Sequence of Formation of Phosphoglycolate and Glycolate in Photosynthesizing Chlorella pyrenoidosa

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ABSTRACT
In Chlorella pyrenoidosa which have been photosynthesizing in either 1.5% 14CO2 or 0.05% 14CO2 in air, gassing with 100% O2 results in rapid formation of phosphoglycolate which is apparently converted to glycolate. However, only about one-third to one-half of the rate of glycolate formation can be accounted for by this route. The remaining glycolate formation may be the result of the oxidation of sugar monophosphates. The rates of formation of both glycolate and phosphoglycolate are about four times greater with algae that have been photosynthesizing in 1.5% 14CO2 than with algae which have been photosynthesizing with air, when the algae are then gassed with 100% O2.

When some species of green plants photosynthesize with 14CO2 and the total CO2 concentration is not higher than in air, glycolate is prominent among the early labeled products (5). However, its formation occurs later than the formation of PGA7 and sugar phosphates (7), which are the first products of CO2 fixation in photosynthesis and are intermediates of the reductive pentose phosphate cycle of photosynthesis (1). This is also true when 14C-labeled glycolate is formed by isolated spinach chloroplasts photosynthesizing with 14CO2 (8). Glycolate formation during photosynthesis is favored by low CO2 concentration (21), high oxygen concentration (4), and high light intensity (18).

Once formed, glycolate may be metabolized via a variety of pathways (for review, see Tolbert [19], including paths which lead to photorespiratory CO2 in higher plants. In some unicellular algae, such as Chlorella pyrenoidosa, glycolate may be excreted into the suspending medium.

The mechanism whereby glycolate is formed from the intermediate compounds of the photosynthetic carbon reduction cycle remains in dispute. Wilson and Calvin (21) suggested that the glycolyl moiety transferred in the transketolase-mediated reactions of the cycle might be oxidized to glycolic acid. Dihydroxyethylthiamine pyrophosphate, the intermediate in the transketolase-catalyzed reaction, can be converted to glycolate with ferricyanide (6). Shain and Gibbs (17) described a reconstituted preparation containing fragments of spinach chloroplasts, transketolase, and cofactors which was capable of rapid conversion of fructose-6-P or dihydroxyethylthiamine pyrophosphate to glycolate in the light.

Recent findings that molecular oxygen competitively inhibits ribulose diphosphate carboxylase, the carboxylation enzyme of the photosynthetic reductive pentose phosphate cycle (13), led to proposals that RuDP is oxidatively split on the enzyme RuDP carboxylase by oxygen, giving phosphoglycolate and phosphoglyceraldehyde, instead of two molecules of phosphoglycenate normally formed by the carboxylation reaction.

There is considerable evidence that glycolate is formed oxidatively from sugar phosphates (10, 11, 15, 17), by one or the other of these paths, although there is also a question of whether the oxidant is oxygen, a peroxide formed by reaction of oxygen with a primary reductant, such as reduced ferredoxin, or an intermediate in the oxidation of water formed by the photosystem 2 of the photoelectron transport path (17).

The present study was undertaken to assess the importance in vivo of phosphoglycolate as an intermediate in glycolate formation. The kinetic curves for the appearance and disappearance of RuDP, phosphoglycolate, and glycolate following the introduction of 100% O2 to Chlorella pyrenoidosa previously photosynthesizing with 14CO2 have been determined.

MATERIALS AND METHODS
In some experiments, Chlorella pyrenoidosa were grown in a continuous culture apparatus aerated with 4% CO2 in air (2). A 0.3% suspension of these algae was withdrawn, the algae were centrifuged from the culture medium and resuspended in 0.1 mM KH2PO4 plus 1 mM KNO3 to a concentration of 1% (v/v). Sixty ml of this suspension were transferred to the algae vessel of the steady state apparatus (3). The pH was adjusted to 5.5 with 0.1 M HNO3 and maintained by periodic automatic addition of 0.1 M HNO3. The pH of the suspension in the steady state apparatus is more rapidly and accurately controlled at pH 5.5 than at higher pH values where there is a larger buffering effect of bicarbonate. This is par-

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1 This work was supported by the United States Atomic Energy Commission.

