The Relationship of Glycolic Acid to Respiration and Photosynthesis in Tobacco Leaves

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Studies with glycolic acid oxidase and glyoxylic acid reductase isolated from green leaves have led to the suggestion that these enzymes play a part in the oxygen uptake of leaf tissue (1-3). Glycolic acid oxidase is a flavoprotein which is capable of directly oxidizing glycolic acid, a substance rapidly produced during photosynthesis (4, 5). As has been demonstrated in model systems, the oxidase can also couple with glyoxylic acid reductase to oxidize reduced pyridine nucleotides (2).

If glycolic acid oxidase participates to a significant extent in respiration, specific inhibition of the oxidase might be expected to cause an accumulation of the substrate at a rate comparable to the normal rate of oxygen uptake. Such experiments provide a suitable means for evaluating the role of this enzyme in respiration. A series of effective and highly specific competitive inhibitors of glycolic acid oxidase has been described (6, 7). These compounds are all aldehyde bisulfite addition compounds of the general structure R-CHOH-SO₂Na, which are α-hydroxyalsonates and thus are analogues of glycolic acid, CH₂OH-COOH. When an excised mature leaf from any of the plant species examined is placed in sunlight with its base in a solution of a suitable α-hydroxyalsonate, the concentration of glycolic acid in the tissue increases at a rate which is similar on a molar basis to the normal rate of oxygen uptake of the tissue in darkness (7). On the assumption that the glycolic acid which accumulates in the presence of α-hydroxyalsonates would normally have been metabolized by glycolic acid oxidase, it has been concluded that a substantial part of the respiration of mature leaves in sunlight takes place by means of this oxidase. This conclusion is supported by a number of observations that appear in the literature, and which have been discussed (7).

Experiments have now been carried out in an attempt to elucidate further the action of the inhibitors of glycolic acid oxidase on the metabolism of the intact tobacco leaf. An α-hydroxyalsonate has been found which is more effective than other inhibitors that were previously tested. With this compound, α-hydroxy-2-pyridinemethanesulfonic acid (α-pyridine aldehyde bisulfite). The last compound, sometimes referred to as 2-pyridylhydroxymethanesulfonic acid, was purchased from Aldrich Chemical Company. Experiments were carried out in the greenhouse on sunny days when the light intensity was at least 3000 foot-candles, and in some experiments the temperature was maintained at 25° by use of a temperature-controlled chamber located in the greenhouse. In comparative studies, each experimental point represents an observation on individual adjacent leaves cut from the same plant.

At the end of the test period, 20 disks 1.2 cm in diameter were quickly cut from the central portion of each leaf according to a fixed arbitrary pattern. The disks (approximately 0.5 g of fresh weight) were transferred to a TenBroeck homogenizer, covered with 0.01 M sodium bisulfite solution previously heated to 90°, and placed in a steam bath for 3 minutes. The killed tissue was then ground and the suspension was clarified by centrifugation. Glycolic acid in the extract was isolated by elution from a Dowex 1-X10 acetate ion exchange column with 4 M acetic acid, and determined colorimetrically in the appropriate fraction. The identity of the glycolic acid which accumulates has been established by independent tests, and the validity of the procedure has been verified by obtaining good recoveries of glycolate added to leaf disks immediately before extraction (7).

Experiments on the effect of light intensity under artificial illumination were conducted by placing leaves at various distances from a bank of fluorescent lights in a temperature-controlled room. The effect of light intensity in sunlight was investigated on a bright day in the greenhouse, the light being controlled by shades of varying thicknesses made from cheesecloth.

Measurements of Oxygen Uptake—The respiration rate in darkness was determined by placing 7 of the disks weighing a total of about 150 mg in 15-m1 Warburg vessels together with 1.8 ml of water; KOH was present in the center well. The rate of oxygen uptake was determined calorimetrically in the appropriate fraction. The identity of the glycolic acid which accumulates has been established by independent tests, and the validity of the procedure has been verified by obtaining good recoveries of glycolate added to leaf disks immediately before extraction (7).

