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# $Mn^{2+}$ AS ELECTRON DONOR IN ISOLATED CHLOROPLASTS

G. BEN-HAYYIM AND M. AVRON

*Biochemistry Department, Weizmann Institute of Science, Rehovot (Israel)*

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SUMMARY

1. Added $Mn^{2+}$ is concluded to serve as an electron donor at a site which precedes Photosystem 2. This conclusion is supported by the following observations: (a) Addition of $Mn^{2+}$ increased the apparent rate of $O_2$ uptake in the presence of 1,1'-ethylene-2,2'-dipyridilium dibromide. (b) $Mn^{2+}$ had little or no effect on $NADP^+$ photoreduction, while the concomitant $O_2$ evolution was severely inhibited. (c) All the reactions in which $Mn^{2+}$ served as the electron donor were as sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea as the Hill reaction and were insensitive to $CN^-$ or $N_3^-$.

2. In the presence of $Mn^{2+}$ and $Fe(CN)_6^{3-}$ high ratios of $P/2e$ were obtained indicating the existence of a cyclic process due to an interaction between the products of the reaction, $Fe(CN)_6^{4-}$ and $Mn^{3+}$.

3. A method was developed for assaying the $Mn^{3+}$ formed. In the presence of both $Mn^{2+}$ and water as electron donors to Photosystem 2, the $Mn^{3+}$ produced accounted for about 80 % of the electron flow observed.

## INTRODUCTION

The role of manganese in photosynthetic organisms is one of the longest studied aspects of photosynthesis. Studies concerning photosynthesis and Hill reaction in manganese-deficient whole plants, algae or chloroplast preparations revealed an absolute requirement for this nutrient[1–10]. With the concept of two light reactions it was possible to demonstrate that the manganese is essential for Photosystem 2-mediated reactions. Thus, $O_2$ evolution[11,12] or fluorescence properties[13–16] which are dependent on Photosystem 2 activity were inhibited in the absence of manganese and the activity was restored by its readdition[17,18]. On the other hand, KESSLER[5,19] showed that photoreduction of $CO_2$ by hydrogen in hydrogen-adapted algae was not affected by manganese deficiency. This reaction was shown to require only Photosystem 1 activity. Thus, manganese is not an essential cofactor in reactions mediated by this photosystem. ANDERSON *et al.*[20] found that the fractionation of chloroplasts by treatment with digitonin yielded two fractions, a light and a heavy fraction corresponding to Photosystem 1 and Photosystem 2, and manganese was found to be tightly bound to the heavy particle.

Abbreviations: diquat, 1,1'-ethylene-2,2'-dipyridilium dibromide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

However, despite ample evidence for a manganese requirement in photosynthetic reactions no report of light-induced changes in endogenous manganese have appeared. It is generally postulated that manganese serves as a catalyst in one of the electron transport steps of the $O_2$-evolving complex.

Several authors observed that exogenous $Mn^{2+}$ was photooxidized by isolated chloroplasts. KENTEN AND MANN[21,22] were the first to show photooxidation of $Mn^{2+}$ to $Mn^{3+}$ and they were followed by others[23,24]. In all these studies it was suggested that $Mn^{2+}$ could serve as an electron donor for Photosystem 2. However, all could not rule out the possibility of an indirect oxidation of $Mn^{2+}$, namely by the $H_2O_2$ which was produced under the reaction conditions employed (see DISCUSSION in ref. 23).

In the present study we examined the effect of added $Mn^{2+}$ on various types of Hill reactions. By introducing $NADP^+$ or $Fe(CN)_6^{3-}$ as electron acceptors rather than viologens or FMN, we could exclude any possible reaction between $Mn^{2+}$ and $H_2O_2$. We investigated the effect of $Mn^{2+}$ on electron transport to 1,1'-ethylene-2,2'-dipyridilium dibromide (diquat), $NADP^+$ and $Fe(CN)_6^{3-}$, its site of action and the rate of its photooxidation. $Mn^{2+}$ was found to interact directly with the electron transport chain serving as electron donor to Photosystem 2.

