ESR and NMR Studies on the Effects of Magnesium Ion on Chloroplast Manganese

RITA KHANNA,[a*] S. RAJAN,[b] K. E. STEINBACK,[a] S. BOSE,[a] GOVINDJEE[a,c,**] AND H. S. GUTOWSKY[b]

[a]Departments of Botany, [c]Physiology & Biophysics, and [b]Chemistry, University of Illinois, Urbana, IL 61801 USA

(Received 16 April 1981)

Abstract. The addition of 1–50 mM $MgCl_2$ to "low salt" thylakoid membranes causes a monotonic decrease in the transverse, aqueous proton relaxation rate ($R_2 \equiv 1/T_2$) and an increase in the 6-line ESR spectrum characteristic of free Mn(II) but does not affect the oxygen evolution rate. These results suggest that magnesium ion at low concentrations replaces Mn(II) from a very loosely bound pool which is not involved in oxygen evolution. The decrease of R_2 is probably due mainly to the smaller molar relaxivity of free compared with bound Mn(II). R_2 can be affected by conformational and structural changes involving the Mn(II) as well as by the amount of Mn(II) and the nature of its binding. However, $MgCl_2$ causes the same decrease in R_2 for trypsin treated thylakoids, which do not undergo the gross structural changes, e.g. grana stacking, that it produces in untreated membranes. R_2 measurements of isolated light harvesting complex preparations indicate the presence of bound Mn in it; neutron activation analysis of these samples shows that they have about one-third of the functional manganese bound in thylakoid membranes — this may be the tightly bound pool of Mn, but the results are not conclusive.

INTRODUCTION

The manganese associated with photosystem II (PS II) in chloroplasts is bound heterogeneously.[1] About 2/3 of the bound Mn is in a loosely-bound pool, the removal of which leads to a proportional decrease in oxygen evolution. The function of the more tightly-bound 1/3 is obscure. Values for the amount of chlorophyll (Chl) per Mn bound in chloroplasts range from 14 to 600 Chl/Mn, with the more recent data[2] converging on 100–200 Chl/Mn. Some of this variability may stem from differences in preparative methods which extract more or less of the most loosely bound Mn. The oxygen evolving activity of chloroplasts is lost in various treatments that lead to release of the loosely-bound Mn; these include Tris washing,[3] hydroxylamine extraction[2] and temperature shock.[4,5] The remaining Mn cannot be released readily by these treatments. The treated chloroplasts are capable of electron flow from artificial electron donors through PS II to the electron acceptors on the reducing side of PS II or I.

Incubation of chloroplasts with chelating agents such as ethylene diamine tetracetic acid (EDTA) does not release manganese associated with O_2 evolution.[6–8] Also, such Mn does not exchange easily with other divalent cations,[6] but it is slowly released[9,10] by incubation with relatively high ($\sim$ 200 mM) concentrations of Mg ion. In the present work we have employed low concentrations of $MgCl_2$ to explore further the heterogeneity of the bound Mn. We have measured not only the O_2 evolution rate but also the proton transverse relaxation rate ($R_2 \equiv 1/T_2$) and ESR spectra for aqueous suspensions of pea chloroplasts as a means of determining changes caused in the Mn by the $MgCl_2$. R_2 is known to be sensitive to the amount and oxidation state of Mn present[10] and to the extent and nature of its binding in a complex.[11] In ESR, free aqueous Mn(II) gives a distinctive 6-line spectrum[12] while other oxidation states and bound Mn(II) give at most very broad, weak absorption. As an aid in determining whether the structural changes[13] caused by $MgCl_2$ contribute to the decrease it produces in R_2, similar experiments were performed on trypsin treated membranes. Also, observations have been made of the manganese in the light harvesting complex (LHC).[14,15]