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and B is a-hydroxy-2-pyridinemethanesulfonic acid. Uptake were measured in Warburg vessels as previously described [1, 8], except that the addition of riboflavin phosphate was found unnecessary with homogenates. Portions of leaf tissue weighing 0.5 g were ground in a TenBroeck homogenizer with 4.5 ml of 0.1 mM tris(hydroxymethyl)aminomethane chloride buffer at pH 8.3 in the cold, and the resulting suspension was assayed directly. In all experiments, the amount of oxygen finally taken up indicated that catalase was present in excess [1, 9], and the stoichiometry of the reaction was consistent with the equation:

\[
\text{Glycolate} + \frac{1}{2} \text{O}_2 \rightarrow \text{glyoxylate} + \text{H}_2\text{O}
\]

Photosynthesis in Presence of C\(^{14}\)O\(_2\) Radioactive CO\(_2\) was supplied to leaves in an air atmosphere in cabinets (volume of 8.7 liters), fitted with air-tight glass windows. The C\(^{14}\)O\(_2\) was liberated by addition of an excess of perchloric acid to tracer amounts of D\(_2\)C\(^{14}\)O\(_2\) at zero time in the closed container. At the end of the experimental period in sunlight, the leaf blade was separated from the midrib, cut into small pieces, and plunged into 25 ml of boiling water. After about 3 minutes at boiling temperature, the killed tissue was ground and extracted as described earlier. Leaf extract representing about 1 g of tissue was placed on a Dowex 1-aceate column. After the nonacidic fraction, containing neutral carbohydrates and most of the amino acids, was obtained by washing the column with water, elution was continued with 4 M formic acid to collect the malic acid, and finally with 4 M formic acid to obtain the citric acid [10]. Formic acid was evaporated in a stream of air at 46°, and the remaining malic and citric acids were titrated before determination of the radioactivity.

Measurements of radioactivity were made by counting the extract and fractions from the Dowex 1 column on infinitely thin samples with a thin window counter.

**RESULTS**

Effect of Light Intensity on Accumulation of Glycolic Acid—Previous experience in this laboratory had indicated that inhibition of glycolic acid oxidase in vivo could best be demonstrated in sunlight at intensities between 2000 and 8000 foot-candles, at which the rate of photosynthesis is maximal for tobacco leaves [11]. The requirement for high light intensities to bring about the accumulation of glycolic acid in leaves placed in sodium bisulfite solution is shown in Fig. 1, which demonstrates that light intensities similar to those necessary for high rates of photosynthesis are essential in order to observe large increases in glycolic acid concentration.

\[\text{\(\alpha\)-Hydroxy-2-pyridinemethanesulfonic Acid as Effective Inhibitor of Glycolic Acid Oxidase in Vivo—}\] In attempts to find \(\alpha\)-hydroxysulfonates more effective than those already reported [6, 7], it was observed that \(\alpha\)-hydroxy-2-pyridinemethanesulfonic acid, an internal salt, was particularly efficient in promoting the accumulation of glycolic acid in leaves in sunlight. This compound, at a final concentration of \(5 \times 10^{-4} M\), was found to inhibit the initial rate of reaction of isolated glycolic acid oxidase 60% when the substrate concentration was at \(5 \times 10^{-3} M\). It is thus about as inhibitory as the other \(\alpha\)-hydroxysulfonates previously tested [6, 7]. The effect of this compound in the intact leaf is shown in Table 1.

It has already been shown that although glycolic acid accumulates rapidly in leaves placed in sunlight in the presence of \(\alpha\)-hydroxysulfonates, the concentration of glycolic acid diminishes when the leaf is transferred to darkness [7]. This probably occurs because glycolic acid is synthesized primarily in sunlight, and, since inhibition of the oxidase in vivo is never complete,
glycolic acid disappears when photosynthesis ceases. It is apparent from Experiment 1 (Table I), that although higher concentrations of disodium sulfoglycolate do not increase the amount of glycolic acid formed in sunlight, the rate of glycolic acid disappearance in the dark is lowered. In Experiment 2, where a longer period of darkness was used, this effect is still more apparent. The variability in the amount of inhibitor taken up and retained in different leaves undoubtedly accounts for the variability of the rate of glycolic acid disappearance in darkness previously observed (7).