## METHODS

Chloroplasts were isolated from lettuce leaves as previously described[25]. Chlorophyll was assayed after ARNON[26]. $O_2$ uptake and evolution were measured with a Yellow Springs Instruments Clark type oxygen electrode. $NADP^+$ photoreduction was measured in a Cary 14 recording spectrophotometer equipped with a scattering attachment. The photomultiplier was protected from the actinic light by 1 cm of a saturated solution of $CuSO_4$ and a corning C.S. 7-60 filter. Illumination was provided by a 500-W projector and was filtered through Baird Atomic sharp cut-off interference filters (all blocked to infinity) peaking at 640 m$\mu$ (25-m$\mu$ half-band width) and 715 m$\mu$ (15-m$\mu$ half-band width). When monochromatic light was not required a corning C.S. 2-58 filter was used. The intensity of the actinic light was measured with a Yellow Springs Instrument Radiometer Model 65 and was varied by using calibrated metal screens. Absorption data were calculated from a curve previously published[27].

$Fe(CN)_6^{3-}$ photoreduction was measured either by following the decrease in absorbance at 420 m$\mu$ in a Cary 14 recording spectrophotometer or with an Aminco-Chance dual-wavelength spectrophotometer following the change 420 — 470 m$\mu$. The photomultiplier was protected from the actinic light by a corning C.S. 4-96 filter. ATP formation was assayed as previously described[25].

Oxidation of $Mn^{2+}$ to $Mn^{3+}$ was measured by reduction of the $Mn^{3+}$ to $Mn^{2+}$ with $Fe(CN)_6^{4-}$ followed by a sensitive determination of the remaining $Fe(CN)_6^{4-}$. MCKENNA AND BISHOP[24] utilized a similar principle for standardization of $Mn_4(P_2O_7)_3$ but found their assay inadequate for estimation of $Mn^{3+}$ formed by illuminated chloroplasts. The procedure used in this paper was as follows: A reaction mixture containing $Mn^{2+}$ and pyrophosphate (to stabilize the oxidized $Mn^{3+}$ as $Mn_4(P_2O_7)_3$) was illuminated for the desired period. 3.0 ml of the suspension were added to 1.0 ml of 1 mM $Fe(CN)_6^{4-}$ solution. After incubation of 3–5 min in the dark at room tem-

perature, 0.4 ml of 30% trichloroacetic acid was added to inactivate the system. The remaining $Fe(CN)_6^{4-}$ in the clear supernatant was determined by the method of AVRON AND SHAVIT[28].

## RESULTS

Addition of $Mn^{2+}$ to illuminated chloroplast preparations which consume $O_2$ in the presence of diquat resulted in an increase of the apparent rate of the $O_2$ uptake (Fig. 1a). Similar effects were observed when the $Mn^{2+}$ was added to an uncoupled system (Fig. 1b). In both cases, the $O_2$ uptake was as sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) as was the $Fe(CN)_6^{3-}$ Hill reaction. The effect of $Mn^{2+}$ as a function of its concentration is shown in Fig. 2. As will be discussed later, the increase of the apparent rate of $O_2$ uptake does not seem to be due to an increase in the rate of electron transport but rather to the fact that $Mn^{2+}$ served as electron donor prior to Photosystem 2. The stoichiometry for calculating

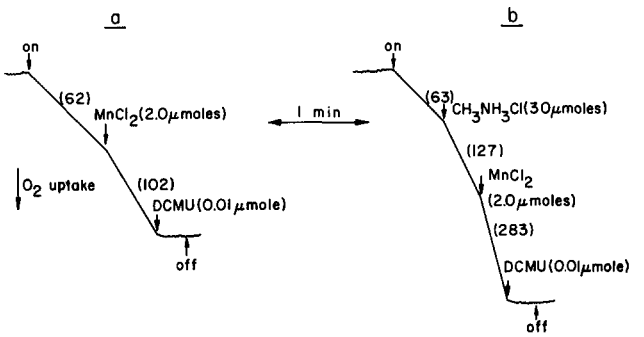



Fig. 1. Effect of $Mn^{2+}$ on $O_2$ uptake in the presence of diquat. Reaction mixture contained: Tricine (pH 8.0), 45 μmoles; NaCl, 60 μmoles; diquat, 0.03 μmole; $NaN_3$, 3 μmoles and chloroplasts containing 73 μg chlorophyll, in a total volume of 3.0 ml. Numbers in parentheses represent rates of $O_2$ uptake expressed in μmoles per mg chlorophyll per h.

