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EFFECTS OF PHYSICAL AND CHEMICAL TREATMENTS ON CHLOROPLAST MANGANESE

NMR AND ESR STUDIES

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Measurements were made of the water proton relaxation rate ($T_2^{-1} = R_2$), electron spin resonance (ESR) six-line signal of 'free' Mn^{2+}, and O_2-evolution activity in thylakoid membranes from pea leaves. The main results are: (1) Aging of thylakoids at 35°C causes a parallel decrease in O_2-evolution activity, in R_2 and in the content of bound Mn, suggesting that R_2 may be related to the loosely bound Mn involved in O_2 evolution. (2) Treatment of thylakoids with tetraphenylboron (TPB) at [TPB] > 2 mM produces a 2-fold increase in R_2, without release of Mn^{2+}. The titration curve exhibits three sharp end points. The first end point occurs at a [TPB]/[chlorophyll] of 1.25, at which the O_2 evolution is completely inhibited. (3) Treatment of thylakoids with NH_2OH also increases R_2 by nearly 2-fold, either by the reduction of the higher oxidation states of Mn to Mn^{2+} and/or by exposing the Mn to solvent protons. Also, progressive release of bound Mn occurs at [NH_2OH] ≥ 1 mM as shown by an increase in the Mn^{2+} ESR signal and a decrease in R_2. (4) Addition of H_2O_2 (0.1–1.0%) to thylakoids causes an enhancement of R_2 similar to that by NH_2OH, but without the release of Mn^{2+}. (5) Heat treatment of thylakoids at 40–50°C releases Mn^{2+} and increases R_2. Conversely, pH values of 7 to 4 release Mn^{2+} without changing R_2 while pH values of 7–9 increase R_2 without releasing Mn^{2+}. Thus, both high and low pH values as well as the heat treatment cause structural changes enhancing the relaxivity of the bound Mn or of other paramagnetic species.

Introduction

Relatively little is known about the molecular mechanism, of O_2 evolution in photosynthesis [1]. Some form of membrane-bound manganese appears to be involved in the process [2–4]. The kinetics of O_2 production have provided considerable information about how the oxidizing equivalents are transferred from the primary photoreactions to the oxygen-evolving site [1,5]. It has been suggested that Mn undergoes redox changes during O_2 evolution [1,4] in successive photoacts. The pattern of Mn release from chloroplast membranes by rapid temperature shock after a series of brief flashes of light, reported by Wydrzynski and Sauer [6], indicates that changes in the oxidation states of bound Mn occur during O_2 evolution. Also, Dismukes and Siderer [7,8] have observed an ESR signal which oscillates with a periodicity of 4, suggesting a cyclic change in the oxidation state of Mn. However, the function in O_2 evolution of such changes has been difficult to establish.

At least some of the difficulty lies in the fact that changes in Mn oxidation state can be accompanied by changes in its binding. We have sought

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Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PS, photosystem; TPB, tetraphenylboron;

to separate the two effects by observing both the water proton transverse relaxation rate R_2 and the ESR spectrum, as well as O_2 evolution, of thylakoids subjected to various treatments. The R_2 is sensitive not only to the amount and oxidation state of Mn present but also to the extent and nature of its binding in thylakoids (see, e.g., Ref. 9). Moreover, changes in binding (including conformational changes) can affect the correlation times governing R_2, or change the accessibility of Mn to the aqueous protons. However, in ESR, the free aqueous Mn^{2+} gives a distinctive six-line spectrum while other oxidation states and bound Mn^{2+} give at most very broad, weak absorption. Therefore, the two types of measurements augment one another.

The functional aspects of the oxygen-evolving system can be modified by a variety of physical and chemical treatments. Aging of chloroplasts at 35°C decreases the rate of O_2 evolution by thylakoids, accompanied by a loss of bound Mn (see, e.g., Ref. 10). The addition of redox reagents may shift the distribution of manganese among its oxidation states and, perhaps, its distribution among the types of binding sites as well. It has been shown that tetraphenylboron (TPB) specifically interacts with the electron transport of PS II by donating electrons to the holes produced by PS II, thereby suppressing O_2 evolution [11,12]. NH_2OH and H_2O_2 can also serve as electron donors to PS II [10,13]. Low concentrations (micromolar) of NH_2OH retard the charge-accumulating S states [10] without destroying the O_2-evolving activity [14]. At high concentrations, however, the O_2-evolving capacity is destroyed but NH_2OH still acts as an electron donor to PS II [15]. The ability of H_2O_2 to donate electrons to PS II is also known [16]; Velthuys and Kok [13] have reported that it reduces the S states.

In the present work, we describe the effects of these treatments upon the proton R_2 and the ESR spectrum of free Mn^{2+} in aqueous suspensions of thylakoid membranes. We also investigated the effects of heating the thylakoids up to 50°C and of both high pH (up to 9) and low (down to 4). The results are compared with those for O_2 evolution in order to characterize further the different types of Mn associated with thylakoid membranes.