Experiment 3 (Table I) demonstrates that a leaf that has been first placed in 0.01 M α-hydroxy-2-pyridinemethanesulfonic acid can subsequently be transferred to water in the light for a considerable period before the glycolic acid concentration diminishes. If the leaf is provided with 0.01 M inhibitor for 2 hours (Experiment 4), the leaf can then be placed in water for at least 2 more hours in sunlight without a decrease in the glycolic acid concentration occurring. With higher concentrations of inhibitor (0.02 M) the accumulated glycolic acid does not decrease after 2 more hours in sunlight in water, and only a slight decrease is observed when the leaf in water is placed in the dark for the same time, thus demonstrating the effectiveness of this inhibitor in the leaf.

**Effect of Concentration of Inhibitor on Glycolic Acid Accumulation in Leaves**—A comparison of the relative effectiveness of various concentrations of sodium bisulfite and α-hydroxy-2-pyridinemethanesulfonic acid when supplied to a tobacco leaf on the increase of glycolic acid in sunlight is illustrated in Fig. 2. A short experimental period, 30 minutes, was used in order to obtain initial rates of increase of glycolic acid. At a concentration of 0.01 M, each inhibitor has its maximal activity, but both the rate and extent of accumulation of glycolic acid is twice as great with α-hydroxy-2-pyridinemethanesulfonic acid. The maximal accumulation of glycolic acid with this inhibitor is also observed in 30 minutes rather than in 60 minutes (Table II), as was reported earlier with less efficient inhibitors (7). Glycolic acid increases in the presence of optimal concentrations of the α-pyridine aldehyde derivative at a rate of 18 μmoles per hour per g of leaf weight.

With purified preparations of glycolic acid oxidase, it was shown that inhibition by sodium bisulfite itself arose from its interaction with the enzymically produced glyoxylate to form an inhibitory α-hydroxy-sulfonate (6). In other experiments, it has been observed that the curve for sodium bisulfite shown in Fig. 2 is very similar to that obtained when glyoxylate bisulfite is provided. This further supports the view that bisulfite also interacts to form an α-hydroxy-sulfonate within the tissue. At concentrations above 0.02 M, the rates of accumulation of glycolic acid are often less than maximal, presumably because the synthesis of glycolic acid as well as its oxidation is interfered with at higher inhibitor levels.

**Effect of Age of Leaves on Inhibition of Glycolic Acid Oxidase in Vivo**—All of the experiments described thus far were carried out with tobacco leaves weighing from 10 to 15 g. As shown in Table II, under conditions where α-hydroxy-2-pyridinemethanesulfonic acid causes a substantial increase in glycolic acid in large older leaves, smaller leaves from the same plant show little change. The concentration normally observed in untreated leaves is from 0.4 to 1.0 μmole per g of leaf weight. Accordingly, glycolic acid oxidase probably functions to a greater extent in more mature leaves.

Other experiments which relate the physiological age of a tobacco leaf to its rate of respiration and to its glycolic acid oxidase activity are presented in Table III. Tissue from young leaves respires at a considerably higher rate in darkness in terms of fresh weight than that obtained from more mature leaves from the same plant. The specific activity of glycolic acid oxidase, however, is slightly higher in older leaves and the total activity per leaf is considerably greater. In such older leaves, more than 11 times as much glycolic acid oxidase activity is present as is needed to account for all of the respiration, if oxygen uptake is assumed to be similar in darkness and in light (12). Even if the rate of respiration of leaves is higher in the light than in the dark,
**Table III**

**Effect of age of tobacco leaves on respiration rate in darkness and on glycolic acid oxidase activity**

<table>
<thead>
<tr>
<th>Leaf No.*</th>
<th>Leaf fresh weight</th>
<th>Rate oxygen uptake</th>
<th>Glycolic acid oxidase activity</th>
<th>Ratio glycolic acid oxidase:oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>2.4</td>
<td>26.3</td>
<td>60.8</td>
<td>2.3</td>
</tr>
<tr>
<td>13</td>
<td>4.6</td>
<td>22.9</td>
<td>70.4</td>
<td>3.1</td>
</tr>
<tr>
<td>0</td>
<td>14.8</td>
<td>7.6</td>
<td>84.0</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* Leaves were numbered on the plant from the bottom upward.

Effect of age of tobacco leaves on respiration rate in darkness and on glycolic acid oxidase activity

Two samples were prepared from the central portions of leaves of 2 similar plants. Opposite sides of each leaf were used for determination of oxygen uptake and glycolic acid oxidase activity respectively. The initial fresh weights of the 2 leaves taken at each position on the plant differed by less than 15%. Duplicate glycolic acid oxidase assays were carried out at 2 enzyme concentrations, one twice the other, and duplicate determinations of oxygen uptake in darkness were made on disks obtained from the other sample. The pairs of determinations differed by less than 10%.

