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EFFECT OF MANGANESE ON THE NUCLEAR MAGNETIC RELAXIVITY OF WATER PROTONS IN CHLOROPLAST SUSPENSIONS

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SUMMARY Field dispersion profiles of the proton spin-lattice relaxation rate, T_1^{-1}, in chloroplast suspensions show a local maximum near 20 MHz, probably due to bound Mn(II); EDTA extraction eliminates, and $MnCl_2$ addition restores, the paramagnetic relaxivity. Since neither treatment affects water oxidation, the Mn(II) site monitored appears to lie outside the water-splitting enzyme. Intense illumination almost totally suppresses the paramagnetic relaxivity through an electron-transport-dependent mechanism. Previous reports that chloroplast nuclear magnetic relaxivity varies cyclically in flash experiments require reevaluation in terms of the probable role of Mn(II) that is nonfunctional in water oxidation.

INTRODUCTION The relaxivity of the water proton resonance in concentrated suspensions of broken (Class II) chloroplasts has been reported to contain a large paramagnetic contribution (1-5). This component of the spin-lattice relaxation rate, $R_1 \equiv 1/T_1$, shows a local maximum in the dispersion profile (plot of R_1 against Larmor frequency) near 20 MHz, which is suggestive of membrane-bound Mn(II)(1). Correlations of R_1 with oxygen evolution activity and chloroplast manganese content were also reported and interpreted as arising from manganese in the water-splitting complex(3). Flash oscillations have been observed in the spin-spin relaxation rate and interpreted in terms of oxidation state changes of manganese, acting as an intermediate in the water-splitting reaction(2,4,5).

The measurements reported in this communication suggest that functional manganese does not contribute significantly to R_1 in dark-adapted chloroplast suspensions. Endogenous nonfunctional Mn(II) is a potent relaxing agent that probably accounts for previously observed effects in both dark-adapted and flash-illuminated chloroplasts.

MATERIALS AND METHODS Broken chloroplasts were prepared from spinach as previously described(6), except that for some experiments, 1 mM EDTA was included in the homogenization buffer; KCN/Hg-inhibited chloroplasts were prepared by the method of Yocum and Guikema(7). The techniques for assay of O_2 evolution and photophosphorylation activity have been reported previously(6). The standard buffer used for chloroplast suspension and storage was 0.4 M sucrose, 20 mM Tricine (pH 8) containing 15 mM NaCl. Samples (200µl) were illuminated where

ABBREVIATIONS nmr, nuclear magnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem.

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indicated with 1 x 10^6 ergs·cm^{-2}·s^{-1} of white light filtered through 5 cm of 0.2% $CuSO_4$. Spin-lattice relaxation times were measured on a Bruker B-KR 322s pulsed nmr spectrometer using an external ^{7}Li field lock and a flux stabilizer. The sample temperature was controlled at 25 ± 1°C, and the sample compartment was carefully shielded from light. Data from the modified triplet sequence (8) were digitized in a Fabri-Tek Model 1064 signal averager and analyzed in a Commodore Model 2001 microcomputer. The measurement sequence was under microprocessor control in the kinetic experiments.

RESULTS Figure 1 shows the effects of EDTA and added Mn^{+2} (as $MnCl_2$) on the relaxivity of chloroplast suspensions. Chloroplasts prepared in the absence of EDTA and washed once in the isolation buffer exhibit a relatively high relaxivity per mg chlorophyll (solid circles of Figure 1). The dispersion profile of R_1 passes through a local maximum near 25 MHz. This type of dis-