Portions of this paper have been presented at various meetings (Biophysical Society of America (1979), American Society of Plant Physiology (1980), and the 5th International Congress on Photosynthesis Research (1980)).

Materials and Methods

Chloroplast preparation. Chloroplasts were isolated from leaves of laboratory-grown 14-day-old pea (*Pisum sativa*) plants in a medium containing 400 mM sucrose, 10 mM NaCl and 50 mM Hepes buffer (pH 7.5). These chloroplasts were given an osmotic shock in the same buffer without sucrose and finally the broken isolated chloroplast fragments (thylakoid membranes) were resuspended in the original medium. In this paper, these preparations are sometimes referred to as thylakoids. Chlorophyll concentration was determined by the method of Mackinney [17].

Aging of thylakoids. The thylakoids were aged by placing them in a medium preequilibrated at 35°C, and containing 25 mM Tris-HCl (pH, 8.3), 5 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, at a chlorophyll concentration of 200 μg/ml as described by Cheniae and Martin [18]. At specific times, aliquots were removed, suspended in buffer at 4°C, centrifuged, and the pellets suspended for 10 min. at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.4), 400 mM sucrose, 10 mM NaCl and 1 mM EDTA (to chelate the free Mn^{2+} released by aging). Then, to remove the chelated Mn^{2+}, the thylakoids were recentrifuged and resuspended in the buffer without EDTA at a chlorophyll concentration of 2.0 or 2.5 mg/ml.

Nuclear magnetic relaxation measurements. The proton spin-spin relaxation rate R_2 was measured from the exponential decay of the echo envelope with time; the spin-echo amplitudes were observed with a Carr-Purcell-Meiboom-Gill modification train of radiofrequency pulses. A 90° radiofrequency pulse was followed by time τ (500 μs) and then a series of 2000 180° pulses spaced 2τ apart were given. An analog-to-digital converter sampled the echo heights and the data were analysed by a PDP-8f minicomputer using a least-squares program. The values so obtained are given without subtracting R_2 of the buffer. All measurements were made at room temperature (23°C). For details see Ref. 9.

Electron spin resonance measurements. ESR spectra were recorded as the first derivative with a Varian E-9 (X band, 9.5 GHz) spectrometer. The microwave power in all experiments was 50 mW, modulation amplitude was 10 G and the spectrometer time constant was 0.3 s with a scan time of 8 min. All spectra were taken at room temperature (23°C) using a flat sample cell held in position with Varian clips.

Electron-transport rates. For the measurement of O_2-evolution rates, a Clark electrode (platinum-Ag/AgCl) and a Yellow Springs oxygen monitor (model 53) were used. The signal was recorded on an Esterline Angus (Model E 11015) recorder. Light from a tungsten lamp was focused on the thylakoid suspension, after being passed through a Corning CS 3-70 glass filter and a 2 inch water filter. The intensity of the continuous incident light was approx. 250 mW/cm^2.

Reduction of DCIP mediated by PS II was measured at 580 nm using a Hitachi Model 100-60 spectrophotometer. The sample, containing 5 μg Chl/ml, was illuminated from the side through a red Corning CS 2-58 filter. A blue Corning CS 4-96 filter was placed in front of the photomultiplier to protect it from the scattered actinic light. Electron-transport rates were calculated from recordings of the absorbance change using the extinction coefficient of DCIP as described by Armstrong [19].

Treatment with redox agents. The details of the experiments involving redox agents are given in the corresponding sections below.

Results and Discussion

Aging of thylakoid membranes at 35°C

Aging of thylakoids results in time-dependent decreases in the rate of O_2 evolution, in R_2 and in the Mn content as shown in Fig. 1. After 3 min at 35°C, the Mn content, as measured by neutron activation analysis [9], decreased by about 70% (from 0.69 to 0.22 μg Mn/mg Chl; Fig. 1, open circles). The steady-state saturation rates of O_2 evolution decreased in parallel as a function of the time of aging; 10 min of incubation at 35°C were enough to inhibit almost all O_2 evolution (Fig. 1, open squares). Also, the aging causes a parallel decrease in R_2 (Fig. 1, filled circles). These results