**Table IV**

**Effect of α-hydroxy-2-pyridinemethanesulfonic acid on glycolic acid formation**

<table>
<thead>
<tr>
<th>Glycolic acid fraction obtained from the Dowex 1-ace- tate column</th>
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<th>Glycolic acid fraction obtained from the Dowex 1-ace- tate column</th>
<th>Glycolic acid fraction obtained from the Dowex 1-ace- tate column</th>
<th>Glycolic acid fraction obtained from the Dowex 1-ace- tate column</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,830 c.p.m.</td>
<td>840 c.p.m.</td>
<td>1,620 c.p.m.</td>
<td>1,620 c.p.m.</td>
<td>1,620 c.p.m.</td>
</tr>
</tbody>
</table>

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<th>Glycolic acid fraction obtained from the Dowex 1-ace- tate column</th>
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<th>Glycolic acid fraction obtained from the Dowex 1-ace- tate column</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,620 c.p.m.</td>
<td>1,620 c.p.m.</td>
<td>1,620 c.p.m.</td>
<td>1,620 c.p.m.</td>
<td>1,620 c.p.m.</td>
</tr>
</tbody>
</table>

Confirmation of High Radioactivity in Glycolic Acid and Degradation of Glycolic Acid—The unusually high activity of the glycolic acid in the inhibited leaf in Experiment 1 (Table IV) prompted a further investigation to test the validity of the methods of isolation employed, and of the radiochemical purity of the glycolic acid isolated. Accordingly, a portion of the leaf extract from this experiment, which was calculated to contain 918 c.p.m. in its glycolic acid, was placed on paper, and the paper chromatogram was then developed in a mixture of ethyl ether-acetic acid-water (13:3:1) (14) together with carrier glycolic acid. After development of the chromatogram and treatment of the paper for several minutes with steam, the paper was sprayed with bromophenol blue indicator. The area of paper outlined by the yellow spot (RFP 0.47) was eluted with water, and the radioactivity thus recovered was 840 c.p.m. (92%). A portion of the paper chromatogram obtained from the Dowex 1-ace- tate column was treated in a similar manner. Of the sample representing 1,830 c.p.m. which was placed on the paper chromatogram, 1,620 c.p.m. (89%) of the activity was recovered after elution of the area which had the same RFP as glycolic acid. Thus the radioactivity in the glycolic acid has been confirmed by two independent methods, and the validity of the isolation procedure on Dowex 1 columns has been reinforced.

It has previously been reported that glycolic acid formed during photosynthesis in C14O2 is labeled equally in both carbon atoms (15). It became of interest to determine whether this was also true for the glycolic acid which accumulates in the presence of glycolic acid oxidase inhibitor. Tolbert et al. (16) have shown that in the glycolic acid oxidase reaction, the carboxyl carbon is converted to CO2 and the methyl carbon to formic acid as follows:

\[ \text{CH}_2\text{COOH} + \text{O}_2 \rightarrow \text{HCOOH} + \text{CO}_2 + \text{H}_2\text{O} \]

A sample of the glycolic acid in Experiment 1 (Table IV) representing 10,600 c.p.m. was treated with purified glycolic acid oxidase from spinach leaves (1) in the presence of carrier glycolate. The reaction was stopped when measurement of oxygen uptake indicated it was 85% complete. There were 4,000 c.p.m. (44%) in the CO2 which was counted as CaCO3, and 5,150 c.p.m. (56%) recovered in the formic acid by difference after counting the reaction mixture and correcting for the unchanged glycolate. Thus the glycolic acid produced in Experiment 1 must have been approximately equally labeled in both carbon atoms.