MATERIALS AND METHODS

Chloroplast Preparation

Chloroplasts were isolated by grinding pea leaves in a Waring blender in a medium containing 0.4 M sorbitol and 0.1 M Tricine-NaOH (pH 7.8). The slurry was filtered through 4, then 12 layers of cheese cloth, and then centrifuged at $1{,}000 \times g$ for 10 min. The pellet was washed once with unbuffered 10 mM NaCl and then resuspended at a concentration of about 3 mg Chl/ml in 10 mM Tricine-NaOH at a predetermined pH in the 6.5 to 7.8 range as specified. Chlorophyll concentration was determined by the method of MacKinney.[16]

Trypsin Treatment

Chloroplasts were isolated, washed once in 10 mM NaCl and resuspended in 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, and 10 mM Tricine-NaOH (pH 7.8). For trypsin treatment, thylakoid membranes were diluted in the same buffer to a final Chl concentration of 100 μg/ml and treated with trypsin (at 21 °C) as described by Steinback et al.[13] Trypsin from bovine pancreas, Type III

* This work is based on the Ph.D. Thesis of Rita Khanna (University of Illinois at Champaign-Urbana, 1980).

** Correspondence should be addressed to: Professor Govindjee, Department of Botany, 289 Morrill Hall, 505 S. Goodwin Ave., University of Illinois, Urbana, IL 61801 USA.

(12,000 units/mg protein), was added to a final concentration of 0.25 μg/ml. Trypsin digestion was stopped at various time intervals by the addition of a twenty-fold excess of trypsin inhibitor (from soybean, Type I-S, Sigma). Chloroplasts were pelleted by centrifuging at $10,000 \times g$ for 10 min at 4 °C and dispersed in 1 mM Tricine-NaOH and 10 mM NaCl (pH 6.0). Polypeptides of trypsin treated membranes were analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described.[13,17]

Isolation and Purification of the Light Harvesting Complex

Light harvesting complex (LHC) was isolated according to the method of Burke et al.[14] The chloroplasts were washed in low salt medium and incubated with 0.5% Triton X-100 for 30 min at 21 °C (Chl, 0.5 mg/ml). The mixture was centrifuged at $40,000 \times g$ to remove unsolubilized membrane fragments and the supernatant was loaded on a linear 0.1–1.0 M sucrose gradient and centrifuged for 15 h at $100,000 \times g$ using a Beckman SW-27 rotor. The region of sucrose gradient showing high fluorescence (containing the LHC) was removed and the LHC was precipitated from this fraction by the addition of 10 mM $MgCl_2$ and 100 mM KCl. The sample was stirred briefly and then centrifuged for 10 min at $9,000 \times g$ over a 500 mM sucrose cushion. The resulting pellet yielded purified LHC.

Measurement of Oxygen Evolution

Oxygen evolved by repetitive flashes was measured with a Clark (platinum/Ag–$AgCl_2$) electrode and a Yellow Spring oxygen monitor (Model 53). Flashes of saturating intensity and short duration (~10 μs) were provided from a xenon flash lamp (General Radio Stroboslave Type 1539-A). For measuring steady-state O_2 evolution, saturating light (175 mW/cm^2) from a tungsten lamp was focused on the thylakoid suspension through a Corning Glass CS 3–70 filter. The voltage from the Clark electrode was recorded on an Esterline Angus (Model E11015) recorder.

Measurements were made at a chlorophyll concentration of 20 μg/ml with 0.5 mM $Fe(CN)_6^{3-}$ and 1 mM NH_4Cl included in the suspension medium.

Nuclear Magnetic Relaxation Measurements

The proton spin-spin (transverse) relaxation rate ($R_2 \equiv 1/T_2$) was measured from the exponential decay of echo amplitudes in a Carr–Purcell (Meiboom–Gill modification) train of rf pulses.[11] A 90° rf pulse was followed by time τ (500 μs), and then a series of 2,000 180° pulses spaced 2τ apart was applied. The R_2 was calculated from the decay of the spin-echo envelope with time. The echo heights of the envelope were sampled with an analog to digital converter and the amplitudes were analysed by a PDP-8f minicomputer using a least-squares program. All measurements were made at room temperature (25 °C) at a frequency of 26.89 MHz.

