The Relation of Glycolic Acid Synthesis to the Primary Photosynthetic Carboxylation Reaction in Leaves

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The studies initiated by Calvin and his colleagues led to the elucidation of a well supported sequence of reactions known as the photosynthetic carbon reduction cycle (1). According to this scheme, which was largely deduced from kinetic experiments, ribulose-1,5-di-P is first carboxylated to give 2 molecules of 3-P-glyceric acid as the first stable carboxylation product (2, 3). The experiments from which the reactions of the photosynthetic cycle were inferred were always conducted at high concentrations of CO₂ in air, 1.0% or more. Wilson and Calvin (4), however, found that on lowering the CO₂ concentration glycolic acid was produced in large amounts, and they suggested that it was derived from an intermediate of the photosynthetic cycle, presumably ribulose-5-P. The recent finding of a specific P-glycolic acid phosphatase in leaves (5) was taken as further evidence that glycolic acid is a secondary product of the cycle.

On the other hand, I have shown that when the oxidation of glycolic acid in leaves is blocked with a suitable α-hydroxysulfonate (6), as much as half of the 14CO₂ fixed during photosynthesis in air may accumulate as glycolic acid (7). In addition, Warburg and Krippahl (8) have reported a 92% conversion into glycolic acid of the CO₂ taken up during photosynthesis by Chlorella when the cells were maintained in an atmosphere containing not more than 0.1 to 0.2% CO₂. This observation implies that glycolic acid may be synthesized by a carboxylation reaction different from that catalyzed by ribulose diphosphate carboxylase. Experiments were therefore carried out to determine the optimal conditions for glycolic acid synthesis in leaves, and to attempt to discover whether glycolic acid is derived by cleavage of a metabolite that is part of the photosynthetic cycle or by an additional carboxylation. The results obtained from measurements of specific activity provide evidence that glycolic acid is synthesized in the light from CO₂ by reactions that are largely separate from those of the photosynthetic cycle, especially at concentrations of CO₂ close to those normally found in air.

EXPERIMENTAL PROCEDURE

Photosynthesis—Tobacco plants (Nicotiana tabacum, var. Havana Seed) were grown in the greenhouse in a subirrigated bench. For each experiment, a leaf weighing 10 to 15 g was excised and first stored for 1 hour in darkness with its base in water. Disks 1.0 cm in diameter, about 40 mg in fresh weight, were cut, avoiding large veins from the central portion of the leaf with a sharp punch, and were floated on water. Disks were selected at random, equal numbers being taken from each side of the midrib, and were placed on a thin layer of water in 75-ml Warburg vessels (six disks per vessel fitted around the center well) in the light (flood lamps provided about 2000 foot-candles) with the stopcocks open to the air. The flasks were illuminated from above while immersed in a water bath at 30° that was fitted with mirrors to reflect light upon the lower surface of the leaf disks. The Warburg vessels were shaken at only 50 oscillations per minute so as not to displace the disks. After 60 minutes, the liquid was quickly removed with a hypodermic syringe and replaced with water or a solution of α-hydroxy-sulfonate. About 30 seconds after changing the fluid, 14CO₂ of known concentration was liberated by adding acid to an NaH14CO₃ solution in a side arm with a double sack, and the Warburg vessel was immediately returned to the water bath, at zero time. At the end of the period of photosynthesis, the leaf disks were removed and rapidly plunged into about 20 ml of boiling 20% ethyl alcohol (within 30 seconds), and were kept at this temperature for 3 minutes. The killed tissue and fluid were transferred to a glass homogenizer and ground, and the suspension was centrifuged. The residue was stirred with water, and the centrifugation repeated. The combined supernatant fluids were used for the separation of the various fractions and compounds.