**Discussion**

As seems evident from these experiments and those previously described (7), studies with purified glycolic acid oxidase have
provided information useful in evaluating the physiological role of this enzyme in leaves. It has now been shown that in the presence of an α-hydroxysulfonate which is relatively stable during the test, the glycolic acid concentration of tobacco leaves may increase at a rate of at least 18 μmoles per hour per gram of leaf weight (Fig. 2, Table II). This is greater than the rate of oxygen uptake by the same tissue in darkness in older leaves, and is about equal to the rate of oxygen uptake in all but the youngest leaves of the tobacco plant (Table III). Glycolic acid oxidase plays a role in the respiration of leaves in sunlight, but the oxidase probably does not function as extensively in younger and rapidly expanding leaves. This conclusion is consistent with the view expressed earlier (7), that oxidative phosphorylation would not be as efficient when mediated by the flavoprotein mechanism of respiration as it is when a system of more positive oxidation-reduction potential such as the cytochromes is involved.

The data in Table III suggest that as a leaf matures, there is a marked synthesis of glycolic acid oxidase. The finding that more mature tobacco leaves contain 11 times the glycolic acid oxidase necessary to account for all of the oxygen uptake does not by itself prove its role in respiration, and may only signify that enough is known about the enzyme to assay it under optimal conditions. On the other hand, many of the oxidases that have been found in leaf tissues can account for only a small fraction of the total respiration if current methods of extraction and assay are reliable.

The rapid increases in glycolic acid concentration in sunlight in the presence of inhibitors of the oxidase have now been confirmed by independent methods with use of C14O2 (Table IV). The increased radioactivity of the glycolic acid in the leaves treated with inhibitor parallels closely the increase in the concentration of accumulated glycolic acid. Thus the glycolic acid must have been synthesized from recently assimilated carbon. It seems evident that at least half of the carbon fixed in photosynthesis in the normal leaf may be metabolized by the glycolic acid oxidase reaction, since when the enzyme is blocked the carbon taken up is converted into glycolic acid to this extent.

Other workers have observed high radioactivity in glycolic acid in photosynthetic tissue supplied with C14O2 (4, 17) but their experiments were always conducted under conditions where the partial pressure of CO2 was very much lower than normal. The experiments described in Table IV were carried out in air under conditions where the normal concentration of CO2 could not have been greatly depleted, especially in Experiment 2 where only a small portion of the C14O2 supplied was fixed. That the α-hydroxysulfonate used specifically blocks glycolic acid oxidase in the leaf, is further borne out by the relatively small effect of the inhibitor on the total C14O2 fixed by the leaf.

Even in short periods of photosynthesis such as 4 seconds, barley leaves in C14O2 produced glycolic acid in which the carbon atoms were uniformly labeled (15). The glycolic acid which accumulates in the presence of inhibitors of glycolic acid oxidase is also essentially uniformly labeled, suggesting that it is synthesized by the normal reactions of the leaf.

Griffith and Byerrum (18) have shown that glycolic acid is produced in young leaves of Nicotiana rustica from ribose-1-C14 in light, and that most of the C14 is in the methyl carbon. This is consistent with the suggestion of Wilson and Calvin (17) that glycolic acid arises from the first two carbon atoms of pentose phosphate. However, the yield of glycolic acid obtained from ribose-1-C14 was only about 0.5% of the radioactive carbon administered (18), perhaps because the glycolic acid oxidase metabolized much of it. More unequivocal information about the origin of glycolic acid in leaves must await further enzymic studies. The central role of this metabolite in respiration and photosynthesis in leaves, however, seems well established.

Moses and Calvin (19) have recently provided further support for the view that glycolic acid plays a role in hydrogen transfer during photosynthesis. In experiments with Chlorella cells carried out in tritiated water in the light, glycolic acid was the most highly radioactive compound isolated.

**SUMMARY**

An inhibitor of glycolic acid oxidase, α-hydroxy-2-pyridine-methanesulfonic acid, which is highly effective when supplied to tobacco leaves, has been described. In the presence of this inhibitor in sunlight, the glycolic acid concentration increases at a rate of at least 18 μmoles per hour per gram of leaf. This rate of accumulation is as large as the rate of oxygen uptake in darkness in all but the youngest leaves, and suggests that this oxidase plays an important role in the respiration of leaves in sunlight, especially in older tissue.

The rapid accumulation of glycolic acid in tobacco leaves in light in the presence of the inhibitor has been confirmed in experiments with CO2. More than 50% of the radioactivity of leaves treated with glycolic acid oxidase inhibitor has been found in the glycolic acid. This suggests that, in the normal leaf in sunlight, more than half of the carbon fixed may be metabolized by this oxidase, and emphasizes the central position of glycolic acid in the processes of respiration and photosynthesis in leaves.

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