Nuclear Magnetic Relaxation by the Manganese in Aqueous Suspensions of Chloroplasts[†]

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ABSTRACT: Proton and oxygen-17 NMR relaxation rate $(T_1^{-1}$ and $T_2^{-1})$ data are presented for aqueous suspensions of dark-adapted chloroplasts. It is concluded from the dependence of the proton relaxation rates (PRR) upon Mn concentration that T_1^{-1} and T_2^{-1} are determined largely by the loosely bound Mn present in the chloroplast membranes. The frequency and temperature dependences of the PRR are characteristic of Mn(II). The effects of oxidants (e.g., ferricyanide) and reductants (e.g., tetraphenylboron) on the PRR indicate that only about one-third to one-fourth of the loosely bound Mn is present in the dark-adapted chloroplasts as Mn(II), the remainder being in a higher oxidation state(s), probably Mn(III). The frequency dependence of the PRR for the chloroplast suspensions was fitted by a simplified form of the Solomon–Bloembergen–Morgan equations, and the following parameters were obtained: $\tau_S = (1.1 \pm 0.1) \times 10^{-8}$ s; $\tau_M = (2.2 \pm 0.2) \times 10^{-8}$ s; and $B = (0.9 \pm 0.09) \times 10^{19}$. The oxygen-17 T_1 and T_2 data for suspensions before and after treatment with a detergent are consistent with the location of the manganese in the interior of the thylakoids. An analysis of the relaxation rates shows that the average lifetime of a water molecule inside a thylakoid is >1 ms.

The involvement of manganese ions in photosynthesis was first suggested in the early studies of Pirson (1937). Much later, the site of its activity was assigned to the oxygen-evolving mechanism of photosystem II (Kessler et al., 1957; Spence and Possingham, 1961; Cheniae and Martin, 1966; Homann, 1968). Subsequently, Kok et al. (1970) proposed a model for oxygen evolution in which a photochemically activated intermediate accumulates up to 4 oxidizing equiv before reacting with water to produce oxygen. Manganese can exist in several oxidation states and thus it could play a central role in the charge accumulation (Cheniae, 1970; Olson, 1970; Renger, 1970; Earley, 1973). However, until recently there has been no direct experimental evidence to show that manganese changes its oxidation state during photosynthesis (Wydrzynski et al., 1976a,b).

The current view is that manganese is complexed to some protein bound to the chloroplast membrane. However, attempts to isolate a manganese protein and to reconstitute oxygen evolution have failed (Cheniae and Martin, 1966; Lagoutte and Duraton, 1975; Henriques and Park, 1976). One possible exception is the stimulation of oxygen evolution by a low-molecular-weight manganese polypeptide isolated from *Phormidium luridium* (Tel-Or and Avron, 1974). In any event, if one wishes to study the role of manganese in oxygen evolution, it is necessary to use an experimental approach which responds to changes in situ of the manganese.

The unpaired electrons of Mn(II) can, in favorable instances, give rise to a detectable electron paramagnetic resonance (EPR[1]) spectrum. However, so far there has been only one report of EPR spectra from Mn bound to chloroplasts (Siderer et al., 1977). Another possibility is that neighboring magnetic nuclei are perturbed enough by the large magnetic moment of the unpaired electrons in Mn(II) to be sensitive to changes in its oxidation state. Mn(II) usually maintains some water ligands even when bound to large macromolecules and the electronic relaxation of Mn(II) is such that the relaxation rates of the protons in the solvation sphere are strongly enhanced. Furthermore, all of the water protons share in the enhancement because of the fast proton exchange between solvation sphere and solvent. For these reasons, it seemed likely to us that the proton relaxation rates (PRR) of the water could be used to detect changes in situ of the Mn in aqueous suspensions of chloroplasts.

Preliminary experiments (Wydrzynski et al., 1975) showed that treatments which affect the amount of bound manganese in chloroplast membranes have a large effect on the spin–lattice or longitudinal relaxation rate (T_1^{-1}) of the water protons. This included washing the membranes with alkaline tris(hydroxymethyl)aminomethane (Tris) buffer or NH_2OH–ethylenediaminetetraacetic acid (EDTA) solution. Also, the addition of oxidants led to a decrease in T_1^{-1}, whereas the addition of reductants caused a large increase, suggesting that the PRR could be used to detect changes in the oxidation state. In a second set of experiments (Wydrzynski et al., 1976a), the aqueous suspensions were exposed to a series of intense light flashes after n of which ($n = 0, 1, 2, \ldots 20$) the proton transverse (spin–spin) relaxation rate (T_2^{-1}) was measured. The response of T_2^{-1} to light flashes is very similar to that of oxygen evolution, both sequences exhibiting a damped oscillation with a periodicity of four flashes.

Thus, the proton relaxation rates are closely associated with the charge-accumulating mechanism for oxygen evolution by

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[1] Abbreviations used are: PRR, proton relaxation rates (i.e., T_1^{-1} and T_2^{-1}); EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; chl, chlorophyll; FID, free induction decay; SBM, Solomon, Bloembergen, and Morgan; chlpts, chloroplasts; T–A chlpts, Tris–acetone washed chloroplasts; Ph$_4$B, tetraphenylboron; FeCy, potassium ferricyanide, $K_3Fe(CN)_6$.

photosystem II. The purpose of the present study was to determine whether the enhanced rates found in aqueous suspensions of chloroplasts, compared to buffered suspensions without chloroplasts, are indeed caused primarily by Mn(II). Several different approaches were used. The total Mn concentration of the chloroplasts was varied, measured, and compared with the proton relaxation rates. The frequency dependence of the proton relaxation was observed for the suspensions and compared with that reported for known Mn(II) systems. Also, the effects of redox agents upon the frequency dependence were studied. And, finally, some measurements of ^{17}O relaxation were made for suspensions in enriched water.

Experimental Procedure

Sample Preparation. The chloroplasts were obtained from dwarf peas (*Pisum sativum,* var. progress no. 9). The seeds were soaked in aerated deionized water for 12 h and planted in vermiculite. At planting, each flat received 1 L of Hoagland's solution (Johri and Varner, 1964) containing 9 μM $MnCl_2$; thereafter, the plants received only deionized water. The plants were grown under a 16-h light (23 °C)/8-h dark cycle (20 °C). Light intensity was 15 W/m^2 (ITT F72512/cool white fluorescence lamps).

