Hydrogen Peroxide Synthesis in Isolated Spinach Chloroplast Lamellae

AN ANALYSIS OF THE MEHLER REACTION IN THE PRESENCE OF NADP REDUCTION AND ATP FORMATION

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ABSTRACT

Light-dependent O₂ reduction concomitant with O₂ evolution, ATP formation, and NADP reduction were determined in isolated spinach (Spinacia oleracea L. var. America) chloroplast lamellae fortified with NADP and ferredoxin. These reactions were investigated in the presence or absence of catalase, providing a tool to estimate the reduction of O₂ to H₂O₂ (Mehler reaction) concomitant with NADP reduction. In the presence of 250 micromolar O₂, O₂ photoreduction, simultaneous with NADP photoreduction, was dependent upon light intensity, ferredoxin, Mn²⁺, NADP, and the extent of coupling of phosphorylation to electron flow.

In the presence of an uncoupling concentration of NH₄⁺, saturating light intensity (>500 watts/square meter), saturating ferredoxin (10 micromolarity) rate-limiting to saturating NADP (0.2–0.9 millimolarity), and Mn²⁺ (50–1000 micromolarity), the maximum rates of O₂ reduction were 13–25 micromoles/milligram chlorophyll per hour, while concomitant rates of O₂ evolution and NADP reduction were 69 to 96 and 134 to 192 micromoles/milligram chlorophyll per hour, respectively. Catalase did not affect the rate of NADPH or ATP formation but decreased the NADP/PHO₂ ratios from 2.3–2.8 to 1.9–2.1 in the presence of rate-limiting as well as saturating concentrations of NADP.

Photosynthetic electron flow at a rate of 31 micromoles O₂ evolved/milligram chlorophyll per hour was coupled to the synthesis of 91 micromoles ATP/milligram chlorophyll per hour, while the concomitant rate of O₂ reduction was 0.6 micromoles/milligram chlorophyll per hour and was calculated to be associated with an apparent ATP formation of only 2 micromoles/milligram chlorophyll per hour. Thus, electron flow from H₂O to O₂ did not result in ATP formation significantly above that produced during NADP reduction.

A significant increment of light-dependent O₂ uptake in photosynthetic tissues and cells is the result of O₂ reduction to H₂O₂ (18, 21, 30) and this reductive reaction is associated with PSI activity coupled to noncyclic electron transport (10, 15, 18, 21, 23, 30). In preparations of NADP-deficient chloroplast lamellae, it has been established that O₂ photoreduction results in the formation of both the superoxide-free radical (O₂−•) as well as H₂O₂ (Mehler reaction) (4, 11–13, 19). Electron flow from H₂O through the photosystems to O₂ was shown to be associated with ATP synthesis and was designated as pseudocyclic photophosphorylation (1, 3, 15). However, recent evidence has indicated that the primary function of O₂ reduction is to prevent over-reduction and proper poising of redox states for components of the photosynthetic electron transport chains (3, 16, 28, 30, 32). Additionally, current evidence strongly suggests that O₂ serves to regulate interaction between the cyclic and noncyclic electron transport systems (3, 16, 28, 32).

One of the questions which has remained is whether or not O₂ photoreduction can be observed with chloroplast preparations in the presence of rate-limiting as well as saturating levels of NADP. The experiments of Elstner et al. (12) indicated that in isolated spinach plastid lamellae, fortified with NADP and Fd, there was not a measurable light-dependent formation of O₂−• or H₂O₂ until 85 to 90% of the total NADP supplied had been reduced. This observation implied that, when NADP levels were saturating, there was no competition between NADP and O₂ for reducing sites on the enzyme Fd-NADP reductase. In contrast, Allen (1) observed a measurable O₂ photoreduction to H₂O₂ in plastid lamellae supplied with Fd and NADP, and these data were interpreted to mean that there existed a constant competition between NADP and O₂ for reducing equivalents.

In this report, we present evidence that there is a significant reduction of O₂ to H₂O₂ concomitant with NADP photoreduction in lamellae fortified with NADP andFd. We show that the rate of O₂ reduction concomitant with NADP reduction was dependent upon light intensity, Fd, Mn²⁺, and NADP and was affected by an uncoupling agent. We also demonstrate and discuss the influence of the Mehler reaction upon the stoichiometric relationships between NADP reduced and O₂ evolved. We found that the Mehler reaction, when functioning simultaneously with NADP photoreduction, was associated with a low rate of photophosphorylation, especially when compared with that amount of ATP concomitantly coupled to NADP reduction.

