# Effect of Manganese Extraction on Oxygen Generation and EPR Signal II in Spinach Chloroplasts

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ABSTRACT

Removal of endogenous manganese from fresh chloroplasts by incubation with solutions of magnesium ion was found to decrease the efficiency of oxygen generation in the Hill reaction and increase the steady-state amplitude of Signal II. The efficiency of oxygen generation in the Hill reaction was found to be directly proportional to the manganese content of the extracted sample, up to about 67% extraction of the endogenous manganese, whereas the steady-state amplitude of Signal II was found to be inversely proportional to the manganese content of the extracted chloroplast sample. These observations suggest that in freshly prepared chloroplasts all lamellae-bound manganese atoms are equally active in catalyzing oxygen generation, and that Signal II may be due to a radical intermediate generated by the photo-oxidation of an electron carrier between the manganese complex and the chlorophyll molecules of PS II.

KEY WORDS

Oxygen generation; manganese content of chloroplasts; photosynthesis; signal II, EPR; photo-oxidation of water.

It is generally accepted that the two light-induced EPR signals in chloroplasts are related to different pigment systems, PS II and PS I [1, 2]. Signal II ($g$-value 2.0046, half-width about 20 G) is probably associated with chlorophyll molecules, $Chl_{II}$, of PS II, [3, 4], whereas Signal I is associated with chlorophyll molecules, $Chl_I$, of PSI [5, 6]. Since both manganese and PS II are required in the photo-synthetic oxygen generation process [7], it may be informative to study the effect of manganese removal on both the intensity of Signal II and the efficiency of oxygen generation in a

Hill reaction [8]. The experimental results of such a study are reported and discussed in this paper.

## EXPERIMENTAL SECTION

### Chloroplasts

Chloroplasts were prepared by homogenizing fresh spinach leaves in a Waring blender, followed by differential centrifugation as described by Avron [9]. Chlorophyll concentration was determined by the precedure of McKinney [10]. For EPR and some oxygen generation studies, the isolated chloroplasts were used immediately after preparation. For experiments with aged chloroplasts, the chloroplast pellet was first frozen in liquid nitrogen, then stored at −20°C for at least three days before use.

### Manganese Extraction

Freshly prepared chloroplasts were first washed with dilute Tris buffer, which contained 0.1 M sucrose, 5 mM NaCl, and 0.01 M Tris plus its hydrochloride at pH 7.9, then incubated with an extraction mixture containing $Mg^{2+}$ for a fixed length of time (from 30 to 90 min) at 0 to 2°C. The incubation mixture for manganese extraction contained 0.1 M sucrose, 5 mM NaCl, 0.01 M Tris, plus its hydrochloride at pH 7.9, 50 or 100 or 200 mM $MgSO_4$, and chloroplasts at a concentration equivalent to about 700 μg chlorophyll per milliliter of the mixture. As control, chloroplast sample containing 0.25 M sucrose, 0.02 M Tris, and its hydrochloride at pH 7.9, 10 mM NaCl but without $MgSO_4$ was incubated under the same conditions for exactly the same length of time. After incubation, each chloroplast sample was divided into two equal parts and centrifuged at 10,000 g for 10 min. The pellet in one centrifuge tube was used for assay of manganese remaining in the chloroplasts; the other pellet was immediately resuspended in Tris buffer, which contained 0.25 M sucrose, 0.02 M Tris, plus its hydrochloride at pH 7.9 and 0.01 M NaCl, either for EPR measurements or for the measurement of oxygen generation in the Hill reaction. The characteristic six-line EPR spectrum due to the liberated $Mn(H_2O)_6^{2+}$ in the sample appeared after incubation with $MgSO_4$, but was removed completely in the subsequent centrifugation and resuspension step. Because the present extracting solution is free of complexing agents for $Mn^{2+}$, the progress of manganese extraction can be monitored precisely by measuring the EPR spectrum due to the liberated $Mn(H_2O)_6^{2+}$. The Hill reaction

activity of chloroplasts generally decreases with the time of storage even without extraction. By fixing the extraction time but varying the concentration of $Mg^{2+}$, we hope to minimize the error due to aging and obtain chloroplasts of different manganese content but otherwise similar integrity. For these reasons, the use of high concentrations of Tris and the so-called "chaotropic reagents" were avoided in this work. Replacing $MgSO_4$ by $MgCl_2$ in the above extraction mixture gave the same experimental results. Manganese content in chloroplasts was determined by the permanganate method [11].

