Mn(II) distribution among the S states (Eqn. 1). If this is the case, then the relative magnitude of the EPR signal (ΔP) after a flash can be expressed as:

$$\Delta P(m) = \sum_{i=0}^{3} [S_i(m)]\, W_i \qquad (2)$$

where W_i is a weighting factor proportional to the amount of the Mn(II) associated with each S_i state after heat treatment and $[S_i(m)]$ is the concentration of the S_i state after flash m. The weighting factors include the original

Mn(II) contribution to the S states plus any Mn(II) contributions that may arise from disproportionation of higher oxidation states upon release from the membranes. $[S_i(m)]$ is given by the following relation:

$$[S_i(m)] = \alpha[S_i(m-1)] + \beta[S_{i-2}(m-1)] + (1-\alpha-\beta)[S_{i-1}(m-1)] \quad (3)$$

where α is the fraction of centers that miss and β is the fraction of centers that are hit twice during the flash.

The concentrations of the S states throughout a flash sequence can be calculated from the O_2 flash yield measurements, if the initial concentrations of the S states and α and β are known. In the original analysis of Kok and coworkers [19,20] a large proportion of the O_2-evolving centers were assumed to start out in the S_1 state in the dark, to account for the peak O_2 yield after the 3rd flash. Kok obtained a good fit to the O_2 yield sequence using the following set of parameters for chloroplasts: $[S_0(0)] = 0.25$, $[S_1(0)] = 0.75$, $[S_2(0)] = [S_3(0)] = 0$, $\alpha = 0.10$ and $\beta = 0.05$.

Recently, it has been suggested that multiple electron acceptors in Photosystem II allow rapid turnover of *P*-680, the reaction center chlorophyll, during the first flash [21]. Thibault [22,23] has since shown that an equally good or better fit to the O_2 flash yield sequence can be achieved by assuming that most centers start out in S_0 rather than S_1 in the dark and that a large number of double hits occur on the first flash. In Thibault's model the starting parameters for chloroplasts are: $[S_0(0)] > 0.94$, $[S_{1,2,3}(0)] < 0.06$, $\alpha = 0.10$, $\beta' = 0.61$ and

TABLE III

THEORETICAL O_2 YIELD PARAMETERS

The parameters were obtained from a least-squares best fit of the measured O_2 flash yield sequence to a synthetic sequence generated from either the Kok et al. [19,20] or Thibault [22,23] model for O_2 evolution. The quadratic deviation measures the agreement between model and experiment and is defined as:

$$\text{Quadratic deviation} = \left[\frac{\sum_{m=1}^{n} [Y_e(m) - Y_c(m)]^2}{\sum_{m=1}^{n} [Y_e(m)]^2}\right]^{1/2}$$

where $Y_e(m)$ is the O_2 yield measured after flash m and $Y_c(m)$ is the calculated O_2 yield. The $Y(m)$ sequence out to 25 flashes was used in the fitting procedure. $[S_0(0)]$ and $[S_1(0)]$ refers to the initial S_0 and S_1 state concentrations, α is the miss parameter, β is the double hit parameter and β' is the double hit parameter on the first flash in the Thibault model only. Other details are given in the text.

O_2 yield parameters	Kok model		Thibault model	
	Untreated chloroplasts	+ 0.5 mM $K_3Fe(CN)_6$	Untreated chloroplasts	+ 0.5 mM $K_3Fe(CN)_6$
$[S_0(0)]$	0.256	0.161	0.866	0.857
$[S_1(0)]$	0.744	0.839	0.134	0.143
α	0.103	0.089	0.093	0.079
β'	—	—	0.643	0.709
β	0.100	0.097	0.083	0.047
Quadratic deviation	0.0270	0.0714	0.0246	0.0604

$\beta = 0.03$, where β' refers to double hits on the first flash and β to double hits on all subsequent flashes.

We used both models to calculate the initial O_2 yield parameters in our samples. The results for the control and $K_3Fe(CN)_6$-treated chloroplasts are given in Table III. The parameters are obtained from a least-squares best fit of the O_2 flash yield sequence (measured out to 25 flashes) to a synthetic sequence generated by either the Kok of Thibault model. The quality of the fits between the models and experiment is measured by the quadratic deviation.

