Effect of Glycidate, an Inhibitor of Glycolate Synthesis in Leaves, on the Activity of Some Enzymes of the Glycolate Pathway

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ABSTRACT

Under conditions where glycolate synthesis was inhibited at least 50% in tobacco (Nicotiana tabacum L.) leaf discs treated with glycidate (2,3-epoxypropionate), the ribulose diphosphate carboxylase activity in extracts and the inhibition of the activity by 100% oxygen were unaffected by the glycidate treatment. [1-14C]Glycidate was readily taken into leaf discs and was bound to leaf proteins, but the binding occurred preferentially with proteins of molecular weight lower than ribulose diphosphate carboxylase. Glycidate added to the isolated enzyme did not inhibit ribulose diphosphate carboxylase activity or affect its inhibition by 100% O2. Thus, glycidate did not inhibit glycolate synthesis by a direct effect on ribulose diphosphate carboxylase/oxigenase.

NADH-glycolate reductase and phosphoglycolate phosphatase activities were also unaffected in extracts of leaf discs supplied with glycidate, but NADPH-glycolate reductase was inhibited about 35% in such extracts. Addition of 10 mM glycidate to isolated NADPH-glycolate reductase inhibited the activity about 25% after 15 minutes of reaction.

The small inhibition of NADPH-glycolate reductase by glycidate may help to explain the increase in glyoxylate concentration found in glycidate-treated leaf discs. Increasing the glyoxylate pool size in leaf discs has been shown to effectively block glycolate synthesis and photorespiration and increase net photosynthesis. Thus, the similar effects brought about by glycolate in leaf discs can be attributed to indirect effects of metabolic regulation.

The loss of CO2 by photorespiration, a process related biochemically to the synthesis and metabolism of glycolate (28), has been shown to decrease net photosynthesis greatly in many species. This has stimulated interest in regulating the glycolate pathway. Supplying 20 mM glycidate (2,3-epoxypropionate), a chemical analogue of glycolate and glyoxylate, to tobacco leaf discs in the light for 1 hr resulted in a 40 to 50% inhibition of both glycolate synthesis and photorespiration and caused a similar percentage increase in net 14CO2 fixation (30). The inhibitor did not affect glycolate oxidase activity either in vivo or in vitro. The products of photosynthetic 14CO2 fixation in leaf discs were altered by floating them on glycolidic solution. Pool sizes of glycine and serine (products of glycolate metabolism) were decreased while carbohydrate and especially the aspartate and glutamate pools were increased.

Since then a number of other glycolate effects have been described. Purolith and Tregunna (18) have shown that floating leaf discs of Nicotiana glutinosa for long periods on 5 mM glycidate inhibited the multiplication of tobacco mosaic virus (in which serine is a major constituent of the coat protein). This inhibition was reversed by supplying glycine or serine to the discs, indicating that the glycolate pathway was affected by glycidate. Chollet (6) observed a 20 to 50% increase in photosynthetic 14CO2 fixation with isolated spinach chloroplasts provided with 3 mM glycidate, although no inhibition of the low rates of glycolate synthesis was observed in this system in the presence of glycidate. Whole cotton leaves wet with glycidate solution did not have a lower CO2 compensation point, and he concluded that his results were at variance with the experiments previously conducted in this laboratory with tobacco leaf discs (30). However, no attempt to repeat our experiments was made. Wildner and Henkel (26) have observed that RuDP oxygense activity, but not carboxylase activity, was severely inhibited by treating the isolated enzyme from spinach leaves for 1 hr with 5 mM glycolate. The interpretation of their results is uncertain because apparently in their experiments the enzyme preparations used for carboxylase and oxygenase assays were not similar activated by prior treatment with Mg ions and CO2 (14) and the assays were carried out under different conditions. With Chromatium cells, Asami and Akazawa (1) found that glycolate had no effect on CO2 fixation, but that the slow rate of glycolate synthesis and excretion from the cells was stimulated by the treatment. They also showed that in crude bacterial extracts 5 mM glycolate did not inhibit RuDP carboxylase/oxygenase activity or the transketolase-catalyzed formation of glycolate, another possible source of glycolate (21).