### Measurement of Hill Reaction Activity

The rate of oxygen generation in Hill reaction at 20.0°C was measured polarographically with a YSI oxygen probe and Model 53 Oxygen Monitoring System. The reaction mixture contained 750 μmoles sucrose, 60 μmoles Tris plus its hydrochloride at pH 7.9, 2 μmoles $K_3Fe(CN)_6$ and chloroplasts equivalent to 50 to 80 μg chlorophyll in a total volume of 4 ml. Red light of saturating intensity was provided by a 500-W projector lamp fitted with a Kodak 600 nm cut-off filter. In some measurements the assay mixture also contained 8.5 mM methylamine as an added uncoupler.

### EPR Measurements

The magnetic resonance measurements were made at room temperature (22°C) with a Varian E-3 EPR Spectrometer and a Varian E-248-2 aqueous solution cell. A microscope illuminator covered with green cellophane paper was used to provide the stray light for operating the instrument and handling the sample in the dark. A 500 W projector lamp with a 2-in. thick cold water shield was used as the actinic light source. The light intensity on the window grid of the resonance cavity during illumination was $1.35 \times 10^6$ ergs/cm$^2$/sec. A constant stream of dry nitrogen gas was flushed through the cavity throughout the experiment to prevent heating of the sample or condensation of moisture. For both the control sample (chloroplasts with natural manganese content) and the extracted sample (chloroplasts with part of their manganese removed by treatment with $Mg^{2+}$ solution), identical EPR conditions were used, which included microwave power, modulation amplitude, microwave frequency, receiver gain, magnetic field strength, and position of the cell. The intensity change of light-induced Signal II with time was followed by fixing the magnetic field at resonance value of Signal II (3350 G under the experimental conditions).

## RESULTS

### Dependence of the Efficiency of Oxygen Generation on Manganese Content

In each of these experiments, two or three identical fresh chloroplast samples (including the control) were simultaneously incubated in a buffered solution containing different concentrations of $Mg^{2+}$ at 0 to 2°C for the same length of time. In this way, two or three samples with the same aging time but containing different amounts of lamellae-bound manganese were prepared. By measuring and comparing the efficiency of oxygen generation of the $Mg^{2+}$-treated chloroplast samples relative to the corresponding control samples, which were treated with $Mg^{2+}$-free buffer solution for exactly the same length of time, it is hoped that errors due to aging during the extraction process can be minimized.

The observed rates of oxygen generation by the Hill reaction with differ-

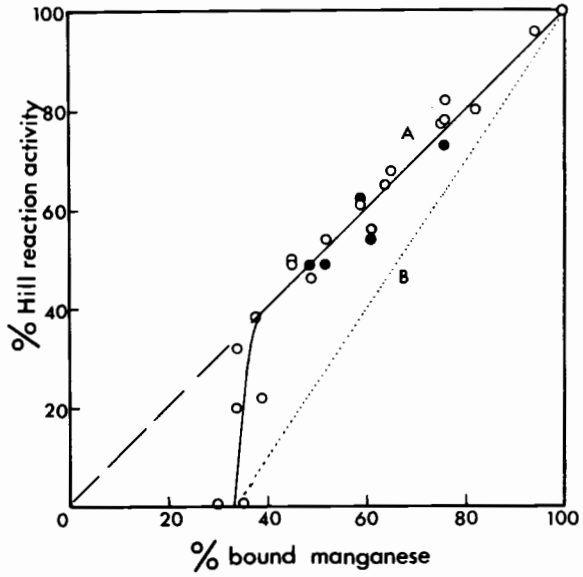


Fig. 1. Dependence of the rate of oxygen generation by the Hill reaction upon the concentration of lamellae-bound manganese in fresh spinach chloroplasts. ○ in the absence of methylamine during the Hill reaction measurement; ● in the presence of methylamine. Both Hill reaction activity and bound manganese are expressed as percent of the corresponding values of the control samples.