2 Abbreviations: PGA: 3-phosphoglycerate; PG: 2-phosphoglycolate; RuDP: ribulose-1,5-diphosphate; HMP: glucose-6-phosphate plus sedoheptulose-7-phosphate.
Table I. Rate of Appearance of Glycolate in O2 Compared with Rate of Disappearance of Phosphoglycolate in N2

In experiment 2, rate of glycolate appearance was measured at 11.3 min, while rate of phosphoglycolate disappearance was at 13.4 min (see Fig. 4). Comparable measurements were made in experiment 3. In experiments 4 and 5, O2 was administered for 2 min instead of 3 min. Rates were again measured at points on the curve where phosphoglycolate concentration was the same in O2 and in N2. In all cases rate of disappearance of phosphoglycolate was measured where it was maximum. Steady state rates of CO2 uptake, prior to O2 flushing, were 18 to 23 μmoles CO2/min·cm² algae, or about 200 μmoles CO2/hr·mg Chl. See text for discussion of k.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>d(Glycolate)/dt</th>
<th>d(PG)/dt</th>
<th>k = d(PG)/dGlycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (Fig. 4)</td>
<td>+2.5</td>
<td>-0.87</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>+2.7</td>
<td>-0.40</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>+3.2</td>
<td>-0.90</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>+3.0</td>
<td>-0.75</td>
<td>2.3</td>
</tr>
</tbody>
</table>

FIG. 1. Effect of 100% O2 on levels of labeled RuDP, phosphoglycolate and glycolate in Chlorella pyrenoidosa after photosynthesis with 1.5% 14CO2.

ticularly important in experiments where the gas phase in equilibrium with the algae suspension is suddenly depleted of CO2. The temperature of the algae was kept at 20°C. CO2 (1.5–2%) in air was bubbled through the suspension, and the lights were turned on. Cells were preilluminated in this way for about 30 min. During this time the photosynthesis rate was tested and found to be about 18 to 22 μg atoms of carbon assimilated/min·cm² wet-packed algae (3).

The gas flow system was closed, and 14CO2 was added so that the concentration of CO2 was about 1.5% with a specific radioactivity of 15 μc/μmole. The cells were allowed to photosynthesize normally for 11 min, by which time the cycle intermediates had time to become "saturated" with 14CO2. After 11 min the system was quickly flushed with 100% O2. Samples were taken before the flushing with O2 and were removed at short intervals after the oxygen flush began.

These samples were killed in methanol and were analyzed by paper chromatography and radioautography (14). Because of the volatility of glycolic acid from paper chromatograms developed with acidic solvents, duplicate samples of the algae were chromatographed in a second pair of chromatographic solvents. The first of these was made up of 754 ml of aqueous phenol (approximately 85% by weight), 224 ml H2O, 22.4 ml of concentrated NH4OH, and 2 ml of 0.5 M EDTA. This solvent is similar to one previously reported (20), but with the EDTA salt added. The second solvent system consisted of 600 ml of l-propanol, 300 ml of concentrated NH4OH, and 100 ml of H2O (12). The papers were developed for 30 hr in the first solvent and, after drying, for 24 hr in the second solvent. Identification of the major labeled compounds was made by cochromatography with unlabeled or 14C-labeled compounds. A 14C-glycolate spot cut from the chromatogram (with these alkali solvents) did not lose any 14C activity during 3 days' hanging in the fume hood, whereas a glycolate spot cut from our conventional acidic chromatogram loses up to 80% of its activity in 3 days. Separation of glycolic acid from other compounds was good, but the general separation of products of photosynthesis from one another was poor, so that all other compounds were analyzed in our usual system (14).

Although phosphoglycolate separates only a small distance from PGA in our usual system, development for 48 hr in each direction gave sufficient separation to allow quantitative results. In those few cases where good separation was not achieved initially, chromatography was repeated using another aliquot portion of the original mixture, and separation was sufficient.

Radioactivity in each compound was determined using an automatic gas-flow Geiger counter (14). The amounts of 14C in each compound are expressed as μg atoms 14C/cm² algae (wet-packed volume of algae after harvesting and centrifugation). One cm² of these packed algae contains about 6 mg of chlorophyll. Thus a typical photosynthesis rate of 20 μmoles CO2 fixed/cm² algae·min (Table I) is equal to 20 × 60/6 = 200 μmoles CO2 fixed/mg chlorophyll·hr.

In four experiments, the period of flushing with O2 was followed by a period of flushing with N2, with times as shown in the figures. In one of these experiments, alternate samples were killed in 80% methanol as usual, while the remaining samples were killed in 80% methanol to which 100 μl of 12 N HCl (per 4 ml methanol) had been added. In another of these experiments, alternate samples were killed in 50% methanol, 27% formic acid, and 23% water.

