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# SITES OF FUNCTION OF MANGANESE WITHIN PHOTOSYSTEM II. ROLES IN $O_2$ EVOLUTION AND SYSTEM II

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SUMMARY

The Mn content of spinach chloroplasts has been decreased by growth deficiency, extraction and by ageing at 35°. We studied the effect of subnormal Mn content upon the chloroplasts capacity to evolve $O_2$ and to photooxidize electron donors other than water *via* Photosystem II. We observed the following:

1. In fresh chloroplasts ascorbate and other reducing agents, if present in sufficient concentration, fully replace water as the System II oxidant and can sustain maximum rates of 1000–1200 equiv/chlorophyll per h.

2. None of the studied donors proved entirely specific for System II; to a variable extent all could react with the oxidant of System I. We therefore considered only the 3-(3,4-dichlorophenyl)-1,1-dimethylurea-(DCMU)-sensitive fraction of the observed rates as pertinent.

3. Normal fresh chloroplasts contained 3 Mn/200 chlorophylls$_{II}$ and showed flash yields of approx. 1 $O_2$/1600 chlorophylls. This indicates that each System II trapping and $O_2$-evolving center contains three Mn atoms.

4. $O_2$ evolution capacity is abolished when about 2/3 of the total Mn pool is removed by way of Tris or hydroxylamine extraction, *i.e.* upon removal of two of the three Mn atoms normally present per reaction center. Between the limits of 1 Mn per trap and 3 Mn per trap $O_2$ evolution capacity is linear with Mn content.

5. Mn removal affects the rates of $O_2$ evolution in strong light and in weak light (quantum yield) in the same fashion. This indicates that complete $O_2$ reaction centers are inactivated.

6. With Mn removal the capacity for donor (ascorbate or *p*-phenylenediamine) photooxidation in strong light declines in a very similar fashion as the $O_2$ evolving capacity. However, after removal of 2/3 of the Mn pool (by Tris or hydroxylamine extraction) 15–20% of the maximum rate remains (100–250 equiv/chlorophyll per h) as previously noticed by other workers. Secondly, the rate in weak light (quantum yield) of these photooxidations remains unaffected by Mn removal. This shows that for donor photooxidation the larger of the two Mn pools is not essential.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q, the quencher of System II fluorescence; $F_0$, the invariant low level of fluorescence observed at onset of illumination; $F_{max}$, maximum level of fluorescence; $DCIPH_2$, 2,6-dichlorophenolindophenol, reduced form; $DH_2$, a reductant capable of donating electrons to light-induced oxidants; A pool, the large electron acceptor pool in association with Q of System II; PMS, *N*-methylphenazonium ion (phenazine methosulfate).

7. Complete removal of Mn (< 1 Mn/4000 chlorophylls) led to 90–95% loss of donor photooxidation in strong light.

8. Removal of 2/3 of the Mn left a low fluorescence yield (variable fraction = 0) which could be fully restored by adding DCMU. After complete removal of Mn (< 1 Mn/4000 chlorophylls) DCMU enhanced the yield of the variable fluorescence to only 1/2 the maximum level but the full maximum could be restored by chemical reduction. This indicates that fluorescence quencher of System II, Q, is not affected by Mn removal.

9. Of the three Mn associated with each trapping center, one is linked more closely to the center than the other two. While all three are essential for $O_2$ evolution, artificial donors can enter with various rate constants at several loci on the oxidant side of System II.

## INTRODUCTION

Although the site of function of Mn in photosynthesis generally has been assigned to the $O_2$-yielding reactions of Photosystem II, this assignment is not undisputed[1,2]. Flash yields of $O_2$ (ref. 3) and luminescence[4] are markedly diminished as a consequence of Mn deficiency. While specifically indicating a function of Mn within System II, such results do not distinguish between sites on the oxidant or the reductant side of this system.

ANDERSON AND THORNE[1] located the functional site of Mn on the reducing side of System II, because deficiency by growth induces a high fluorescence yield. Alternate explanations, however, are available[2,3].

We have varied the chloroplast Mn pool by growth deficiency, extractions[10,11], and with ageing at 35° and have studied the effect upon $O_2$ evolution activity, System II-sensitized photooxidations[5–10,41] and fluorescence of spinach chloroplasts. Preliminary accounts of these results have been given previously[12].

## METHODS

### *Rate measurements of $O_2$ evolution and donor photooxidation*

Rate measurements of $O_2$ exchange were made polarographically[3,13]. Light from a 750-W projection lamp was filtered through 12 inches of water and an OG-3 filter (Schott), then focussed with suitable condensing lenses onto the polarographic vessel of 1.0-ml capacity. Light intensity was varied with neutral density filters. Reaction mixtures for the assay of spinach chloroplast $O_2$ evolution contained in $\mu$moles: Tris–HCl (pH 7.5), 50; methyl viologen, 0.1; KCN, 0.1; methylamine, 30; and chloroplasts equivalent to 10 or 20 $\mu$g chlorophyll in a total volume of 1.13 ml. For assay of donor photooxidation the following additions were made to the reaction mixture: ascorbate (5.5 $\mu$moles); or ascorbate (0.37 $\mu$mole) and recrystallized *p*-phenylenediamine (0.037 $\mu$mole)[10]. The photooxidation of ascorbate or *p*-phenylenediamine, with fresh, Mn-deficient, extracted or aged chloroplasts, showed no inductions or "over-shoots" as has been observed for photooxidation of 2,3-diketogulonate[9]. Neither were the rates enhanced by addition of $Mn^{2+}$ (2–100 $\mu$M). Thus the photo-

oxidations of these compounds differ markedly from the photooxidation of 2,3-diketogulonate[9].

When $Fe(CN)_6^{3-}$ was used as electron acceptor, KCN and viologen were omitted and $Fe(CN)_6^{3-}$ (1.3 $\mu$moles) was included. Scenedesmus chloroplast particles were assayed similarly except for the use of 20 $\mu$moles phosphate buffer, pH 6.8. Quinone-Hill activity of Anacystis cells was determined as described previously[13].

For measurements of $O_2$ exchange stoichiometries of the viologen–water and viologen–donor reactions, KCN was omitted from the reaction mixture. With suitable amplification of the polarograph, about full-scale recorder deflection was obtained in saturating light within 30–45 sec. The $H_2O_2$ formed was decomposed 20–30 sec after removing the light by addition of catalase (5 $\mu$l of Sigma product, 15000 units/ml), through a small hole in the vessel cap[41].

*Measurement of the flash yields of $O_2$*

$O_2$ flash yields were measured in the polarographic vessel described above. The flash lamp (Amglo U-35-O, yielding 1-J flashes, half-duration 2 $\mu$sec) was mounted above the vessel. A monochromatic beam (5 nm half-band width) entered the front of the vessel. Intensity of the flash or background light was varied with neutral density filters. For yield measurements, the flash lamp was fired repetitively, maximum yields were obtained with darktimes $\geqslant$ 100 msec ($t_d$) between the saturating flashes.

For these measurements chloroplasts (20 $\mu$g/ml) were suspended in the regular viologen or $Fe(CN)_6^{3-}$ assay medium, except for omission of methylamine. At the concentration (30 mM) of methylamine routinely used in $v_{max}$ measurements, flash yields and Hill-reaction quantum yields were decreased by 25–35%. This effect of methylamine may possibly be related to an inhibition of System II as described previously by VERNON AND ZAUGG[31] and IZAWA *et al.*[32]. The integrated rates of $O_2$ evolution in flashing light under these conditions ($t_d$ approx. 100 msec) were linear only for about 20 sec and corresponded to 6–10% of $v_{max}$ rates.

*Fluorescence measurements*

Fluorescence measurements were made at room temperature essentially as described by MALKIN AND KOK[14]. Chloroplasts were diluted to 3 $\mu$g/ml in 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4) and either kept in darkness for 10 min or illuminated for 3 min with 740-nm light to oxidize pools between the photoacts. The time-course of fluorescence ($\lambda > 700$ nm) upon admission of a blue exciting light was recorded either with a Clevite 250 recorder or a storage oscilloscope.

*Source of $^{54}Mn$-labeled chloroplasts and Mn-deficient spinach*

Spinach (soil grown) was obtained from the greenhouse except for plants made Mn-deficient and $^{54}Mn$-labeled. The latter plants were cultured in nutrient solution in the following composition: $Ca(NO_3)_2$, 5 mM; $KH_2PO_4$, 0.5 mM; $MgSO_4$, 2 mM; $K_2SO_4$, 2.5 mM; KCl, 0.02 mM; $A_5$ solution, prepared with Specpure chemicals, 1 ml/l; and Specpure $FeSO_4$–EDTA, 9 $\mu$M. Precautions to exclude Mn have been described[3]. $^{54}Mn$-labeled plants were obtained by culturing seedlings in covered blackened-plastic pans containing 14 l of the above solution supplemented with $MnCl_2$ (1.8 $\mu$M) and 200 $\mu$C carrier-free $^{54}MnCl_2$ (New England Nuclear). Spinach

seed (Hybrid No. 7) was germinated in vermiculite, the seedlings washed free of vermiculite, then transferred to the aerated solution and maintained on a 12-h light regime in the greenhouse.