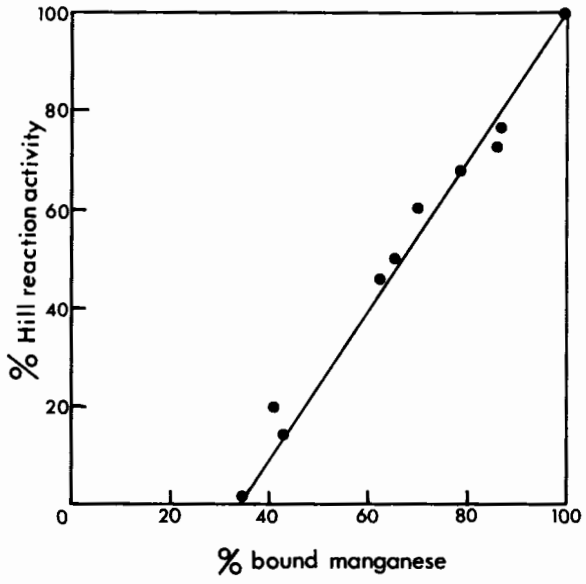


Fig. 2. Dependence of the rate of oxygen generation by the Hill reaction upon the concentration of lamellae-bound manganese in spinach chloroplasts which has been kept frozen for several days. Both Hill reaction activity and bound manganese are expressed as percent of the control samples.

ent batches of chloroplasts are summarized in Table 1. The data of Table 1 are plotted in percentages in Fig. 1, which shows that before two-thirds of the manganese are removed, all the lamellae-bound manganese atoms are equally reactive in catalyzing oxygen generation. After nearly two-thirds of the total bound manganese are extracted, the oxygen generating efficiency in the Hill reaction drops rapidly to zero. The remaining one-third of the manganese atoms are very resistant to extraction by the present method. The Hill reaction was also measured in the presence of the uncoupler methylamine for both the control and $Mg^{2+}$-treated chloroplast samples. Curve B in Fig. 1 is the theoretical straightline according to the "two-pool hypothesis" of Cheniae and Martin [12] which predicts an intercept of 33.3% of total bound manganese.

The observed dependence of the efficiency or oxygen generation in Hill reaction on the manganese content of aged chloroplasts is shown in Fig. 2, which corresponds very well with Curve B in Fig. 1. Cheniae and Martin [12] carried out Tris-washing on both fresh and freezer-stored chloroplasts and obtained results similar to that shown in Fig. 2, which prompted them

TABLE 1

Effect of Manganese Extraction on the Efficiency of Oxygen Generation by Hill Reaction with Fresh Chloroplasts[a]

| Expt. no. | $Mg^{2+}$ treatment | Manganese content[c] | | Hill reaction activity | | | |
|---|---|---|---|---|---|---|---|
| | | | | With $CH_3NH_2$[b] | | Without $CH_3NH_2$ | |
| | | moles Chl/ g-atom Mn | % | moles $O_2$/ mg Chl/hr | % | moles $O_2$/ mg Chl/hr | % |
| 1 | Control | | 100 | | | 106 | 100 |
| | Treated | | 94 | | | 102 | 96 |
| 2 | Control | 57 | 100 | | | 128 | 100 |
| | Treated | 75 | 76 | | | 104 | 82 |
| | Treated | 76 | 75 | | | 100 | 77 |
| 3 | Control | | 100 | 241 | 100 | 125 | 100 |
| | Treated | | 76 | 177 | 73 | 98 | 78 |
| | Treated | | 61 | 130 | 54 | 70 | 56 |
| 4 | Control | | 100 | 153 | 100 | 100 | 100 |
| | Treated | | 59 | 96 | 63 | 61 | 61 |
| | Treated | | 49 | 75 | 49 | 46 | 46 |
| 5 | Control | 47 | 100 | | | 81 | 100 |
| | Treated | 73 | 65 | | | 55 | 68 |
| | Treated | 74 | 64 | | | 52 | 65 |
| 6 | Control | | 100 | 127 | 100 | 46 | 100 |
| | Treated | | 52 | 62 | 49 | 25 | 54 |
| 7 | Control | | | 243 | 100 | 80 | 100 |
| | Treated | | | 229 | 94 | 76 | 95 |

