Influence of Varying CO₂ and Orthophosphate Concentrations on Rates of Photosynthesis, and Synthesis of Glycolate and Dihydroxyacetone Phosphate by Wheat Chloroplasts

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ABSTRACT

Intact chloroplasts of wheat (Triticum aestivum) were isolated from mesophyll protoplasts. With decreasing concentrations of bicarbonate from 10 to 0.3 millimolar (pH 8.0), the optimal concentration of orthophosphate (Pi) for photosynthetic O₂ evolution decreased from a value of 0.1 to 0.2 millimolar to 0 to 0.025 millimolar. The extremely low Pi optimum for photosynthesis at the low bicarbonate levels of 0.3 millimolar was increased by lowering the O₂ concentration from 253 (21% gas phase) to 72 micromolar (6% gas phase). The relative amount of glycolate and dihydroxyacetone phosphate (DHAP) synthesized under high and low levels of bicarbonate and varying levels of Pi was determined. At low levels of bicarbonate, glycolate was the main product, whereas at high bicarbonate levels, DHAP was the main product. Most of the DHAP and glycolate was found in the extrachloroplast fraction.

The rate of photosynthesis at low levels of bicarbonate (0.3 millimolar) was as high as 75 to 95% of that at high levels of bicarbonate (10 millimolar) at the respective optimal levels of Pi. At low bicarbonate levels, and without Pi, there was little lag in photosynthetic O₂ evolution upon illumination in comparison to that of high bicarbonate levels and optimal levels of Pi. It is proposed that conditions which favor glycolate synthesis allow photosynthesis to continue without a depletion of internal Pi, whereas consumption of Pi may occur in the chloroplast during the net synthesis of organic phosphates under high levels of bicarbonate and without addition of Pi. At low bicarbonate levels, the extreme susceptibility of photosynthesis to inhibition by Pi may be due to excessive export of carbon from the chloroplast in the form of both glycolate and triose phosphate.

Photosynthesis by isolated chloroplasts is dependent on an optimum level of orthophosphate in the medium. The stoichiometric uptake of Pi into and export of DHAP from the chloroplasts and metabolism of DHAP to sucrose in the cytoplasm are an established part of photosynthetic carbon assimilation. High levels of exogenous Pi inhibit photosynthesis by causing excessive exchange of triose-P from the chloroplast which limits the accumulation of intermediates for function of the cycle (17, 18). Studies on the Pi dependence of photosynthesis of chloroplasts have normally been conducted at relatively high bicarbonate levels, which repress glycolate synthesis. In the present study, characteristics of photosynthetic O₂ evolution of isolated chloroplasts under low bicarbonate levels are considered.

MATERIALS AND METHODS

Plant Material. The seeds of wheat were obtained from Old's Seed Company (Madison, WI) or kindly provided by Dr. T. Yoshida of the Central Research Station of the Upland Farming Research Center, Japan. In the experiments of Figures 1 and 2 wheat (Triticum aestivum L.) was grown in a growth chamber with a light/dark period of 15/9 h and a day/night temperature regime of 20/15°C. The photon flux density at plant height was approximately 400 μE/m²·s between 400 to 700 nm from a mixture of fluorescent and incandescent lamps.

In all other experiments, wheat was grown under similar conditions except the light intensity at plant height was approximately 100 μE/m²·s between 400 to 700 nm. In this case, light was provided by fluorescent lamps (Toshiba FL 20 BRF) specifically developed for plant growth and having peaks of light energy at approximately 460 and 660 nm.

Chemicals. Cellulase (Onozuka R-10) and pectinase (Maceroyzyme R-10) were obtained from Yakult Biochemical Co., Ltd., Nishinomiya, Japan. Other reagents were of the highest purity available from several sources.

