Influence of pH upon the Warburg Effect in Isolated Intact Spinach Chloroplasts

II. INTERDEPENDENCY OF GLYCOLATE SYNTHESIS UPON pH AND CALVIN CYCLE INTERMEDIATE CONCENTRATION IN THE ABSENCE OF CARBON DIOXIDE PHOTOASSIMILATION

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ABSTRACT

The light-dependent synthesis of glycolate derived from fructose 1,6-diphosphate, ribose 5-phosphate, or glycerate 3-phosphate was studied in the intact spinach (Spinacia oleracea) chloroplasts in the absence of CO₂. Glycolate yield increased with an elevation of O₂, pH, and the concentration of the phosphorylated compound supplied. No pH optimum was observed as the pH was increased from 7.4 to 8.5. The average maximal rate of glycolate synthesis was 50 μmoles per milligram chlorophyll per hour while the highest rate observed was 92 with 2.5 mM fructose 1,6-diphosphate in 100% O₂. The highest yields of glycolate synthesized from fructose 1,6-diphosphate, ribose 5-phosphate, or glycerate 3-phosphate were 0.14, 0.24, and 0.36, respectively, on a molar basis.

In the previous paper (9), data were presented which established that increasing the pH from 7.55 to 8.40 enhanced the O₂ inhibition of chloroplast CO₂ assimilation (the Warburg effect). The enhancement of the Warburg effect at higher pH was thought to be due to an increase in the synthesis of glycolate formed during CO₂ assimilation resulting in a diminution of phosphorylated intermediates needed to maintain the ribulose 1,5-diP pool. The effect of more alkaline pH on glycolate synthesis was shown not be due to a lowering of the CO₂ level (9). A number of laboratories have established that glycolate is derived during the photoassimilation of CO₂ from intermediates of the photosynthetic carbon reduction cycle (2, 14). The question remained whether pH influenced glycolate formation in the isolated chloroplast when the mechanism functioned in the absence of CO₂ assimilation. The purpose of the work described in this report has been to examine the conditions required for glycolate synthesis in the absence of CO₂ assimilation but in the presence of supplied intermediates of the photosynthetic carbon reduction cycle. Data will be presented to indicate that glycolate can be derived from PGA, R5P, or FDP supplied to the intact plastid, and that the rate of synthesis is dependent on their concentration, light, O₂ concentration, and pH.

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2 Postdoctoral trainee of National Institutes of Health Grant BM-1586-09.
3 Abbreviations: FDP: fructose 1,6-diphosphate; R5P: ribose 5-phosphate; PGA: glycerate 3-phosphate.

MATERIALS AND METHODS

Plant Material. Spinacia oleracea (var. Winter Bloomsdale) leaf tissue was obtained from plants grown in local fields in the period from September to November.

Chloroplast Isolation. Intact chloroplasts were prepared employing modifications of the method of Gibbs and Robinson (5). Deveined spinach leaf tissue was diced and 60 to 70 g of this tissue was homogenized in a Waring Blender with 200 ml of chilled (5 C) medium containing 0.05 M HEPES (pH 6.8), 0.33 M sorbitol, 1 mM Na₄P₂O₇, and 1 mM DTT. All solutions were prepared with CO₂-free glass-distilled H₂O, and the pH was attained by titration with CO₂-free 12 N NaOH. Two consecutive, 3-sec homogenizations at full line voltage produced complete tissue maceration. The resulting homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged at 755g for 50 sec. The resulting chloroplast pellet was resuspended and combined with 60 ml of the homogenizing medium. This resuspension was centrifuged at 755g for 50 sec, and the pellet containing the intact plastids was resuspended in 7 to 9 ml of the homogenizing medium. Chl was estimated as previously indicated (5).

In these preparations, 80% of the chloroplasts were intact as monitored by the osmotic disruption technique described earlier (9) except that aldolase was employed as the marker enzyme. At saturating levels of CO₂, (pH 8), and 0.25 mM O₂, these preparations fixed CO₂ at rates of 35 to 40 μmol/mg Chl·hr.