Using the Kok model for analysis, the presence of $K_3Fe(CN)_6$ has little effect on β, but does cause a small decrease in α and an increase in the $S_1(0)/S_0(0)$ compared with the control. This result is consistent with the analysis of Bouges-Bocquet [24] who has suggested that $K_3Fe(CN)_6$ oxidizes S_0 to S_1. By contrast, in the Thibault model $S_1(0)/S_0(0)$ does not change significantly in the presence of $K_3Fe(CN)_6$, while β' increases and β decreases. The extent of the decrease in α, however, is about the same as in the Kok analysis. The increased β' can be explained by the chemical oxidation of the electron acceptors associated with Photosystem II. But the decrease in β can only be due to some additional effect of $K_3Fe(CN)_6$; e.g. oxidation of intermediate donors. The relatively large number of double hits in our measurements is probably due to the long pulse duration (approx. 20 μs) of the xenon flash lamp used. As might be expected, the Thibault model yields a better fit to the O_2 yield data than does the Kok model, although the difference in the quadratic deviations between the two models is not very large.

To fit the manganese release data according to Eqn. 2 the S state concentrations throughout the flash sequence are given fixed values using the parameters from Table III. The weighting factors for the S_0, S_1, S_2, and S_3 states are then allowed to vary to give the best least-squares fit to the ΔP flash patterns. Any S_4 contribution to the manganese release is ignored because S_4 deactivates within a few ms. The weighting factors are restricted to positive values. The fits to the ΔP flash patterns are shown in Fig. 5, and the resulting weighting factors and quadratic deviations are given in Table IV.

In Fig. 5 the solid circles are the data points taken from Fig. 4. The open circles show the theoretical fit using all the data points, while the other symbols ($\diamond$, $\square$, $\triangle$) represent fits in which the first three data points ($\Delta P(0)$, $\Delta P(1)$, $\Delta P(2)$) are successively excluded in the fitting procedure. Although there is an obvious difference in the behaviour of the first two flashes between the control and $K_3Fe(CN)_6$-treated samples, the quality of the fits from $\Delta P(3)$ to $\Delta P(8)$ do not substantially improve as the initial data points are excluded. For the Kok model the quadratic deviation (Table IV) remains relatively constant as the initial points are excluded, while for the Thibault model the quadratic deviation decreases, the worst fit being when all data points are used. However, due to the large standard deviation in the data (see Fig. 4) it is difficult to exclude either model on the basis of these results. The best fit to all data points is obtained with the Kok model on the $K_3Fe(CN)_6$-treated sample.

For the control sample neither the Kok nor the Thibault model could satisfactorily describe the behavior on the first two flashes. This may indicate that not all of the manganese photooxidized by Photosystem II is associated

TABLE IV

THEORETICAL Mn(II) WEIGHTING FACTORS

The weighting factors were obtained from a least-squares best fit of the Mn(II) release data in Fig. 4 to a synthetic sequence calculated according to Eqn. 2 in the text and the O_2 yield parameters for the Kok and Thibault models given in Table III. W_0, W_1, W_2 *and* W_3 refer to the weighting factors for the S_0, S_1, S_2 and S_3 states, respectively. 0, ◇, □, and △, various ranks of the $\Delta P(m)$ flash sequence used in the fitting procedure and correspond to the fits shown in Fig. 5. The values in parentheses are the weighting factors normalized so that $W_0 = 1$. The quadratic deviation is defined in Table III. Other details are given in the text.

