MANGANESE AND CHLORIDE: THEIR ROLES IN PHOTOSYNTHESIS

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Introduction

Manganese [4] and chloride [9] ions have long been implicated to play important roles in oxygen evolution during photosynthesis. Chloride-free chloroplasts and thylakoid membranes depleted of bound manganese fail to evolve O_2, but are capable of electron flow from artificial donors (such as diphenylcarbazide, feeding electrons to an intermediate (Z) between the oxygen evolving intermediate (S) and the reaction centre II (P 680)) to Hill oxidants. There has, however, been no experimental observation to show functional changes in either manganese or chloride ions during oxygen evolution. Manganese and/or chloride ions could simply be parts of a structural component.

Joliot and coworkers and Kok and coworkers (see ref. [10]) suggested the existence of a charge-accumulating species (S), which according to Kok and coworkers, undergoes changes as follows (also see Mar and Govindjee, [11]):

$$S_0 \xrightarrow{h\nu} S_0' \longrightarrow S_1 \xrightarrow{h\nu} S_1' \longrightarrow S_2 \xrightarrow{h\nu} S_2' \longrightarrow S_3 \xrightarrow{h\nu} S_3' \longrightarrow S_4 \qquad (1)$$

$$S_4 \longrightarrow S_0 \quad (O_2 + 4H^+ \quad 2H_2O)$$

where each S_{n+1} state is assumed to differ from the previous S_n state by the accumulation of an additional oxidizing equivalent. The prime states refer to the initial

states created as a consequence of light reactions, and $S_n' \longrightarrow S_{n+1}$ steps refer to the dark relaxation steps which lead to the recovery of the reaction centre complex in a state that can undergo a light reaction. This scheme predicts a periodicity of four in O_2 evolution/flash when chloroplasts are given a series of brief light flashes separated by dark periods long enough to allow $S_n' \longrightarrow S_{n+1}$ reactions, but not long enough to allow deactivation of S states. The periodicity of 4 in the O_2 pattern was first observed by Joliot and coworkers (see ref. [10]), and the maximum was in the 3rd flash suggesting that dark-adapted chloroplasts start mainly from the S_1 state. There are no other steps in photosynthesis which oscillate with a periodicity of four. Thus, an oscillation of 4 is restricted to the O_2 evolving side of photosynthesis. (However, an oscillation with a period of two has been located on an intermediate R (or B) on the system I side of photosystem II [2,7,18].)

To our knowledge, only two other signals, besides O_2, oscillate with a period of 4 in brief flashes of light: (1) transverse (spin-spin) relaxation rates ($1/T_2$) of water protons (not to be confused with H^+ evolved in the scheme shown above, but all protons whether as H^+ or in H_2O etc. in the sample which are related to bound Mn) [25]; and (2) an absorbance change in the near ultraviolet region [15]. The chemical identity of the latter signal is not yet known.

In this paper, we shall review our recent observations [20,22-24] which strongly support the thesis that water proton relaxation rates monitor changes in contribution of paramagnetic Mn(II) as related to the O_2 evolving mechanism. In addition, data will be presented to show the possible role of halogen ions (i.e. chloride) in O_2 evolutior

Proton relaxation measurements

In order to appreciate our conclusions, it is necessary

to provide a brief background of the measurements of the water proton relaxation rates $1/T_1$ (longitudinal or spin-lattice) and $1/T_2$ (transverse or spin-spin) [5,6]. When a sample is placed in a strong magnetic field, $\overline{H_o}$ parallel to the z axis (see Fig. 1 (a)), the individual nuclear magnetic dipoles tend to align with it and precess about the z axis. In a rotating frame of reference (x', y', z', Fig. 1 (b)) moving at the precessional frequency of the nuclear dipoles, the individual dipoles appear stationary to an observer rotating along with the frame in the x'y' plane. The vector sum of all nuclear dipoles gives the net magnetization, $\overline{M}$, of the spin system. Since more dipoles are aligned with $\overline{H_o}$ rather than against it, $\overline{M}$ appears parallel along the +z' direction (Fig. 1 (c)). If another field, $\overline{H_1}$, is applied orthogonally to $\overline{H_o}$ in the x' direction (see Fig. 1 (d)), $\overline{M}$ will move about $\overline{H_1}$. If $\overline{H_1}$ is app-

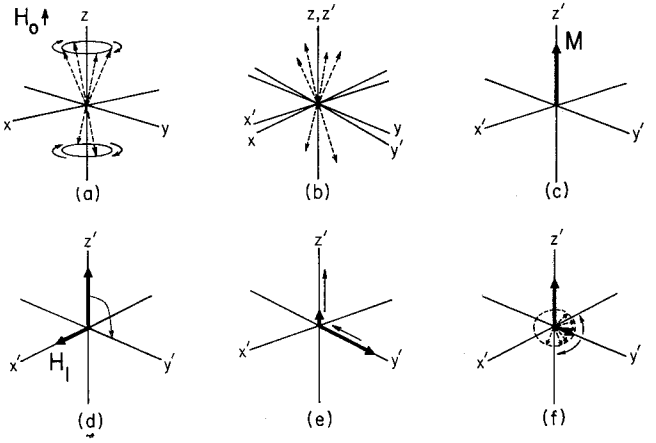

Fig. 1. Definition of $1/T_1$ and $1/T_2$ relaxation in the rotating frame. (a) Precession of nuclear spins (I=1/2) about the applied field, $\overline{H_o}$. $\overline{H_o}$ is defined along the z-axis. (b) Spins appear stationary in the rotating frame (x',y',z'). (c) Net magnetization, $\overline{M}$, of the nuclear spins. (d) Application of an rf field, $\overline{H_1}$, in the rotating frame. (e) After rf is turned off, $\overline{M}$ relaxes back to equilibrium condition. The growth of $\overline{M}$ along the +z axis is called longitudinal relaxation and is characterized by a time constant T_1 while the decay of $\overline{M}$ along the y axis is called the transverse relaxation characterized by a time constant T_2. (f) Contribution of the dephasing of the individual nuclear dipoles in the x'y' plane to $1/T_2$. (See ref. [5]).

lied for a long enough time $\overline{M}$ will tip to the x'y' plane (this is called a 90° pulse). After $\overline{H_1}$ is turned off, then $\overline{M}$ tends to return to its equilibrium position *via* relaxation mechanisms. The rate of build-up of $\overline{M}$ along the z' axis is defined as $1/T_1$ or the longitudinal relaxation while the decay of $\overline{M}$ along the y' axis (the direction of the detection system) is defined as $1/T_2$ or the transverse relaxation (Fig. 1 (e)). The decay of $\overline{M}$ along the y' axis arises from a dephasing of the individual nuclear dipoles in the x'y' plane (Fig. 1 (f)); thus, $1/T_2$ is always $\geq 1/T_1$.