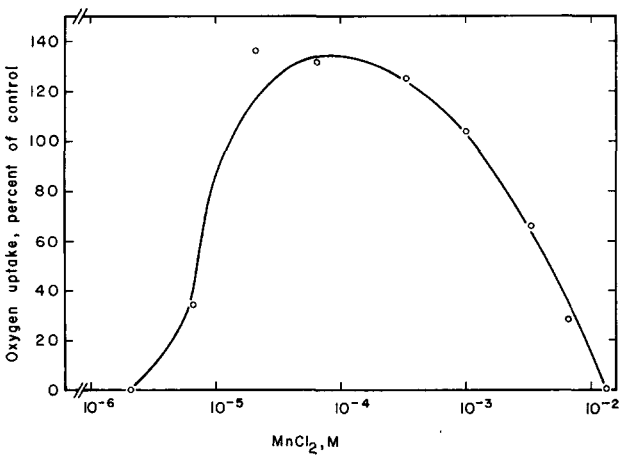



Fig. 2. Concentration curve for the effect of $Mn^{2+}$. Reaction mixture as described for Fig. 1. The control was measured in the absence of $MnCl_2$, its activity being 18 μmoles $O_2$ per chlorophyll per h. Chloroplasts contained 100 μg chlorophyll.

the electron transport rate in the absence and presence of $Mn^{2+}$ can be depicted by Eqns. 1, 3, 4 and 2, 3, 5, respectively.

1. $H_2O + DQ \xrightarrow{light} (DQH_2) + 1/2\ O_2$

3. $(DQH_2) + O_2 \longrightarrow H_2O_2 + DQ$

4. $H_2O_2 + 1/2O_2 \xrightarrow{light} H_2O_2$

2. $2\ Mn^{2+} + DQ + 2\ H^+ \xrightarrow{light} 2\ Mn^{3+} + (DQH_2)$

3. $(DQH_2) + O_2 \longrightarrow H_2O_2 + DQ$

5. $2\ Mn^{2+} + 2\ H^+ + O_2 \xrightarrow{light} 2\ Mn^{3+} + H_2O_2$

Thus one molecule of $O_2$ taken up represents the photoinduced transfer of 4 electrons in the absence of $Mn^{2+}$ but of only 2 electrons in the presence of $Mn^{2+}$. With DQ as an electron acceptor $O_2$ is involved both at the donor and the acceptor sites. By using $NADP^+$ as an electron acceptor one can measure separately the effect of $Mn^{2+}$ on the donor and the acceptor ends of the electron transport chain. As seen in Fig. 3, the effect of $Mn^{2+}$ on $NADP^+$ photoreduction was different from that on $O_2$ evolution. While the $O_2$ evolution was strongly inhibited, $NADP^+$ photoreduction stayed essentially the same.

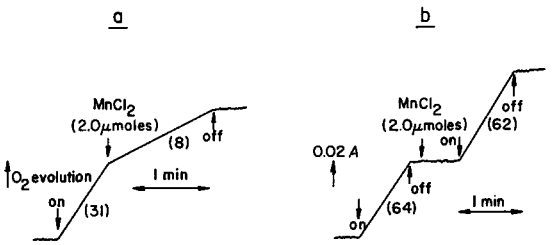



Fig. 3. Effect of $Mn^{2+}$ on electron transport to $NADP^+$. Reaction mixture contained: Tricine (pH 8.0), 45 $\mu$moles; NaCl, 60 $\mu$moles; $NADP^+$, 0.5 $\mu$mole; saturating amount of ferredoxin and chloroplasts containing 65 $\mu$g chlorophyll in 3.0 ml. $NADP^+$ photoreduction was followed by increase in absorbance at 350 m$\mu$ in a Cary 14 spectrophotometer as described under METHODS. Numbers in parentheses are initial rates of $O_2$ evolution and $NADP^+$ photoreduction expressed in $\mu$moles $O_2$ per mg chlorophyll per h and $\mu$moles NADPH per mg chlorophyll per h, respectively.

The effect of $Mn^{2+}$ on the electron transport to either diquat or $NADP^+$ was highly specific for these ions. $Mg^{2+}$ or $Ca^{2+}$ at the same concentration was totally inactive or even slightly inhibitory. As seen in Table I, $Mn^{2+}$ increased the apparent rate of $O_2$ uptake mediated by diquat to about the same extent as it decreased the $O_2$ evolution concomitant to $NADP^+$ photoreduction. Under all tested conditions it was not possible to fully inhibit the $O_2$ evolution accompanying $NADP^+$ reduction by the addition of $Mn^{2+}$.

*The site of action of $Mn^{2+}$*

The sensitivity of the $Mn^{2+}$ effect to DCMU indicated that its site of action was relatively close to Photosystem 2. Quantum requirement measurements of $NADP^+$ photoreduction in the presence and absence of $Mn^{2+}$ exhibited the 'red drop' phenomenon characteristic of light-induced reactions in which Photosystem 2 participates.