Leaves were harvested 4–6 weeks from date of sowing except for Mn-deficient plants which were harvested after 4 weeks. At harvest-time the $^{54}Mn$-sufficient plants showed no signs of Mn deficiency as judged by appearance and Mn content of chloroplasts of comparable Hill activity of plants cultured in soil or in nutrient solution with a 3-fold higher concentration of $Mn^{2+}$. Chloroplast obtained from such plants thus contained Mn of high specific activity (30–45 counts/min per $\mu$g chlorophyll). Mn-deficient spinach was obtained by culturing plants in the same medium with the exception that $MnCl_2$ was added to yield concentrations of 9 or 70 nM.

*Preparation, extraction and ageing of chloroplasts*

Chloroplasts from spinach were prepared as described by SCHWARTZ[15], and Scenedesmus particles were isolated as described by KOK AND DATKO[16]. Chlorophyll determinations were made as described by ARNON[17]. In a few instances chloroplasts, resuspended in 0.4 M sucrose–0.05 M Tris–maleate–0.01 M NaCl (pH 6.8), were stored under liquid $N_2$.

Chloroplast extractions were routinely made by suspending chloroplasts (5 mg chlorophyll) in 200 ml extractant[11]. When buffered hydroxylamine was used as extractant, the concentration routinely used was 1 mM. Higher concentrations resulted in more rapid losses, the loss with time saturating at 5 mM. At times designated (RESULTS) chloroplasts were recovered by centrifugation (1100 $\times$ *g* for 10 min) and resuspended in 200 ml 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl, pH 7.4. After centrifugation at 300 $\times$ *g* for 1 min the chloroplasts were recovered (1100 $\times$ *g* for 10 min, pellet) and resuspended in 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4). Extraction with Tris by this procedure or that of YAMASHITA AND BUTLER[10] yielded essentially similar results. For short exposures in extractants, chloroplasts were recovered by high-speed centrifugation in a Model RC-2B Sorvall centrifuge set for top speed. 30 sec after starting, the power was cut and hand-braking applied. Subsequent washing and recovery of chloroplasts were made as described above. Preliminary experiments with hydroxylamine as an extractant determined that this washing procedure was sufficient to remove the hydroxylamine from the chloroplasts.

Aged (35°) chloroplasts were obtained by blowing chloroplasts (in 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4)) into a medium containing 25 mM Tris chloride–5 mM $MgSO_4$–10 mM $(NH_4)_2SO_4$ (pH 8.3) preequilibrated at 35°, to yield a concentration of 200 $\mu$g/ml. At specified times 1.3-ml aliquots were removed and blown into 8 ml of 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4)–1 mM EDTA at 4°. Chloroplasts were recovered by centrifugation at 10000 $\times$ *g* for 5 min, washed with 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4), recovered by centrifugation, and resuspended in 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4).

*Manganese analyses*

Manganese analyses were made by atomic absorption (Perkin Elmer, Model 303, acetylene flame) equipped with DCR 1 concentration readout.

Samples (chloroplasts and Mn standards) for analyses were dried at 100° in

porcelain crucibles, charred at 170°, then dry-ashed at 530° for 15 h. The ash was dissolved in a few drops of conc. HCl. The resulting solution and rinses were transferred to beakers and adjusted to pH 5. 1 ml 2% ammonium pyrrolidino dithiocarbamate was added and extraction was made using water-saturated methyl isobutyl ketone[18]. Volumes were adjusted to 10 ml before analyses. Linearity of instrument read-out *vs.* Mn concentration was obtained up to at least 1 $\mu$g Mn/ml with 85–90% overall recovery of Mn as determined by radioactivity. No corrections were necessary for possible Mn contaminants in the chloroplast preparation medium. Radioactivity was measured (5% efficiency) with a NaI scintillation well counter calibrated for the $^{54}$Mn $\gamma$-emission. Such measurements therefore yielded the specific radioactivity of the chloroplast Mn.

*Mass spectrometer measurements of $O_2$ exchange*

A mass spectrometer inlet system similar to that described by HOCH AND KOK[42] was employed. The semi-permeable membrane (MEM-213) used was a product of General Electric Co. (Schenectady, N.Y.). In these experiments the water was enriched with $^{18}$O. The reaction mixtures for Hill activity and electron donor photooxidation were the same as used in polarographic measurements. Chloroplasts (10 $\mu$g/ml) were allowed to settle on the membrane before onset of measurements of light-induced rates of $O_2$ production (mass 34) or of $O_2$ uptake (mass 32).

## RESULTS

*Stoichiometry of $O_2$ exchange; abolishment of $O_2$ evolution by ascorbate*

In most of the experiments reported here viologen[19] has been used as the electron acceptor. Interpretation of the results required measurements of the stoichiometry of $O_2$ exchange for the two assay systems exemplified in Table I. We observed for the

TABLE I

STOICHIOMETRY OF $O_2$ EXCHANGE BY UNEXTRACTED AND EXTRACTED SPINACH CHLOROPLASTS

Cofactor requirements and stoichiometry of $O_2$ exchange for unextracted and Tris-extracted spinach chloroplasts. The routine extraction procedure (METHODS) was used. Extractants used were 0.8 M Tris chloride (pH 8.0) and 0.8 M KCl–0.05 M Tris, pH 8.0. For other details see METHODS.

| *Line* | *Type of chloroplasts* | *Electron donor* | *Electron acceptor* | *Rate ($\mu$moles $O_2$/mg chlorophyll per h)* | | $H_2O_2/O_2$ |
|---|---|---|---|---|---|---|
| | | | | *1.8% I* | *100% I* | |
| 1 | Unextracted | Water | Viologen | 5.6 | 203 | 1.96 |
| | | | None | 4.3 | 55.6 | — |
| 2 | Unextracted | Ascorbate | Viologen | — | 203 | 0.96 |
| 3 | Extracted (Tris) | Water | Viologen | 0.0 | 27.2 | 2.00 |
| | | | None | — | 14.1 | — |
| 4 | Extracted (Tris) | Water | $Fe(CN)_6^{3-}$ | — | 30.0 | — |
| 5 | Extracted (Tris) | Ascorbate | Viologen | 4.5 | 53.5 | 1.02 |
| | | | None | 1.97 | 19.9 | — |
| 6 | Extracted (KCl–Tris) | Water | Viologen | — | 18.2 | — |
| | | | None | — | 12.1 | — |
| 7 | Extracted (KCl–Tris) | Ascorbate | Viologen | 4.7 | 36.4 | 0.88 |
| | | | None | 3.0 | 22.5 | — |

Hill reaction the expected formation of 2 $H_2O_2$ per net $O_2$ consumed, both with unextracted chloroplasts as well as the extracted ones which yielded only 13% of the original Hill activity. The data of Table I (Line 4 *vs.* Line 3) also show that the extent of loss of Hill activity with chloroplast extraction was independent of the Hill reagent ($Fe(CN)_6^{3-}$ *vs.* viologen).

However, addition of 5 mM ascorbate, *p*-phenylenediamine[10] or 2,6-dichlorophenolindophenol, reduced form ($DCIPH_2$), to either extracted or unextracted chloroplasts resulted in a stoichiometry ratio ($H_2O_2/O_2$) of 0.9–1.0. Under the conditions employed, namely high light, the ascorbate and *p*-phenylenediamine photooxidations were inhibited about 90% by 1–2 $\mu$M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). These results therefore suggested that in high enough concentration ascorbate and *p*-phenylenediamine completely replace water as a source of electrons for System II. This interpretation could be confirmed by mass spectrometric observation of the $^{18}O_2$ evolution from $^{18}O$-enriched water in presence or absence of 5 mM ascorbate. In the absence of ascorbate, $^{18}O_2$ production and $^{16}O_2$ uptake were observed as expected for the viologen-Hill reaction[19]. In the presence of 5 mM ascorbate no $^{18}O_2$ production from $H_2{}^{18}O$ was observed only $^{16}O_2$ uptake. These results were obtained both at low and high light intensity. This conclusively showed that ascorbate rapidly reduces the System II oxidant which normally results in water photooxidation. Despite the fact that this result was suggested years ago by HABERMANN AND BROWN[20] and HABERMANN AND VERNON[21] and more recently by TREBST *et al.*[7] and BÖHME AND TREBST[8], it came as a surprise to us, particularly since the stoichiometries of Table I were observed with chloroplasts yielding 800–1200 equiv/mg per h. It thus is not surprising that the same stoichiometry (1 $H_2O_2/O_2$) was obtained in weak light (1/5 saturating intensity) for all donor systems (ascorbate, *p*-phenylenediamine, $DCIPH_2$). Thus we assumed this stoichiometry in all our measurements of $O_2$ uptake in the photooxidation in the presence of viologen and divided the observed uptake rates by 2 to obtain the correct electron equivalency.