In preliminary reports (32, 34) we presented further evidence that glycolate is an effective and specific inhibitor of glycolate synthesis in tobacco leaf discs, and showed that the inhibitor acted without directly affecting the RuDP carboxylase/oxygenase activity. Increasing the pool sizes in leaf discs of some common metabolites (such as glutamate, aspartate, or glyoxylate) blocks glycolate synthesis and photorespiration and greatly increases net CO2 assimilation (16, 17). This paper shows that glycolate does not block glycolate synthesis by directly affecting RuDP carboxylase/oxygenase activity, but that glycolate inhibits NADPH-glycolate reductase and may thereby increase the glyoxylate pool size. This increase together with the inhibitory effect of glycolate on glutamate:glyoxylate aminotransferase reported in the accompanying paper (13), indicate that glycolate inhibits glycolate synthesis by increasing the glutamate and glyoxylate concentrations in the tissue.

MATERIALS AND METHODS

Treatment of Leaf Discs and Preparation of Leaf Disc Extracts for Enzyme Assays. Leaf discs of tobacco (var. Havana

1 Abbreviation: RuDP, ribulose 1,5-diphosphate.
Seed) 1.6 cm in diameter were cut with a sharp punch from plants grown in a greenhouse. The discs were assigned to each sample by means of a Latin square design, and were floated top side up on 2.5 ml of water in 50-ml beakers, three discs per beaker (with a total fresh wt of about 120 mg). A total of three to 12 discs constituted a sample in different experiments. The beakers were held in place in a cutout Plexiglas sheet in a 28 C water bath and were illuminated from above and with a mirror beneath the beakers so that 300 μE m-2sec-1 (400-700 nm) of irradiance were provided. All of the beakers were loosely covered with a Plexiglas sheet for a preliminary period of 60 min. In glycolate experiments, the water was then replaced with either glycolate solution or water for an additional period of 60 min or longer with occasional shaking. When glycolate synthesis was measured, the solutions were removed, the discs were washed with water, and 10 mm α-hydroxy-2-pyridinethiolene-sulfonic acid (Fluka AG, Buchs, Switzerland) was added to inhibit glycolate oxidase (29). The discs were killed by plunging them into boiling 20% ethanol and keeping them at this temperature several min, and the glycolate accumulated in the presence of sulfonate was determined colorimetrically after anion exchange chromatography as previously described (29).

When extracts of leaf discs were prepared for enzyme assays, at the end of the experimental period the discs were quickly immersed in fresh water and homogenized in a TenBroeck homogenizer with a 2.25 ml of ice-cold grinding medium containing 0.05 M Tris-HCl buffer at pH 7.4, 1 M NaCl, 1 mM EDTA, 2 mM MgCl2, and 80 mM 2-mercaptoethanol (5). The homogenates were centrifuged at 2,500g for 10 min and the soluble proteins were collected by placing the supernatant fluid on a column (2 × 13 cm) of Sephadex G-25 (Pharmacia Fine Chemicals), which had previously been equilibrated with 0.05 M Tris-HCl buffer at pH 7.4 containing 0.2 M NaCl and 0.5 mM EDTA at 5 C (5), and eluting in the cold with the same solution. After a void volume of 17 ml was discarded, proteins were eluted in the next 10 ml.

In some experiments, the proteins eluted from Sephadex G-25 were further concentrated, to about 2 ml, by ultrafiltration using an Amicon Diaflo (model 12) apparatus fitted with a PM-10 membrane (which retains mol wt above 20,000). The proteins were then fractionated by chromatography on a column (2 × 21 cm) of Sephadex G-100, at 5 C. Elution was carried out with the same buffer system as above. After a void volume of about 20 ml was discarded, the first 10 ml fraction coincided with the elution of dextran blue (mol wt about 2 × 106). A second fraction of 5 ml and a third fraction of 10 ml were collected. Finally a fourth fraction of 10 ml which contained the early portion of horse heart Cyt c (mol wt about 12 × 106) was eluted. Protein was determined colorimetrically (15).