Preliminary reports concerning these observations have been presented (24, 25).

MATERIALS AND METHODS

Plant Material. Plants (Spinacia oleracea L. var. America) were propagated from seed sown in vermiculite in circular plastic dishes approximately 30 cm deep and 50 cm in diameter. The plants were irrigated twice weekly with Rapid-Gro fertilizer solution (3.5 g/L). Mature leaves were selected from 6- to 10-week-old plants which had been grown on a cycle of 8 h illumination at 25°C and...

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16 h dark at 18°C. Average light energy on the plants was 300 to 500 w/m².

Chloroplast Lamellar Membranes. Twelve to 19 g diced spinach leaf tissue was placed in a Micro-Monel homogenizer with 75 to 80 ml blending medium (Solution 'H') and macerated with two, 1-s bursts at full line voltage. Solution H consisted of 50 mm Hepes-NaOH (pH 6.8); 0.33 mM sorbitol; 2 mm Na₂-EDTA; 1 mm MgCl₂, and 1 mm MnCl₂. Alternatively, plastids were prepared in a homogenizing medium containing 50 mm Hepes-NaOH (pH 6.8) and 0.33 mM sorbitol. The homogenate was filtered through one layer of Miracloth and centrifuged at 755g for 50 s. The resulting supernatant was discarded and the pellet consisting of intact plastids was resuspended in 40 ml 50 mm Hepes-NaOH (pH 7.0) and kept at 4°C in ice for 5 to 10 min to ensure complete osmotic shock. Following centrifugation at 5090g for 5 min, from the pellet was resuspended in 40 ml 50 mm Hepes-NaOH (pH 7.0) and recentlyrifuged at 5090g for 5 min. The supernatant was discarded and the pellet was suspended in 1.5 to 2.5 ml of the sorbitol/homogenizing medium at a concentration of 50 to 60 µg Chl/50 µl of suspension. Chl was estimated in 80% acetone as previously described (26).

Ferredoxin. Spinach Fd was prepared including Step 3 of the purification of Buchanan and Arnon (8). Ferredoxin was quantitated at 465 nm employing the mm extinction coefficient value 8.24 (20). These preparations, suspended in 50 mm Hepes-NaOH (pH 7.6), contained approximately 11 to 22 nmol Fd/100 µl.

Ferredoxin-NADP Reductase. Fd-NADP reductase was partially purified according to the method of Shin et al. (27). These preparations were obtained from the 0.20 M Cl⁻ DEAE-cellulose elution step (just prior to Fd elution), and the resulting saline eluate was dialyzed at 5°C against 50 mm Hepes-NaOH (pH 7.8) with five changes of dialysis solution over 48 h. The reductase activity was assayed by its transhydrogenase activity using a procedure similar to that described by Nelson and Neumann (20).

The assay of 1.05 ml contained 50 mm Hepes, pH 7.6, 8.6 µM NADP, 2.44 mM NAD, 1 mM MgCl₂, 1.5 units glucose-6-P dehydrogenase, and 0.10 ml reductase, and the reaction was started by addition of 5 mm glucose-6-P. In this system, a catalytic level of NADP was converted to NADPH by glucose-6-P dehydrogenase which in turn, reduced NAD to NADH with concomitant recycling of NADH. The reaction was monitored at 340 nm. One hundred µl of the reductase preparation converted NADPH to NADH at a rate of 22.4 nmol/min. Using the data of Nelson and Neumann (20), it was calculated that 100 µl represented approximately 0.50 nmol Fd-NADP reductase assuming a mol wt of 40,000 for this protein (27).

It must be emphasized that the influence of Fd in enhancement of O₂ photoreduction was not associated with over-reduction of Fd at sites where Fd-NADP reductase was lost from lamellae during isolation. The lamellar preparations used here were characterized by displaying the same rate of NADPH photoreduction in the presence of exogenously supplied Fd-NADP reductase as they did in the absence of this flavoprotein. For example, in the presence of approximately 0.10 µM Fd-NADP reductase preparation, the rate of NADPH photoreduction was 222.3 µmol NADPH formed/mg Chl-h, and in the absence of the supplied reductase, it was 229.8; thus, the reductase was not deficient in our plastid preparations.