*Number of atoms of Mn per System II trap*

In previous reports[3,24] we have expressed the amount of chloroplast Mn in relation to total chlorophyll on an arbitrary basis (Mn/50 or Mn/400 chlorophylls). To relate the concentration of chloroplast Mn to the actual concentration of $O_2$-yielding centers, we have measured flash yields of $O_2$ as well as the Mn content of isolated spinach chloroplasts. $Fe(CN)_6^{3-}$ and viologen were used as electron acceptors and yielded identical results (Fig. 1).

Spacing between flashes was 0.1 sec; doubling this dark interval had no effect upon the yields. Weak long-wave background light also yielded no improvement of the yields. With the onset of the flashing light the $O_2$ evolution rate was essentially linear for approx. 20-sec interval. Over such intervals we obtained with active winter chloroplasts, flash yields of $O_2$ ranging between 1 $O_2$/1400 and 1 $O_2$/1600 total chlorophyll. With a given chloroplast batch maximum yields decreased about 25–35% during 4–5 h after their preparation. The above yields could not be obtained with chloroplasts from summer spinach. However, the Mn content of such chloroplasts was decreased to 2–2.5 Mn/200 total chlorophylls (see later section). We believe this suboptimal concentration of Mn contributes to the low flash yields and high quantum requirement for $O_2$ evolution which we consistently observe with summer

chloroplasts. For a quantum requirement of 8 $h\nu/O_2$ these values (1 $O_2$/1400–1600 total chlorophylls) yield a concentration of $O_2$-evolving centers of approx. 1/400 total chlorophylls. Assuming equal distribution of the chlorophyll between the two photosystems, each System II trap has 200 chlorophylls$_{II}$. The Mn concentration of such chloroplasts was 2.5–3 Mn/200 total chlorophylls, and if we assume that all of this Mn is associated with System II (ref. 25), we conclude that 2.5–3 Mn are associated with each System II trapping center. Thus in the following sections the Mn concentration of chloroplasts is expressed per 200 total chlorophylls, since this number of chlorophylls approximates most closely the System II unit size.

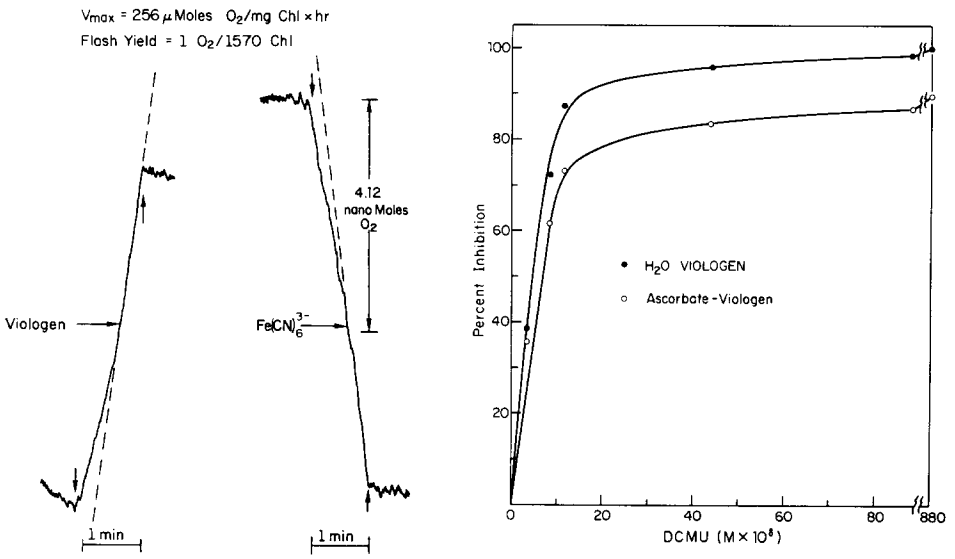



Fig. 1. Flash yields of $O_2$ of spinach chloroplasts. For details see METHODS.

Fig. 2. Sensitivities to DCMU at saturating light intensity of water (unextracted chloroplasts) and ascorbate photooxidation by Tris-extracted chloroplasts. Unpoisoned photooxidation rates of water (unextracted) and ascorbate (Tris extracted) were 290 and 71 μmoles $O_2$/mg chlorophyll per h, respectively.

*Effect of System II inhibitors upon $DH_2$ photooxidations; effect of light intensity*

DCMU and *o*-phenanthroline block the oxidation of the primary reductant $Q^-$ (ref. 22) of System II by the larger pool of oxidants located between the two photoacts. Accordingly, electron flow from water and donors ($DH_2$) which are specific for System II should be inhibited equally by these inhibitors at all intensities.

In Fig. 2 we compared the sensitivity to DCMU of $O_2$ evolution by unextracted chloroplasts and of ascorbate photooxidation of Tris-extracted chloroplasts[10,11]. A similar comparison is made in Fig. 3 except *p*-phenylenediamine[10] was used as the donor and *o*-phenanthroline as the inhibitor. Results of such comparisons show notable differences between the inhibitions of $O_2$ evolution and $DH_2$ photooxidation by extracted chloroplasts: (1) water photooxidation proved slightly more sensitive than $DH_2$ photooxidation and significant rates of $DH_2$ photooxidation remained at inhibitor concentrations which virtually abolished $O_2$ evolution. (2) $DH_2$ photo-

oxidation proved somewhat less sensitive at low than at high light while the reverse was true for water photooxidation (Fig. 3, Curve 4 *vs.* Curve 3; Curve 1 *vs.* Curve 2).

The effect of light intensity upon the degree of inhibition by DCMU (2 $\mu$M) of *p*-phenylenediamine photooxidation[10] by Tris-extracted chloroplasts is shown in Fig. 4, Curve 1. In weak 650-nm light rates of *p*-phenylenediamine or ascorbate photooxidation by extracted chloroplasts showed a near linear dependency upon the rate of quantum absorption up to 30 neinsteins absorbed per min per 6.5 nmoles chlorophyll per ml. With fresh chloroplasts water photooxidation was abolished under these conditions by 2 $\mu$M DCMU. We observed, however, that the rates of $DH_2$

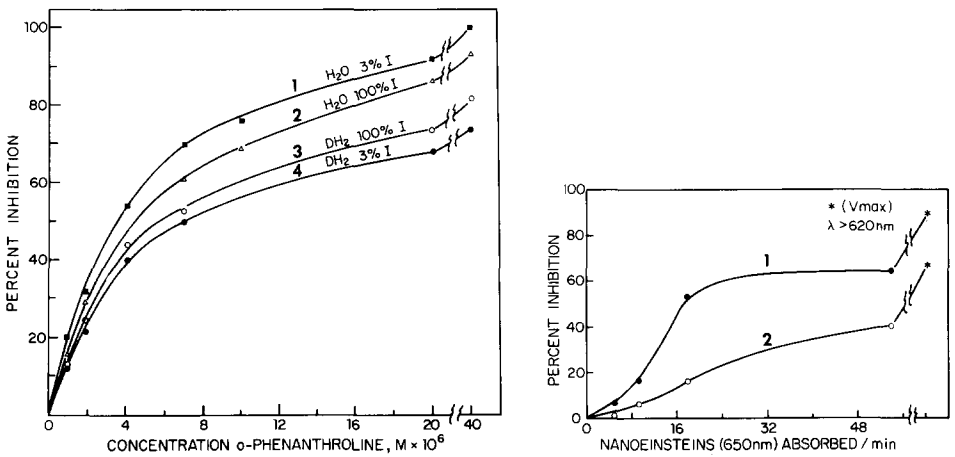



Fig. 3. Sensitivities to *o*-phenanthroline of water (Curves 1 and 2) and *p*-phenylenediamine photooxidation (Curves 3 and 4) by Tris-extracted chloroplasts at low and at saturating light intensities. Hill reaction: Curves 1 and 2, at 3% and saturating light intensity, respectively; *p*-phenylenediamine photooxidation: Curves 4 and 3, at 3% and saturating light intensity, respectively. Absolute rates of Hill activity of unextracted chloroplasts were 246 and 42.5 $\mu$moles $O_2$/mg chlorophyll per h at the two intensities. *p*-Phenylenediamine photooxidation rates (extracted chloroplasts) were 62.6 and 28.4 $\mu$moles $O_2$/mg chlorophyll per h at the two intensities.