In practice $1/T_1$ is measured by the inversion recovery method, where a 180°, τ, 90° rf pulse sequence is applied. In the first 180° pulse, $\overline{M}$ is inverted to the -z' direction. The succeeding 90° pulse then tips $\overline{M}$ to the y' axis, the direction in which the magnetization signal can be measured. The extent of build-up of $\overline{M}$ along the z' axis back to its equilibrium position is dependent upon the time, τ, between the 180° and 90° pulse. The slope of a plot of $\ln \frac{\overline{M_o}-\overline{M_z}(\tau)}{2M_o}$ *versus* τ is proportional to $-1/T_1$, where $\overline{M_o}$ is the equilibrium value of $\overline{M}$ and $M_z(\tau)$ is the value of the z component of M at a time τ.

The measurement of $1/T_2$ is somewhat more complex in that field inhomogeneities in $\overline{H_o}$ also lead to dephasing in the x'y' plane and thus obscure the true $1/T_2$ rate. The Carr-Purcell-Meiboom-Gill pulse sequence overcomes errors due to field inhomogeneities. This method can be described as a 90°, τ, 180°, 2τ, 180°, 2τ...pulse sequence. The first 90° pulse tips $\overline{M}$ to the +y' direction (Fig. 1). $\overline{M}$ starts to decay because of $1/T_2$ processes and field inhomogeneities. The succeeding 180° pulse inverts the dipoles to the -y' axis. The dipoles which dephase due to field inhomogeneities tend to refocus back along the y' axis while the dephasing due to $1/T_2$ processes continues. By applying a series of 180° pulses until $1/T_2$ relaxation is complete, errors due to field inhomogeneities are

essentially overcome and the decay of the magnetization signals along the y' axis gives the true $1/T_2$. The Meiboom-Gill phase modification of the H_1 pulse after the initial 90° pulse is used to overcome pulse width errors. The slope of a plot of $\ln \frac{\overline{M}_z(\tau)}{M_o}$ *versus* τ gives $-1/T_2$.

Influence of paramagnetic ions on proton relaxation rates

Paramagnetic ions are known to influence strongly both $1/T_1$ and $1/T_2$ [5]. Neglecting the contributions outside the first coordination sphere of paramagnetic ion-H_2O complexes, the paramagnetic contribution to the water proton relaxation rates ($1/T_{1,2(P)}$) is given as:

$$\frac{1}{T_{1,2(P)}} = \frac{pq}{T_{1,2(M)}+\tau m} \tag{2}$$

where, p is the mole fraction of paramagnetic ion (i.e., [Mn]/55.6 for Mn-HOH complex), q is the number of bound nuclei (i.e., $6 \geq q \geq 0$ for Mn-HOH), $T_{1,2(M)}$ are the relaxation times for the bound nuclei and τ_m is the lifetime of the nuclei bound to paramagnetic species. The $1/T_{1,2(M)}$ are related to the distance (r) between the paramagnetic ion and the nuclei, the nuclear Larmor frequency (ω_I), and the dipolar correlation time (τ_c) by the following simplified Solomon-Bloembergen equation:

$$\frac{1}{T_{1(M)}} = \frac{2}{15} \frac{S(S+1)(\gamma_I)^2 g^2 \beta^2}{r^6} \left[\frac{3\tau_c}{1+\omega_I^2 \tau_c^2}\right] \tag{3a}$$

$$\frac{1}{T_{2(M)}} = \frac{1}{15} \frac{S(S+1)(\gamma_I)^2 g^2 \beta^2}{r^6} \left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2 \tau_c^2}\right] \tag{3b}$$

where, S is the total electron spin, γ_I is the nuclear magnetogyric ratio, g is the electronic g factor, β is the Bohr magneton, and other symbols are as defined above (The hyperfine (contact) contribution has been neglected in the above equations.)

The $1/\tau_c$ itself is a function of the molecular processes which modulate the dipolar fields:

$$\frac{1}{\tau_c} = \frac{1}{\tau_s}\frac{1}{\tau_m} + \frac{1}{\tau_r} \qquad (4)$$

where, τ_s = electronic spin relaxation time, τ_m = lifetime of the paramagnetic ion water complex and τ_r =rotational correlation time. In macromolecular and membrane systems, $1/\tau_r$ is negligible and thus $1/\tau_s$ and/or $1/\tau_m$ will govern the relaxation rates. When τ_s dominates all other relaxation processes, $1/T_1$ and $1/T_2$ follow a predicted frequency dependence [5] which is given by the Bloembergen-Morgan equation:

$$\frac{1}{\tau_s} = B\left[\frac{\tau_v}{1+\omega_s^2\tau_v^2} + \frac{4\tau_v}{1+4\omega_s^2\tau_v^2}\right] \qquad (5)$$

where, ω_s is the 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 by molecular collisions.

Proton relaxation rates and manganese

(1) Conditions which are known to remove most of the bound manganese from thylakoid membranes (NH_2OH-EDTA treatment [14], TRIS-washing [26], TRIS-acetone washing [27], heat treatment + EDTA) decrease $1/T_1$ of chloroplasts significantly. This is shown in Table 1. (EDTA itself has some effect because it binds paramagnetic ions.) These data indicate that the proton relaxation rates monitor bound Mn.