Symbols corresponding to fits in Fig. 5	Rank of $\Delta P(m)$ used in fitting procedure	Mn(II) weighting factors									
		Untreated chloroplasts					+0.5 mM $K_3Fe(CN)_6$				
		W_0	W_1	W_2	W_3	Quadratic deviation	W_0	W_1	W_2	W_3	Quadratic deviation
(a) Kok model											
○	$\Delta P(0)$—$\Delta P(8)$	0.26	0.17	0.13	0.15	0.0790	0.21	0.15	0.12	0.18	0.0596
		(1.00)	(0.65)	(0.50)	(0.58)	—	(1.00)	(0.71)	(0.57)	(0.86)	—
◇	$\Delta P(1)$—$\Delta P(8)$	0.28	0.17	0.11	0.16	0.0734	0.20	0.17	0.11	0.18	0.0510
		(1.00)	(0.61)	(0.39)	(0.57)	—	(1.00)	(0.85)	(0.55)	(0.90)	—
□	$\Delta P(2)$—$\Delta P(8)$	0.27	0.13	0.11	0.16	0.0728	0.20	0.18	0.08	0.19	0.0544
		(1.00)	(0.48)	(0.41)	(0.59)	—	(1.00)	(0.90)	(0.40)	(0.95)	—
△	$\Delta P(3)$—$\Delta P(8)$	0.25	0.13	0.11	0.20	0.0626	0.22	0.17	0.10	0.16	0.0518
		(1.00)	(0.52)	(0.44)	(0.80)	—	(1.00)	(0.77)	(0.45)	(0.73)	—
Average of normalized weighting factors		(1.00)	(0.56)	(0.44)	(0.63)		(1.00)	(0.81)	(0.48)	(0.86)	
(b) Thibault model											
○	$\Delta P(0)$—$\Delta P(8)$	0.22	0.18	0.12	0.17	0.0911	0.16	0.21	0.08	0.21	0.0805
		(1.00)	(0.82)	(0.54)	(0.77)		(1.00)	(1.31)	(0.50)	(1.31)	
◇	$\Delta P(1)$—$\Delta P(8)$	0.27	0.18	0.09	0.15	0.0878	0.21	0.18	0.10	0.18	0.0560
		(1.00)	(0.67)	(0.33)	(0.55)		(1.00)	(0.86)	(0.48)	(0.86)	
□	$\Delta P(2)$—$\Delta P(8)$	0.29	0.15	0.09	0.15	0.0699	0.21	0.18	0.08	0.19	0.0560
		(1.00)	(0.52)	(0.31)	(0.52)		(1.00)	(0.86)	(0.38)	(0.90)	
△	$\Delta P(3)$—$\Delta P(8)$	0.25	0.15	0.09	0.22	0.0546	0.23	0.18	0.10	0.15	0.0533
		(1.00)	(0.60)	(0.36)	(0.88)		(1.00)	(0.78)	(0.43)	(0.65)	
Average of normalized weighting factors		(1.00)	(0.65)	(0.38)	(0.68)		(1.00)	(0.83)	(0.43)	(0.80) (excludes $\Delta P(0)$—$\Delta P(8)$)	

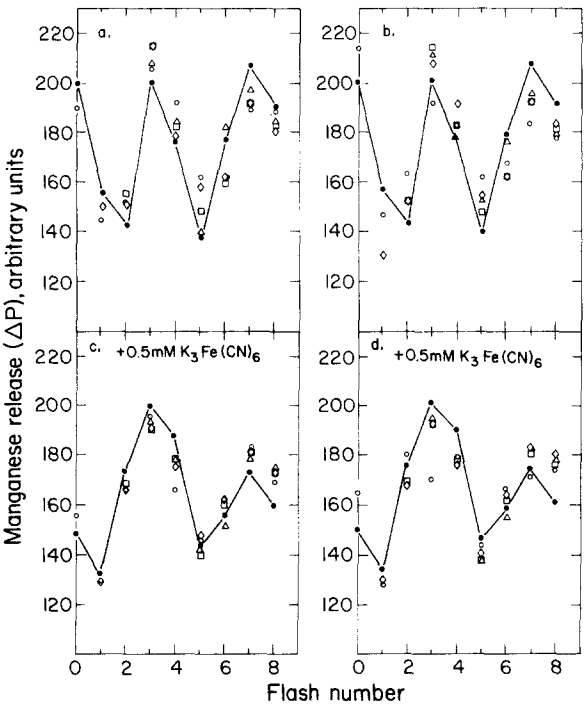


Fig. 5. Theoretical fits to the manganese release data. (a) Control chloroplasts, Kok model; (b) control chloroplasts, Thibault model; (c) +0.5 mM $K_3Fe(CN)_6$, Kok model; (d) +0.5 mM $K_3Fe(CN)_6$, Thibault model. •, the data points taken from Fig. 4; ○, the theoretical fit using all data points in the fitting procedure. The symbols (◇, □, △) represent fits in which the first three data points ($\Delta P(0)$, $\Delta P(1)$, $\Delta P(2)$) were successively excluded from the fitting procedure. The weighting factors obtained from the fits and the quadratic deviations are given in Table IV. Details are described in the text.

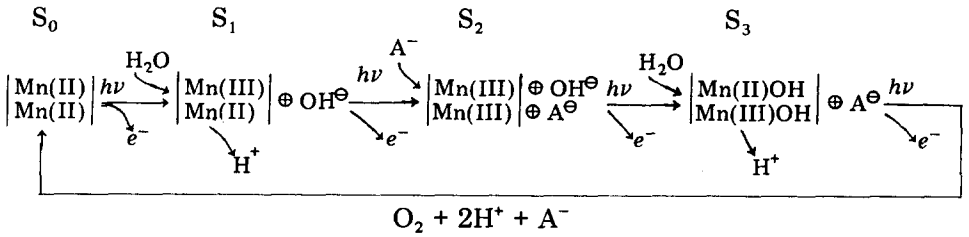
with the O_2 mechanism. Clearly, the presence of $K_3Fe(CN)_6$ alters the behavior on the first two flashes to give a better fit with the predicted values. The measured ΔP after the first two flashes in the control sample is not due to an aging phenomenon, because $\Delta P(2)$ was consistently observed to be smaller than $\Delta P(1)$ in several fresh sample preparations.