| | | | | |
|---|---|---|---|---|
| ) | | | | |
| 8 | Control | 100 | 80 | 100 |
| | Treated | 45 | 40 | 50 |
| | Treated | 39 | 18 | 22 |
| 9 | Control | 100 | 75 | 100 |
| | Treated | 45 | 37 | 49 |
| | Treated | 34 | 15 | 20 |
| 10 | Control | 100 | 86 | 100 |
| | Treated | 38 | 33 | 38 |
| | Treated | 34 | 28 | 32 |
| 11 | Control | 100 | 102 | 100 |
| | Treated | 82 | 82 | 80 |
| 12 | Control | 100 | 73 | 100 |
| | Treated | 35 | 0 | 0 |
| | Treated | 30 | 0 | 0 |

[a] Composition of the reaction mixtures and the reaction conditions are described in the Experimental Section. The concentration of $MgSO_4$ in the extractant, the concentration of chloroplasts and the incubation time for each experiment are listed below.

(1) 0 and 200 mM (90 min), 1 mg CHl/ml.
(2) 0 and 120 and 240 mM (120 min), 1.36 mg Chl/ml.
(3) 0 and 150 and 300 mM (60 min), 1.2 mg Chl/ml.
(4) 0 and 150 and 300 mM (60 min), 1.47 mg Chl/ml.
(5) 0 and 150 and 200 mM (60 min), 0.7 mg Chl/ml.
(6) 0 and 250 mM (60 min), 0.9 mg Chl/ml.
(7) 0 and 125 mM (60 min), 1 mg Chl/ml.
(8) 0 and 48 and 120 mM (150 min), 0.5 mg Chl/ml.
(9) 0 and 72 and 144 mM (100 min), 0.67 mg Chl/ml.
(10) 0 and 120 and 240 mM (120 min), 0.64 mg Chl/ml.
(11) 0 and 142 mM (90 min), 1.4 mg Chl/ml.
(12) 0 and 120 and 205 mM (120 min), 0.58 mg Chl/ml.

[b] 8.5 mM methylamine is present in the reaction mixture.

[c] The average manganese content of four control samples was found to be 1.03 ± 0.10 g-atoms manganese/50 chlorophyll molecules. The corresponding values reported in the literature are 1.04 ± 0.09 (16), 0.95 ± 0.10 (22) and 0.82 (22), respectively.

to propose the "two-pool hypothesis". According to them, manganese atoms in the larger pool (⅔ of the total bound manganese) are easily extractable and are directly responsible for oxygen generation. Manganese atoms in the smaller pool are tightly bound and only serve as electron carrier. The different behavior of fresh chloroplasts and aged chloroplasts toward $Mg^{2+}$ treatment observed in the present work suggests that this problem deserves further investigation.

## Dependence of Light-induced EPR Signal II on Manganese Content

In spite of the considerable overlap of the light-induced EPR Signal I and Signal II in chloroplasts, changes in the amplitudes of these two signals can still be followed separately [1]. For example, Fig. 3 shows that at

