LIGHT-DEPENDENT OXYGEN METABOLISM OF CHLOROPLAST PREPARATIONS. II. STIMULATION BY MANGANOUS IONS ¹, ²

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The importance of manganese for photosynthesis was first suggested by McHargue (17) and Bishop (2). Pirson (21) found that photosynthesis in manganese-starved Chlorella cells was inhibited despite the absence of visible chlorosis; this inhibition could be relieved by adding manganese to the medium. Emerson and Lewis (6) observed that manganese deficiency affected the maximum quantum yield of photosynthesis. Gerretsen (7) found that manganese deficiency caused a reduction in the rate of photosynthesis in oats. He surmised that manganese is specifically involved in the oxygen-liberating stage of photosynthesis and went on to study the effect of manganese salts on the potentials of electrodes immersed in chloroplast suspensions (8). Working with chloroplasts in the absence of Hill reagents, Gerretsen found that the electrode potential was changed very markedly on addition of manganese and he suggested that peroxide is formed in the presence of manganese but not in its absence.

More recently Kessler (15, 16), from his studies of photosynthesis and photo reduction in manganese-deficient algae, provided convincing proof that the role of manganese in photosynthesis is mainly, if not exclusively, concerned with oxygen evolution.

Aside from the implication of manganese, we know very little about the process by which oxygen is evolved from the oxidized product of the splitting of water in photosynthesis. There is a comparable lack of experimental evidence concerning the role of manganese in photochemical oxygen production by isolated chloroplasts.

Mehler (18) was the first to show that oxygen could function as a Hill reagent, i.e., as an oxidant which is effective in promoting photochemical oxygen production by chloroplast preparations. In the Mehler reaction there is a light-dependent consumption of oxygen which is a consequence of the simultaneous reduction of oxygen by a photochemically generated reductant and evolution of oxygen from the oxidized product of photolysis. Added ethanol and excess catalase act as a "sink" for the peroxide produced by the reduction of molecular oxygen (13). The stoichiometry of the over-all reaction is such that two molecules of oxygen are consumed for every one produced (Mehler and Brown, 20).

More recently, Brown and Good (3) have shown that chloroplasts illuminated without added ethanol can produce and consume oxygen simultaneously. Both production and consumption are light dependent processes. The stoichiometry being 1:1, the chloroplasts thus effect an exchange of the oxygen of water for molecular oxygen dissolved in the suspending medium. In this exchange reaction (which differs from the Mehler reaction only in the method of removal of peroxide formed in the reduction of molecular oxygen), each mole of oxygen reduced by chloroplast preparations in the light is exactly balanced by the production of 1/2 mole from the oxidized product of photolysis and 1/2 mole from the decomposition of peroxide by the endogenous catalase of the chloroplasts. Because there is no net change of oxygen tension in the exchange reaction, it can be followed only by tracer oxygen techniques.

Mehler (19) tested the effect of added manganous ions on the Hill reaction and found no effect on the rate of oxygen evolution when quinone was used as the oxidant (whether or not catalase and ethanol were present). In the case of the Mehler reaction, however, he found that Mn⁺⁺ increased the rate of net oxygen consumption. The manganese-stimulated rates were approximately the same as those which Mehler (19) had observed following quinone reduction. This stimulatory effect of manganese was catalytic; marked stimulation was given by 10⁻⁴ M Mn⁺⁺ and the maximum effects were produced by a 10⁻³ M concentration.

Andreae (1) has reported a flavin-sensitized photooxidation of manganese ions and has suggested that the cyclic oxidation-reduction of manganese might explain the effects of manganese ions on oxygen uptake by illuminated chloroplast suspensions. Also, Kenten and Mann (14) have demonstrated a light-dependent oxidation of manganese in chloroplast preparations and have discussed its possible role in photosynthesis.

Mehler's observation of the manganese stimulation has been confirmed by Good and Hill (9) and extended to preparations from chard and Chenopodium.

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Fig. 1 (top left). Effect of Mn$^{++}$ concentration on rates of net oxygen uptake in the Mehler reaction. Vessel contents: 3 cc chloroplast suspension (containing 0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50% ethanol, 0 to 4 µM MnCl$_2$.