Table IV gives the actual weighting factors obtained from the various fits, while the values in parentheses are the weighting factors normalized to $W_0 = 1$. In general, the weighting factors decrease from S_0 to S_1 to S_2 and then increase for S_3. The one exception is the fit using the Thibault model for all data points in the $K_3Fe(CN)_6$-treated sample. Since the Thibault model predicts that most centers start out in S_0, to accommodate the low $\Delta P(0)$, W_0 is made small with respect to W_1. Nevertheless, the general trend in the weighting factors is consistent with a successive photooxidation of manganese through the first two S state transitions, and then a partial reduction accompanying the $S_2 \rightarrow S_3$ transition, the oxidation levels of S_3 being about the same as S_1. If manganese in the O_2 mechanism leads directly to a reaction with water, then this result indicates that water undergoes a partial oxidation prior to the release of O_2.

The same conclusion was reached previously based on proton release measurements, which also show period four oscillations in brief flashes of light [25—27]. Analysis of the proton release measurements in terms of the four-

step mechanism (Eqn. 1) led Fowler [25] to suggest that the majority of the O_2 centers exhibit a 1,0,1,2 proton release pattern for the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4 \rightarrow S_0$ transitions, respectively.

We have attempted to construct a model to describe the S state transitions taking into account both the manganese oxidation state changes and the proton release data. Analytical data suggest that there may be as many as four manganese atoms involved in each oxygen-evolving center [1,3]. Govindjee et al. [28] have proposed a model based on proton magnetic resonance relaxation rate measurements in which the O_2 evolving complex was suggested to contain four Mn atoms, and the Mn in the S_3 state was considered to be more reduced than the Mn in the S_0 state. However, the involvement of as many as three or four manganese cycling between Mn(II) and Mn(III) or higher oxidation states would involve significantly smaller oscillations in the Mn(II) level than we observe. Correspondingly, a single manganese atom per site would involve oscillations much larger than those observed. Thus, we have chosen a binuclear manganese complex as the best model to reconcile the data. The following scheme is in reasonably good agreement with the quantitative evidence available.



In the first step ($S_0 \rightarrow S_1$) the charge increase of the complex is compensated by binding a hydroxide anion from water and releasing a proton in the process. In the second step ($S_1 \rightarrow S_2$) a counterion A^- is involved and no H^+ is released. A^- could be an inorganic anion such as chloride. Recent evidence suggests that chloride binds to the manganese-protein complex [29]. In the third step ($S_2 \rightarrow S_3$) another hydroxide is bound and two electrons are transferred to the manganese atoms, thus decreasing their oxidation level and increasing that of the bound oxygens to the level of peroxide. The final step ($S_3 \rightarrow S_4 \rightarrow S_0 + O_2$) forms molecular oxygen by removing two more electrons from the bound oxygens, releasing the two remaining protons and returning the two manganese atoms to the Mn(II) state.

Assuming that Mn(III) disproportionates upon release from the membrane, the model above predicts a pattern of normalized Mn(II) weighting factors: $W_0 = 1.00$, $W_1 = 0.75$, $W_2 = 0.50$, $W_3 = 0.75$. These values are in fair agreement with the experimentally derived values presented in Table IV. They account nicely for the observation that $W_1 \approx W_3$. The magnitude of W_2 is also in quite reasonable agreement with the experiment, which is the strongest justification for the assumption that two manganese atoms are involved.

As with all models, however, caution must be taken in viewing these conclusions. Because of lack of precision in the measurements we cannot, for example, rule out completely the possibilities that higher oxidation states or larger complexes of manganese are involved. In addition, as Fowler points out, other proton release patterns may be involved [25].

The major features of the model are that only Mn(II) and Mn(III) oxidation states need to be involved in the stabilized S state and that water-splitting reactions involve hydroxyl ligand partial oxidation in a manganese-protein complex. Our results implicate a role for manganese in the cycling of the S states, but a more explicit manganese model for O_2 evaluation must await further details.

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