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NMR Studies on Chloroplast Membranes

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In green plant photosynthesis the mechanism by which water is photo-oxidized and oxygen is produced still remains largely unsolved (see review 1). However, it is known that manganese is directly involved (2). Inasmuch as the unpaired electron spin of Mn(II) can lead to large increases in magnetic relaxation rates of nuclei bound near the ion, it appeared to us that manganese would be a natural paramagnetic probe and that proton magnetic relaxation could be used to study the oxygen evolving mechanism. In this communication we present our initial findings, some of which have been reported earlier (3, 4). The results indicate that a significant contribution to proton relaxation rates of chloroplast membrane suspensions does arise from interactions with membrane-bound manganese. Furthermore light-induced changes in the relaxation rates suggest that proton relaxation is monitoring the oxygen evolving system.

Materials and Methods

Chloroplast Preparation. Chloroplast thylakoid membranes were isolated either from commercial spinach (Spinacea oleracea) or green house grown peas (Pisum sativa) in a medium consisting of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer adjusted to pH 7.5 with NaOH, 400 mM sucrose and 10 mM NaCl. The chloroplasts were given an osmotic shock in a similar medium containing 100 mM sucrose and finally resuspended in the original isolation medium. Chlorophyll concentration was adjusted to 3 mg Chl/ml in all samples.

Nuclear Relaxation Measurements. The inversion recovery method ($180^0 - \tau - 90^0$ sequence) was used to determine the spin-lattice relaxation rate (T_1^{-1}). The spin-spin relaxation rate (T_2^{-1}) was measured from the exponential decay of the echo amplitudes in a Carr-Purcell (Meiboom-Gill modification) (CPMG) train of rf pulses. The experimental uncertainties in T_1^{-1} and T_2^{-1} data are within $\pm 5\%$.

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In order to measure light-induced changes the nmr probe was designed to provide the best optical geometry while still maintaining a good signal-to-noise ratio. A tight fitting Plexiglas plug was inserted into the bottom of a 12 mm nmr tube to support a thin layer of sample ($\sim$100 µl total volume) in the region of the nmr coils. Illumination was from the top. This arrangement allowed for a large surface area and hence maximum absorption of light by the whole sample.

In the flashing light experiments T_2^{-1} was measured after a sequence of light flashes. The CPMG train was initiated simultaneously with the last light flash of the sequence. The time interval between successive flashes in a sequence was 2 sec. A dark adaptation period of 7 min was allowed between each sequence of light flashes. Although this procedure is somewhat modified from the one usually employed to measure oxygen, we found that it did not affect the oxygen yield pattern.

Light flashes were obtained from a strobe light (Strobotac type 1538-A, General Radio Co.) and were of short duration (2.4 µsec at half height with an extended tail up to 10 µsec).

Results and Discussion

Paramagnetic Contributions to the Water Proton Relaxation Rates of Chloroplast Membrane Suspensions. Suspensions of dark-adapted chloroplast membranes have a large effect on the water proton relaxation rates. Upon washing the membranes twice in buffer medium T_1^{-1} decreases in general by about 50%. Simple washing usually has little effect on chloroplast activity but undoubtedly serves to remove loosely bound paramagnetic ions. However, not all ions are removed; for example, it has been reported that about 35% of the manganese is lost upon repeated washings (5). Washed chloroplasts represent the control in the following experiments.

In washed chloroplasts any paramagnetic contribution to water proton relaxation will depend on the accessibility of water to the tightly bound metal ions in the membrane. When EDTA is added to washed chloroplasts the relaxation rates decrease. For example, at 26 MHz and 26°C 1 mM EDTA reduces T_1^{-1} to about one fourth of the control value (TABLE I). As shown in TABLE I the magnitude of the effect of EDTA depends to a large degree on temperature and nmr frequency. It appears from these results that the tightly bound paramagnetic ions do have a major influence on the relaxation rates in washed chloroplasts.