Assays of Isolated Enzymes. Ribulose diphosphate carboxylase was assayed in Warburg flasks that contained Tris-HCl buffer at pH 8.3 (100 μmol), GSH (6 μmol), MgCl2 (10 μmol), EDTA (0.6 μmol), NaH2C1O3 (5 μmol), and enzyme (either an extract eluted from a Sephadex G-25 column or partially purified spinach leaf enzyme) in a final volume of 1 ml (32). After incubating for 15 min at 30 C, the reaction was initiated by the addition of RuDP (Sigma Chemical Co., 0.7 μmol) from the sidearm. The reaction was terminated after 6 or 12 min by injecting 0.5 ml of 1 M HCl, and the radioactivity incorporated was determined by scintillation counting. Under these conditions the enzymic reaction rate was constant between 3 and 12 min. The effect of O2 on this reaction was determined by flushing the reaction mixture containing all of the components except RuDP with O2 for 3 min before the flasks were closed and incubated at 30 C for 15 min prior to RuDP addition. Thus, the enzyme was activated to the same extent when assayed in 21% O2 or 100% O2 (14).

Phosphoglycolate phosphatase was assayed (19) in a reaction mixture that contained Tris-acetate buffer at pH 6.3 (100 μmol), MgCl2 (1 μmol), and the crude enzyme extract in a final volume of 0.75 ml. At zero time, P-glycolate (prepared as described (27); 10 μmol) was added, and after 10 to 30 min the reaction was stopped by the addition of 0.25 ml of 10% trichloroacetic acid. The Pi produced was determined colorimetrically and compared with that found in control reaction mixtures without enzyme.

The NADPH-glyoxylate reductase activity was determined spectrophotometrically as the glyoxylate-dependent oxidation of NADPH at 30 C with 1 mm glyoxylate as described previously (33) and in Figure 1. NADH-glyoxylate reductase was assayed in an analogous manner with 16.7 mm glyoxylate. These reductases were assayed in extracts of leaf discs prepared as detailed above or in some experiments in enzyme extracts from whole tobacco leaves (33) precipitated with ammonium sulfate to 0.8 of saturation and dissolved in 1/10 the original extract volume with 10 mM K-phosphate buffer at pH 7 containing 5 mM EDTA.

SYNTHESIS OF GLYCIDATE AND [1-14C]GLYCIDATE

Nonradioactive potassium glycidate hemihydrate was prepared from 3-chlorolactate (3, 30). The synthesis of [1-14C]glycidate began with [1-14C]pyruvic acid which was converted to 3-chloro-[1-14C]pyruvic acid (25), and then to 3-chloro-[1-14C]lactic acid (24) before the final step. In a typical preparation, 0.25 MCI of sodium [1-14C]pyruvate (7.2 MCI/mmol, New England Nuclear) was mixed with 3.6 Mmol of pyruvic acid (Alrich Chemical Co.) which had been freshly distilled in vacuo. The total 14C was 411 × 104 cpn; specific radioactivity was about 113,000 cpn/mmol. An excess of sulfuric chloride (Alrich) was added dropwise to the [1-14C]pyruvic acid over a period of several hr with constant stirring at 0 C in a fume hood, and the viscous slightly yellow material was allowed to react for at least 24 hr longer at room temperature. The progress of the formation of [1-14C]chloropyruvic acid was followed by carrying out high voltage (2500 v) electrophoresis on small samples of the reaction mixture on Whatman 3MM paper for 30 min in 0.05 M formic acid-NH4OH buffer at pH 3.4. Pyruvate moved about 18 cm
and chloropyruvate 10 cm in this system (the acids were detected by spraying the steamed and dried paper with alkaline brom phenol blue solution). The reaction mixture was dried in vacuo over soda lime.

