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THE ROLE OF MANGANESE IN THE OXYGEN EVOLVING MECHANISM OF PHOTOSYNTHESIS

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INTRODUCTION

It is generally accepted that the ultimate source of O_2 in photosynthesis is water, although how O_2 is released from water is not known. In the past, we have made several attempts to understand the O_2 evolving side of system II but did not have much success.

First, we prepared antibodies against chloroplast suspension[1] or against an extract from frozen and thawed preparations of washed thylakoids[2]; these antibodies were tested for their specificity against the O_2 evolving side of photosynthesis. We succeeded in obtaining one preparation[2] which gave us specific inhibition of the O_2 evolving side, but the inhibition was only upto 30%. This could be taken to confirm what was already surmised before, that a protein may be somehow involved in O_2 evolution and that the O_2 evolving components may lie on the inner side of the thylakoid membrane (see Trebst[3], Fowler and Kok[4] and Babcock and Sauer[5]).

Next, we started with the hope that bicarbonate (or CO_2) may have something to do with O_2 evolution, even if in a catalytic fashion (see Metzner[6]). Initially, we thought that the site of stimulation of the Hill reaction by CO_2 was located on the water side (see Stemler and Govindjee[7-9]) but later experiments did not support[10] this conclusion. In fact, all our efforts[11] to find an effect on the water side failed. On the other hand, we were able to show that a major effect of CO_2 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 chlorophyll (Chl) a fluorescence yield, after a flash, was reversibly slowed down by about five fold in CO_2-depleted chloroplasts (Jursinic et al.[11]), and this qualitatively explained the reversible slowing down[12] of the relaxation of S_n' to S_{n+1} states involved in O_2 evolution kinetics (S being the charge accumulator for oxygen evolution, see Joliot and Kok[13]). More recently, we have shown[14,15] that the major effect of CO_2 depletion is in the electron flow from R(or B), the secondary two electron acceptor, to the PQ pool. The ^{14}C binding studies of Stemler[16] also support a site of CO_2 effect on the electron acceptor side of system II. Independent biochemical studies of Khanna et al.[17] provided further confirmation that the bicarbonate (or CO_2) effect lies somewhere between Q and the PQ pool. Thus, if CO_2 must play a direct part in O_2 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 O_2 evolution (see review[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 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.[19]). When thylakoids were depleted of their bound manganese either by alkaline TRIS or NH_2OH/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.[20]). These maxima correspond with those in the O_2/flash as a function of flash number in a series of flashes. We, thus, suggested[20] that $1/T_2$ monitors the O_2 evolving mechanism. Significant differences in the $1/T_2$ and O_2 patterns were not explained at that time.

In this paper we present a brief review of our recent data[21,22] on proton relaxation rates (PRR) showing (1) the relationship of $1/T_1$ and $1/T_2$ to manganese concentration and 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 O_2 evolution patterns as a function of flash number under a variety of conditions which show that O_2 evolution can be uncoupled from the intermediate monitored by PRR. Finally, we present a hypothetical manganese model of O_2 evolution.

MATERIALS AND METHODS

Chloroplast membrane fragments were prepared by homogenizing leaves in a medium consisiting of 50 mM N-2-hydroxyethylpiperazineN-2 ethane solfonic 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 filteration 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.

O_2 evolution/flash was measured on a Joliot-type electrode. The light source was a Phase-R Model DL 2100 A dye laser (λ, 590 nm) having a pulse width of 0.6 μs at half height, terminating in about 2 μs. 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.[21] for details.)

