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Inhibition of Glutamate:Glyoxylate Aminotransferase Activity in Tobacco Leaves and Callus by Glycidate, an Inhibitor of Photorespiration[1]

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ABSTRACT

The effect of glycidate (2,3-epoxypropionate), an inhibitor of glycolate synthesis and photorespiration in leaf tissue, was studied on glutamate:glyoxylate and serine:glyoxylate aminotransferases and glycine decarboxylase activities in particulate preparations obtained from tobacco (*Nicotiana tabacum* L.) callus and leaves. Glycidate specifically and effectively inhibited glutamate:glyoxylate aminotransferase. The inhibition was dependent on glycidate concentration and, to a lesser extent, on substrate concentration. The enzyme was not protected by either substrate. Even with saturating substrate concentrations the glycidate inhibition was only partially reversed. Under the *in vitro* assay conditions, glycidate inhibition of the aminotransferase was reversible. Glutamate:glyoxylate aminotransferase is the only enzyme of the glycolate pathway thus far examined which is severely inhibited by glycidate. However, in leaf discs, pretreatment with glycidate decreased both glutamate:glyoxylate and serine:glyoxylate aminotransferase activities suggesting binding by glycidate *in vivo*.

Glycidate increased the pool sizes of both glutamate and glyoxylate in leaf discs. It has been shown that increases in concentration of either of these metabolites decrease photorespiration and glycolate synthesis and increase net photosynthesis. It is proposed that glycidate inhibits photorespiration indirectly by increasing the internal concentrations of glutamate and glyoxylate, as a consequence of the inhibition of glutamate:glyoxylate aminotransferase activity.

Glycidate (2,3-epoxypropionate) has been shown to decrease glycolate synthesis and photorespiration, and increase net photosynthetic CO_2 fixation in tobacco leaf discs (20). Glycidate did not inhibit purified ribulose diP carboxylate or its dependence on O_2 concentration (5, 21, 22), although Wildner and Henkel (17) did observe a time-dependent inhibition of the partially activated ribulose diP oxygenase activity by glycidate. In the accompanying paper (22), glycidate is shown to have little inhibitory effect on the activities of other enzymes of the glycolate pathway with the exception of a slight, time-dependent inhibition of NADPH-glyoxylate reductase. Glycidate had no effect on purified glycolate oxidase activity and glycolate did not accumulate in the presence of glycidate in tobacco leaf discs (20), an effect that would be expected if glycolate oxidase were inhibited.

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Oliver and Zelitch (11, 12) showed that increasing the pool sizes of glutamate or glyoxylate in tobacco leaf discs by floating them on solutions of the metabolites caused decreases in glycolate synthesis and photorespiration, and stimulated net photosynthetic CO_2 fixation. The effect of glyoxylate was reversed when the glyoxylate solution was removed from the leaf discs after a 1-hr treatment (11). The inhibitory effect of glutamate, on the other hand, was not reversible even 3 hr after the glutamate solution had been removed from the leaf discs (12). Thus, a temporary increase in glutamate can produce the effect of decreasing photorespiration irreversibly.

The reactions catalyzed by glutamate:glyoxylate aminotransferase and serine:glyoxylate aminotransferase are responsible for the conversion of glyoxylate to glycine in the photorespiratory pathway (13). Inhibition of glutamate:glyoxylate aminotransferase in metabolically active tissue would therefore be likely to increase the pool sizes of both glutamate and glyoxylate. As suggested above, accumulations of these metabolites would then decrease the rate of glycolate synthesis in these tissues. Accordingly, the effects of glycidate on glutamate:glyoxylate aminotransferase, serine:glyoxylate aminotransferase, and glycine decarboxylase activities in both tobacco callus and leaves were investigated. The results demonstrate a specific inhibition of glutamate:glyoxylate aminotransferase by glycidate in both systems.

MATERIALS AND METHODS

Plant Material. Callus tissues were derived from anther cultures of *Nicotiana tabacum* L., variety John Williams Broadleaf, *su* (1) and maintained on Linsmaier and Skoog medium (8) in 1% agar with a modified Fe-EDTA solution, 1.6 μM naphthaleneacetic acid, 1.5 μM isopentenylaminopurine, and 2% sucrose (1). The callus was grown in Petri plates sealed with rubber bands and kept in a 27 C room under an irradiance of 120 to 235 $\mu E \cdot m^{-2} \cdot sec^{-1}$(400–700 nm) provided by fluorescent and Gro-lux lamps.

The tobacco leaves (var. Havana Seed) used for isolating particulate preparations were grown in sand in a subirrigated greenhouse bench. The tobacco leaves used for the metabolite accumulation experiments were excised from John Williams Broadleaf plants regenerated from callus cells and grown under the same conditions.