**Reduction Step.** To the above chloropyruvic acid in 11 ml of water was added a 15-fold excess (54 mmol) of sodium borohydrate (Sigma Chemical Co.) in 7.5 ml of water over a 20-min period with stirring at 0°C. The excess borohydrate was destroyed by dropwise addition of excess 10 n acetic acid. To remove some of the boric acid, the reaction mixture was then added to a column (4.3 x 5 cm) of Dowex 50-H+ cation exchange resin. The column was washed with 10 10-ml portions of water and the radioactivity in the eluate was monitored until all of the 14C was eluted. The combined eluate was taken to dryness in vacuo at 50°C. The residue was suspended in six successive 10-ml portions of ice-cold water, and each extract was filtered through a Whatman 42 filter paper. The total extract contained 350 x 10^6 cpm.

The [1-14C]chlorolactic acid was separated by placing the solution in a column (4.3 x 4.8 cm) of Dowex 1-acetate anion exchange resin. Chlorolactic acid is eluted with the “malic acid” and “citric acid” fraction (27). The column was first eluted with water, and then with 420 ml of 4 n acetic acid. Elution with 600 ml of 1 n formic acid and 900 ml of 2 n formic acid removed the radioactive chlorolactic acid (total of 209 x 10^6 cpm). A radiochemical purity of at least 93% was indicated by high voltage electrophoresis on Whatman 3MM paper in the above buffer system (chlorolactic acid moved about 11 cm and lactic acid 8 cm). The volume was reduced in vacuo, and as the dried viscous material did not crystallize, it was taken to dryness in vacuo and again chromatographed on a column (0.7 x 6 cm) of Dowex 1-acetate. The eluate containing the chlorolactic acid, when dried in vacuo over concentrated H2SO4, crystallized to give [1-14C]chlorolactic acid of at least 95% radiochemical purity by high voltage electrophoresis (total of 157 x 10^6 cpm; 96,300 cpm/μmol).

**Epoxy Formation.** The 3-chloro[1-14C]lactic acid (1.63 mmol by weight) was converted to potassium [1-14C]glycidate by treatment with two equivalents of KOH in absolute methanol as described by Blau et al. (3). The radioactive glycidate was precipitated by slowly adding a total of 2 volumes of ethyl ether at room temperature and placing the solution in an ice bath for several hr. The precipitate was washed by centrifugation with ether:methanol solution (2:1) at room temperature and then dried in vacuo over concentrated H2SO4. The glycidate in 1.7 ml of absolute methanol was reprecipitated by addition of 3 volumes of ether at room temperature. The colorless solid was dried in vacuo over concentrated H2SO4. The yield of potassium [1-14C]glycidate was 0.87 mmol by weight; 76.7 x 10^6 cpm; 89.600 cpm/μmol. This represented an overall radiochemical yield of 19% based on the starting [1-14C]pyruvate.

The neutral equivalent of the potassium [1-14C]glycidate hemihydrate after passage through Dowex 50-H+ was 138.7 (theoretical 135.0). By chromatography on Dowex 1-acetate, 92% of the 14C was eluted in the “malic acid” fraction, and a total of 98% in the combined glycolic, “malic,” and “citric acid” fractions as shown in Table II (27); this is a typical distribution for pure glycidate. Paper chromatography of the radioactive glycidate was carried out in the solvent system used to separate glycolate from glyceraldehyde (27). Chlorolactate (Rf = 0.48) and glycolate (Rf = 0.31) were well separated in this system, and the [1-14C]glycidate accounted for 96% of the total 14C in the sample. Degradation of the [1-14C]glycidate with ceric sulfate in H2SO4 (27) showed at least 95% of the 14C was in the C-1 position.