Chloroplasts were isolated from 12–16 day old plants. Leaves were homogenized in a Waring blender for ~15 s in a medium containing 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. Also, the homogenization medium included 0.5% bovine serum albumin along with 10 mM sodium ascorbate. The homogenate was filtered through four layers of cheesecloth and a 10 μm mesh nylon cloth, centrifuged, and finally resuspended in the complete medium. Chlorophyll was determined by the method of Arnon (1949), and the suspensions were adjusted to a final concentration of 3 mg of chlorophyll (chl)/mL.

Manganese was extracted from the chloroplast membranes either by magnesium exchange according to the procedure of Chen and Wang (1974) or by washing in Tris–acetone according to the procedure of Yamashita and Tomita (1974). For extraction by magnesium exchange, chloroplasts were incubated in the isolation media containing $MgCl_2$ at various [Mg]/[chl] ratios (0; 167; 332; 667; 2500; 10 000) for 2 h at 4 °C. The chloroplasts were then centrifuged and resuspended in the isolation medium to a final concentration of 3 mg of chl/mL. For Tris–acetone extraction the chloroplasts were suspended in 0.8 M Tris buffer, pH 8.2, containing 20% acetone at 200 μg of chl/mL for 20 min at 4 °C. The chloroplasts were centrifuged and resuspended in the isolation medium as described above.

Manganese Determination. The manganese content of the chloroplast membranes was generally measured by the atomic absorption method. Preparation of the samples was after Blankenship and Sauer (1974). One milliliter of the sample (3 mg of chl/mL) was digested with 2 mL of 85% concentrated HNO_3 and 15% concentrated $HClO_4$, v/v, under gentle heating. The digested sample was filtered through Whatman no. 50 filter paper and brought to a final volume of 5 mL with distilled water. Measurements were made with a Perkin-Elmer Model 303 atomic absorption spectrometer. Standards at 0.2, 0.4, 0.6, 0.8, and 1.0 ppm Mn were prepared in the buffer medium and carried through the same digestion procedure as the sample. Manganese determinations of the samples were interpolated from the standard curve which was linear and passed through the origin.

Alternatively, the manganese content was determined on

sample aliquots by neutron activation analysis. The samples were heat sealed in precleaned polyethylene containers. Standards for comparison were prepared by pipetting 1 μL of solutions of known manganese concentrations onto a Whatman no. 42 filter paper in precleaned vials. The samples and standards were then irradiated for 1 h at a flux of 2×10^{12} neutrons/cm²-s in the Illinois Advanced TRIGA Reactor facility. After a delay of 2 or 3 h to permit the decay of ^{38}Cl, each sample was counted for 900 s using a 10% efficient Ge(Li) detector (Canberra) having a FWHM resolution of 2.1 keV at 1333 keV. The γ-ray data were reduced to elemental concentrations using the program PIDAQ (developed by J. P. Maney, J. L. Fasching, and P. K. Hopke, University of Illinois). Two different γ-ray lines of ^{56}Mn were used to determine a weighted mean.

In both methods, several determinations were made of each preparation. The reproducibility was ±1%.

NMR Relaxation Measurements. Longitudinal (T_1^{-1}) and transverse (T_2^{-1}) relaxation rates were measured over a 4.7- to 70-MHz range with a variable frequency pulsed spectrometer. The instrument employed a Varian (Model V-7400) 15-in. electromagnet with an operating range up to 24 kG. A Varian flux stabilizer (Model V-3508) and field-homogeneity unit (Model V-7530) provided adequate field stability and homogeneity. The output of the frequency synthesizer (Rockland Model 5600, 0.1 to 160 MHz) is split into two channels by the gating unit and is phase shifted for the pulse sequence of Carr and Purcell (1954) with the modification of Meiboom and Gill (1958). The A and B channels are gated by a pulse programmer through double-balanced mixers. The gated low-level rf is amplified using an Arenberg Model PG 650 tunable oscillator operated in the gated amplifier mode (this unit puts out up to 400 W). The probe design (single coil, 6.5-mm diameter) features interchangeable plug-in insert, vacuum Dewar assemblies. The 90° pulse widths were ~2 μs. The receiver is broadbanded (3–150 MHz) and employs a Watkins–Johnson preamplifier (28-dB gain) and Kay Elemetrics amplifier (40 dB). A double-balanced mixer is the phase-sensitive detector, the output of which is applied to an operational amplifier for further gain and low-pass filtering.

The T_1^{-1} measurements were made with the 180°–τ–90° pulse sequence. Free induction decays (FID) following the 90° pulse were collected on a storage oscilloscope for measurement of their amplitudes as a function of τ. At least 8 τ values were sampled and acquisitions were repeated at intervals of no less than 5T_1. The signal-to-noise ratio of the FID was sufficiently good even at the lowest frequency, so that semilog plots of the magnetization, ln $[M_0 - M(\tau)]$ vs. τ, showed essentially no scatter and were linear over their whole range. The T_1 values are estimated to have an experimental uncertainty of about ±5% and a deviation on a particular sample of not more than ±3%.

The T_2^{-1} values were obtained from the decay of the echo envelope plotted on an oscillographic recorder. We used a delay of 2τ = 1 ms between 180° pulses. The T_2^{-1} results have approximately the same error limits as T_1^{-1}.

Probe temperature was controlled by a thermostatic flow of N_2. A calibrated thermocouple encased in the NMR tube was used to measure the sample temperature before and after relaxation measurements. These readings varied by less than 1 °C after the temperature was stabilized.

Oxygen-17 Measurements. Chloroplast suspensions were prepared with water enriched to about 28% (w/w) in ^{17}O. The water was purchased from the Mound Laboratory of the Monsanto Research Corp., Miamisburg, Ohio. The T_1 was measured by the inversion recovery method on the instrument

described above. As a check, several measurements were repeated on a similar pulsed NMR spectrometer kindly made available to us by Dr. J. Jonas and his co-workers. Some continuous-wave (i.e., wide line) T_2 measurements were performed on a Varian WL-115 spectrometer. All measurements were made at room temperature. The pulsed NMR measurements were made at 8.1 and 12.8 MHz and the wide-line measurements at 12.8 MHz.