Isolation of Mesophyll Chloroplasts. Mesophyll protoplasts were isolated enzymically, as described previously, using a combination of Cellulase and Maceroyzyme (2). The protoplasts of wheat were suspended in 0.4 M sorbitol, 50 mM Hepes-KOH (pH 8.0), and 5 mM EDTA (medium A). In the preparation of wheat chloroplasts, protoplasts were ruptured by four passes through a 20 μm nylon net (Tetko, Inc., 420 Saw Mill River Road, Elmsford, NY) attached to a 5-ml syringe as described previously (2). The protoplast extracts were centrifuged for 1 min at 650 g to pellet the chloroplasts. The chloroplasts were then suspended in medium A. The percentage of intact chloroplasts as determined by ferricyanide-dependent O₂ evolution before and after osmotic shock (12) was greater than 85%. Chl was determined by the method of Wintermans and De Mots (19).

CO₂-Dependent O₂ Evolution. CO₂-dependent oxygen evolution of wheat chloroplasts was measured polarographically in a 200 cm electrode (Rank Bros., Cambridge, United Kingdom) at 25°C. In the experiments of Figures 1 and 2, the basal assay medium contained 0.4 M sorbitol, 50 mM Hepes-KOH (pH 8.0), and 5 mM EDTA. Assay volume was 1 ml and contained 30 μg Chl. The quantum flux density at the surface of the cuvette of 1,800 μE·m⁻²·s⁻¹ between 400 to 700 nm was provided by a 150 W Sylvania flood lamp.

In all other experiments, the basal assay medium contained 0.4 M sorbitol, 50 mM Hepes-KOH (pH 8.2), and 0.1 mM EDTA. The

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2 Abbreviations: DHAP, dihydroxyacetone phosphate; PCA, perchloric acid; PGA, 3-phosphoglyceric acid; RuBP, ribulose 1,5-bisphosphate.
basal medium was flushed with CO₂-free air for at least 1 h prior to use. The assay volume was 1 to 2 ml and contained 43 to 80 μg Chl/ml. In assays with 0.3 mM NaHCO₃ over 6 min (Figs. 3, 4, and Table I), calculations indicate that at least 50% of the inorganic carbon remained at the end of the assay (considering a maximum of 2 CO₂ fixed/1.5 O₂ evolved during glycocolate formation, see under “Discussion”). EDTA is essential for bicarbonate-dependent photosynthetic O₂ evolution by wheat chloroplasts; and the concentration of chelator required decreases with increasing pH from 7.2 to 8.4 (3). Inasmuch as EDTA interferes with the determination of glycocolate by the Calkins method (9, 13), the minimum level of EDTA (0.1 mM) needed to satisfy the chelation requirement was used in experiments subsequent to those of Figures 1 and 2. With this amount of EDTA, its interference in the assay of glycocolate determination was not significant. The quantum flux density at the surface of the cuvette of approximately 6,000 μE/m²·s between 400 to 700 nm was provided by a 500 W incandescent lamp.

**Determination of the Amount of DHAP and Glycolate.** In determining the amount of DHAP and glycocolate synthesized during photosynthesis, samples were taken prior to illumination and from a separate treatment after 6 min in the light. The values from the respective dark treatments were very low and these were subtracted from the values obtained in the light. For each treatment in determining DHAP, a sample of 0.6-ml was withdrawn from a 2-ml assay volume and 36 ml 20% PCA was added to each sample. After 3 min, 30 ml 2.5 M K₂CO₃ was added to bring the mixture to neutral pH. The precipitate was removed by centrifugation. The amount of DHAP was determined enzymically using L-glycerol-3-P dehydrogenase (6). This procedure gave approximately 100% recovery of authentic DHAP.

For glycocolate determination, two samples of 0.6 ml/treatment were withdrawn from a 2-ml assay volume and were heated for 3 min at 100°C in the presence or absence of 60 nmol authentic glycocolate. After centrifugation, an aliquot was applied to a 0.6 × 3-cm column of Dowex 1 × 8 (100–200 mesh), acetate form. The column was washed thoroughly with water, and the glycolic acid was eluted with 4 N acetic acid. The eluates were dried under vacuum. The glycolic acid content of the residue was then determined by Calkins’ method (1). On the average, the percentage of recovery of glycocolate was 56% (a range of 47–62% over all experiments).

