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Inhibition of Glycine Decarboxylation and Serine Formation in Tobacco by Glycine Hydroxamate and Its Effect on Photorespiratory Carbon Flow[1]

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ABSTRACT

Glycine hydroxamate is a competitive inhibitor of glycine decarboxylation and serine formation (referred to as glycine decarboxylase activity) in particulate preparations obtained from both callus and leaf tissue of tobacco. In preparations from tobacco callus tissues, the K_i for glycine hydroxamate was 0.24 ± 0.03 millimolar and the K_m for glycine was 5.0 ± 0.5 millimolar. The inhibitor was chemically stable during assays of glycine decarboxylase activity, but reacted strongly when incubated with glyoxylate. Glycine hydroxamate blocked the conversion of glycine to serine and CO_2 *in vivo* when callus tissue incorporated and metabolized [1-^{14}C]glycine, [1-^{14}C]glycolate, or [1-^{14}C]glyoxylate. The hydroxamate had no effect on glyoxylate aminotransferase activities *in vivo*, and the nonenzymic reaction between glycine hydroxamate and glyoxylate did not affect the flow of carbon in the glycolate pathway *in vivo*. Glycine hydroxamate is the first known reversible inhibitor of the photorespiratory conversion of glycine to serine and CO_2.

Hydroxamic acid analogs of amino acids are synthesized by reacting the aliphatic esters of amino acids with hydroxylamine in absolute alcohol (12). Their use as biological probes of metabolism has been limited to a few examples. An analog of glutamic acid, γ-glutamylhydroxamate, inhibited growth of *Streptococcus faecalis* (17), and β-aspartylhydroxamate acted as a feedback inhibitor and repressed asparagine synthetase in *Lactobacillus arabinosus* (16). Serine hydroxamate inhibited protein synthesis in *Escherichia coli* by competitively inhibiting seryl-tRNA synthetase (19). Inhibition by serine hydroxamate was reversed by the addition of L-serine or by removal of the analog (20). Mutants resistant to serine hydroxamate were selected in *E. coli* and some had seryl-tRNA synthetases with decreased affinity for serine hydroxamate. One mutant had 3-P-glycerate dehydrogenase activity which was no longer sensitive to inhibition by L-serine (19). Of the amino acid hydroxamates tested, only L-serine hydroxamate and L-lysine hydroxamate reduced the growth rate of *E. coli* (20). L-Lysine hydroxamate has been used to select for high lysine-excreting mutants of *Lactobacillus* (18).

An investigation on the effect of glycine hydroxamate on tobacco cells was undertaken because, as an analog of glycine, it was hoped that the hydroxamate would be useful in studying the photorespiratory conversion of glycine to serine and CO_2 in the cells. Since glycine decarboxylase activity is involved in photorespiratory CO_2 production (6, 21), such an inhibition could be expected to increase the intracellular concentration of glycine in the cells and perhaps decrease photorespiratory CO_2 release.

We describe here the effects of glycine hydroxamate on the activities of glycine decarboxylation and serine formation, glutamate:glyoxylate aminotransferase, and serine:glyoxylate aminotransferase in particulate preparations obtained from tobacco callus and leaf tissue. Studies were also carried out on the effect of the inhibitor on the flow of carbon through the glycolate pathway of photorespiration in tobacco callus cells.

MATERIALS AND METHODS

Plant Material. Haploid callus tissues were derived from anther cultures of *N. tabacum* L., var. John Williams Broadleaf, *su* (4). Callus was maintained on Linsmaier and Skoog medium (13) using salt mixtures ("Murashige and Skoog Mixture", Flow Laboratories, Rockville, Md.) in 1% agar with 0.3 mg/l naphthaleneacetic acid, 0.3 mg/l isopentenylaminopurine, 100 mg/l inositol, 1 mg/l thiamine, and 2% sucrose (4). The callus was grown in Petri dishes sealed with rubber bands and kept in a 27 C room under an irradiance of 120 to 235 $\mu E \cdot m^{-2}\ s^{-1}$ (400–700 nm) provided by fluorescent and Gro-lux lamps. The tobacco leaves (var. Havana Seed) used for isolating particulate preparations were excised from plants grown in the greenhouse.

