THE ROLE OF MANGANESE IN THE
OXYGEN EVOLVING MECHANISM OF PHOTOSYNTHESIS

Govindjee, T. Wydrzynski and S. B. Marks
Departments of Physiology & Biophysics and Chemistry
University of Illinois, Urbana, Illinois 61801
U.S.A.

INTRODUCTION

It is generally accepted that the ultimate source of O₂ in photosynthesis is
ewater, although how O₂ is released from water is not known. In the past, we have
made several attempts to understand the O₂ evolving side of system II but did not
have much success.

First, we prepared antibodies against chloroplast suspension¹ or against an
extract from frozen and thawed preparations of washed thylakoids²; these antibod-
ies were tested for their specificity against the O₂ evolving side of photosynthe-
sis. We succeeded in obtaining one preparation² which gave us specific inhibition
of the O₂ evolving side, but the inhibition was only up to 30%. This could be ta-
ten to confirm what was already surmised before, that a protein may be somehow
involved in O₂ evolution and that the O₂ evolving components may lie on the inner
side of the thylakoid membrane (see Trebst³, Fowler and Kok⁴ and Babcock and
Sauer⁵).

Next, we started with the hope that bicarbonate (or CO₂) may have something to
do with O₂ evolution, even if in a catalytic fashion (see Metzner⁶). Initially,
we thought that the site of stimulation of the Hill reaction by CO₂ was located
on the water side (see Steiner and Govindjee⁷-⁹) but later experiments did not
support¹⁰ this conclusion. In fact, all our efforts¹¹ to find an effect on the
water side failed. On the other hand, we were able to show that a major effect of
CO₂ lies in stimulating electron flow from Q⁻ (Q being the "primary" acceptor of
system II) to the plastoquinone (PQ) pool. The halftime of the decay of chloro-
phyll (Chl) a fluorescence yield, after a flash, was reversibly slowed down by a-
bout five fold in CO₂-depleted chloroplasts (Jursinic et al.¹¹), and this qualifi-
tatively explained the reversible slowing down¹² of the relaxation of Sn¹ to Sn¹⁺
states involved in O₂ evolution kinetics (S being the charge accumulator for oxy-
genesis, see Joliot and Kok¹³). More recently, we have shown¹⁴,¹⁵ that the
major effect of CO₂ depletion is in the electron flow from R(or B), the secondary
two electron acceptor, to the PQ pool. The ¹⁴C binding studies of Steiner¹⁶ also
support a site of CO₂ effect on the electron acceptor side of system II. Independ-
ent biochemical studies of Khanna et al.¹⁷ provided further confirmation that the
bicarbonate (or CO₂) effect lies somewhere between Q and the PQ pool. Thus, if
CO₂ must play a direct part in O₂ evolution, it still remains to be discovered.
Attempts to isolate "oxygen evolving components" have thus far failed. However, there is clear evidence that manganese is required for \( \text{O}_2 \) evolution (see review\textsuperscript{18}). But there has been no evidence, until recently, that manganese undergoes dynamic changes during oxygen evolution. This is what we shall describe here.

Our present approach to understand the \( \text{O}_2 \) evolution mechanism became promising when we recognized that water proton longitudinal (or, spin-lattice) relaxation rate, \( 1/T_1 \), of thylakoid membranes was strongly dependent upon the presence of bound manganese (Wydrzynski et al.\textsuperscript{19}). When thylakoids were depleted of their bound manganese either by alkaline TRIS or \( \text{NH}_2\text{OH/EDTA} \) treatment, the \( 1/T_1 \) was reduced to 0.4 of its original value. Furthermore, reductants such as tetraphenylboron (TPB) increased and oxidants, such as potassium ferricyanide, decreased the \( 1/T_1 \) of thylakoids. We suggested that \( 1/T_1 \) can monitor bound-manganese and that it exists as a mixture of oxidation states. Soon thereafter, we measured the water proton transverse (or spin-spin) relaxation rates, \( 1/T_2 \), as a function of light-flash number, and discovered oscillations with a period of four, with maxima at the 3rd, 7th, 11th, 15th etc. flashes (Wydrzynski et al.\textsuperscript{20}). These maxima correspond with those in the \( \text{O}_2/\text{flash} \) as a function of flash number in a series of flashes. We, thus, suggested\textsuperscript{20} that \( 1/T_2 \) monitors the \( \text{O}_2 \) evolving mechanism. Significant differences in the \( 1/T_2 \) and \( \text{O}_2 \) patterns were not explained at that time.