Fig. 2 (top right). Effect of adding Mn$^{++}$ on time courses of normal and tracer oxygen in the Mehler reaction (Exp. 1). Vessel contents: 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 mg catalase, 0.1 cc 50% ethanol, 6 µM MnCl$_2$ in sidearm. To convert partial pressure units of the ordinate to microliters oxygen multiply by 1.12.
They have suggested that increased oxygen uptake must have been due to hydrogen peroxide formation because it did not occur when the hydrogen peroxide-trapping catalase and ethanol were omitted.

This paper describes experiments in which tracer oxygen techniques were used to measure the effects of manganous ions on the rates of consumption and production of oxygen in the Mehler reaction and the related exchange reaction. The data indicated that manganese stimulation resulted from a reaction with the oxidized product of photolysis and there was indirect evidence of peroxide formation. The stimulation of light-dependent chloroplast reactions by manganous ions is compared with the stimulation by quinone and ascorbic acid previously described (Habermann, 10; Habermann and Brown, 11) and a scheme for the action of manganese is proposed.

**Methods**

Chloroplasts were prepared from mature leaves of *Phytolacca americana* L. according to procedures previously described (Habermann, 10). All reactions were run in conventional rectangular Warburg vessels. Light from a bank of fluorescent bulbs mounted vertically behind the water bath was reflected onto the bottoms of the experimental vessels by a mirror mounted at a 45° angle. Incident light intensity was approximately 400 ft-c. Manometric experiments were run at 15.6° C. isotope experiments at either 14.5° or 23° C. Adaptation of the mass spectrometer (CEC model 21-201) has been described by Brown, Nier, and Van Norman (4), and further modifications of the apparatus have been described by Johnston and Brown (12).

The gas phase for all manometric experiments was air. In experiments using tracer oxygen, the vessel containing 3 cc buffered chloroplast suspension plus addenda was attached to the leak assembly of the mass spectrometer and flushed with helium for 5 minutes. Oxygen enriched with mass 34 (O\(^{16}\) O\(^{18}\)) was then added so that the resulting gas phase was composed of 2 to 3% oxygen in helium. The rates of oxygen consumption and evolution were calculated from time course records of the concentrations of mass 32 (ordinary oxygen) and mass 34 (O\(^{16}\) O\(^{18}\)) according to methods discussed by Brown and Weis (5).

**Results**

**Effect of Mn\(^{++}\) Concentration:** Figure 1 shows the rate dependence of the manganese-stimulated Mehler reaction on the amount of Mn\(^{++}\) added to the reaction mixture. Considerable stimulation of net oxygen uptake was observed at even the lowest concentration of added manganous ions and maximally stimulated rates were observed at concentrations above 10\(^{-4}\) M in the reaction mixture.

**Tracer Oxygen Experiments:** 1. **Effect of Mn\(^{++}\) on the rates of oxygen consumption and production in the Mehler reaction:** A number of experiments were run in which MnCl\(_2\) in solution was tipped from the sidearm of the experimental vessel after a period of unstimulated Mehler reaction in the light. The time courses of uptake of tracer and normal oxygen in a typical experiment are shown in figure 2. Following the addition of manganous ions to the reaction mixture, the rates of net consumption of both species were accelerated. The calculated rates of oxygen consumption and production in this experiment are given in table I (Exp. 1). Also in table I (Exp. 2) are data of a second representative experiment run with a different chloroplast preparation and at a higher temperature. In both experiments, the rates of uptake and production of oxygen in the Mehler reaction were accelerated following addition of Mn\(^{++}\) ions to the reaction mixture. In this case, uptake was stimulated much more than production (the rate of oxygen uptake increased an average of 83% after adding Mn\(^{++}\), while the rate of oxygen production increased only 40%).

2. **Effect of Mn\(^{++}\) on the rates of uptake and production of oxygen in the exchange reaction:** Experiment 3 (table I) was run with an aliquot of the chloroplast suspension used in experiment 1. Three cc of chloroplast suspension was pipetted into the main compartment of the vessel and MnCl\(_2\) in solution was tipped from the sidearm after a period of unstimulated oxygen exchange in the light. The time courses of tracer and normal oxygen pressure changes in this experiment are shown in figure 3 and the calculated rates of oxygen consumption and production are summarized in table I (Exp. 3). In this case also, the rates of both uptake and production of oxygen were accelerated by the addition of manganous ions. In the exchange reaction, however, in contrast to the changes observed in the Mehler reaction, production of oxygen was stimulated more than uptake (the rate of uptake increased only 34%, while the rate of production increased 61%).