Chemical Separations—When the basic compounds were to be collected, the leaf extract was first passed through a column of Dowex 50-H⁺ resin, and the compounds retained on the column were eluted with 2.0 N ammonium hydroxide. The extract, or the water effluent from the Dowex 50 column, was placed on a chromatographic column 0.7 cm in diameter packed with Dowex 1-acetate resin to a height of 6 cm. The method of separation used was based on work from this laboratory (9, 10) and that by Bartlett (11). The location of known compounds was determined by titration (9). The column was washed three times with water and elution of acidic compounds was then carried out at a flow rate of about 1 ml per minute, starting with 4.0 N acetic acid. The first 4 ml of effluent were discarded and the next 10 ml were collected. This fraction contains glycolic acid and glycine. Elution with 1.0 N formic acid followed, and 20 ml were retained, comprising the malic acid fraction. The separation was continued by addition of 30 ml of 2.0 N formic acid to obtain a fraction known to contain citric acid, ribose-5-P, and glucose-6-P. This was followed by elution with 6.0 N formic acid; the first 20 ml were discarded, and the next 44 ml...
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included the 3-P-glyceric acid (Fig. 1). The next 10 ml were discarded, and the subsequent 64 ml of effluent were used for the isolation of phosphoglycolic acid (Fig 1). The solution was finished with 20 ml of 0.1 N HCl to obtain a fraction including fructose-1,6-di-P and P-enolpyruvate.

Glycolic acid and glyc eric acid were separated from each other, when necessary, by descending chromatography on Whatman No. 3MM paper. The solvent system was somewhat modified from that described by Jones, Dowling, and Skraba (12) and consisted of n-butyl alcohol, 95% ethyl alcohol, water, and diethylamine in the proportions 80:10:20:1. Samples of the glycolic acid fraction from the Dowex 1-acetate column were dried at the origin at room temperature with a fan. About 2 μmoles each of carrier glycolic acid and glyc eric acid were added, and the chromatogram was developed for about 20 hours. The solvents were steamed from the paper, and the diethylamine salts of the acids were located with a spray consisting of 50 mg acid is 0.24. Excellent recoveries of radioactive glycolic acid, which is somewhat volatile as the free acid, were obtained by this procedure after elution of the salt with water.

Radioactivity Measurements—All determinations on fractions and compounds were made on infinitely thin samples with a thin window counter. In the degradation procedures employed for the various radioactive compounds, the percentage of 14C in a particular carbon atom was usually determined by conversion of that carbon to 14CO2, which was recovered in alkali. The radioactivity was then determined on a 0.2-ml sample of the alkaline solution by scintillation counting in the presence of 5 mg of bromophenol blue and 200 mg of citric acid in 100 ml of water (12). The RF of glycolic acid is about 0.32 and of glyc eric acid 0.24. Excellent recoveries of radioactive glycolic acid, which is somewhat volatile as the free acid, were obtained by this procedure after elution of the salt with water.

Glycolic Acid—To determine its concentration, samples of 10 ml were taken from the 10-ml glycolic acid fraction obtained from the column, and the colorimetric reagent consisting of 0.01% 2,7-dihydroxynaphthalene in concentrated sulfuric acid was used (10).

The degradation of glycolic acid was carried out by the following reactions.

\[
\text{CH}_2\text{OH} - \text{COOH} + \text{O}_2 \xrightarrow{\text{glycolate oxidase, catalase}} \text{CHO} - \text{COOH} \xrightarrow{\text{ceric sulfate}} \text{HCOOH} + \text{CO}_2
\]

glycolic acid

glyoxylic acid

The radioactive glycolic acid to be degraded was placed in a Warburg vessel with two side arms. To the main compartment was also added carrier potassium glycolate, 10 μmoles, Tris-chloride buffer at pH 8.0, 40 μmoles; and water to make the final volume 3.0 ml. One side arm contained 0.2 ml of an enzyme preparation containing glycolate oxidase and catalase dissolved in buffer (the precipitate obtained from an aqueous extract of tobacco leaves brought to 0.4 of saturation with ammonium sulfate and stored as a suspension in the cold with the same concentration of ammonium sulfate) and 0.05 mg of riboflavin phosphate. The second side arm had 0.3 ml of 0.2 μmole of ceric sulfate in 2.0 N sulfuric acid. The center well contained 0.2 ml of 2.0 N sodium hydroxide.

The reactions were carried out at 30°, and the first step was started by tipping in the glycolate oxidase preparation. After oxygen uptake ceased (about 60 minutes) and indicated about 5 μmoles of oxygen uptake (13, 14), the cemic sulfate solution was tipped in from the second side arm (15). The second reaction was allowed to proceed for 120 minutes to ensure completion. Radioactivity measurements were then made on samples taken from the center well and the main compartment by scintillation counting. The validity of the degradation procedure was confirmed with samples of C-1- and C-2-labeled glycolic acid.