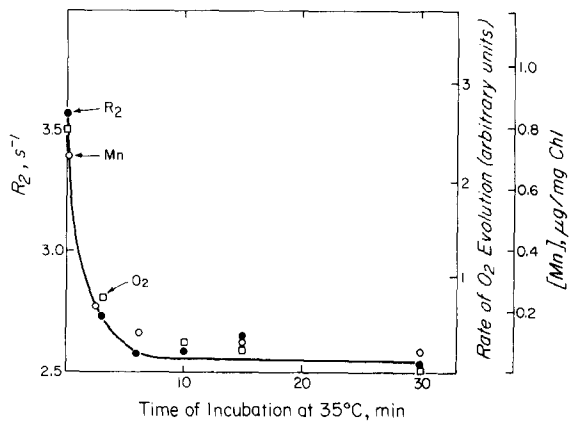

Fig. 1. Effect of time of incubation at 35°C of thylakoid membranes upon $R_2 (= T_2^{-1})$, O_2 evolution and Mn content. For R_2 measurements, the suspension medium contained 50 mM Hepes (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 2 mg/ml. R_2 was measured at 27 MHz and 25°C. For O_2 evolution, 0.5 mM $[Fe(CN)_6]^{3-}$, 1 mM NH_4Cl and 0.1 μM gramicidin were included in the reaction mixture; the chlorophyll concentration was 10 μg/ml. Mn content was determined by neutron activation analysis as described elsewhere [9,20]. The maximum rate of O_2 evolution was 92 μmol O_2/mg Chl per h.

establish a clear proportionality between R_2, the Mn content and the rate of O_2 evolution in thylakoids. A similar qualitative correlation between R_2, Mn and O_2 evolution was observed by Wydrzynski et al. [9,20] in thylakoids from which Mn was removed by incubation with high concentrations of $MgCl_2$, followed by washing.

The effect of aging on O_2 evolution and Mn content of thylakoids are in agreement with the results of Cheniae and Martin [18], who found no clear demarcation between removal of the loosely and tightly bound Mn. Moreover, the amount of Mn remaining after 15–30 min of aging is only about 10% of that originally present. This implies that aging also releases part of the very tightly bound Mn, which is about one-third of the initial content. This relatively nonselective removal of Mn by aging contrasts with the progressive removal of the different pools of Mn by incubation with $MgCl_2$.

Effects of tetraphenylboron

TPB was selected as a redox agent because it does not form free radical intermediates [21] that

would have complicated the interpretation of the R_2 data. In our experiments, thylakoid suspensions with a fixed [Chl] of 2 mg/ml were incubated for 30 min with different concentrations of TPB. An aliquot of the incubated sample was diluted 100-fold for the measurements of O_2 evolution and 200-fold for the reduction of DCIP. R_2 was measured without dilution, since high concentrations of thylakoids are needed for the NMR measurements. The results are presented in Fig. 2 as a function of log ([TPB]/[Chl]), the ratio being the same for each set of three measured properties.

The plot of R_2 (filled squares) as a function of added TPB shows three distinct regions of increase related, perhaps, to successive reduction of three different pools of ions titratable with TPB. This result is similar to that obtained for the spin-lattice relaxation rate R_1 [20]. In the experiments shown in Fig. 2, the increases in R_2 are centered at [TPB]/[Chl] ratios of 1,25, 4 and 12.5 mM/mg per ml. There is no increase in R_2 until [TPB]/[Chl] $\gtrsim$ 1.

The O_2 evolution is inhibited by low concentrations of TPB (Fig. 2, open circles), becoming completely inhibited when [TPB]/[Chl] = 1. This is expected if TPB acts as a competitive electron donor and donates electron more readily than does water [11]. The data for DCIP reduction are the initial rates (Fig. 2, filled circles) because progressive inhibition of electron transport occurs during illumination, probably by the oxidation products of TPB. A comparison of the rates of O_2 evolution and DCIP reduction shows that for incubation at [TPB]/[Chl] = 0.5 there is 80% inhibition in O_2 evolution but only a 25% reduction in electron flow. Similarly, the ratio of [TPB]/[Chl] required for 50% inhibition (I_{50}) is much less for O_2 evolution (0.25) than for DCIP reduction (1.25).

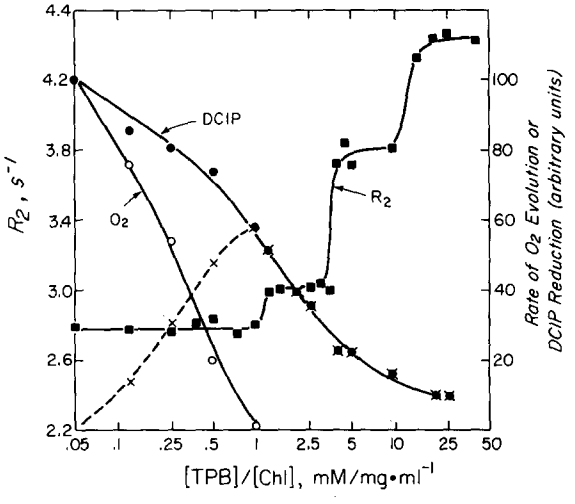

Fig. 2. The effect of incubation with TPB on R_2, O_2 evolution and DCIP reduction in pea thylakoids. The [Chl] was 2 mg/ml for the R_2 measurements, 20 μg/ml for O_2 evolution and 10 μg/ml for DCIP reduction; in the latter, [DCIP] was 0.03 mM. The difference between DCIP reduction and O_2 evolution (crosses) indicates electron donation by TPB.