**RESULTS**

**Effect of Glycidate on Glycolate Synthesis and RuDP Carboxylase Activity.** Glycolate synthesis was inhibited 50% in leaves floated on 20 μM glycidate for 1 hr in light, and about 60% inhibition was obtained with 40 μM glycidate (30). Since RuDP carboxylase/oxygenase activity is believed to be an important reaction in glycolate synthesis in vivo (2, 22), the effect of glycidate on the activity of this enzyme in leaf discs was investigated. Table I shows that under conditions where glycolate synthesis in leaf discs was inhibited 74% (40 μM glycidate for 90 min), the RuDP carboxylase activity in extracts was essentially unchanged. The inhibition of this activity by 100% O2, a measure of the oxygenase activity, was also unaffected by the glycidate treatment.

**Experiments with Glycidate and [1-14C]Glycidate on RuDP Carboxylase Activity.** Glycolate synthesis was inhibited about 60% when leaf discs were floated on 20 μM glycidate or [1-14C]glycidate for 2.5 hours (Table II). Under these conditions of 14C uptake by the leaf tissue and the specific radioactivity of the inhibitor, the final concentration of glycidate was calculated to be about 8 μM in experiment 1 and 5 μM in experiment 2 (Table II). This indicates that glycidate was readily taken into the leaf discs under these conditions. As indicated under “Materials and Methods,” pure glycidate is eluted mainly but not exclusively in the “malic acid” fraction obtained by ion exchange chromatography on Dowex 1-acetate. The difference in radioactivity in the isolated “malic acid” fraction from the Dowex 1 column compared with the values in the nonmetabolized glycidate controls, indicated that during a 2.5-hr exposure 16% of the glycidate was metabolized by the leaf discs. The rate of glycidate metabolism was therefore about 0.5 μmol/g fresh wt of leaf/hr.

The specific activity of RuDP carboxylase was essentially the same in extracts from leaf discs floated on 20 μM [1-14C]glycidate and [1-14C]glycolate, even though the glycolate was synthesized from 14C-labeled glycidate in vivo. Table II shows that [1-14C]glycidate was eluted almost totally from the ion exchange resin, whereas [1-14C]glycolate was eluted as a minor spot at the 80% ethanol front.

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**Table I. Effect of Supplying Glycidate to Tobacco Leaf Discs in Light on Glycolate Synthesis and RuDP Carboxylase Activity**

<table>
<thead>
<tr>
<th>Glycolate Synthesis</th>
<th>Discs on 40 μM Water 90 min Glycolate 90 min Effect of Glycidate</th>
<th>µmol per g fresh wt-hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>90.1</td>
<td>10.2</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table II. Metabolism of [1-14C]Glycidate by Tobacco Leaf Discs in the Light and the Effect of the Radioactive Inhibitor on Glycolate Synthesis**

Leaf discs (6 per sample) were floated for 1 hr at 28°C in light as described in Materials and Methods before the treatment with glycidate as shown. The discs were killed in boiling 20% ethanol, and leaf extracts were fractionated by column chromatography on Dowex-I acetate. The [1-14C]glycidate had a specific radioactivity of 89.100 cpm/μmol, and a total of 2.48 x 10^6 cpm were supplied to 6 leaf discs. In the unincubated control, 37,000 cpm were recovered in the eluate of RuDP in the blot 178,000 cpm; in the leaf extract of Experiment 1, 168,000 cpm (6.7% of the residue); [C-14C]glyceraldehyde (110,000 cpm, 12.35 μmol in the residue). Glycolate synthesis was determined as described in Materials and Methods.

