

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. [^{14}C]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% O_2 . Thus, glycidate did not inhibit glycolate synthesis by a direct effect on ribulose diphosphate carboxylase/oxygenase.

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

The small inhibition of NADPH-glyoxylate 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 glycidate in leaf discs can be attributed to indirect effects of metabolic regulation.

The loss of CO_2 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 $^{14}\text{CO}_2$ fixation (30). The inhibitor did not affect glycolate oxidase activity either *in vivo* or *in vitro*. The products of photosynthetic $^{14}\text{CO}_2$ fixation in leaf discs were altered by floating them on glycidate 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 glycidate effects have been described. Purohit 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 $^{14}\text{CO}_2$ 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 CO_2 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 described. Wildner and Henkel (26) have observed that RuDP¹ oxygenase activity, but not carboxylase activity, was severely inhibited by treating the isolated enzyme from spinach leaves for 1 hr with 5 mM glycidate. The interpretation of their results is uncertain because apparently in their experiments the enzyme preparations used for carboxylase and oxygenase assays were not similarly activated by prior treatment with Mg ions and CO_2 (14) and the assays were carried out under different conditions. With *Chromatium* cells, Asami and Akazawa (1) found that glycidate had no effect on CO_2 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 glycidate 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 glycidate 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 CO_2 assimilation (16, 17). This paper shows that glycidate does not block glycolate synthesis by directly affecting RuDP carboxylase/oxygenase activity, but that glycidate inhibits NADPH-glyoxylate reductase and may thereby increase the glyoxylate pool size. This increase together with the inhibitory effect of glycidate on glutamate:glyoxylate aminotransferase reported in the accompanying paper (13), indicate that glycidate 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 $300 \mu\text{E m}^{-2}\text{sec}^{-1}$ (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 glycidate experiments, the water was then replaced with either glycidate 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-pyridinemethanesulfonic 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 MgCl_2 , 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 \times 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 \times 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 2×10^6). 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 12×10^3) 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), MgCl_2 (10 μmol), EDTA (0.6 μmol), $\text{NaH}^{14}\text{CO}_3$ (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 N 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 O_2 on this reaction was determined by flushing the reaction mixture containing all of the components except RuDP with O_2 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% O_2 or 100% O_2 (14).

Phosphoglycolate phosphatase was assayed (19) in a reaction

mixture that contained Tris-acetate buffer at pH 6.3 (100 μmol), MgCl_2 (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- ^{14}C]GLYCIDATE

Nonradioactive potassium glycidate hemihydrate was prepared from 3-chlorolactate (3, 30). The synthesis of [1- ^{14}C]glycidate began with [1- ^{14}C]pyruvic acid which was converted to 3-chloro[1- ^{14}C]pyruvic acid (25), and then to 3-chloro[1- ^{14}C]lactic acid (24) before the final step. In a typical preparation, 0.25 mCi of sodium [1- ^{14}C]pyruvate (7.2 mCi/mmol, New England Nuclear) was mixed with 3.60 mmol of pyruvic acid (Aldrich Chemical Co.) which had been freshly distilled *in vacuo*. The total ^{14}C was 411×10^6 cpm; specific radioactivity was about 113,000 cpm/ μmol . An excess of sulfuric acid (Aldrich) was added dropwise to the [1- ^{14}C]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- ^{14}C]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- NH_4OH buffer at pH 3.4. Pyruvate moved about 18 cm

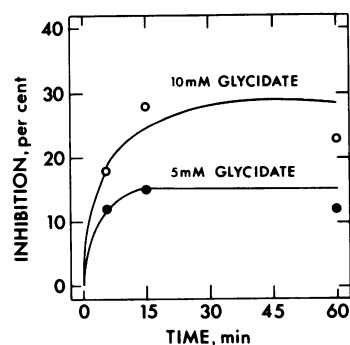


FIG. 1. Effect of time and glycidate concentration on the inhibition of isolated NADPH-glyoxylate reductase activity. The enzyme preparation was prepared from tobacco leaf extracts by ammonium sulfate precipitation. The reaction mixture contained K-phosphate buffer at pH 6.4 (50 μmol), 0.05 ml enzyme diluted with 0.1 M buffer, and NADPH (0.15 μmol) in a final volume of 3 ml. At zero time the reaction was initiated by the addition of glyoxylate (3 μmol), and the oxidation of NADPH at 30 C was followed at 340 nm in a Gilford spectrophotometer. The specific activity in the controls without glycidate was about 18 $\mu\text{mol}/\text{mg}$ of protein \cdot hr. When glycidate was added it was supplied to the reaction mixture containing buffer and enzyme for the times shown at 30 C before the addition of NADPH and glyoxylate at zero time. The values shown are the means of from two to eight separate determinations for each point.

and chloropyruvate 10 cm in this system (the acids were detected by spraying the steamed and dried paper with alkaline bromophenol 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 borohydride (Sigma Chemical Co.) in 7.5 ml of water over a 20-min period with stirring at 0 C. The excess borohydride 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 × 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 × 10⁶ cpm.