A plot of the difference between DCIP reduction and O_2 evolution (Fig. 2, crosses) represents the net electron flow from TPB to DCIP. The decline in the net electron flow at [TPB]/Chl] > 1.25 could be due to (a) an inhibition caused by the oxidation product of TPB as suggested by Homann [11] or (b) a reduction of other components of the electron-transport chain thereby preventing electron flow to DCIP. The latter is lent some support by the occurrence of the first break in the titration curve of R_2 vs. [TPB]/[Chl] when O_2 evolution is completely inhibited, i.e., at a ratio of greater than approx. 1.

This leads to the following possible interpretation of Fig. 2. At low concentrations ([TPB]/[Chl] < 1), TPB is not a strong enough reducing agent to reduce Mn to Mn^{2+} or produce other paramagnetic species of high relaxivity that increase R_2. At these concentrations, TPB may donate electrons to PS II in competition with H_2O. At higher concentrations, it may not only supplant H_2O completely in the functioning of PS II (no O_2 evolution) but also reduce Mn to Mn^{2+} (which remains bound). However, we cannot rule out the possibility that the TPB-induced increase in R_2 may be due to a change in the accessibility of Mn in TPB-treated membranes. The occurrence of three breaks in the titration curve could reflect the heterogeneity of the Mn or other species affected. Or there may be different mechanisms by which TPB affects R_2. In this connection, however, our ESR experiments show that TPB has little or no effect upon the binding of Mn by the membranes, producing only a slight increase in the six-line spectrum of free Mn^{2+}. Further work is needed to establish why there are three end points.

Effects of hydroxylamine

NH_2OH is known to serve as an electron donor to PS II [22,23]. Millimolar NH_2OH releases Mn from the chloroplasts and inhibits the O_2 evolution in proportion [15,18]; concentrations of greater than approx. 5 mM cause nearly complete inhibition in a few minutes. The mechanism of the electron donation by NH_2OH has been determined by Radmer [24]. The effects of NH_2OH upon PS II Mn are less well characterized. Therefore, we have measured R_2 and the ESR-detectable Mn^{2+} for thylakoids treated with NH_2OH under a variety of conditions, obtaining the results presented in this section.

The addition of NH_2OH to thylakoid suspensions increases R_2 and releases Mn^{2+}. These changes occur on a time scale of several minutes. At 25°C the suspensions attain equilibrium (or a steady state) in 30 min or less after the NH_2OH is added, and the results in Figs. 3–5 were obtained after such equilibration.

Fig. 3 shows that for our standard thylakoid suspensions (closed circles) R_2 increases by 50% as $[NH_2OH]$ increases from 0.01 to 1.0 mM. However, much of this increase in R_2 is reversed as $[NH_2OH]$ approaches 100 mM. In Fig. 4 it is seen that the addition of NH_2OH produces the six-line ESR spectrum characteristic of aqueous Mn^{2+}, the intensity of which is appreciable by 0.5 mM NH_2OH and increases further at higher $[NH_2OH]$.

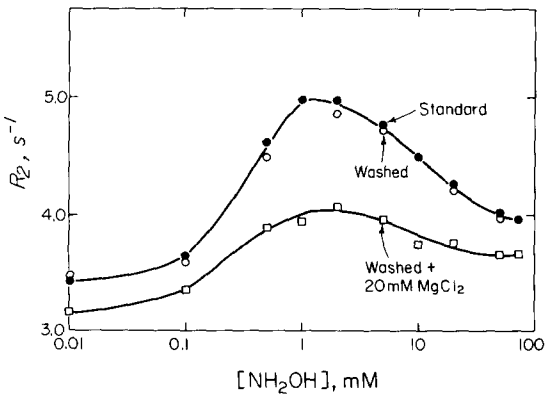

Fig. 3. R_2 of thylakoid membranes (2.5 mg Chl/ml) incubated for 30 min at 25°C with different concentrations of NH_2OH. Other conditions for the standard samples were as in Fig. 1. Washing was with 10 mM NaCl. The bottom curve is for washed samples to which 20 mM $MgCl_2$ was added.