(2) As one replaces Mn with Mg according to Chen and Wang [3], O_2 evolution decreases. However, O_2 evolution becomes nil even when about 1/3 of Mn is still in the chloroplast. Figs 2 (left) and (right) show that as O_2 evolution decreases due to lowered Mn, both $1/T_1$ and $1/T_2$ also decrease but then they attain a fixed background value at lowered rates of O_2 evolution. The details of

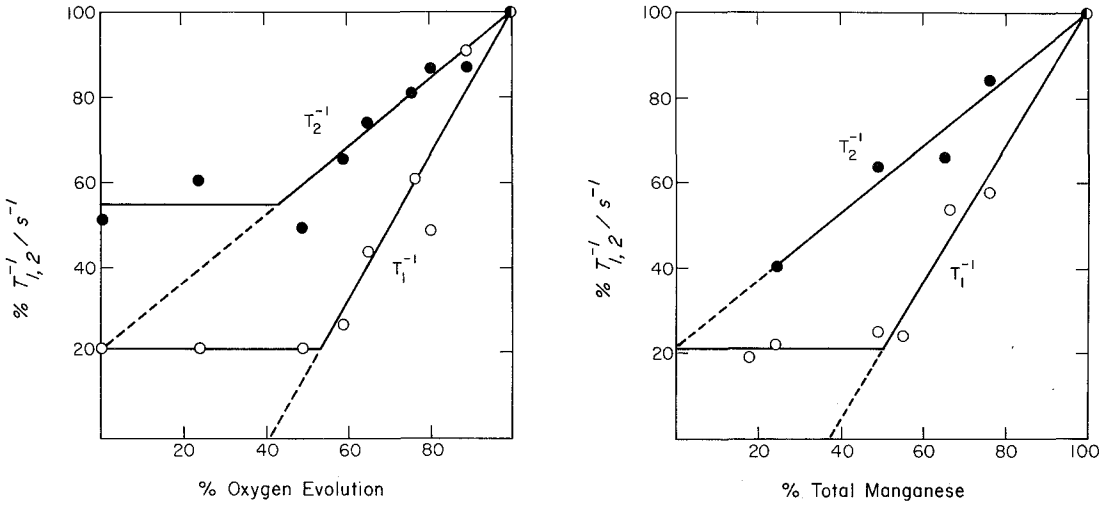

Fig. 2. (Left). Plot of percent $1/T_1$ and $1/T_2$ *versus* percent O_2 activity. (Right). Plot of percent $1/T_1$ and $1/T_2$ *versus* percent Mn content of the membrane. Mn extraction was achieved by incubating pea chloroplasts for 2 h in dark at 4°C in HEPES buffer medium (50 mM HEPES, pH 7.5, 400 mM sucrose and 10 mM NaCl) 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 the pellet resuspended to 3 mg Chl/ml in HEPES buffer medium. Mn content was determined by neutron activation analysis. For the control the manganese content was 0.62±0.03 μg Mn/mg Chl and the rate of O_2 evolution was 120 μmoles O_2/mg Chl-hr. (After refs. [20,23].)

TABLE 1

Effect of various treatments which alter the environment of native bound manganese on the $1/T_1$ of chloroplast membranes. (After ref. [20].)

Condition	T_1^{-1}/s^{-1}
Spinach	
(a) Washed control	1.04
(b) NH_2OH-EDTA washed	0.54
(c) TRIS washed	0.41
+ 10^{-4} M EDTA	0.74
Peas	
(a) Washed control	0.82
(d) TRIS-acetone washed	0.21

(a) Spinach or pea chloroplasts were washed in HEPES buffer medium (50 mM HEPES, pH 7.5, 400 mM sucrose and 10 mM NaCl) and resuspended to a final concentration of 3 mg Chl/ml. $1/T_1$ was measured at 27 MHz, at 25°C.

(b) Chloroplasts were incubated in HEPES buffer medium containing 2 mM $MgCl_2$, 1 mM EDTA and 3 mM NH_2OH for 20 min in dark at 25°C, centrifuged and resuspended in HEPES buffer medium, according to Ort and Izawa [14].

(c) Chloroplasts were incubated in 0.8 M TRIS, pH 8.2, for 20 min in dark at 4°C, centrifuged and resuspended in HEPES buffer medium, according to Yamashita and Butler [26].

(d) Chloroplasts were incubated in 0.8 M TRIS, pH 8.2, 20% acetone (v/v) for 20 min at 4°C, centrifuged and resuspended in HEPES buffer medium, according to Yamashita and Tomita [27].

these results are not easily understood but two points are clear (i) in the region of 50-100% Mn content, there is a linear relationship between $1/T_1$, $1/T_2$, O_2 evolution and Mn content; and (ii) there are some background $1/T_1$ and $1/T_2$ signals.

(3) Fig. 3 (a) shows $1/T_1$ as a function of frequency of the rf pulse from several different samples of chloro-

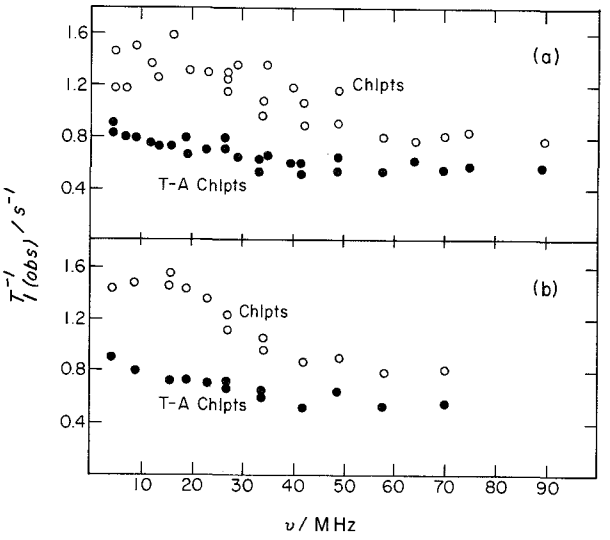

Fig. 3. Frequency dependence of $1/T_{1(obs)}$ for dark-adapted chloroplast membranes (Chlpts). (a) Uncorrected observed rates for several different samples. (b) Observed rates normalized to the same Mn concentration. Samples of pea chloroplasts contained 3 mg Chl/ml. T-A Chlpts refers to TRIS-acetone washed chloroplasts in which the loosely bound fraction of Mn is removed. $1/T_1$ was measured at room temperature (23-25°C). (After refs. [20,23]).

plasts. We can see that the scatter in data is very large. However, when corrections are made for differences in [Mn], much of the scatter is smoothed out (Fig. 3 (b)). The reduction in the scatter upon correction for [Mn] again shows the relationship between $1/T_1$ and [Mn].