Light-Dependent O₂ Evolution and Consumption and H₂O₂ Synthesis. Light-dependent O₂ evolution and consumption was monitored with a Clark-type O₂ electrode polarograph-amplified system designed and built by George J. Johnson, Baltimore, MD. O₂ electrodes were constructed by William Lauer of Catonsville, MD. In these determinations, the reaction media usually consisted of 50 mM Tricine-NaOH (pH 8.1), 0.33 mM sorbitol, 1 mM MnCl₂, lamellae equivalent to 35 to 98 µg Chl, 1 or 4 mM MgCl₂; 2 mM Na₂-EDTA; 0.2 to 0.9 mM NADP, and 2 to 11 µM Fd. Additionally, the mixtures contained 5 mM NH₄Cl and/or 1 mM ADP plus 1 mM Pi. Where indicated, 1100 units of catalase were added. The rate of O₂ uptake was estimated from the difference between the rate of O₂ evolution in the presence compared with the rate in the absence of catalase.

The Clark-type electrode reduces O₂ to H₂O₂ as part of its detection mechanism, but normally, H₂O₂ is instantly decomposed to form hydroxyl radical and hydroxide ion (9). In order to be certain that all of the H₂O₂ in our reaction mixtures was due to the chloroplastic activity (9), levels of O₂ up to approximately 500 µl were introduced into mixtures consisting of 50 mM Tricine-NaOH (pH 8.1), 0.33 mM sorbitol, 2 mM Na₂-EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 11 µM Fd, and 1 mM NADP. Neither in the light nor in the dark did the addition of catalase produce any O₂ 'bursts,' indicating that H₂O₂ was not accumulating in these solutions due to nonplastid O₂ reactions.

When O₂ photoreduction was examined as a function of light intensity, copper- or stainless steel-meshed screens were positioned in series between the O₂ electrode chamber and the projector lamp lens. A Lucite water reservoir was placed directly in front of the O₂ electrode chamber. The projector lamp, maintained at full voltage, was either a General Electric or a Sylvania DAK bulb. The light energy was estimated with a Yellow Springs Instrument Co. model 65A radiometer. Energy readings were taken at the face of the electrode chamber.

Estimation of NADP, NADPH, and ATP. To determine NADPH, samples of 0.2 ml were removed from the Clark O₂ electrode chamber at a given time point and pipetted into 1.0 or 2.8 ml of 50 mm Hepes-NaOH (pH 7.6) containing 1 to 5 units yeast glutathione reductase. NADPH was estimated from the decrease in A at 340 nm upon addition of 1.5 µmol oxidized glutathione. Measurements were made within 30 s after sampling.

ATP was estimated by pipetting an 0.20 ml aliquot of reaction mixtures into 1.0 or 2.80 ml 50 mm Hepes-NaOH (pH 7.6) with 2 units glucose-6-P dehydrogenase, 2 units hexokinase, 5 µmol MgCl₂, and 0.5 µmol NADP and then recording the increase in A at 340 nm by supplying 2 µmol glucose in 3.1 ml final volume. Measurements were made within 30 to 90 s after sampling.

NADP was assayed in 50 mm Hepes-NaOH (pH 7.6), 1 mM MgCl₂, 2 units glucose-6-P dehydrogenase, 0.7 mM glucose-6-P (to initiate), and sample containing NADP in a total volume of 1.0 ml. NADP was estimated from the increased A at 340 nm due to NADPH formation.

ATP, ADP, NAD(H), NADP(H), glutathione and its oxidized form, glucose-6-P, glucose-6-P dehydrogenase, hexokinase, glutathione reductase, and catalase (thymol-free) were obtained from Sigma Chemical Co.

RESULTS

When measured under anaerobic conditions, the stoichiometry of NADPH formation and O₂ evolution associated with the photosynthetic cleavage of H₂O has been observed to be 2.0 (3, 6). When this relationship was examined in the presence of air, Ben Hayyim and Avron (7) reported that Mn²⁺ diminished O₂ evolution without affecting NADPH reduction. In their study (7), presumably the decrease in O₂ evolution was due to O₂ consumption resulting in an NADPH/O₂ ratio higher than 2.0.