Particulate Preparations from Tobacco Callus. All assays of enzyme activity from callus tissue were carried out on particulate preparations isolated using modifications of the methods of Bird *et al.* (2). These preparations contained mixtures of mitochondria, peroxisomes, chloroplasts, and other cellular debris. Two to five g fresh wt of callus tissue were ground with a pestle in a small, ice-cold mortar containing a small quantity of sand

and from 20 to 30 ml of cold grinding medium containing 0.4 M sucrose, 33 mM KH_2PO_4, 20 mM sodium citrate, 5 mM disodium EDTA, and 7 mM 2-mercaptoethanol (prepared daily), at pH 7. The resulting slurry was passed through a Miracloth filter (Chicopee Mills, Milltown, N. J.) on a Büchner funnel and the filtrate was centrifuged for 10 min at 38,000*g* and 4 C. The resulting pellet was then slowly suspended with the aid of a glass rod in sufficient cold assay medium to make the final concentration about 0.4 g initial fresh wt/ml of medium. The assay medium contained 0.4 M sucrose, 33 mM KH_2PO_4, 20 mM sodium citrate, and 4 mM $MgCl_2$ at a final pH of 7.2.

Particulate Preparations from Tobacco Leaves. All assays of enzyme activity from tobacco leaves used preparations isolated by modifications of the methods of Tolbert *et al.* (15, 16). From 5 to 12 g of washed tobacco leaves with midribs removed were ground in an ice-cold mortar containing sand and 35 ml of the grinding medium described above. The resulting slurry was pressed through eight layers of cheesecloth. Differential centrifugation of this crude preparation at 120*g*, 6,000*g*, and 38,000*g* resulted in pellets containing primarily the intact chloroplasts, broken chloroplasts and peroxisomes, and mitochondria, respectively (15). The 6,000*g* pellet, containing the majority of the peroxisomes, was suspended in assay medium as above to give a final concentration of approximately 1 g of initial fresh wt/ml. This suspension was used to assay aminotransferase activities. Glycine decarboxylase activity was assayed in a suspension of the 38,000*g* pellet that contained most of the mitochondria. The final concentration of the suspension was about 1 g initial fresh wt/ml.

Experiments in which the peroxisomal location of glutamate:glyoxylate aminotransferase was determined used a modification of the method of Tolbert *et al.* (15, 16). The 6,000*g* peroxisomal pellet was suspended and 2 ml of this suspension was placed on top of a discontinuous sucrose gradient which contained 6 ml of 2.3 M sucrose, 6 ml of 1.8 M sucrose, 6 ml of 1.5 M sucrose, and 10 ml of 1.3 M sucrose. Each layer was buffered with 33 mM KH_2PO_4 at pH 7.2. The gradient was placed in a Beckman SW 25 swinging bucket rotor and centrifuged for 3 hr at 20,000 rpm (41,000*g*) in a Beckman model L ultracentrifuge at 4 C. The interfaces between the 1.3 and 1.5 M, 1.5 and 1.8 M, and 1.8 and 2.3 M sucrose layers primarily contained the broken chloroplasts, intact chloroplasts and mitochondria, and peroxisomes, respectively (16). The layers were separately removed with a pipette and 0.1-ml samples were assayed for glutamate:glyoxylate aminotransferase activity. Protein determinations followed a modification of the Lowry method (9).

Enzyme Assays. Particulate preparations from an initial fresh wt of 0.4 g of callus or 1 g of leaves were used in each assay of glutamate:glyoxylate aminotransferase (EC 2.6.1.4), serine:glyoxylate aminotransferase (EC 2.6.1.45), and glycine decarboxylase (glycine synthase, EC 2.1.2.10). All reactions were carried out in Warburg flasks with double sidearms and center walls. The substrates were placed in one sidearm, inhibitor in the second sidearm, the particulate preparation and cofactors in the main compartment, and a paper wick moistened with 150 μmol of ethanolamine in the center well when required for the assay. The flasks were shaken in a water bath at 30 C for 5 min with the system closed. The reactions were initiated by tipping in the substrates, inhibitors were added 5 to 15 min before the substrates, and reactions were terminated by adding 0.15 ml of 0.8 N H_2SO_4.

The glycine decarboxylase assays normally contained the particulate preparation in 1 ml of assay medium (containing 400 μmol of sucrose, 33 μmol of KH_2PO_4, 20 μmol of sodium citrate, and 4 μmol of $MgCl_2$). The reaction mixture also contained 1 μmol of NAD^+, 0.1 μmol of pyridoxal-5-P, and from 1 to 20 μmol of [1-^{14}C]glycine (approximately 1.5×10^6 dpm/assay) in 0.2 ml. The $^{14}CO_2$ released during the reaction was collected in the center well and the radioactivity determined with a scintillation counter. A correction was made for nonenzymic $^{14}CO_2$ formation in control assay mixtures containing all reaction components except the particulate preparation. This correction usually accounted for less than 5% of the $^{14}CO_2$ released enzymatically.

In rapidly photorespiring plants glycine decarboxylation is coupled to serine formation by serine hydroxymethyltransferase (6). To insure that [^{14}C]serine formation was associated with $^{14}CO_2$ production from [1-^{14}C]glycine, radioactive serine was isolated at the end of the reaction. The reaction mixture was passed through a column (0.7 × 6 cm) of Dowex 50-H^+ cation exchange resin, the weak bases eluted with 10 ml of 2 N NH_4OH, and the eluate taken to dryness. The glycine and serine were then separated by paper electrophoresis as described earlier for the purification of [1-^{14}C]glycine and the radioactivity in serine was determined.