Glycolate Synthesis. Glycolate synthesis as a function of FDP, R5P, or PGA and pH was carried out in a reaction medium containing 50 mM Tricine (pH 7.4–8.5), 330 mM sorbitol, 1 mM Na₄P₂O₇, 1 mM DTT in a final volume of 25 ml. This medium was prepared in CO₂-free H₂O, and brought to the appropriate pH by titration with CO₂-free 12 N NaOH. In addition, the reaction medium was supplied with PGA, R5P, FDP, and intact chloroplasts. In the studies with FDP, each mixture also contained 20 units (2.5 mg protein) of purified rabbit muscle aldolase (Sigma Chem.). The pH of the final reaction mixture was determined potentiometrically.

The experiments were carried out at 25 C in sealed 55 ml "lollipops" each of which possessed a 1-cm light path. Samples (10–12 ml) were withdrawn at appropriate intervals from the sealed "lollipops" with 12-ml hypodermic syringes. Reactions were terminated by addition of 0.1 ml 12 N HCl to the sample.

To examine the O₂ requirement for glycolate synthesis, [U-¹⁴C]FDP was supplied to the chloroplasts in the absence of CO₂. Chloroplasts for this study were also prepared according to the method of Gibbs and Robinson (5). Reactions were carried out at 25 C and light intensity of 1,000 w/m² in 2.1-ml mixtures containing 97 nmol (0.63 μCi) of FDP (New England
Nuclear). The other components of the mixtures were identical to those used in the [14C]glycolate experiments except that they contained 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, and 136.5 μg Chl. The mixtures were aerated with 100% O₂, CO₂-free 21% O₂, or 100% N₂. Samples of 1.05 ml were withdrawn and added to 0.05 ml 12 N HCl to terminate the reaction.

**Glycolate Estimation.** Prior to quantitation, glycolate (¹²C or ¹⁴C) was purified from the reaction mixtures using a previously reported method (8, 9). The Dowex AG 1-X8 acetate resin beds measured 0.8 x 7 cm.

[¹⁴C]Glycolate was determined in the 4 n acetic acid fraction as previously described (8) except that 0.15 aliquots were reacted with 2,7-naphthalenediol (4), and the color complex was determined at 540 nm according to the modification of Takahashi (10). Glycolate synthesis rates were calculated on the assumption that the FDP was uniformly labeled, and that each glycolate carbon possessed the same specific radioactivity as that of the initially supplied [U-¹⁴C]FDP. These rates were most likely underestimated due to isotopic dilution by unlabeled endogenous compounds in the chloroplast preparations.

**Photosynthetic Intermediates.** Aliquots for the estimation of PGA, A, and R5P were taken from the HCl-treated extracts analyzed for glycolate. FDP and triose-P were estimated by the method of Bucher and Hohorst (3), R5P by the method of Racker (3), and PGA according to Czok and Eckert (3). All determinations were carried out in 3-m1 reaction mixtures buffered by 0.10 M HEPES (pH 7.6).

**RESULTS**

**Light and O₂.** In the absence of CO₂, glycolate accumulation from either [U-¹⁴C]FDP (data not shown) or from the addition of unlabeled FDP, PGA, or R5P had an absolute requirement for light (Fig. 1). Although there was a small endogenous level of glycolate associated with the plastids initially, this level did not increase in the 15-min dark periods (Table 1).

In the absence of CO₂, glycolate synthesis from FDP was also dependent upon O₂ concentration. Plastids incubated with 0.05 M [U-¹⁴C]FDP, 1,000 w/m² light, at pH 8.5 were monitored for the formation of [¹⁴C]Glycolate. During aeration with 100% N₂, 21% O₂, or 100% O₂ the rate of glycolate synthesis at pH 8.5 was 0.3, 1.8, and 2.7 μmol/mg Chl-hr, respectively.