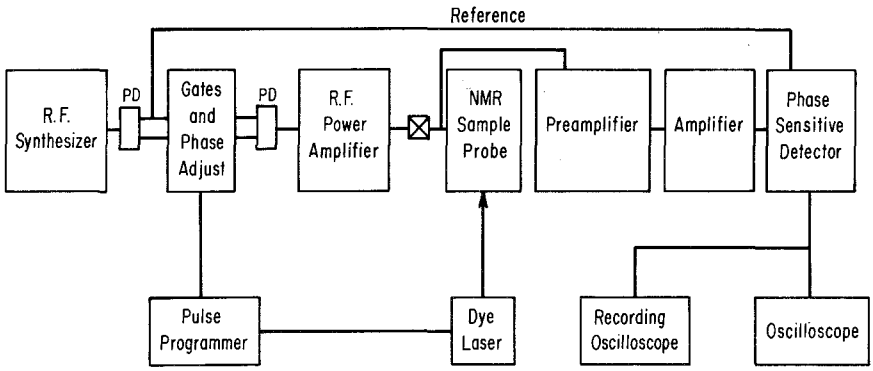

Fig. 1 Generalized Block Diagram of a Pulsed NMR Spectrometer. The rf transmitter consists of rf synthesizer, gating and phase adjust unit and rf power amplifier. The rf receiver consists of appropriate amplifiers and a phase sensitive detector. PD refers to power divider. The NMR sample probe is positioned in strong magnetic field. A dye laser (λ, 590 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 (τ). The $1/T_1$ was calculated from a plot of $\ln \frac{Mo-Mz(\tau)}{2\ Mo}$ as a function of τ, where Mo is the equilibrium magnetization and $Mz(\tau)$ is the magnetization at time τ 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 τ and then a series of 2,100 180° pulses spaced 2τ apart (τ= 500 μs) were given. The $1/T_2$ was calculated from the decay of spin echo envelopes with time (τ); the slope of $\ln \frac{Mz(\tau)}{Mo}$ as a function of τ is proportional to $1/T_2$.

RESULTS AND DISCUSSION

1. Proton Relaxation Rates Monitor Bound Manganese

Using the method of Chen and Wang[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 O_2 evolution is thus established. It appears that $1/T_1$ monitors mainly the loosely bound manganese related to O_2 evolution (c.f. ref. 18), where $1/T_2$ may monitor the total manganese content of the membrane due to greater non-site specific contributions to $1/T_2$ (see Wydrzynski[23] for further details).

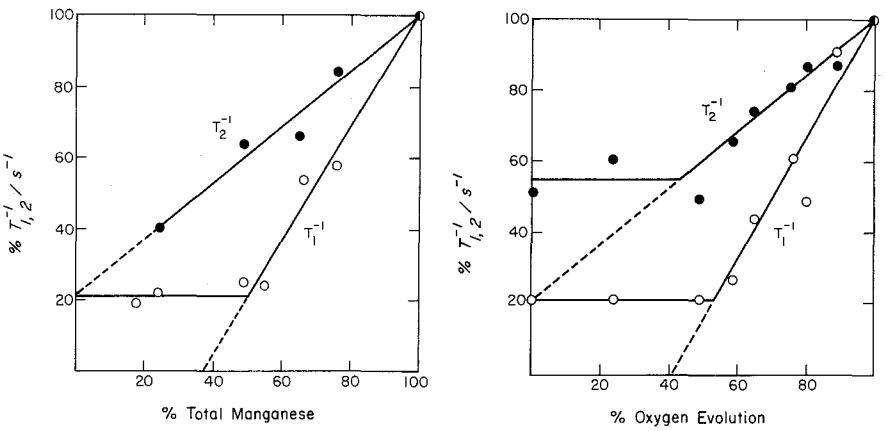

Fig. 2 (left):Plot of Percent $1/T_1$ and $1/T_2$ versus percent total Mn content. (right):Plot of Percent $1/T_1$ and $1/T_2$ versus percent O_2 activity. Mn extraction was achieved by incubating pea chloroplasts for 2 hr in dark at 4°C in HEPES buffer medium containing $MgCl_2$ at the following [Mg]/[Chl] 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 O_2 evolution for control was 120 μmoles O_2/mg Chl-hr. (After ref. 21.)