The glutamate:glyoxylate and serine:glyoxylate aminotransferase assays, in a final volume of 1.2 ml, contained the particulate preparation in 1 ml of assay medium. The reaction also contained from 1.5 to 15.5 μmol of [1-^{14}C]glyoxylate (usually from 1.5 to 3×10^5 dpm/assay), and 20 μmol of either L-serine or L-glutamate. The [^{14}C]glycine formed during the reaction was isolated by placing the entire reaction mixture on a column (0.7 × 6 cm) of Dowex 50-H^+ cation exchange resin, eluting the acidic and neutral compounds with water, and eluting the radioactive glycine with 10 ml of 2 N NH_4OH. Aliquots were taken from the eluate and their radioactivity determined in a scintillation counter. To insure further that [^{14}C]glycine was synthesized during the reactions, the glycine-containing fractions were occasionally concentrated, the glycine separated by paper electrophoresis as described above for [1-^{14}C]glycine, and the radioactivity determined.

NADH-glyoxylate reductase was assayed as described elsewhere (22) using particulate preparations from tobacco leaves obtained by centrifugation at 6,000*g*.

$^{14}CO_2$ Incorporation by Leaf Discs in the Presence of Glycidate. In order to determine if glyoxylate accumulated in the presence of glycidate, experiments were carried out with $^{14}CO_2$ supplied to tobacco leaf discs in the light. Photosynthetic $^{14}CO_2$ fixation in leaf discs was measured by rapidly passing $^{14}CO_2$ of constant specific radioactivity over six leaf discs floating in 1.2 ml of either water or glycidate solution in large Warburg flasks (12). The $^{14}CO_2$ had a high specific radioactivity to facilitate measurement of the relatively small glyoxylate pool. After the leaf discs were floated on water for 1 hr at 28 C in light (270 $\mu E \cdot m^{-2} \cdot sec^{-1}$), the water was removed and replaced with an equal volume of either 20 mM potassium glycidate or fresh water. After another hr at 28 C, the $^{14}CO_2$ (1.08×10^6 dpm/μmol) was supplied for 5 min at a concentration of 600 μl/l, and the discs were killed by rapidly plunging them in boiling 20% (v/v) ethanol.

Metabolites present in the supernatant fluid after centrifuging the homogenates were separated by chromatography on Dowex 1-acetate columns (0.7 × 6 cm). After the basic and neutral compounds were collected by elution with water, the fractions containing aspartic and glutamic acids, glycolic and glyceric acids, and glyoxylic acid were eluted successively with 4 N acetic acid (the aspartic and glutamic acids fraction was eluted with the first 4 ml) (20). The strong acids were then eluted with 10 ml of 1 N HCl. Aliquots of the fractions were assayed for radioactivity.

The radiochemical purity of the glyoxylic acid fraction (collected between 20 and 50 ml of 4 N acetic acid) was determined. The fraction was taken to dryness *in vacuo* at 40 C and the residue dissolved in 1 ml of nonradioactive glyoxylic acid solution, 2 mg/ml. This mixture was then reacted with 3 ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine in 2 N HCl, to form the glyoxylic acid 2,4-dinitrophenylhydrazones. After 1 hr at room

temperature, the resulting phenylhydrazones were extracted with 1 ml of ethyl acetate, and aliquots were spotted on a thin layer plate (Silica G, 250 μm thick, Uniplate, N.J.) and chromatographed in a solvent system of 1-butanol:1-propanol:benzene:6% v/v NH_4OH (15:50:15:20) (4). The plate was dried at room temperature and successive sections were removed for determination of radioactivity. The glyoxylic acid dinitrophenylhydrazones had R_F values of about 0.5 and 0.7.

Chemicals. The [1-^{14}C]glycine (Schwarz/Mann) was further purified by electrophoresis on Whatman 3MM paper for 45 min at 3,000 v and 1 C in a formic acid:acetic acid:water (12:48:340) system at pH 1.9. The glycine was eluted from the paper and the solution stored in a freezer. This procedure removed a radioactive impurity present in the original [1-^{14}C]glycine which produced small quantities of $^{14}CO_2$ nonenzymically under the conditions of the glycine decarboxylase assay. The [1-^{14}C]glyoxylate was prepared from [1-^{14}C]glycolate (Amersham/Searle) with spinach glycolate oxidase as previously described (18). The [1-^{14}C]glyoxylate was purified by passing the entire reaction mixture through a column (0.7 × 6 cm) of Dowex 1-acetate anion exchange resin. The glyoxylate was eluted in a fraction between 20 and 50 ml of 4 N acetic acid (19). The glyoxylic acid fraction was then taken to dryness *in vacuo* at 40 C. Nonradioactive sodium glyoxylate was added to make the final solution 10 mM, and N_2 was bubbled through the solution before storage in the freezer. Potassium glycidate was prepared as described earlier (20) by the method of Blau *et al.* (3).