In this paper we present a brief review of our recent data\textsuperscript{21,22} on proton relaxation rates (PRR) showing (1) the relationship of \( 1/T_1 \) and \( 1/T_2 \) to manganese concentration and \( \text{O}_2 \) evolution; (2) the frequency dependence of \( 1/T_1 \) and \( 1/T_2 \), which suggests that the electron spin relaxation (1/\( \tau_s \)) of Mn[II] dominates the PRR, and (3) the relationship of \( 1/T_2 \) and \( \text{O}_2 \) evolution patterns as a function of flash number under a variety of conditions which show that \( \text{O}_2 \) evolution can be uncoupled from the intermediate monitored by PRR. Finally, we present a hypothetical manganese model of \( \text{O}_2 \) evolution.

**Materials and Methods**

Chloroplast membrane fragments were prepared by homogenizing leaves in a medium consisting of 50 mM N-2-hydroxyethylpiperazineN-2 ethane sulfonic acid (HEPES) buffer, adjusted to pH 7.5 with NaOH, 400 mM sucrose, and 10 mM NaCl. 0.5% bovine serum albumin and 10 mM sodium ascorbate were included during the grinding procedure. After appropriate filtration and differential centrifugation, chloroplast fragments were given osmotic shock and then suspended in the medium described above, but adjusted to appropriate pH. For PRR measurements, thylakoid suspensions containing 2-3 mg Chl/ml were used.

\( \text{O}_2 \) evolution/flash was measured on a Joliot-type electrode. The light source was a Phase-R Model DL 2100 A dye laser (\( \lambda \), 590 nm) having a pulse width of 0.6 \( \mu s \) at half height, terminating in about 2 \( \mu s \). The dark time between flashes was 4s. The same illuminating conditions were used for the measurements of \( 1/T_2 \) as a function of flash number. Manganese concentrations were measured with a Perkin-
Elmer Model 303 Atomic Spectrometer or by neutron activation analysis.

Proton relaxation rates were measured in a pulsed nuclear magnetic resonance spectrometer (constructed in the laboratories of Drs. Paul Schmidt and of H.S. Gutowsky). (See Fig. 1 for the basic design and Wydrzynski et al.\textsuperscript{21} for details.)

![Diagram](image)

Fig. 1 Generalized Block Diagram of a Pulsed NMR Spectrometer. The rf transmitter consists of rf synthesizer, gating and phase adjust unit and rf power amplifier. The rf receiver consists of appropriate amplifiers and a phase sensitive detector. PD refers to power divider. The NMR sample probe is positioned in strong magnetic field. A dye laser (\(\lambda, 590 \text{ nm}\)) is used for light excitation of the sample. A pulse programmer determines the rf and light pulse sequence. Signals are recorded by an oscilloscope and oscillographic recorder. (after ref. 23.)

The \(1/T_1\) was measured by the inversion recovery method. The sample was placed in magnetic field, then a 180° radiofrequency (rf) pulse was given, followed by 90°rf pulses after various times (\(\tau\)). The \(1/T_1\) was calculated from a plot of \(\ln \frac{M_0 - M_z(t)}{M_0}\) as a function of \(\tau\), where \(M_0\) is the equilibrium magnetization and \(M_z(t)\) is the magnetization at time \(\tau\) after the 180° pulse. The \(1/T_2\) was measured as follows. A Carr-Purcell-Meiboom-Gill pulse sequence was given: a 90° rf pulse was followed by time \(\tau\) and then a series of 2,100 180° pulses spaced 2\(\tau\) apart (\(\tau = 500 \mu\text{s}\)) were given. The \(1/T_2\) was calculated from the decay of spin echo envelopes with time (\(\tau\)); the slope of \(\ln \frac{M_z(t)}{M_0}\) as a function of \(\tau\) is proportional to \(1/T_2\).

