Photosynthesis Research **80:** 125–135, 2004.

Minireview

Early indications for manganese oxidation state changes during photosynthetic oxygen production*: a personal account

Thomas J. Wydrzynski
Photobioenergetics, Research School of Biological Sciences, The Australian National University, Canberra, ACT 0200, Australia (e-mail: tom@rsbs.anu.edu.au; fax: +61-2-61258056)

Received 1 May 2003; accepted in revised form 10 June 2003

Key words: Herbert Gutowsky, manganese, NMR, O_2 evolution, Photosystem II, proton relaxation rates

Abstract

One of the major questions yet to be answered in photosynthesis research today is what is the chemical mechanism for the oxidation of water into molecular oxygen. It is well established that an inorganic cluster of four manganese ions and at least one calcium ion form the catalytic core. As the oxidation potential generated by the Photosystem II reaction center is accumulated over the four sequential steps needed to produce O_2, changes in the oxidation state of the catalytic manganese occur, though the formal oxidation states that are involved are still a matter of considerable debate. Much of what is currently known has come from direct measurements of the catalytic manganese using electron paramagnetic resonance (EPR) and X-ray spectroscopy. However, in the early attempts to attack this problem, the catalytic manganese was monitored indirectly by its paramagnetic effect on the nuclear magnetic resonance (NMR) relaxation rates of solvent water protons. In this contribution, a description of the proton relaxation rate phenomenon and its use to indicate manganese oxidation state changes during O_2 production is presented.

Introduction

One of the remaining, largely unresolved problems in modern-day photosynthesis research concerns the molecular mechanism in which atmospheric O_2 is produced from the oxidation of water by Photosystem II (PS II) (see Renger, 2003, these historical issues). A major contribution to the understanding of this mechanism came from the elegant, kinetic O_2 measurements made by Pierre Joliot in Paris, France (see Joliot 1993; also see Joliot, 2003, these historical issues). In particular, Pierre showed that the light activation step needed for O_2 evolution (Allen and Franck 1955; Whittingham and Brown 1958) is mediated by the same component that produces the O_2. This observation led Pierre and others to postulate the existence of a 'charge accumulating' intermediate on the oxidizing side of PS II, which reacts with water to produce O_2. In later studies, when the O_2 yields of dark-adapted samples were measured in a series of brief saturating light flashes, a very unique oscillatory pattern could be observed (Joliot et al. 1969; Kok et al. 1970). It was found that very little (or no) O_2 is produced after the 1st and 2nd flashes in the sequence while the maximum O_2 is produced after the 3rd flash. Peaks in the O_2 yield then appear after the 7th, 11th, etc. flashes, following a basic periodicity of four, until the oscillations damp out to a steady state level after the third or the fourth cycle.

Although several kinetic models were proposed to explain the O_2 flash yield pattern (Mar and Govindjee 1972), it was the model of Bessel Kok (1918–1979) at the Martin Marietta Corporation in Baltimore, Maryland (Kok et al. 1970; Forbush et al. 1971) that has withstood the test of time and forms the modern-day framework to describe PS II function. In Kok's model, a special component on the oxidizing side of PS II cycles through a series of five intermediate states,

*In memory of Herbert S. Gutowsky.

termed the S_n states (where $n = 0, 1, 2, 3, 4$), before reacting to produce O_2. In particular, each S_n state differs from the previous S_{n-1} state by the accumulation of one oxidizing equivalent until S_4 is reached, at which point O_2 is released and the S_0 state is regenerated. Other fundamental assumptions in Kok's model include: (1) the S_1 state is stable in the dark; (2) the S_2 and S_3 states eventually deactivate to the S_1 state if not advanced by another incoming photon; (3) upon reaching the S_4 state, O_2 is released through an instantaneous dark reaction; (4) after activation there is a finite time before an S_n state can be advanced by another photon; and (5) there is a certain small probability for a double hit (i.e., where an O_2 evolving center advances twice) or a miss (i.e., where an O_2 evolving center fails to advance) to occur after an activating light flash.

Upon promulgation of Kok's *S*-state hypothesis in the early 1970s, the quest began to identify the chemical nature of the 'charge accumulating' intermediate. At the time, the electron transport chain in photosynthesis was recognized to rely largely on special protein-bound co-factors, such as chlorin pigments, quinones, and metal centers. With his tremendous insight, Eugene Rabinowich at the University of Illinois was one of the first to discuss the possible involvement of peroxides in the liberation of O_2 during photosynthesis (Rabinowitch 1945, pp 281–299). Since it was known that Mn is a necessary micronutrient for plant growth (Pirson 1937; see Pirson 1994) and can take on a number of stable oxidation states, various manganese-peroxo intermediates were postulated (Wang 1970; Early 1973). Thus, it was suspected for a long time that Mn is the co-factor directly involved in O_2 evolution. However, it was not until the late 1950s and early 1960s that the Mn requirement in photosynthesis could be localized to PS II (Kessler et al. 1957; Spencer and Possingham 1961; Cheniae and Martin 1966). Nevertheless, it was difficult to obtain evidence for a direct role of Mn in O_2 evolution. Indeed, the Mn site in PS II is arguably the most labile of all of the bound cofactors in photosynthetic electron transport. Although many early attempts were made to biochemically isolate a unique Mn-containing protein (see Wydrzynski 1982), nearly a quarter of a century later we are still unable to completely identify all of the Mn ligation sites (Debus 2001; Diner 2001) or even to know if more than one subunit is involved in its binding (Zouni et al. 2001; Kamiya and Shen 2003). Thus, the only approaches to obtain information on the role of Mn in O_2 evolution had to rely on biophysical measurements of intact samples.