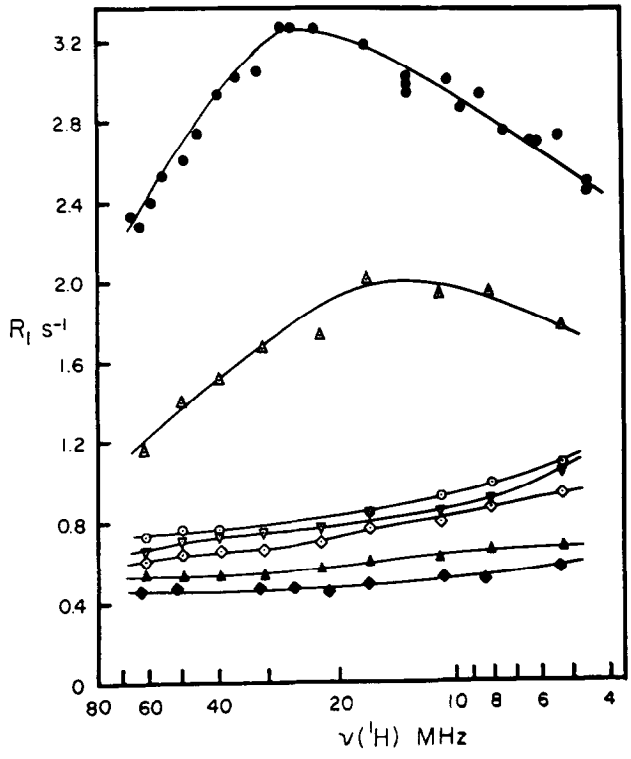

Fig. 1. Dispersion profiles of chloroplast suspensions and the suspending buffer. From top to bottom, these profiles represent: Chloroplasts isolated without EDTA (closed circles); chloroplasts isolated in EDTA, to which $MnCl_2$ (25 μM final concentration) was added (open triangles); chloroplasts isolated in EDTA, then washed (open circles); chloroplasts isolated in EDTA and washed, to which $MnCl_2$ and EDTA (25 μM and 1 mM final concentrations) were added (inverted triangles); chloroplasts isolated in EDTA and resuspended in 1 mM EDTA (open diamonds); resuspending buffer containing 25 μM $MnCl_2$ (closed triangles); resuspending buffer (closed diamonds). The chlorophyll concentration was 2.3 mg/ml in all cases save for the experiment represented by closed circles, where the concentration was 2.5 mg/ml.

persive feature invariably results from a paramagnetic contribution to the relaxation rate and is highly characteristic of macromolecular complexes of divalent manganese(1). Similar measurements on other chloroplast preparations have shown that the relaxivity (per mg chlorophyll) of chloroplast suspensions in the absence of EDTA is quite variable. Addition of EDTA during resuspension produces a drastic reduction in R_1 and eliminates the local maximum near 20 MHz (Figure 1, diamonds). This concentration of EDTA has little if any, effect on oxygen evolution rates or photophosphorylation (Table I); EDTA is nearly as effective in suppressing the paramagnetic contribution to R_1 when it is included during homogenization of leaves and then removed by two subsequent washes in the isolation procedure (Figure 1, open circles).

Experiments have been undertaken to determine whether exogenous Mn(II), added at concentrations of 25-50μM, can bind to external sites on the thylakoid membrane and produce a maximum in the dispersion profile that is similar to the endogenous effect seen for chloroplasts prepared in the absence of EDTA. In these experiments, a final concentration of 25μM $MnCl_2$ was added to chloroplasts that were prepared in 1mM EDTA and washed free of EDTA in two subsequent sedimentation and resuspension steps. The resulting dispersion profile (triangles in Figure 1) shows the enhanced relaxivity and local maximum near 20 MHz that is characteristic of bound Mn(II). Free hexaquo manganese, when added at an equal concentration to the isolation buffer, produces virtually no enhancement of the relaxivity (Figure 1, solid triangles). Consequently, much, if not all, of the added Mn^{+2} must bind to sites on the thylakoid membrane. Manganese bound to these sites remains chelatable by EDTA (Figure 1, inverted triangles).

The effect of steady-state illumination on chloroplast relaxivity has been studied in the presence and absence of added $MnCl_2$, where chloroplast suspensions were illuminated in saturating white light for one minute outside the nmr

Table I

Effect of EDTA on chloroplast oxygen evolution and photophosphorylation[a]

Addition to assay	Rates of: ATP Synthesis (μmoles/hr.mg chlorophyll)	Rates of: Oxygen Evolution (μmoles/hr.mg chlorophyll)	P/e_2
-	448	193	1.16
1 mM EDTA	427	183	1.17

[a]The reaction mixtures contained chloroplasts prepared in EDTA and washed (20 μg chlorophyll/ml); assay conditions are given in Reference 6. The electron acceptor was methylviologen (66 μM).

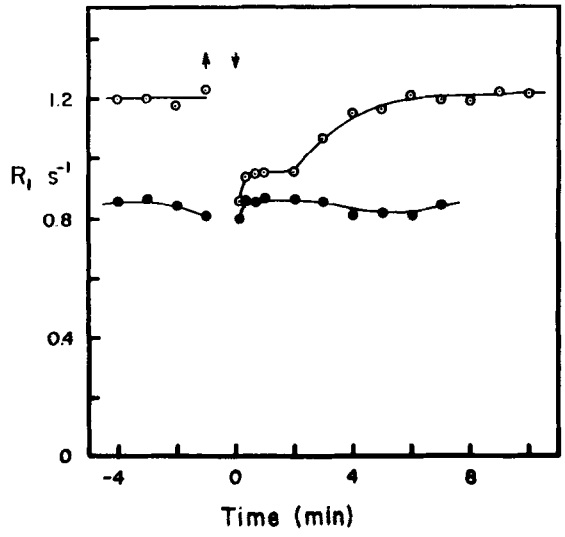

Fig. 2. Effect of illumination on the relaxivity of chloroplast suspensions. The open circles represent changes observed with chloroplasts not exposed to EDTA; the closed circles show data from a comparable experiment with chloroplasts isolated in EDTA and then washed. The arrows indicate initiation (up) and cessation (down) of illumination. The chlorophyll concentration was adjusted to 2.3 mg/ml in both experiments, and methylviologen (500 μM (open circles); 50 μM (closed circles)) was present as an electron acceptor.