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**Effect of Glycidate on Glycolate Synthesis and RuDP Carboxylase Activity.** Glycolate synthesis was inhibited 50% in leaves floated on 20 μM glycidate for 1 hr in light, and about 60% inhibition was obtained with 40 μM glycidate (30). Since RuDP carboxylase/oxygenase activity is believed to be an important reaction in glycolate synthesis in vivo (2, 22), the effect of glycidate on the activity of this enzyme in leaf discs was investigated. Table I shows that under conditions where glycolate synthesis in leaf discs was inhibited 74% (40 μM glycidate for 90 min), the RuDP carboxylase activity in extracts was essentially unchanged. The inhibition of this activity by 100% O2, a measure of the oxygenase activity, was also unaffected by the glycidate treatment.
Fraction of 5 mm glycidate on the activity of partially purified RuDP carboxylase isolated from spinach leaves was also investigated. When assayed at pH 8.3, or at pH 9.3, which favors the oxygenase activity (22), glycidate had a negligible effect on the enzymic activity (Table I in ref. 32). The inhibition by 100% O₂ was unchanged when glycidate was added at either pH value. Thus, neither the in vivo or isolated carboxylase nor oxygenase activities were affected by glycidate.

Fractionation of Glycolate Pathway Enzymes. RuDP carboxylase is an unusually large protein with a mol wt of about 560,000 (12). Most, and probably all, of the other enzymes of the glycolate pathway have lower mol wt. The pure NADH-glyoxylate reductase from spinach leaves, for example, has a mol wt of 97,500 ± 5,000 (11), and P-glycolate phosphatase from pea leaves had mol wt of 50,000 and 100,000 (9). Accordingly, proteins differing so greatly in mol wt from RuDP carboxylase could readily be fractionated by chromatography on Sephadex G-100, as described under "Materials and Methods."

Table III shows the arbitrary separation of the soluble proteins into the four fractions previously described places about 70% of the protein extracted in fraction 1 (including proteins with mol wt above 100,000), and about 10% in each of fractions 2, 3, and 4 (the last fraction contains protein with mol wt approaching 12,000). As expected, about 95% of the RuDP carboxylase activity was located on fraction 1 (Tables III and IV), and the specific activity was greatest in fraction 1. Fraction 2 contained the highest total and specific activity for NADH- and NADPH-glyoxylate reductases and P-glycolate phosphatase.

Table III. Distribution of Protein and Some Enzymes of the Glycolate Pathway on Fractionation by Chromatography on Sephadex G-100

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction No.</td>
<td>Protein Conc.</td>
<td>RuDP Carboxylase Activity</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>[1-14C]mol/ hr</td>
</tr>
<tr>
<td>1</td>
<td>3.77</td>
<td>6.29</td>
</tr>
<tr>
<td>2</td>
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<td>1.08</td>
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<td>0.47</td>
<td>0.71</td>
</tr>
<tr>
<td>4</td>
<td>0.47</td>
<td>—</td>
</tr>
</tbody>
</table>

Table IV. Effect of [1-14C]Glycidate and Prior Glycidate Treatment on the Distribution of Protein Labeling in Tobacco Leaf Discs in the Light

In Experiment 1, a preliminary period on water, 6 leaf discs were floated on a total of 1.5 ml of 20 mM [1-14C]glycidate (a total of 1.58 x 10⁶ cpm; 52,500 cpm/mol) for 2.5 hr (A), or for 2.5 hr on nonradioactive glycidate followed by an additional 2.5 hr with [1-14C]glycidate (B). Control experiments showed RuDP carboxylase activity was not inhibited by glycidate treatment. In (A), the RuDP carboxylase activity in fraction 1 decreased 10.1 and in fraction 2 0.25 [1-14C]mol fixed per fraction- hr. In (B), these values were 10.1 and 0.21, respectively.