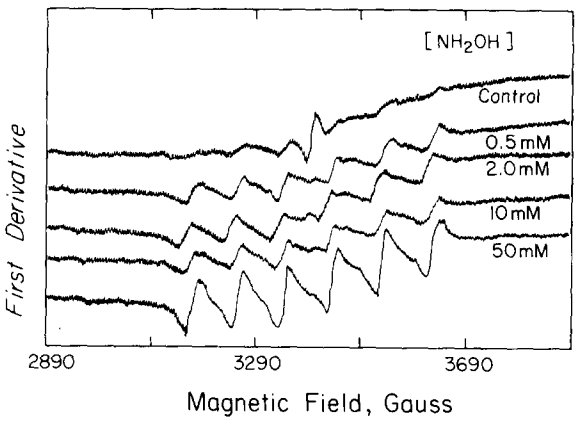

Fig. 4. ESR spectra of thylakoid membranes washed with 10 mM NaCl and incubated for 30 min at 25°C with different concentrations of NH_2OH. The control was a washed sample without NH_2OH.

It seems likely that the enhancement of R_2 at lower $[NH_2OH]$ is caused by the reduction of higher oxidation states of bound Mn to Mn^{2+} and/or to an increase in the accessibility of H_2O to bound Mn. Moreover, the downturn in R_2 at $[NH_2OH]$ above 1 mM can be attributed to release of the Mn^{2+}, which has a lower molar relaxivity in the free than in a bound state.

The data shown in Figs. 3 and 5 suggest that the Mn^{2+} released by NH_2OH includes loosely as well as very loosely bound Mn. Washing of the thylakoids in 10 mM NaCl does not change the effect of NH_2OH upon R_2 (Fig. 3, open circles), indicating that this washing removes very little of whatever is reduced by the NH_2OH. However, the addition of 20 mM $MgCl_2$ to the washed thylakoids decreases substantially the enhancement of R_2 by NH_2OH (Fig. 3, open squares), the curve of which still turns down at high $[NH_2OH]$. The 20 mM $MgCl_2$ has been shown to displace the very loosely bound Mn^{2+} [25], not involved in O_2 evolution [25,26]. Thus, the difference between the upper and lower curves in Fig. 3 can be attributed to removal of the very loosely bound Mn by the $MgCl_2$, and the downturn in R_2 at high $[NH_2OH]$ of $MgCl_2$-treated samples to release of loosely bound Mn^{2+}.

The ESR spectra in Fig. 5 support this interpretation. The spectrum of thylakoids with 10 mM NH_2OH shows somewhat more free Mn^{2+} than

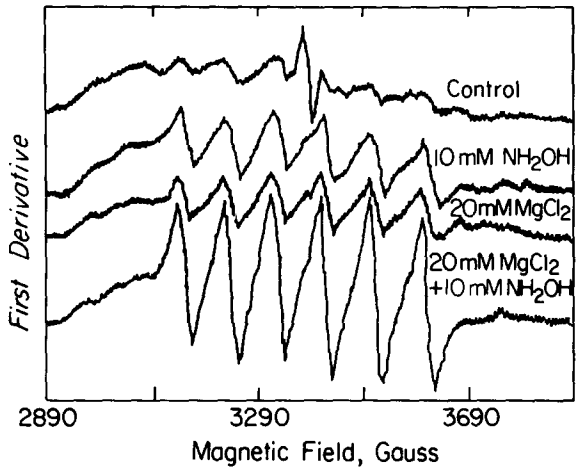

Fig. 5. The individual and combined effects of NH_2OH and $MgCl_2$ upon the ESR spectrum of thylakoid membranes. Conditions otherwise as in Fig. 4.

that with 20 mM $MgCl_2$ (and no NH_2OH) while that with both has appreciably more free Mn^{2+} than the sum of the two separately. An equivalent nonadditivity is apparent in Fig. 3 where the curves of R_2 vs. [NH_2OH] with and without 20 mM $MgCl_2$ would otherwise have more nearly the same shape. Thus, the presence of one of these two reagents enhances in some way the ability of the other to release Mn^{2+}. A likely explanation is that the reduction of bound Mn to Mn^{2+} or its exposure to the solvent by NH_2OH enables the $MgCl_2$ to release more Mn^{2+}.

Several experiments were performed to explore the time dependence of the effects of NH_2OH (at 25°C) during the 30 min following its addition to the thylakoids. Fig. 6 gives R_2 observed as a function of time for seven concentrations of NH_2OH ranging from 0.01 to 100 mM. At [NH_2OH] < 0.1 mM (1 NH_2OH to 20 Chl) the R_2 increases monotonically, while for higher concentrations it goes through a maximum and then decays. The time dependence of the ESR spectrum as well as that of R_2 was followed for 0.5 mM NH_2OH, with the results given in Fig. 7. The release of Mn^{2+} became evident as soon as the first measurement was made, 3 min after adding the NH_2OH, with the amplitude increasing more slowly thereafter.