Fig. 4. Effect of rate of quantum absorption upon DCMU inhibition of ascorbate photooxidation by Tris-extracted chloroplasts. Concentration of DCMU was 2.0 $\mu$M; chlorophyll concentration was 6.5 $\mu$g/ml in standard assay medium (METHODS). Curve 1 and Curve 2 were obtained with Tris-extracted chloroplasts from Mn-sufficient and Mn-deficient spinach (see Table III, Expt. 1), respectively. Absorption measurements were made in a split-beam integrating sphere. Rates of $O_2$ uptake (corrected for $H_2O_2$ and dark rates) in absence of DCMU at 18.2 neinsteins absorbed/min were 1.64 and 1.06 nmoles/min for Curve 1 and 2, respectively.

photooxidation in the presence of 2 $\mu$M DCMU were not greatly decreased at rates of quantum absorptions less than 1.5 $h\nu \cdot$chlorophyll$^{-1} \cdot$min$^{-1}$. With increased rates of quantum absorption of weak 650-nm light the extent of inhibition increased and eventually reached a maximum at 650 nm 18.2 neinsteins absorbed per 6.5 nmoles chlorophyll per min (Fig. 4). The unpoisoned rate of $O_2$ uptake at this rate of quantum absorption corresponded to 15 $\mu$moles $O_2$/mg chlorophyll per h. This maximum level of inhibition remained constant up to 54 neinsteins absorbed per min, then attained 90% in strong saturating red light (> 620 nm). The results of Curve 1, Fig. 4, can be explained if one assumes: (1) a lack of specificity of *p*-phenylenediamine (or ascorbate) for the oxidants of Systems II and I; (2) a quantum distribution of

60/40 between System II and System I (ref. 23); and (3) an early light saturation of $DH_2$ entry into System I.

Curve 2 (Fig. 4) was obtained with Tris-extracted chloroplasts prepared from extremely Mn-deficient plants (see Expt. 1, Table III). The unpoisoned rate of $DH_2$ photooxidation (1.06 nmoles $O_2$/min per 6.5 nmoles chlorophyll) by such chloroplasts was 65% of the rate obtained with chloroplasts of Curve 1 at 18.2 neinsteins absorbed per min. In contrast to results of Curve 1, 2 $\mu$M DCMU did not decrease the observed rates except at the higher rates of quantum absorption. Such contrasting behavior may indicate a loss of System II units relative to System I units as a consequence of the long growthperiod under suboptimal Mn concentrations. Such a loss of System II units may have bearing upon the peculiar fluorescence characteristics of growth-induced Mn-insufficient chloroplasts[1] or whole cells[4].

We conclude from results of Figs. 2–4: (1) neither donor is specific for System II, they also enter System I at a smaller rate; (2) Mn-deficient long-term growth leads to loss of System II units.

*Specificity of extractants upon loss of water and $DH_2$ photooxidations; effect of Mn deficiency upon the photooxidations*

NAKOMOTO *et al.*[11] observed that extraction of chloroplasts with Tris caused a loss of $O_2$ evolution without affecting *N*-methylphenazonium ion (phenazine methosulfate) (PMS)-mediated phosphorylation. More recently the effect of this and other chloroplasts treatments upon System II activities has been investigated by GOOD *et al.*[26] and particularly by YAMASHITA AND HORIO[27] and YAMASHITA AND BUTLER[10].

In Table II we have compared several different extraction media for their

TABLE II

COMPARISON OF EXTRACTION MEDIA ON LOSS OF SYSTEM II ACTIVITY OF SPINACH CHLOROPLASTS

Specificity of extractant for loss of System II activities of spinach chloroplasts. The routine extraction procedure (METHODS) was used. Adjustments of pH were made at 23°. Rates of ascorbate photooxidation have been corrected for the small rate (see Table III) insensitive to 2 $\mu$M DCMU. Rates of Hill activity (water donor) were measured both with $Fe(CN)_6^{3-}$ and viologen. Rates with either acceptor were essentially equivalent. Rates of $DH_2$ (ascorbate or *p*-phenylenediamine, see METHODS) photooxidation were measured with viologen as acceptor. Control unextracted chloroplasts yielded rates of electron flow of 1000–1200 $\mu$equiv/mg chlorophyll per h. The results reported represent averages of 5–6 experiments.

| *Extraction medium* | *Activity remaining (%)* | | *Ratio* |
|---|---|---|---|
| | *Donor* *Water* | *$DH_2$* | |
| 0.8 M Tris–HCl, pH 8.0 (0.43 M $Cl^-$) | 12.4 | 32.3 | 2.5 |
| 0.8 M Tricine, pH 8.0 (0.43 M $Cl^-$) | 69.2 | 65.6 | 1 |
| 0.8 M glycine, pH 8.0 (0.43 M $Cl^-$) | 70.2 | 72.3 | 1 |
| 0.8 M glycylglycine, pH 8.0 (0.43 M $Cl^-$) | 79.1 | 73.6 | 0.9 |
| 0.8 M Tris–HCl, pH 7.8 (0.49 M $Cl^-$) | 5.0 | 13.0 | 2.5 |
| 0.8 M KCl + 50 mM Tricine, pH 7.8 | 29.7 | 33.2 | 1 |
| 0.8 M KCl + 50 mM phosphate, pH 7.8 | 29.6 | 35.0 | 1 |
| 0.8 M KCl + 50 mM glycine, pH 7.8 | 24.2 | 30.0 | 1.2 |
| 0.8 M KCl + 50 mM Tricine, pH 8.1 | 5.3 | 15.0 | 3 |
| 0.4 M sucrose–50 mM Tris–maleate–10 mM NaCl, pH 6.5 | 100 | 100 | 1 |
| 0.4 M sucrose–50 mM Tris–maleate–10 mM NaCl–1 mM hydroxylamine, pH 6.5 | 3 | 18 | 6 |

effectiveness in causing both loss of $O_2$ evolution and DCMU-sensitive $DH_2$ photooxidation. None of these treatments affected the DCMU-insensitive System I photooxidation of $DCIPH_2$. The rates of $DH_2$ photooxidation in Table II have been corrected for a small (approx. 10%) DCMU-(2 $\mu$M)-insensitive rate (see previous section) to obtain a meaningful comparison between the two activities. With increase of the ionic strength significant loss of both activities was observed with all buffers. Extraction with Tricine (50 mM, pH 8.1) in 0.8 M KCl or with 0.8 M Tris (pH 8.0) resulted in a substantial (95%) loss of $O_2$ evolution. Also the rates of $DH_2$ photooxidation were greatly decreased (87%) by these extractants, however, because of the greater sensitivity of the $O_2$ evolution process the resulting preparations showed a 3–6-fold greater rate of $DH_2$ than water photooxidation. With additional extraction, rates of $O_2$ evolution diminished still relatively more which resulted in even greater differences between the two rates.

A similar and actually the most distinct discrimination was observed with sucrose–Tris–maleate–NaCl (pH 6.5) containing 1 mM hydroxylamine. This inhibitor of System II (ref. 28) also caused a loss of the quinone-Hill reaction in whole (Ana-

TABLE III

EFFECT OF Mn DEFICIENCY ON RATES OF ELECTRON FLOW OF HILL ACTIVITY AND DONOR PHOTOOXIDATION

Effect of Mn deficiency on rates of $O_2$ exchange of Hill activity and $DH_2$ photooxidation. Assays were made as described in METHODS. The source of chloroplasts and the extraction procedures were as follows: Expt. 1, Mn deficiency was obtained by culturing plants in 9 nM $MnCl_2$; extraction of both Mn-sufficient and Mn-deficient chloroplasts was made at 4° with 10-min centrifugation at 1100 × *g* to recover chloroplasts. Expt. 2, Mn deficiency was obtained by culturing plants in 70 nM $MnCl_2$. Extraction (0.8 M Tris chloride, pH 8.0) was for about 11 min including a fast centrifugation (see METHODS) for chloroplast recovery. Concentration of DCMU where indicated was 2 $\mu$M.

| *Chloroplast preparation* | *Rate of electron flow ($\mu$equiv/mg chlorophyll per h) or (%)* | | | | | |
|---|---|---|---|---|---|---|
| | *$O_2$ evolution* | | *$DH_2$ photooxidation* | | *Corrected $DH_2$ photooxidation* | |
| *Expt. 1* (*p*-phenylenediamine donor) | | | | | | |
| Spinach + Mn | 1024 | (100) | 1068 | | 1026.0 | (100) |
| + Mn + DCMU | 0 | | 42.0 | | — | |
| − Mn | 263.2 | (25.7) | 300.4 | | 272.8 | (26.8) |
| − Mn + DCMU | 0 | | 26.7 | | — | |
| 23-min extracted spinach + Mn | 38.8 | (3.8) | 260 | | 232 | (22.6) |
| + Mn + DCMU | 0 | | 28 | | — | |
| − Mn | 18 | (1.8) | 110.0 | | 74.0 | (7.2) |
| − Mn + DCMU | 0 | | 36.0 | | — | |
| *Expt. 2* (ascorbate donor) | | | | | | |
| Spinach + Mn | 1240 | (100) | 1332 | | 1288 | (100) |
| + Mn + DCMU | 0 | | 45.2 | | — | |
| − Mn | 728 | (58.8) | 788 | | 757.6 | (58.9) |
| − Mn + DCMU | 0 | | 30.4 | | — | |
| 11-min extracted spinach + Mn | 424 | (34.2) | 326.8 | | 290 | (22.5) |
| + Mn + DCMU | 0 | | 36.8 | | — | |
| − Mn | 183.2 | (14.7) | 187.2 | | 164 | |
| − Mn + DCMU | 0 | | 23.2 | | — | |

cystis) cells. Despite repeated washes to remove the inhibitor, $O_2$ evolution was not restored unless the cells were preilluminated [31,24].