2. Proton Relaxation Rates Monitor Bound Mn[II]

Fig. 3 shows dependence of $1/T_1$ and $1/T_2$ as a function of NMR frequency. The $1/T_1$ shows a broad peak in the 10-20 MHz range (open circles, lower curve), whereas $1/T_2$ 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 $1/T_1$ and $1/T_2$ are related to the dipolar correlation time (τ_c) by Solomon-Bloembergen equation[25]. The major molecular processes which lead to dipolar interactions include the electron spin relaxation ($1/\tau_s$), rotational motions ($1/\tau_r$) and the rate of chemical changes ($1/\tau_m$). The $1/\tau_c$ is related to these processes as follows: $\frac{1}{\tau_c} = \frac{1}{\tau_s} + \frac{1}{\tau_r} + \frac{1}{\tau_m}$. For macromolecules, τ_r is long ($\sim 10^{-6}$ s) and thus

$1/\tau_r$ is insignificant with respect to $1/\tau_s$ and $1/\tau_m$. If τ_s dominates τ_c, one expects (see ref. 25) the frequency behavior as shown in Fig. 3 since τ_s itself changes with frequency. The frequency dependence of τ_s is given by the Bloembergen and Morgan equation: $\frac{1}{\tau_s} = B\left(\frac{\tau_v}{1+\omega_s^2\tau_v^2} + \frac{4\tau_v}{1+4\omega_s^2\tau_v^2}\right)$ where, τ_s is electronic Larmor frequency, B is a constant related to the zero field splitting parameters and τ_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 $B = 0.9\times10^{19}(\text{rad/s})^2$. These values compare favorably with those for Mn[II]-pyruvate kinase systems, $\tau_v = 14\pm4\times10^{-12}$ s, $\tau_m = 0.4\pm1.04\times10^{-8}$ s, and $B = 0.8\pm0.1\times10^{19}(\text{rad/s})^2$ (after Navon[26]). 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.

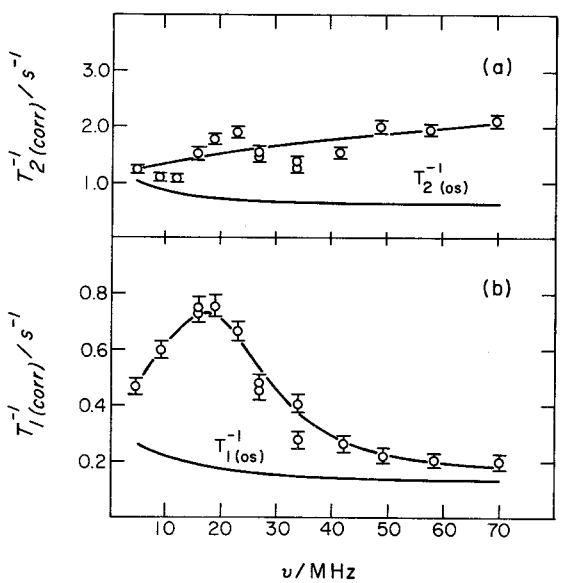

Fig. 3. "Best" Fit Theoretical Curves to the Frequency Dependence of the Relaxation Rates for Dark-Adapted Chloroplast Membranes. (a) $1/T_2$ relaxation ; (b) $1/T_1$ relaxation; $1/T_{1,2(corr)} = 1/T_{1,2(obs)} - 1/T_{1,2(T-A)}$. Rates were measured at room temperature (23-25°C) and normalized to the same Mn concentration at each frequency. $1/T_{1,2(os)}$ 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[28], 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.