The curves in Figs. 6 and 7 could result from

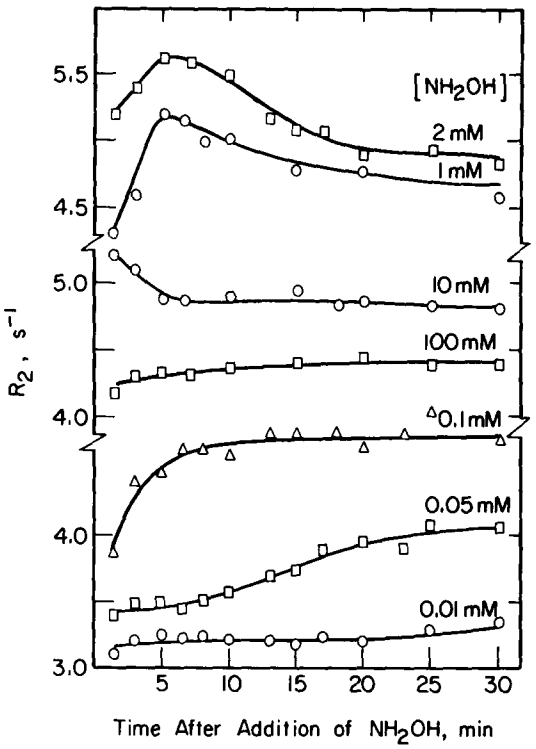

Fig. 6. R_2 of thylakoids (2 mg Chl/ml) washed with 10 mM NaCl, as a function of time after the addition of 0.01–100 mM NH_2OH. Conditions otherwise as in Fig. 1.

two consecutive, first-order reactions: (i) the reduction of bound Mn in higher oxidation states ($n \geq 3$) to Mn^{2+} or its exposure to the solvent, and

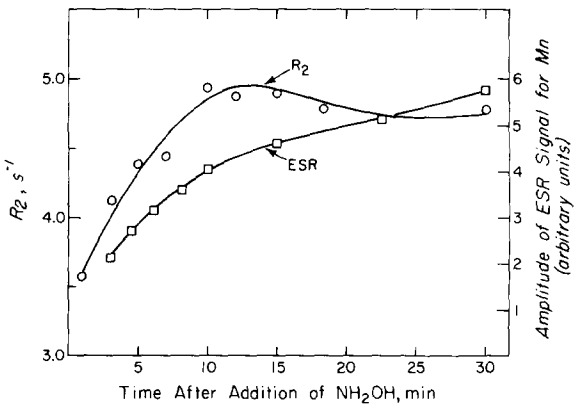

Fig. 7. R_2 and amplitude of ESR signal for thylakoids (2 mg Chl/ml) as a function of time after the addition of 0.5 mM NH_2OH. The ESR amplitude was taken to be the area under the second peak of the six-line spectrum for free Mn^{2+}. Conditions otherwise as in Fig. 1.

(ii) the release of the bound Mn^{2+} to the ESR-detectable free Mn^{2+}. The occurrence of these reactions is consistent with measurements of O_2 yield in light flashes [14,27]. The normal O_2-yield pattern has the first peak in O_2 evolution after the third flash [5]. Incubation of thylakoid membranes with low concentrations (0.5 mM) of NH_2OH delays O_2 evolution until the fifth flash, after which it oscillates with the usual periodicity of 4. Higher concentrations of NH_2OH decrease the O_2 yield and at concentrations much greater than 1 mM the O_2-evolving capacity is destroyed.

It has been suggested [14,28] that the delay in maximum yield of O_2 by low concentrations of NH_2OH is caused by the donation of two electrons to the PS II complex or by the binding to it of two molecules of NH_2OH. Our results with 50 and 100 μM NH_2OH (Fig. 6) are consistent with the suggestion that NH_2OH acts by reducing higher oxidation states of bound Mn to Mn^{2+}, which may be involved in the progression of the S states. However, the increase in R_2 could arise also from changes in the accessibility of the bound Mn to H_2O.

Recently, Robinson et al. [29] reported the effects of up to 1 mM NH_2OH on the proton R_1 (spin-lattice) or chloroplast suspensions. Their independent results, while qualitatively similar to ours, are shifted to 10-fold higher [NH_2OH]. Their curve for 1 mM NH_2OH (Fig. 2 in Ref. 29) is nearly identical to ours for 0.1 mM (Fig. 6). This shift most likely comes from their use of 1 mM EDTA to remove the very loosely bound Mn, a procedure not employed by us. Wydrzynski et al. (see Ref. 30) have found that EDTA can have complex effects on R_2. In the present instance, it appears that the very loosely bound Mn removed by EDTA is affected more readily by NH_2OH than is that more tightly bound.

The experiments of Robinson et al. [29] did not extend to high enough [NH_2OH] for R_1 to exhibit the maximum we found in R_2. Also, it is noteworthy that they observed R_1 in chloroplast suspensions treated with 0.5 and 1.5 mM TPB (at comparable [Chl]) and found no effect. This, however, is consistent with our R_2 data (Fig. 2) which show no change in R_2 until [TPB] > 2 mM.