P-glyceric Acid—The appropriate fraction from the Dowex 1-acetate column was taken to dryness in a vacuum in a rotary evaporator with the water bath at 45°. The residue was dissolved in 3 ml of water. Potassium acetate buffer (1.0 M, pH 5.0), 0.05 ml, and potato phosphatase (Sigma), 0.1 ml of a solution containing 10 mg per ml, were added. The hydrolysis was allowed to proceed at 30° for 90 minutes. The solutions were heated on a steam bath for 3 minutes and filtered, and the filtrate and washings were placed on a Dowex 1-acetate column as described before. The glyceric acid fraction was collected and the glyceric acid concentration was determined on a 1.0-ml sample that was first taken to dryness in a colorimeter tube in a steam of air blown on the surface while the tube was immersed in a water bath at 46°. The colorimetric reagent consisted of 2.0 ml of a freshly prepared solution of 0.01% naphthoresorcinol in concentrated sulfuric acid (16), and the color was developed at 100° for 60 minutes. A control sample consisting of 1.0 μmole of 3-P-glyceric acid was carried through the entire procedure and served as an internal standard in the analytical determination.

A sample of the glyceric acid thus obtained from the P-glyceric acid was used to determine the radioactivity in the carboxyl carbon atom as follows.

\[
\text{CH}_2\text{OH} - \text{CHOH} - \text{COOH} \xrightarrow{\text{cemic sulfate}} \text{CH}_3\text{COOH} + \text{CO}_2
\]

The reaction was carried out at 30° for 2 hours in Warburg vessels containing 10 μmoles of carrier glyceric acid and 0.2 ml of 0.2 M cemic sulfate in 2.0 N sulfuric acid in a final volume of 2.0 ml. The center well contained 0.2 ml of 2.0 N sodium hydroxide. These conditions produced a quantitative evolution of 1.0 mole of CO2 per mole of glyceric acid added in control experiments. Since Schou et al. (17) have shown that the C-2 and C-3 of

*Fig. 1. Separation of 3-P-glyceric acid and P-glyceric acid on a column of Dowex 1.*
P-glyceric acid are equally labeled in short times of photosynthesis, the determination of the percentage of radioactivity remaining in the main compartment of the Warburg vessel at the end of the reaction was divided by 2 to estimate the specific activity of C-3 of phosphoglyceric acid.

To test its validity, the degradation procedure was carried out with a sample of 3-P-glyceric acid-1-14C kindly provided by Dr. M. Gibb and Dr. E. S. Bamberger. They found 99.5% of the 14C in the carboxyl carbon by their procedure, which was based on the periodate degradation described for serine (18). The ceric sulfate oxidation method described above showed 99.0% of the 14C to be present in C-1 of this sample.

Preparation of Phosphoglycolic Acid—Because of failure to obtain satisfactory samples of salts of this substance by methods described in the literature, the procedure of Neuhauß and Korkes (19) for the phosphorylation of serine with monochlorophosphoric acid was adapted. The reaction was carried out as described for serine, starting instead with 19 g of glycolic acid. After the polyphosphates were hydrolyzed, phosphate was removed with ammoniacal magnesia mixture (20), and the filtrate and washings were brought to pH 1.5 with HCl. Then solid Ba(OH)2 was added to raise the pH to 7.8. 40% of the volume of 95% ethyl alcohol was added, and the suspension was refrigerated for several days. The barium salt was collected and dried at 105° for 3 days, yielding 9.8 g of product corresponding to PO3H2--CH2--COOBA1.5.2H2O. The analysis showed

\[
\text{C}_3\text{H}_4\text{O}_3\text{PBA}_{1.5} \\
\text{Calculated: Ba 52.15, P 7.84} \\
\text{Found: Ba 53.0, P 7.54}
\]

A sample (3.0 g) of the dry barium salt was stirred into a column 1.8 cm in diameter packed with Dowex 50-H+ resin to a height of 10 cm. The column was then washed with small portions of water until the effluent was neutral. The solution of free phosphoglycolic acid was concentrated in a rotary evaporator with the water bath at 45°. The resulting syrup was refrigerated. Large colorless needles in rosettes formed overnight, and the crystallization was allowed to continue for another 24 hours, by which time an almost solid crystalline mass had been produced. The colorless crystals were collected and dried in a vacuum oven over P2O5 at 75°. The yield was 0.75 g. The free acid, PO3H2--CH2--COOH, melts at 110–112° in a Fisher-Johns melting point apparatus. The analysis showed

\[
\text{C}_3\text{H}_4\text{O}_3\text{P} \\
\text{Calculated: P 19.85, Neutral equivalent 52.01} \\
\text{Found: P 19.9, Neutral equivalent 50.7}
\]

The free phosphoglycolic acid was treated with potato phosphatase under the conditions described for the hydrolysis of the phosphate ester of P-glyceric acid. The glycolic acid thus produced was separated on a Dowex 1-acetate column and its concentration determined colorimetrically. The yield of glycolic acid obtained in this way was 92% based on the starting quantity of phosphoglycolic acid.