RESULTS

Enzyme Assays. The assays of glycine decarboxylase as well as those of glutamate:glyoxylate aminotransferase and serine:glyoxylate aminotransferase increased linearly with enzyme concentration up to the concentration normally used and were linear with respect to time, from 15 to 60 min (*e.g.* Table I). A number of experiments have shown that the above three enzymes had pH optima between pH 7 and 7.5.

Glycine Decarboxylase Activity. The V_{max} and K_m constants for glycine decarboxylation activity in particulate preparations derived from callus tissue, obtained from reciprocal plots (1/v *versus* 1/[S]), were 0.05 ± 0.01 μmol CO_2/g fresh wt of tissue·hr and 5.5 ± 1.2 mM, respectively (Table II). The absence of either NAD^+, pyridoxal-5-P, or 2-mercaptoethanol in assays of tobacco callus decreased glycine decarboxylase activity 17 to 50% (Table III). However, the presence of ADP had no effect on the enzyme assay. This contrasts with the stimulation of glycine decarboxylase by ADP and the lack of NAD^+ and pyridoxal phosphate dependence in tobacco leaf preparations described by Bird *et al.* (2), even though the reaction mixtures and preparations used here were a modification of their leaf procedure.

The glycine decarboxylase reaction with [1-^{14}C]glycine produced [^{14}C]serine as a second product in addition to $^{14}CO_2$. The synthesis of [^{14}C]serine was clearly established as described under "Materials and Methods." The formation of 1 mol of $^{14}CO_2$ and 1 mol of [1-^{14}C]serine would be expected for every 2 mol of [1-^{14}C]glycine utilized on the basis of the reported coupling in tobacco mitochondria of glycine synthase and serine hydroxymethyltransferase (2). In a preliminary experiment with a callus preparation, a ratio of 1.0 $^{14}CO_2$ to 1.5 [^{14}C]serine was observed.

Aminotransferase Activities. The V_{max} and K_m values obtained for glutamate:glyoxylate and serine:glyoxylate aminotransferases in particulate preparations are given in Table II. V_{max} values obtained from experiments using callus preparations were 0.48 ± 0.11 and 0.39 ± 0.11 μmol/g fresh wt·hr for glutamate:glyoxylate and serine:glyoxylate aminotransferases, respectively, whereas the K_m (glyoxylate) values obtained were 1.08 ± 0.23 and 1.48 ± 0.50 mM. On a fresh wt basis, the V_{max} value obtained for glutamate:glyoxylate aminotransferase in particulate preparations derived from leaves was 1.7 ± 0.1 μmol/g fresh wt of leaf·hr, more than three times the value obtained in callus preparations. In leaf preparations, the K_m value for glutamate (5.7 mM) was much larger than the K_m value obtained for glyoxylate (1.1 mM) in glutamate:glyoxylate aminotransferase assays.

Glutamate:glyoxylate and serine:glyoxylate aminotransferase activities are distinct in preparations from tobacco since their activities are additive when assayed at saturating levels of both amino donors. Aminotransferase activity was little affected by adding either catalase (6 μmol of H_2O_2/min) or pyridoxal-5-P (0.1 μmol) or by eliminating the 330 mM sucrose in the assay mixture. Radioactive glycine was the only product detected in the reaction mixture at the end of the assay. Small amounts of $^{14}CO_2$ were also produced during the assays but this production

Table I. Dependence of Glutamate:Glyoxylate Aminotransferase Activity Assay on Time and Enzyme Concentration

The complete reaction mixture contained particles obtained from 0.43 g of tobacco callus, 10.5 µmol [1-^{14}C]glyoxylate (specific radioactivity 24,800 dpm/µmol), and 20 µmol glutamate in a final volume of 1.2 ml. The tobacco leaf preparation was obtained from 1.05 g of leaf and contained 1.6 µmol [1-^{14}C]glyoxylate (specific radioactivity 40,400 dpm/µmol), and 20 µmol glutamate in a final volume of 1.5 ml. Non-enzymatic activity determined in control reaction mixtures containing boiled enzyme and no glutamate was subtracted.

Reaction Mixture	Time	Callus Particulate Preparation		Leaf Particulate Preparation	
		^{14}C-Glycine Produced	Enzymic Activity	^{14}C-Glycine Produced	Enzymic Activity
	min	dpm	µmol/g fresh wt·hr	dpm	µmol/g fresh wt·hr
Complete	60	7200	0.72	53,300	1.26
Complete	30	3760	0.75	28,300	1.34
Complete with one-half enzyme	60	4080	0.81	31,400	1.46

Table II. Kinetic Values of Glutamate:Glyoxylate and Serine:Glyoxylate Aminotransferases and Glycine Decarboxylase in Particulate Preparations of Tobacco Leaf and Callus

The standard methods of assay are described under Materials and Methods. The kinetic values were obtained from Lineweaver-Burk plots (1/v vs. 1/[S]) with three substrate concentrations per experiment and a minimum of five experiments for callus. The data for tobacco leaf were obtained from two experiments. Standard deviations are given.