Electron Spin Resonance (ESR) Spectra

The ESR spectra were recorded as the first-derivative with a Varian E-9 spectrometer (X band, 9.5 GHz). The cavity was continuously flushed with dry N_2 gas. Unless otherwise stated, the instrument conditions were: microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; time constant, 0.3 s; scan rate, 250 G/min. Thylakoid samples (2.0–3.0 mg Chl/ml) were placed in a flat cell positioned with clips and all the spectra were recorded at room temperature (23 °C). Approximate concentrations of free Mn(II) were estimated from the area under the second peak from low field in the 6-line spectrum under the same conditions.

Manganese Determination

The manganese content was determined by neutron activation analysis using the procedures described in detail previously.[10]

RESULTS AND DISCUSSION

Effects of Mg Ion on Cation-Depleted Thylakoid Membranes

Figure 1 (solid circles) shows the effect of increasing concentrations of added $MgCl_2$ on the proton relaxation rate R_2 of thylakoid membrane suspensions at pH 6.5, depleted of cations by washing once with 10 mM NaCl. There is a significant decrease in R_2 from 4.3 to 3.3 s^{-1} as the $MgCl_2$ concentration is increased to 20 mM, above which there is no appreciable further change (at least up to 50 mM). The concentration of $MgCl_2$ required to produce half of the maximum change in R_2 is ~3.5 mM. A similar decrease in R_2 is seen in unwashed thylakoid membranes upon the addition of $MgCl_2$, except that a much higher concentration of $MgCl_2$ (~20 mM) is required to attain the half-maximal change (data not shown). At pH 7.0 and 7.5 R_2 also is decreased by the addition of $MgCl_2$; however, the change is only about half as large (10–15%) as that at a pH of 6.5 and requires higher concentrations of $MgCl_2$ (6 mM half-change at pH of 7.5).

Figure 2 gives ESR spectra of the membrane suspensions at a pH of 6.5 as a function of added $MgCl_2$. It is seen that the amplitude of the 6-line spectrum ascribed[12] to free Mn(II) increases with increasing concentration of $MgCl_2$. Relative concentrations of Mn(II) in the suspensions were estimated from the spectra and are plotted (solid triangles) in Fig. 1. The increase in [Mn(II)] is a mirror image of the decrease in R_2. Also, the increase in Mn(II) is less at pH's of 7.0 and 7.5, and occurs at higher $MgCl_2$ concentrations. The sharp, central line at $g = 2.0$ in Fig. 2 is that designated[18] as Signal II; it is unaffected.

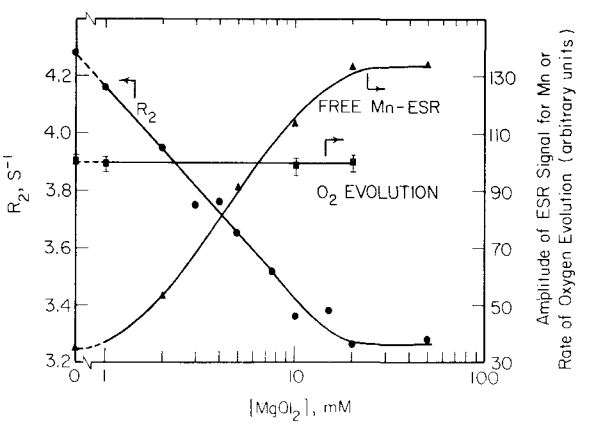

Fig. 1. Effect of $MgCl_2$ in washed thylakoids on the water proton relaxation rate R_2, the O_2 evolution, and the free manganese (as estimated from ESR spectra in Fig. 2). Thylakoid membranes were washed once in unbuffered 10 mM NaCl and resuspended in 10 mM Tricine (pH, 6.5), 400 mM sorbitol, 10 mM NaCl and $MgCl_2$ at the concentrations shown. R_2 was measured at a chlorophyll concentration of 3 mg/ml.