Phosphoglycolic Acid Isolation—After separation of this fraction in the Dowex 1-acetate column (Fig. 1), it was taken to dryness in a rotary evaporator with the water bath at 45°. The phosphoglycolic acid was hydrolyzed with potato phosphatase under the same conditions used for phosphoglyceric acid, and the resulting glycolic acid was separated on Dowex-1 acetate. A sample of phosphoglycolic acid was carried through the procedure and served as the standard for the analytical determination.

Glucose-6-P—The appropriate fraction from the Dowex 1-acetate column was taken to dryness in a rotary evaporator at a temperature not exceeding 45°. The following sequence of reactions served both for the quantitative determination and for the measurement of radioactivity in C-1.

\[
\text{PO}_4\text{H}_2--\text{CH}_2--(\text{CHOH})_n--\text{COOH} + \text{NADP} \rightarrow \\
\text{PO}_4\text{H}_2--\text{CH}_2--(\text{CHOH})_n--\text{COOH} + \text{NADPH} + \text{H}^+ \\
\]

An enzyme preparation containing both glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase was obtained from Candida utilis by following the method of Pontremoli et al. (21) through the charcoal treatment and heat denaturation steps. The determination of the glucose-6-P was made on samples placed in cuvettes in a Beckman DU spectrophotometer under the conditions described for the assay of 6-P-gluconic dehydrogenase (21). The molecular extinction coefficient 6.22 $\times$ 104 $\text{cm}^{-1} \text{mole}^{-1}$ (22) was used to determine the total NADPH produced in the two successive reactions.

The degradation to isolate C-1 of glucose-6-P was carried out at 30° in Warburg vessels fitted with two side arms. The main compartment contained the following in a final volume of 3.0 ml: triethanolamine chloride buffer at pH 7.5, 800 $\mu$moles; the sample to be degraded; carrier glucose-6-P, 5 $\mu$moles; and the enzyme preparation containing both glucose-6-P and 6-P-gluconate dehydrogenase activities. The center well contained 0.2 ml of 2.0 N sodium hydroxide. The reactions were initiated by tipping in NADP, 0.25 mg, and N-methylphenazonium sulfate, 10 mg, from the side arm. The reactions were allowed to proceed until oxygen uptake ceased, and then 0.2 ml of 10 N sulfuric acid was tipped in from the side arm to liberate any bound CO2. Radioactivity of the 14CO2 thus produced was determined by scintillation counting on a sample from the center well.

RESULTS

Optimal Conditions for Glycolic Acid Synthesis—Leaves and algae have been shown to produce glycolic acid best in light at concentrations of CO2 in air not exceeding 0.1 to 0.2% (8, 23, 24). Oxygen in the gas phase is also necessary for maximal rates of synthesis to be achieved (24–26). Wilson and Calvin (4) observed a large increase in the pool size of glycolic acid when the CO2 concentration in the air provided to Chlorella cells was lowered from a steady state of 1.0% to 0.003%, and they assumed that the glycolic acid arose from intermediates of the photosynthetic cycle that also increased under these conditions.

$\alpha$-Hydroxy-2-pyridinemethanesulfonic acid (7), an inhibitor of glycolate oxidase, caused maximal glycolic acid formation when supplied to leaf disks at a concentration of 0.01 M (24). Table I shows representative experiments on the effect of the length of time disks were kept in air (about 0.03% CO2) in the light on the subsequent glycolic acid formation. The accumulation of glycolic acid was investigated during a 15-minute period on
inhibitor solution in either air, CO₂-free air, or air containing 1.8% CO₂.