Tobacco Callus Particulate Preparations		
Glycine Decarboxylase		
Vmax	0.05 ± 0.01	µmol/g fresh wt·hr
Km (glycine)	5.5 ± 1.2	mM
Serine:Glyoxylate Aminotransferase		
Vmax	0.39 ± 0.11	µmol/g fresh wt·hr
Km (glyoxylate)	1.48 ± 0.50	mM
Glutamate:Glyoxylate Aminotransferase		
Vmax	0.48 ± 0.11	µmol/g fresh wt·hr
Km (glyoxylate)	1.08 ± 0.23	mM
Tobacco Leaf Particulate Preparations		
Glutamate:Glyoxylate Aminotransferase		
Vmax	1.7 ± 0.1	µmol/g fresh wt·hr
Km (glyoxylate)	1.13	mM
Km (glutamate)	5.7	mM

Table III. Assay Requirements for Glycine Decarboxylase Activity in Preparations from Tobacco Callus

The complete assay system contained 10 µmol of [1-^{14}C]glycine (specific radioactivity 1.5 x 10^5 dpm/µmol), 1 µmol NAD^+, 0.1 µmol pyridoxal-5-phosphate, 4 µmol $MgCl_2$, and 7 µmol 2-mercaptoethanol; the $^{14}CO_2$ released was determined. Experiment 1 contained particles obtained from 0.42 g fresh weight of callus in a final volume of 1.2 ml. Experiment 2 contained particles from 0.23 g of callus, and 4 µmol ADP were added where indicated.

Expt. No.	Assay Conditions	Activity	Effect of Change
		µmol/g fresh wt·hr	%
1	Complete	0.138	—
	- Mercaptoethanol	0.115	-17
	- NAD^+	0.063	-54
	- Pyridoxal Phosphate	0.066	-52
2	Complete	0.048	—
	- Mercaptoethanol	0.028	-42
	- NAD^+	0.031	-35
	- Pyridoxal Phosphate	0.019	-60
	- $MgCl_2$	0.051	+ 6
	+ ADP	0.047	- 2

did not correlate with enzyme concentration or activity and was probably due to a nonenzymic decarboxylation reaction.

The subcellular location of glutamate:glyoxylate aminotransferase was determined by methods described by Tolbert *et al.* (16). Table IV shows that most of the enzymic activity of leaf homogenates sedimented at 6,000*g* which coincides with the "broken chloroplasts and peroxisomes" fraction (16). When the 6,000*g* pellet was further fractionated on a discontinuous sucrose gradient as described under "Materials and Methods," the fraction with the highest glutamate:glyoxylate aminotransferase activity, and highest specific activity was obtained in the interface between 2.3 M and 1.8 M sucrose layers, referred to as the "peroxisome" fraction (16). The total aminotransferase activity recovered in the three sedimented fractions obtained by differential centrifugation was 9% of the original activity present in the crude preparation. About 85% of the activity from the 6,000*g* pellet was recovered in fractions obtained from the discontinuous sucrose gradient (Table IV). These recoveries are similar to those described by previous workers (16). These results confirm that the particulate preparations, even though they contain a mixture of organelles, were similar in activity to those used by other workers and were therefore adequate to examine the nature of the inhibition by glycidate.

Inhibition of Glutamate:Glyoxylate Aminotransferase by Glycidate. Glycidate strongly inhibited glutamate:glyoxylate aminotransferase activity in callus particulate preparations. Under favorable conditions for demonstrating the inhibition (15.4 mM glycidate, 1.25 mM glyoxylate, and 16.6 mM glutamate) aminotransferase activity was inhibited 78 ± 2% (Fig. 1). The inhibition in callus preparations was dependent on glycidate concentration, and was partially competitive since increasing glyoxylate concentration decreased the extent of inhibition. For example, by raising the glyoxylate concentration from 1.25 mM to 13 mM, under conditions identical to those given above, the inhibition was decreased from 78 ± 2% to 46 ± 2%. Strong inhibition was observed even at low glycidate concentrations (Fig. 1). With 3.1 mM glycidate, glutamate:glyoxylate aminotransferase activity was inhibited 64 ± 3% while with 15.4 mM glycidate the inhibition only increased to 78 ± 2%. The kinetic data show mixed inhibition since glycidate inhibition was not eliminated even at saturating substrate levels. Added glycidate decreased the maximal velocity of the aminotransferase reaction, and lowered the affinity of glyoxylate for the enzyme.

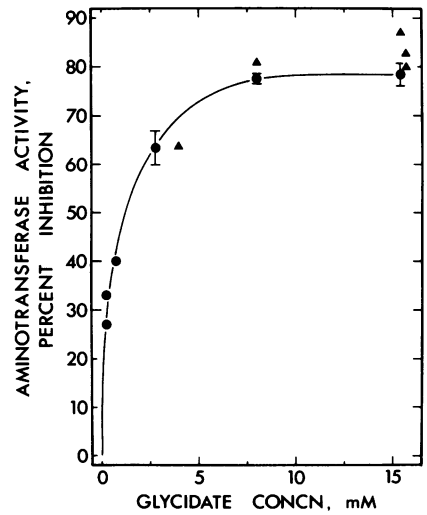

FIG. 1. Effect of glycidate concentration on inhibition of glutamate:glyoxylate aminotransferase activity. About 1.3 mM glyoxylate, and 16.7 mM glutamate were used in assay conditions as described under "Materials and Methods" for particulate preparations derived from tobacco callus (●) and leaves (▲). Bars represent standard deviations of three or more determinations.