For a system such as chloroplast membranes the measured relaxation rate, $T_1^{-1})_{obs}$ (or $T_2^{-1})_{obs}$) can be considered as the sum of contributions from all sites in the membrane accessible the solvent water, plus the relaxation rate of free water:

$$T_1^{-1})_{obs} = \sum_i \frac{P_i}{T_{1,i}} + T_1^{-1})_{free} \qquad (1)$$

TABLE I. Effect of EDTA and Tris-Acetone Washing on Water Proton Relaxation Rates of Pea Chloroplasts

Conditions	$^{a}T_1^{-1}$ (sec^{-1})					$^{a}T_2^{-1}$ (sec^{-1})				
	16 MHz		26 MHz			16 MHz		26 MHz		
	26°	8°	38°	25°	8°	26°	8°	38°	24°	9.5°
[b]Washed Chloroplasts	0.50	0.44	0.56	0.75	0.78	2.80	2.94	2.69	3.02	3.34
Washed Chloroplasts + 1 mM EDTA	0.14	0.22	0.16	0.15	0.40	2.45	2.89	2.34	2.42	2.64
[c]Tris-Acetone Washed Chloroplasts	0.20	0.18	0.18	0.19	0.21	2.30	2.24	2.29	2.27	2.39

[a]Relaxation rates corrected by subtracting rates of buffer medium from observed rates of chloroplast suspensions.

[b]After isolation chloroplasts were washed twice in buffer medium and resuspended to a concentration of 3 mg chlorophyll/ml.

[c]Chloroplasts were treated with tris-acetone medium according to procedure of Yamashita and Tomita (9) and then washed once with buffer media.

where P_i is the fraction of water in site i. Most water is free ($P_{free} \cong 1$) and $T_1^{-1})_{free}$ is taken as the relaxation rate in the buffer medium without chloroplasts. The quantity $T_1^{-1})_{obs} - T_1^{-1})_{free}$ is therefore the relaxation contribution due to the membranes and is denoted simply T_1^{-1} (or T_2^{-1}) in this communication.

In macromolecular systems T_1^{-1} of H_2O is usually influenced most strongly by paramagnetic sites. The relaxation rate T_{1m}^{-1} of water at such a site is usually dominated by electron nuclear dipole-dipole interactions:

$$\frac{1}{T_{1m}} = \frac{2}{15} \frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6} \left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right] \qquad (2)$$

where γ_I is the nuclear magnetogyric ratio, S is the total electron spin, g is the electronic g-factor, β is the Bohr magneton, r is the distance between the nucleus and the paramagnetic ion, ω_I and ω_S are the nuclear and electronic Larmor frequencies respectively, and τ_c is the correlation time.

The dipole-dipole interaction may be modulated by any of several time-dependent processes such that:

$$\frac{1}{\tau_c} = \frac{1}{\tau_s} + \frac{1}{\tau_R} + \frac{1}{\tau_M}$$

where τ_S is the electronic relaxation time, τ_R is the rotational correlation time and τ_M is the exchange lifetime. The shortest of these correlation times dominates.

An expression similar to (2) can be obtained for the spin-spin relaxation rate, T_{2m}^{-1}, but contains additional terms associated with scalar coupling not usually important for T_1^{-1}.

$$\frac{1}{T_{2,m}} = \frac{1}{15} \frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6} \left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right) +$$

$$\frac{1}{3} S(S+1)\left(\frac{A}{\hbar}\right)^2 \left(\frac{\tau_e}{1+\omega_S^2\tau_e^2} + \tau_e\right) \qquad (3)$$

where A is the electron-nuclear hyperfine coupling constant and τ_e is the correlation time for the scalar interaction.

Chloroplast Manganese and Water Proton Relaxation. Several treatments are known to affect chloroplast managnese (e.g. see ref. 6). For example, washing chloroplasts with 0.8 M tris (hydroxymethyl) aminomethane (tris) buffer at pH>8 alters the environment of manganese such that a Mn(II) esr signal appears (7). The current hypothesis is that some of the manganese is released to the inside of the membrane vesicle (6), but is not removed

from the chloroplasts. However, the amount of manganese affected is still a matter of controversy (see ref. 6). Nevertheless, tris washing inactivates the oxygen evolving mechanism, but leaves the rest of the electron transport chain intact and functional to the extent that photoreduction of $NADP^+$ can be restored by adding exogenous electron donors (8). This suggests that tris washing does not affect other paramagnetic centers in the electron transport chain up to the primary acceptor of Photosystem I. We find that tris washing generally changes T_1^{-1} of chloroplasts, but that the magnitude and direction of the change vary with the source of the plant material. Some examples are shown in TABLE II.