We have re-examined the NADPH/O₂ stoichiometry resulting from the photoreduction of NADP under aerobic conditions in order to determine whether the Mehler reaction, the photoreduction of O₂ resulting in the formation of H₂O₂ is concomitant with NADPH formation. The simultaneous reductions of O₂ and NADP would be a critical factor in arriving at an explanation of...
Table I. Effect of Light Intensity and Catalase upon the Stoichiometry of NADP Reduction and O₂ Evolution

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Catalase</th>
<th>Light Intensity ( w/m^2 )</th>
<th>NADP Reduction ( \mu mol/mg \text{ Chl-}h )</th>
<th>( O_2 ) Evolution ( O_2 ) Uptake</th>
<th>NADPH:O₂ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>+</td>
<td>1290</td>
<td>186.5</td>
<td>95.3</td>
<td>1.96</td>
</tr>
<tr>
<td>1B</td>
<td>-</td>
<td>1290</td>
<td>195.5</td>
<td>79.8</td>
<td>15.5</td>
</tr>
<tr>
<td>2A</td>
<td>+</td>
<td>1290</td>
<td>164.2</td>
<td>86.0</td>
<td>1.91</td>
</tr>
<tr>
<td>2B</td>
<td>-</td>
<td>1290</td>
<td>165.5</td>
<td>73.6</td>
<td>12.4</td>
</tr>
<tr>
<td>3A</td>
<td>+</td>
<td>99</td>
<td>83.9</td>
<td>44.8</td>
<td>1.87</td>
</tr>
<tr>
<td>3B</td>
<td>-</td>
<td>99</td>
<td>91.2</td>
<td>44.8</td>
<td>0</td>
</tr>
<tr>
<td>4A</td>
<td>+</td>
<td>99</td>
<td>98.3</td>
<td>50.0</td>
<td>1.97</td>
</tr>
<tr>
<td>4B</td>
<td>-</td>
<td>99</td>
<td>99.2</td>
<td>46.7</td>
<td>3.3</td>
</tr>
</tbody>
</table>

It was found that a higher light energy was required to saturate \( O_2 \) consumption than to saturate \( O_2 \) evolution. A threshold saturation of 300 \( w/m^2 \) was determined for \( O_2 \) evolution while concomitant \( O_2 \) uptake required approximately 500 \( w/m^2 \) (data not shown).

**NADP Concentration.** NADP at 0.8 to 0.9 mm in the presence of approximately 11 µm Fd (saturating) was sufficient to yield the maximum rate of NADPH formation in our preparations. In the presence of this concentration of NADP and at high light, the usual \( O_2 \) uptake rates were in the order of 13 to 15 µmol/mg Chl-h but in a few experiments rates of 20 to 26 µmol/mg Chl-h were observed. In contrast, in the absence of NADP or at low (0.2 mm) levels, \( O_2 \) uptake was routinely doubled to 25 to 30 µmol/mg Chl-h (Fig. 1). The data illustrated in Figure 1 are an example of \( O_2 \) evolution and reduction followed polarographically as the NADP was reduced totally from an initial level of 0.2 mm. From the onset of illumination, the rate of \( O_2 \) reduction in the 1st min was 22.4 µmol/mg Chl-h. In the 30 s just prior to the inflection point, where \( O_2 \) evolution balanced \( O_2 \) reduction and the NADP supply was 90% reduced as determined in a separate experiment, the rate of \( O_2 \) reduction increased only slightly to 25.5 µmol/mg Chl-h. After 1.5 min, the NADP became totally reduced, \( O_2 \) uptake was linear, and its rate remained unchanged at approximately 25 µmol/mg Chl-h. Regardless of the point of sampling, the NADPH:O₂ ratio was 2.0 in the presence of catalase but in its absence, these ratios reached values of 3.1.

**Ferredoxin Concentration.** It was well established that chloroplast preparations without NADP and fortified with Fd will reduce \( O_2 \) to \( H_2O_2 \) with \( O_2^- \) as an intermediate (11). We observed that, in the presence of 2.1 µm Fd, the rate of \( O_2 \) evolution was 48.9 µmol/mg Chl-h in the presence of and 35.9 in the absence of catalase or 13.0 µmol \( O_2 \) reduced/mg Chl-h. When the concentration of Fd was increased to 11.0 µm, the rate of \( O_2 \) evolution increased to 95.6 µmol/mg Chl-h in the presence of and 70.0 in the absence of catalase or a rate of 25.6 µmol \( O_2 \) consumed/mg Chl-h. The level of Fd which produced threshold saturation for \( O_2 \) evolution was similar for that of \( O_2 \) reduction (data not shown).

