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Proton relaxation and charge accumulation during oxygen evolution in photosynthesis

(nuclear magnetic resonance/manganese in chloroplasts)

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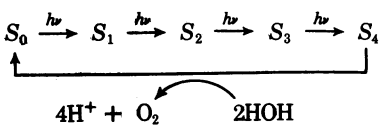
ABSTRACT **The water proton spin-spin (transverse) relaxation rate of chloroplast suspensions has been measured after each of a series of 2.4 μsec light flashes. The sequence of relaxation rates shows a damped oscillatory pattern with a period of four and peaks after the 3rd, 7th, 11th, and 15th flashes. This result indicates that water proton relaxation can be used to monitor the charge-accumulating states as postulated by Kok and coworkers for the oxygen-evolving mechanism in green plants [(1970) *Photochem. Photobiol.* 11, 457–475]. Other experiments [Wydrzynski *et al.* (1975) *Biochim. Biophys. Acta* 408, 349–354] have shown that the proton relaxation rate is strongly influenced by membrane-bound manganese in various oxidation states, suggesting that manganese participates in the charge accumulation process during oxygen evolution.**

In a series of microsecond light flashes, the yield of oxygen evolved from isolated chloroplasts or intact algal cells shows a damped oscillatory pattern, having a period of four with peaks after the 3rd, 7th, and 11th flashes (for review see ref. 1). Based on this unique pattern, Joliot and Kok (1) and Mar and Govindjee (2) have discussed a four-step model in which some chemical intermediate accumulates up to four oxidizing equivalents upon successive photoactivations of the oxygen-evolving centers:



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 P680, which is oxidized concomitantly with the reduction of the primary electron acceptor Q; $P680^+$ then receives an electron from the S intermediate, perhaps via another intermediate labeled Z (see ref. 3).

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 (4–7). Manganese is known to be essential for oxygen evolution (4) and can take on a number of relatively stable oxidation states, making it a likely candidate. However, there has been no direct experimental evidence to show that chloroplast manganese undergoes changes in oxidation state during photosynthesis.

The rate of proton relaxation is influenced by the presence of paramagnetic ions, such as manganese, bound to macromolecules and accessible to the solvent water protons (8, 9). In a previous communication (10) we reported results indicating that a large component of the proton relaxation rate of water in chloroplast suspensions can be attributed to interactions with membrane-bound manganese. In this paper we show that the proton spin-spin relaxation follows a pattern after a series of light flashes similar to the one for oxygen evolution, indicating that the proton relaxation is also monitoring the S states of the charge-accumulating intermediate.

Abbreviations: NMR, nuclear magnetic resonance; rf, radio frequency.

MATERIALS AND METHODS

Chloroplast Preparation. Chloroplasts were isolated from commercial spinach (*Spinacea oleracea*) in a medium consisting of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, adjusted to pH 7.5 with NaOH, 400 mM sucrose, and 10 mM NaCl. They were given an osmotic shock in a similar medium containing 100 mM sucrose and finally resuspended in the original isolation medium. The chlorophyll concentration in the samples was adjusted to 3.0 mg/ml.

Nuclear Relaxation Measurements. The proton transverse relaxation rate ($1/T_2$) was measured from the spin echoes in a Carr–Purcell (11) sequence of radio frequency (rf) pulses on a pulsed nuclear magnetic resonance (NMR) spectrometer operating at 26.89 MHz. $1/T_2$ is given by the exponential decay rate of the peak echo amplitudes. The Meiboom-Gill phase shift modification (12) after the initial 90° pulse was used to correct for diffusion and pulse adjustment errors.

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

Fig. 1 gives an outline of the experimental procedure. $1/T_2$ was measured after a sequence of n_1 light flashes, where n_1 was varied from 1 to 21. 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 flashes. Although this procedure is somewhat modified from the one usually used to measure the oxygen, we found that it had no effect on the oxygen yield pattern. The light pulses were obtained from a strobe light (Strobotac Type 1538-A, General Radio Co.) and were of short enough duration (2.4 μsec at half height, with an extended tail up to 10 μsec) to reduce the possibility of double hits on the reaction center chlorophyll complex.

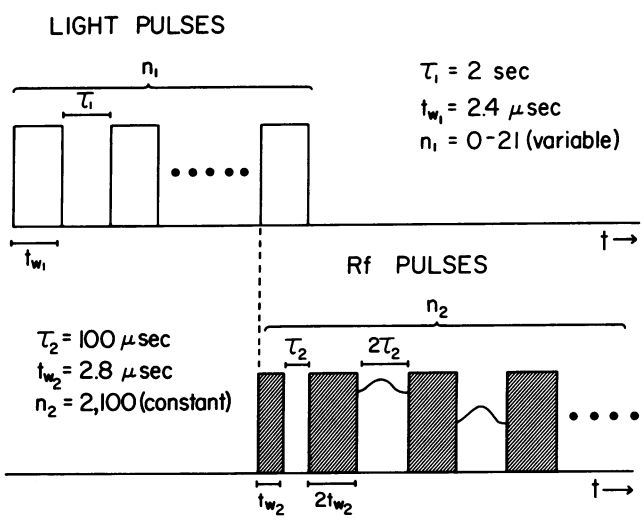


FIG. 1. Experimental procedure used to measure $1/T_2$ after a sequence of light pulses. $1/T_2$ is the decay rate for the exponential decrease of the proton spin echoes in the Carr-Purcell rf pulse sequence.