In Experiment 2, 12 discs were used on a total of 3.0 ml of 20 mM [1-14C]glycidate (a total of 5.6 x 10⁶ cpm; 94,400 cpm/mol). Control experiments showed that in Experiment 1 glycolate synthesis was inhibited 51% after 2.5 hr with glycidate and 80% after 5.0 hr. In Experiment 2, glycolate synthesis was inhibited 56% after 2.5 hr with glycidate and 66% after 5.0 hr.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Chromatography on Sephadex G-100</th>
<th>Glycidate 2.5 hr,</th>
<th>Glycidate 2.5 hr,</th>
<th>Total [14C]-Bound</th>
<th>Total [14C]-Bound</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[1-14C]Glycidate 2.5 hr</td>
<td>[1-14C]Glycidate 2.5 hr</td>
<td>per Fraction</td>
<td>per mg Protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(A) - (B)</td>
<td>(A) - (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cpm [14C]-Bound</td>
<td>cpm [14C]-Bound</td>
<td>cpm [14C]-Bound</td>
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<td>mg protein</td>
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glycolate phosphatase was investigated. Under conditions where glycidate would severely inhibit glycolate synthesis, extracts of leaf discs were essentially unaffected in their NADH-glyoxylate reductase or P-glycolate phosphatase activity (Table V). NADPH-glyoxylate reductase, however, was consistently inhibited about 35% in extracts from leaf discs treated with 20 mM glycolate for 2.5 hr compared with extracts from control discs on water and with other enzyme activities assayed.

**Table V. Effect of Treating Tobacco Leaf Discs with Glycidate in the Light on the Activity of Some Extractable Enzymes of the Glycolate Pathway**

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Enzyme Assayed</th>
<th>Dishes on</th>
<th>Water</th>
<th>Glycolate</th>
<th>Effect of Glycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NADH-Glyoxylate Reductase</td>
<td>10 64.8 10 64.4</td>
<td>+11 15 10 64.8</td>
<td>0 10 64.8</td>
<td>-25 0 10 64.8</td>
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<tr>
<td>2</td>
<td>NADH-Glyoxylate Reductase</td>
<td>10 64.8 10 64.4</td>
<td>+11 10 64.8 10 64.4</td>
<td>0 10 64.8</td>
<td>-25 0 10 64.8</td>
</tr>
<tr>
<td>3</td>
<td>P-Glycolate Phosphatase</td>
<td>10 64.8 10 64.4</td>
<td>+11 10 64.8 10 64.4</td>
<td>0 10 64.8</td>
<td>-25 0 10 64.8</td>
</tr>
<tr>
<td>4</td>
<td>P-Glycolate Phosphatase</td>
<td>10 64.8 10 64.4</td>
<td>+11 10 64.8 10 64.4</td>
<td>0 10 64.8</td>
<td>-25 0 10 64.8</td>
</tr>
</tbody>
</table>

| 2 | umol/mg fresh wt. | 2 | umol/mg protein-hr |

NADPH-glyoxylate reductase, but not NADH-glyoxylate reductase, was inhibited in leaf discs treated with glycolate, the effect of the inhibitor on partially purified enzyme from tobacco leaves was also examined. Figure 1 shows a concentration- and time-dependent inhibition of isolated NADPH-glyoxylate reductase by glycolate. About 15% inhibition was obtained after 15 min with 5 mM glycolate and 25% inhibition was found with 10 mM glycolate. Some further characteristics of this inhibition were studied. Adding glycolate (as much as 16.7 mM) or NADPH to the enzyme in the reaction mixture before adding glycolate did not protect the enzyme or alter the degree of inhibition; there was no effect of enzyme concentration on the extent of inhibition by glycolate; and little difference was found in the extent of inhibition by glycolate at pH 8 compared with pH 6.4.

In contrast to the results in Figure 1, NADH-glycolate reductase activity in the same preparation (specific activity, about 5 μmol/mg of protein-hr) was inhibited an average of only 2.5% (in four experiments) with 5 mM glycolate and 5.1% with 10 mM glycolate.