**Distribution of DHAP and Glycolate Between the Chloroplasts and Assay Medium.** The distribution of DHAP and glycocolate was investigated by rapid separation of the chloroplasts from the medium in a Beckman microfuge B. Samples of 1 ml were withdrawn from the 1.5-ml assay volume and centrifuged for 10 s at 10,000g in a 1.5-ml microfuge tube. The supernatant (extrachloroplast fraction) and pellet (chloroplasts) were immediately separated.

For the determination of the amount of DHAP, 60 μl 20% PCA were added to the supernatant fraction and 60 μl 20% PCA and 1 ml of 0.4 M sorbitol and 50 mM Hapes-KOH (pH 8.2) were added to the pellet. After 3 min, 50 μl 2.5 M K₂CO₃ were added to both fractions, and the amount of DHAP was determined as described above.

For the determination of glycocolate, 1 ml 0.4 M sorbitol and 50 mM Hapes-KOH (pH 8.2) was added to the pellet. Both the supernatant and chloroplast fractions were incubated at 100°C for 3 min, and the amount of glycocolate was determined as described above. Corrections were made for cytoplasmic contamination of the chloroplast fraction by addition of 20 mM exogenous sucrose to the chloroplast suspension, followed by centrifugation and determination of the distribution between the pellet and supernatant fraction. Sucrose, which is considered nonpermeant to intact chloroplasts, was determined enzymically according to the method of Jones et al. (7) after heating the samples in 0.04 N NaOH at 95°C for 30 min. Corrections for loss of metabolites from the chloroplasts due to breakage of the organelles were made by determining the distribution of NADP-glyceraldehyde 3-P dehydrogenase (as assayed in ref. 10) in the chloroplast pellet and supernatant fractions.

**RESULTS**

**Photosynthetic O₂ Evolution with Varying Levels of Bicarbonate, Pi, and O₂.** As shown in Figure 1, the Pi optimum for photosynthetic O₂ evolution with wheat chloroplasts decreased with decreasing concentration of bicarbonate. In this experiment, the highest rate of photosynthesis in the presence of 0.3 mM bicarbonate was without addition of Pi. Under this condition, photosynthesis was 75% of the maximum rate obtained at high bicarbonate levels and optimal levels of Pi.

The increased sensitivity of photosynthetic O₂ evolution to Pi at low bicarbonate concentration was largely eliminated by reducing the O₂ level from 253 μM (21% in gas phase) to 72 μM (equivalent to 6% in gas phase) (Fig. 2). Maximum rates of photosynthesis under 0.3 mM bicarbonate and 21% O₂ were again obtained at low Pi levels (in this experiment at approximately 25 μM Pi).

At 10 mM bicarbonate, and in the absence of Pi, there was an initial rate of O₂ evolution upon illumination which rapidly diminished with time. Addition of 0.1 mM Pi restored O₂ evolution to the initial rate (Fig. 3a). With 10 mM bicarbonate and 0.2 mM Pi there was a substantial lag following illumination, after which the rate of O₂ evolution became linear (Fig. 3c). These photosynthetic responses at high bicarbonate are similar to those previously observed with wheat and spinach chloroplasts (2, 17). In contrast, at low bicarbonate, photosynthetic O₂ evolution was maintained at a near linear rate over the 6-min assay period in the absence of

![Figure](image-url)

**FIG. 1.** Effect of NaHCO₃ concentration on the Pi optimum for photosynthetic O₂ evolution of isolated chloroplasts of wheat.

![Figure](image-url)

**FIG. 2.** Effect of NaHCO₃ concentration and O₂ on the Pi optimum for photosynthetic O₂ evolution of isolated chloroplasts of wheat. For assays at 72 μM O₂ (equivalent to 6% gas phase) nitrogen gas was passed over the reaction media in the O₂ electrode for several minutes until the O₂ tension reached about 25 μM. The electrode chamber was immediately closed. The reaction mixture was illuminated and the rate of O₂ evolution was calculated when the O₂ concentration reached 72 μM.
Pi (Fig. 3b), whereas there was little activity at 0.2 mM Pi (Fig. 3d).

Synthesis of DHAP and Glycolate with Varying Levels of Pi and Bicarbonate. Under high levels of bicarbonate (10 mM), the net synthesis of DHAP by the wheat chloroplasts tends to parallel the net rate of 0\textsubscript{2} evolution at varying levels of Pi over the 6-min assay period. There is little synthesis of glycolate under the high bicarbonate concentration (Fig. 4).