Treatment of the Data. The proton relaxation rates observed as a function of frequency were fitted to a theoretical model whose details are outlined in the Theory section. A theoretical relaxation rate was calculated from the model, as a function of five adjustable parameters, for the frequency of each appropriately corrected experimental rate. Best-fit values were obtained for the five parameters by systematically varying them to minimize the differences between calculated and experimental rates. This least-squares type calculation was performed on an IBM 360/70 computer using the program MINROS, developed by Duane Steidinger, available in the computer program library at the University of Illinois.

Theory

The factors governing the proton relaxation rates (PRR) of aqueous systems containing low concentrations of paramagnetic species have been reviewed by Dwek (1973). In the dilute solutions of interest here, the paramagnetic contribution, i.e., the enhancement of the PRR, is given by

$$\left(1/T\right)_p = \frac{pq}{T_M + \tau_M} + \frac{pq_{os}}{T_{os}} = (1/T)_{obsd} - (1/T)_d \quad (1)$$

where T stands for either T_1 or T_2, the $(T^{-1})_{obsd}$ is the observed, exchange averaged PRR, $(T^{-1})_d$ is the PRR for the system in the absence of the paramagnetic species, p is the mole fraction of the paramagnetic species, q is the hydration number of the paramagnetic species, T_M is the relaxation time of protons in the hydration sphere of the paramagnetic site, τ_M is the mean lifetime of a proton in that sphere, and T_{os} is the relaxation time for the so-called "outer-sphere" protons, for which q_{os} is the effective "hydration number."

The frequency dependences of T_M are given by the Solomon, Bloembergen, and Morgan (SBM) equations (Dwek, 1973) which are presented in a simplified form appropriate to the system studied here:

$$\frac{1}{T_{1M}} = C \left[\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2 \tau_c^2} \right] \quad (2)$$

and

$$\frac{1}{T_{2M}} = \frac{C}{2} \left[4\tau_c + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{13\tau_c}{1 + \omega_S^2 \tau_c^2} \right] \quad (3)$$

where ω_I and ω_S are the nuclear and electronic Larmor frequencies, respectively, and $C = (2/15)S(S + 1)\gamma_I^2 \gamma_S^2 r^{-6}$. In the latter, S is the resultant electron spin-angular momentum, γ_I and γ_S are the nuclear and electronic magnetogyric ratios, respectively, and r is the distance between a proton in the hydration sphere and the paramagnetic ion. In these equations, a term due to the electronic–nuclear scalar coupling has been omitted, since it has been shown to be negligible for Mn(II) bound to macromolecules (Navon, 1970).

The correlation time τ_c is given as:

$$(1/\tau_c) = (1/\tau_r) + (1/\tau_S) + (1/\tau_M) \quad (4)$$

where τ_r is the characteristic time for rotational reorientation of the paramagnetic complex and τ_S is the lifetime of an electronic spin state. The correlation times τ_r and τ_M are in-

dependent of frequency. However, τ_S is usually given by the following equation:

$$\frac{1}{\tau_S} = B \left[\frac{\tau_v}{1 + \omega_S^2 \tau_v^2} + \frac{4\tau_v}{1 + 4\omega_S^2 \tau_v^2} \right] \quad (5)$$

where τ_v is the characteristic time for symmetry distortion of the complex and B is a constant dependent upon the zero-field splitting of the paramagnetic complex. Therefore, τ_S will be frequency dependent unless $\omega_S^2 \tau_v^2 \ll 1$.

The temperature dependence of τ_M is expected to show Arrhenius behavior, i.e.:

$$(1/\tau_M) = A \exp(-\Delta E^{\ddagger}/RT) \quad (6)$$

The correlation times τ_r and τ_v are expected to obey a similar exponential relation, e.g., for τ_r:

$$\tau_r = \tau_r^0 \exp(E_a/RT) \quad (7)$$

Outer sphere relaxation was found to contribute significantly to T_P^{-1} and could not be neglected in the treatment of the frequency-dependence data reported here. Two limiting cases (Dwek, 1973) have been suggested for the frequency dependences of T_{os}^{-1}: (1) T_{os}^{-1} are dominated by the correlation time τ_S and hence their frequency dependences are identical to those of τ_M, and (2) T_{os}^{-1} are controlled by a correlation time which is related to the relative diffusional motion of the paramagnetic ion complex and the ligand protons. In the latter case, the values of T_{os}^{-1} are given by the following equations:

$$(1/T_{1os}) = D[7f_t(\omega_S \tau_S) + 3f_t(\omega_I \tau_S)] \quad (8)$$

and

$$(1/T_{2os}) = D \left[\frac{13}{2} f_t(\omega_S \tau_D) + 3f_t(\omega_I \tau_D) + 2 \right] \quad (9)$$

where τ_D is the characteristic time for diffusional motion of a proton, f_t is a function of ω and τ_D, and D is a constant.

The above theory applies to dilute aqueous solutions of a single paramagnetic species. However, the chloroplast suspensions contain several paramagnetic species, so some thought need to be given to the separability of their effects on the PRR. Inspection of eq 1–3 shows that the effects of two paramagnetic centers (of the same or different species) will be additive if the presence of one does not affect the hydration (q, q_{os}, τ_M, r, τ_r) or spin-lifetime (τ_S) of the other. This is the case at infinite dilution and it is a good approximation in "dilute" solutions. However, at higher concentrations, direct or indirect interactions among the paramagnetic centers will lead to PRR that are nonlinear in the concentration(s) p of the species involved.

Consider, for example, τ_S which is probably the parameter most likely to be affected significantly by other paramagnetic centers. Besides the zero-field splitting term given in eq 5, there is diffusional, dipole–dipole relaxation among the centers, which adds a concentration-dependent term, that is:

$$(1/\tau_S) = (1/\tau_S)_v + Ap \quad (10)$$

where A is a phenomenologically determined constant. Substitution of this expression in eq 1–4 shows that for high concentrations of paramagnetic species $(1/T)_p$ becomes proportional to p^2. There can be a similar, rotational, dipole–dipole contribution to τ_S if two paramagnetic centers are held close to one another by tight binding to the same macromolecule.