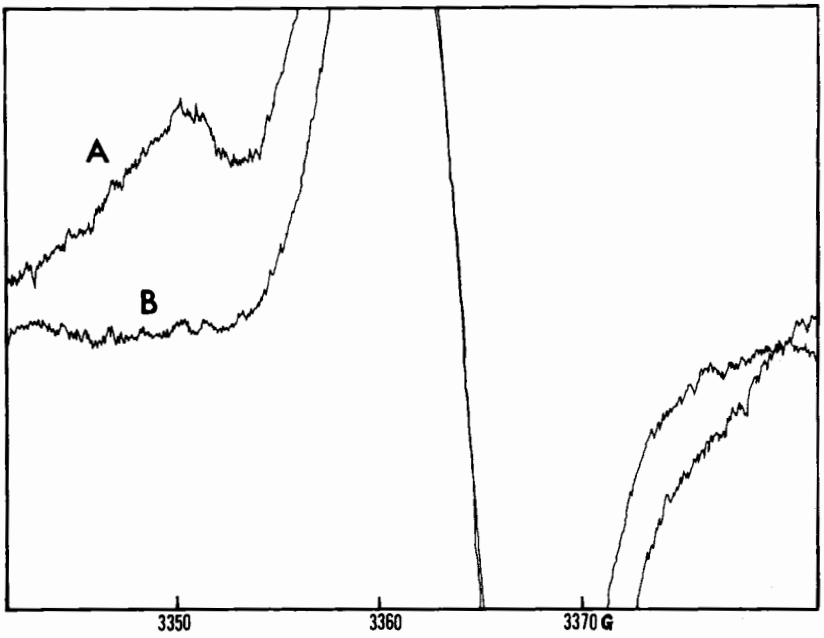


Fig. 3. Light-induced EPR signals of spinach chloroplasts, Curve A: lamellae-bound manganese partially removed by extraction with $Mg^{2+}$ solution. Curve B: Same as Curve A but in the presence of 5 mM TPB. Chloroplast concentration, 2.6 mg chlorophyll/ml. EPR settings, Microwave frequency 9.522 GHz, microwave power 25 mW, modulation amplitude 5 G, receiver gain $1.25 \times 10^6$, scan rate 25 gauss/min.

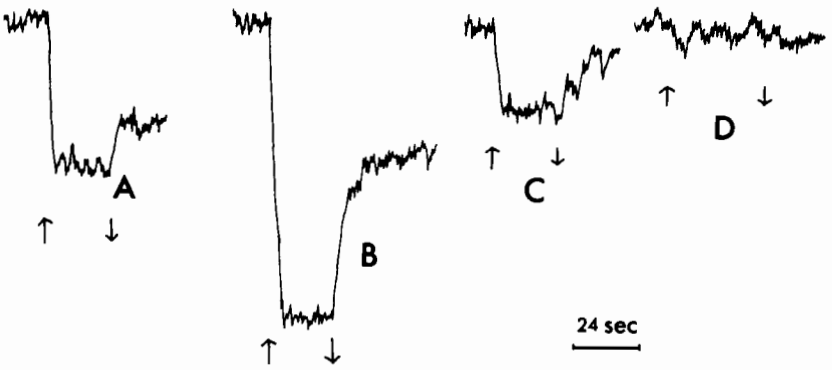


Fig. 4. Effects of manganese extraction and TPB respectively on the steady-state amplitude of Signal II. Curve A: The control chloroplast sample. Curve B: An equivalent chloroplast sample with its manganese extracted by treatment with $Mg^{2+}$ solution. Curve C: same as Curve B but in the presence of $5 \times 10^{4}$ M TPB. Curve D: same as Curve B but with $5 \times 10^{-3}$ M TPB. EPR settings: Microwave frequency, 9.525 GHz, microwave power 40 mW, modulation amplitude 8 G, receiver gain $1.25 \times 10^{6}$, magnetic field fixed at 3350 G. ↑ indicates light on, ↓ indicates light off.

steady-state under constant illumination the contribution of Signal I is negligible at 3350 G, and consequently changes in the amplitude of Signal II can be followed at this field setting. Curve A in Fig. 3 represents the EPR spectrum of illustrated chloroplasts, with lamellae-bound manganese partially removed by $Mg^{2+}$ treatment, which includes contributions from both Signal I and Signal II. Curve B is similar to Curve A, but in the presence of 5 mM sodium tetraphenylboron (TPB) as an added electron donor which totally eliminates Signal II but leaves Signal I unaffected [13]. The corresponding EPR spectra of unextracted chloroplasts show a similar but less pronounced difference in amplitude at 3350 G than that between Curve A and Curve B in Fig. 3.

Figure 4 illustrates the effects of manganese extraction and the addition of TPB respectively on the steady-state amplitude of light-induced Signal II. The observed effects of various redox reagents on the steady-state amplitude, $l_{II}$, of light-induced Signal II in extracted chloroplasts are summarized in Table 2. It seems that while exogenous reductants generally suppress Signal II, exogenous oxidants have little effect.

Figure 5 shows that steady-state amplitude, $l_{II}$, of the extracted chloroplasts sample is inversely proportional to the manganese content of the extracted chloroplast sample. These observations suggest that manganese in chloroplasts behave as a photoreductant to the radical species responsible for Signal II.