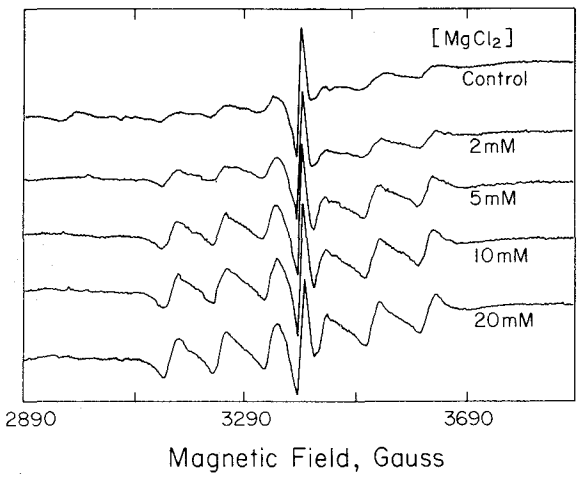

Fig. 2. Effect of $MgCl_2$ on ESR spectra of thylakoid membranes washed as in Fig. 1. Instrumental conditions were as in Materials and Methods except for receiver gain of 5×10^3 and Chl concentration of 3 mg/ml.

ESR spectra were also run of aqueous 10 μM $MnCl_2$ solution with low concentrations of $MgCl_2$ to determine whether the changes observed in Fig. 2 could be attributed to the effects of $MgCl_2$ upon the small amount of free Mn(II) in the control suspension. There was a small increase (~15%) in the peak amplitudes of the 6-line spectrum, probably because of line-narrowing due to decreased electron or nuclear relaxation rates of the Mn(II); but the effect saturated at ~1 mM $MgCl_2$ and is negligible compared with the several-fold increase in Fig. 2 for chloroplasts. A comparison of the two sets of spectra gives an approximate calibration for the amount of Mn(II) released from the chloroplasts, the value at 20–50 mM $MgCl_2$ corresponding to ~0.15 μg Mn/mg Chl. The total Mn content of the untreated membranes is 0.80 μg Mn/mg Chl.

The Mn(II) released by $MgCl_2$ at these low concentrations has little or no effect upon the oxygen evolution. This is shown in Fig. 1 by the steady-state evolution observed for the washed membranes at pH 6.5 with various amounts of $MgCl_2$. The O_2 evolved per flash is also unaffected. Measurements with a Clark-type electrode gave a value of 1 O_2 molecule per 2,600 ± 250 Chl molecules per flash, with and without 10 mM $MgCl_2$ at a pH of 7.5. This value is comparable with the photosynthetic unit size of 2,500 chlorophyll molecules/O_2 reported in Chlorella.[19] These results are consistent with the work of Govindjee et al.[20] on O_2 yield/flash and on charge separation at reaction center II (as measured by 515 and 320 nm absorption changes). The contrary findings of Bose and Arntzen[21] that O_2 evolution was *enhanced* by low $MgCl_2$ concentrations appears to be an artifact caused by its effect upon the rate at which chloroplasts sediment.[22]

Thus, from our results it appears that thylakoid suspensions can be prepared with about 20% of their total Mn bound very loosely and not required for O_2 evolution. Removal of additional bound Mn by more drastic treatments is known to affect O_2 evolution. Up to 2/3 or so of such Mn can be removed by incubation of thylakoids in 100–200 mM $MgCl_2$ for 20 to 90 min at 0 to 2 °C, with a corresponding decrease in O_2 evolution by the Hill reaction and an increase in the steady-state amplitude of ESR Signal[9] II. Similarly, progressive removal of Mn by incubation of thylakoids with increasing ratios of [Mg(II)]/[Chl] for 2 h at 4 °C decreases[10] R_1 and R_2 as well as O_2 evolution.[23]

In the latter experiments, the chloroplasts were prepared in a different medium (HEPES instead of Tricine) and had a total Mn content[10] of 0.62 μg of Mn/mg of Chl. This corresponds to the total Mn in the present preparations (0.80) less the maximum amount released (~0.15) by the low concentrations of $MgCl_2$. Removal of the very loosely bound Mn can readily occur during preparation of the chloroplasts, depending upon the conditions employed. For example, we have found from ESR spectra that low [$MgCl_2$] does not release Mn(II) from chloroplasts prepared with HEPES or EDTA. Also, very loosely bound Mn was not found by Chen and Wang[9] who washed their chloroplasts with dilute Tris buffer before extracting Mn with $MgCl_2$.