As can be seen in Table II, $Mn^{2+}$ somewhat lowered the quantum requirement at 640 m$\mu$ but did not change the quantum requirement at 715 m$\mu$. Therefore, the

'red drop' in the presence of $Mn^{2+}$ became even more pronounced than that with water.

$Mn^{2+}$ failed to reactivate $NADP^+$ photoreduction in Tris-treated chloroplast preparations, possibly indicating that their site of action not only precedes Photosystem 2 but is rather close to the $O_2$ evolution step itself.

TABLE I

THE SPECIFICITY OF THE EFFECT OF $Mn^{2+}$ ON PHOTOREACTIONS TAKING UP OR EVOLVING $O_2$

Reaction mixture contained: Tricine (pH 7.8), 45 $\mu$moles; NaCl, 60 $\mu$moles; chloroplasts containing 70 $\mu$g chlorophyll in a total volume of 3.0 ml. 0.03 $\mu$mole diquat and 3 $\mu$moles $NaN_3$ were added in the reaction with diquat and 0.5 $\mu$mole $NADP^+$ with a saturating amount of ferredoxin were added in the $NADP^+$ reaction. The activity of $O_2$ evolution with $NADP^+$ and $O_2$ uptake in presence of diquat and in absence of $Mn^{2+}$ (taken as 100%) were 20 and 44 $\mu$moles $O_2$ per mg chlorophyll per h, respectively.

| *Electron acceptor* | *Ion added* | *Activity (% of control)* |
|---|---|---|
| $NADP^+$ | None | (100) |
| | $Mn^{2+}$ | 36 |
| | $Mg^{2+}$ | 100 |
| Diquat | None | (100) |
| | $Mn^{2+}$ | 170 |
| | $Mg^{2+}$ | 71 |
| | $Ca^{2+}$ | 82 |

TABLE II

QUANTUM REQUIREMENT OF $NADP^+$ PHOTOREDUCTION IN THE PRESENCE OF $Mn^{2+}$

Reaction mixture contained: Tricine (pH 7.8), 45 $\mu$moles; NaCl, 60 $\mu$moles; aminomethane·HCl, 18 $\mu$moles; $NADP^+$, 1 $\mu$mole; saturating amount of ferredoxin and chloroplasts containing 39 $\mu$g chlorophyll in 3.0 ml. 2.0 $\mu$moles of $Mn^{2+}$ were added where indicated. $NADP^+$ photoreduction was measured as described under METHODS. Quantum requirement data represent values extrapolated to zero intensity (see ref. 27).

| *Electron donor* | *Quanta/electron* | | *Ratio 715 mμ/640 mμ* |
|---|---|---|---|
| | *640 mμ* | *715 mμ* | |
| $H_2O$ | 5.1 | 12.6 | 2.5 |
| $Mn^{2+}$ | 3.7 | 14.2 | 3.8 |

The photoreduction of $NADP^+$ in the presence of $Mn^{2+}$ was as sensitive as $NADP^+$ Hill reaction to all the inhibitors tested including DCMU, *o*-phenanthroline, ioxynil and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO).

In contrast to $NADP^+$ photoreduction, $Mn^{2+}$ inhibited both $Fe(CN)_6^{3-}$ photoreduction and the concomitant $O_2$ evolution to the same degree. The inhibition of $O_2$ evolution was believed to arise from the competition between $Mn^{2+}$ and water, in a manner similar to that described for the case of $NADP^+$. However, since (as will be shown later) $Mn^{2+}$ is photooxidized to $Mn^{3+}$, this $Mn^{3+}$ is rereduced by the $Fe(CN)_6^{4-}$ which is formed when $Fe(CN)_6^{3-}$ is the electron acceptor. Since it is impossible to separate the two processes, we studied the $Fe(CN)_6^{3-}$-dependent photo-

phosphorylation as an indirect measurement of the rate of electron flow from either water or $Mn^{2+}$ to $Fe(CN)_6^{3-}$. If indeed a cyclic electron flow of the type just described occurs, one would expect the rate of ATP formation to remain constant while the apparent rate of $Fe(CN)_6^{3-}$ reduction is curtailed, resulting in an apparent increase in the ATP/2*e* ratio. As shown in Table III, this was indeed the case. As can be expected, the effect was most striking at the lower $Fe(CN)_6^{3-}$ concentrations, where the $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$ ratio would be highest.