Results

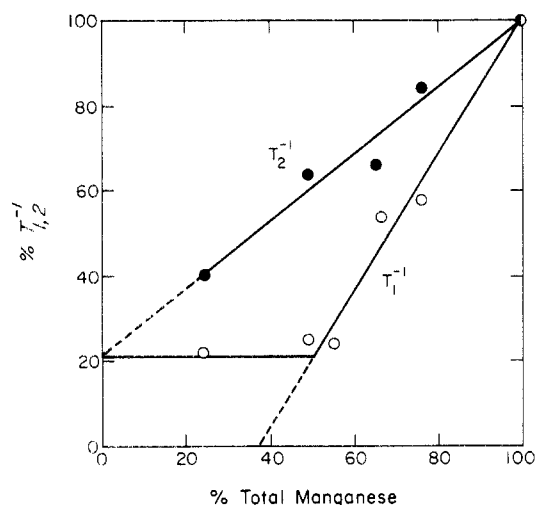*Dependence of the PRR upon Mn Content.* Chloroplast membranes contain two pools of manganese (Cheniae, 1970),

FIGURE 1: Proton relaxation rates as a function of manganese content, plotted as percent of the values for an unwashed control containing 0.62 ± 0.03 μg of Mn/mg of chl. The PRR were measured at 27 MHz and 25 °C on dark-adapted pea chloroplast suspensions containing 3 mg of chl/mL, and corrected for buffer contributions. The manganese content was varied by Mg exchange.
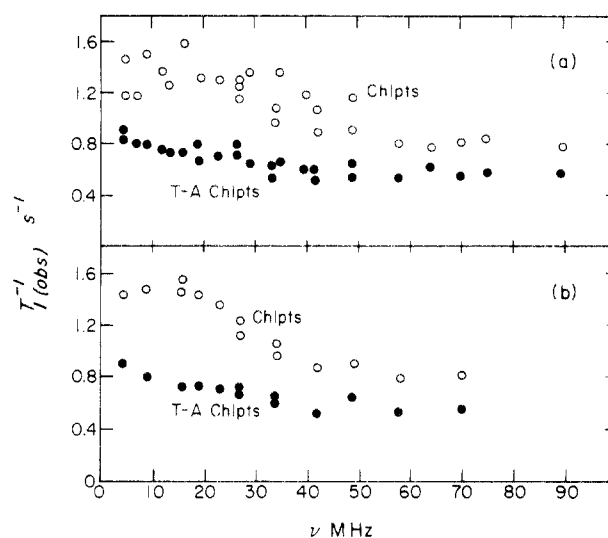

FIGURE 2: Frequency dependence of the proton T_1^{-1} observed for dark-adapted pea chloroplast suspensions (chlpts) containing 3 mg of chl/mL, at room temperature (23–25 °C). (a) Uncorrected rates observed for several different samples. (b) Observed rates adjusted to the same Mn concentration. T–A chlpts refers to Tris–acetone washed chloroplasts in which the loosely bound fraction of Mn is removed.

a "tightly" bound and a "loosely" bound fraction. The latter, which represents approximately two-thirds of the total manganese, has been associated with oxygen evolution. Several treatments cause the release of the loosely bound manganese from the membrane and the concurrent appearance of a Mn(II) EPR signal (Lozier et al., 1971); these include incubation with alkaline Tris buffer (Yamashita and Butler, 1968), hydroxylamine (Cheniae and Martin, 1971), or high concentrations of MgCl₂ (Chen and Wang, 1974). In our experiments the chloroplasts were incubated at different [Mg]/[chl] ratios, thereby removing an adjustable fraction of the initial manganese.

Figure 1 shows the relaxation rates that result from this treatment. The normalized enhancement of the proton relaxation in the treated suspensions is plotted as a function of the residual fraction of manganese, in percent. The normalized enhancement was obtained by subtracting the relaxation rate of the buffer from the relaxation rate observed for the treated suspension, per eq 1, and dividing by the enhancement of the untreated suspension. If all of the enhancement were due to manganese which is homogeneous in its effects upon the relaxation rates, the data would fall upon a single straight line extending from the origin to the upper right hand corner of the plot. Instead, the T_2^{-1} data in Figure 1 lie above such a line, while the T_1^{-1} data are below.

A simple interpretation of these results is the following: (1) About 20% of the enhancement in the untreated suspensions is caused by species other than manganese in the chloroplasts. (2) The tightly and loosely bound fractions of manganese are of comparable effect, per atom, upon T_2^{-1}. (3) The tightly bound fraction of manganese has little effect upon T_1^{-1}. Point 1 is based upon extrapolation of the T_1^{-1} and T_2^{-1} data to zero manganese, both of which give an intercept of 20% enhancement. Point 2 is indicated by the linearity of the T_2^{-1} plot, including concentrations at which some tightly bound Mn has been removed. Point 3 is based upon the two straight-line portions in the T_1^{-1} plot.

Figure 2 shows the values of T_1^{-1} observed for dark-adapted chloroplast suspensions as a function of NMR frequency. Part a of the figure shows the original T_1^{-1} data, which are affected by small differences in Mn concentration. The manganese

content was determined for seven of the chloroplast preparations, five by atomic absorption and two by neutron activation. The average values for these preparations ranged from 0.400 to 0.496 μg of Mn/mg of chl, a variation comparable with that reported by Blankenship and Sauer (1974). In part b of Figure 2, the T_1^{-1} data for these seven preparations have been multiplied by the appropriate factors to adjust them to a single Mn concentration, 2.7×10^{-5} M (0.5 μg of Mn/mg of chl). (Some of the data in part a are from early preparations for which the Mn content was not determined.) It is clear that most of the scatter of the data in part a is due to variations in the Mn content of the different preparations.

This observation and the data in Figure 1 demonstrate that the major part of the enhancement in the PRR is caused by the loosely bound Mn. Our next step is to consider separation of the proton relaxation by the loosely bound Mn from that of the other paramagnetic species in the chloroplasts. If this is feasible, information about the nature of the complex containing the loosely bound Mn can be sought from the frequency and the temperature dependences of its effects.