A few observations can be made about the nature of this very loosely bound pool. The amount of Mn released corresponds to a concentration of ~8 μM free Mn(II). It is noteworthy that it is released more readily under acidic conditions. Some might be associated with Mn-superoxide dismutase, but the amount of the latter is small.[24] Finally, the decrease in R_2, ~10^5 s^{-1}/mole Mn(II) released, is comparable with the enhancement found in the binding of Mn(II) to small proteins.[11]

Effects of Mg Ion on Trypsin-Treated Thylakoid Membranes

Cations cause major structural and functional changes in thylakoid membranes. Addition of low concentrations of divalent cations to low-salt thylakoids containing unstacked lamellae results in the formation of stacked regions (grana).[25] This structural change is usually accompanied by an increase in quantum yield of PS II and a decrease in quantum yield of PS I under low light conditions.[26] It is known that the LHC of chloroplasts is necessary for cation mediated grana formation and energy redistribution between the two pigment systems.[25] The proteolytic enzyme trypsin modifies the LHC at the external surface of the membrane such that the membranes do not exhibit cation induced structural changes.[13] Such structural changes could lead to a change in the environment of the loosely bound Mn, thereby affecting the relaxation of water protons in the hydration sphere of the Mn complex. In order to verify that the decrease in R_2 upon $MgCl_2$ addition (Fig. 1) is due to the release of Mn(II) and its lower relaxivity (Fig. 2) and not due to the structural changes, we performed experiments on trypsin-treated thylakoid membranes. Electrophoretic analysis of the latter, of control membranes and of the LHC isolated from them gave the polypeptide compositions and differences reported by Steinback et al.[13]

The results for R_2 and O_2 evolution are summarized in Table 1. R_2 decreases when 20 mM $MgCl_2$ is added to the

Table 1. Effect of $MgCl_2$ on R_2 and O_2 Evolution of Trypsin Treated Thylakoid Membranes[a]

[$MgCl_2$] mM	R_2 s^{-1}		O_2 evolution μ equiv/mg Chl h^{-1}	
	Control	Trypsin	Control	Trypsin
0	1.97	1.96	418 ± 8	408 ± 9
20	1.70	1.73	415 ± 7	414 ± 8

a. Chlorophyll concentration for R_2 was 1.8 mg/ml.

membranes. However, the decrease for trypsin-treated membranes is the same as for control membranes which were carried through the same incubation without trypsin. Moreover, in both samples the $MgCl_2$ releases Mn(II), as shown by an increase in the 6-line ESR spectrum for free aqueous Mn(II) (Fig. 3). Also, the Mg ion does not change the rate of O_2 evolution in membranes treated with trypsin for ~ 12 min (Table 1). Thus, the effects of Mg ion persist in thylakoids that do not undergo structural changes, confirming our earlier view that low concentrations of $MgCl_2$ release Mn from a very loosely bound pool not required for O_2 evolution.

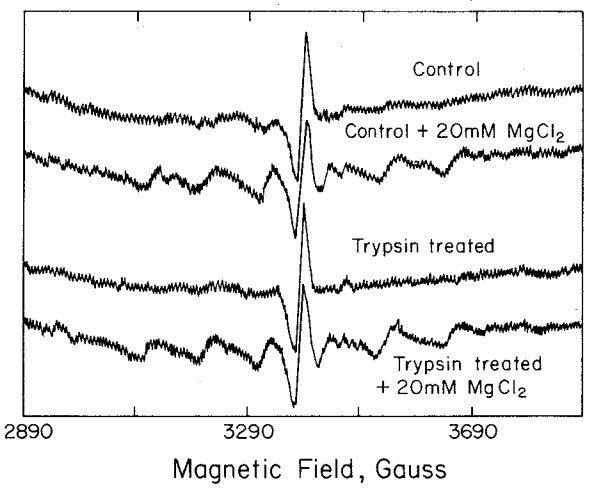

Fig. 3. ESR spectra of control and trypsin-treated thylakoid membranes in the absence and the presence of 20 mM $MgCl_2$. Instrumental conditions as in Fig. 2 except for time constant of 0.1 s; receiver gain of 10^4 and Chl concentration of 2.4 mg/ml.