TABLE 2

Effect of Redox Reagents on the Steady-State Amplitude of Signal II in Extracted Chloroplasts

| Reductant | | | Oxidant | | |
|---|---|---|---|---|---|
| Formula | Concentration[a] (mM) | $l_{II}$ | Formula | Concentration (mM) | $l_{II}$ |
| $H_2NOH$ | 18 | 0.48 | $NaIO_4$ | 20 | 2.0 |
| $Na_2S_2O_4$ | 5 | 0.46 | $K_3Fe(CN)_6$ | 7.5 | 1.7 |
| $NaBH_4$ | 25 | 0.56 | | | |
| TPB | 0.5 | 0.52 | | | |
| TPB | 5 | 0.0 | | | |

[a] Concentrations refer to the final chloroplast suspension which contained 2 to 3 mg chlorophyll per milliliter. $l_{II}$ denotes the amplitude increase at 3350 G due to light-induced Signal II.

$l_{II}$ of native chloroplasts = 1; $l_{II}$ of extracted chloroplasts = 1.85.

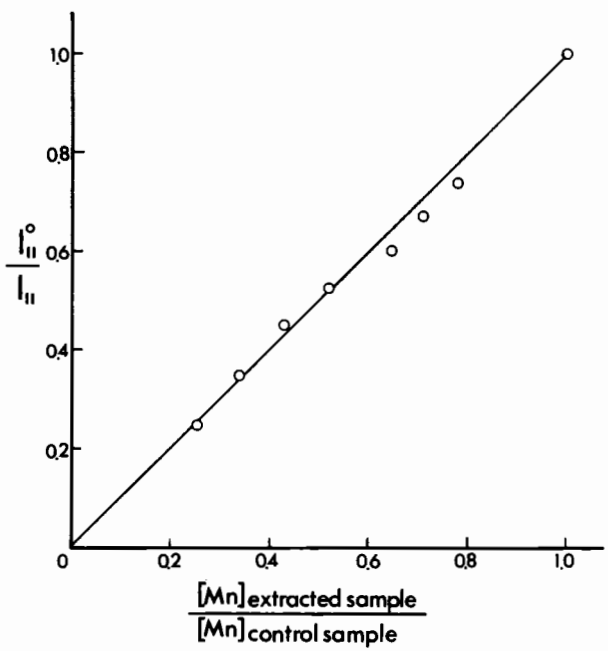


Fig. 5. Dependence of the steady-state amplitude, $l_{II}$, of Signal II on the remaining manganese content of extracted chloroplasts. $l_{II}$ was measured as the amplitude increase at 3350 G (microwave frequency, 9.522 GHz) due to light-induced Signal II. $I^0_{II}$ represents the corresponding amplitude increase of the control chloroplast sample.

For Gaussian or Lorentzian type of absorption curves of a given line width, the area under the curve is proportional to the amplitude of its derivative curve. With the approximation that under the present experimental conditions the amplitude of Signal II at 3350 G is proportional to the concentration of Signal II radicals, we conclude from the data in Fig. 5 that the steady-state concentration of these light-induced radicals is inversely proportional to the amount of lamellae-bound manganese.

## DISCUSSION

The generation of an $O_2$ molecule requires the extraction of four electrons from two water molecules. A very efficient way to fulfill this requirement is to let the photochemically produced oxidizing equivalents accumulate in a catalyst system until a total of four equivalents are stored, then the system returns to its initial oxidation state by oxidizing water to oxygen. Such a mechanism was strongly suggested by the work of Kok and coworkers [14] and by Joliot et al. [15]. These investigators also showed that the storage of the four positive charges occurs separately in each photosynthetic unit. Since manganese has multi-oxidation states (from 2+ to 7+), manganese complexes are particularly attractive candidates for such a catalyst system. The observed proportionality between the Hill reaction activity and the manganese content of the $Mg^{2+}$-treated samples (Fig. 1) shows that all manganese atoms in fresh chloroplast function equivalently in catalyzing the photo-oxidation of water to oxygen and are equally tightly bound in the native state. But as soon as about ⅔ of the manganese atoms are removed, a rearrangement may take place at the active center which renders the remaining ⅓ of the manganese atoms both catalytically inert and difficult to extract. Figure 2 suggests that the same rearrangement could take place during prolonged storage of chloroplasts in the frozen state.