**Concentration of Substrates and pH.** The data in Figure 1 illustrate the time course of glycolate formation as a function of the concentration of added FDP, R5P, and PGA. Sections A and B of this figure also reveal that endogenous pools of the photosynthetic carbon reduction cycle, depending on the previous history of the leaf tissue, could sustain a high initial rate of glycolate synthesis. Leaf tissue (Fig. 1, A and B) was taken from the field during optimal growing conditions (September) while the tissues supplied with R5P (part C) were from plants at the end of the growing season (mid-November). Beyond 5 min, glycolate synthesis responded positively to both levels of PGA and R5P but only to the lesser concentrations (1.85 and 2.5 mM) of FDP. In contrast, when FDP was supplied at 3.75 or 4.35 mM, glycolate synthesis was initially enhanced in comparison to the control and subsequently inhibited.

In terms of initial velocity (0-5 min) of glycolate accumulation, the endogenous rate rose from 38 to approximately 50 μmol/mg Chl-hr with 3.75 or 4.35 mM FDP (Fig. 2A). PGA at 0.5 to 2 mM produced a 1.5-fold increase while R5P at 0.5 to 2 mM brought about a 10-fold increase over the endogenous rate. The rate of glycolate synthesis in the presence of 2.5 mM PGA, R5P, or FDP increased with increasing pH (Fig. 3). Of the three compounds tested, the response was most striking with PGA. There was an approximate 3.4-fold increase with an elevation of pH from 7.4 to 8.4.

![Figure 1](image-url)  
**Fig. 1.** Time course of glycolate synthesis in intact spinach plastids as a function of supplied FDP, PGA, or R5P. Reaction mixtures contained 0.05 mM Tricine (pH 8) 0.33 mM sorbitol, 1 mM Na₃PO₄, and 1 mM DTT in a final volume of 25 ml. Additionally, in A, the mixtures contained 20 units rabbit muscle aldolase, FDP, and 323 μg Chl; the dark control contained 4.35 mM FDP. In B, the mixtures contained PGA and 329 μg Chl; the dark control contained 2 mM PGA. In C, the mixtures contained R5P and 514 μg Chl; the dark control contained 2 mM R5P. Prior to addition of plastids, the solutions were aerated with 100% O₂ in sealed “lollipops” but at the point of addition of plastids, and for the remainder of the experimental period, O₂ aeration was shifted to the chamber space just above the solution surface. Illuminated samples received an incident intensity of approximately 1500 w/m². Each time point represents the average of two identical treatments. All values are corrected for glycolate formed in the dark (---): illuminated reactions.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Substrate consumed</th>
<th>Glycolate made</th>
<th>μmol glycolate formed</th>
<th>μmol substrate consumed</th>
<th>ratio</th>
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<tr>
<td>None</td>
<td>0.13</td>
<td>0.31</td>
<td>2.28</td>
<td>0.93</td>
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<tr>
<td>Glyceral-3-P</td>
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<td>1.64</td>
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<tr>
<td>Ribose-5-P</td>
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<td>0.77</td>
<td>1.71</td>
<td>0.31</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 1. The photosynthesis of glycolate by intact chloroplasts compared with the disappearance of supplied photosynthetic intermediates.

The reaction mixture and conditions for the reactions are those reported in Fig. 1 except the 25 ml solutions initially contained 12.5 μmol PGA, R5P, or FDP where indicated, and in experiments A and B, the mixtures contained 90μg Chl and 775μg Chl, respectively. In experiment A, initially and after 15 min dark the mixtures contained 0.22μmol glycolate, and in experiment B this value was 0.23μmol. The time interval in the light was approximately 7.7 min.

Stoichiometry of Glycolate Synthesis. The yield of glycolate per mol of PGA, R5P, or FDP consumed is recorded in Table 1. The molar ratio of glycolate synthesized to carbon source consumed did not exceed 0.3. The lowest yield was obtained with the FDP and the highest with PGA.