The [¹⁴C]chlorolactic acid was separated by placing the solution in a column (4.3 × 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 × 10⁶ 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 × 6 cm) of Dowex 1-acetate. The eluate containing the chlorolactic fraction, when dried *in vacuo* over concentrated H₂SO₄, crystallized to give [¹⁴C]chlorolactic acid of at least 95% radiochemical purity by high voltage electrophoresis (total of 157 × 10⁶ cpm; 96,300 cpm/μmol).

Epoxide Formation. The 3-chloro[¹⁴C]lactic acid (1.63 mmol by weight) was converted to potassium [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 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 [¹⁴C]glycidate was 0.87 mmol by weight; 76.7 × 10⁶ cpm; 89,100 cpm/μmol. This represented an over-all radiochemical yield of 19% based on the starting [¹⁴C]pyruvate acid.

The neutral equivalent of the potassium [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 glycerate (27). Chlorolactate (R_F = 0.48) and glycidate (R_F = 0.31) were well separated in this system, and the [¹⁴C]glycidate accounted for 96% of the total ¹⁴C in the sample. Degradation of the [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 tobacco

leaf discs floated on 20 mM glycidate for 1 hr in light, and about 60% inhibition was obtained with 40 mM 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 mM 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 [¹⁴C]Glycidate on RuDP Carboxylase Activity. Glycolate synthesis was inhibited about 60% when leaf discs were floated on 20 mM glycidate or [¹⁴C]glycidate for 2.5 hours (Table II). From the amount of ¹⁴C taken up by the leaf tissue and the specific radioactivity of the inhibitor, the final concentration of glycidate was calculated to be about 8 mM in experiment 1 and 5 mM 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 mM [¹⁴C]

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

RuDP carboxylase activity was assayed with 6 leaf discs on the protein fraction obtained after elution of a leaf extract from a Sephadex G-25 column; the enzymic reaction was initiated by the addition of RuDP to the otherwise complete reaction mixture at pH 8.3. The values in parentheses show the percentage inhibition caused by 100% O₂. Glycolate synthesis was measured on different discs in the presence of sulfonate.

Glycolate Synthesis			Effect of Glycidate %
Discs on Water 90 min	Discs on Glycidate 90 min	Discs on 40 mM Glycidate 90 min	
μmol per g fresh wt·hr			
58.7	15.2		-74
RuDP Carboxylase Activity			Effect of Glycidate %
Discs on Water 90 min	Discs on Glycidate 90 min	Discs on 40 mM Glycidate 90 min	
μmol ¹⁴ CO ₂ fixed per mg protein·hr			
Assayed in air	10.8	10.7	-1
Assayed in 100% O ₂	6.3 (-42%)	5.3 (-50%)	-16

Table II. Metabolism of [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-1 acetate. The [¹⁴C]glycidate had a specific radioactivity of 89,100 cpm/μmol, and a total of 2.68 × 10⁶ cpm were supplied to 6 leaf discs. In the unheated control, 875,000 cpm were placed on the column; in the heated control, 178,000 cpm; in the leaf extract of Experiment 1, 166,000 cpm (6,300 cpm in the residue); and in the leaf extract of Experiment 2, 104,000 cpm (2,350 cpm in the residue). Glycolate synthesis was determined as described in Materials and Methods.

Fraction	Distribution of ¹⁴ C in Various Fractions by Chromatography on an Anion Exchange Column		Extract of Killed Leaf Discs on 20 mM [¹⁴ C]Glycidate for 2.5 hr	
	[¹⁴ C]Glycidate Directly on Column	[¹⁴ C]Glycidate First in Boiling 20% Ethanol	Experiment 1	Experiment 2
% of Total Radioactivity Placed on Column				
Neutral and Basic	0.13	0	7.1	3.2
Aspartic Acid	0.15	0.09	0.76	0.98
Glycolic Acid	3.5	5.7	6.5	14.0
Malic Acid	92.0	89.0	76.0	71.0
Citric Acid	2.0	2.3	2.7	2.9
Strong Acid	—	2.3	2.2	2.9
Glycolate Synthesis (Experiment 1, above)				
Discs on Water 2.5 hr	Discs on 20 mM Nonradioactive Glycidate 2.5 hr	Discs on 20 mM [¹⁴ C]Glycidate 2.5 hr		
μmol per gram fresh weight·hr				
61.7	21.9 (-65%)	27.6 (-55%)		