**Effect on Rates of Quinone-Hill Reaction when Catalase and Ethanol are Present:** The

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**Fig. 3 (bottom left).** Effect of adding Mn\(^{++}\) on time courses of normal and tracer oxygen in the exchange reaction (Exp. 3). Vessel contents: 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 \(\mu\)M MnCl\(_2\) in sidearm. To convert partial pressure units of the ordinate to microliters oxygen multiply by 1.53.

**Fig. 4 (bottom right).** Effect of Mn\(^{++}\) concentration on rates of oxygen production in the quinone reaction and on rates of net oxygen consumption of the subsequent quinone-stimulated Mehler reaction. Vessel contents: 3 cc chloroplast suspension (containing 0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50% ethanol, 6 \(\mu\)M quinone, 0 to 8 \(\mu\)M MnCl\(_2\).
Table I

Effects of Added Manganese Ions on Rates of Oxygen Uptake and Production in Mehler and Exchange Reactions

<table>
<thead>
<tr>
<th></th>
<th>Exp. 1</th>
<th></th>
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<th>Exp. 2</th>
<th></th>
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<th>Exp. 3</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxygen</td>
<td>Oxygen</td>
<td>Net</td>
<td>Oxygen</td>
<td>Oxygen</td>
<td>Net</td>
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<tr>
<td></td>
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<td>production</td>
<td>rate</td>
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<td></td>
<td>(μL/min)</td>
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<td>(μL/min)</td>
<td>(μL/min)</td>
</tr>
<tr>
<td>Initial dark period</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-0.27</td>
<td>+0.01</td>
<td>-0.26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unstimulated light reaction</td>
<td>-1.18</td>
<td>+0.44</td>
<td>-0.74</td>
<td>-1.64</td>
<td>+0.69</td>
<td>-1.04</td>
<td>-1.48</td>
<td>+1.34</td>
</tr>
<tr>
<td>Mn²⁺ stimulated light reaction</td>
<td>-2.27</td>
<td>+0.69</td>
<td>-1.58</td>
<td>-2.86</td>
<td>+0.74</td>
<td>-2.12</td>
<td>-1.98</td>
<td>+2.16</td>
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<tr>
<td>Final dark period</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>34 %</td>
<td>61 %</td>
</tr>
</tbody>
</table>

Vessel contents and experimental conditions: Exp. 1. 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μM MnCl₂ in sidearm; temp. 14.5° C. Exp. 2. 3 cc chloroplast suspension (containing 0.42 mg chlorophyll), other addenda the same as in Exp. 1; temp. 23° C. Exp. 3. 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 μM MnCl₂ in sidearm; temp. 14.5° C.

Experimental data summarized in figure 4 show the effects of increasing amounts of manganese ions in reaction mixtures containing identical amounts of chloroplast suspension, quinone, catalase, and ethanol. Low concentrations of added Mn²⁺ had no effect on either the rate of oxygen evolution in the quinone reaction or on the rate of net oxygen consumption in the Mehler reaction which followed quinone reduction. When amounts of Mn²⁺ greater than 4 micromoles were added, however, the rates of oxygen production in the quinone reaction were reduced by approximately 20 %.

Discussion

Stimulation of the Mehler reaction by manganese ions is similar in its effect on net oxygen uptake to the effects of quinone and ascorbic acid. Although net uptake of molecular oxygen is stimulated by all three substances, their effects on the actual rates of uptake and production of oxygen are different. These differences, then, must serve as the basis for any hypothesis concerning the nature of their action. In contrast to quinone, which stimulates the rates of uptake and production equally so that the stoichiometry of the over-all reaction remains unchanged (10), both ascorbic acid (11) and manganese ions produce an unequal stimulation of oxygen consumption and production with greater increments in the rates of oxygen uptake. Unlike the ascorbic acid effect, which lasts only so long as unoxidized ascorbic acid remains in the reaction mixture and in which the net amount of oxygen taken up in excess of the unstimulated rate is equivalent to the ascorbic acid initially added (11), there is no tendency for manganese-stimulated rates to revert to those of the controls. In this respect, manganese stimulation resembles that of quinone. Stimulated rates have been followed for several hours using the stable chloroplasts prepared from Phytoeca.