In all instances with the different extractants the emergence of a greater rate of System II-sensitized $DH_2$ than water photooxidation occurred only after about 80–90% of $O_2$ evolution had been abolished. Such obtained rates of $DH_2$ photooxidation were, however, only 15–20% of rates obtainable with unextracted chloroplasts.

It is not clear which of the many possible factors such as pH, ionic strength, *etc.* inactivate water and $DH_2$ photooxidation. Generally, the sensitivity of the two processes judged from rates in strong light, appears quite similar and preparations which show a substantially higher rate of donor photooxidation than $O_2$ evolution cannot be obtained without a severe loss of both. As a result such preparations retain only approx. 20% of the maximum possible (strong light and uncoupled) System II activity (approx. 100–250 equiv/chlorophyll per h *vs.* 800–1200 equiv/chlorophyll per h).

In Table III we summarize the effect of Mn deficiencies obtained through growth upon water and $DH_2$ photooxidation before and after Tris extraction. In Expts. 1 and 2, $O_2$ evolution was decreased to about 74 and 41%, respectively, as a consequence of the growth deficiency. After correction for the DCMU-insensitive (System I) component of the $DH_2$ photooxidation, the data reveal closely identical decreases of the rates of $DH_2$ photooxidation. Moreover, the absolute uncoupled rates of water and $DH_2$ photooxidation were equivalent in all instances. These results show that chloroplast Mn is required not only for $O_2$ evolution but also for $DH_2$ photooxidation by System II.

Tris extraction of both Mn-deficient and Mn-sufficient chloroplasts resulted in pronounced decreases of water and $DH_2$ photooxidation, the extent depending upon the duration of extraction. Relatively short (Table III, Expt. 2) exposures (11 min) of chloroplasts from Mn-sufficient and moderately Mn-deficient plants resulted in identical losses of water and $DH_2$ photooxidation in both cases. Irrespective of the initial Mn content, long (23-min) extractions (Table III, Expt. 1) yielded chloroplasts which were essentially inactive in $O_2$ evolution but retained a (4–6 times) higher rate of DCMU-sensitive $DH_2$ photooxidation. Though these results are consistent with those of Table II, we note a deleterious effect of Mn deprivation during growth upon the DCMU-sensitive $DH_2$ photooxidation which is expressed after extraction.

*Effect of extraction upon Mn content, $O_2$ evolution and fluorescence of chloroplasts: heterogeneity of Mn pool*

The extractants of Table II which cause a loss of $O_2$ evolution and a marked decrease in $DH_2$ photooxidations also cause a loss of Mn. Fig. 5 shows the correlation between $v_{max}$ of $O_2$ evolution and Mn/200 total chlorophylls. These data were obtained by repetitive brief extractions with small amounts of extractant to minimize the loss occurring with each extractant. The values given represent the extremes of variation we observed within eleven such determinations. Curves 2 and 3 were obtained with Tris extractions of freshly isolated spinach chloroplasts; Curve 1 by extraction (KCl–Tris or Tris) of spinach and Scenedesmus particles that had been stored at −198° in sucrose–Tris–maleate–NaCl, pH 6.75. With stored or summer chloroplasts, but not with winter chloroplasts, loss of water and $DH_2$ photooxidation

and Mn were observed even with 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4), a commonly used chloroplast preparation medium.

Such data of Fig. 5 show that a 90% loss of $O_2$ evolution ($v$ or $v_{max}$) is correlated with a loss of 2/3 of the Mn (from 2.5–3 Mn/200 total chlorophylls to 1–1.25 Mn/200 total chlorophylls, the latter value obtained from the clear intercepts on the abscissa). Evaluation of the results from eleven such experiments with fresh chloroplasts showed a linear relation between this larger pool of Mn and $O_2$ evolution.

With the loss of the larger pool of Mn a residual rate of water and $DH_2$ photooxidation remained. The residual water photooxidation ($Fe(CN)_6^{3-}$ or viologen) showed a high quantum requirement (at 650 nm $\geqslant$ 100 $h\nu/O_2$) and only 10% of the original $v_{max}$ rates. In contrast $DH_2$ photooxidations by chloroplasts containing 1–1.25 Mn/200 total chlorophylls showed a low quantum requirement in 650-nm light (unpublished results) which was partially sensitive to DCMU (Fig. 4); however, the $v_{max}$ of $DH_2$ photooxidation was only 1/3–1/5 of that of the unextracted chloroplasts.

The small residual pool of Mn, as well as the residual $v_{max}$ rates of $DH_2$ and water photooxidations, proved difficult to abolish with further repetitive and prolonged (60-min) extractions. However, with many additional exhaustive extractions the small residual pool of Mn was decreased further as well as the residual $v_{max}$ rates of water and $DH_2$ photooxidations. The small rate of water photooxidation declined

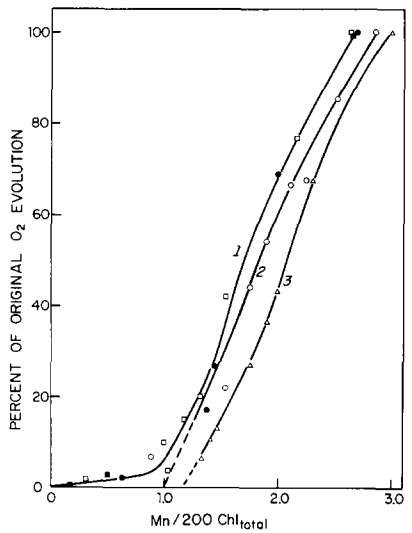



Fig. 5. Relation of $v_{max}$ Hill activity to chloroplast Mn content. Curves 2 ($v_{max}$ of 250 $\mu$moles $O_2$/mg chlorophyll per h) and 3 ($v_{max}$ of 310 $\mu$moles $O_2$/mg chlorophyll per h): data obtained by Tris extraction of two different preparations of freshly isolated spinach chloroplasts; Curve 1: data obtained from extraction of spinach chloroplasts (squares, $v_{max}$ of 90 $\mu$moles $O_2$/mg chlorophyll per h) and Scenedesmus particles (circles, $v_{max}$ of 64 $\mu$moles $O_2$/mg chlorophyll per h) which had been stored about 3 months at $-198°$. For Curve 1 the extractants for spinach chloroplasts were 0.8 M Tris chloride (pH 8.0) (closed squares) and 0.8 M KCl–0.05 M Tris chloride (pH 8.0) (open squares); for Scenedesmus particles the extractants were 0.8 M Tris chloride (pH 8.0) (closed circles) and 0.8 M KCl–0.05 M Tris chloride (pH 8.0) (open circles). Results were obtained by successive 10-min extractions (200 $\mu$g chlorophyll/ml extractant) followed by 1100 $\times$ $g$ centrifugation for chloroplast recovery. For other details see METHODS.

more sharply than the DCMU-sensitive $DH_2$ photooxidation thus resulting in a 5–7 times greater $v_{max}$ rate of $DH_2$ than water photooxidation. Nevertheless the quantum yield of DCMU-sensitive $DH_2$ photooxidation remained high. The results indicate that $O_2$-yielding reactions were far more susceptible to inactivation than System II trapping centers.