Manganese Content and R_2 of the Light Harvesting Complex

The LHC, a pigment protein complex,[14] contains at least three polypeptides (two major polypeptides of 23,000 and 25,000 daltons and one minor polypeptide of 29,000 daltons) and Chl *a* and *b*. The LHC *in situ* can undergo self association in the presence of cations and bring about cation mediated grana formation.[25] Thus, to further assess the possible effects of structural changes on the observed R_2, we measured R_2 and Mn content of the isolated LHC. However, the effect of Mg ion on the R_2 of purified LHC could not be measured because isolation of LHC entails high concentrations of $MgCl_2$.[14]

The R_2, corrected for relaxation of the buffer, was found to be 0.74 and 0.66 s^{-1}, respectively, for thylakoid membranes and for the purified LHC preparations obtained from them (both with 0.3 mg Chl/ml). The corresponding total Mn content was 0.8 and 0.4 ± 0.4 μg Mn/mg Chl. The R_2 and Mn content of the LHC are unexpectedly high when expressed on a mg Chl basis, indicating that the LHC preparation contains an appreciable portion of the Mn. Moreover, the molar relaxivity of Mn in the LHC is nearly twice (1.8) that in the whole membrane.

The Mn content of the LHC corresponds to 6.6 moles Mn/mole Chl. For isolated LHC the average mole ratio of Chl/polypeptide (23,000 molecular wt) has been determined to be 13.4 with about 6 polypeptides/LHC,[14] i.e., ~ 80 Chl/LHC. This gives 0.53 Mn/LHC. Foyer and Hall[26] have also reported the presence of Mn in LHC. Their analyses correspond to $\lesssim 1/2$ as much Mn/LHC as we find. However, there are several differences in the materials and procedures.

The origin of the 0.4 μg Mn/mg Chl we find in the LHC depends upon the nature of the heterogeneity of Mn binding in the whole membranes. For example, suppose that all of the tightly bound Mn is associated with and retained by Chl in the LHC. The amount of tightly bound Mn is about a third of the total in whole membranes,[1,2] excluding the very loosely bound Mn we report here, i.e. $\sim \frac{1}{3}(0.80\text{–}0.15) = 0.22$ μg Mn/mg Chl. However, at most 60% of the total Chl is in the LHC[27] so the ratio of tightly bound Mn to Chl in the LHC would be $\gtrsim (0.22/0.6) = 0.37$ μg Mn/mg Chl. This, in fact, is consistent with the 0.40 figure actually found. On the other hand, if the binding sites are distributed uniformly with respect to Chl in the LHC and the rest of the whole membranes, one would conclude that half of the 0.8 μg Mn/mg Chl originally in the LHC had been lost during its isolation, presumably the 0.15 very loosely bound and 0.25 of the 0.44 loosely bound.

It seems unlikely that the high Mn content of the LHC is due to nonspecific association of free Mn during the isolation procedure. The buffer for initial isolation of the thylakoids included 5 mM EDTA. Also, dialysis of the purified LHC with 1 mM EDTA did not remove Mn. The R_2 of dialysed LHC is lower (0.3 s^{-1} for 0.6 mg Chl/ml) as compared to the undialyzed sample (0.66 s^{-1} for 0.3 mg Chl/ml) but analysis of the total Mn content gave 0.38 ± 0.03 μg Mn/mg Chl after dialysis versus 0.40 before. The decrease in R_2 is probably because EDTA replaces water ligands on the bound Mn.

In any case it appears that as much as 60–100% of the tightly bound Mn is associated with the LHC. No role has been assigned to the tightly bound pool of Mn. Possingham et al.[28] showed that Mn deficiency had a large effect on the structure of spinach chloroplasts, leading to a progressive disorganization of the lamellar system. A loss of structure could arise directly if Mn acts as an essential structural link in the membranes, or indirectly if Mn participates in some reaction which is required for the production of molecules essential for the formation of these membranes. It is not possible to distinguish between these alternatives. It seems likely that the tightly bound Mn in LHC could play an important structural role in chloroplasts.

