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PULSED NUCLEAR MAGNETIC RESONANCE AND THYLAKOID MEMBRANES*

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Recent data obtained on the first application of the Nuclear Magnetic Resonance (nmr) technique to photosynthesis research is briefly reviewed here. It has been shown in our laboratory that water proton transverse relaxation rates (T_2^{-1}) monitor the manganese [II] content and the oxygen evolving mechanism in thylakoid membranes. These results now allow us to monitor the individual steps involved in O_2 evolution in photosynthesis.

All life on earth depends ultimately on green plant photosynthesis which converts solar energy into chemical energy on a large scale. The efficiency of energy conversion by plants ranges from about 0.1 to 2.5%. However, in actively growing cells of algae in controlled laboratory conditions, this efficiency may reach a value of 30%. One estimate for the total carbon fixed per year for the entire world is of the order of 10^{11} tons. The end products of photosynthesis O_2 and food are used by mankind for its survival.

Photosynthesis can be considered as a tripartite process involving (i) production of reducing power from H_2O ; (ii) fixation of CO_2 into carbohydrates by the reducing power produced in step (i); and (iii) evolution of O_2 from H_2O . The mechanism of fixation of CO_2 into carbohydrates and of the electron flow from H_2O to produce the reducing power are fairly well established now. However, the mechanism of O_2 evolution from H_2O is still unknown. This aspect of photosynthesis is unique to green plants as it is not shared by bacterial photosynthesis. (For general discussions on photosynthesis, see refs. 1,2).

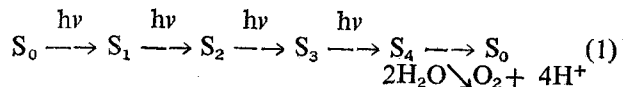
The present letter deals with the question of O_2 evolution and how the technique of pulsed nmr has helped us to make an inroad in the understanding of this problem. In our nmr experiments, samples of broken chloroplasts, containing the basic photosynthesizing membranes—the thylakoids (which are membranous vesicles containing all the machinery for the O_2 evolution and the production of the reducing power), are placed in a magnetic field of an nmr spectrometer. Then, a radio frequency pulse

is applied at right angles to the permanent magnetic field. This rf pulse provides a strong magnetic field and the nuclear spins begin to align themselves to this new field; as this rf pulse is over, the nuclei relax to their original position. The relaxation in the transverse direction (T_2^{-1}), mainly due to spin-spin interactions, and in the longitudinal direction (T_1^{-1}), due to spin-lattice interactions, are measured³.

The O_2 System—the O_2 Clock.

All what has been known about O_2 evolution can be summarized as follows⁴: (i) O_2 evolution is sensitive to heating—its activity drops remarkably if thylakoids are heated to $45^\circ C$ for 5-10 minutes; (ii) manganese and certain anions are necessary for its activity; and (iii) some sort of charge accumulation is required because O_2 evolution occurs only after the 3rd light flash when thylakoids are exposed to a series of brief (μs) light flashes spaced ~ 1 second apart.

The discussion of the last experiment requires further elaboration (see ref. 5). First, there is a periodicity of 4 with O_2 yield/flash having maxima at 3rd, 7th, 11th flashes, and second, there is a damping of the yield until O_2 yield is $\sim 1/2$ of that in the 3rd flash. These and other data led to the suggestion that O_2 is evolved by the following scheme (we call it the O_2 clock):



where the S's refer to the state of the oxygen evolving system (not to be confused with the excited states

*Talk delivered at Midwest Photosynthesis Conference, Turkey Run, Indiana, Oct. 20, 1977.

of molecules) with the subscripts referring to the number of positive oxidizing equivalents. (Note that $S_n \rightarrow S_{n+1}$ reaction does not use light ($h\nu$) directly, but, indirectly through its action on the reaction centre chlorophyll *a* of this system. In each light reaction, there is a charge separation, the positive charges being stored on the "S" complex and the negative charges (electrons) being diverted to produce the reducing power). Several additional assumptions are needed to fit the above model with the experimental data on O_2 yield/flash as a function of flash number. These include (i) the ratio $S_1 : S_0$ in dark-adapted thylakoids is 3:1 and the other states (S_2, S_3, S_4) do not exist in darkness ; (ii) all the quanta of light, absorbed by the system, do not advance the S states forward ; there is an approximate inefficiency of $\sim 10\%$. A great deal of data—all on O_2 evolution kinetics, which have been accumulated over the last five years, fit the above scheme, but no insight into the chemical nature and the functioning of the "S" intermediate has been forthcoming.

First Application of Nuclear Magnetic Resonance to Thylakoid Membranes : Water Proton Relaxation Rates Monitor Manganese in Thylakoids.