Particulate preparations from leaves also showed mixed and partially competitive inhibition by glycidate for both glyoxylate and glutamate. With similar concentrations of substrate and inhibitor, glycidate inhibition of glutamate:glyoxylate aminotransferase activity in leaf preparations was similar to that in callus particulate preparations (Fig. 1). Because of this similarity and the relatively higher activities found in the leaf preparations, leaf particulate preparations were used for most of the studies on the nature of glycidate inhibition. Glutamate:glyoxylate aminotransferase activity located in the peroxisomal fraction of the discontinuous sucrose gradient (Table IV) was inhibited 81 ± 3% (with 3.9 mM glycidate, 1.7 mM glyoxylate, and 17 mM glutamate), a value comparable to the inhibition found in the particulate preparations (Fig. 1).

Glutamate:glyoxylate aminotransferase activity was not protected from glycidate inhibition by pretreatment of the enzyme with either substrate. Prior substrate addition had no effect on the percentage inhibition by glycidate in an experiment in which either glycidate (3.8 mM), glutamate (17 mM), glyoxylate (13.5 mM), glycidate and glutamate, or glycidate and glyoxylate were added to the leaf particulate preparation for 30 min at 30 C before addition of the remaining assay components.

Under the assay conditions used, glycidate inhibition was reversible. When the particulate preparation was treated with glycidate for increasing periods of time up to 1 hr at 30 C before the substrates were added, no increase in aminotransferase inhibition was observed even at high levels of substrate and low levels of glycidate. The reversibility of the inhibition was also demonstrated by treating the particulate preparation with glycidate at 30 C for 20 min, and then recentrifuging the mixture in order to dilute the inhibitor. When the resulting sediments were resuspended and assayed for aminotransferase activity, no inhibition of enzyme activity was observed. In the same experiment, an undiluted sample of enzyme was inhibited 67% by glycidate.

Inhibition of glutamate:glyoxylate aminotransferase by glycidate was pH-dependent and was increased at lower pH (Table

Table IV. Location of Glutamate:Glyoxylate Aminotransferase Activity in the Peroxisomal Fraction of Tobacco Leaves

The assays contained 2 μmol [1-^{14}C]glyoxylate (81,600 dpm/μmol) and 20 μmol glutamate in 1.5 ml of assay medium. Of the total original activity in the crude preparation 9% was recovered in the differential centrifugation pellets. Of the total activity in the 6000*g* pellet, 85% was recovered in the discontinuous sucrose gradient.

Fraction	Total Protein Per Fraction	Enzyme Activity	Specific Activity Relative to Initial Preparation	Distribution of Activity Recovered from Pellets	Distribution of Activity Recovered from Sucrose Gradient
	mg	μmol/mg protein·hr		%	%
Initial Preparation	275.0	0.199	1.0	—	—
Differential Centrifugation					
120*g* Pellet (Chloroplasts)	10.6	0.030	0.15	6	—
6,000*g* Pellet (Broken chloroplasts + peroxisomes)	7.6	0.333	1.67	50	—
38,000*g* Pellet (Mitochondria)	13.4	0.167	0.81	43	—
Supernatant fraction	195.0	0.439	2.21	—	—
Discontinuous Sucrose Gradient on 6,000*g* Pellet					
Broken Chloroplasts	0.18	3.520	17.7	—	29
Chloroplasts, Mitochondria	0.13	4.410	22.2	—	26
Peroxisomes	0.12	7.840	39.4	—	45

V). The pH optimum for glutamate:glyoxylate aminotransferase activity was from pH 7 to pH 7.5, hence most assays were carried out at pH 7.2. Glycidate inhibition was more than twice as great at pH 6.2 as at pH 7.2. A 1-hr treatment with glycidate at the lower pH indicated that this increased inhibition was also reversible.

The possibility still remains that *in vivo* glycidate may act in an irreversible manner. To explore this possibility, experiments were carried out with leaf discs floated for 2.5 hr in the light on solutions of either 20 mM potassium glycidate or water. Particulate preparations were then obtained and analyzed for changes in glutamate:glyoxylate aminotransferase, serine:glyoxylate aminotransferase, and NADH-glyoxylate reductase activities. Under similar experimental conditions, glycidate effectively decreased glycolate synthesis and photorespiration in leaf discs and had little effect on NADH-glyoxylate reductase activity (22). However, glycidate decreased the activities of both aminotransferases by approximately 35% under these conditions. The NADH-glyoxylate reductase activity was unaffected. These experiments suggest that *in vivo* glycidate may bind irreversibly not only to glutamate:glyoxylate aminotransferase but to serine:glyoxylate aminotransferase as well.