TABLE II. Effect of Tetraphenylboron and Tris-Washing on Water Proton T_1^{-1} of Spinach Chloroplasts

Conditions	[a] T_1^{-1} (sec) Sample No. 1	2	3	4
[b] Washed Chloroplasts	0.86	0.90	1.04	1.03
Washed Chloroplasts + 5 mM TPB^-	1.64	1.70	-	-
[c] Tris Washed Chloroplasts	0.82	-	1.36	0.41
Tris Washed Chloroplasts + 5 mM TPB^-	0.88	-	-	-

[a] Rates corrected by subtracting rates of buffer medium from observed rates of chloroplast suspensions. Measurements were made at 26 MHz, 24°C.

[b] As in TABLE I.

[c] Tris washing according to procedure of Yamashita and Butler (8).

Recently Yamashita and Tomita (9) have found that a more complete extraction of manganese from the membrane is obtained when 20% acetone is included during tris washing. Again photoreduction of $NADP^+$ can be restored with added electron donors. When chloroplasts are treated in this way T_1^{-1} and T_2^{-1} is considerably reduced (TABLE I). Interestingly, the rates do not show either a marked frequency or temperature dependency.

Although these results indicate that bound manganese does influence the proton relaxation, contributions from other paramagnetic centers cannot be ruled out; however, they probably do not have a dominating effect. For example, the copper bound in plastocyanin, a component of the electron transport chain, is not accessible to the bulk water and has little effect on observed water proton relaxation rates (10). With respect to iron, high spin Fe(II) and high and low spin Fe(III) have much faster electronic relaxation rates than Mn(II) and are less efficient in relaxation by comparison (11).

Water Proton Relaxation as a Monitor of Manganese Oxidation States. It is not known what oxidation states of manganese exist in chloroplast membranes. The electronic relaxation rate, however, is strongly dependent on the oxidation state. For example, the values of τ_S for Mn(II) are generally 10^{-8} - 10^{-9} sec, depending on the nmr frequency and chemical environment (11). Mn(III), on the other hand, has a much shorter electron spin relaxation time. A recent study by Villafranca et. al. (12) yielded a value of $\tau_S \simeq 3 \times 10^{-11}$ sec for Mn(III) bound to a superoxide dimutase from E. coli. This difference in τ_S is sufficient to account for a much greater relaxation effect by Mn(II) than Mn(III). If the electronic relaxation of metal ions is dominating the proton relaxation in chloroplast membranes, then changes in oxidation state will be reflected in the relaxation rates.

Oxidation states of bound ions can be shifted by adding redox reagents. But many redox reagents upon oxidation or reduction give rise to free radical intermediates which could interfere with the proton relaxation rates. One reductant which does not appear to form free radical intermediates is the tetraphenylboron anion (TPB^-). The oxidation of TPB^- is a two electron transfer (13):

$$B(C_6H_5)_4^- \xrightarrow{0.7v} (C_6H_5)_2 + B(C_6H_5)_2^+ + 2e^-$$

$$B(C_6H_5)_2^+ + HOH \longrightarrow (C_6H_5)_2BOH + H^+$$

TPB^- is known to act as a reductant in the oxygen evolving system of chloroplasts (14, 15). When TPB^- is added to the chloroplast suspension, T_1^{-1} increases (TABLE II). Figure 1 shows T_1^{-1} as a function of TPB^- concentration in unwashed chloroplasts. The titration curve shows several plateaus which may be indicative of several fractions of ions being successively reduced by TPB^-. TPB^- itself has no effect on the buffer medium. Interestingly, TPB^- also has no effect in tris-washed chloroplasts (TABLE II) suggesting that it is acting on manganese involved in O_2 evolution.

In a number of cases it has been found (11) that τ_S of Mn(II) and other paramagnetic ions depends on the strength of the applied magnetic field. The value of τ_S is determined by crystal lattice field fluctuations having a correlation time, τ_ν, such that:

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

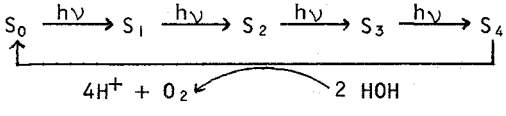
where B is a constant containing the value of the resultant electronic spin and the zero field splitting parameters.