In the early 1970s electron paramagnetic resonance (EPR) spectroscopy was being widely used in photosynthesis research and was employed to study Mn, since Mn is a paramagnetic ion. Unfortunately, under standard X-band measuring conditions, Mn bound to a protein is largely EPR silent at room temperatures (for a possible exception, see Siderer et al. 1977). In contrast, unbound aqueous Mn^{2+} ions give rise to a very distinct, broad 6-line signal. With the advent of treatments to inactivate O_2 evolution activity, such as incubation in high concentrations of Tris buffer (Yamashita and Butler 1968), addition of hydroxylamine (Cheniae and Martin 1970), and non-denaturing temperature shocks (e.g., 55 °C) (Katoh and San Pietro 1967), it was found that the appearance of the EPR-detectable Mn 6-line signal could be inversely related to the O_2 evolving activity (Lozier et al. 1971). Robert (Bob) Blankenship and Kenneth Sauer at the Laboratory of Chemical Biodynamics, at the University of California Berkeley, were able to quantify the Mn released by Tris inactivation using EPR and identified two pools of bound Mn located towards the inner part of the thylakoid membrane (Blankenship and Sauer 1974). However, direct measurements of the intact, bound Mn still could not be made and had to wait until the discovery of the low-temperature (e.g., ~10 K), S_2-state Mn multi-line EPR signal by Charles Dismukes and Yona Siderer at Princeton University (Dismukes and Siderer 1981) and the first applications of Mn X-ray absorption near edge spectroscopy (XANES) by Mel Klein and co-workers at the Lawrence Berkeley National Laboratory in Berkeley (Kirby et al. 1981).

Nevertheless, it was in the 1970s at the University of Illinois at Urbana-Champaign that the first attempts were made to *indirectly* probe bound Mn based on its paramagnetic properties, by measuring the solvent-water proton relaxation rates (PRR) of aqueous suspensions of thylakoid membranes using pulsed nuclear magnetic resonance NMR techniques. It is this experimental approach that forms the topic of this historical minireview.

Proton NMR relaxation rate measurements

Basic background

To appreciate the type of information available from PRR measurements, a brief summary of the NMR theory and the definitions for the longitudinal ($1/T_1$) or spin–lattice relaxation and the transverse ($1/T_2$) or

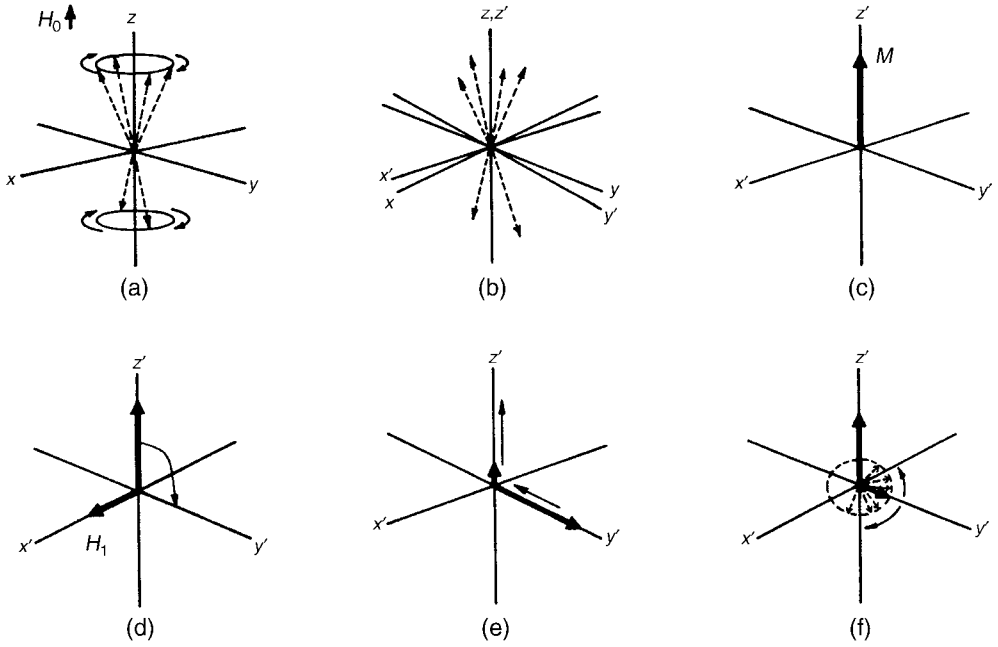

Figure 1. Definition of $1/T_1$ and $1/T_2$ relaxation phenomena in the rotating frame. (a) Precession of nuclear spins ($I = \frac{1}{2}$) about an applied field, H_0, which is defined along the z-axis. (b) Spins appear stationary in the rotating frame (x', y', z'). (c) Net magnetization, M, of the nuclear spins. (d) Application of a radio-frequency field, H_1, orthogonal to M in the rotating frame. (e) After the radio-frequency field is turned off, M relaxes back to the equilibrium condition. The growth of M along the $+z'$-axis is called the longitudinal or spin–lattice relaxation and is characterized by a time constant T_1, while the decay of M along the y' axis is called the transverse or spin–spin relaxation characterized by a time constant T_2. (f) Contribution of the dephasing of the individual nuclear dipoles in the x', y' plane to the $1/T_2$ relaxation. See text for details (taken from Wydrzynski 1977).

spin–spin relaxation are presented for students unfamiliar with this technique (for details, see Dwek 1973). In general, any mechanism that gives rise to a fluctuating magnetic field at an atomic nucleus is a possible nuclear magnetic relaxation mechanism. In classical terms, an atomic nucleus can be considered as a spinning positive charge, which gives rise to a magnetic dipole moment. In the absence of an external magnetic field, the individual magnetic dipoles will be randomly oriented and the net magnetization, M, defined as the vector sum of all nuclear dipoles, will be zero. When an external magnetic field, H_0, is applied, the individual nuclear dipoles will tend to align with H_0. The consequent torque acting upon alignment causes the nuclear dipoles to rotate or precess about the H_0 axis at a characteristic Larmor frequency. The Larmor frequency depends on the strength of H_0 and the nature of the atomic nucleus of interest. Thus, the most important condition for nuclear magnetic relaxation to occur is that any interacting fields fluctuate at the nuclear Larmor frequency.