There is substantial evidence that the different paramagnetic species give additive contributions to the PRR. For example, it has been pointed out (Bloembergen and Morgan, 1961) that interactions among Mn²⁺ ions play a negligible role in the proton relaxation of aqueous solutions at concentrations below 0.05 N, which is a hundred times more concentrated than Mn in the chloroplast suspensions. In Figure 1, removal of Mn from the chloroplasts reduces their enhancement of the PRR in linear proportion. Also, the proton T_1^{-1} was found to be linear in the amount of chlorophyll over a tenfold concentration range, from 0.5 to 5.0 mg of chl/mL, at 27 MHz and 25 °C, using pea chloroplasts. Such linear behavior would be expected for additive contributions in a homogeneous system, as pointed out in the discussion of eq 10. However, the chloroplast suspensions are heterogeneous and the observed linearities do not eliminate a rotational contribution to τ_S by interactions between paramagnetic centers selectively bound to neighboring sites on a macromolecule. But any such term (analogous to eq 5) is unlikely to be significant because the tumbling time τ_r for Mn bound to a membrane is expected to be relatively long
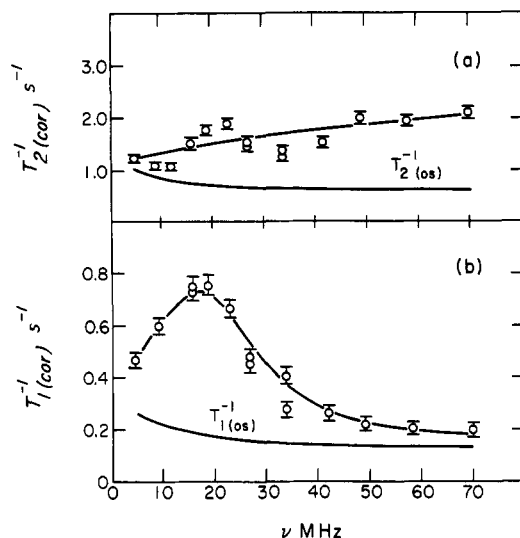
FIGURE 3: Frequency dependence of the PRR for the loosely bound paramagnetic species in dark-adapted suspensions of pea chloroplasts (3 mg of chl/mL). The open circles are experimental values measured at room temperature (23-25 °C), corrected for the tightly bound species and adjusted to the same Mn concentration. The upper, solid lines are theoretical best fits of the data corresponding to the parameters in Table II. The lower solid lines are theoretical outer-sphere contributions based on a diffusional model.

TABLE I: Relaxation Parameters Found for Protons in Aqueous Solutions of Chloroplasts and in Several Known Mn(II) Systems.

	τ_S^0 $(10^{-8}$ s)	τ_M $(10^{-8}$ s)	B $(10^{19}$ rd^2 s$^{-2})$
Chlpts	1.1 ± 0.1	2.2 ± 0.2	0.90 ± 0.09
Chlpts + Ph$_4$B	1.1 ± 0.1	1.0 ± 0.1	0.90 ± 0.09
Chlpts + FeCy	2.0 ± 0.2	0.1 ± 0.01	1.1 ± 0.1
Aqueous Mn(II)[a]	0.95 ± 0.1	2.3	10
Mn(II)-carboxy-peptidase A[b]	0.92 ± 0.1	0.25 ± 0.3	3.1 ± 0.4
Mn(II)-pyruvate kinase[b,c]	2.3 ± 0.6 2.3	0.4 ± 0.04 0.5 ± 0.1	0.8 ± 0.1 1.46

[a] Bloembergen and Morgan (1961). [b] Navon (1970). [c] Reuben and Cohn (1970).

(Dwek, 1973). This leads to $\omega_S^2 \tau_r^2 \gg 1$ and a negligible contribution to τ_S.

Nature of the Loosely Bound Mn. The evidence just cited makes it reasonable to attempt to separate the effects of the loosely bound Mn from those of the other paramagnetic species. The open circles in both parts of Figure 2 are T_1^{-1} for untreated chloroplasts. The T_1^{-1} data labeled T-A chlpts (Tris-acetone washed chloroplasts) are for chloroplasts which have had the loosely bound fraction of Mn removed by washing with Tris buffer containing 20% acetone. The difference between the two rates should be a good approximation to the enhanced relaxation caused by the loosely bound Mn. Such "corrected" relaxation rates (T_2^{-1} as well as T_1^{-1}) have been obtained by subtracting the two sets of rates, adjusted to the same Mn concentration, i.e.,

$$(1/T)_{cor} = (1/T)_{obsd} - (1/T)_{T-A \text{ chlpts}} \qquad (11)$$

The results for $(1/T)_{cor}$ are given in Figure 3. In general appearance they are quite similar to those for Mn(II) complexes, i.e., a severalfold value for T_2/T_1 and a broad maximum in T_1^{-1} at lower frequencies ($\nu \approx 20$ MHz). In contrast, for the residual paramagnetic species, e.g., $(1/T_1)_{T-A \text{ chlpts}}$ in Figure 2, we find $T_2/T_1 \approx 2$; also, both T_2^{-1} (not shown) and T_1^{-1} are virtually independent of frequency except for a small increase in T_1^{-1} at lower frequencies. This behavior is characteristic of paramagnetic ions with a short τ_S ($\sim 10^{-10}$ s) such as Fe(III) (Villafranca et al., 1974).

A quantitative treatment of the data in Figure 3 can be accomplished with the simplified SBM equations for the case in which τ_S is frequency dependent and makes a sizable contribution to τ_c^{-1}. Attempts to fit the data assuming only τ_S relaxation (i.e., $\tau_c^{-1} = \tau_S^{-1}$) failed and it was necessary to include an additional frequency independent correlation time in the analysis (i.e., either τ_M, τ_r, or both). However, as pointed out at the end of the preceding section, τ_r is probably relatively long and will have little effect on τ_c; therefore, it was assumed that the frequency-independent term in τ_c^{-1} arises from τ_M so that $\tau_c^{-1} = \tau_S^{-1} + \tau_M^{-1}$. While this approach fits the qualitative features of the T_1^{-1} and T_2^{-2} dependences upon

frequency, it does not account for the large T_1^{-1} at high frequencies (i.e., $\nu \geq 50$ MHz) or the large values of T_2^{-1} at low frequencies ($\nu \leq 30$ MHz).