Effects of hydrogen peroxide

Velthuys and Kok [13] have shown that H_2O_2 can reduce the higher S states to a state designated as S_{-1}, the formation of which is favored at high pH. To see if this involved the formation and/or release of Mn^{2+}, as in the case of NH_2OH, we have studied the effect of H_2O_2 on R_2 and on the ESR spectrum of thylakoid membranes at pH 8.8. In these experiments, a catalase inhibitor, sodium azide, was added to the sample to avoid consumption of H_2O_2 by the endogenous catalase.

Fig. 8 gives the measurement of R_2 as a function of time for thylakoid membranes treated with 0.1 and 1.0% (v/v) H_2O_2. At 0.05% H_2O_2, results similar to those for 0.1% were obtained. The changes shown in R_2 are in addition to the very rapid increases caused by high pH values (see, e.g., Fig. 12). ESR spectra are reproduced in Fig. 9 for samples incubated with H_2O_2 for 40 min or more. The control (pH 8.8) has a trace of free Mn^{2+}, which is consistent with the pH dependence (Fig. 12).

It is seen that the H_2O_2 enhances R_2 by an amount (approx. 50%) and on a time scale ($t_{1/2} \approx 5$ min) comparable with that for 0.1 mM NH_2OH. However, the ESR spectra show that, unlike the results for NH_2OH, the treatment of thylakoids with 0.1 and 1.0% H_2O_2 does not release any appreciable amount of Mn^{2+}. Because of this it is tempting to relate the changes caused by H_2O_2 in

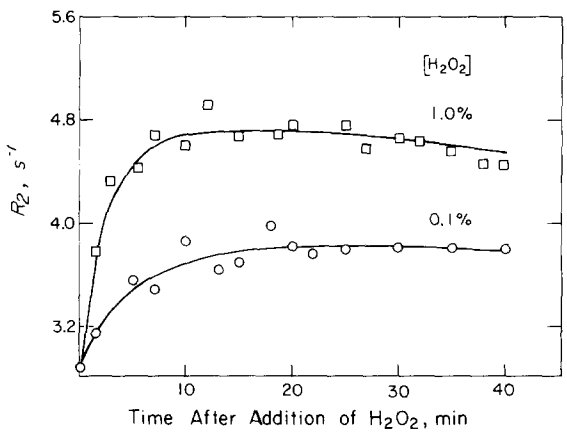

Fig. 8. R_2 of thylakoids washed with 10 mM NaCl, as a function of time after the addition of H_2O_2. The suspension medium contained 0.5 mM sodium azide and was at a pH of 8.8; conditions otherwise as in Fig. 1.

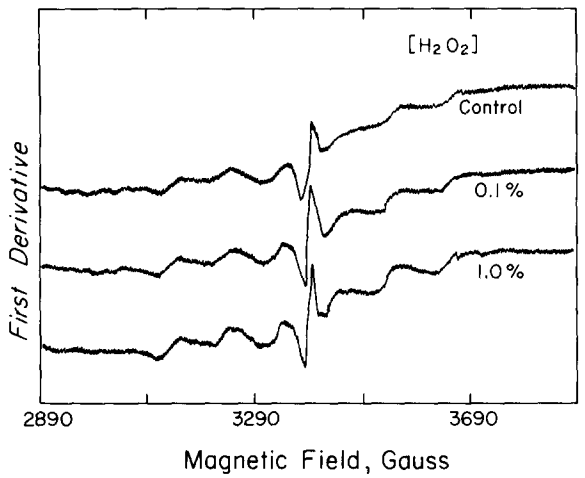

Fig. 9. ESR spectra of thylakoid membranes incubated for 40 min at 25°C with 0.1 and 1.0% H_2O_2. Conditions otherwise as in Fig. 8; the control was a sample at pH 8.8 without H_2O_2.

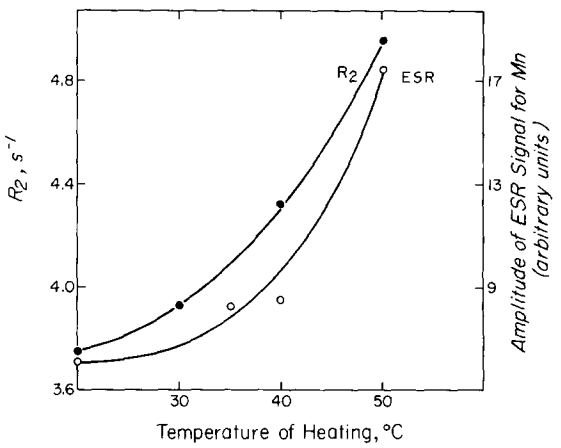

Fig. 10. Effect of heating of thylakoid membranes upon R_2 and the ESR amplitude of free Mn^{2+} (as in Fig. 7). In the heat treatment, thylakoids were incubated for 5 min at the specified temperature, then stored on ice until warmed to room temperature for the measurements. Conditions as in Fig. 1 except 2.5 mg Chl/ml.

the S states at these concentrations to the reduction of Mn to Mn^{2+} in the loosely bound Mn pool. However, if H_2O_2 exposes this pool to the solvent water similar results would be obtained.