^{14}C]glycidate for 2.5 hr, or 20 mM glycidate for 2.5 hr followed by 20 mM $[1-^{14}\text{C}]$ glycidate for 2.5 hr (Table 3 in ref. 32). This was true whether the results were expressed on the basis of leaf fresh wt or protein concentration. Similarly, the lengthy exposure of the leaf discs to glycidate did not alter the sensitivity of the RuDP carboxylase activity to 100% O_2 .

The effect of 5 mM glycidate on the activity of partially purified RuDP carboxylase isolated from spinach leaf 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 1 in ref. 32). The inhibition by 100% O_2 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 $97,500 \pm 5,000$ (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 that 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.

Labeling of Leaf Proteins with $[1-^{14}\text{C}]$ Glycidate. Since epoxide groups have been shown to inactivate enzymes by reacting covalently with carboxyl (20) and sulfhydryl groups (10) on proteins, ^{14}C -labeled glycidate was used to determine whether a chemical reaction occurred with some of the enzymes associated with glycolate synthesis and the glycolate pathway. In order to minimize the measurement of nonspecific and reversible binding between $[1-^{14}\text{C}]$ glycidate and proteins, the labeling of proteins in leaf discs first treated with nonradioactive glycidate for 2.5 hr and then with $[1-^{14}\text{C}]$ glycidate for 2.5 hr was subtracted from radioactivity bound to proteins on labeling with $[1-^{14}\text{C}]$ glycidate alone for 2.5 hr (Table IV). This is a conservative procedure, because the increased time used for the pretreated discs increased the percentage inhibition of glycolate synthesis slightly (legend of Table IV).

Although column chromatography with Sephadex G-100 isolated almost all of the RuDP carboxylase activity in fraction 1 (Tables III and IV), the greatest amount of ^{14}C fixed from the radioactive glycidate was in fraction 2 (Table IV, experiments 1 and 2). The highest specific radioactivity on a mg protein basis (or based on a conservative estimate of the moles of protein per fraction) was also found in fraction 2, showing that $[1-^{14}\text{C}]$ glycidate reacted more with a protein or proteins of lower mol wt than with larger proteins such as RuDP carboxylase.

Effect of Glycidate in Leaf Discs on the Activity of Some Extracted Enzymes. A number of independent experimental approaches described above were all consistent with the view that glycidate does not inhibit glycolate synthesis by directly affecting RuDP carboxylase/oxygenase activity. Therefore, the possible effect of glycidate on the glyoxylate reductases and P-

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

Samples of 9 leaf discs were floated in the light on water for 1 hr, and then on 20 mM glycidate (Experiment 1) or on water (Experiments 2 and 3) for an additional 2.5 hr before proteins were extracted. The soluble proteins were collected by chromatography on Sephadex G-25. The proteins were then either first concentrated by ultrafiltration (Experiment 1) before further fractionation, or a sample of protein solution was directly fractionated (Experiments 2 and 3) by Sephadex G-100 chromatography. The fractions were assayed as described under Materials and Methods. The results in Experiment 2 are the means of two separate experiments.

Fraction No.	Experiment 1		Experiment 2		Experiment 3	
	Protein Concentration	RuDP Carboxylase Specific Activity	NADH-Glyoxylate Reductase Activity	NADPH-Glyoxylate Reductase Activity	Protein Concentration	P-Glycolate Phosphatase Activity
	mg	$\mu\text{mol } ^{14}\text{CO}_2/\text{mg protein}\cdot\text{hr}$	$\mu\text{mol NADH/hr per Fraction}$	$\mu\text{mol NADPH/hr per Fraction}$	mg	$\mu\text{mol P/mg protein}\cdot\text{hr}$
1	3.77	6.29	4.2	2.0	7.25	4.2
2	0.35	1.08	8.1	2.1	0.73	40.6
3	0.47	0.71	2.9	1.2	0.60	18.9
4	0.47	—	0	—	—	—

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

In Experiment 1, after a preliminary period on water, 6 leaf discs were floated on a total of 1.5 ml of 20 mM $[1-^{14}\text{C}]$ glycidate (a total of 1.58×10^6 cpm; 52,500 cpm/ μmol) for 2.5 hr (A), or for 2.5 hr on nonradioactive glycidate followed by an additional 2.5 hr with $[1-^{14}\text{C}]$ glycidate (B). Control experiments showed RuDP carboxylase activity was not inhibited by glycidate treatment. In (A), the RuDP carboxylase activity in Fraction 1 was 10.1 and in Fraction 2 0.25 $\mu\text{mol } ^{14}\text{CO}_2$ fixed per Fraction $\cdot\text{hr}$. 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 $[1-^{14}\text{C}]$ glycidate (a total of 5.64×10^6 cpm; 94,400 cpm/ μmol). 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.