The discrepancies cannot be removed by adjusting the scale of the "background" correction, $(1/T)_{T-A \text{ chlpts}}$ in eq 11. Instead, the data were successfully fitted by including the terms T_{os} of eq 1 in the analysis and assuming that the diffusional model correctly describes their frequency dependence. The solid lines passing through the data points in Figure 3 have been calculated on this basis from eq 1, 9, 10, and the SBM equations. The calculated curves marked T_{1os}^{-1} and T_{2os}^{-1} show the contributions of these terms. However, the source of this contribution is uncertain, and the treatment presented here, although reasonable, is empirical. For this reason, no physical significance is attached to the parameters D and τ_D of eq 8 and 9, obtained in the fitting process. Similarly, no attempt was made to interpret the best-fit value of C (eq 3 and 4).

Table I lists the values of τ_S^0, τ_M, and B for dark-adapted chloroplasts, obtained from the fitting process for the data of Figure 3. The superscript 0 designates the value of τ_S at zero field, i.e., $\omega_S = 0$ in eq 5. Also listed in Table I are these parameters evaluated by other workers from data on aqueous systems containing known macromolecular Mn(II) complexes. There are close similarities among the several sets of values. On the other hand, Mn(III) is quite different. In particular, $\tau_S^0 \approx 10^{-8}$ s for Mn(II) (Dwek, 1973), while it has been estimated to be $\approx 10^{-11}$ s for Mn(III) (Villafranca et al., 1974). The latter value is too small to account adequately for the observed relaxation data; e.g., it does not predict the maximum in $(1/T_1)_{cor}$ at ~ 17 MHz. Therefore, we conclude that Mn(II) is the species of manganese governing the PRR in the chloroplast suspensions.

With the values given above for the various parameters, T_M and τ_M were calculated and it was found that $\tau_M \ll T_M$ at all frequencies employed. This result is confirmed by the temperature dependence of the PRR shown in Figure 4. The data exhibit the non-Arrhenius type of behavior that occurs when more than one rate process contributes significantly to the relaxation, τ_S (or τ_v) and τ_M in this case. The changing slope of the curved lines gives an estimated activation energy of 2-3 kcal/mol. This range is intermediate to the values expected for electronic relaxation via τ_v (~ 1 kcal/mol) and for chemical exchange (5-15 kcal/mol). The limited temperature range available to the system (5-38 °C) prevents one from obtaining accurate activation energies for τ_S and τ_M separately.

Effects of Redox Agents. The addition of redox agents to the chloroplast suspensions produces changes that are in accord with loosely bound Mn(II) being the chief source of the proton relaxation. The higher oxidation states of manganese are not
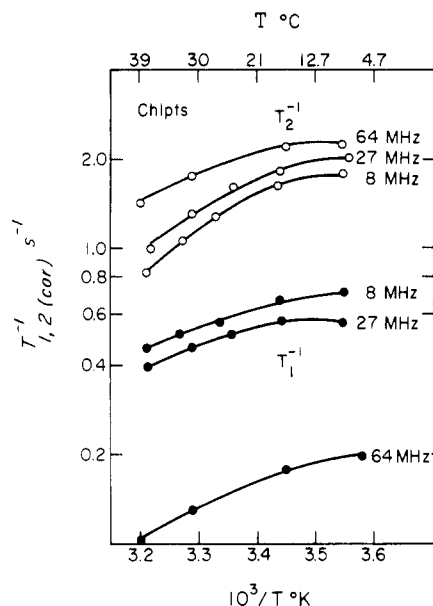
FIGURE 4: A semi-log plot of the temperature dependence of the PRR for the loosely bound paramagnetic species in dark-adapted suspensions of pea chloroplasts (3 mg of chl/mL) at several frequencies. The experimental values have been corrected for the tightly bound species and adjusted to the same Mn concentration.
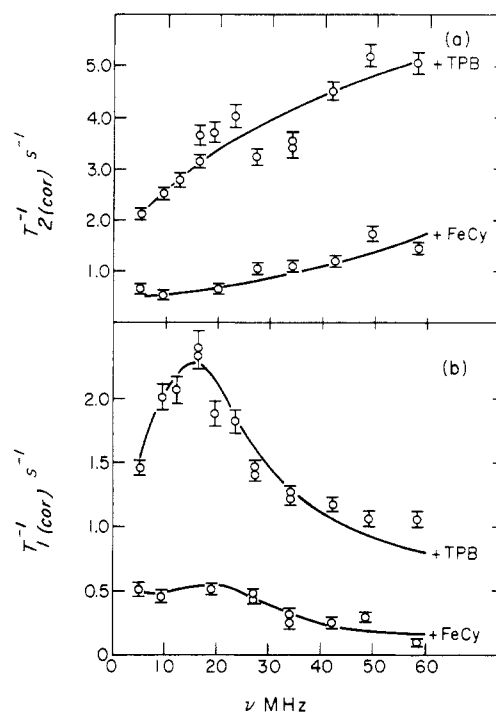

FIGURE 5: Frequency dependence of the PRR for the loosely bound paramagnetic species in dark-adapted suspensions of pea chloroplasts (3 mg of chl/mL) in the presence of redox agents, with conditions otherwise as in Figure 3. The Ph_4B (TPB) was 5 mM and the FeCy was 50 mM. The parameters from the best-fit, theoretical solid lines are given in Table II.

readily reduced beyond Mn(II), but the latter is oxidized quite easily. Thus, if the Mn were present in dark-adapted chloroplasts as a mixture of Mn(II) and some higher oxidation state(s), e.g., Mn(III), the addition of a reducing agent could convert the Mn(III) to Mn(II), thereby increasing the PRR. On the other hand, an oxidizing agent could convert the Mn(II) to Mn(III), decreasing the PRR.