Relationship of proton relaxation rates to Mn[II]

For normal chloroplasts in Fig. 3 (b), a broad peak in the 10-25 MHz region is apparent whereas only a flat curve is obtained for TRIS-acetone washed chloroplasts in which the large pool of Mn is removed. The broad peak in the control curve indicates that the dipolar correlation time governing the rates is itself frequency dependent and hence is dominated by electronic relaxation [5]. Since this frequency dependence is absent in TRIS-acetone (T-A)

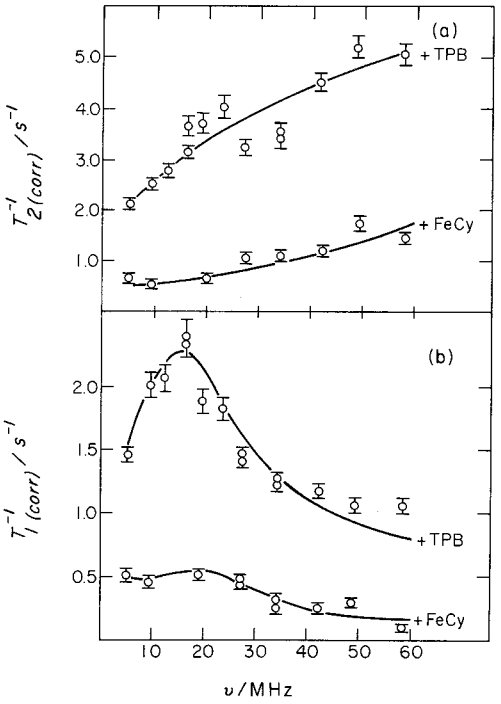

Fig. 4. "Best fit" theoretical curves to the frequency dependence of the relaxation rates for dark-adapted chloroplast membranes in the presence of redox reagents. (a) $1/T_2$ relaxation; (b) $1/T_1$ relaxation. $1/T_{1,2(corr)} = [1/T_{1,2(obs)} - 1/T_{1,2(T-A)}]$. T-A = TRIS-acetone washed chloroplasts. Rates were measured at room temperatures and normalized to the same Mn concentration at each frequency. Either 5 mM TPB or 50 mM FeCy was used. Pea chloroplasts at 3 mg Chl/ml were used. (After refs. [20,23]).

washed chloroplasts, this contribution must arise from the large pool of Mn. Analysis of Fig. 3 (b) (see refs [8,23]) led us to the conclusion that $1/T_1$ and $1/T_2$ monitor mainly Mn[II]. This analysis was extended to chloroplasts treated with the reductant tetraphenylboron (TPB) and the oxidant ferricyanide (FeCy). These results are shown in Fig. 4. Both $1/T_1$ and $1/T_2$ were corrected by subtracting off the value of $1/T_1$ and $1/T_2$ for TRIS-acetone washed chloroplasts to obtain the contributions of

TABLE 2

"Best Fit" NMR parameter values for chloroplast membranes and other Mn(II) systems. (After ref. [20].)

	$\tau_v/s\times10^{12}$	$\tau_m/s\times10^{8}$	$B/(rad\ s^{-1})^2\times10^{-19}$	$\tau_s/s\times10^{9}$
Chlpts	20	2.2	0.90	1.11
Chlpts+TPB	20	1.0	0.9	1.11
Chlpts+FeCy	9.3	0.1	1.1	1.96
Aqueous Mn(II)	[a] 2.1	2.3	10	0.95
Mn(II)-carboxy-peptidase A	[b] 7±0.6	0.25±0.3	3.1±0.4	0.92
Mn(II)-pyruvate-kinase	[a] 6	0.5±0.1	1.46	2.28
	[b] 14±4	0.4±0.04	0.8±0.1	1.78

[a] Bloembergen and Morgan [1].

[b] Navon [13].

[c] Reuben and Cohn [16].

the large pool of Mn. The $1/T_2$ increases with frequency, whereas $1/T_1$ shows a peak in the 10-20 MHz range (open circles) as has been observed for control chloroplasts. The solid lines are theoretical lines obtained by fitting the various NMR parameters to theoretical equations. A master equation was obtained by combining equations (5),(4), and (3). Then, at each frequency, B, τ_v and τ_m were varied until curves $1/T_1$ and $1/T_2$ were obtained which fit the experimental points (note that $1/\tau_r$ was assumed to be negligible in our case); an outersphere contribution had to be also included to obtain the "best" fits shown by solid lines in Fig. 4.

Table 2 gives the calculated parameter values. τ_v ranged from 9.3×10^{-12}s (FeCy) to 20×10^{-12}s (TPB), B from $.1\times10^{19}$ rad·s^{-1} (FeCy) to 1.0×10^{19} rad·s^{-1} (TPB), and τm from 0.1×10^{-8}s (FeCy) to 1×10^{-8}s (TPB). These values are not too different from those of control chloroplasts and are in the same order of magnitude as for other Mn[II]

systems [1,13,16,19]. We note that the correlation time (τ_s) for Mn[II] calculated to be ~10^{-9}s from Eq. 5 (at zero field) is two orders of magnitude larger than for Mn[III] and high spin iron [5]; hence, these ions have only a small effect on the proton relaxation rates in comparison to Mn[II[.