The catalytic nature of the manganese stimulation and the unequal stimulation of the rates of consumption and production in no way argue against a cyclic oxidation and reduction of manganese such as that proposed by Andreae (1) and also by Kenten and Mann (14). A chlorophyll-sensitized photooxidation of manganese ions superimposed on the Mehler reaction might be plausible via a scheme analogous to that proposed by Andreae (1) for his non-biological system. This is improbable, however, because of the observed enhanced rates of oxygen production. A more likely reaction mechanism would involve an alternate pathway mediated by Mn²⁺ in the reaction sequence of the oxidized product of photolysis. Such a pathway could conceivably lead to oxygen production via peroxide formation. Under the conditions of the Mehler reaction, peroxide thus formed would be removed in the coupled oxidation of ethanol to acetaldelhyde. A more efficient removal of the oxidized product would decrease the probability of a back reaction, or recombination of the products of photolysis. The enhanced reduction of molecular oxygen by the reduced product of photolysis could thus be a consequence of the more efficient removal of one of the products of photolysis. This reaction mechanism is consistent with all the experimental data.

Let us then examine the experimental results in terms of the proposed mechanism: Adding manganese ions to a Mehler reaction mixture resulted in increased rates of net oxygen uptake which could be
observed manometrically. Tracer studies showed that this enhanced rate of net uptake was the result of a stimulation of the rate of oxygen uptake which was considerably greater than the stimulation of its rate of production. In the presence of manganous ions, removal of the oxidized product of photolysis (other than by back reaction with the reduced product) could follow two possible pathways: i.e., the usual pathway to oxygen of the unstimulated Mehler reaction or peroxide formation mediated by manganous ions. An effective peroxide trapping mechanism (the catalase and ethanol in the Mehler reaction mixture) removes the peroxide thus formed. In accordance with the proposed mechanism then, uptake was enhanced and the ratio of uptake to production was greater than in the unstimulated reaction.

Adding manganous ions to chloroplast preparations with no other addenda (which on illumination carry on an oxygen exchange reaction) resulted once again in enhanced rates of both uptake and production of oxygen. In this case, the peroxide trap had not been added to the reaction mixture but the endogenous catalase of the chloroplasts was able to decompose peroxide catalytically by dismuting it to $H_2O$ and $O_2$. The ratio of uptake to production should theoretically have remained constant because the alternate pathway for the oxidized product of photolysis results in oxygen production, as does the normal pathway. The calculated rates of oxygen uptake and production showed that their ratio changed from slightly more to slightly less than the theoretical value of one.

According to the proposed reaction mechanism, the presence of manganous ions in a Hill reaction mixture would not enhance the rate of oxygen production. Quinone seems to be efficient in its reaction with the reduced product of photolysis. Evidence for this is the observation that the rates of oxygen production with quinone as the oxidant are as a rule twice to several times those observed when oxygen is used as the Hill reagent. If a peroxide trap were present in the reaction mixture, however, we would anticipate that manganese-mediated peroxide production from the oxidized product of photolysis could decrease the rate of oxygen production. When catalase and ethanol were present during the quinone reaction and sufficiently high concentrations of Mn$^{++}$ were added, a decrease in the rate of oxygen production was observed.

These experiments support the possibility that manganese-mediated peroxide formation is involved in the evolution of oxygen in photosynthesis. They do not, however, tell us whether oxygen evolution in vivo proceeds via peroxide formation similar to that which is indicated in vitro, or by the apparently normal pathway to oxygen which exists in unstimulated chloroplast preparations.

**Summary**

Tracer oxygen techniques were used to measure simultaneously the rates of consumption and production of oxygen in unstimulated and manganese-stimulated light-dependent chloroplast reactions. Both rates were stimulated on addition of manganous ions to the Mehler system and a similar stimulation of the related exchange reaction was observed.

These experiments indicate that the action of manganese is primarily involved with the oxidized product of photolysis. A reaction mechanism has been postulated whereby manganese mediates peroxide formation from the (OH)-product of the splitting of water. This provides an alternate pathway which results in the more efficient removal of this product and accounts for the stimulation of net oxygen uptake by manganous ions.

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**Literature Cited**