The individual nuclear dipoles are spin quantized and take on $(2I+1)$ orientations. For protons with $I = \frac{1}{2}$, two orientations are possible – parallel and antiparallel with respect to the H_0. At thermal equilibrium a Boltzmann distribution is established between the energy levels associated with each dipole orientation. This is illustrated in Figure 1a where the lowest energy level is associated with the dipoles oriented parallel along the positive direction of the H_0 axis.

There are two types of magnetic relaxation phenomena, the longitudinal ($1/T_1$) or spin–lattice relaxation and the transverse ($1/T_2$) or spin–spin relaxation. The two relaxation phenomena can be visualized using a classical rotating frame analysis. In a frame of reference (x', y', z') rotating at the nuclear Lamor frequency, the individual nuclear dipoles appear stationary to an observer that is rotating with the frame in the x', y' plane (see Figure 1b). The vector sum of all nuclear dipoles in the rotating frame gives the net magnetization, M, for the nuclear spin system of interest. Since slightly more dipoles are aligned with H_0 than against it at thermal equilibrium, M will appear parallel along the $+z'$-direction (see Figure 1c). If another magnetic field, H_1, is introduced orthogonally to H_0 along the x'-direction (see Figure 1d) and perturbs the thermal equilibrium, M will rotate about H_1. If H_1 is applied for a long enough time, M will

tip to the x', y' plane (this is called a 90° pulse). After H_1 is turned off, M tends to return to its thermal equilibrium position via various relaxation processes. The rate of build-up of M along the z'-axis is characterized by the longitudinal or $1/T_1$ relaxation rate, while the decay of M along the y'-axis (i.e., the direction of the observer) is characterized by the transverse or $1/T_2$ relaxation rate (see Figure 1e). All magnetic interactions that lead to $1/T_1$ relaxation also lead to $1/T_2$ relaxation. However, the decay of M along the y'-axis is also influenced by a de-phasing (i.e., the loss of directional coherency) of the individual nuclear dipoles in the x', y' plane due to asymmetry in the local magnetic environment and inhomogeneities in the applied fields (see Figure 1f). Thus, it is always true that $1/T_2 \geq 1/T_1$.

In addition to the frequency profile for an interacting field, the rate of magnetic relaxation will also depend upon the magnitude of the interacting field. The electronic magnetic dipole is about a 1000× larger than the nuclear magnetic dipole. Thus, electron/nuclear dipole–dipole interactions will always dominate over smaller nuclear/nuclear dipole–dipole interactions and the introduction of a paramagnetic species (i.e., an ion with one or more unpaired electrons) will greatly enhance the nuclear relaxation of interest.

Manganese in its +2 oxidation state contains five unpaired electrons ($S = 5/2$) and is a particularly strong paramagnetic relaxer. The Mn^{2+} ion usually retains octahedral geometry with six co-ordination sites, some of which are often ligated to water. The extent of the magnetic interaction between the unpaired electrons of a Mn ion and bound water protons is governed by the fluctuations in the local magnetic interactions. These fluctuations are usually defined in terms of a correlation time, τ_C, and depend upon a range of time-dependent processes, e.g.,

$$\frac{1}{\tau_C} = \frac{1}{\tau_S} + \frac{1}{\tau_M} + \frac{1}{\tau_R},$$

where $1/\tau_S$ is the inherent rate of the electronic relaxation for the unpaired electrons, $1/\tau_M$ is the rate of chemical exchange between bound and unbound nuclear sites, and $1/\tau_R$ is the rate of rotational tumbling of the paramagnetic ion complex as a whole. Obviously, the fastest rate process will dominate.

For aqueous Mn^{2+} ions, $1/\tau_R$ dominates the PRR. In this case, the frequency dispersion profile for the fluctuations in the local magnetic interactions contains minimal components at the proton Larmor frequencies typically obtained with available NMR instruments and the measurable paramagnetic relaxation effect is reduced. On the other hand, when Mn^{2+} ions are bound to a macromolecular complex, the slower tumbling rate causes $1/\tau_R$ to drop out, in which case $1/\tau_S$ then usually dominates. Since the electronic relaxation for the unpaired electrons in the Mn^{2+} ion in particular contains more frequency components at the usual proton Larmor frequencies, especially at low resonance frequencies (e.g., ∼20 MHz), the measurable paramagnetic effect on the PRR is considerably enhanced.

The paramagnetic effect varies with the inverse 6th power of distance between the center of the paramagnetic ion and the relaxing nucleus; thus, it is largest on the nuclei bound directly to the paramagnetic ion and rapidly diminishes as the distance from the ion center increases. However, the mole fraction of protons bound at paramagnetic ions in aqueous solutions or suspensions is very small compared with concentration of solvent water protons (∼110 M) and is well below the sensitivity of the measurement. Since the NMR experiment determines the macroscopic magnetization of the system, the protons bound at a paramagnetic ion must be in fast exchange with the solvent water protons in order to distribute the magnetization effect throughout the system as a whole. Thus, the paramagnetic effect on the PRR is often described as:

$$\frac{1}{T_{1,2P}} = \frac{pq}{T_{1,2M} + \tau_M}$$

where $1/T_{1,2P}$ is the paramagnetic contribution to the proton spin–lattice ($1/T_1$) and the spin–spin ($1/T_2$) relaxation rates of the system as a whole; $1/T_{1,2M}$ is the paramagnetic contribution to the spin–lattice and spin–spin relaxation rates for the protons bound within the inner coordination sphere of the paramagnetic ion (and is governed by the correlation time τ_C for the electron/nuclear dipole–dipole interactions – see above); p is the molar concentration of the paramagnetic ion; q is the proton co-ordination number for the paramagnetic ion; and, τ_M is the residence time for the protons bound at the paramagnetic ion. When $\tau_M < 1/T_{1,2M}$, the paramagnetic effect dominates and the system is said to be in the fast exchange region. When $\tau_M > 1/T_{1,2M}$, chemical exchange dominates and the system becomes slow exchange limited. The condition $\tau_M \approx 1/T_{1,2M}$ or the intermediate exchange region could also arise, in which case both paramagnetic and chemical exchange contributions govern the overall relaxation rates. Thus, the paramagnetic en-

hancement of the solvent water PRR (i.e., $1/T_{1P}$ and $1/T_{2P}$) in aqueous suspensions of Mn-containing macromolecular complexes becomes an indirect probe for bound Mn. This feature of PRR enhancement was widely applied in the early 1970s to get structural information around enzymatic sites containing divalent metal ions that could be replaced with Mn^{2+} (Mildvan and Cohn 1970).