\( Mn^{2+} \). Publications from several laboratories have established that \( Mn^{2+} \) stimulated \( O_2 \) reduction in illuminated broken chloroplast preparations (4, 7, 13, 31) when NADP was absent. Apparently, the cation participates in superoxide dismutation (17). In the presence of NADP photoreduction, we observed that the addition of \( Mn^{2+} \) at 1.0 mm stimulated \( O_2 \) photo-induced consumption (Table II). For example, the rate of \( O_2 \) reduction in the absence of \( Mn^{2+} \) was usually 0 to 6 µmol/mg Chl-h while it was 10 to 13 in the presence of 1.0 mM \( Mn^{2+} \). Concentrations as low
Table II. Effect of Mn²⁺ and Catalase upon the Stoichiometry of NADP Reduction and O₂ Evolution

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Catalase</th>
<th>Mn²⁺</th>
<th>NADP Reduction</th>
<th>O₂ Evolution</th>
<th>O₂ Uptake</th>
<th>NADPH:O₂†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>μmol/mg Chl-h</td>
<td></td>
<td></td>
<td>ratio</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>−</td>
<td>109.4</td>
<td>51.7</td>
<td>5.5</td>
<td>2.11</td>
</tr>
<tr>
<td>1B</td>
<td>−</td>
<td>−</td>
<td>114.6</td>
<td>46.2</td>
<td>5.5</td>
<td>2.48</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>112.1</td>
<td>52.5</td>
<td>13.0</td>
<td>2.13</td>
</tr>
<tr>
<td>2B</td>
<td>−</td>
<td>+</td>
<td>113.1</td>
<td>39.5</td>
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<td>2.86</td>
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</table>

Table III. Stoichiometry of NADP Reduction and O₂ Evolution in Coupled and Uncoupled Chloroplasts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Catalase</th>
<th>NH₄Cl</th>
<th>NADPH Reduction</th>
<th>O₂ Evolution</th>
<th>O₂ Uptake</th>
<th>NADPH:O₂†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>μmol/mg Chl-h</td>
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<td>ratio</td>
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<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>133.8</td>
<td>69.0</td>
<td>12.8</td>
<td>1.91</td>
</tr>
<tr>
<td>1B</td>
<td>−</td>
<td>+</td>
<td>132.3</td>
<td>56.2</td>
<td></td>
<td>2.35</td>
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<td>−</td>
<td>107.0</td>
<td>55.5</td>
<td></td>
<td>1.87</td>
</tr>
<tr>
<td>2B</td>
<td>−</td>
<td>−</td>
<td>110.9</td>
<td>48.5</td>
<td></td>
<td>2.27</td>
</tr>
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</table>

as 50 μM Mn²⁺ were sufficient to produce the maximum rate of O₂ photooxidation (data not shown).

We also observed that 1 mM Na₂-EDTA stimulated O₂ consumption in the presence or absence of NADP but this stimulation was half of that observed with Mn²⁺. When Mn²⁺ and Na₂-EDTA were supplied in equimolar levels, the stimulation of O₂ uptake equaled that caused by Mn²⁺ alone.

The Uncoupler NH₄⁺. We examined the possibility as to whether phosphorylating conditions affected O₂ photooxidation. As seen in Table III, the rate of O₂ evolution in preparations supplied with ADP and Pi were 8 times higher than the rate of O₂ reduction 55.7 versus 7.0 μmol/mg Chl-h. However, uncoupling of phosphorylation from electron flow with NH₄⁺ not only stimulated O₂ evolution and NADPH formation, but the cation also stimulated O₂ uptake.

The rate of O₂ reduction was increased approximately 1.8-fold in the presence of, as compared to the absence of, the uncoupler, but the rate of O₂ evolution and NADPH formation increased only 1.25-fold (Table III, compared Experiment 1 with 2). In other experiments (not shown), the uncoupler increased the rate of O₂ reduction from 5 to 22 μmol/mg Chl-h and O₂ evolution from 70 to 90 μmol/mg Chl-h.

**Contribution of ATP Formation from Noncyclic and Pseudocyclic (O₂ Reduction) Phosphorylation.** To account for ATP/2e ratios higher than 1.0, investigators have invoked a contribution from pseudocyclic photophosphorylation to provide the extra ATP (14). With respect to pseudocyclic photophosphorylation, ATP is formed by a noncyclic system where O₂ is taken up and evolved in a 1:1 ratio, and cannot be readily distinguished from the linear type of noncyclic photophosphorylation associated with NADP reduction. We have taken advantage of a suggestion of Hall (14) and determined the rates of O₂ evolution, NADP reduction, ATP formation, and O₂ consumption in the presence of 1 or 4 mM MgCl₂ with the purpose of distinguishing between the two kinds of phosphorylation sequences. The data presented in Table IV show that an increase in Mg²⁺ from 1 to 4 mM resulted in a 50% increase in O₂ evolution and NADPH formation with a 500% increase in ATP synthesis but only a very small positive change in O₂ consumption.