Specificity of Glycidate Inhibition of Glutamate:Glyoxylate Aminotransferase. Under optimal conditions, when glycidate strongly inhibited glutamate:glyoxylate aminotransferase activity by 80%, both serine:glyoxylate aminotransferase and glycine decarboxylase activities were only inhibited 20% or less (Table VI). This specificity was observed in particulate preparations from callus and leaves. Other papers (5, 21, 22) have shown that glycidate has no effect on ribulose diP carboxylase/oxygenase, P-glycolate phosphatase, glycolate oxidase, and NADH-glyoxylate reductase, with only a slight effect on NADPH-glyoxylate reductase. The strong inhibition of only one enzyme of the photorespiratory pathway suggests that glutamate:glyoxylate aminotransferase is an important site of glycidate inhibition.

Glycidol (2,3-epoxypropanol), the carbinol analogue of glycidate, was also tested for its inhibitory effect on glutamate:glyoxylate aminotransferase activity. Under conditions where glycidate, at 15 mM, inhibited the aminotransferase activity about 82%, glycidol, at 20 mM (with 1.5 mM glyoxylate and 17 mM glutamate) inhibited the aminotransferase only 9% and 5% in particulate preparations from callus and leaf, respectively. This contrast between glycidol and glycidate provides further support for the specific nature of glycidate inhibition.

Glyoxylate Accumulation in Leaf Discs in the Presence of Glycidate. The effect of glycidate on increasing the pool sizes of glutamate and aspartate was reported earlier (20). If glutamate:glyoxylate aminotransferase is important in the photorespiration pathway, and the flow of carbon is from glyoxylate to glycine, both glutamate and glyoxylate pools would be expected to increase when the aminotransferase is inhibited. In order to characterize further the effects of glycidate inhibition, the changes in glyoxylate concentration were studied in leaf discs. Because of the relatively small glyoxylate pool size, the earlier data on the effect of glycidate on glyoxylate concentration were not previously presented (20). Experiments were therefore carried out in which tobacco leaf discs floating on either 20 mM potassium glycidate or water were exposed to a steady stream of $^{14}CO_2$ of high specific radioactivity. As shown in Table VII, net CO_2 fixation was increased by 30% in the presence of 20 mM glycidate. The radioactivity of the fraction containing neutral and basic compounds decreased in the presence of glycidate, whereas that of acidic fractions increased as was previously observed (20). Aspartate and glutamate pool sizes increased and the glyoxylate pool, though very small, increased 40% in

Table V. Effect of pH on Glutamate:Glyoxylate Aminotransferase Activity and Glycidate Inhibition in Tobacco Leaf Preparations

The 6,000g pellet from 5.7 g fresh weight of tobacco leaf was suspended in 2.25 ml of assay medium at various pH values. One ml samples were assayed in a system containing 15.5 µmol [1-^{14}C]glyoxylate (10,500 dpm/µmol) and 20 µmol glutamate in a final volume of 1.2 ml. The glycidate (4.6 µmol) was added 15 min before the substrates.

Assay Conditions	^{14}C-Glycine Produced	Amino-transferase Activity	Control Rate Compared to pH 7.2	Inhibition by Glycidate
	dpm	µmol/g fresh wt·hr		%
pH 6.2 without Glycidate	11,800	1.55	0.85	—
pH 6.2 with 3.9 mM Glycidate	3,640	0.48	—	69
pH 7.2 without Glycidate	13,800	1.83	1.0	—
pH 7.2 with 3.9 mM Glycidate	9,390	1.24	—	32
pH 8.1 without Glycidate	12,000	1.58	0.87	—
pH 8.1 with 3.9 mM Glycidate	9,440	1.25	—	21

Table VII. Effect of Glycidate on ^{14}C-Glyoxylate Accumulation During Photosynthetic $^{14}CO_2$ Fixation in Tobacco Leaf Discs

Leaf discs were floated on water or 20 mM glycidate in the light for 60 min in air, and $^{14}CO_2$ (1.08 x 10^6 dpm/µmol; 600 µl/l) was then passed over the discs for 5 min. The ^{14}C-labeled products were separated as described under Materials and Methods. The photosynthetic rates obtained in this experiment were 24.5 and 31.8 µmol $^{14}CO_2$/g fresh wt·hr for discs floated on water and glycidate respectively, a 30% stimulation of net photosynthesis in the presence of glycidate. The data shown are the mean of duplicate sets of flasks where the variation between any set was less than 5% for $^{14}CO_2$ uptake and the various fractions, except the glyoxylate fraction in the glycidate set which varied by 13%.

Fraction	Leaf Discs on Water	Leaf Discs on Glycidate	Effect of Glycidate
	Percent of total $^{14}CO_2$ fixed		%
Basic and Neutral Compounds	47.3	35.2	-26
Aspartate and Glutamate	5.02	6.19	+23
Glycolate and Glycerate	3.18	4.34	+38
Glyoxylate	1.84	2.58	+40
Strong Acids	31.4	36.5	+17

Table VI. Comparison of Glycidate Inhibition of Glutamate:Glyoxylate Aminotransferase, Serine:Glyoxylate Aminotransferase, and Glycine Decarboxylase Activities in Tobacco Callus and Tobacco Leaf

In Experiment 1 the assay systems contained 15.4 mM glycidate, 1.25 mM glyoxylate, and 16.6 mM glutamate or serine with particles obtained from 0.46 g fresh weight of callus per assay. Experiments 2 and 3 contained 15.4 mM glycidate, 1.25 mM glyoxylate, 16.6 mM glutamate or serine, and 0.83 mM glycine with 0.41 g fresh weight of callus. Experiment 4 contained 8.0 mM glycidate, 1.38 mM glyoxylate, 16.5 mM glutamate or serine, and 0.83 mM glycine with particles from 1.0 g fresh wt of leaf per assay.