My personal involvement

As a doctoral student in the early 1970s at the University of Illinois at Urbana-Champaign, I first learned about the PRR enhancement phenomenon in a biophysics techniques course taught by Paul Schmidt. I discussed the PRR technique with my thesis advisor Govindjee and, realizing that Mn was a natural component of PS II, we approached Paul about the possibility of using PRR measurements to probe the functional manganese. As this approach had never been tried before in photosynthesis research, we became quite excited about the idea but did not have an instrument at the time to make PRR measurements at the low resonance frequencies needed to observe the optimal Mn paramagnetic effect. So we decided to approach Herb Gutowsky, one of the pioneers in NMR research, to use his old permanent iron magnet set up which he had used to take the very first NMR spectrum back in 1951. It was an ideal instrument to measure PRR enhancements due to Mn since it operated at a proton resonance frequency of ~27 MHz. With a twinkle in his eye, Herb particularly liked the idea to determine light-induced changes in the NMR and he gave us his full support. He even asked Nick Zumbulyadis, and later Steve Marks, (both postdoctoral associates working with him) to collaborate with us on the measurements.

PRR and manganese in thylakoids

In a relatively short space of time we were able to show that removal of the Mn from thylakoid membrane samples by both Tris and NH_2OH/EDTA extraction procedures led to a decrease of about two-thirds in the $1/T_1$ or spin–lattice PRR. Upon addition of reductants to dark-adapted samples there was 2–3-fold increase in the $1/T_1$ while oxidants caused about a one-half decrease compared with the $1/T_1$ of the untreated controls. These observations led us to suggest that there exists a mixture of Mn oxidation states in dark-adapted thylakoid samples, since at the time the other likely Mn oxidation state considered to be involved, Mn^{3+} ($S = 2$), has only a weak effect on the PRR (Wydrzynski et al. 1975). By using a simplified Solomon–Bloembergen–Morgan analysis of the frequency dispersion profiles for both $1/T_1$ and $1/T_2$ in the presence of an oxidant and a reductant, we concluded that the PRR enhancement in aqueous suspensions of thylakoid membranes was basically reflecting the relative Mn^{2+} content in the sample (Wydrzynski et al. 1978).

PRR as a function of flash number

The important experiment of course was to determine the PRR of thylakoids in a series of light flashes, to see if it could be related to Kok's *S*-states. After some difficulty to interface laser flash illumination of the sample *in situ* in the NMR probe, we were able to obtain flash-induced $1/T_2$ patterns for thylakoid samples having a periodicity of four (Wydrzynski et al. 1976; Govindjee et al. 1978). Figure 2 shows the results from Wydrzynski et al. (1976) re-plotted as $\Delta[1/T_2]$ (open circles) or the difference between the $1/T_2$ after a particular flash and the $1/T_2$ in the dark. Since in this particular experiment the $1/T_2$ in the dark has the highest value, $\Delta 1/T_2$ appears negative as light flashes are applied. As discussed above, we assumed that the PRR enhancement reflects the relative Mn^{2+} content of the sample. Thus, as the *S*-state cycle advances, the relative Mn^{2+} content appears to decrease (or the net

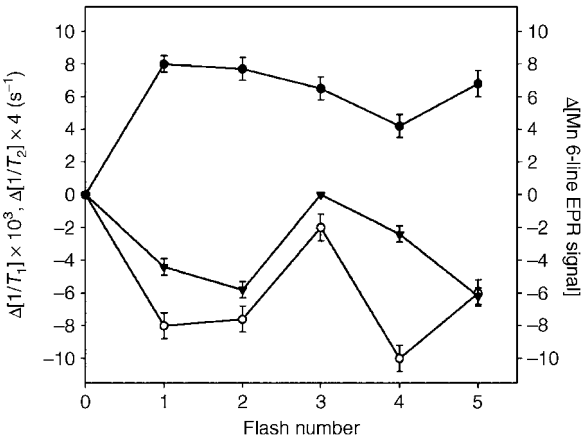

Figure 2. Plots of the $1/T_2$ enhancements (○) and the heat-induced Mn 6-line EPR signals (▼) for spinach thylakoid membrane samples and the $1/T_1$ enhancements (●) for spinach PS II-enriched membrane fragments as a function of brief light flashes. The data was adapted from Wydrzynski et al. (1976), Wydrzynski and Sauer (1980), and Srinivasan and Sharp (1986), respectively. See text for details.

Mn oxidation level appears to increase) until the accumulated oxidation level reacts to produce O_2 on the 3rd and subsequent flashes where the O_2 yield peaks. The oscillations eventually damp out as the *S* states loose synchrony through the accumulation of misses and double hits during the flash sequence. At the peak O_2 yields, the catalytic Mn appear to exhibit its most reduced level. Unfortunately, later attempts at the University of Illinois to obtain $1/T_2$ flash patterns were not always successful (see Govindjee and Wydrzynski 1981). However, the original $1/T_2$ flash pattern was supported by measurements of the Mn 6-line EPR signal that arise from the aqueous Mn^{2+} formed in spinach thylakoid samples subjected to a temperature shock. When the Mn 6-line signal was measured under these conditions following a sequence of light flashes, an oscillatory pattern could be observed (Wydrzynski and Sauer, 1980). These results are also replotted in Figure 2 (inverted triangles) for comparison. In this case, in order to account for the oscillatory behavior, it was assumed that the higher oxidation states of the catalytic Mn disproportionate in part to Mn^{2+} (e.g., $2Mn^{3+} \rightarrow Mn^{2+} + Mn^{4+}$, where the released Mn^{4+} would react with solvent water to form the EPR-silent MnO_2 precipitate) upon heat treatment. These early results therefore represented some of the first indications that Mn oxidation state changes occur during photosynthetic oxygen production.