It had been assumed that manganese is specifically important for O_2 evolution but not for the other reaction of photosynthesis. Since manganese (a paramagnetic ion) has large effects on the relaxation of bound water protons, T. Wydrzynski, N. Zumbulyadis, P. Schmidt and Govindjee⁶ attempted to see if they can monitor manganese in thylakoids by measuring water proton relaxation rates by the method described above. The very first experiments showed that the longitudinal relaxation rate T_1^{-1} can indeed monitor the presence of membrane bound manganese in thylakoids because the extraction of manganese led to decreases in this rate. Furthermore, addition of reductants increased T_1^{-1} and oxidants decreased it suggesting that a mixture of oxidation states of manganese exists in chloroplasts.

Light Effects : Proton Relaxation Rates Monitor the O_2 Clock.

Our interest in the use of nmr techniques took a

quantum jump when Wydrzynski, Zumbulyadis, Schmidt, H. S. Gutowsky and Govindjee⁷ showed that T_2^{-1} as a function of number of light flashes oscillates with a periodicity of 4 just like O_2 yield/flash does—maximum at the 3rd, 7th, 11th flashes. Thus, these authors paved the way for the conclusion that nmr techniques can be used to monitor the "S" states in the O_2 clock. This then became the first new method to study O_2 evolution besides measuring the O_2 evolution itself. However, there were many differences between the patterns of O_2 yield and T_2^{-1} as function of flash numbers, and further analysis of these differences has now led to a better understanding of the O_2 clock as we realized that T_2^{-1} monitors the individual S states whereas O_2 monitors only the last step in equation (1).

Relationship of the O_2 Clock to a Manganese Clock.

Wydrzynski, S. B. Marks, Schmidt, Gutowsky and Govindjee⁸ have now established a quantitative relationship between O_2 evolution, T_2^{-1} and the manganese content of thylakoid membranes. As the Mn content of thylakoid membrane was decreased by exchange with magnesium, both O_2 evolution and T_2^{-1} decreased almost in parallel. Furthermore, Wydrzynski, Govindjee Zumbulyadis, Schmidt and Gutowsky⁹ and later Wydrzynski *et al.*⁸ showed that T_1^{-1} increases, reaches an optimum and then declines as the frequency of the pulsed radio waves is increased ; on the other hand, the latter authors⁸ showed that T_2^{-1} increases monotonously with increasing frequency. These data suggest that the electronic spin relaxation rates dominate the measured relaxation rates and the electronic spin relaxation lifetime (τ_s) needed to fit the experimental data, which is of the order of 1-2 ns, agrees with that of the Mn [II], but not that of Mn [III], Fe [II] or Fe [III] which is of the order of ~ 10 ps¹⁰.

Wydrzynski, Marks, Govindjee, Schmidt and Gutowsky¹¹ are now attempting to explain the T_2^{-1} and O_2 yield/flash as a function of flash numbers in terms of a Mn clock. First, the data on the O_2 yield/flash as a function of flash number is used to calculate, as was done by Kok *et al.* (see ref. 5), the relative concentrations of S_0 and S_1 in dark-adapted chloro-

plants. Based on these initial conditions, the concentrations of S_0 , S_1 , S_2 and S_3 after each flash are calculated. Then each S state is assigned a weighting factor, and the weighted sum for the third flash is normalized to the T_2^{-1} value after the 3rd flash. The

weighting factors needed to fit the experimental data of T_2^{-1} as a function of flash number, in thylakoids at pH 6.7, is found to be 2, 1, 1, 3 for S_0 , S_1 , S_2 , S_3 , respectively. (We are still working to obtain more precise fits than obtained thus far). We speculate that these weighting factors simply imply the relative concentrations of Mn [II] in each S state.

As a working hypothesis (Fig. 1), we have assumed that there are 4 Mn atoms associated with one O_2 evolving center, then in S_0 , two of these atoms are as Mn [II] and the other two as Mn [III] giving a weighting factor of 2, the transition of S_0 to S_1 is clear in this model as S_1 has one more oxidizing equivalent than S_0 and thus there are three Mn [III] and one Mn [II]. The next step in the model shows no change (as the weighting factor for both S_1 and S_2 are 1). However, a quantum of light must have caused further oxidation, *i.e.*, Mn [II] concentration for S_2 should have been zero; a return to the one Mn [II] condition is explained by suggesting that an electron is picked up from outside the Mn complex. Since, there is not enough energy in a quantum of light to extract directly an electron from H_2O^{12} , it must directly arise from it, perhaps, through a hypothetical oxygen evolving enzyme, which may be complexed with 2 water molecules. The extra plus charge from this complex could be released as a proton (H^+) leaving the enzyme H_2O