probe. Changes in the relaxivity at 20.7 MHz were monitored as a function of time before and after illumination. The relaxivity of chloroplasts prepared in the absence of EDTA decreases upon illumination (Figure 2, open circles). The minimum relaxivity measured six seconds after cessation of illumination, is very near the value found for dark-adapted EDTA-treated chloroplasts. The return of R_1 to the dark-adapted state occurs over a time scale of several minutes.

Chloroplasts from leaves that were homogenized in 1 mM EDTA and subsequently sedimented and resuspended twice in EDTA-free buffer do not show a light-dependent relaxivity (Figure 2). This indicates that only the paramagnetic, EDTA-chelatable portion of the relaxivity is light-sensitive. To reinforce this conclusion, the experiment was repeated after addition to a final concentration of 50μM $MnCl_2$ to these chloroplasts. Most of the relaxivity arising from added Mn(II) is light-sensitive (Figure 3). The return to the dark-adapted state occurs again over a time scale of several minutes and, interestingly, appears to follow complex kinetics.

Illumination experiments have also been performed in the presence of DCMU, which inhibits reoxidation of the primary acceptor of PS II. Transient electron transport through PS I that is associated with removal of reducing equivalents from the quinone pool would not be inhibited under these conditions.

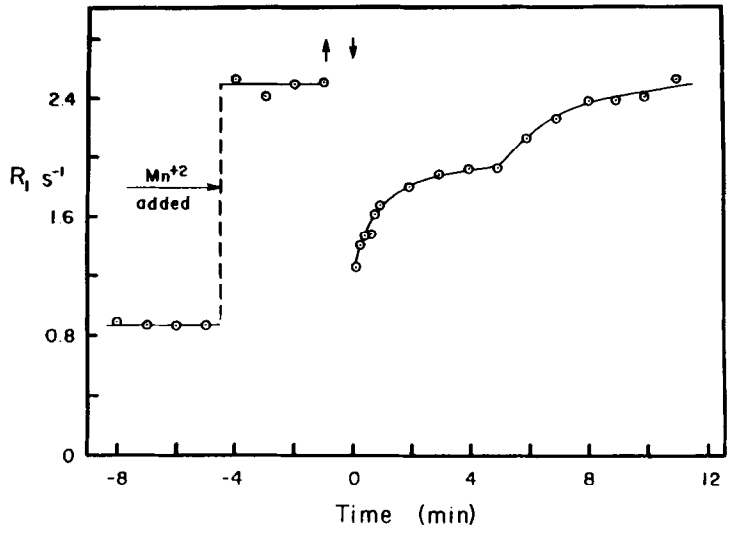

Fig. 3. Reconstitution by $MnCl_2$ of light-induced relaxivity changes in chloroplasts isolated in the presence of EDTA. The reaction mixture contained 50 μM $MnCl_2$, added as shown in the Figure, along with 50 μM methylviologen. The chlorophyll concentration was adjusted to 2.3 mg/ml; the arrows indicate the illumination period.

Figure 4a shows that DCMU diminishes, but does not eliminate, the light-dependent relaxivity. Inhibition of PS I activity by the combination of KCN and Hg, however, leads to a complete loss of the light effect (Figure 4b). Chloroplasts thus inhibited are devoid of the light-induced signals arising from either endogenous or added Mn(II).