RESULTS AND DISCUSSION

1. Proton Relaxation Rates Monitor Bound Manganese

Using the method of Chen and Wang\textsuperscript{24}, we were able to vary the concentration of chloroplast manganese by replacement with magnesium. Fig. 2 (left) shows \(1/T_1\) and \(1/T_2\) as a function of manganese concentration. Both \(1/T_1\) and \(1/T_2\) decrease as manganese concentration is decreased till about 50% of manganese is left. The \(1/T_2\) continues to decrease linearly, whereas \(1/T_1\) reaches a constant value representing some background contribution. A similar pattern is observed if \(1/T_1\) and \(1/T_2\) are plotted as a function of \(O_2\) (Fig. 2 (right)). A direct relationship be-
tween PRR, manganese content and O₂ evolution is thus established. It appears that 1/T₁ monitors mainly the loosely bound manganese related to O₂ evolution (c.f. ref. 18), where 1/T₂ may monitor the total manganese content of the membrane due to greater non-site specific contributions to 1/T₂ (see Wydrzynski 23 for further details).

Fig. 2 (left): Plot of Percent 1/T₁ and 1/T₂ versus percent total Mn content. (right): Plot of Percent 1/T₁ and 1/T₂ versus percent O₂ activity. Mn extraction was achieved by incubating pea chloroplasts for 2 hr in dark at 4°C in HEPES buffer medium containing MgCl₂ at the following [Mg]/[Chl] ratios: 0, 167, 332, 667, 2,500 and 10,000. After incubation the chloroplasts were centrifuged and pellet was resuspended to 3 mg Chl/ml. Mn content was determined by neutron activation analysis. For control, Mn content was 0.62±0.03 μg Mn/mg Chl. Rate of O₂ evolution for control was 120 μmoles O₂/mg Chl-hr. (After ref. 21.)

2. Proton Relaxation Rates Monitor Bound Mn[II]

Fig. 3 shows dependence of 1/T₁ and 1/T₂ as a function of NMR frequency. The 1/T₁ shows a broad peak in the 10-20 MHz range (open circles, lower curve), whereas 1/T₂ shows only an increase (open circles, upper curve). This characteristic frequency behavior is obtained when the electronic relaxation of bound paramagnetic ions dominate the PRR. The solid lines through the experimental points in Fig. 3 are the "best" fit theoretical curves based on Solomon-Bloembergen-Morgan analysis for relaxation in paramagnetic systems.

Both 1/T₁ and 1/T₂ are related to the dipolar correlation time (τ₀) by Solomon-Bloembergen equation 25. The major molecular processes which lead to dipolar interactions include the electron spin relaxation (1/τₑₑ), rotational motions (1/τ₀) and the rate of chemical changes (1/τᵢₘ). The 1/τ₀ is related to these processes as follows: 1/τ₀ = 1/τₛ + 1/τᵢ + 1/τᵢₘ. For macromolecules, τᵢ is long (~10⁻⁶ s) and thus
$1/\tau_r$ is insignificant with respect to $1/\tau_s$ and $1/\tau_m$. If $\tau_s$ dominates $\tau_C$, one expects (see ref. 25) the frequency behavior as shown in Fig. 3 since $\tau_s$ itself changes with frequency. The frequency dependence of $\tau_s$ is given by the Bloembergen and Morgan equation:

$$\frac{1}{\tau_s} = B\left(\frac{\tau_{V}}{1+\tau_{S}} + \frac{4\tau_{V}}{1+4\tau_{S}2\tau_{V}}\right)$$

where, $\tau_s$ is electronic Larmor frequency, $B$ is a constant related to the zero field splitting parameters and $\tau_V$ is the correlation time for the modulation of the zero field splitting. The NMR parameter values obtained from the "best" fit curves in Fig. 3 are $\tau_V=20\times10^{-12}$, $\tau_m=2.2\times10^{-8}$ s and $B=0.9\times10^{19}$ (rad/s)$^2$. These values compare favorably with those for Mn[II]-pyruvate kinase systems, $\tau_V=14.4\times10^{-12}$ s, $\tau_m=0.4\times1.04\times10^{-8}$ s, and $B=0.8\times0.1\times10^{19}$ (rad/s)$^2$ (after Navon26). The correlation times for Mn[III] and high spin Fe[II] and Fe[III] are 2-3 orders of magnitude different (lower) than for Mn[II]. And, since the copper in plastocyanin has no effect on PRR (see ref. 27), the PRR of chloroplasts, thus, must be monitoring only Mn[II] contributions.