Based upon the observed changes in the molar amounts of O₂, ATP, and NADPH, the stoichiometry of electron flow coupled to phosphorylation was calculated to be 1 O₂ evolved:2.9 ATP:2 NADPH:0.02 O₂ consumed. This stoichiometry indicated that 39.2% of the measured electron flow from H₂O → NADP was associated with ATP formation. Assuming an ATP/2e ratio of 1.47 (Table IV), the rate of O₂ reduction and ATP formation coupled with this oxidative reaction would be 0.6 and 1.8 μmol/mg Chl-h, respectively. Clearly, 90% of the observed O₂ uptake appeared not to be associated with ATP synthesis.

**DISCUSSION**

Our experiments showed that photosynthetic electron flow in preparations of spinach chloroplast lamellae fortified with satu-
H₂O₂ SYNTHESIS AND NADP PHOTOREDUCTION

Table IV. Influence of Mg²⁺ Concentration upon O₂ Evolution, NADP Reduction, ATP Formation, and Concomitant O₂ Photoreduction in Plastid Lamellae

<table>
<thead>
<tr>
<th>Catalase</th>
<th>O₂ evolved</th>
<th>ATP formed</th>
<th>NADPH formed</th>
<th>O₂ consumed</th>
<th>NADPH/O₂</th>
<th>Observed Rates</th>
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</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>mm</td>
<td>nm</td>
<td>nmol</td>
<td>ratio</td>
<td>μmol/mg Chl-h</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>1</td>
<td>113.8</td>
<td>222.7</td>
<td>1.96</td>
<td>52.3</td>
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</tr>
<tr>
<td>Absent</td>
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<td>101.4</td>
<td>39.5</td>
<td>2.20</td>
<td>47.1</td>
<td></td>
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<tr>
<td>Present</td>
<td>4</td>
<td>167.0</td>
<td>327.0</td>
<td>1.96</td>
<td>77.5</td>
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<td>Absent</td>
<td>4</td>
<td>153.4</td>
<td>334.0</td>
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Part B

Derived Stoichiometry

<table>
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<tr>
<th>O₂↑ (catalase present)</th>
<th>ATP</th>
<th>NADPH</th>
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<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>O₂↓</td>
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</table>

Actual Rates of Syntheses Associated with Photophosphorylation

<table>
<thead>
<tr>
<th>O₂↑</th>
<th>ATP</th>
<th>NADPH</th>
<th>O₂↓</th>
<th>ATP₀↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol/mg Chl-h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.9</td>
<td>91.3</td>
<td>60.8</td>
<td>0.6</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*nm = not measured (see table caption, Part B, 2).

rating NADP and Fd was associated with light-dependent O₂ reduction (Fig. 1; Tables I–III). Examination of O₂ evolution, NADP reduction, and photosynthetic phosphorylation in the presence of catalase provided a method for monitoring concomitant O₂ reduction to H₂O₂. In our plastid preparations with catalase absent, an increment of the evolved O₂ was consumed, but the presence of catalase resulted in complete recovery of the reduced O₂. This meant that O₂ reduction was mediated with reducing equivalents derived only from the photolytic cleavage of H₂O₂. Based on the repeated observation that the presence of catalase resulted in NADP%H₂O₂ values of approximately 2, but the absence of catalase resulted in values from 2.3 to 3.1 (Fig. 1; Tables I–III), it was clear that the addition of catalase resulted in causing the net O₂ uptake of the Mehler reaction to balance the release of O₂ from the H₂O₂ produced, e.g.

\[ 2H₂O + O₂ \xrightarrow{\text{plastid lamellae}} 2H₂O₂ \]

\[ 2H₂O₂ \xrightarrow{\text{catalase}} 2H₂O + O₂↑ \]

The result was that the only measurable noncyclic electron flow was H₂O₂ → NADP.