Heating and pH effects

In our heating experiments, thylakoid membranes were incubated for 5 min in a water bath maintained at a specific temperature. This treatment is expected to release Mn from its bound state [6]. In contrast to the aging experiment described above, the released Mn was not extracted with an EDTA buffer, but was directly measured by ESR. Incubation at 35–50°C was found to release Mn^{2+} and to increase R_2, the amounts increasing rapidly with the temperature (Fig. 10). Free Mn^{2+} is less efficient in relaxing water protons than is bound Mn^{2+}, so there should have been a decrease in R_2 paralleling the release of Mn^{2+} as the temperature is raised from 30 to 50°C.

Thus, the heating must produce other changes that increase R_2 enough to more than compensate for the release of Mn^{2+}. These might be structural changes that increase the accessibility and/or otherwise enhance the relaxivity of the remaining bound Mn or of other paramagnetic species such as the Cu^{2+} in plastocyanin or polyphenol oxidase [31]. This view is supported by the results of fixing the thylakoid membranes with glutaraldehyde [32] before heat treatment and by incubation of other samples with 60 mM KCN (a Cu chelator [33]) for 90 min after heat treatment. The former reduces the heat-induced increase in R_2 and the latter eliminates it, as shown in Fig. 11.

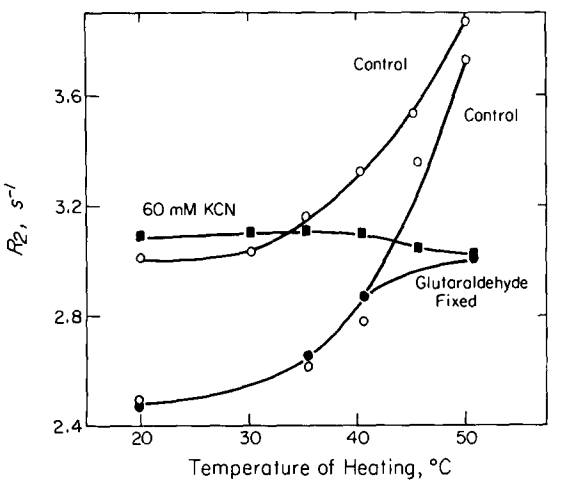

Fig. 11. Modification of the effects of heat treatment upon R_2, obtained by postincubation of the heated thylakoids with 60 mM KCN for 90 min at 4°C (top set) or by fixing them in glutaraldehyde before heat treatment (bottom set). The two sets of curves are displaced because the top set has 2 mg Chl/ml and the bottom set, 1.8. Conditions otherwise as in Fig. 1 and heat treatment as for Fig. 10.

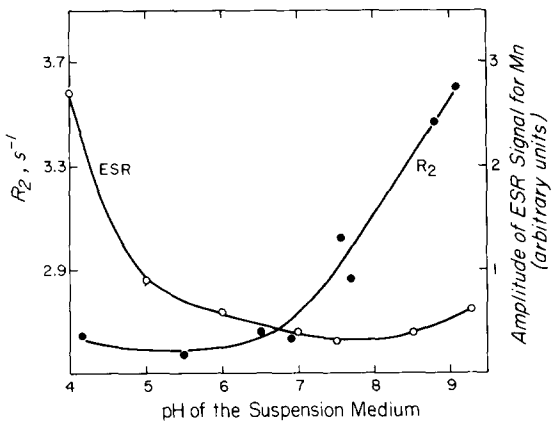

Fig. 12. Effect of pH of the suspension medium on R_2 and the ESR amplitude of free Mn^{2+} for thylakoid membranes. Conditions as in Fig. 1 and ESR amplitude as in Fig. 7.

Extreme pH values also affect R_2 and release Mn^{2+}. The Mn^{2+} release occurs with decreasing pH, starting at about 7 and increasing sharply to 4 (Fig. 12). This is consistent with the data of Cheniae and Martin [3] on the effect of pH on loss of chloroplast-bound Mn measured by the retention of ^{54}Mn in the membrane. In a control experiment, this change in pH increased the ESR amplitude of a 10 μM $MnCl_2$ solution by 30–40%; however, this is negligible compared with the 10-fold increase found with the membranes. On the other hand, R_2 increases linearly with increasing pH, starting at 7 and extending up to 9. This increase in R_2 is caused probably by structural changes as in the case of heating, but less extensive. It is likely that such structural changes occur at low as well as high pH; otherwise R_2 would decrease from pH 7 to 4 because of the Mn^{2+} release, instead of remaining almost constant.

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