Proton relaxation rates and oxygen evolving mechanism

Wydrzynski *et al.* [25] discovered the oscillations in $1/T_2$ as a function of flash number shown in Fig. 5 (top) for spinach chloroplasts. Fig. 5 (bottom) shows the O_2

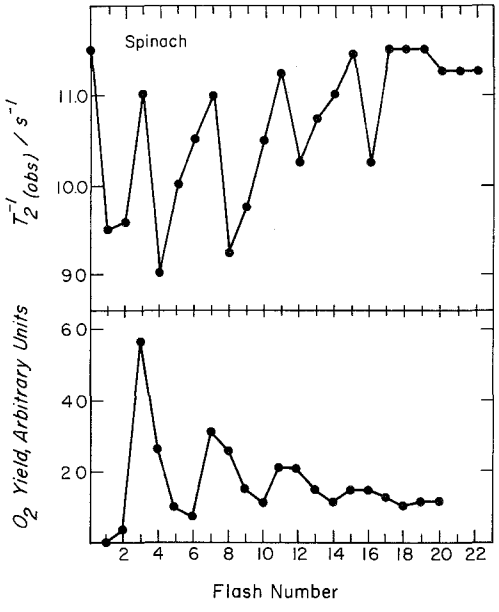

Fig. 5. $1/T_{2(obs)}$ and O_2 yield flash patterns for spinach chloroplast membranes. (Top) $1/T_2$ relaxation; (bottom) O_2 yield. $1/T_2$ was measured at 27 MHz, 25°C, on sample aliquots in HEPES buffer medium, pH 7.5, at 3 mg Chl/ml while O_2 was measured on sample aliquots at 1 mg Chl/ml. Flash procedure: Flashes spaced 2 s apart were given in sequence from 1 to 22 flashes. The Carr-Purcell-Meiboom-Gill pulse train was initiated at the last flash in the sequence. A 7 min dark period was allowed between each flash sequence. The same flash procedure was used to measure the O_2 yield. Light flashes were obtained from a xenon strobe flash lamp. The results in the top figure are from ref. [25] and in the bottom figure from ref. [20].

flash pattern for spinach chloroplasts under identical conditions. The following similarities in the two curves are observed: (a) peaks at 3rd, 7th, etc. flashes; (b) periodicity of four; and (c) damping of oscillations at about the 17th flash. However, there are several dissimilarities: (a) high dark $1/T_2$ *versus* no O_2 in darkness; (b) upward trend in $1/T_2$ pattern and downward trend in O_2; (c) minima are at 4th, 8th etc. flashes in $1/T_2$ and at 6th, 10th etc. flashes in O_2; and (d) when $1/T_2$ increases from 4th to 5th to 6th flashes, O_2 yield decreases. Clearly, the two ($1/T_2$ and O_2) do not monitor exactly the ame thing. However, the periodicity of 4 establishes the relationship of $1/T_2$ to the water side of pigment system II. The differences could be due to several reasons

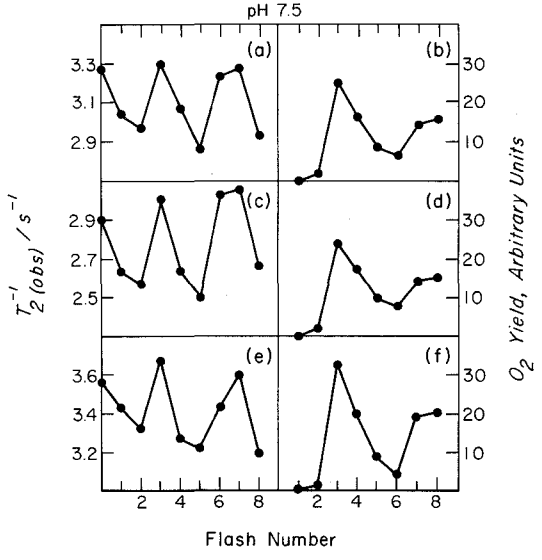

Fig. 6. $1/T_{2(obs)}$ and O_2 yield flash patterns at pH 7.5. (a), (c) and (e) are the $1/T_2$ relaxation and (b), (d) and (f) are the O_2 yield for three different samples. $1/T_2$ was measured at 27 MHz, 25°C, on sample aliquots in HEPES buffer medium at 2 mg Chl/ml while O_2 as measured on sample aliquots of 1 mg Chl/ml. $1/T_2$ and O_2 yield were measured after each flash in a pulse sequence of 2 μs saturating flashes obtained from a pulsed dye laser (λ, 590 nm). Flashes were spaced 4 s apart to allow for complete $1/T_2$ relaxation.(After refs. [20,22].)

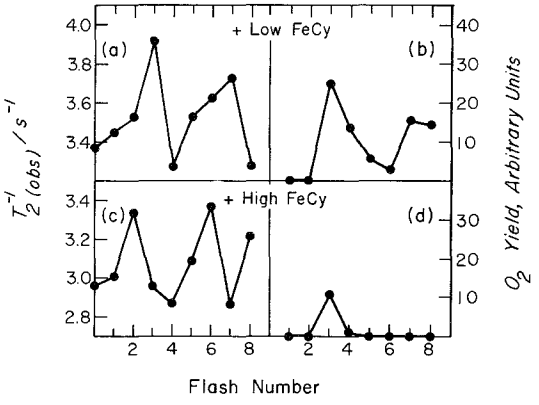

Fig. 7. $1/T_{2(obs)}$ and O_2 yield flash patterns in the presence of ferricyanide (FeCy). (a) [FeCy]/[Chl] = 0.222; (b) [FeCy]/[Chl] = 22.2. Flash procedure and conditions for pea chloroplasts were as described in the legend of Fig. 6. (After refs. [20,22].)