As the preliminary period in the light in air was increased from 30 minutes to 120 minutes, the rate of glycolic acid synthesis increased (Experiment 1). The amount of glycolic acid produced by leaf disks in CO₂-free air was always less than that formed in normal air (see also Table III). If the interpretations

![Table I](https://i.imgur.com/5Q8zQ.png)

**Effect of time in air on subsequent synthesis of glycolic acid by leaf disks in light**

> A tobacco leaf was kept in darkness for 1 hour. Then three leaf disks were floated on water in each 25-ml Erlenmeyer flask, which was closed with a rubber serum stopper. The disks were kept at 30°C in the light with the disks in the atmosphere indicated and for the length of time shown. The gas was passed through the disks at a rate of several volumes per minute. The glycolic acid concentration in zero time samples (always less than 0.5 μmole per g, fresh weight) was subtracted to show the amount that accumulated.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time in air</th>
<th>Time in inhibitor</th>
<th>Glycolic acid formed μmoles/g, fresh wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>min</td>
<td>mix</td>
<td>μmoles/g, fresh wt.</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>15, air</td>
<td>3.0</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>15, no CO₂</td>
<td>2.6</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>15, air</td>
<td>5.0</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>15, no CO₂</td>
<td>3.0</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
<td>15, air</td>
<td>6.9</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
<td>15, no CO₂</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>15, air</td>
<td>7.3</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>15, 1.8% CO₂</td>
<td>0.83</td>
</tr>
</tbody>
</table>

![Fig. 2](https://i.imgur.com/6Q8zQ.png)

**Fig. 2 (left).** ¹⁴CO₂ uptake by tobacco leaf disks in the light. For 1 hour, six leaf disks in each of four Warburg vessels were floated on water in the light and shaken slowly with the taps open to the air. Then the stopcocks were closed and 5 μmoles of ¹⁴CO₂ (total of 6 μmoles in the gas phase to make the final concentration about 0.18%) containing 1.80 × 10⁶ c.p.m. were liberated in each vessel. At the end of the times shown, the leaf disks were killed and the radioactivity in the homogenates determined. The radioactivity in the fluid surrounding the disks was less than 0.5% of the total fixed by the tissue.

![Fig. 3](https://i.imgur.com/6Q8zQ.png)

**Fig. 3 (right).** Specific activity of carboxyl carbon of glycolic acid in leaf disks as a function of time. The photosynthesis experiments were carried out in inhibitor solution as described in Fig. 2. Each symbol on the curve represents a result obtained in a separate experiment with different leaf material. It was assumed that the carboxyl carbon and methyl carbon of glycolic acid were equally labeled. The percentage of ¹⁴C in the carboxyl carbon actually ranged from 45 to 53.
The largest change occurred in the radioactivity of the glycolic acid as was previously observed in whole leaves (7). In this experiment, 0.9% of the $^{14}C$ incorporated was found in glycolic acid when the leaf disks were floated on water, compared with 35% in the presence of inhibitor. The other fractions separated on the Dowex 1 column showed that relatively smaller changes were brought about by the inhibitor.

Requirement for CO$_2$ for Glycolic Acid Synthesis—It seemed clear that a high proportion of the $^{14}CO_2$ taken up was fixed in glycolic acid in the presence of glycolate oxidase inhibitor. The experiment was then repeated, but in the reverse manner. The endogenous sources of carbon were first labeled by exposure of the leaf disks to $^{14}CO_2$ for 40 minutes (see Fig. 2) followed by an additional period of 20 minutes in an atmosphere of normal air. The vessels were closed again, and the disks were permitted to photosynthesize in the presence of $\alpha$-hydroxysulfonate for 10 minutes either in CO$_2$-free air or in 0.18% CO$_2$ (Table III).

Less glycolic acid was formed without CO$_2$ in the atmosphere, and the specific activity was higher when external CO$_2$ was eliminated. Therefore, in this reversal of the normal experiment, it was also shown that CO$_2$ uptake is essential for maximal rates of glycolic acid synthesis.

Since the addition of 6 μmoles of CO$_2$ diminished the specific activity of C-1 of glycolic acid to half the value in CO$_2$-free air, it seems fair to assume that six leaf disks contained an equal concentration, or about 6 μmoles of endogenous CO$_2$-bicarbonate available from respiration (30). With use of this figure, one can therefore estimate the specific activity of the $^{14}CO_2$ actually available to the leaf disks in such a closed system.