Expt. No.	Fraction No. Chromatography on Sephadex G-100	Discs on $[1-^{14}\text{C}]$ Glycidate 2.5 hr		Total ^{14}C -Bound per Fraction (A) - (B)	Total ^{14}C -Bound per mg Protein (A) - (B)
		(A)	(B)		
		cpm ^{14}C -Bound	cpm ^{14}C -Bound	cpm ^{14}C -Bound	cpm ^{14}C -Bound/mg protein
1	1	645	453	192	—
	2	342	69	273	—
	3	264	93	171	—
	4	102	63	39	—
2	1	202	169	240	33
	2	1107	243	375	864
	3	292	250	50	42
	4	217	256	0	-39

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

Tobacco leaf discs (9 per sample) were floated on water for 1 hr and then on water or 20 mM glycidate for 2.5 hr. Extracts were then prepared and the enzymes assayed on the eluate obtained after Sephadex G-25 chromatography.

Expt. No.	Enzyme Assayed	Specific Activity of Enzyme		Effect of Glycidate %
		Discs on Water	Discs on Glycidate	
1	NADH-Glyoxylate Reductase	325 ^a	361 ^a	+11
	NADPH-Glyoxylate Reductase	80.1 ^a	60.1 ^b	-25
2	NADH-Glyoxylate Reductase	15.5 ^b	15.3 ^b	- 1.3
	NADPH-Glyoxylate Reductase	4.36 ^b	3.18 ^b	-27
3	P-Glycolate Phosphatase	249 ^a	217 ^a	-13
	NADPH-Glyoxylate Reductase	140 ^a	55.0 ^a	-61
4	P-Glycolate Phosphatase	9.79 ^b	9.07 ^b	- 7.4
	NADH-Glyoxylate Reductase	15.8 ^b	16.4 ^b	+ 3.8
	NADPH-Glyoxylate Reductase	1.59 ^b	1.32 ^b	-17

^a $\mu\text{mol/g fresh wt}\cdot\text{hr}$
^b $\mu\text{mol/mg protein}\cdot\text{hr}$

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 glycidate for 2.5 hr compared with extracts from control discs on water and with other enzyme activities assayed.

Glycidate Effect on Activity of Isolated NADPH- and NADH-Glyoxylate Reductases. Since the activity of NADPH-glyoxylate reductase, but not NADH-glyoxylate reductase, was inhibited in leaf discs treated with glycidate, 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 glycidate. About 15% inhibition was obtained after 15 min with 5 mM glycidate and 25% inhibition was found with 10 mM glycidate. Some further characteristics of this inhibition were studied. Adding glyoxylate (as much as 16.7 mM) or NADPH to the enzyme in the reaction mixture before adding glycidate did not protect the enzyme or alter the degree of inhibition; there was no effect of enzyme concentration on the extent of inhibition by glycidate; and little difference was found in the extent of inhibition by glycidate at pH 8 compared with pH 6.4.

In contrast to the results in Figure 1, NADH-glyoxylate reductase activity in the same preparation (specific activity, about 5 $\mu\text{mol/mg}$ of protein $\cdot\text{hr}$) was inhibited an average of only 2.5% (in four experiments) with 5 mM glycidate and 5.1% with 10 mM glycidate.

DISCUSSION

Several different kinds of experiments have shown that the treatment of tobacco leaf discs with glycidate (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]Glycidate 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. Glycidate had no effect on the activity of isolated RuDP carboxylase or its inhibition by 100% O₂ (32). Thus, glycidate 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 pretreatment 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 glycidate (30), and similar inhibitions are caused by glutamate (17) or glyoxylate (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 glycidate. Isolated NADPH-glyoxylate reductase was also inhibited 25% by reaction with 10 mM glycidate for 15 min. The chemical nature of the binding between glycidate and the enzyme has not been investigated, although reactions between the epoxide group of glycidate 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). Glycidate 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]glyoxylate 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 glyoxylate and inhibit glycolate synthesis by a feedback mechanism similar to that apparently observed when glyoxylate itself is supplied to leaf discs (16).

In an accompanying paper (13) it is shown that glycidate 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 glycidate 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 glycidate.

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