Fig. 3. "Best" Fit Theoretical Curves to the Frequency Dependence of the Relaxation Rates for Dark-Adapted Chloroplast Membranes. (a) $1/T_2$ relaxation; (b) $1/T_1$ relaxation: $1/T_{1,2}(\text{corr})=1/T_{1,2}(\text{obs})-1/T_{1,2}(T-A)$. Rates were measured at room temperature (23-25°C) and normalized to the same Mn concentration at each frequency. $1/T_{1,2}(\text{os})$ refers to the theoretical outer sphere contribution on a translational diffusion model. Pea chloroplasts at 3 mg Chl/ml were used. (After ref. 21.)

3. The $1/T_2$ Monitors the $S$ States in $O_2$ Evolution

Fig. 4 shows $1/T_2$ and $O_2$/flash as a function of flash number in a series of
flashes for three different samples of pea chloroplasts, at pH 6.7. The absence of O_2 in the second flash indicates that there are no double hits with our laser flashes. The main peak in O_2 or 1/T_2 curve is at the 3rd flash, the second peak is at the 7th flash in 1/T_2 (curves c & e), but for O_2 yields, the 7th and 8th flashes have about the same value. When manganese is extracted from chloroplasts by TRIS-acetone wash method, both oscillations in 1/T_2 and O_2 disappear. Addition of DCMU to chloroplasts which stops turnover of reaction center II, after one flash, also eliminates 1/T_2 oscillations (Fig. 5). These results suggest that 1/T_2 is monitoring the O_2 evolving mechanism.

Differences between 1/T_2 and O_2 patterns are: (a) the ratio of 1/T_2 in the 3rd to the 2nd flash is very low as compared to that for O_2; (b) a minimum in 1/T_2 is at the 5th flash, whereas in O_2 it is at the 6th flash and (c) the dark 1/T_2 level is significantly higher than in the first flash, whereas there is no O_2 in the first flash as in the darkness.

Fig. 4 (left) Observed 1/T_2 Rates and O_2 Yield Measured as a Function of Saturating Flashes of Light for three samples of Pea Chloroplast Membranes. The 1/T_2 and O_2 measured after each flash in a pulse sequence. Saturating light flashes obtained from a pulsed dye laser (λ, 590 nm) were spaced 4s apart. 1/T_2 was measured at 27 MHz, 23°C. Sample contained 2 mg Chl/ml. (After ref. 22.)

Fig. 5 (right) Effect of DCMU and Manganese Extraction on 1/T_2(obs) Flash Pattern. (a) [DCMU]/[Chl]=0.089; (b) TRIS-acetone washed chloroplasts. Flash procedure and conditions for pea chloroplast in Fig. 4 were used. (After ref. 22.)

Note also that 1/T_2 in dark is decreased by DCMU. Data reported by Wydrzynski show that the dark 1/T_2 level can also be decreased by (a) ferricyanide,
(b) fixation of chloroplasts with glutaraldehyde; and (c) removal of sucrose from the suspension medium. Furthermore, unpublished observations of Rita Khanna (in our laboratory) show that in intact cells of blue-green alga Phormidium luridum the dark level is also low. These observations suggest that the significance of the high dark level of $1/T_2$ in isolated chloroplasts should not be exaggerated although it still needs to be explained.

When tetraphenylboron (TPB), hydroxylamine (NH$_2$OH) or carbonyl cyanide m-chlorophenylhydrazone (CCCP) is added to chloroplasts, the flash pattern of $1/T_2$ is changed with the peaks appearing at the 2nd and 6th flashes instead of the 3rd and 7th flashes (Fig. 6). No O$_2$ is evolved in the TPB case in the first 10 flashes, but then slowly increases with succeeding flash, with NH$_2$OH the O$_2$ peaks are on the 6th and 10 flashes, and no O$_2$ is evolved when CCCP is present. All of the above suggest that $1/T_2$ oscillation can be uncoupled from the O$_2$ evolution. The differences in $1/T_2$ and O$_2$ patterns suggest that $1/T_2$ is monitoring more than just the final O$_2$ evolving step. It is easy to qualitatively understand this difference because O$_2$ comes off only during the last step of the following set of reactions, according to Kok et al. (see ref. 13):

\[
S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4 \\
2H_2O \longrightarrow O_2 + 4H^+
\]

where, the subscripts refer to the number of oxidizing equivalents on the intermediate $S_i$, PRR may be monitoring the $S_i$ intermediate directly. Since PRR is suggested (in section 2) to monitor Mn[II], it is logical to propose that $1/T_2$ changes induced by light flashes indicate dynamic changes in Mn[II] concentration during O$_2$ evolving process. The relationship is, however, complex (see section 4).