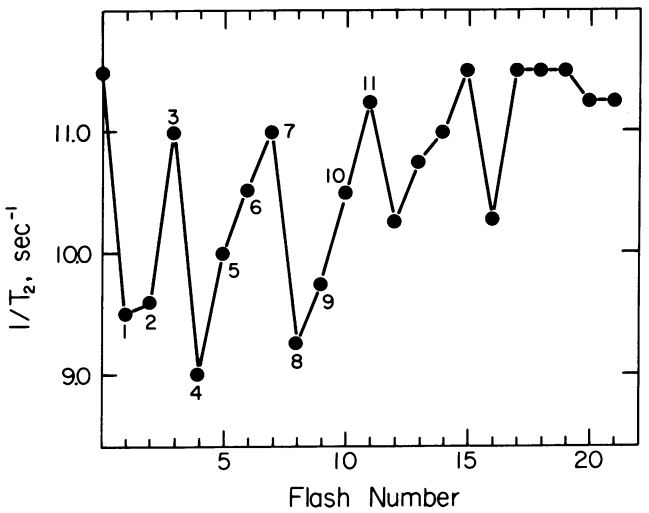


FIG. 2. The spin-spin relaxation rate ($1/T_2$) as a function of flash number in a chloroplast suspension from spinach. The procedure for measuring $1/T_2$ is given in Fig. 1. Measurements were made on the same sample at room temperature; 7 min dark time was allowed between each measurement.

Nonexponentiality in the decay of the NMR spin-echo amplitudes was not observed. The time scales for the formation ($t_{1/2} \sim 600$ μsec) and life-times ($t_{1/2} \sim 10$–30 sec) of the individual S states (1) are sufficiently different from the spin-spin relaxation time ($T_2 \sim 100$ msec) so as not to introduce a complex behavior of the echo envelope.

The reproducibility of $1/T_2$ for a particular sample and a particular flash number was found to be within ±3%. The values of $1/T_2$, however, were found to vary for different preparations, in agreement with our previous finding (10) that $1/T_1$ depends on the physiological age and Hill activity of the chloroplast membranes.

The changes found in $1/T_2$ 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. This amount was calculated to have less than a 1% effect on $1/T_2$, whereas the light-induced changes are about 20%.

RESULTS AND DISCUSSION

The proton spin-spin relaxation rate measured as a function of flash number is shown in Fig. 2. Similar data have been obtained from five other preparations of spinach and lettuce chloroplasts. The oscillatory pattern followed by $1/T_2$ 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 $1/T_2$ 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 the NMR relaxation rate is a sensitive monitor of the charge-accumulating apparatus.

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 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 the $1/T_2$ and oxygen yield patterns can be attributed to the fact that the relaxation rates may differ significantly for each of the various S states, whereas oxygen evolution only takes place during the S_4 to S_0 transition.

As shown by our earlier studies (10), it is very likely that the spin-lattice relaxation rate of water protons in chloroplast suspension is dominated by paramagnetic interactions with membrane-bound manganese. $1/T_2$ also contains these contributions from manganese and in addition may have contributions from other sources (e.g., nuclear–nuclear dipole interactions) not important for $1/T_1$. There will no doubt be effects on proton relaxation from manganese atoms in different sites and assigning a detailed mechanism for the paramagnetic induced relaxation is not feasible at the present time. It is important, however, to note that for dark-adapted chloroplasts, $1/T_1$ and $1/T_2$ are essentially constant over the 11–35°C range, suggesting that the system is in the fast chemical exchange region. Other experiments on dark-adapted chloroplasts (10) led us to propose that the electron spin relaxation time (τ_s) was primarily responsible for modulating the electron–nuclear interactions leading to spin lattice relaxation and we suggested that manganese existed in a mixture of oxidation states in dark-adapted chloroplasts.

If manganese is indeed involved in charge accumulation, the oscillations in $1/T_2$ may also be understood in terms of the different electron spin relaxation times, τ_s, of manganese in different oxidation states. For manganese(II), values of τ_s are generally 10^{-8}–10^{-9} sec, the exact value being dependent on the NMR frequency and chemical environment (9). Correlation times in this range lead to highly efficient relaxation of nuclear spins near the paramagnetic center. Manganese(III), on the other hand, has a much shorter electron spin relaxation time. A recent study by Villafranca *et al.* (13) yielded a value of $\tau_s = 3 \times 10^{-11}$ sec for manganese(III) bound to a superoxide dismutase from *Escherichia coli*. Thus, manganese(III) is considerably less efficient in relaxing the water protons.

From four to six atoms of manganese are known to be associated with each oxygen-evolving center (4). As suggested by Earley (7), the charge-accumulating intermediate may be a multiple manganese complex. If this is the case, then in

those flashes which show a decrease in $1/T_2$, the accumulation of charge can be attributed to the oxidation of a fraction of manganese(II) to manganese(III). For this model, in those flashes in which the maximum oxygen yield is observed, the four oxidizing equivalents that have accumulated react immediately with water to produce oxygen, restoring the manganese complex back to a +2 oxidation state. Since oxygen evolution is completed within a few milliseconds (1), $1/T_2$ would monitor the efficient relaxation by the manganese(II) complex. The eventual damping in the oscillations then arises from the gradual mixing of states during the flash sequence.

While changes in the electron-spin relaxation time, τ_s, provide the simplest qualitative explanation of the changes observed in the proton relaxation rate, other mechanisms could conceivably lead to different relaxation rates for the higher oxidation states of the charge-accumulating intermediate. Such possibilities include differences in the access of water to the paramagnetic center because of conformation changes, and modifications in the chemical exchange processes for similar reasons. Further experiments are needed to develop a quantitative interpretation of the flash-induced changes in relaxation rate, including measurements of the action spectrum with monochromatic light for a range of proton resonance frequencies.

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