A second series of experiments were performed using Chlorella pyrenoidosa grown on air not enriched with CO2. For this purpose the algae are grown in a low form culture flask on a shaking apparatus in a temperature-controlled bath with illumination through the transparent bottom of the flask (2). A sintered glass bubbler provides for better aeration. Other conditions for culturing and for the exposure to 14CO2 in the steady state apparatus were as described above for the algae grown in 1.5 to 2% CO2. Gassing times and regimes are described under "Results."

RESULTS

A comparison of the changes in levels of RuDP, phosphoglycolate, and glycolate after the introduction of O2 (Fig. 1) shows that the RuDP pool and the phosphoglycolate pool each increase rapidly in amount and then fall. The phosphoglycolate pool level tends to follow the level of the RuDP pool, and peaks about 90 sec after the peaking of the RuDP pool. This is clearly consistent with the idea that phosphoglycolate formation depends on O2 and RuDP concentration. Since the only likely fate of phosphoglycolate is its conversion to glycolate, this suggests that some of the glycolate is formed by the
sequence RuDP → phosphoglycolate → glycolate. From the slope of the glycolate curve compared with the level of phosphoglycolate at various times, it is apparent that the rate of formation of glycolate only partly depends on the level of phosphoglycolate.

Among other effects of the addition of O₂ (and removal of CO₂) were the following: The levels of PGA dropped sharply while the levels of HMP, UDP-glucose, and of fructose-6-P (not shown) first dropped and then rose (Fig. 2). The level of sucrose begins to decline soon after the addition of O₂ suggesting that this reserve sugar is mobilized to supply carbon to the cells’ metabolism. The later rise in levels of hexose monophosphates, following the initial drop, also suggests that carbon flows into the reductive pentose phosphate cycle from endogenous sugars. This appears to be some kind of compensatory regulatory mechanism to keep the chloroplasts “primed” for the return of CO₂. This mechanism keeps the RuDP level from dropping below its steady state level during the time of the experiment, in spite of the conversion of RuDP to phosphoglycolate, which will continue in the presence of O₂.

The levels of several amino acids including alanine dropped, while the level of glycine rose (Fig. 3). This rise in glycine level may indicate that some of the glycolate is metabolized via glyoxylate to give glycine.

An analysis of these kinetic curves for phosphoglycolate, RuDP, and glycolate concentrations as shown in Figure 1 suggested that the rate of glycolate formation changes substantially during the first 2 min of O₂ gassing. Therefore, we decided to attempt to evaluate the rate of phosphoglycolate hydrolysis under a set of conditions in which phosphoglycolate formation should have stopped. The sequence of gas changes was altered to include a second change from 100% O₂ to 100% N₂ after 3 min, when phosphoglycolate is still at a high level. Since O₂ is required for the formation of phosphoglycolate from RuDP, we reasoned that the disappearance rate of phosphoglycolate after a few seconds of N₂ flushing would represent the absolute rate of phosphoglycolate hydrolysis. Presumably this rate should be dependent on phosphoglycolate concentration. Provided that the rate constant for the hydrolysis of phosphoglycolate remains constant during the time the algae are with O₂ and the time they are with N₂, the rate of hydrolysis of phosphoglycolate at a given concentration of phosphoglycolate during the N₂ regime should be the same as the rate of hydrolysis during the O₂ regime. It might be argued that there is still appreciable phosphoglycolate formation when the algae are being flushed with N₂. The presumption in such an argument would be that O₂ evolution is still occurring (since the light is still on), and that even with N₂ flushing, the locally generated O₂ causes some oxidation of RuDP to give phosphoglycolate. However, with N₂ flushing there can be no net CO₂ uptake and hence no net O₂ evolution (since the electron acceptors, ferredoxin and NADP, would not be able to accept electrons without reoxidation). Further evidence that the rate of phosphoglycolate production becomes small or zero under N₂ flushing is the fact that the glycine level (which is affected by glycolate formation by all pathways including phosphoglycolate hydrolysis) no longer increases after about 1 min. The increase during the 1st min of N₂ flushing is in part the consequence of phosphoglycolate hydrolysis, and perhaps in part due to sugar monophosphate oxidation.

The concentration of phosphoglycolate in this experiment (experiment 2, Fig. 4 and Table I) was the same (0.37 μg atoms ¹³C/cm³ algae) at 11.3 min as it was at 13.4 min, and the rates of phosphoglycolate disappearance at 13.4 min thus can be compared with the rate of glycolate formation at 11.3 min. The rate of disappearance of phosphoglycolate at 13.4 min was at 15.3 min, can be compared with the rate of glycolate formation at 11.3 min. The rate of disappearance of phosphoglycolate at 13.4 min, can be compared with the rate of glycolate formation at 11.3 min.