At low magnetid fields τ_S is often the shortest correlation

time and dominates the relaxation of nuclei bound to the paramagnetic sites (Eq. 3 and 4). At higher field strengths τ_S can increase and τ_R or τ_M may then become the dominant correlation time. T_1^{-1} reaches a maximum as the field increases and then declines. On the other hand, T_2^{-1} has terms depending directly on τ_C (Eq. 3). For the case of a field dependent τ_S, T_2^{-1} is found to increase to a plateau as the magnetic field increases.

Figure 2 shows the frequency dependence for T_1^{-1} and T_2^{-1} for a normal chloroplast suspension and for one containing 5 mM TPB^-. The T_1^{-1} for normal chloroplasts shows a broad maximum and then a slow decline as the nmr frequency is increased. However, the T_2^{-1} increases significantly at the higher frequencies. This behavior does suggest that electronic relaxation dominates the proton relaxation in normal chloroplasts. However, the lack of a distinct peak in T_1^{-1} is peculiar. This may indicate the existence of a distribution of correlation times. On the other hand when TPB^- is added to the chloroplasts, T_1^{-1} and T_2^{-1} show a frequency dependence distinctly characteristic of electronic domination of proton relaxation. The correlation time calculated at the peak in T_1^{-1} with TPB^- is approximately 6×10^{-9} sec at 24 MHz, which is within the expected range of τ_S for Mn(II). This result is consistent with the idea that TPB^- reduces a fraction of manganese in a higher oxidation state to a lower oxidation state which is more efficient in proton relaxation.

Light Effects on Water Proton Relaxation Rates of Chloroplast Membranes: Relationship to the Oxygen Evolving Mechanism. In a series of microsecond light flashes the yield of oxygen evolved from isolated chloroplasts or whole algal cells shows a dampled oscillatory pattern, having a period of four with peaks after the 3rd, 7th, and 11th flashes (1). Based on this unique pattern Kok and co-workers (16) have proposed a four step model in which some chemical intermediate accumulated up to four oxidizing equivalents upon successive photoactivations of the oxygen evolving centers:



(5)

Here S indicates the oxidation state of the intermediate; S_4 represents the most oxidized state. The primary photoreaction of the oxygen evolving system is the excitation of the reaction center chlorophyll molecule P_{680}, which is oxidized upon reduction of the primary electron acceptor Q; P_{680}^+ then receives an electron from the S intermediate, perhaps via another intermediate labeled Z (for details see review, 17). When four oxidizing equivalents have accumulated and the S_4 state is formed, two water molecules react to produce oxygen and the original S_0

state.

The identity of the charge accumulating intermediate is unknown, although it has been suggested to involve manganese (2, 18-20). However, there has been no direct experimental evidence to show that chloroplast manganese undergoes changes in oxidation state during photosynthesis. Data from previous sections indicate that proton relaxation monitors membrane-bound manganese and suggest that the relaxation rates are sensitive to changes in oxidation states. To determine whether proton relaxation could be related to the oxygen evolving mechanism we measured the spin-spin relaxation rate in brief flashes of light.

Figure 3 shows T_2^{-1} of chloroplast membranes as a function of flash number (4). Similar data have been obtained from seven other preparations of spinach and lettuce chloroplasts. The oscillatory pattern for T_2^{-1} shows some striking similarities to the oxygen yield pattern. As in oxygen measurements, maxima occur after the 3rd, 7th, 11th and 15th flashes. Also, the T_2^{-1} oscillations damp out after the 17th flash, corresponding to a similar damping of the oscillations in the oxygen yield. These important parallels in the two types of data strongly imply that proton relaxation is monitoring the oxygen-evolving mechanism.

However, there are some significant differences. After the first flash where no oxygen is evolved, the relaxation rate shows a large decrease which has no subsequent counterpart. Minima in the relaxation rates then occur after the 4th, 8th, 12th, etc. flashes. Minima in the oxygen yield, on the other hand, occur after the 6th, 10th, 14th, etc. flashes. The relaxation rates steadily increase from the 4th to the 7th flash, from the 8th to the 11th flash and so on, while the trend is the opposite for oxygen evolution, the yield steadily dropping from the 3rd to the 6th flash and from the 7th to the 10th flash. These differences in T_2^{-1} and oxygen yield patterns may be explained on the basis that the relaxation rates differ for each of the S states whereas oxygen evolution only takes place during the S_4 to S_0 transition.