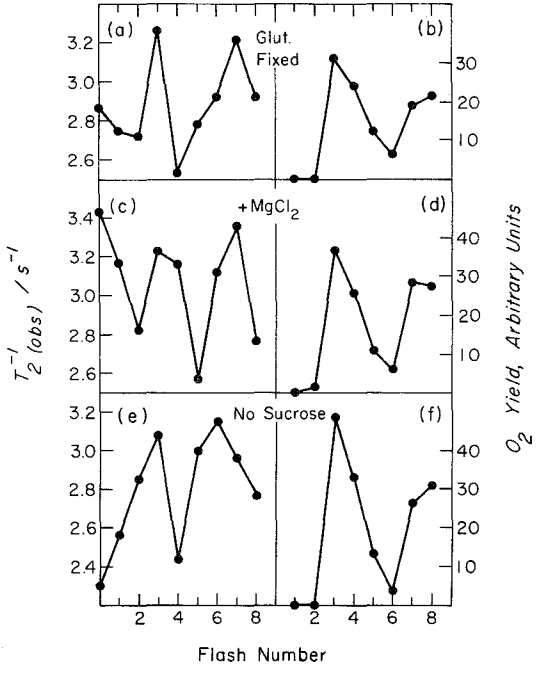

Fig. 8. (For legend see opposite page.)

including (a) $1/T_2$ monitors the individual S states, whereas O_2 only monitors the $S_4 \rightarrow S_0$ transition; and (b) the $1/T_2$ unrelated to Mn not involved in O_2 evolution, which has not been subtracted, influences the $1/T_2$ but not O_2 results. We believe that exploitation of the differences between $1/T_2$ and O_2 yield should lead to a better understanding of the O_2 evolving mechanism.

Fig. 6 shows $1/T_2$ and O_2 yield as a function of flash number in 3 different samples of pea chloroplasts at pH 7.5. (Data for chloroplasts at pH 6.7 were presented at an earlier conference [8].) Here, again, the similarities etween $1/T_2$ and O_2 are (a) peaks are at the 3rd flash in both cases; (b) an approximate periodicity of 4 is observed in both cases. And, the differences are (a) $1/T_2$ has a minimum at the 5th flash, whereas O_2 has the usual minimum at the 6th flash; (b) the 1st and 2nd flashes decrease $1/T_2$ from its high dark level where O_2 shows the opposite trend; and (c) the $1/T_2$ for the 3rd and 7th flashes are about the same, but O_2 shows a clear decrease at the 7th flash. For the same pea chloroplasts at pH 6.7, there was no O_2 in the 2nd flash under our illumination (brief 2 μs laser flash, λ=590 nm). Thus, O_2 in the second flash, observed at pH 7.5, should not have been due to a technical double hit.

The question of high dark $1/T_2$

(1) Various conditions were shown to lower the high dark level of $1/T_2$ without significantly interfering with the main patterns of O_2 and $1/T_2$ (Figs 7 and 8). This includes (a) treatment of chloroplasts with low concen-

Fig. 8. $1/T_{2(obs)}$ and O_2 yield flash patterns after various treatments which affect membrane conformations. (a), (c) and (e) are $1/T_2$ relaxations, (b), (d) and (f) are O_2 yields. (a) and (b) chloroplasts were fixed in glutaraldehyde (98.5% fixation) according to procedure given in ref. [28]; (c) and (d), $[MgCl_2]/[Chl]$ = 2.22; (e) and (f) chloroplasts suspended in medium with no sucrose. Flash procedures and conditions for pea chloroplasts in Fig. 6 were used. (After refs. [20,22].)

tration of ferricyanide (Fig. 7 (a)); (b) treatment with low osmoticum (absence of sucrose, see Fig. 8 (e)); this treatment does cause the second peak to be after the 6th flash; and (c) fixation of chloroplasts with glutaraldehyde [28] (Fig. 8 (a)). Addition of $MgCl_2$ ($[MgCl_2]/[Chl]$ = 2.22) did not decrease the $1/T_2$ in darkness.

(2) Certain conditions which abolish O_2 evolution, e.g., addition of DCMU and very high concentrations of ferricyanide, also decreased $1/T_2$ in dark, the former eliminating $1/T_2$ flash pattern in light [8,20] and the latter retaining it but changing it to show peaks at 2nd and 6th flashes (Fig. 7 (b)).

(3) Unpublished results of R. Khanna in our laboratory show that in blue-green alga *Phormidium luridum*, $1/T_2$ in dark is low. Thus, the high dark $1/T_2$ in isolated chloroplasts is not a universal feature of all the samples, although it still needs to be explained when it exists.

A model to explain $1/T_2$ flash pattern

We have begun to explain, at least, qualitatively $1/T_2$ flash pattern in terms of a manganese model [8,12,21]. Using Kok *et al.*'s model (see ref. [10]) as a starting point, the concentration of $[S_0]$ and $[S_1]$ in darkness, α(misses) and β (double hits) were calculated from O_2 data. At pH 6.7, $[S_0] = 0.30$, $[S_1] = 0.70$, $\alpha = 0.10$ and $\beta = 0$ were obtained. From these data, the concentration of S_0, S_1, S_2 and S_3 were calculated after each flash. Then, each S state was assigned a certain weighting factor which we assume is proportional to Mn[II] contribution and $1/T_2$ was calculated from the net sum of the contributions of all S states after each flash. The calculated $1/T_2$ was normalized at flash 3. The "best" fit curves for calculated $1/T_2$ for chloroplasts at pH 6.7 gave the model to be 2, 1, 1, 3 for S_0, S_1, S_2, S_3 states with the qualification that S_0 in darkness has a contribution of 4, the numbers indicating the contribution of Mn[II] to each S state.

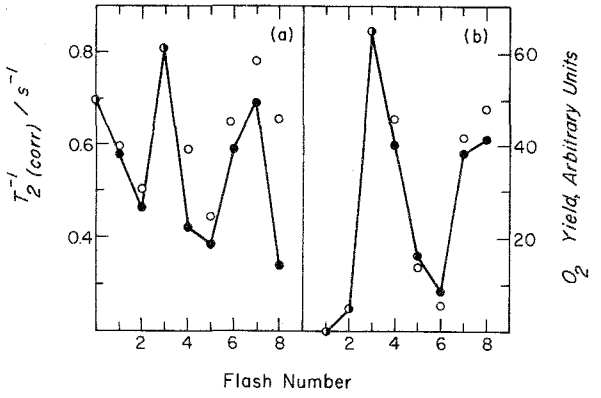

Fig. 9. Theoretical fit to the $1/T_2$ and O_2 yield flash patterns at pH 7.5. (a) $1/T_2$ relaxation and (b) O_2 yield. Closed circles are experimental data points [$1/T_{2(corr)}$], open circles are theoretical points. For the theoretical fit, $[S_0] = 0.30$, $[S_1] = 0.67$, $[S_2] = 0.03$, $\alpha = 0.10$, $\beta = 0$, and weighting factors for S_0, S_1, S_2 and S_3^+ are 2,1,2,1 on the first cycle, 1,3,2,1, on succeeding cycles, except that $S_0 = 4$ in the dark. (After refs. [20,22].)