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II. Table

The indicated 0.05% glycolate

II.

Table II. Rate of Appearance of Glycolate Compared with Level of Phosphoglycolate

Rates are calculated from slopes of curve shown in Figure 5 at the indicated times.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Condition</th>
<th>Phosphoglycolate (μg atom 14C/cm³ algae)</th>
<th>d(Glycolate)/dt (μg atom 14C/min·cm³ algae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>0.05% 14CO₂ in air</td>
<td>0.100</td>
<td>0.0115</td>
</tr>
<tr>
<td>21</td>
<td>100% O₂</td>
<td>0.256</td>
<td>0.434</td>
</tr>
<tr>
<td>34</td>
<td>100% O₂</td>
<td>0.125</td>
<td>0.324</td>
</tr>
</tbody>
</table>

In another experiment, with similar conditions to those just described (experiment 3, Table I), the rate of appearance of glycolate at 11.3 min and of disappearance of phosphoglycolate at 13.4 min were comparable to the values just given. In experiments 4 and 5 (Table I), the O₂ was replaced by N₂ after only 2 min. In experiment 4, the rate of disappearance of phosphoglycolate was somewhat higher, but still well below the rate of appearance of glycolate at 11.3 min, the time of the same phosphoglycolate concentration during O₂ gassing. In each case we are assuming that the rate of hydrolysis of phosphoglycolate is the same at a given concentration of phosphoglycolate during the O₂ flushing as during the N₂ flushing. No additional PG was detected in samples killed with methanol plus HCl or with methanol and formic acid (27%).

With air-adapted algae, photosynthesizing in 0.05% 14CO₂, there is a measurable rate of appearance of glycolate and a measurable steady state level of phosphoglycolate before the addition of 100% O₂ (Fig. 5). After the air and 14CO₂ is replaced by 100% O₂, the rate of appearance of both phosphoglycolate and glycolate increases. However, the rate of appearance of glycolate is much less than it was in the algae switched from 1.5% 14CO₂ to 100% O₂. The rate of appearance of glycolate after the switch to 100% O₂ is much greater in relation to phosphoglycolate concentration than before (Table II), but this may be misleading, since the rate of both formation and utilization of glycolate may change when 0.05% 14CO₂ in air is replaced by O₂. The data suggest again that phosphoglycolate concentration is not very dependent on glycolate concentration.

When algae photosynthesizing in 0.05% 14CO₂ are switched first to O₂ and then after 3 min to N₂ (Fig. 6), it can be seen that the rate of glycolate utilization is in this case significant compared to rate of appearance of glycolate. Thus in O₂, glycolate increased at a rate of +0.26 μg atoms/min·cm³ algae. During the 1st min in N₂, the glycolate concentration dropped at a rate of 0.60 μg atoms/min. If we assume that this rate of decrease in glycolate during the 1st min in N₂ represents the approximate rate of glycolate utilization in O₂, then the total rate of formation of glycolate in O₂ was at least 0.26 —(-0.60) = +0.86 μg atoms/min·cm³ algae. This is to be compared with a rate of disappearance of phosphoglycolate in the 1st minute in N₂ of 0.22 μg atoms/min·cm³ algae.

DISCUSSION

Although the rise and fall in phosphoglycolate concentration follows closely after the rise and fall in RuDP concentration (Fig. 1), it is not likely that the rate of phosphoglycolate formation is linearly dependent on RuDP concentration if the enzyme ribulose diphosphate carboxylase is catalyzing the conversion of RuPD to phosphoglycolate in the presence of O₂. The enzyme is normally saturated with respect to RuDP under conditions of normal in vivo photosynthesis, and the level of RuDP goes still higher when the O₂ gassing commences, due to removal of CO₂. Thus the rise and fall in phosphoglycolate concentration is taken as an indication of a changing rate of conversion of enzyme-RuDP complex to PGA and phosphoglycolate in the presence of O₂.