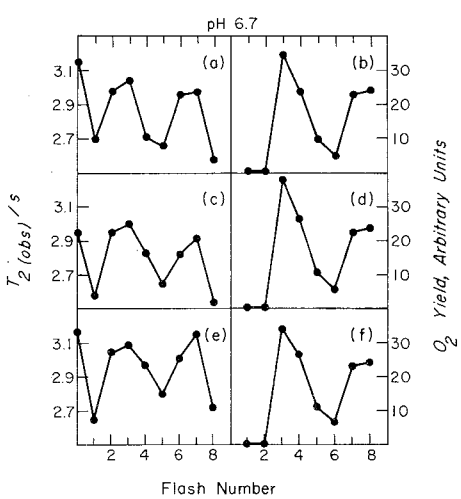

Fig. 4 (left) Observed $1/T_2$ Rates and O_2 Yield Measured as a Function of Saturating Flashes of Light for three samples of Pea Chloroplast Membranes. The $1/T_2$ and O_2 measured after each flash in a pulse sequence. Saturating light flashes obtained from a pulsed dye laser (λ, 590 nm) were spaced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

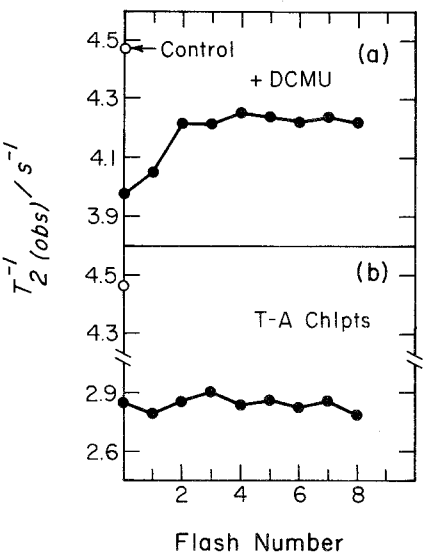

Fig. 5 (right) Effect of DCMU and Manganese Extraction on $1/T_{2(obs)}$ Flash Pattern. (a) [DCMU]/[Chl]=0.089; (b) TRIS-acetone washed chloroplasts. Flash procedure and conditions for pea chloroplast in Fig. 4 were used. (After ref. 22.)

Note also that $1/T_2$ in dark is decreased by DCMU. Data reported by Wydrzynski[23] 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_2OH) or carbonylcyanide 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_2OH 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):

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4 \longrightarrow S_0$$

$$2H_2O \longrightarrow O_2 + 4H^+$$

where, the subscripts refer to the number of oxidizing equivalents on the intermediate S; PRR may be monitoring the S 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[20] the flash number dependance 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 upto 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).

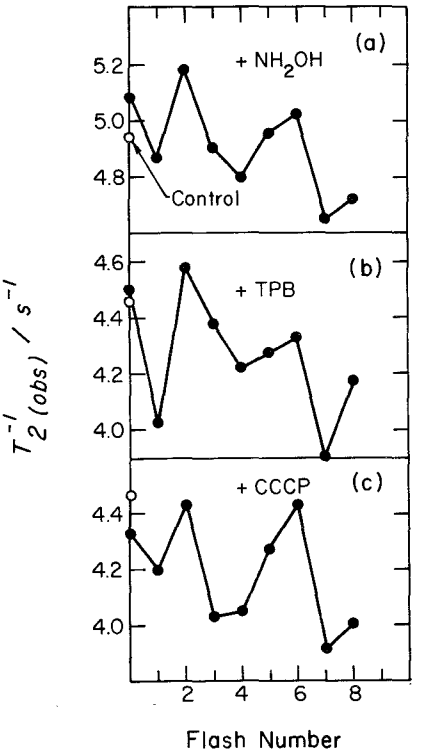

Figure 6. $1/T_{2(obs)}$ Flash Pattern in the Presence of NH_2OH, TPB and CCCP. (a) $[NH_2OH]/[Chl]=0.44$; (b) $[TPB]/[Chl]=0.016$; (c) $[CCCP]/[Chl]=0.089$. The $1/T_2$ for the dark-adapted controls are shown with open circles. Flash procedure and conditions for pea chloroplasts in Figure 4 were used. (After ref. 22.)