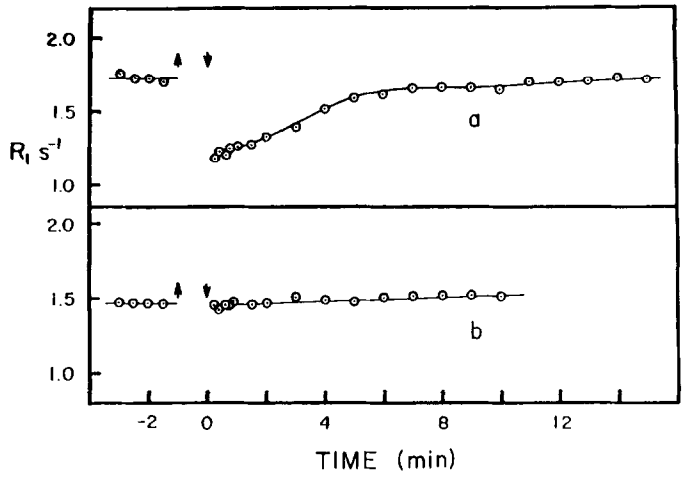

Fig. 4. Effect of electron transport inhibition on light-induced relaxivity changes. In (a) the reaction mixture contained chloroplasts isolated in EDTA and then washed (3.0 mg chlorophyll/ml) along with $MnCl_2$, methylviologen and DCMU, all at concentrations of 50 μM; in (b), the reaction mixture contained KCN/Hg-inhibited chloroplasts (2.6 mg chlorophyll/ml) and 50 μM methylviologen. No $MnCl_2$ was added in the experiment shown.

DISCUSSION The data in Figure 1 and Table I show conclusively that 1 mM EDTA, which has no effect on chloroplast oxygen evolution or photosynthetic phosphorylation activity, can dramatically influence the relaxivity of these preparations. The identity of the relaxing species affected by EDTA as Mn(II) is supported by the presence of a paramagnetic maximum near 20 MHz in the dispersion profile of R_1, observed with chloroplasts not exposed to EDTA, and by the appearance of a similar maximum in chloroplasts, prepared with EDTA, to which Mn(II) has been added (Figure 1). The observations presented here as well as other unpublished results shows that the dispersion profiles of chloroplasts not exposed to EDTA are subject to wide variation. We ascribe this variability to the presence of contaminating nonfunctional Mn(II). The dispersion profile of fully functional chloroplasts prepared in the presence of EDTA, and then washed free of the chelator shows no paramagnetic maximum, suggesting that the relaxivity of isolated chloroplast suspensions must be due to loosely bound, nonfunctional Mn(II).

Figures 2 and 3 demonstrate that illumination can produce a change in relaxivity only if loosely-bound EDTA-chelatable Mn(II) is present, either as a contaminant (from chloroplast preparations conducted in the absence of EDTA), or after intentional addition of the ion (to fully functional chloroplasts prepared in the presence of EDTA). In both cases, illumination reduces the relaxivity to a value approximating that seen in EDTA-treated chloroplasts, and the kinetics of dark recovery of relaxivity are essentially the same in both experiments. The use of selective inhibitors of photosynthetic electron transport (DCMU, KCN/Hg) reveals that inhibition of PS I, rather than PS II, is required to block light-induced changes in relaxivity.

We conclude, from the data presented here, that nonfunctional, EDTA-chelatable Mn(II) is the predominant species responsible for the relaxivity of chloroplast suspensions, and that previous reports (1-5) ascribing this phenomenon to functional Mn(II) associated with the water-splitting enzyme of chloroplasts are in error. This conclusion is strengthened by our data showing that light-induced changes in relaxivity require the function of PS I rather than PS II.

We cannot presently explain the origin of the light-induced change in relaxivity by a simple mechanism. Certainly, the effect must be due to oxidation of Mn(II) in a process which involves PS I, not PS II. To our knowledge there is no evidence that Mn(II) is photooxidized directly by PS I; electron transport catalyzed by this photoreaction does, however, generate superoxide ion (9,10), and it may be that this species serves as the oxidant of Mn(II). Experiments to test this hypothesis are now in progress.

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REFERENCES

1. Wydrzynski, T. J., Marks, S. B., Schmidt, P. G., Govindjee, and Gutowsky, H. S. (1978) Biochemistry 17, 2155-2162.
2. Wydrzynski, T., Zumbulyadis, N., Schmidt, P. G., Gutowsky, H. S., and Govindjee (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1196-1198.
3. Wydrzynski, T., Zumbulyadis, N., Schmidt, P. G. and Govindjee (1975) Biochim. Biophys. Acta 408, 349-354.
4. Govindjee, Wydrzynski, T. and Marks, S. B. (1978) Photosynthetic O_2 Evolution, pp. 321-344, Academic Press, New York.
5. Govindjee (1978) Nat. Acad. Sci. Letters 1, 3-6.
6. Robinson, H. H. and Yocum, C. F. (1980) Biochim. Biophys. Acta, in press.
7. Yocum, C. F. and Guikema, J. A. (1977) Plant Physiol. 59, 33-37.
8. Sharp, R. R. (1972) J. Chem. Physics 12, 5321-5330.
9. Epel, B. L., and Neumann, J. (1973) Biochim. Biophys. Acta 325-520-529.
10. Lumsden, J. and Hall, D. O. (1975) Biochem. Biophys. Res. Comm. 64, 595-602.