Expt. No.	Tissue	Glutamate:Glyoxylate Aminotransferase Activity			Serine:Glyoxylate Aminotransferase Activity			Glycine Decarboxylase Activity		
		Without Glycidate	With Glycidate	Inhibition	Without Glycidate	With Glycidate	Inhibition	Without Glycidate	With Glycidate	Inhibition
		µmol/g fresh wt·hr		%	µmol/g fresh wt·hr		%	µmol/g fresh wt·hr		%
1	Callus	0.0290	0.0058	80	0.026	0.019	22	—	—	—
2	Callus	0.190	0.041	78	—	—	—	0.0039	0.0035	10
3	Callus	0.300	0.053	82	0.17	0.12	29	0.0049	0.0040	19
4	Leaf	1.26	0.24	81	1.01	0.97	3	0.047	0.041	14

the presence of glycidate. This accumulation is consistent with an inhibition of glutamate:glyoxylate aminotransferase by glycidate.

To assure that the change in the [^{14}C]glyoxylic acid fraction obtained by column chromatography with Dowex 1-acetate was entirely due to glyoxylic acid, the 2,4-dinitrophenylhydrazones of glyoxylic acid were prepared from this fraction and further separated as described under "Materials and Methods." By this procedure it was shown that the increase in the glyoxylic acid fraction in the presence of glycidate was due to [^{14}C]glyoxylic acid exclusively.

DISCUSSION

Glycidate specifically and effectively inhibits glutamate:glyoxylate aminotransferase activity in tobacco particulate preparations. Under the assay conditions used, the inhibition is reversible as discussed under "Results." Even when assayed at pH 6.2, where glycidate inhibition is over twice that observed at pH 7.2, increasing the period of glycidate pretreatment for up to 1 hr did not increase the inhibition. Despite the apparent reversibility, the inhibition does not follow the kinetics of classical competitive inhibition with either substrate, and neither substrate protects the enzyme against the glycidate inhibition. Glycidate, with its reactive epoxide group, might be expected to react irreversibly with carboxyl (10, 14), or sulfhydryl (7) groups on proteins, and may react in such a manner over longer periods of time *in vivo*. When tobacco leaf discs were floated on glycidate for 2.5 hr in the light, both glutamate:glyoxylate and serine:glyoxylate aminotransferase activities were decreased by about 35%, suggesting that irreversible binding may occur *in vivo*. Binding experiments using radioactive glycidate may provide further insight into the nature of this glycidate binding (22).

The effect of glycidate on a number of enzymes related to the photorespiratory pathway has been studied. Only glutamate:glyoxylate aminotransferase and NADPH-glyoxylate reductase are inhibited by glycidate, though the effect on the reductase is small. There is almost no effect on ribulose diP carboxylase/oxygenase, P-glycolate phosphatase, glycolate oxidase, NADH-glyoxylate reductase (22); or on serine:glyoxylate aminotransferase, and glycine decarboxylase (Table VI). The inhibition of glutamate:glyoxylate aminotransferase could result in a severe decrease in the rate of carbon flow through the glycolate pathway. This is consistent with the decreases in the pool sizes of glycine and serine observed in the presence of glycidate (20) (Table VII).

Increases in either glyoxylate or glutamate concentrations in tobacco leaf discs inhibit glycolate synthesis and photorespiration, and increase net photosynthesis (11, 12). The glutamate pool size increased about 3-fold in the presence of glycidate in leaf discs fixing CO_2 photosynthetically (20). In the present paper, glyoxylate pool sizes have also been observed to increase about 40% in the presence of glycidate (Table VII). A 40% increase in the glyoxylate pool size effectively inhibited glycolate synthesis when glyoxylate was supplied to tobacco leaf discs (11). The effect of glycidate in decreasing glycolate synthesis is therefore probably an indirect one, resulting from the inhibition of glutamate:glyoxylate aminotransferase activity which causes the accumulation of both substrates, glutamate and glyoxylate, in leaf tissue. If NADPH-glyoxylate reductase is active in normal cell metabolism, inhibition of its enzymic activity may also result in accumulation of glyoxylate in those tissues (22).

This indirect mechanism of glycidate inhibition of photorespiration accounts for the results described earlier in this laboratory (20). The irreversible effect of glycidate inhibition on glycolate synthesis for 2 hr after the glycidate solution is removed from leaf discs (20) is consistent with the essentially irreversible decrease in glycolate synthesis observed when glutamate concentration increases (12). Thus, even when glycidate solution is removed from the discs and glutamate levels return to normal, the temporary increase in glutamate concentrations would still inhibit glycolate synthesis for several hr. Glyoxylate accumulation caused by glycidate may also contribute to the inhibition of photorespiration by metabolic regulation.

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