Earlier work (Wydrzynski et al., 1975) has shown that reductants do increase the PRR of untreated chloroplasts, while oxidants cause decreases. However, if the loosely bound Mn is removed by washing with Tris–acetone, redox agents have little effect. In the present work, the T_1^{-1} for a washed chloroplast suspension was found to be 0.21 s^{-1} compared with 0.82 s^{-1} for an unwashed control. The presence of the reductant tetraphenylboron (Ph_4B) at 5 mM in the washed sample gave 0.28 s^{-1} and the oxidant potassium ferricyanide (FeCy) at 50 mM gave 0.23 s^{-1}. Furthermore, the frequency dependences of T_1^{-1} and T_2^{-1} were found to be the same for T–A washed chloroplasts with and without FeCy.

Frequency-dependence studies were made of the PRR for dark-adapted suspensions of unwashed pea chloroplasts, in one case with PH_4B at 5 mM and in the other with FeCy at 50 mM. The results are given in Figure 5. The behavior in the presence of Ph_4B is very similar to that for the control (the suspensions without Ph_4B in Figure 3), except that the rates are three to four times greater. This is also the case for the temperature dependences of $(1/T)_{cor}$ at 8, 24, and 64 MHz for chloroplasts with Ph_4B added. In contrast, the rates in the presence of FeCy are somewhat less than those of the control, and the frequency dependences differ. Analysis of the data gives the best fit parameters included in Table I for the Mn(II) contribution.

Separation of the outer-sphere terms in the fitting process was only semiquantitative because they are not much larger than experimental error. But it is clear that both the T_1^{-1} and T_2^{-1} contributions are increased by Ph_4B, the T_1^{-1} severalfold and T_2^{-1} less than twofold. Addition of FeCy, on the other hand, causes T_2^{-1} to decrease but seems to have little effect upon T_1^{-1}. It seems likely that much of what we lump together

in the outer-sphere terms is caused by Mn(II). However, the terms are still relatively large for suspensions in which most of the enhancement characteristic of Mn(II) has been removed by FeCy. Therefore, the terms for the untreated chloroplasts probably include appreciable contributions from paramagnetic species other than Mn(II) that are removed by the Tris–acetone washing. Furthermore, Mn(III) is an unlikely candidate for this role because of its small molar relaxivity (Villafranca et al., 1974).

These experiments with redox agents confirm that the PRR are determined primarily by the amount of Mn(II) in the chloroplast suspensions. In addition, the results show that the loosely bound Mn in untreated chloroplasts is a mixture of Mn(II) and a higher oxidation state(s), perhaps Mn(III). About one-fourth of the loosely bound Mn occurs as Mn(II) in the dark-adapted, untreated chloroplasts. There is some evidence for several types of Mn sites and/or states. A titration curve for spinach chloroplasts (3 mg of chl/mL) of T_1^{-1} vs. Ph_4B, at 27 MHz and 25 °C, shows two distinct plateaus, one at 1–2 mM and the other above 4 mM, with perhaps a third plateau above 15 mM (Wydrzynski et al., 1976b).

Exchange of Water across Thylakoid Membranes. Oxygen-17 NMR data can complement the PRR because relaxation of the oxygen-17 is governed by exchanges of whole water molecules between the solvent and solvation sphere of a paramagnetic center, while proton exchange dominates the PRR. One would expect different exchange rates for the two species in at least some circumstances, e.g., across membranes. Results obtained for oxygen-17 that bear on this point are summarized in Table II. In no case was there any evidence of other than a single relaxation time. The T^{-1} values observed for the various samples have been corrected by subtracting from them the relaxation rates found for buffers containing the same species except the chloroplasts.

TABLE II: Results of Oxygen-17 NMR Relaxation Rate Measurements for Chloroplast Suspensions.[a,b]

Sample	rf pulse expts			Wide line expts[c]	
	$(T_1^{-1})_{obsd}$ (s^{-1})	$(T_1^{-1})_{cor}$ (s^{-1})	Freq (MHz)	$(T_2^{-1})_{obsd}$ (s^{-1})	$(T_2^{-1})_{cor}$ (s^{-1})
Control	199[d]	11	12.8		
Ph$_4$B (5 mM)	192	4	12.8		
T–A washed	198	10	12.8		
Control	182[d]	14	8.1	613[d]	0
Triton-X^R	246	37[e]	8.1	683	157[e]

[a] All measurements were with the sample at 25 ± 1 °C. [b] The reproducibility of the values given is ±5%. [c] The wide line experiments were at a frequency of 12.8 MHz. [d] The control sample contained 2 mg of Chl/mL of untreated chloroplasts plus the usual buffer. [e] In determining these values, the same amount of detergent (v/v) was added to the buffer and to the suspension.

It is seen that, within experimental error, the addition of redox agents has no effect upon the oxygen-17 T_1^{-1} of the chloroplast suspensions. This indicates that the water molecules in the solvent phase exchange too slowly with the small fraction of water molecules in the solvation sphere of the loosely bound Mn(II) to be affected by them. The limiting rate might, in principle, be either the chemical exchange itself or slow exchange of water across the membranes to Mn(II) located inside the thylakoids. In order to distinguish between these two possibilities, the chloroplasts were treated with the detergent Triton-X^R (1%, v/v) which solubilizes the thylakoid membranes and exposes their interior to the bulk water. This treatment caused the oxygen-17 T_1^{-1} and T_2^{-1} to increase appreciably, as shown by the data in Table II.

A similar set of experiments was performed on solutions of 6×10^{-5} M Mn^{2+} in the buffer, with and without the detergent. The oxygen-17 T_2^{-1} from the line width (500 s^{-1} in the buffer alone) was found to be only slightly broader (960 s^{-1}) with the detergent than without (900 s^{-1}), well within experimental error. This indicates that the detergent has little or no direct effect upon τ_M or the relaxation rates for oxygen-17 in Mn(II) complexes. We conclude therefore that the detergent increases the relaxation rates of the chloroplast suspensions by exposing the bulk water population to the Mn(II) originally inside the thylakoids.