Controversy

As with all new experimental approaches, groups with access to similar techniques often repeat and elaborate on the original observations, thereby expanding and refining the interpretation. So it was with Robert (Bob) Sharp, in collaboration with Charles (Charlie) Yocum, at the University of Michigan at Ann Arbor, who repeated the NMR-PRR measurements on thylakoid samples. Interestingly, Bob found that in the presence of EDTA (Ethylene Diamine Tetra Acetic acid), the PRR enhancement in $1/T_1$ by thylakoids largely disappears. Since EDTA has little effect on O_2 evolution activity, Bob suggested that the bulk of the PRR enhancement in thylakoids must arise from adventitiously, non-functionally bound Mn^{2+} ions (Robinson et al. 1980; Sharp and Yocum 1980). In Bob's view, EDTA can only interact as a chelator of monomeric ions and thus scoops up adventitious Mn^{2+} ions bound to the outer surface of the thylakoid membrane and eliminates their contribution to the PRR measurement. In our studies, we were aware of the EDTA effect (Wydrzynski 1977) but found it difficult to explain the large influence of oxidants and reductants on the PRR in the absence of EDTA simply in terms of effects from adventitiously bound Mn^{2+} ions.

On a visit to the University of Illinois in 1979, Melvin Calvin, who received the 1961 Nobel Prize in Chemistry for the 'Path of Carbon in Photosynthesis', offered a compromise explanation. He suggested that adventitiously bound Mn ions on the surface of the membrane 'sensed' the paramagnetism of the functional Mn at the O_2 evolving site and communicated the paramagnetic effect to the solvent water protons.

In subsequent work we reported that EDTA has complicated effects on pea thylakoid samples (Govindjee and Wydrzynski 1981; Wydrzynski and Renger 1986). We showed that most of the adventitiously bound Mn in our samples could be removed by thorough washing in standard buffer medium or in a low osmotic buffer medium. After the washings we determined the Mn content of our samples to be 3.24 ± 0.25 Mn ions/400 Chl or PS II reaction center, close to the expected value for the catalytic site. Oddly the addition of EDTA to the sample did not facilitate the removal of the bound Mn but did largely suppress the PRR enhancement in both $1/T_1$ and $1/T_2$. And more surprisingly the PRR enhancement in EDTA-treated samples could be restored to that for untreated samples by thoroughly washing out the EDTA. We interpreted these results to indicate that the presence of EDTA must cause microenvironment changes within the thylakoid membrane, which restricts the accessibility of internally bound paramagnetic sites to the solvent phase protons on the NMR time scale. Other PRR measurements of thylakoids made as a function of pH and heat treatment also indicated structural perturbations to the PS II complex and strongly influenced the PRR enhancement (Khanna et al. 1983). In other words, depending upon the experimental conditions, τ_M could increase such that the paramagnetic effect of bound ions on the PRR is obscured. This is not an unreasonable assumption as the temperature dependence of the PRR of thylakoids indicated that the paramagnetic enhancement is probably in the intermediate exchange region (Wydrzynski et al. 1978), where small changes in the exchange rate can obscure or enhance paramagnetic effects (see above).

The use of EDTA, and of the other commonly used chelator EGTA, has otherwise been shown to have subtle effects on PS II structure and function. Bob Blankenship first observed that EDTA readily elim-

inates the aqueous Mn^{2+} 6-line EPR signal in Tris-treated thylakoid samples (Blankenship and Sauer 1976). Since the Mn 6-line signal in the absence of EDTA cannot be washed away, Bob suggested that EDTA must surprisingly be able to penetrate the membrane and interact with internal Mn^{2+} ions that are somehow trapped in the thylakoid. More recently it has been suggested from pulsed EPR measurements that EGTA can bind close to or at the catalytic site to give rise to modified Mn EPR signals in calcium deficient samples (Zimmermann et al. 1993). In contrast, under similar conditions, FTIR (Fourier Transform Infra Red) measurements have indicated that EDTA and EGTA may reversibly replace a carboxylate ligand associated with the catalytic site but without affecting the magnetic properties of the Mn EPR signals (Kimura and Ono 2001).

Subsequently, Bob Sharp and co-workers were able to obtain evidence to indicate that the catalytic Mn can indeed be monitored by PRR measurements. Using PS II-enriched membrane fragments (i.e., so-called Berthold–Babcock–Yocum (BBY) samples, see Berthold et al. 1981), Sharp, with Srinivansan, could measure relatively small enhancements in $1/T_1$ ($<1\%$ of background) in the presence of EDTA as a function of light flashes (Srinivansan and Sharp 1986a, b). These measurements are re-plotted in Figure 2 as $\Delta 1/T_1$ (closed circles) for comparison with the earlier results. In this case the values are positive with respect to the dark value and oscillate weakly with peaks after the 1st and 5th flashes. Bob noted that this pattern mimicked the flash-induced pattern for the S_2-state Mn multi-line EPR signal identified with the catalytic Mn cluster (Dismukes and Siderer 1981). He therefore interpreted the increase in the PRR after the first flash (i.e., the predominant S_1 to S_2 transition) as the formation of a hydrated Mn^{4+} ion ($S = 3/2$) (Sharp 1992) since this was how the EPR results were interpreted. Although hydrated Mn^{4+} complexes do not normally exist since the Mn^{4+} ion is a poor Lewis base with a strong oxidation potential (and as such prefers to take on fully deprotonated oxo-ligands – see Hillier and Wydrzynski 2001), Bob estimated that its electron spin relaxation time (τ_S) should nevertheless be similar to that for Mn^{2+}. Thus, he predicted that there was an oxidation of a hydrated Mn^{+3} to hydrated Mn^{+4} on the S_1 to S_2 transition based on his $1/T_1$ flash pattern (Sharp 1992). This proposal was consistent with the interpretations of the EPR and XANES measurements at the time (for a summary, see Hasegawa et al. 1999). However, according to Bob's interpretation the oxidation state changes reflected in the $1/T_1$ flash pattern should reside on a monomeric Mn ion. In an elegant theoretical analysis, Bob clearly showed that the relaxation behavior in a spin-coupled, polynuclear Mn cluster containing a weakly paramagnetic Mn^{3+} ion would be the same as a monomeric Mn^{3+} ion, regardless of whether or not the other Mn ions in the cluster were in a highly paramagnetic relaxing oxidation state (i.e., Mn^{2+} or Mn^{4+}). Thus, based on Bob's PRR measurements, the organization of the catalytic Mn at the O_2-evolving site would have to fit into a monomer/trimer model where the oxidation state change occurs at an uncoupled monomeric Mn ion. In contrast, most current models predict that the redox-active Mn ion(s) is located in a cluster that is spin coupled (e.g., see Hasegawa et al. 1999; Carrell et al. 2002; Messinger et al. 2001).