The high value of S_0 in darkness was rationalized in view of Velthuys's [17] suggestion that a special dark-adapted state S_{-1} may exist in isolated chloroplasts.

Fig. 9 shows the experimental data (solid points) for $1/T_2$ and O_2 patterns for a particular preparation of chloroplasts at pH 7.5. Since no technical double hits exist with our laser flashes (no O_2 in the 2nd flash for chloroplasts at pH 6.7), we assumed that starting states are $[S_0] = 0.30$, $[S_1] = 0.67$, $[S_2] = 0.03$, $\alpha = 0.10$ and $\beta = 0$. The first cycle $1/T_2$ is fitted with a model (open circles in Fig. 9 (a)) in which the weighting factors are 2,1,2,1 for S_0, S_1, S_2, and S_3 (quite different from the previous fit at pH 6.7 [8]), *but* in the remaining cycles the contributions *are* 2,1,1,3 and S_0 in dark is 4 (as before). ualitatively, the fit is quite good except for the 8th flash where the model gives a much higher value than observed experimentally. Obviously, a finer "tuning" of the model remains to be accomplished.

It is not difficult to generate qualitatively similar (i.e., with a periodicity of 4), but quite different curves, e.g., peaks at 2nd and 6th flashes, as observed when O_2 evolution is inhibited in the first cycle with TPB, NH_2OH or CCCP [8], or curves with peaks at 4th and 8th flashes. A low dark level is simple to generate by eliminating the additional condition that S_0 in dark has a very high Mn[II] contribution. Also, minima can be

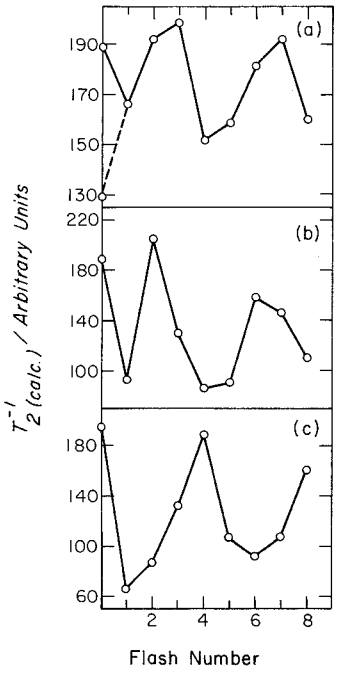

Fig. 10. Several theoretical patterns that qualitatively fit various experimental $1/T_2$ flash patterns. (a) $[S_0] = 0.30$, $[S_1] = 0.70$, $\alpha = 0.10$, $\beta = 0$; weighting factors for S_0, S_1, S_2 and S_3 are: 2,1,2,2, except $S_0 = 4$ in dark (solid line) or $S_0 = 2$ in dark (dashed line). (b) $[S_0] = 0.30$, $[S_1] = 0.70$, $\alpha = 0.10$, $\beta = 0$; weighting factors are: 0,1,1,3 except $S_0 = 4$ in dark (c) $[S_0] = 1.00$, $\alpha = 0.10$, $\beta = 0$; weighting factors are: 0,1,2,3 except $S_0 = 4$ in dark. The pattern in (a) is similar to the flash pattern for spinach chloroplasts (Fig. 5) and glutaraldehyde fixed chloroplasts (Fig. 8a); the dashed line in (a) is similar to that for pea chloroplasts with low FeCy (Fig. 7a). The pattern in (b) is similar to the flash pattern for pea chloroplasts containing either NH_2OH, TPB or CCP (see ref. [8]). And, the pattern in (c) is similar to the flash pattern for peas at pH 4 (Fig. 22b in ref. [20]). (After refs. [20,22].)

shifted from 5th to 4th flash, and so on.

Fig. 10 (a) shows a curve generated with 2,1,2,2, pattern with S_0=4 in dark (solid line) and S_0=2 (as in light, dashed line); the minimum is at the 4th flash as in spinach chloroplasts (Fig. 5) and the dark $1/T_2$ is low as in pea chloroplasts with low concentration of ferricyanide (Fig. 7 (a)). Fig. 10 (b) shows a $1/T_2$ curve with 0,1,1,3 pattern except that S_0=4 in dark. Such a curve is experimentally obtained when O_2 evolution is inhibited with TPB (in the first cycle), with CCCP or with low concentration of NH_2OH (see refs. [8,20,22]). Fig. 10 (c) was generated by assuming that $[S_0] = 1.00$ with 0,1,2,3 pattern except that S_0=4 in dark. Such a curve in $1/T_2$ is experimentally observed for chloroplasts at pH 4.0 when there is no O_2 evolution (see ref. [20]).

The above discussion indicates that using different weighting factors for S_0, S_1, S_2, S_3, almost all of our light effects on $1/T_2$ can be generated. What does this mean? We suggest that each specific S state when it is monitored in the time scale of 200-300 ms after the flash is either not in the same average redox state of manganese, or that differences in the number of exchangeable protons in bound water cause the apparent Mn[II] contribution to be different. However, what is clear is that in a great majority of cases, S_3 appears more reduced than S_2 in contrast to Kok's picture. Thus, we have suggested [8,12,21] that H_2O may be reducing the S states before the time scale of NMR measurements. This is consistent with the suggestion that H^+ may be released in steps prior to the S_4-S_0 transition. Such H^+ releases have now been shown by Fowler (prsonal communication) and Crofts and coworkers (personal communication).