At low levels of bicarbonate (0.3 mM) and low Pi, glycolate was a major product and its synthesis paralleled the rate of photosynthesis at varying Pi concentrations. Under low bicarbonate, the highest rate of photosynthesis and glycolate synthesis occurred in the absence of Pi. At low levels of bicarbonate, the synthesis of DHAP was relatively low at all Pi levels (Fig. 4).

Distribution of DHAP and Glycolate Between the Chloroplast and Extrachloroplastic Assay Medium. As indicated in Table 1, most of the DHAP and glycolate appeared in the supernatant fraction following removal of chloroplasts by centrifugation, indicating export of these metabolites from the chloroplasts. There was little contamination of the chloroplast pellets by the extrachloroplastic fraction as indicated by partitioning of the exogenous sucrose. Since about 30% of NADP-dependent glyceraldehyde-3-P dehydrogenase appeared in the supernatant fraction, this figure was assumed to represent the degree of chloroplast breakage and the distribution of both metabolites was corrected accordingly. This had little influence on the corrected values since most of the glycolate and DHAP was exported from the chloroplasts.

**DISCUSSION**

Photosynthesis in \textit{C}\textsubscript{3} plants is closely linked to sucrose synthesis and photorespiratory metabolism, both of which occur outside the chloroplasts. Triose-P, exported from the chloroplasts in exchange for Pi, is metabolized to sucrose in the cytoplasm. Glycolate, synthesized in the chloroplasts, is metabolized to glyceraldehyde-3-phosphate through the glycolate pathway. There is little subsequent utilization of DHAP and/or low Pi favors glycolate synthesis while high bicarbonate and/or low 

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![Typical traces of photosynthetic O\textsubscript{2} evolution of isolated chloroplasts of wheat under varying levels of NaHCO\textsubscript{3} and Pi. L. Light on.](image)

![Effect of NaHCO\textsubscript{3} concentration (\textbullet, 10 mM; \textbullet, 0.3 mM) on the optimum Pi concentration for photosynthetic O\textsubscript{2} evolution, and the formation of glycolate and DHAP by isolated chloroplasts of wheat. Photosynthetic O\textsubscript{2} evolution is expressed as the total amount of O\textsubscript{2} evolved during the 6-min assay, and as the maximum rate obtained during the assay period.](image)
Under low levels of Pi, photosynthesis continues at a near linear rate under low bicarbonate which is consistent with internal Pi being continually available during glycolate synthesis. Under high levels of bicarbonate and low Pi, photosynthesis ceases after a few minutes of illumination (Fig. 3).

If glycolate were the only net product of photosynthesis by isolated chloroplasts under conditions most favorable for its synthesis, then the expected stoichiometry is 1.5 $O_2$ evolved per glycolate synthesized.

$$2\text{RuBP} + 2\text{CO}_2 + 2\text{H}_2\text{O} \rightarrow 4\text{PGA}$$

(RuBP carboxylase)  
$$\text{RuBP} + \text{O}_2 \rightarrow \text{PGA} + \text{P}-\text{glycolate}$$

(RuBP oxygenase)  
$$\text{P}-\text{glycolate} + \text{H}_2\text{O} \rightarrow \text{Pi} + \text{glycolate}$$

(P-glycolate phosphatase)  
$$5\text{PGA} + \text{Pi} \rightarrow 3\text{RuBP} + 2.5\text{O}_2 + \text{H}_2\text{O}$$

(reductive + regenerate phase of photosynthesis)

NET: $2\text{CO}_2 + 2\text{H}_2\text{O} \rightarrow 1.5\text{O}_2 + \text{glycolate}$

In the experiment of Table 1, with 0.3 mM bicarbonate and no Pi added, the ratio of $O_2$ evolution to glycolate synthesis calculated over 6 min was $3.1\mu\text{mol} O_2$ evolved/1.58 $\mu\text{mol}$ glycolate formed or 1.96 $O_2$ evolved per glycolate formed (average of two replications). In the experiment of Figure 4, in the absence of Pi and with 0.3 mM bicarbonate, the calculated ratio of $O_2$ evolved per glycolate formed over a 6-min period was also 2.0. Thus, glycolate synthesis can largely account for the $O_2$ evolved. Formation of a relatively small amount of other products, e.g. triose-P and starch, can account for the ratio being greater than 1.5.