Acknowledgements. We thank Dr. C. J. Arntzen for discussions. The research was supported by NSF grants PCM 11657 (to G) and CHE 04585 (to HSG).

REFERENCES

1. R. Radmer and G. Cheniae in J. Barber, ed., *Topics in Photosynthesis*, Elsevier/North-Holland Biomedical Press, Amsterdam, 1977, Vol. 2, p. 303.
2. G. M. Cheniae and I. F. Martin, *Biochim. Biophys. Acta*, **197**, 219 (1970).
3. T. Yamashita and W. L. Butler, *Plant Physiol.*, **43**, 1978 (1968).
4. S. Katoh and A. San Pietro, *Arch. Biochem. Biophys.*, **122**, 144 (1967).
5. P. H. Homann, *Biochem. Biophys. Res. Commun.*, **33**, 229 (1968).
6. B. Kok and G. M. Cheniae in R. Sanadi, ed., *Current Topics in Bioenergetics*, Academic Press, New York, 1966, Vol. 1, p. 1.

7. J. V. Possingham and D. Spencer, *Aust. J. Biol. Sci.*, **15**, 58 (1962).
8. P. H. Homann, *Plant Physiol.*, **42**, 997 (1967).
9. K.-Y. Chen and J. H. Wang, *Bioinorg. Chem.*, **3**, 339 (1974).
10. T. Wydrzynski, S. B. Marks, P. G. Schmidt, Govindjee and H. S. Gutowsky, *Biochemistry*, **17**, 2155 (1978).
11. R. A. Dwek, *N.M.R. in Biochemistry*, Clarenden Press, Oxford, 1975, p. 247.
12. B. B. Garrett and L. O. Morgan, *J. Chem. Phys.*, **44**, 890 (1966).
13. K. E. Steinback, J. J. Burke and C. J. Arntzen, *Arch. Biochem. Biophys.*, **195**, 546 (1979).
14. J. J. Burke, C. L. Ditto and C. J. Arntzen, *Arch. Biochem. Biophys.*, **187**, 252 (1978).
15. D. Wong, H. Merkelo and Govindjee, *FEBS Lett.*, **104**, 223 (1979).
16. G. MacKinney, *J. Biol. Chem.*, **140**, 215 (1941).
17. V. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
18. E. C. Weaver, *Ann. Rev. Plant Physiol.*, **19**, 283 (1968).
19. R. Emerson and W. Arnold, *J. Gen. Physiol.*, **15**, 391 (1932).
20. Govindjee, P. Mathis, C. Vernotte, D. Wong, S. Saphon, T. Wydrzynski and J.-M. Briantais, *Z. Naturforschung*, **34C**, 826 (1979).
21. S. Bose and C. J. Arntzen, *Arch. Biochem. Biophys.*, **185**, 567 (1978).
22. R. Khanna and P. Jursinic, *FEBS Lett.*, **126**, 318 (1981).
23. Govindjee, T. Wydrzynski and S. B. Marks in L. Packer, G. C. Papageorgiou and A. Trebst, eds., *Bioenergetics of Membranes*, Elsevier/North-Holland, Amsterdam, 1977, p. 305.
24. K. Asada, S. Kanematsu, S. Okada and T. Hayakawa in *Active Oxygen in Biological Species*, 1980, in press.
25. C. J. Arntzen in D. R. Sanadi and L. P. Vernon, eds., *Current Topics in Bioenergetics*, Academic Press, New York, 1978, Vol. 8, p. 111.
26. C. H. Foyer and D. O. Hall, *FEBS Lett.*, **101**, 324 (1979).
27. J. P. Thornber, *Ann. Rev. Plant Physiol.*, **26**, 127 (1975).
28. J. V. Possingham, M. Vesk and F. V. Mercer, *J. Ultrastruct. Res.*, **11**, 68 (1964).