**Specific Activity of Glycolic Acid Carbon—**Photosynthesis experiments of from 5- to 20-minute duration were then carried out on leaf disks floated on 0.01 M $\alpha$-hydroxy-2-pyridinemethanesulfonic acid. At zero time, each lot of six disks was supplied with 6 μmoles of $^{14}CO_2$ containing 1.80 × 10$^6$ c.p.m. Since it seems fair to assume that six leaf disks contained an equal concentration of $^{14}CO_2$ actually available to the leaf, it was of interest to compare the specific activity of the $^{14}CO_2$ actually available to the leaf disks in such a closed system.

**Table II**

**Distribution of $^{14}C$ in leaf disks on water or inhibitor solution after 10 minutes of photosynthesis in $^{14}CO_2$**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Leaf disks in water</th>
<th>Leaf disks in inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{14}C$ c.p.m.</td>
<td>$^{14}C$ c.p.m.</td>
</tr>
<tr>
<td>Homogenate</td>
<td>11.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Insoluble residue</td>
<td>1.50</td>
<td>0.776</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>10.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Neutral compounds</td>
<td>2.94</td>
<td>1.15</td>
</tr>
<tr>
<td>Basic compounds</td>
<td>3.81</td>
<td>1.72</td>
</tr>
<tr>
<td>Glycolic acid fraction</td>
<td>0.222</td>
<td>4.24</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>0.0860</td>
<td>3.49</td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>0.123</td>
<td>0.271</td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.209</td>
<td>0.0600</td>
</tr>
<tr>
<td>Citric acid, ribose-5-P, glucose-6-P</td>
<td>0.370</td>
<td>0.388</td>
</tr>
<tr>
<td>3-P-glyceric acid</td>
<td>0.685</td>
<td>0.457</td>
</tr>
<tr>
<td>P-glycolic acid</td>
<td>0.0220</td>
<td>0.0275</td>
</tr>
<tr>
<td>Fructose-1,6-di-P, P-enolpyruvate</td>
<td>0.450</td>
<td>0.690</td>
</tr>
</tbody>
</table>

For 1 hour, six tobacco leaf disks were floated on water in the light in each of three Warburg vessels with the taps open to the air. Then the stopcocks were closed and 6 μmoles of $^{14}CO_2$ containing 1.80 × 10$^6$ c.p.m. were liberated from the side arm of each vessel. After 40 minutes of photosynthesis, the taps were opened for an additional 20 minutes. The water was withdrawn from the vessels with a hypodermic syringe, and replaced with a solution of 0.01 M $\alpha$-hydroxy-2-pyridinemethanesulfonic acid. At zero time, leaf disks from the first vessel were removed and killed. Sodium hydroxide solution was placed in the center well of the second vessel, the stopcocks were closed, and 6 μmoles of nonradioactive CO$_2$ (0.18% CO$_2$ in the gas phase at the start) were released in the third vessel. At the end of 10 minutes in the light, the disks were killed, and the glycolic acid concentration in the tissue and the specific activity of the carboxyl carbon were determined as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>CO$_2$-free air</th>
<th>0.18% Non-radioactive CO $^{14}CO_2$ in homogenate, 10$^6$ c.p.m.</th>
<th>Glycolic acid formed in 10 minutes by 240 mg of tissue (fresh wt.), μmoles</th>
<th>Total $^{14}C$ increase in glycolic acid, c.p.m.</th>
<th>Glycolic acid specific activity, c.p.m. per μmole of carboxyl carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.4</td>
<td>17.8</td>
<td>1.06</td>
<td>22,300</td>
<td>16,600</td>
</tr>
</tbody>
</table>

* Based on zero time control.

**Table III**

**Synthesis of glycolic acid from added CO$_2$ compared with endogenous sources of carbon**

Under these conditions there was about an equal concentration of endogenous CO$_2$ the specific activity of the available $^{14}CO_2$ in these experiments was calculated to be 1.50 × 10$^6$ c.p.m. per μmole of carbon.

As shown in Fig. 3, the specific activity of the individual carbons of glycolic acid is essentially equal to that of the $^{14}CO_2$ available. The specific activity was almost maximal at the shortest time taken, 5 minutes. Thus the synthesis of glycolic acid in leaves must be closely related to the primary carboxylation reaction in photosynthesis.