*Photooxidation of $Mn^{2+}$*

The $Mn^{2+}$ effect on electron transport was interpreted as a result of $Mn^{2+}$ serving as an electron donor. If so, oxidation of $Mn^{2+}$ should occur. Such oxidation, of $Mn^{2+}$ to $Mn^{3+}$, was already demonstrated by others[23,24] though at very low rates. $Mn^{3+}$ in aqueous solution is extremely unstable, but addition of pyrophosphate to form $Mn_4(P_2O_7)_3$ yields a more stable complex. A method for assaying $Mn^{3+}$ was developed (see METHODS) which enabled us to follow a time-course of the photooxidation of $Mn^{2+}$ in a chloroplast preparation which consumed $O_2$ in the presence of diquat. $NaN_3$ was replaced by ethanol and catalase to avoid any possible complication due to a chemical reaction between $Mn^{3+}$, peroxide and the added $Fe(CN)_6^{4-}$. As

TABLE III

APPARENT P/2*e* RATIOS OF $Fe(CN)_6^{3-}$ PHOTOREDUCTION IN THE PRESENCE OR ABSENCE OF $Mn^{2+}$

Reaction mixture contained: Tricine (pH 8.0), 45 $\mu$moles; NaCl, 60 $\mu$moles; $MgCl_2$, 12 $\mu$moles; phosphate buffer (pH 8.0), 12 $\mu$moles (containing $9 \cdot 10^6$ counts/min $^{32}P$; ADP, 4 $\mu$moles; chloroplasts containing 43 $\mu$g chlorophyll and 2.0 $\mu$moles $MnCl_2$ where indicated, in a total volume of 3.0 ml. $Fe(CN)_6^{3-}$ reduction was measured in a dual-wavelength spectrophotometer as described under METHODS. Each sample was illuminated for 2 min and later analyzed for its ATP content.

| *Initial $Fe(CN)_6^{3-}$ concn. ($\mu M$)* | *ATP formation ($\mu$moles per mg chlorophyll per h)* | | *Apparent P/2e* | |
|---|---|---|---|---|
| | $-Mn^{2+}$ | $+Mn^{2+}$ | $-Mn^{2+}$ | $+Mn^{2+}$ |
| 500 | 292 | 240 | 1.0 | 2.5 |
| 170 | 167 | 134 | 1.1 | 2.5 |
| 50 | 58 | 52 | 1.0 | 4.3 |
| 17 | 26 | 17 | 1.8 | 7.9 |

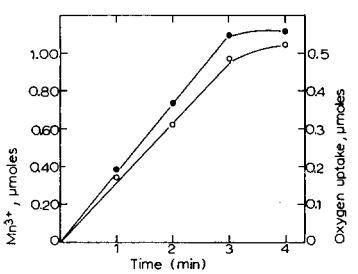



Fig. 4. Photooxidation of $Mn^{2+}$ by isolated chloroplasts. Reaction mixture contained: Tricine (pH 7.8), 90 $\mu$moles; NaCl, 120 $\mu$moles; diquat, 0.1 $\mu$mole; $Na_4P_2O_7$ (pH 7.8), 60 $\mu$moles; ethanol, 1020 $\mu$moles; catalase, 100 $\mu$g; $MnCl_2$, 4 $\mu$moles and chloroplasts containing 129 $\mu$g chlorophyll in 6.0 ml. $Mn^{3+}$ content was determined as described under METHODS. The $O_2$ uptake in absence of $Mn^{2+}$ was 0.14 $\mu$mole for the first 2 min. Full dots indicate $O_2$ uptake.

can be seen in Fig. 4, the photooxidation of $Mn^{2+}$ was linear with time during the first few minutes and it accounted for about 80 % of the $O_2$ uptake activity in the presence or absence of $Mn^{2+}$. Thus, it seems that in the presence of $Mn^{2+}$ the donation of electrons by these ions is dominant over the water-donating reaction.

## DISCUSSION

Previous communications dealing with photooxidation of $Mn^{2+}$ by chloroplast preparations presented two alternative hypotheses to explain the mechanism of this reaction: (a) direct effect of $Mn^{2+}$ *i.e.* oxidation *via* the electron transport chain, or (b) indirect effect, namely a chemical reaction between $Mn^{2+}$ and some oxidized component produced during illumination, such as $H_2O_2$.