To further examine the Mn requirement of this extraction-resistant, residual $DH_2$ photooxidation, chloroplasts were extracted first with Tris for 10 min to decrease water photooxidation by about 90% (from 206 to 18.6 μmoles $O_2$/mg chlorophyll per h); $DH_2$ photooxidation by 73% (from 207 to 62 μmoles $O_2$/mg chlorophyll per h), and Mn from 3.0 to 1.7 Mn/200 total chlorophylls. These partially extracted chloroplasts were then subjected to a mild 35° treatment (see later section) which resulted in the decline of the residual $DH_2$ photooxidation rate (in strong light) as shown by Curve 1 (Fig. 6); the loss of chloroplast Mn followed Curve 2 (Fig. 6). Curve 1 (Fig. 6, inset) shows the decrease of DCMU-sensitive $DH_2$ photooxidation of extracted chloroplasts (expressed as percent of $DH_2$ photooxidation activity of extracted chloroplasts) *versus* the remaining chlorophyll Mn; Curves 2 and 2′ represent $DH_2$ and water photooxidation, respectively, of extracted chloroplasts expressed as percent activity of unextracted chloroplasts. These results indicate that additional loss of Mn by mild 35° treatment results in further decline of the residual $DH_2$ (ascorbate) photooxidation, eventually obtaining a value of only 4% (1 Mn/4000 total chlorophylls) of the original unextracted chloroplasts. Such results suggested that the

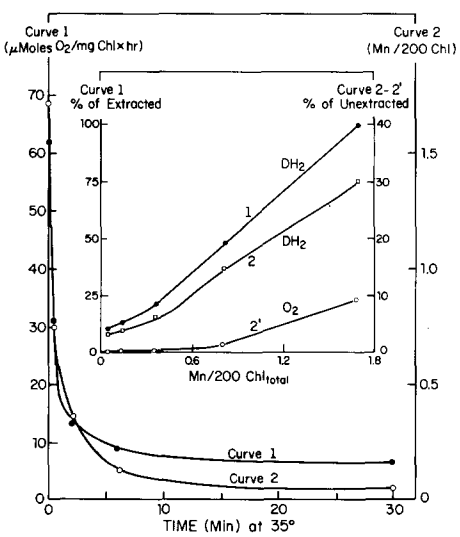
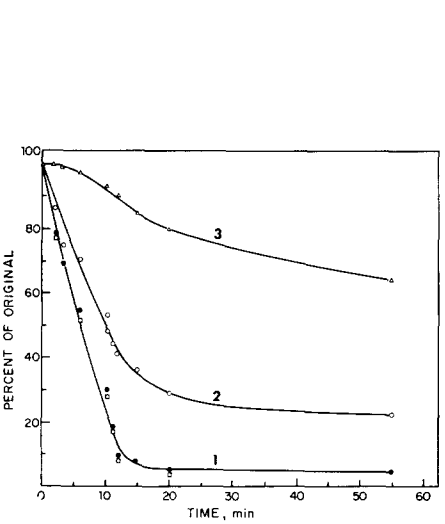



Fig. 6. Effect of 35° ageing upon residual rate of ascorbate photooxidation and residual chloroplast Mn following Tris extraction of spinach chloroplasts. Chloroplasts were extracted with Tris for 10 min, washed with STN buffer (0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl, pH 7.4) and then resuspended in STN. Hill activity (or ascorbate photooxidation) rate of original chloroplasts was 206 μmoles $O_2$/mg chlorophyll per h. Original chloroplast Mn content was 3 Mn/200 chlorophylls. Following Tris extraction Hill activity was 18.6 μmoles $O_2$/mg chlorophylls per h and chloroplast Mn was 1.7 Mn/200 chlorophylls. Curve 1 (closed circles), ascorbate photooxidation; Curve 2 (open circles), chloroplast Mn content. Inset: Curve 1, $DH_2$ photooxidation, expressed as percent of rate with extracted chloroplasts; Curve 2, $DH_2$ photooxidation expressed as percent of rate with unextracted chloroplasts; Curve 2′, water photooxidation expressed as percent of rate with unextracted chloroplasts.

smaller pool of Mn (approx. 1/3 of total Mn) was functional. Other means were sought to clarify this point.

To more clearly define the locus or loci affected by the extractions we studied the time-course of extraction (Tris and hydroxylamine) upon chloroplast activities and Mn content. The summer spinach chloroplasts (var. America) used for extraction with hydroxylamine had relatively low rates of $O_2$ evolution (106 $\mu$moles $O_2$/mg chlorophyll per h) and a somewhat decreased chloroplast Mn content. Nevertheless, the results obtained with extraction of winter (Fig. 7, Tris) or summer (Fig. 8, hydroxylamine) chloroplasts yielded essentially similar results.

As noted earlier (Tables II and III), the rates of $DH_2$ and water photooxidation remained equivalent until the extractions resulted in 80–90% loss of water photo-



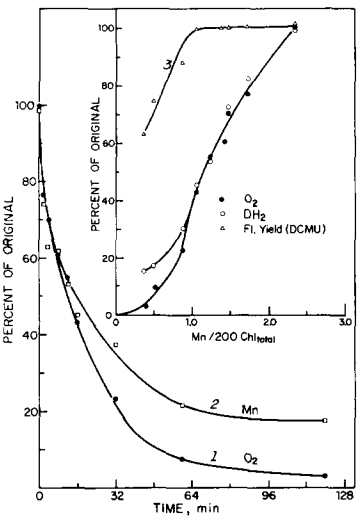




Fig. 7. Time-course of Tris extraction of spinach chloroplasts; effect upon Hill activity, variable fluorescence, fluorescence yield and chloroplast Mn content. Curve 1: Hill activity (closed circles) or variable fluorescence (open squares); Curve 2: chloroplast Mn; Curve 3: yield of variable fluorescence in presence of DCMU. For these experiments chloroplasts were suspended in 0.8 M Tris (pH 8.0) to yield a concentration of 50 $\mu$g/ml. The time of exposure to Tris is indicated on the abscissa. Rapid centrifugation (METHODS) was used to recover chloroplasts and the times indicated include time of centrifugation. Live fluorescence (METHODS) was measured in absence of acceptor at 23°. Original chloroplasts, containing 2.8 Mn/200 total chlorophylls, yielded a rate of water photooxidation of 256 $\mu$moles $O_2$/mg chlorophyll per h.

Fig. 8. Time-course of hydroxylamine extraction of spinach chloroplasts; effect upon water and $DH_2$ photooxidation, fluorescence yield and chloroplast Mn content. Curve 1: $O_2$ evolution; Curve 2: chloroplast Mn; Curve 3: yield of variable fluorescence in presence of DCMU. For these experiments chloroplasts from summer-greenhouse spinach (*Spinaceae oleracea*, var. *America*) were isolated in 0.4 M sucrose–0.05 M Tricine–0.01 M NaCl, pH 7.3. Chloroplasts (200 $\mu$g/3 ml) were incubated at 4° in 0.4 M sucrose–0.05 M Tris–maleate–0.01 M NaCl (pH 6.5) containing 1.0 mM hydroxylamine for the times designated on the abscissa. Then 5 ml 0.4 M sucrose–0.05 M Tris chloride–0.01 M NaCl (pH 7.4) were added and the chloroplast pellet recovered by rapid centrifugation (METHODS). The pellet was resuspended in 8 ml sucrose–Tricine–NaCl (pH 7.3) centrifuged at 300 × *g* for 1 min, then the chloroplasts were recovered at 1500 × *g* for 7 min. This washing procedure proved sufficient to remove the hydroxylamine. Chloroplasts were resuspended in sucrose–Tricine–NaCl for the various determinations (METHODS). $v_{max}$ of water and ascorbate photooxidation of original chloroplasts was 106 $\mu$moles $O_2$/mg chlorophyll per h. Incubation of chloroplasts for 60 min in the absence of hydroxylamine resulted in 8% loss of either photooxidative activity.

oxidation. From here on $O_2$ evolution declines more rapidly than DCMU-sensitive $DH_2$ photooxidation. This result is clearly shown in the inset of Fig. 8. These data were obtained by extraction with hydroxylamine; however, similar results were obtained also with Tris or KCl–Tris extractions (see Tables II and III). Divergence between water and $DH_2$ photooxidation occurred when the Mn content approached 0.8–1.0 Mn/200 total chlorophylls (extrapolated value to intercept Fig. 8, inset). Such results suggest more than one site of $DH_2$ entry into the oxidant side of System II.

Returning to the time-course of extraction upon chloroplast activities, we note from Curve 1 (Fig. 7) that the loss of $O_2$ evolution (closed circles) and the decrease of the steady-state level of variable fluorescence (corrected for $F_0$ (the invariant low level of fluorescence observed at onset of illumination) which did not change during course of the extraction) parallel each other. With increasing time of extraction the steady-state level of fluorescence decreased, and the rise-time of the variable fluorescence (beginning from a fluorescence-restored state) became increasingly slower. Since System I activity is not affected by these extractions, such behavior is consistent, in the series formulation of photosynthesis, with a perturbation only of the oxidant side of System II.