**DISCUSSION**

Zelitch (14) noted that the rate of photoregressive CO₂ release in C-3 plants was in the order of 76 μmol/mg Chl-hr in 21% O₂. Our average maximal rates were 50 μmol/mg Chl-hr in 100% O₂, and in one instance, the mixture of 2.5 mM FDP caused a rate of 92. While these rates do not approach that of 274 reported by Vandor and Tolbert (11), nonetheless, our intact plastids possess the potential to produce glycolate at rates...
approaching those required to account for photosynthetic processes in the intact plant.

Similar to the photosynthetic conversion of CO₂ to glycolate, glycolate derived from RSP, FDP, and PGA supplied to the chloroplast in the absence of CO₂ was enhanced by high alkalinity of the reaction medium (Fig. 3), by high levels of O₂, and by light. In contrast, while glycolate formation is inversely related to the CO₂ concentration (8), the reverse was observed when the concentration of the Calvin cycle intermediates was elevated to 2 to 2.5 mM (Figs. 1 and 2). Inhibition of glycolate synthesis was observed at the higher concentrations of FDP tested, but only after an initial enhancement (Fig. 1A).

Our data have identified pH and the level of intermediates of the photosynthetic carbon reduction cycle as critical factors in glycolate synthesis. Examination of Figure 3 reveals that the maximum rates of glycolate formation were realized when the pH of the reaction mixture was 8.5. No pH optimum was observed and it may well be that the pH optimum of this process in the intact chloroplast lies above 8.5. Clearly, the hydrogen ion concentration is a factor limiting glycolate formation in the chloroplast since the stromal pH in the illuminated organelle has been reported to be approximately 8.1 (12).

The rate of glycolate accumulation responded differently to increasing concentrations of the three compounds provided (Figs. 1 and 2). In contrast to PGA and FDP, increasing the concentration of RSP beyond 0.5 mM had no effect on glycolate formation. This difference may reflect the more rapid entry of triose-P and PGA into the stroma by means of the phosphate translocator (6) or the previous history of the leaves. Interestingly, the highest concentrations of FDP added eventually resulted in a stoppage of glycolate formation. The underlying reason for this inhibitory effect needs study but it should be pointed out that concentrations of FDP up to 8 mM had no effect on CO₂ assimilation by intact spinach chloroplasts (Y. W. Kow, unpublished data). On the other hand, 2 mM PGA which enhanced glycolate accumulation is known to inhibit CO₂ fixation (1).

An attempt was made to determine the stoichiometric yield of glycolate from FDP, PGA, or RSP when the conditions consisted of 100% O₂, no CO₂, and high pH to maximize glycolate synthesis (Table I). A molar ratio in the order of unity could be the result when FDP or RSP was supplied but roughly 0.5 of that value in the case of PGA. The actual value would depend upon whether the immediate donor of the glycolate was a 5- or 6-carbon sugar, the compound supplied, and the route of metabolic interconversions. The highest molar yield of glycolate observed was 0.3. A large part of the consumed carbon was found in glucose-6-P, fructose-6-P, and PGA (data not shown). Most likely starch was another depot for the carbon consumed. Clearly, glycolate formation was limited in our preparations by competing reactions even under the conditions most highly favorable to its formation.

Finally, there are ample data indicating a causal relationship between glycolate synthesis from CO₂ and the Warburg effect (13). The concentration of O₂, CO₂, and the hydrogen ion have been identified as factors decisive in the photoconversion of CO₂ to glycolate (9, 13, 14). Our data suggest that glycolate was derived from supplied intermediates of the photosynthetic carbon reduction cycle and that their concentration in addition to light, pH, and O₂ were critical factors in the synthesis of this two-carbon acid. That glycolate formation can be regulated by carbon compounds other than CO₂ was indicated by our data with the higher concentrations of FDP (Fig. 1A) and also with certain amino acids as reported recently by Oliver and Zelitch (7). In addition, the molar yield results recorded in Table I point to reactions competing for the glycolate-yielding intermediates even under conditions presumably optimal for glycolate synthesis. Clearly, there are many factors regulating glycolate formation in the chloroplast, and in turn, the Warburg effect and photorespiration, of which the concentrations of CO₂ and O₂ are but two.

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