Two factors which significantly enhanced the O₂ reduction rate were high light intensity and uncoupling of phosphorylation from electron flow (Fig. 1; Tables I and III). These conditions increase electron pressure (30), and we conclude that O₂ reduction may be a reflection of prevention of over-reduction of the electron transport chain rather than the delivery of ATP beyond that provided by the H₂O₂ → NADP series of reactions. A similar conclusion has been reached in several laboratories where estimation of O₂ photoreduction was made in preparations of intact chloroplasts photosynthesizing in saturating CO₂ (10, 16, 30, 32) or in broken chloroplast preparations consuming O₂ (3). Under conditions where CO₂ was saturating in studies with whole chloroplasts, O₂ was required for production of additional ATP so that the total adenylate requirement for CO₂ assimilation could be satisfied (28, 30, 32), but it appeared likely that the ATP originated not from pseudocyclic electron transport, but from cyclic and noncyclic electron transport (3, 16, 30, 32). It has been postulated that O₂ reduction serves to maintain the proper poised of electron transport such that cyclic and noncyclic electron flow may coexist optimally (28, 32). Our data (Table IV) do not confirm or deny the existence of cyclic electron transport and cyclic photophosphorylation, but it does indicate that if O₂ serves to poised electron transport components, it does so through a Mehler type reaction. O₂ photoreduction in Scenedesmus obliquus cells and isolated spinach leaf mesophyll cells and chloroplasts exhibits the highest
rates when CO₂ is rate-limiting to photosynthesis and in the initial lag phase associated with 1 to 2 min postillumination (18). In the lag phase of photosynthesis, which appears to represent a period of electron transport chain over-reduction, Marsho et al. (18) found that the O₂ photoreduction rate balanced the O₂ evolution rate. Their reaction conditions appear comparable with that shown in Figure 1 where NADP was essentially totally reduced to NADPH at the inflection point, and this presumably would reflect a maximal tendency for over-reduction of photosynthetic electron transport chain components.

Our results indicate that O₂ and NADP compete for reducing equivalents. When O₂ levels are 250 μM O₂ (air level), O₂ is saturating with respect to the O₂ reduction sites in the chloroplast lamellae, in intact plastids, and in whole cells (5, 18, 22, 24). Additionally, the Kₐₐ of Scenedesmus cells and isolated spinach leaf mesophyll cells for O₂ photoreduction was approximately 96 μM (18, 23), and a value of Kₐₐ of 75 μM was found for reduction in intact spinach chloroplast preparations under conditions favoring full photosynthetic competence (30). In preparations of plastid lamellae, the Kₐₐ for O₂ reduction has been estimated to be as low as 2 to 3 μM (5). We found that lamellae O₂ reduction was saturated fully with respect to O₂ levels as low as 155 μM and our measurements yielded Kₐₐ values of 13.5 to 22.2 μM (24). O₂ concentrations in the illuminated intact chloroplast may rise as high as 1 mM (29), such that in vivo it is likely that the sites where O₂ may bind to be reduced are strongly competed for by other reducible moieties, e.g., NADP and nitrite. Our results indicate that this competition is complete at NADP levels of at least 0.2 mM, because the rate of O₂ reduction at that concentration of NADP followed to complete reduction was associated with a continuous rate of O₂ photoreduction of 22 to 25 μmol/mg Chl (Fig. 1). At 0.9 mM NADP, rates of O₂ reduction were usually 50% less than those rates observed at 0.2 mM NADP (Fig. 1; Tables I-III). We conclude that O₂ has a strong enough affinity for reducing sites so that there is always a potential for O₂ reduction even when NADP is saturating.

Finally, we must note that O₂ reduction was stimulated by Mn²⁺, although concomitant NADP reduction was not affected (Table II). Manganese ion has been reported to enhance O₂ reduction (7, 13, 31) by an increase in dismutation of O₂⁻ to H₂O₂ (13). Mn²⁺ has been shown to catalyze oxidation of H₂O₂ to O₂ (31), and the net effect of adding Mn²⁺ to plastid lamellae preparations appears to result in a Mn²⁺/H₂O₂ cyclical superoxide dismutase system (4). In conjunction with these observations, a Mn²⁺-dependent, plastid lamellae-bound superoxide dismutase has been observed (17). Since the chloroplast stromal Mn²⁺ level has been estimated at 400 μM (4) and we found that 50 μM Mn²⁺ enhanced maximally O₂ photoreduction, we conclude that Mn²⁺ could play a physiological role in driving O₂ reduction when that process is required to compensate for over-reduction of electron transport components.

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