Chloride depletion and ^{19}F NMR

It can be shown that chloride can be replaced by fluoride. This permits us to use ^{19}F as a tool to look at the

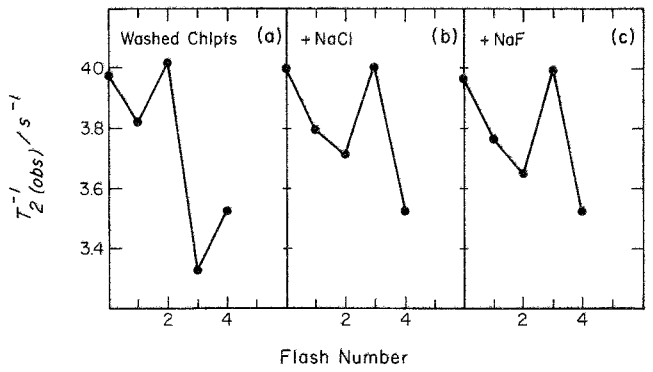

Fig. 11. $1/T_{2(obs)}$ flash pattern in the presence of NaCl and NaF. (a) Chloroplasts washed free of chloride; (b) [NaCl]/[Chl] = 4.44; (c) [NaF]/[Chl] = 4.44. Flash procedures and conditions for pea chloroplasts in Fig. 6 were used except NaCl was eliminated from the medium. (After ref. [20].)

question of the role of chloride in photosynthesis. Fig. 11 shows that chloride-depleted chloroplasts have their $1/T_2$ peak on the 2nd flash (in fact, 3rd flash gives the lowest $1/T_2$); such chloroplasts do not evolve O_2. Addition of either NaCl or NaF restores the $1/T_2$ pattern, i.e., maximum on the 3rd flash; also the Hill reaction is restored. Thus, fluoride replaces chloride well. Using such chloroplasts, ^{19}F spectra were measured. The $1/T_2$ values, calculated from bandwidths of the resonant peak, are shown in Table 3. Addition of chloroplasts to buffer enhances $1/T_2^*$, addition of reductant TPB to chloroplasts increases $1/T_2^*$, and removal of manganese by TRIS-acetone washing brings back the $1/T_2^*$ to that of the buffer control. These results parallel the measurements on water proton relaxation. This establishes a relationship between manganese related to O_2 evolution to the fluoride (and thus chloride). Whether this means that chloride is a ligand to manganese involved in O_2 evolution and thus may help stabilize the higher oxidation states of Mn (Mn[III]?) remains to be shown.

TABLE 3

^{19}Fluorine relaxation measurements of chloroplast membranes[a] (After ref. [20].)

Condition	T_2^{*-1}/s^{-1}
[b] Buffer	13.7
[c] Chlpts	34.0
Chlpts + 5 mM TPB	60.2
TRIS-acetone washed Chlpts	15.6

[a] ^{19}F spectra were measured on a Jeol FX-60 Fourier Transform NMR spectrometer. Relaxation rates were calculated from the half bandwidth ($\Delta\nu$) of the resonance peak, $1/T_2^* = \pi\Delta\nu$.

[b] HEPES buffer medium containing 20 mM NaF was used.

[c] Dark-adapted pea chloroplasts, 3 mg Chl/ml, were washed free of Cl^- and suspended in buffer medium containing NaF.

Summary and concluding remarks

(1) Water proton relaxation rate (PRR) is an excellent monitor of manganese bound to thylakoid membranes as (a) removal of Mn by TRIS-acetone washing or by NH_2OH-EDTA treatment dramatically decreases PRR; (b) PRR is approximately linearly related to [Mn] in chloroplasts with different [Mn] obtained by replacing Mn with Mg.

(2) The major species of Mn which influences PRR is Mn[II] according to an analysis of the dependence of PRR on the frequency of the rf pulse.

(3) The $1/T_2$ (transverse relaxation) rates of thylakoids as a function of flash number show a periodicity of 4 suggesting their correlation to the O_2 evolving mechanism.

(4) The particularly high dark $1/T_2$ is eliminated by (a) treatment with ferricyanide; (b) glutaraldehyde fixation; and (c) low osmoticum. This dark level is explained on the basis that in isolated chloroplasts, a very reduced state (high Mn[II] contribution) may exist (for details

see ref. [20]).

(5) A theoretical model, which assumes that in pea chloroplasts, suspended at pH 7.5, the Mn[II] contribution for S_0, S_1, S_2 and S_3 is 2,1,2,1, respectively, in the first flash sequence and then 2,1,1,3 in the succeeding cycles except that S_0=4 in the dark, fits in quite well with the experimental data. A model assuming a 0.1,1,3 Mn[II] contribution with S_0=4 in dark explains $1/T_2$ flash pattern under conditions when O_2 evolution has been inhibited by CCCP, NH_2OH or TPB.

(6) ^{19}F relaxation rate ($1/T_2^*$) was shown to behave in parallel with the PRR suggesting that chloride (which was replaced by fluoride) might play a role in stabilizing the Mn complex involved in O_2 evolution.

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Footnote:

Abbreviations:

β, Bohr magneton; B, constant in Bloembergen-Morgan equation containing the value of the resultant electronic spin and the zero field splitting parameters; γ, nuclear magnetogyric ratio; $\bar{H}_o$, external applied magnetic field; $\bar{M}$, net magnetization of a spin system; $\bar{M}_o$, equilibrium magnetization; ν, frequency (Hz); ρ, mole fraction of ligand nuclei bound; PRR, proton relaxation rate; q, number of ligand nuclei bound; rf, radio frequency; S, total electron spin; $1/T$, longitudinal or spin-lattice relaxation rate, corrected with buffer medium rate; $1/T_2$, trans-

verse or spin-spin relaxation rate, corrected with buffer medium rate; $1/T_{1,2(M)}$, relaxation rates at the bound site; $1/T_{1,2(obs)}$, observed relaxation rates; $1/T_{1,2(T-A)}$, relaxation rates of TRIS-acetone washed chloroplast membranes; τ_c, dipolar correlation time; τ_m, chemical exchange lifetime; τ_r, rotational correlation time; τ_s, electron spin relaxation time; τ_v, correlation time for the modulation of zero field splitting, ω_I, nuclear Larmor frequency; ω_s, electronic Larmor frequency; all other abbreviations see list on page xxx.

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