4. The Model

A search for a model to explain $1/T_2$ as a function of flash number began as soon as we observed the flash number dependence of $1/T_2$ in spinach chloroplasts. Many differences between $1/T_2$ and O$_2$ had to be recognized. First, the minima were at 4th, 8th, 12th flashes in $1/T_2$ in contrast to minima at 6th, 10th etc. flashes in O$_2$ evolution. Second, the dark $1/T_2$ was as high as that at 3rd etc. flashes. Third, when O$_2$ yield decreased in going from 4th to 6th flash, $1/T_2$ increased.

Fourth, overall the O$_2$ yield decreased with increasing flash number, but the $1/T_2$ increased. In these experiments, chloroplasts stayed at room temperature for a long time, particularly for flash numbers greater than 9, because $1/T_2$ was measured only at the end of a series of flashes. The data on peas reported here ($1/T_2$ measured after each flash in a series of flashes) and lettuce (measured as in the spinach case) show essentially the same major features uptil the 8th flash as observed in spinach except that the $1/T_2$ after the first flash is almost the same as dark (lettuce) or is intermediate between that for spinach and lettuce (peas).
Another difference was that the minimum in lettuce or peas was on the 5th flash in contrast to the 4th flash in spinach. No significant difference in O₂ flash pattern was, however, observed. Many of these differences could be accounted for by variations in the following model.

We used the basic features of Kok et al.'s model and procedure (ref. 29) to calculate the concentrations of S₀ and S₁, α (misses) and β (double hits) from O₂/flash as a function of flash number for a sample at pH 6.7. This gave us [S₀] = 0.30, [S₁] = 0.70, α = 0.10 and β = 0. From these initial conditions, we calculated the concentration of all the S states after each flash. Then, we assigned various weighting factors to each S state and multiplied these by the concentration for that S state after each flash. These values were then summed to give the relative theoretical 1/T₂ values after a flash and normalized to the experimental point (corrected for non-specific 1/T₂ value in TRIS-acetone washed chloroplasts) at the 3rd flash. The weighting factors which best fit the 1/T₂ oscillations for pea chloroplasts at pH 6.7 were 2, 1, 1, 3 for S₀, S₁, S₂, S₃ respectively, but, in addition, we had to assume that S₀ in darkness had a value of 4 to
fit the high $1/T_2$ in darkness (Fig. 7). This high value of Mn[II] contribution in darkness may be due to the existence of a special dark-adapted state in isolated chloroplast described as $S_{-1}$ by Velthuys\textsuperscript{30}.  

![Graph showing $T_2$ relaxation and O$_2$ yield flash patterns at pH 6.7.](image)

Figure 7. Theoretical fit to the $1/T_2$ and O$_2$ yield flash patterns at pH 6.7. (a) $1/T_2$ relaxation; (b) O$_2$ yield. Closed circles are experimental data points, $1/T_2$(corr); open circles are theoretical points. For the theoretical fit $[S_0]=0.30$, $[S_1]=0.70$, $\nu=0.10$, $\mu=0$, and weighting factors for $S_0$, $S_1$, $S_2$, $S_3$ are 2, 1, 1, 3 respectively except $S_0=4$ in the dark (After ref. 22.)