The increase in rate after the onset of O₂ gassing is easily explainable as being the result of several seconds being required for the effective dissolved concentration of O₂ to rise as a result of the switch from atmospheric O₂ to 100% O₂. We attribute the subsequent fall in the level of phosphoglycolate to a decreased rate of formation of phosphoglycolate resulting
from some change in the activity of the enzyme. Chu and Bassham (9) found that the carboxylation activity of the enzyme greatly decreases (presumably as the result of conforma-
tional change), when the enzyme is presented with RuDP in the absence of CO₂. We suspect that this change, which must occur when the O₂ gassing takes place, also inactivates the enzyme with respect to the oxidative reaction.

Given the low concentration of phosphoglycolate and the high reported activity of the enzyme phosphoglycolate phosphatase (16), it seems reasonable to suppose that the rate of hydrolysis of phosphoglycolate exhibits first order dependence on phosphoglycolate concentration under the conditions of our experiments. This assumption is implicit in our comparison of the rate of disappearance of phosphoglycolate under N₂ in experiments 2 to 5 with the maximum rates of appearance of glycolate in those experiments. We wish to use this assumption also in the analysis of the phosphoglycolate and glycolate curves in experiment 1 (Fig. 1).

There are two points in experiment 1, Figure 1, at which the rate of change of phosphoglycolate concentration is at least momentarily zero. These are at 13.5 min, when the phosphoglycolate concentration is 0.98 μg atoms of ¹⁴C, and again at 20 min, when the phosphoglycolate concentration is 0.33 μg atoms of ¹⁴C. We can assume that at each of these two points the rate of hydrolysis of phosphoglycolate (equal to its rate of formation) is given by k(PG). The second assumption can be that glycolate formation is given by K + k(PG). This rate experimentally is +3.30 μg atoms ¹⁴C/min⁻¹ and +1.9 μg atoms ¹⁴C/min⁻¹ at 13.5 min and at 20 min, respectively. Solving the two simultaneous equations for these two times, we get K = 1.34 μg atoms ¹⁴C/min⁻¹ and k = 1.7 min⁻¹. In other words, rate of glycolate formation = 1.34 + 1.7 [PG]. Thus there would be formation of glycolate by a pathway independent of phosphoglycolate at a rate of 1.34 μg atoms/min⁻¹ and formation as the result of phosphoglycolate formation and hydrolysis at rates varying from 1.7 μg atoms/min⁻¹ when phosphoglyco-
late formation is maximal to 0.56 μg atoms ¹⁴C/min⁻¹ at 20 min.

These values may be compared with the results for experiments 2 through 5 where the measured rates of phosphoglyco-
late disappearance varied from 0.4 to 0.9 μg atoms/min⁻¹, and the calculated values for k varied from 2.0 to 2.6 (Table 1). Considering these measurements and calculations for experiments 1 through 5, it appears that under the conditions of O₂ gassing of Chlorella previously photosynthesizing in 1.5% CO₂, glycolate formation takes place both by way of phosphoglyc-olate (presumed to be formed by oxidation of RuDP) and by an independent pathway, which may be the oxidation of sugar monophosphates. During the first 90 sec of O₂ gassing, the pathway via phosphoglycolate may account for one-half or more of the glycolate formation, but during a subsequent period the rate of formation of the phosphoglycolate decreases, and this route to glycolate accounts for only about a third of the glycolate formation.

It will be noted that no allowance has been made for the conversion of glycolate to other metabolic products, despite the evidence (glycine formation, Fig. 3) that such conversion does occur. The actual rate of formation of glycolate is the sum of the rate of its appearance plus the rate of its conversion, so that even less of the total glycolate production can be ac-
counted for in terms of phosphoglycolate hydrolysis.

To summarize, it appears that with Chlorella, photosynthe-
sizing either in 1.5% CO₂ or in air, addition of 100% O₂ re-

sults in a rapid formation of phosphoglycolate which is con-
verted to glycolate, but that only about one-third to one-half of the glycolate formed as a result of O₂ addition is formed by this route. The remaining glycolate formation may be the re-

sult of the oxidation of sugar monophosphates. The rates of formation of both phosphoglycolate and glycolate appear to be about four times greater when the algae had been photosynthe-
sizing in 1.5% CO₂ than when they had been photosynthe-
sizing with air.

These conclusions apply to a transient state imposed by re-

moving CO₂ and increasing O₂ to 100%. This provides strong

evidence that some, but not all, glycolate formed in vivo in Chlorella pyrenoidosa is also be formed by the sequence RuDP → phosphoglycolate → glycolate. The relative amounts formed by this pathway (4, 13, 16) compared to oxidation of sugar monophosphate (6, 17, 21) under "normal" physiological conditions (i.e. in air) need not be the same as found in this study.

LITERATURE CITED

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