**Specific Activity of Several Metabolites during Photosynthesis at Different Concentrations of $^{14}CO_2$—**Since the specific activity of the carbon atoms of glycolic acid produced in the presence of $\alpha$-hydroxy-sulfonate is approximately equal to that of the $^{14}CO_2$, available to the leaf, it was of interest to compare the specific activities of some metabolites that are assumed to be closely related to the photosynthetic cycle (Table IV). Experiment 1 shows that after 5 minutes of photosynthesis at an initial CO$_2$ concentration of 0.18% CO$_2$, C-1 of glycolic acid had a specific activity 75% as high as that of the $^{14}CO_2$. The specific activity of glycolic acid in disks floated on water could not be determined in any of these experiments because of the difficulty in determining its concentration accurately when present in small amounts together with glyceric acid. The specific activity of the carboxyl carbon of P-glyceric acid in this experiment was about one-third as high as that of the carbons of glycolic acid. With-
at 0.786 to 0.244 in Experiment 5B).

<table>
<thead>
<tr>
<th>Experi-</th>
<th>Leaf disks</th>
<th>Time in</th>
<th>CO₂ concent-</th>
<th>Total radiactiv-</th>
<th>Estimated specific</th>
<th>Rate of</th>
<th>Specific activity of C-1 of P-</th>
<th>Specific activity of C-1 of P-</th>
<th>Estimated specific</th>
<th>Specific activity of C-1 of glucose-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ment</td>
<td>floated on</td>
<td>in ¹⁴CO₂</td>
<td>in air at start</td>
<td>in ¹⁴CO₂</td>
<td>activity in</td>
<td>¹⁴CO₂ fixed in homogenate</td>
<td>activity</td>
<td>P-glyceric acid</td>
<td>P-glyceric acid</td>
<td>activity</td>
</tr>
<tr>
<td>1</td>
<td>Water</td>
<td>5</td>
<td>0.18</td>
<td>54.0</td>
<td>1.50</td>
<td>15.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.308</td>
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* Calculated from the concentrations of CO₂ in the atmosphere together with the endogenous CO₂ estimated from Table III.

** The carboxyl carbon and methyl carbon were assumed to be equally labeled. The amount of activity in C-1 actually varied from 48 to 53%.

* It was assumed that C-2 and C-3 were equally labeled (I).

* Amount too small for accurate determination.

out inhibitor, the specific activity of C-1 of P-glyceric acid was also significantly lower than that of C-1 of glycolic acid.

In Experiment 2, in which a longer exposure to ¹⁴CO₂ was used in the presence of inhibitor, the specific activity of P-glyceric acid C-1 was very much lower. Phosphoglycolic acid has been suggested as a likely precursor of glycolic acid (5), yet in this experiment as well as in Experiments 3 and 4, under a variety of conditions the specific activity of phosphoglycolic acid carbon was generally one-tenth or less of that of the carbons in glycolic acid.

Experiment 3 compares the specific activities and the rates of production of glycolic acid starting with 0.11% CO₂ as compared with 1.10% CO₂. At the lower concentration of CO₂ there was a 4-fold greater rate of glycolic acid synthesis, and the specific activity of the glycolic acid was close to that of the available ¹⁴CO₂. At the higher concentration of CO₂, the specific activity of the glycolic acid was lower than that of the ¹⁴CO₂ supplied.

Experiments 4 and 5 required so much leaf tissue that they were each carried out in two parts, designated as A and B, with two adjacent leaves taken on the same day. If one compares the specific activity of C-1 of P-glyceric acid to that of C-1 of glycolic acid, it is apparent that, at low concentrations of CO₂, glycolic acid is considerably higher (0.639 compared with 0.334 in Experiment 4B; 1.64 compared with 0.798 in Experiment 5B).

At the higher concentration of CO₂, however, the relative specific activity in P-glyceric acid was more nearly equal to that in glycolic acid (0.312 to 0.198 in Experiment 4A and 0.660 to 0.938 in Experiment 5B).

In Experiments 4 and 5, the specific activity of C-1 of glucose-6-P was determined, and this was compared with the estimated specific activity of C-3 of P-glyceric acid from which C-1 of glucose-6-P is presumed to be derived (Fig. 4). In every instance but one, the specific activity of the glucose-6-P carbon was similar to but lower than that of the precursor P-glyceric acid carbon.