Future directions

During the evolution of the application of NMR–PRR measurements to the study of the O_2 evolving site in PS II, all of those involved I believe were a little bit naïve as to how much could be gleaned from the results. As a biochemist I did not fully appreciate the intricacies of the NMR theory and techniques while I think the chemists did not fully realize the complexity of PS II. The PRR enhancement depends on a number of parameters, including the number of protons bound at the paramagnetic site, the accessibility of the bound protons to the solvent protons, and the necessity for fast proton exchange to achieve magnetization transfer. Any changes in these parameters can alter the PPR outside of the paramagnetic effect due to oxidation state changes. In PS II we still do not know the exact ligand structure around the catalytic Mn (Zouni et al. 2001; Kamiya and Shen 2003) nor exactly how proton transfer from the substrate water is coupled to the *S*-state cycle (Rappaport and Lavergne 2001). Similarly, we do not know the location of the catalytic Mn in relation to the solvent phase, if it is shielded by a hydrophobic region, or whether or not solvent accessibility plays a regulatory role in the water oxidation reaction (Wydrzynski et al. 1996). Clearly the temperature dependency in the PRR indicates that the paramagnetic effect is in the intermediate exchange region, which means that slight changes in the exchange rate can determine whether a paramagnetic enhancement due to an oxidation state change is observed or not.

The question remains as to where the substrate water binds in the O_2 evolving complex and how does it interact with the catalytic Mn. Some insight into this question has been gained from recent rapid ^{18}O exchange measurements (Messinger et al. 1995). In particular, it has been shown that one substrate water molecule is bound at the beginning of the *S* state cycle in the S_0 state and the second substrate water molecule is bound in at least the S_3 (Hillier and Wydrzynski 2000) and S_2 (Hendry and Wydrzynski 2002) states. Comparison of the ^{18}O exchange rates for the O_2 evolving site with known metal complexes seem to indicate that the substrate water may be bound to Mn^{3+} ions throughout the *S* states (Hillier and Wydrzynski 2001). More recently, deuterium isotope effects on S_2/S_1 difference FTIR spectra have indicated that the two OH groups of an *S*-state active water molecule is H-bonded (Noguchi and Sugiura 2000, 2001). It has been shown from *ab inito* calculations that in the S_2 state, one of the OH groups is strongly H-bonded and the other one much more weakly, whereas in the S_1 state the H-bonding for both OH groups is more similar (Fischer and Wydrzynski 2001).

With the newer information from the mass spectrometric and FTIR studies, plus the use of various PS II mutants (Debus 2001), proton release measurements (Rappaport and Lavergne 2001), and low resolution crystal structures (Zouni et al. 2001; Kamiya and Shen 2003), it is perhaps time to re-evaluate the NMR–PRR results. The NMR–PRR measurements can give dynamic information on time scales that other techniques cannot give and provide still another approach in solving one of nature's most unique chemical reactions.

Finally, I show a photograph of Herb Gutowsky in Figure 3, to whom this paper is dedicated, a photograph of the author in Figure 4, and a series of photographs of the research group in Govindjee's laboratory at the time when the NMR measurements were being made in Figure 5.

Figure 3. A photograph of Herbert S. Gutowsky (1920–2000). Herbert S. Gutowsky was a Research Professor of Chemistry at the University of Illinois at Urbana-Champaign. He took his PhD in Chemistry at Harvard University and joined the University of Illinois in 1947. During his career, he served for many years as Head of the Department of Chemistry and Director of the School of Chemical Sciences. He and his students made great discoveries in the early days of NMR, shaping the development of NMR and setting the stage for its central role in modern day chemistry, medicine, physics, and material science. He won the 1983/1984 Wolf Prize in Chemistry. In 1984, he returned to teaching and research until his death in January 2000 at the age of 80. (Adapted from the preamble to the Herbert S. Gutowsky Memorial Symposium, Urbana, Illinois, June 24, 2000 – http://www.scs.uiuc.edu/chem/gutowsky/.)

Figure 4. A 1991 photograph of the author (TW) with his dog Diets while he was in Germany (1985–1986 and 1988–1991). Photo by Govindjee.

Figure 5. A photograph of Govindjee's research group contemporary with the author (TW) at the University of Illinois at Urbana-Champaign (approximate date, 1976). *Top* (*from left to right*): Mike Slovacek (visiting student from Tom Bannister's lab in Rochester, New York), Ralph Schooley (MS student), Barbara Zilinskas (class of 1975), David van der Meulen (class of 1977; behind Barbara), Thomas Wydrzynski (author; class of 1977), Daniel Wong (class of 1979) and Alan Stemler (class of 1974). *Middle*: Rita Khanna (center; class of 1980) with Jean-Marie Briantais (right; post-doc from CNRS, Gif-sur-Yvette, France), and Govindjee; photo taken at a Gordon Conference. *Bottom*: Paul Jursinic (class of 1977) examining the historical instrument of Robert Emerson, that was used to discover the Enhancement effect in photosynthesis leading to the two-light reaction and two-photosystem concept of photosynthesis. Photographs provided by Govindjee.