The time scales for the formation ($t_{1/2} \sim 600$ μsec) and lifetimes ($t_{1/2} \sim 10$-30 sec) of the individual S states are sufficiently different (1) from the spin-spin relaxation times ($T_2 \sim$ 100 msec) so as not to introduce a complex behavior in the T_2 data. The spin echo amplitudes always yield a single exponential decay.

The changes in T_2^{-1} are not caused by the oxygen produced in photosynthesis. We estimated that the amount of oxygen produced after the third flash is less than 4% of the total oxygen present in the sample when equilibrated as it is with the air. The amount was calculated to have less than 1% effect on T_2^{-1} whereas the maximum light-induced changes are about 20%.

Figure 4 shows T_2^{-1} as a function of flash number for tris washed chloroplasts. There is an initial light-induced decrease in T_2^{-1}, but the oscillations are absent. As pointed out earlier,

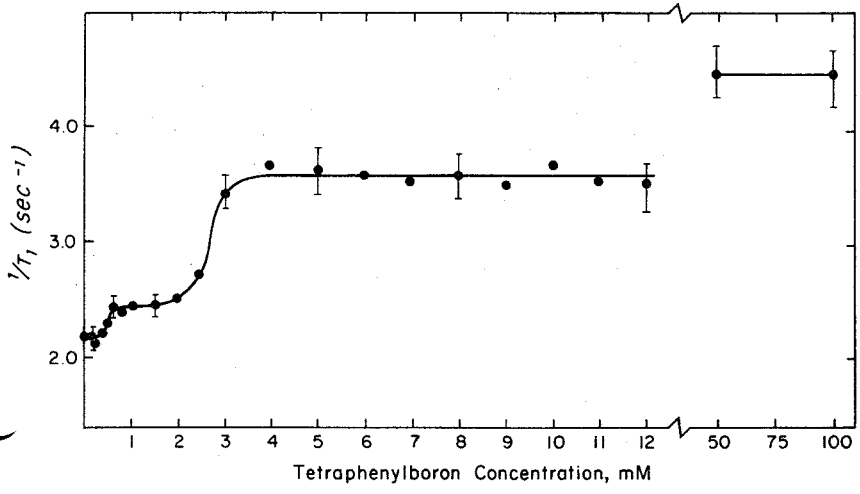


Figure 1. T_1^{-1} *measured as a function of tetraphenylboron (TPB⁻) concentration in unwashed spinach chloroplasts. Measurements were made at 26.9 MHz, 24°C.*

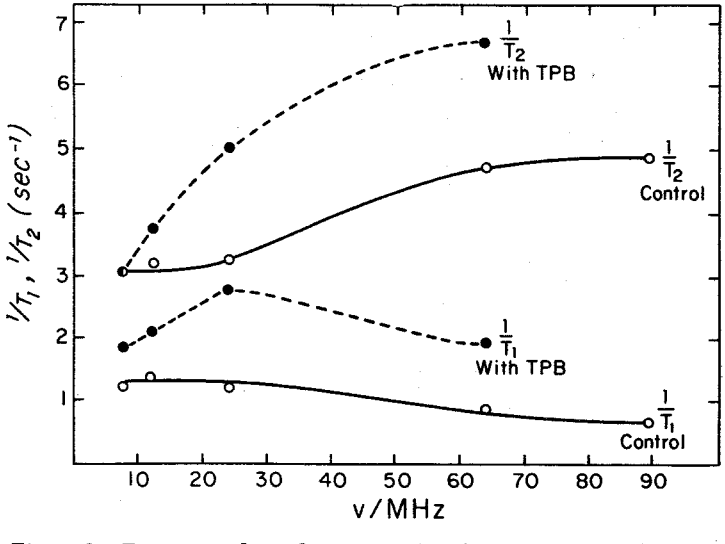


Figure 2. Frequency dependence of T_1^{-1} *and* T_2^{-1} *for a spinach chloroplast suspension and for one containing 5mM tetraphenylboron (TPB⁻); 26°C.*