**DISCUSSION**

Although the synthesis of glycolic acid is severely inhibited when the concentration of CO₂ in the air reaches 0.5% (24), several of the lines of evidence presented indicate that the carbon atoms of glycolic acid come fairly directly from CO₂ when this is supplied at low concentrations. Racker has shown (31) that the Michaelis constant of the ribulose diphosphate carboxylase for CO₂ is a high one. Therefore in experiments carried out at 1.0% CO₂ or higher, this carboxylation would be favored. However, even at concentrations of 1.0 to 2.0% CO₂ in kinetic experiments with *Chlorella*, it was sometimes found that 30% of the CO₂ was taken up by undefined carboxylation reactions (32).

It has now been shown that at lower concentrations of CO₂, the carbon atoms of glycolic acid have the same specific activity as that of the ¹⁴CO₂ available to the leaf tissue (Fig. 4). The specific activities of the carboxyl carbon of P-glyceric acid are
lower that of glycolic acid and only approach equality with the $^{14}$CO$_2$ when the concentration of CO$_2$ in the gas phase is raised. Previous work on the path of carbon in photosynthesis involved a kinetic approach, and these are the first experiments in which a carbon balance has been attempted by measurement of specific activities.

The possibility existed that the results arose from an artifact, and that there was a small but highly radioactive pool of P-glyceric acid and a larger inactive one which were mixed when the tissue was killed. Since C-3 of P-glyceric acid is converted to C-1 of glucose-1-P in normal carbohydrate synthesis, the specific activities of these two related carbon atoms were compared (Table IV). The specific activity of C-1 of glucose-6-P is similar, but lower; hence it seems unlikely that the results obtained can be explained by the presence of pools of P-glyceric acid with differing activities.

Previous suggestions that glycolic acid is produced directly from a compound in the photosynthetic cycle such as ribulose-1,5-di-P or ribulose-5-P (Fig. 1) are not supported by the results obtained. This cannot be a major pathway because the specific activity of glycolic acid is higher than that of P-glyceric acid, and also because the carbons of phosphoglyceric acid have about one-tenth the specific activity of glycolic acid. Labeling experiments with radioactive glucose that have been interpreted as demonstrating direct synthesis of glycolic acid from a member of the photosynthetic cycle (33) have not considered at all the amounts of carbon involved.

The possible role of the oxidative metabolism of glycolic acid in oxygen uptake by higher plants in the light has recently been discussed (30). The high rates of accumulation under the optimal conditions defined here, several fold in excess of the dark respiration, support this view. The importance of glycolic acid metabolism in the process of stomatal opening in leaves has also been demonstrated (24, 27).

Recently (28, 29), glycolic acid has been shown to be converted into carbohydrate in higher plants by a series of reactions presumably involving the formation of glycine, serine, and P-glyceric acid. Accordingly, the undefined carboxylation (Fig. 4) by which glycolic acid is synthesized and which functions well at concentrations of CO$_2$ such as are normally found in air may be as important as the ribulose diphosphate carboxylase in photosynthesis in higher plants.

**SUMMARY**

Tobacco leaf disks were floated on a solution of α-hydroxy-2-pyridinemethanesulfonic acid, an inhibitor of glycolate oxidase.

In the light when low concentrations of CO$_2$ were present in the atmosphere, glycolic acid accumulated in the tissue at an initial rate of about 40 μmoles per hour per g, fresh weight. Under these conditions there was no significant effect on the amount of $^{14}$CO$_2$ taken up, and the rate of glycolate synthesis was several fold in excess of the rate of oxygen uptake by such leaves in the dark.

The specific activity of the carbon atoms of the glycolic acid synthesized under these conditions was the same as that of the $^{14}$CO$_2$ available, considerably higher than that of the carboxyl carbon of phosphoglyceric acid, and greatly in excess of that found in phosphoglycolic acid. It was therefore concluded that glycolic acid does not arise from the cleavage of a compound that is part of the photosynthetic cycle as has previously been suggested. The slightly lower specific activity of C-1 of glucose 6-phosphate in these experiments as compared with C-3 of phosphoglyceric acid supports the view that the results obtained were not an artifact resulting from the presence of two pools of phosphoglyceric acid with different activities.

These experiments suggest that, especially at concentrations of CO$_2$ close to those normally found in air, glycolic acid is synthesized in the light by a carboxylation reaction different from that catalyzed by ribulose diphosphate carboxylase.

**Acknowledgment**—I am grateful to Isabelle Namanworth for skillful technical assistance and for the preparation of the free phosphoglycolic acid.

**REFERENCES**

The Relation of Glycolic Acid Synthesis to the Primary Photosynthetic Carboxylation Reaction in Leaves

Israel Zelitch