In previous studies the effect of $Mn^{2+}$ was tested with either viologens or FMN as electron acceptors. Peroxidase in the presence of $Mn^{2+}$ and $H_2O_2$ catalyze the oxidation of $Mn^{2+}$ to $Mn^{3+}$ at the expense of the peroxide. An illuminated preparation in the presence of FMN or a viologen under aerobic conditions contains all the components needed for this reaction. Chloroplasts are known to contain a sufficient amount of endogenous peroxidase[24], and the product of electron transport mediated by FMN or viologen was shown[29] to be $H_2O_2$. However, while the reaction of peroxidase is known to be sensitive to either $N_3^-$ or $CN^-$ (ref. 30), the Hill reaction is rather insensitive[31]. This was used as a tool to determine which one of the alternative hypotheses accounted for the $Mn^{2+}$ effect. McKENNA AND BISHOP[24] reported that the photooxidation of $Mn^{2+}$ was sensitive to $CN^-$, though at higher concentrations than required for a soluble catalase system. On the other hand, no such sensitivity could be observed by either BACHOFEN[23] or by us. Moreover, all our measurements of $O_2$ uptake in the presence of diquat were carried out in the presence of $N_3^-$ which by itself is a good inhibitor of the peroxidase reaction.

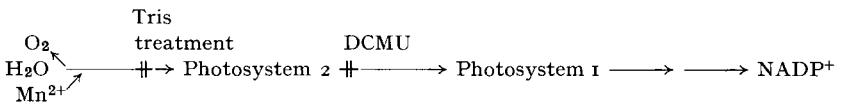
Further evidence pointing to a direct effect of $Mn^{2+}$ came from the experiments with other electron acceptors where $H_2O_2$ was not formed at all. The best indication was obtained with $NADP^+$ as the electron acceptor. The results presented in Fig. 3 together with the fact that $Mn^{2+}$ was found to be oxidized by the same system lead to the conclusion that there is a strong competition between $Mn^{2+}$ and water for the donation of electrons which eventually reduce $NADP^+$. By analogy, the same picture should have been found for the photoreduction of $Fe(CN)_6^{3-}$. However, the interaction between the products $Fe(CN)_6^{4-}$ and $Mn^{3+}$ somewhat obscured the result. Indirect evidence derived from the rate of ATP formation coupled to $Fe(CN)_6^{3-}$ photoreduction in the presence or absence of $Mn^{2+}$ confirmed the hypothesis that the actual rate of electron flow was not affected by $Mn^{2+}$. The measured rate was the net rate, reflecting the total rate of $Fe(CN)_6^{4-}$ production *minus* its reoxidation by the $Mn^{3+}$ formed at the same time. Therefore, the high P/2*e* ratios observed in the presence of $Mn^{2+}$ are meaningless in regard to the number of coupling sites but rather indicate the existence of a cyclic electron flow induced by the $Mn^{2+}$.

The DCMU sensitivity of the effect of $Mn^{2+}$ suggested that the site of its action must be close to Photosystem 2. Conclusive evidence for the site of action of $Mn^{2+}$ was drawn from quantum requirement measurements (Table II). The existence of the 'red drop' phenomenon in the $Mn^{2+}$-induced electron transport must mean that the site of donation of electrons by $Mn^{2+}$ precedes Photosystem 2. Further support

for this conclusion was provided by the interaction between $Fe(CN)_6^{3-}$ photoreduction and $Mn^{2+}$ photooxidation. It was previously shown[27] that $Fe(CN)_6^{3-}$ reduction occurs by activation of Photosystem 2 alone. Thus, in order to establish a cyclic flow of electrons as described above, $Mn^{2+}$ must donate electrons prior to Photosystem 2.

Analysis of fluorescence properties of normal and manganese-deficient chloroplast preparations[15] also suggests that the endogenous manganese site of interaction with the electron transport chain is localized on the reducing side of Photosystem 2.

The data presented in this paper could be summarized in the following scheme:




In conclusion, the photooxidation of $Mn^{2+}$ seems to be competitive with the photooxidation of $OH^-$ derived from water. This competition explains the increase in the apparent rate of $O_2$ uptake in the presence of diquat and the inhibition of $O_2$ evolution in the presence of either $NADP^+$ or $Fe(CN)_6^{3-}$. The photooxidation of $Mn^{2+}$ accounts for 70–80 % of the overall activity. It is hard to correlate the activity of added $Mn^{2+}$ with that of the endogenous manganese which is bound to a protein, since no change of the redox state of the latter was reported. It is of interest, that their site of action with respect to Photosystem 2 seem rather similar. Free $Mn^{2+}$ may replace the bound manganese extracted by Tris treatment[15], but they were found to be inactive as electron donors for $NADP^+$ photoreduction.

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