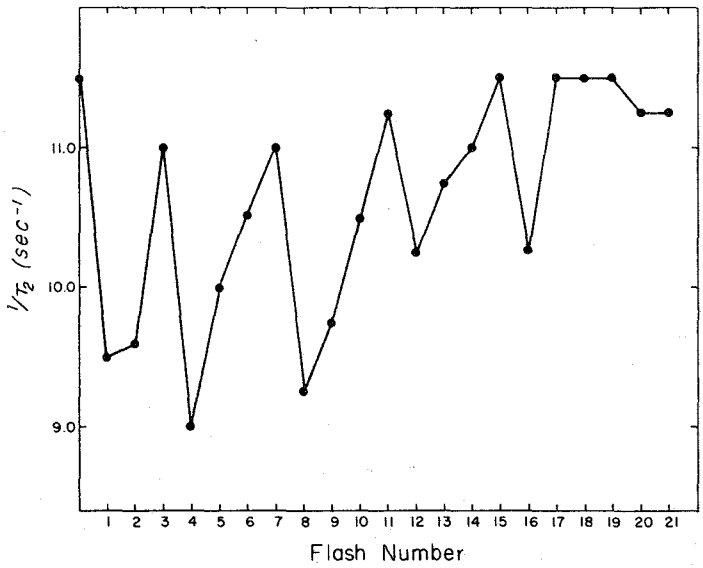


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Figure 3. T_2^{-1} *measured as a function of number of light flashes in unwashed spinach chloroplasts. The procedure is given in "Materials and Methods." Measurements were made at 26.9 MHz, 24°C (5).*

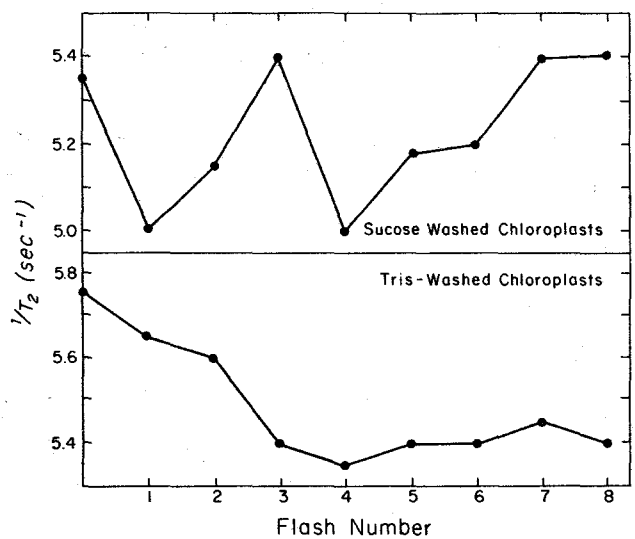

Figure 4. T_2^{-1} *measured as a function of number of light flashes in washed and tris-washed lettuce chloroplasts. Measurements were made at 26.9 MHz, 24°C.*

tris washing inactivates the oxygen-evolving apparatus, but leaves the rest of the electron transport chain intact and functional.

Although stepwise changes in manganese oxidation states and consequent changes in electronic relaxation time could provide a simple qualitative explanation of the flashing light results, other mechanisms could lead to oscillations in T_2^{-1}. Such possibilities include differences in the access of water to the bound paramagnetic ions and modifications in chemical exchange rates as an indirect result of change accumulation. We hope that further experiments will clarify the mechanism involved.

Concluding Remarks

Chloroplast membranes represent an extremely complex system r physical chemical studies. Unfortunately, experiments on the oxygen evolving mechanism are confined to the use of intact membranes as the oxygen evolving capacity is rapidly lost in attempts to isolate submembrane protein fragments.

NMR relaxation measurements of water in chloroplast suspensions in part reflect the system's complexity. On the other hand, some simplification is achieved in that the major contribution to relaxation enhancement appears to be membrane bound manganese ions. Most importantly the pattern of relaxation rate (T_2^{-1}) in flashing light bears close similarities to the oxygen yield demonstrating that nmr can serve as a probe of the oxygen evolving mechanism. The details underlying this observation are the subject of our current work.

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