In vivo $O_2$ inhibition of photosynthesis is thought to occur through competitive inhibition with $CO_2$ through RuBP carboxylase and the photorespiratory loss of $CO_2$ through the glycolate pathway. Up to three-fourths of the carbon of the glycolate pathway is proposed to return to the chloroplast in the form of glyceraldehyde (15, 16) which would minimize the loss of carbon from the C3 cycle.

With isolated chloroplasts, there is substantial inhibition of photosynthesis by $O_2$ which can be overcome by high bicarbonate levels (4). As in vivo, at least part of the inhibition may be through $O_2$ as a competitive inhibitor of RuBP carboxylase. With isolated chloroplasts, the photorespiratory component through glycolate metabolism would be absent. However, the synthesis of glycolate may be an additional component of $O_2$ inhibition by isolated chloroplasts if it results in excessive removal of carbon from the C3 cycle. Inhibition of photosynthesis by $O_2$ with isolated chloroplasts by depletion of carbon from the C3 cycle is most likely under conditions of high Pi which favors export of carbon from the chloroplasts as triose-P. For example, with wheat chloroplasts at 0.15 mM Pi and 0.3 mM bicarbonate, 253 $\mu\text{mol}$ $O_2$ (atmospheric level) caused a 55% inhibition of photosynthesis in comparison to the rate at 72 $\mu\text{mol}$ $O_2$ (Fig. 2). Conversely, at low levels of Pi, atmospheric levels of $O_2$ showed no inhibitory effects in compar-

Table 1. Distribution of Glycolate and DHAP Between the Chloroplast Pellet and Supernatant Fraction After Assay Under Varying Levels of Pi and NaHCO3

<table>
<thead>
<tr>
<th>Determination</th>
<th>Condition</th>
<th>Maximum Rate of $O_2$ Evolution</th>
<th>$O_2$ Evolved</th>
<th>Metabolite Formed</th>
<th>Measured Values of Metabolite or Enzyme Distribution</th>
<th>Corrected Value for Distribution of Metabolites</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Precipitate ($P$)</td>
<td>Supernatant ($S$)</td>
</tr>
<tr>
<td></td>
<td>Pi NaHCO3</td>
<td>$\mu\text{mol/mg Chi-h}$</td>
<td>$\mu\text{mol/mg Chi-6 min}$</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Glycolate</td>
<td>0 0.3</td>
<td>40.0</td>
<td>3.08</td>
<td>1.69</td>
<td>8.5</td>
<td>91.5</td>
</tr>
<tr>
<td>Glycolate</td>
<td>0.025 0.3</td>
<td>39.0</td>
<td>3.11</td>
<td>1.48</td>
<td>11.1</td>
<td>88.9</td>
</tr>
<tr>
<td>Glycolate</td>
<td>0.025 0.3</td>
<td>28.7</td>
<td>2.06</td>
<td>0.87</td>
<td>5.1</td>
<td>94.9</td>
</tr>
<tr>
<td>Glycolate</td>
<td>0.1 10</td>
<td>99.0</td>
<td>7.10</td>
<td>0.56</td>
<td>2.6</td>
<td>97.4</td>
</tr>
<tr>
<td>DHAP</td>
<td>0.1 10</td>
<td>89.2</td>
<td>6.43</td>
<td>0.51</td>
<td>2.3</td>
<td>97.7</td>
</tr>
</tbody>
</table>

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ison to low O₂ (Fig. 2). In some cases at low Pi, the rates of photosynthesis were higher under low bicarbonate than under high bicarbonate levels (Figs. 1 and 2). Apparently, with little or no Pi, O₂ has a beneficial effect by allowing product formation through glycolate synthesis, which offsets the reduction in photosynthesis through O₂ inhibition of carboxylation.

LITERATURE CITED

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