Acknowledgments

The author sincerely thanks Govindjee for his strong encouragement and support during the writing of this article, Taka-Aki Ono for reading the manuscript, and Joel Freeman for assistance in the preparation of the manuscript. This manuscript was edited by Govindjee.

References

Allen FL and Franck J (1955) Photosynthetic evolution of oxygen by flashes of light. Arch Biochem Biophys 58: 124–143

Berthold DA, Babcock GT and Yocum CF (1981) A highly resolved, oxygen-evolving Photosystem II preparation from spinach thylakoid membranes. FEBS Lett 13: 231–233

Blankenship RE and Sauer K (1974) Manganese in photosynthetic oxygen evolution I. Electron paramagnetic resonance study of the environment of manganese in tris-washed chloroplasts. Biochim Biophys Acta 357: 252–266

Carrell TG, Tyryshkin AM and Dismukes GC (2002) An evaluation of structural models for the photosynthetic water-oxidizing complex derived from spectroscopic and X-ray signatures. J Biol Inorg Chem 7: 2–22

Cheniae GM and Martin IF (1966) Studies on the function of manganese in photosynthesis. Brookhaven Symp Biol 19: 409–417

Cheniae GM and Martin IF (1970) Sites of function of manganese within photosystem II. Roles in O_2 evolution. Biochim Biophys Acta 197: 219–239

Debus RJ (2001) Amino acid residues that modulate the properties of tyrosine Y_Z and the manganese cluster in the water oxidizing complex of Photosystem II. Biochim Biophys Acta 1503: 164–186

Diner BA (2001) Amino acid residues involved in the coordination and assembly of the manganese cluster of Photosystem II. Proton-coupled electron transport of the redox active tyrosines and its relationship to water oxidation. Biochim Biophys Acta 1503: 147–163

Dismukes GC and Siderer Y (1981) Intermediates of a polynuclear manganese cluster involved in the photosynthetic oxidation of water. Proc Natl Acad Sci USA 78: 274–278

Dwek RA (1973) Nuclear Magnetic Resonance (NMR) in Biochemistry: Application to Enzyme Systems. Claredon Press, Oxford

Early JE (1973) Oxygen evolution: a molecular model for the photosynthetic process based on an inorganic example. Inorg Nucl Chem Lett 9: 487–490

Fischer G and Wydrzynski T (2001) Isotope effects in FTIR difference spectra of the photosynthetic oxygen-evolving catalytic site determined by *ab inito* calculations on model compounds. J Phys Chem B 105: 12894–12901

Forbush M, Kok B and McGloin M (1971) Cooperation of charges in photosynthetic O_2 evolution 2. Damping of flash yield oscillation and deactivation. Photochem Photobiol 14: 307–321

Govindjee and Wydrzynski T (1981) Oxygen evolution, manganese, ESR and NMR. In: Akoyunoglou G (ed) Proceedings of the Fifth International Congress on Photosynthesis Vol II, pp 307–305. Balaban International Science Services, Philadelphia

Govindjee, Wydrzynski T and Marks SB (1978) Manganese and chloride: their roles in photosynthesis. In: Metzner H (ed) Photosynthetic Oxygen Evolution, pp 321–344. Academic Press, New York

Hasegawa K, Ono T-A, Inoue Y and Kusunoki M (1999) How to evaluate the structure of a tetranuclear Mn cluster from magnetic and EXAFS data: case of the S_2-state Mn-cluster in photosystem II. Bull Chem Soc Japan 72: 1013–1023

Hendry G and Wydrzynski (2002) The two substrate-water molecules are already bound to the oxygen-evolving complex in the S_2 state of Photosystem II. Biochemistry 41: 13328–13334

Hillier W and Wydrzynski T (2000) The affinities for the two substrate water binding sites in the O_2 evolving complex of Photosystem II vary independently during S-state turnover. Biochemistry 39: 4399–4405

Hillier W and Wydrzynski T (2001) Oxygen ligand exchange at metal sites. Implications for the O_2 evolving mechanism of Photosystem II. Biochim Biophys Acta 1503: 197–209

Joliot P (1993) Early researches on the mechanism of oxygen evolution: a personal account. Photosynth Res 38: 214–233

Joliot P (2003) Period-four oscillation of the flash-induced oxygen formation in photosynthesis. Photosynth Res 76: 65–72

Joliot P, Barbieri G and Chabaud R (1969) Un nouveau modele des centres photochimiques du systeme II. Photochem Photobiol 10: 309–329

Kamiya N and Shen J-R (2003) Crystal structure of oxygen-evolving Photosystem II from *Thermosynechococcus vulcanus* at 3.7-Å resolution. Proc Natl Acad Sci USA 100: 98–103

Katoh S and San Pietro A (1967) Ascorbate-supported NADP photoreduction in heated *Euglena* chloroplasts. Arch Biochem Biophys 122: 144–152

Kessler E, Arthur W and Brugger JE (1957) The influence of manganese and phosphate on delayed light emission, fluorescence, photoreduction and photosynthesis in algae. Arch Biochem Biophys 71: 326–335

Khanna R, Rajan S, Govindjee and Gutowsky HS (1983) Effects of physical and chemical treatments on chloroplast manganese: NMR and ESR studies. Biochim Biophys Acta 725: 10–18

Kimura Y and Ono T-A (2001) Chelator-induced disappearance of carboxylate stretching vibrational modes in S_2/S_1 FTIR spectrum in oxygen-evolving complex of Photosystem II. Biochemistry 40: 14061–14068

Kirby JA, Goodin DB, Wydrzynski T, Robertson AS and Klein MP (1981) State of manganese in the photosynthetic apparatus 2. X-ray absorption edge studies of manganese in photosynthetic membranes. J Am Chem Soc 103: 5537–5542

Kok B, Forbush M and McGloin M (1970) Cooperation of charges in photosynthetic O_2 evolution 1. Photochem Photobiol 11: 457–475