Discussion

There are several paramagnetic species besides Mn(II) in chloroplast membranes which may contribute to the PRR. However, we expect the effects of these ions to be small at the concentrations present in the samples used in this study. Mn(II) is probably the most efficient proton relaxer of all ions in the first-row transition elements (Mildvan and Cohn, 1970) due to its relatively long electronic correlation time. High-spin Fe(II), Fe(III) (Dwek, 1973), and Mn(III) (Villafranca et al., 1974) have short electronic relaxation times ($\tau_S \approx 10^{-10}$ s) and the effects of these ions are comparatively small even though they may be present at higher concentrations than the Mn(II). On the other hand, the electronic relaxation time of Cu(II) is comparable to Mn(II), but in chloroplasts at least half of the copper is located in plastocyanin which itself has no effect on the PRR, since the bound copper is not accessible to the solvent water (Blumberg and Peisach, 1966). The remaining copper in chloroplasts is mostly located in polyphenyloxidase, an enzyme not associated with photosynthesis and perhaps is washed out during the chloroplast preparation method used here. The contributions of these ions could nonetheless account for the 20% background in the PRR found in Figure 1.

Also, the results in Figure 1 indicate that T_1^{-1} is sensitive primarily to the loosely bound fraction of Mn (about two-thirds

of the total Mn content of the membrane), while the T_2^{-1} appears to reflect the total Mn content. This difference in proton relaxation by loosely and tightly bound Mn is not too surprising. In fact, the insensitivity of T_1^{-1} to frequency for the tightly bound Mn (the T–A chloroplast curve in Figure 2b) shows that, whatever its oxidation state, its τ_S differs substantially from that for the loosely bound Mn. Moreover, differences would be expected in τ_M, the time required for protons to exchange from the solvation sphere to the Mn. In turn, such differences in τ_S and τ_M could cause the tightly bound Mn to have a small effect on T_1^{-1} but a large effect on T_2^{-1}.

The exchange of whole water molecules across biological membranes has been calculated to be relatively slow. For example, the average lifetime of a water molecule inside human erythrocytes has been given as 17 ms (Vieira et al., 1970; Shporer and Civan, 1975; Andrasko, 1976). It has been noted that such slow exchange could limit nuclear relaxation by paramagnetic species inside the membranes (Case, 1975). The suspensions of chloroplast vesicles contain three water fractions: internal and external fractions plus the solvation sphere of the Mn(II). Nuclei in the solvation sphere of the Mn(II) have greatly enhanced relaxation rates (eq 1 with $pq = 1$). Sharing of the enhancement with the other solvent nuclei depends upon the exchange rates among the three fractions and upon their relative populations (Gutowsky et al., 1965). Current models of chloroplast thylakoids place the manganese associated with oxygen evolution on the inside of the chloroplast vesicles (Trebst, 1974). The results of the preceding section confirm this location and show that exchange of water (^{17}O) across the membrane is too slow to average the different relaxation rates of ^{17}O in the external and internal water. However, inside the vesicles, chemical exchange does average the bulk water with that in the solvation sphere of the Mn(II).

These observations may be used to place a lower limit upon the average lifetime τ_i of water molecules inside the vesicles. The exchange across the membranes will not average out the difference in the relaxation rates provided that (Gutowsky et al., 1965) the average rate constant for exchange is less than the difference between external and internal relaxation rates; that is

$$k_{av} \equiv 0.5[(1/\tau_e) + (1/\tau_i)] < |T_e^{-1} - T_i^{-1}| \quad (12)$$

where $k = 1/\tau$ is a rate constant for exchange across the membrane, the subscripts e and i refer to nuclei external to and inside of the vesicles, and the subscript av designates an average over the two fractions. The exchange rate across the membrane is the same in both directions,

$$p_e/\tau_e = p_i/\tau_i \quad (13)$$

Furthermore, even though the suspensions contained high chlorophyll concentrations (2 mg of chl/mL), the volume of internal water is only about 3–4% of the total, giving $p_e \approx 1$ and $1/\tau_i \gg 1/\tau_e$. Thus, we have

$$k_{av} \approx 0.5/\tau_i \qquad (14)$$

The right-hand side of eq 12 can be evaluated by noting that the relaxation rates T_b^{-1} for the buffer without chloroplasts are T_e^{-1}, while T_i^{-1} is determinable from the exchange-averaged relaxation rates T_d^{-1} for the detergent-treated chloroplasts by the relation

$$T_d^{-1} = T_{av}^{-1} = p_e T_e^{-1} + p_i T_i^{-1} \approx T_b^{-1} + p_i T_i^{-1} \qquad (15)$$

By combining eq 12–15 and introducing the definition $T_{cor}^{-1} = T_d^{-1} - T_b^{-1}$, we obtain the following approximation

$$\tau_i > p_i/T_{cor}^{-1} \qquad (16)$$

The largest lower bound is put upon τ_i by the value of $(T_1^{-1})_{cor}$ from Table II. With p_i taken to be 0.04, we find that $\tau_i(H_2O) > 1$ ms, which is consistent with the 17-ms estimate for water inside human erythrocytes.

A similar analysis can be made of the exchange-averaged PRR to obtain an *upper* bound upon the lifetime of protons inside the thylakoids. In this case the inequality of eq 16 is reversed. When used with the values of T_{cor}^{-1} in Figure 3, it gives $\tau_i(H^+) < 20$ ms.

In summary, we conclude that the PRR of chloroplast suspensions are determined largely by the Mn(II) loosely bound to the inside of the membranes. Furthermore, the PRR are sensitive to changes in oxidation state of the loosely bound manganese. The effects of redox agents upon the PRR indicate that about one-fourth to one-third of the loosely bound Mn occurs as Mn(II) in dark-adapted (untreated) chloroplasts, the balance being in a higher oxidation state(s), the most likely candidate being Mn(III). This fraction of Mn(II) is comparable with the S_0/S_1 population ratio in the model for oxygen evolution proposed by Kok et al. (1970). A forthcoming publication will present further evidence about the relationship between the PRR and the oxygen-evolving mechanism in photosynthesis. In light-flash experiments, the PRR were found to exhibit a damped oscillation with a periodicity of four, similar to that for oxygen evolution, thereby enabling one to explore the occurrence of changes in the oxidation state of manganese during oxygen evolution.

Acknowledgments

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