Comparison of Curve 1 ($O_2$ evolution) with Curve 2 (chloroplast Mn) of Tris- or hydroxylamine-extracted chloroplasts (Figs. 7 and 8, respectively) shows that after 12–15 min (0.8 M Tris) or 35 min (1 mM hydroxylamine) about 85–90% of $O_2$ evolution and 60–65% of chloroplast Mn was lost. This loss of Mn did not result in a substantial loss ($\leq$ 10–15%) of the DCMU fluorescence yield (Curve 3, Figs. 7 and 8), nor were the half-times or the initial slopes for the rise curves significantly altered. YAMASHITA AND BUTLER[10] similarly have observed that short-term Tris extraction does not greatly alter the DCMU fluorescence yield. We observed, however, that with increasing time of extraction with either Tris (Fig. 7) or hydroxylamine (Fig. 8) both the DCMU fluorescence yield and chloroplast Mn decreased further. This decrease of the fluorescence yield set in when the chloroplast Mn content was decreased to about 1/3 of the original amount. At this amount of Mn, divergence between residual initial rates of $DH_2$ and water photooxidations in strong light also occurred (Fig. 8, inset). With prolonged (120 min) Tris extraction, the DCMU fluorescence yield ultimately attained a 50% value (Mn $<$ 1 Mn/3000 total chlorophylls) and approached this same level with prolonged extraction with hydroxylamine. Addition of hydrosulfite to such chloroplasts returned the fluorescence to a level only 10% less than that obtained with hydrosulfite addition to unextracted chloroplasts. Moreover, addition of hydroxylamine (50 mM) to such chloroplasts yielded a DCMU fluorescence rise curve kinetically indistinguishable from that obtainable upon addition of hydroxylamine to unextracted chloroplasts. Since the $F_0$ level of unextracted chloroplasts was not changed regardless of duration of extraction, we interpret these results to indicate that Q, the quencher of fluorescence, was not removed by extraction, and that all of the Mn, although heterogeneous within System II, functioned on the oxidant side of System II.

*Entry sites of $DH_2$ into System II*

The site of inhibition of $O_2$ evolution by hydroxylamine (in contrast to that of DCMU or *o*-phenanthroline) presumably is somewhere on the oxidant side of

System II (refs. 29–31). Since this compound itself can serve as an electron donor to System II (refs. 29, 32), it was difficult in our assay system to ascertain accurately the extent of direct water oxidation inhibition by this compound. With ascorbate or *p*-phenylenediamine, instead of water as donor, hydroxylamine did not affect the $O_2$ exchange stoichiometry. Moreover with fresh chloroplasts the photooxidation of ascorbate or *p*-phenylenediamine was about 2-fold greater under the specified conditions than that obtainable with hydroxylamine (2–5 mM). This allows a reasonable estimate of the inhibitory effect of hydroxylamine upon ascorbate or *p*-phenylenediamine photooxidation. Fig. 9, Curve 1, shows that with fresh untreated chloroplasts, the inhibition reaches a plateau at 2–5 mM hydroxylamine. These concentrations decreased the initial rates of $DH_2$ photooxidation by about 60%, thus yielding a rate essentially identical to the initial rate of $O_2$ uptake seen with hydroxylamine only.

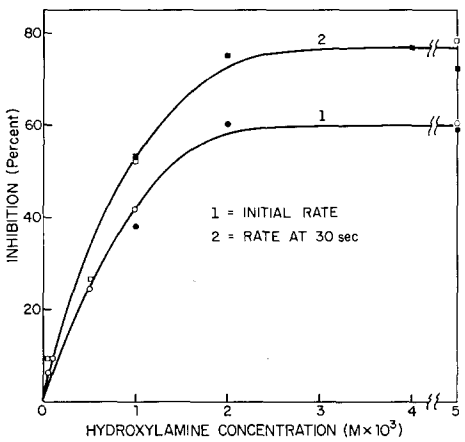



Fig. 9. Effect of hydroxylamine on $DH_2$ photooxidation by spinach chloroplasts. Open circles and squares: ascorbate as electron donor; closed circles and squares: *p*-phenylenediamine as electron donor. Hydroxylamine–HCl was adjusted to pH 7.5 with NaOH just prior to the experiments and kept at 4°. Addition of hydroxylamine was made through polarograph cap 60 sec before illumination. Chloroplast concentration was 10 μg/ml. Curve 1, percent inhibition of rate at onset of illumination; Curve 2, percent inhibition of rate after 30 sec of illumination.

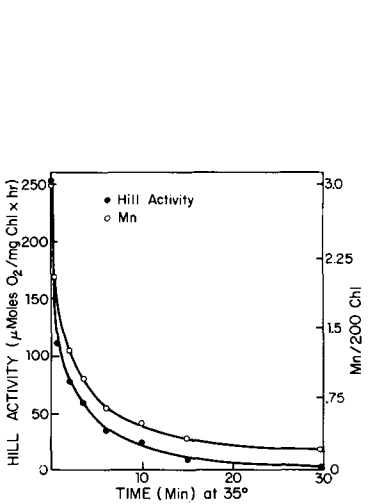
A more severe inhibition (Fig. 9, Curve 2) which is observed after 30 sec illumination will be shown later to result from secondary processes. We observed as others[30,31,37] that a System I (DCMU–DCIP–viologen) photooxidation was not inhibited by 10 mM hydroxylamine. Thus we conclude from the data of Fig. 9 that hydroxylamine poisons a site on the oxidant side of System II, decreasing not only $O_2$ evolution but also $DH_2$ photooxidation.

The data show that concentrations of hydroxylamine (3 mM) which were shown by KATOH AND SAN PIETRO[30] and JOLIOT[36] to inhibit $O_2$ evolution by 80–100% also inhibit System II-sensitized $DH_2$ photooxidations. These results confirm the observations of KATOH AND SAN PIETRO[30] and VERNON AND ZAUGG[31] but are in contrast to results of IZAWA *et al.*[32] who assumed concurrent water and $DH_2$ photooxidation. Without this latter assumption, the results of IZAWA *et al.*[32] also show hydroxylamine inhibition of $DH_2$ photooxidation.

Let us now return to Curve 1, Fig. 9, which showed a residual hydroxylamine-insensitive (40% of maximum) initial rate of $DH_2$ photooxidation. Our data cannot

distinguish whether ascorbate (or *p*-phenylenediamine) or hydroxylamine itself serves as a donor for this residual hydroxylamine-insensitive $DH_2$ photooxidation. In previous sections, however, we have noted effects of Tris and hydroxylamine extraction upon $DH_2$ photooxidations. Here we describe effects of such treatments as well as 35° ageing upon the hydroxylamine sensitivity of $DH_2$ photooxidation.

Ageing of chloroplasts at 35° resulted in a time-dependent loss of $O_2$ evolution and chloroplast Mn as described in Fig. 10. With chloroplasts aged at 35° for different durations, the sensitivity of $DH_2$ photooxidation to hydroxylamine was examined (Fig. 11). After 2 min at 35° the Mn content was decreased to about 40% (from 3.0 to 1.25 Mn/200 total chlorophylls, see Fig. 10), the $DH_2$ photooxidation was decreased to 48% and now was fully insensitive to hydroxylamine. Since the remaining photooxidation proved to be largely inhibited by DCMU (2 $\mu$M) (Fig. 11), a System II



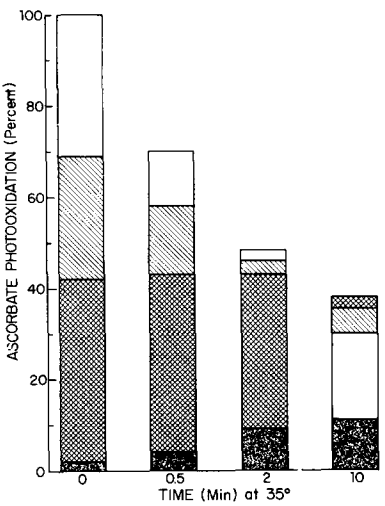




Fig. 10. Effect of 35° ageing of spinach chloroplasts upon Hill activity and chloroplast Mn. For details of methodology see METHODS.

Fig. 11. Effect of 35° ageing on the sensitivity of ascorbate photooxidation to hydroxylamine. Open areas, no hydroxylamine; hatched areas, 0.5 mM hydroxylamine; crosshatched areas, 2 mM hydroxylamine; shaded areas, with 2 $\mu$M DCMU in presence or absence of hydroxylamine. Specific photooxidation rates of ascorbate and hydroxylamine (2 mM) alone without ageing were 317 and 133 $\mu$moles $O_2$/mg chlorophyll per h, respectively. For other details see METHODS.

feed was indicated. We note also (Fig. 11) that after 10 min ageing at 35° the DCMU-sensitive $DH_2$ photooxidation rates actually were greater in the presence of hydroxylamine. This result suggests that hydroxylamine actually is a better donor for this chloroplast state than ascorbate itself. In data not shown, we similarly observed that Tris extraction of chloroplasts (loss of about 2/3 of their Mn and 80% of their $DH_2$ rate) resulted in no loss of the hydroxylamine-sensitive $DH_2$ photooxidation.