Lozier R, Baginsky M and Butler WL (1971) Inhibition of electron transport in chloroplasts by chaotropic agents and the use of manganese as an electron donor to Photosystem II. Photochem Photobiol 14: 323–328

Mar T and Govindjee (1972) Kinetic models of oxygen evolution in photosynthesis. J Theor Biol 36: 427–446

Messinger J, Badger M and Wydzynski (1995) Determination of one slowly exchanging substrate water molecule in the S3 state of Photosystem II. Proc Natl Acad Sci USA 92: 3209–3213

Messinger J, Robblee JH, Bergmann U, Fernandez C, Glatzel P, Visser H, Cinco RM, McFarlane KL, Bellacchio E, Pizarro SA, Cramer SA, Sauer K, Klein MP and Yachandra VK (2001) Absence of Mn-centered oxidation in the S_1 to S_2 transition. Implications for the mechanism of photosynthetic water oxidation. J Am Chem Soc 123: 7804–7820

Mildvan AS and Cohn M (1970) Aspects of enzyme mechanisms studied by nuclear spin relaxation induced by paramagnetic probes. Adv Enzymol 33: 1–70

Noguchi T and Sugiura M (2000) Structure of an active water molecule in the water-oxidizing complex of Photosystem II as studied by FTIR spectroscopy. Biochemistry 36: 10943–10949

Noguchi T and Sugiura M (2001) Flash-induced FTIR difference spectra of the water-oxidizing complex in moderately hydrated Photosystem II core films. Effect of hydration extent on S-state transitions. Biochemistry 40: 1497–1502

Pirson A (1937) Ernährungs- und stoffwechselphysiologische Untersuchungen an Fontinalis und Chlorella. Z Bot 31: 193–272

Pirson A (1994) Sixty years in algal physiology and photosynthesis. Photosynth Res 40: 207–222

Rabinowich E (1945) Photosynthesis and Related Topics Vol I. Wiley (Interscience), New York

Rappaport F and Lavergne J (2001) Coupling of electron and proton transfer in the photosynthetic water oxidase. Biochim Biophys Acta 1503: 246–259

Renger G (2003) Apparatus and mechanism of photosynthetic oxygen evolution: a personal historical reflection of 35 years research activity and current state of the art. Photosynth Res 76: 269–288

Robinson HH, Sharp RR and Yocum CF (1980) Effect of manganese on the nuclear magnetic relaxivity of water protons in chloroplast suspensions. Biochem Biophys Res Comm 93: 755–761

Sharp RR (1992) Proton NMR relaxation due to the photosynthetic oxygen evolving center. In: Pecoraro VL (ed) Manganese Redox Enzymes, pp 177–196. VCH Publishers, New York

Sharp RR and Yocum CF (1980) Field dispersion profiles of the proton spin–lattice relaxation rate in chloroplast suspensions. Effect of manganese extraction by EDTA, tris and hydroxylamine. Biochim Biophys Acta 592: 185–195

Siderer Y, Malkin S, Poupko R and Luz Z (1977) Electron spin resonance and photoreaction of Mn(II) in lettuce chloroplasts. Arch Biochem Biohys 179: 174–182

Spencer D and Possingham JV (1961) The effect of manganese deficiency on photophosphorylation and the O_2-evolving sequence in spinach chloroplasts. Biochim Biophys Acta 52: 379–381

Srinivasan AN and Sharp RR (1986a) Flash-induced enhancements in the proton NMR relaxation rate of Photosystem II particles. Biochim Biophys Acta 850: 211–217

Srinivasan AN and Sharp RR (1986b) Flash-induced enhancements in the proton NMR relaxation rate of Photosystem II particles. Response to flash trains of 1–5 flashes. Biochim Biophys Acta 851: 369–376

Wang JH (1970) Synthetic biochemical models. Acc Chem Res 3: 90–97

Whittingham CP and Brown AH (1958) Oxygen evolution from algae illuminated by short and long flashes of light. J Exp Bot 9: 311–319

Wydrzynski TJ (1977) The role of manganese in photosynthetic oxygen evolution. PhD thesis. University of Illinois at Urbana-Champaign, Illinois

Wydrzynski TJ (1982) Oxygen evolution in photosynthesis. In: Govindjee (ed) Photosynthesis – Energy Conversion by Plants and Bacteria, Vol I, pp 469–506. Academic Press, New York

Wydrzynski T and Renger G (1986) On the interpretation of the NMR water–proton relaxivity of photosynthetic membrane samples: ramifications in the use of EDTA. Biochim Biophys Acta 851: 65–74

Wydrzynski T and Sauer K (1980) Periodic changes in the oxidation state of manganese in photosynthetic oxygen evolution upon illumination with flashes. Biochim Biophys Acta 589: 56–70

Wydrzynski T, Zumbulyadis N, Schmidt PG and Govindjee (1975) Water proton relaxation as a monitor of membrane-bound man-

ganese in spinach chloroplasts. Biochim Biophys Acta 408: 349–354

Wydrzynski T, Zumbulyadis N, Schmidt PG, Gutowsky HS and Govindjee (1976) Proton relaxation and charge accumulation during oxygen evolution in photosynthesis. Proc Natl Acad Sci USA 73: 1196–1198

Wydrzynski TJ, Marks SB, Schmidt PG, Govindjee and Gutowsky HS (1978) Nuclear magnetic relaxation by the manganese in aqueous suspensions of chloroplasts. Biochemistry 17: 2155–2162

Wydrzynski T, Hillier W and Messinger J (1996) On the functional significance of substrate accessibility in the photosynthetic water oxidation mechanism. Physiol Plant 96: 342–350

Yamashita T and Butler WL (1968) Inhibition of the Hill reaction by tris and restoration by electron donation

Zimmermann J-L, Boussac A and Rutherford AW (1993) The manganese center of oxygen-evolving and Ca^{2+}-depleted Photosystem II: a pulsed EPR spectroscopy study. Biochemistry 32: 4831–4841

Zouni A, Witt H-T, Kern J, Fromme P, Krauss N, Saenger W and Orth P (2001) Crystal structure of Photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. Nature 409: 739–743