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 O_2 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 S_0 and S_1, α (misses) and β (double hits) from O_2/flash as a function of flash number for a sample at pH 6.7. This gave us $[S_0] = 0.30$, $[S_1]= 0.70$, $\alpha = 0.10$ and $\beta = 0$. 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 $1/T_2$ values after a flash and normalized to the experimental point (corrected for non-specific $1/T_2$ value in TRIS-acetone washed chloroplasts) at the 3rd flash. The weighting factors which best fit the $1/T_2$ oscillations for pea chloroplasts at pH 6.7 were 2, 1, 1, 3 for S_0, S_1, S_2, S_3 respectively, but, in addition, we had to assume that S_0 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[30].

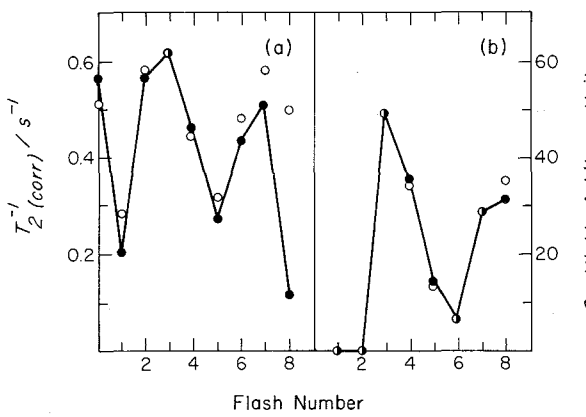

Figure 7. Theoretical Fit to the $1/T_2$ and O_2 Yield Flash Patterns at pH 6.7. (a) $1/T_2$ relaxation; (b) O_2 yield. Closed circles are experimental data points, $1/T_2$(corr); open circles are theoretical points. For the theoretical fit $[S_0]=0.30$, $[S_1]=0.70$, $\alpha=0.10$, $\beta=0$, and weighting 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--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_2O 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--→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_2O 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--→S_1 (0.7 H^+); S_1→S_2 (0 H^+); S_2→S_3 (1.3 H^+) and S_3→S_0 (2 H^+). It is possible to produce a model

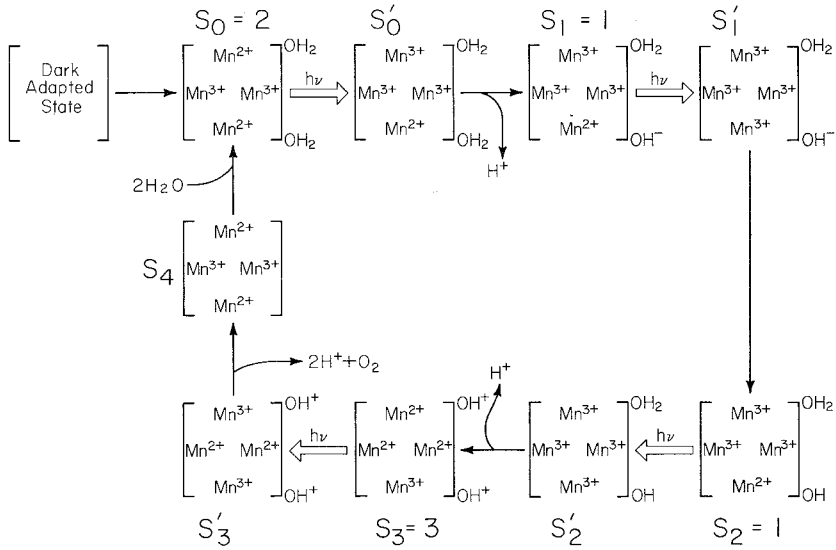

Figure 8. Working Hypothesis for Oxygen Evolution in Photosynthesis.

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 S_0, S_1, S_2 and S_3, respectively. This does not change the model significantly except that S_3 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 S_0 and S_1 are mixtures of different redox states of Mn (see ref. 19). A titration of $1/T_1$ as a function of [TPB] shows[31] four plateaus as if it reflects the four reducible Mn[II] in the system. The chemistry of H_2O 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 O_2 evolving mechanism of photosynthsis.

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 O_2 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 (S_n, 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 O_2, at times proton relaxation rates are measured (~200-300 μs after the flash).

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