We suggest from these data that 35° ageing or extraction results in loss of the primary entry site of ascorbate (or *p*-phenylenediamine) into System II; loss of the primary entry site thus renders these photooxidations insensitive to hydroxylamine. From these experiments we cannot evaluate whether other sites of $DH_2$ entry into System II (see previous sections) also are inhibited by hydroxylamine.

## DISCUSSION

In these studies rigorous attempts were made to exclude "non-functional" Mn, *i.e.* the Mn readily removable from chloroplasts by washing with EDTA[33,35] without affecting parameters of System II activity. Under the conditions of spinach growth described here, rarely did we observe more than about 10% non-functional Mn. Thus we believe that we concern ourselves only with the functional Mn of System II.

It should be emphasized that all procedures to modify the chloroplast Mn content had no deleterious effect upon either the quantum yield or $v_{max}$ of System I and thus we dealt only with effects upon System II. However, we cannot be certain that any of the treatments affects specifically and only the Mn of System II. We can, however, exclude cofactors such as $Cl^-$ (ref. 32), since the effect of this cofactor is reversible and $Cl^-$ was maintained in preparations and assays. The protein (OEF factor), which has been suggested by HUZISIGE *et al.*[34] to be involved in $O_2$ evolution, did not increase rates of $O_2$ evolution of extracted preparations when added at the concentrations used by these workers. Effects of other unknown catalysts and possible structural deformations presently cannot be excluded however. With these reservations we believe that the site affected by the discussed treatments is the functional Mn.

With untreated chloroplasts of high activity (1000–1200 μequiv/mg chlorophyll per h) it was shown by $O_2$ exchange stoichiometry and confirmed by mass spectrometry that $DH_2$ photooxidation through System II completely over-rides the $O_2$-yielding reactions, thus confirming early results[38,20,21] obtained with chloroplasts of rather low activity (132–272 μequiv/mg chlorophyll per h).

Our general experience and the data of mild ageing (35°) show that $O_2$ evolution is easily affected. We have experienced considerable difficulty of this kind with greenhouse spinach material grown in summer months as evidenced by low flash yields, quantum yields, and saturation rates of water photooxidation. However, with fresh untreated winter chloroplasts containing 2.5–3 Mn/200 total chlorophylls we consistently observed with eighteen different preparations flash yields of 1 $O_2$/1400–1600 total chlorophylls and $v_{max}$ values of 1000–1200 equiv/chlorophyll per h. These flash yields were somewhat greater than reported by EMERSON AND ARNOLD[39] for whole algae and more comparable to those found by JOLIOT[40]. Such yields indicate a concentration of System II traps of 1/175–200 chlorophyll (assuming half of the chlorophyll is in this system) with each trap containing 3 atoms Mn (3 Mn/System II).

To interpret our data concerning the Mn of System II we must explain and reconcile the following observations:

(A) The uncoupled maximal photooxidation rates of water and two different System II donor systems are identical (> 1000 equiv/chlorophyll per h). This agrees with the concept that the normally rate-limiting step of the overall process is in the chain connecting the two photoacts and not on the oxidant side of System II.

(B) Any decrease of the Mn pool results in a loss of rate which in extraction and growth-deficiency experiments is identical for $v_{max}$ and $\Phi$ of $O_2$ evolution as well as for $v_{max}$ of $DH_2$ photooxidation (Fig. 8, Table III).

(C) In the extraction experiments these rates are linear with the Mn/trap which is in excess over the residual pool of 1 Mn/trap (Fig. 5).

(D) The quantum yield of $DH_2$ photooxidation is not affected over this range of loss of Mn and $v_{max}$ of this process is not zero but about 15–20% of the normal rate when all but the residual Mn (1 Mn/trap) is removed (Figs. 6 and 8).

Observation (B), the immediate loss of $v_{max}$ upon slight Mn loss, can only be reconciled with conclusion (A) if one assumes: (a) that with each loss of Mn a complete $O_2$ evolution center is lost; and (b) that each System II unit is connected with only one System I unit. The "independent chain" model predicts, since an entire chain is out of operation, that loss of a center decreases $\Phi$ as well as $v_{max}$, regardless where the normally rate-limiting step occurs.

Observation (C), the linear relation between $n-1$ Mn/trap and $Q_{O_2}$, seen in the extraction experiments suggests that the two easily extractable Mn atoms leave the center simultaneously. If each Mn were dislodged by itself, one might expect the loss of $O_2$ evolution not to be proportional to some fraction of $n-1$ Mn/trap and not identical to the loss of $DH_2$ photooxidation in strong light. The hypothesis that during extraction pairs of Mn atoms are removed from the $O_2$ center might imply the loss of an entire enzyme containing two Mn, an implication we presently are trying to verify.

Observation (D) requires the assumption that ascorbate and *p*-phenylenediamine, which feed rapidly into the easily extractable Mn pool also feed (though slowly) into the extraction-resistant, residual Mn atom. Possibly also this site can be bypassed, in a still slower feed directly in chlorophyll$_{II}^+$. These conclusions are summarized in Fig. 12.

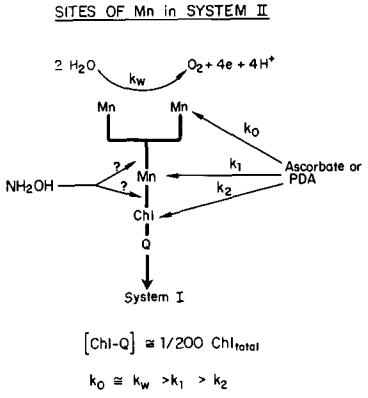



Fig. 12. Suggested functional sites of Mn in the Photosystem II complex. A bound form of Mn is equated with Z, a symbol often used to notate the oxidant of water.

The fluorescence observations showed that removal of the easily extractable Mn pool changed neither the yield of the invariant fluorescence, $F_0$, nor that of the variable fluorescence in the presence of DCMU. Only with further extraction of the residual pool did the DCMU fluorescence maximum decline to half the original value, also the initial slope of the rise curve declined to half. Since hydrosulfite raised this low fluorescence level to the original maximum we must tentatively assume that after complete removal of Mn there occurs a rapid back reaction of photoproducts in System II. Apparently the small residual Mn pool, 1 Mn/trap, prevents this back reaction and stabilizes the charge separation. This same fluorescence behavior was

observed with chloroplasts aged at 35° for 30 min and this points to an unsuspected stability of the photochemical System II conversion centers.

The fluorescence data therefore supports the earlier reached conclusions that Mn functions solely on the oxidant side of System II[12,38] and corroborate the heterogeneity of the Mn pool. We should point out, however, that the loss of Mn and of rate induced by 35° ageing shows different kinetics from those described for extraction. With ageing no clear demarcation of two Mn pools was obtained and the results have proved difficult to interpret without undue speculation.

The effects of extractants upon water and $DH_2$ photooxidation reported here might appear contradictory to the results of YAMASHITA AND BUTLER[10]. While these authors claimed substantial restoration, our data suggest that ascorbate or ascorbate–*p*-phenylenediamine restore only a fraction ($\leq 1/5$) of the maximal electron flow through System II in Tris-extracted chloroplasts. Actually, there is no disagreement; our ascorbate or *p*-phenylenediamine-restored rates ranged between 100–250 equiv/chlorophyll per h while the rates in fresh chloroplasts were 600–1200 equiv/chlorophyll per h. YAMASHITA AND BUTLER[10] reported rates for fresh chloroplasts of approx. 140 equiv/chlorophyll per h, about identical to the extraction-resistant rates.

Like these authors, we also arrived at the conclusion that brief Tris extraction leaves the photochemical trapping centers essentially intact, however, this conclusion now rests on the observations that the quantum yield of $DH_2$ photooxidation and the variable fluorescence yield in the presence of DCMU, are unaffected.

A final remark may be devoted to the rather complicated effects of hydroxylamine. In contrast to other donors hydroxylamine causes a rapid loss of the easily extractable Mn pool. In addition it shows an inhibitory effect upon $DH_2$ photooxidation decreasing the initial rates to about 60% of maximum. This inhibition seems unrelated to the Mn removal effect and also to a more severe damage of Photosystem II which occurs upon longer exposure of chloroplasts to hydroxylamine and light (unpublished results).

The 60% inhibition of $DH_2$ photooxidation by hydroxylamine at concentrations sufficient to completely inhibit $O_2$ evolution and the hydroxylamine- or Tris-induced loss of Mn with accompanying declines in rates of water and $DH_2$ photooxidations, we feel lends support for our hypothesis of two functional Mn catalysts in chloroplasts, one more intimately associated with $O_2$ evolution, one more intimately associated with the photochemical center (Fig. 12). Other means are currently being sought to evaluate further this hypothesis.

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