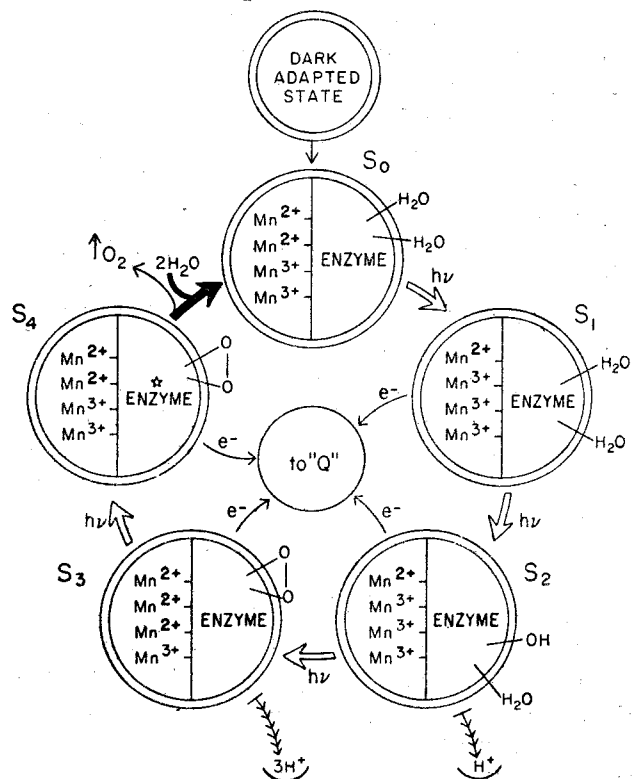


Fig. 1

Legend: One of the working hypotheses for O_2 evolution.

The S states of Kok, which originally represented the state of the O_2 evolving system with subscripts representing the number of positive equivalents, are shown here as Mn-Enzyme- H_2O complexes. Also shown is a special dark-adapted state. $h\nu$ represents a light quantum, but the reactions shown are not direct light reactions; the transformation of $S_n \rightarrow S_{n+1}$ occurs only as a consequence of light reaction in the reaction center chlorophyll *a* of pigment system II. "Q" (in the center of the diagram) represents the electron acceptor of this reaction. The initial charge separation caused by light absorption leads to reduction of Q (a quinone molecule) to Q^- and an oxidation of Mn^{2+} to Mn^{3+} . This step of Mn^{2+} to Mn^{3+} change is not shown but the resultant intermediates are shown. (The immediately formed intermediates, after light reactions, are denoted below as the primed states). In the S_0 to S_1 transformation, no further

change occurs and thus the Mn [II] content of the immediately formed S'_0 is the same as of S_1 . In the transformation of S_1 to S_2 , the Mn^{3+} formed in S'_1 is converted back to Mn^{2+} ; this conversion is accompanied by a release of a proton H^+ . The transformation of S_2 to S_3 is accompanied by the most critical steps; here, not only the Mn^{3+} formed in S'_2 is converted to Mn^{2+} , but two other Mn^{3+} s are changed to Mn^{2+} ; these steps are accompanied by "release"

of $3H^+$, and the formation of Enzyme $\begin{matrix} \diagup O \\ | \\ \diagdown O \end{matrix}$ complex. (The $2H^+$ remain attached to this complex). The next step from S_3 to S_4 like S_0 to S_1 is not accompanied by any further change after the S'_3 is formed which has one more Mn^{3+} than S_3 , except that the enzyme is in a different conformational (indicated by *) state ready to evolve O_2 , and, $2H^+$ are released. (Scheme modified by Govindjee and Wydrzynski after Govindjee, Wydrzynski and Marks, ref. 16).

complex as $\text{EnZ} \begin{array}{l} / \text{H}_2\text{O} \\ | \\ \backslash \text{OH} \end{array}$. The transition of S_2 to S_3

in the model is accompanied by a change from [Mn [II]] of 1 to 3. This is the most crucial step in the model. Here, in order to have 3 Mn [II] in S_3 , 3H^+ must leave the system; the enzyme complex

becomes $-\text{EnZ} \begin{array}{l} / \text{O} \\ | \\ \backslash \text{O} \end{array}$. In order to fit our theoretical prediction of H^+ release (when one Mn [III] must change to one Mn [II]) with the experimental data of Ausländer and Junge¹³, we must speculate that these 3H^+ may be released to the inside of the thylakoid in two steps of 1 and 2H^+ . The 2H^+ must still be somehow bound to the Enzyme complex, and be released only when the next light flash causes a "conformational" change in the complex. This model, however, does not account for the observation of a H^+ release in the transition of S_0 to S_1 and none in the transition of S_1 to S_2 suggested by the data of Fowler¹⁴ and Saphon and Crofts¹⁵. Finally the last flash produces ultimately S_0 ($2\text{Mn}[\text{II}] + 2\text{Mn}[\text{III}]$) + O_2 , and 2H^+ . Two molecules of H_2O are added to bring back the starting condition. Note that already in

S_3 $-\text{EnZ} \begin{array}{l} / \text{O} \\ | \\ \backslash \text{O} \end{array}$ exists, but oxygen comes off only when

S_4 is produced. We speculate that a certain conformational change (indicated by *) is required before the O_2 can come off from the complex. The above model is clearly a speculative one, but it is a starting point for further experiments and theory in order to formulate¹¹ the real Mn clock involved in O_2 evolution.

I am thankful to Drs. T. Wydrzynski, S. B. Marks, P. G. Schmidt and H. S. Gutowsky for collaboration. When our model is finalized, it will be published, in details, with the above-mentioned coworkers and colleagues.

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