Now, the question is: what is the significance of 2, 1, 1, 3 contribution of Mn[II] for $S_0$, $S_1$, $S_2$, $S_3$, respectively. First, it shows heterogeneity of the S states at the time $1/T_2$ measurements are made. The contribution of Mn[II] for $S_1$, being smaller than $S_0$ is in line with Kok et al.'s model that $S_1$ is more oxidized than $S_0$ having more Mn[II] character than $S_1$. However, the following step of $S_1$ to $S_2$ is different because at the time of $1/T_2$ measurement (200-300 μs after the flash) $S_2$ becomes equivalent in terms of Mn[II] contribution. This is possible if the initial oxidation of $S_1$ to $S_2$ is followed by another reaction (not recognized in Kok et al.'s model) in which H$_2$O is used to reduce Kok's $S_2$ (which is more oxidized than $S_1$) to our version of $S_2$ (Figure 8) which has the same oxidizing equivalent as $S_1$ in terms of Mn[II]. The next step from $S_2$ to $S_3$ has more dramatic differences. In Kok's picture, $S_3$ is clearly the most oxidized species among $S_0$ to $S_3$, but in our picture, $S_3$ attains the most reducing character at the time of $1/T_2$ measurement--its Mn[II] character is maximal except, perhaps, for the dark-adapted $S_0[S_{-1}]$. This implies reduction steps following (or simultaneously to) the oxidation steps of the S states. A possible consequence of the reduction steps is that when H$_2$O reduces the S state, protons may be released. This hypothesis is consistent with the recent observations of C.F. Fowler (personal communication) and of A.R. Crofts and coworkers (personal communication) which show that protons may be released in steps much earlier than shown in Kok et al.'s original scheme.

Fowler suggests that the release of H$^+$ is as follows: $S_0\rightarrow S_1$ (0.7 H$^+$); $S_1\rightarrow S_2$ (0 H$^+$); $S_2\rightarrow S_3$ (1.3 H$^+$) and $S_3\rightarrow S_0$ (2 H$^+$). It is possible to produce a model
which would have similar H⁺ releases, if not identical, and retain the 2, 1, 1, 3 picture (Fig. 8). If we assume that Mn[II] contributions, that we have been discussing, are proportional to both the concentration of Mn[II] and the number of exchangeable protons, then 2, 1, 1, 3 pattern would imply a Mn[II] concentration of 0.5, 0.3, 0.3, 1.5 (or, 1.67, 1.0, 1.0, 5.0) for S₀, S₁, S₂ and S₃, respectively. This does not change the model significantly except that S₃ has still greater Mn[II] contributions. We have no way yet to make the distinction between the "pure" effect of Mn[II] concentration and the combined effect of Mn[II] concentration and the number of exchangeable protons.

A glaring difference between the 2, 1, 1, 3, model and the experimental data is the poor fit at the 8th flash. This needs further analysis.

Fig. 8 assumes that there are 4 Mn atoms, and that S₀ and S₁ are mixtures of different redox states of Mn (see ref. 19). A titration of 1/T₁ as a function of [TPB] shows three plateaus as if it reflects the four reducible Mn[II] in the system. The chemistry of H₂O oxidations is unknown, and, therefore, in our scheme only the charges have been balanced, the species shown are only symbolic. Data of Fig. 6 can be simulated by proposing a 0, 1, 1, 3 model. Other variations in the patterns can be generated by altering the weighting factors. We, thus, hope that the extension of the present approach would lead to a further understanding of the O₂ evolving mechanism of photosynthesis.
5. SUMMARY AND CONCLUDING REMARKS

(1) The water proton relaxation rates monitor bound Mn[II] in chloroplast membranes. (2) A mixture of manganese oxidation states exists in dark-adapted chloroplasts. (3) Bound manganese participates directly in the S intermediate, the precursor to photosynthetic O₂ evolution. (4) A special state of the S intermediate, having a high Mn[II] contribution, exists in the dark-adapted isolated chloroplasts. (5) The cycling of the various states of the S intermediate (Sₙ, where n = 0...4) in the light involves changes in the manganese oxidation states. (6) Water, apparently, donates electrons to the S intermediate in reactions prior to the release of O₂, at times proton relaxation rates are measured (~200-300 μs after the flash).

ACKNOWLEDGEMENTS

We thank Drs. Paul G. Schmidt and H. S. Gutowsky for collaboration in this research. Results summarized here will be published jointly with these colleagues in separate publications²¹,²². We are thankful to research grants from the National Science Foundation (to G. and H.S.G.), the National Institutes of Health (to P.G.S.) and the Office of Naval Research (to H.S.G.). T.W. was supported by a U.S. Public Health Service Training Grant in Cellular and Molecular Biology (GM-7283-1 Sub. Proj. -604) and G. by a National Science Foundation Grant (PCM 76-11657).

REFERENCES