**DISCUSSION**

Several different kinds of experiments have shown that the treatment of tobacco leaf discs with glycolate (which could be expected to bind to proteins and inactivate them irreversibly) strongly inhibits glycolate synthesis without affecting the activity of RuDP carboxylase/oxygenase extracted from the tissue (Table I and ref 32). [1-14C]Glycolate combined mainly with proteins of smaller mol wt than RuDP carboxylase whether the results are expressed on a mg protein basis (Table IV) or calculated on the basis of moles of protein. Glycolate had no effect on the activity of isolated RuDP carboxylase or its inhibition by 100% O2 (32). Thus, glycolate must inhibit glycolate synthesis either by affecting RuDP oxygenase activity indirectly, or it interferes with other biochemical reactions responsible for most glycolate synthesis.

The finding of an oxygenase activity associated with RuDP carboxylase (4) stimulated the hypothesis that this was the exclusive or at least the major pathway for glycolate biosynthesis and hence photorespiration (2, 22). There are a number of experimental difficulties associated with accepting this hypothesis with regard to observations about glycolate synthesis in vivo. For example, the rates of RuDP oxygenase activity reported are too low to account for the rapid synthesis of glycolate in leaves in normal air. P-glycolate has not been found to function as an important intermediate in glycolate synthesis in kinetic experiments with whole tissues, and glycolate is synthesized in chloroplasts even at saturating CO2 levels when the RuDP oxygenase cannot function (8, 31). Also, it has not yet been possible to inhibit the oxygenase activity without also inhibiting the carboxylase activity when the enzyme is activated by pre-treatment with CO2 and Mg ions (7). Nevertheless, we have shown (Tables I, II, IV) that glycolate synthesis in tobacco leaf discs may be inhibited 50% or more by floating illuminated leaf discs on solutions of glycolate (30), and similar inhibitions are caused by glutamate (17) or glycolate (16), while net CO2 assimilation is greatly increased. This indicates that RuDP carboxylase activity was not inhibited when glycolate synthesis was blocked.

Glycidate is readily taken up by leaf discs under the conditions described and is slowly metabolized (Table II). Extracts of leaf discs previously treated with glycidate have activities of RuDP carboxylase/oxygenase, NADH-glyoxylate reductase, and P-glycolate phosphatase similar to the activities of extracts from untreated discs (Table I and V). In contrast, NADPH-glyoxylate reductase activity was inhibited about 35% when discs were floated on glycolate. Isolated NADPH-glyoxylate reductase was also inhibited 25% by reaction with 10 mM glycolate for 15 min. The chemical nature of the binding between glycolate and the enzyme has not been investigated, although reactions between the epoxide group of glycolate and exposed carboxyl groups (20) or sulfhydryl groups (10) on proteins would be expected.

Glycidate inhibited NADPH-glyoxylate reductase but had almost no effect on NADH-glyoxylate reductase activity. NADPH-glyoxylate reductase was shown to be a protein distinct from NADH-glyoxylate reductase by conventional fractionation techniques (33). The NADPH-enzyme was later located in chloroplasts (23), while the NADH-enzyme was found in leaf peroxisomes (23). Glycolate may therefore block glycolate synthesis by inhibiting the glyoxylate reductase of the chloroplast. This enzyme may play a role in the biosynthesis of glycolate as shown by the conversion of [2-14C]glycolate to [2-14C]glycolate in leaf discs (29). Alternatively, blocking the NADPH-glyoxylate reductase activity may bring about an increase in the pool size of glycolate and inhibit glycolate synthesis by a feedback mechanism similar to that apparently observed when glycolate itself is supplied to leaf discs (16).

In an accompanying paper (13) it is shown that glycolate is a strong inhibitor of glutamate:glyoxylate aminotransferase and that the inhibitor increases the pool size of glyoxylate in tobacco leaf discs. Blocking this aminotransferase or NADPH-glyoxylate reductase with glycolate could contribute to the increased glyoxylate concentration observed. Inhibition of the aminotransferase also explains the large increase in pool size of glutamate and aspartate in leaf discs treated with glycolate.

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GLYCIDATE ON GLYCOLATE PATHWAY ENZYMES