Signal II is believed to be associated with PS II, since it is absent in mutant algae and photosynthetic bacteria which lack the capability of generating oxygen [3, 4]. According to Itoh and coworkers [16], the segment of photosynthetic electron transport chain near PS II of green plants may be represented by

$$H_2O \rightarrow \text{Mn-complex} \rightarrow Y \rightarrow \text{Chl}_{II} \rightarrow Q \rightarrow PQ \rightarrow \ldots$$

where the arrows denote the direction of electron transfer, $Y$ represents a hypothetical intermediate between the manganese complex and the primary reaction center of PS II, and $Q$ a hypothetical component of the electron transport chain between this primary reaction center and plastoquinone (PQ). It has been speculated that Signal II may be due to a semiplasto-

quinone or another semiquinone type of radical generated by one-electron reduction of $Q$ [2, 17, 18].

On the other hand, Lozier and Butler [19] as well as Chen [13] have independently suggested that Signal II may be due to an oxidized electron transfer intermediate on the water side of PS II. Malkin and Bearden [20] observed another EPR signal when a sample of spinach chloroplasts and ferricyanide was illuminated with 680 nm actinic light at 77°K. It seems very likely that the signal observed by Malkin and Bearden is also due to an oxidized radical intermediate. Recently Babcock and Sauer [21] also concluded from their studies of Signal II by both continuous and flashing techniques that Signal II arise from an oxidized radical on the water side of PS II.

The present data show that Signal II may be a radical intermediate between the manganese complex and the chlorophyll molecules of PS II and generated by the photo-oxidation of $Y$ by $\mathrm{Chl_{II}}$. Such an interpretation is consistent with the observed increase in the amplitude of Signal II after manganese extraction, because the removal of manganese tends to decrease the rate of electron supply from water and hence increase the steady-state concentration of oxidized $Y$.

Let us now assume that Signal II is due to the oxidized form of $Y$ represented by $Y^+$ and consider the following reactions which affect the steady-state concentration of $Y^+$.

$$Y + Q \xrightarrow[\text{chlorophyll}]{h\nu} Y^+ + Q^-$$

$$Y^+ + \mathrm{Mn^{II}} \underset{}{\overset{K_1}{\rightleftharpoons}} Y + \mathrm{Mn^{III}}$$

$$Y^+ + \mathrm{Mn^{III}} \underset{}{\overset{K_2}{\rightleftharpoons}} Y + \mathrm{Mn^{IV}}$$

where $K_1$, $K_2$ etc., represent the equilibrium constants of the corresponding oxidation-reduction reactions.

In the absence of Hill oxidants, $[Q^-]$ increases after the light is turned on until a steady-state is reached when the rate of generation of $Q^-$ by light is equal to the rate of its dissipation due to charge leakage. At constant light intensity, the steady-state concentration of $Q^-$ is equal to the total oxidizing capacity $A$ of the system given by

$$A = [Y^+] + [\mathrm{Mn^{III}}] + 2\,[\mathrm{Mn^{IV}}] + \ldots \tag{1}$$

Assuming that under the experimental conditions

$$[\mathrm{Mn^{III}}] \gg [Y^+] + 2\,[\mathrm{Mn^{IV}}] + \ldots,$$

$$[\mathrm{Mn^{II}}] \gg [\mathrm{Mn^{III}}],$$

and

$$[Y] \approx [Y]_{\text{total}} \gg [Y^+],$$

we have the approximate equilibrium relationship

$$\frac{[Y^+]}{[Y]} = \frac{1}{K_1} \cdot \frac{[\text{Mn}^{\text{III}}]}{[\text{Mn}^{\text{II}}]} \approx \frac{1}{K_1} \cdot \frac{A}{[\text{Mn}]_{\text{total}}},$$

or

$$[\text{Mn}]_{\text{total}} \approx \left(\frac{A[Y]_{\text{total}}}{K_1}\right) \frac{1}{[Y^+]} \tag{2}$$

Figure 5 shows that Eq. 2 is satisfied by our experimental data.

Further experimental support for attributing Signal II to an oxidized radical between the manganese complex and PS II came from the study of the response of Signal II in $Mg^{2+}$-treated chloroplasts to various redox reagents. According to the above scheme of electron transport, PS II reductants tend to decrease $[Y^+]$. On the other hand, PS II oxidants may increase $[Y^+]$ by oxidizing $Q^-$. The data in Table 2 is quite compatible with these considerations.

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