Enzyme Assays. Assays for glycine decarboxylation and serine production (referred to as glycine decarboxylase), serine:glyoxylate aminotransferase, and glutamate:glyoxylate aminotransferase activities were carried out as described previously using particulate preparations containing a mixture of organelles in 1-h radiochemical assays (11). The following modification of the glycine decarboxylase assay was made: the paper wick in the center well was moistened with 25 μl of 5 M ethanolamine and 30 min after terminating the reaction the wick was placed in a precounted scintillation vial containing 10 ml of counting solution and 100 μl of Protosol (New England Nuclear). Protein determinations were carried out by the dye-binding methods of Bradford (7) as supplied by Bio-Rad Laboratories (Richmond, Calif.).

The stability of [1-^{14}C]glycine hydroxamate during enzymic assays was examined after 1 h by passing the acidified reaction mixtures through columns of Dowex 1-acetate and Dowex 50-H^+ to obtain the acidic, neutral, and basic compounds as described below. The basic compounds, including glycine, serine, and glycine hydroxamate, were separated by paper electrophoresis as described below under [1-^{14}C]glycine hydroxamate incorporation.

[1-^{14}C]Glycine Hydroxamate Incorporation and Metabolism by Tobacco Callus Cells. The rate of [1-^{14}C]glycine hydroxamate

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incorporation and metabolism in callus tissues was determined with approximately 1.0-g fresh weight samples of callus tissue placed on Miracloth (Chicopee Mills, Milltown, N.J.) filters moistened with 0.3 ml of 5.3 mM [1-^{14}C]glycine hydroxamate (specific radioactivity about 2.4×10^5 dpm/μmol) in 25-ml Warburg flasks containing center wells. The tissue was first separated into small clumps and all of the pieces were moistened with the inhibitor solution. Ethanolamine, 25 μl of a 5 M solution, was placed on paper wicks in the center wells, and the flasks were stoppered and shaken slowly for 2 h at 30 C. Flasks containing zero time controls were analyzed immediately after the pieces of callus tissue were moistened with the inhibitor solution (approximately 3 min). At the end of the incubation periods, the wicks in the center wells were removed and placed in vials containing 10 ml of scintillation counting solution and 100 μl of Protosol, and the radioactivity was determined.

The cells were washed with water onto a No. 42 Whatman filter paper on a Büchner funnel, then washed twice with 10 ml water and three times with 10 ml nonradioactive 5 mM glycine hydroxamate, waiting 15 s each time before withdrawing the fluid by vacuum. The cells were then quickly transferred to a 15-ml TenBroeck tissue grinder and homogenized with 1 ml of 95% ethanol-1 N HCl (4:1, v/v). The homogenate was brought to 25 ml with water and, after removing two 0.2-ml aliquots for radioactive determinations, the homogenates were centrifuged twice, resuspending the residue in 10 ml of H_2O. The combined supernatants were passed through columns (0.7 × 6 cm) of Dowex 1-acetate. The neutral and basic compounds were eluted with water, and the acidic compounds were then eluted with 10 ml of 1 N HCl. The neutral and basic compounds were passed through columns (0.7 × 6 cm) of Dowex 50-H^+, the neutral compounds eluted with water, and the basic compounds with 10 ml of 2 N NH_4OH. Aliquots were removed for determination of radioactivity. The basic fractions were decreased to less than 1 ml with a stream of air at 40 C, and their radioactivity was redetermined. Portions of the basic fractions (containing about 1,500 dpm) were spotted on 3MM Whatman paper (in thin streaks of 2-cm length) together with 0.25 μmol of carrier glycine, serine, and glycine hydroxamate. The adsorbed fractions were subjected to electrophoresis for 1 h at 3,000 v and 1 C in a formic acid-acetic acid-water (12:48:340, v/v) system at pH 1.9. The radioactivity in glycine, serine, and glycine hydroxamate was determined by placing sections of the paper into vials containing 10 ml of scintillation counting solution and 0.2 ml water.

Effect of Glycine Hydroxamate on ^{14}C-Metabolite Incorporation and Metabolism in Tobacco Callus Cells. The effect of glycine hydroxamate on the metabolism of callus cells was studied in experiments using tobacco callus tissue grown either under normal conditions or incubated on culture medium supplemented with 2 mM glycine hydroxamate for 24 h previous to the experiment. The experimental procedures used were similar to those described above for [1-^{14}C]glycine hydroxamate with the following changes. Large (75 ml) Warburg flasks were used, fitted with Miracloth filters, and wet with 0.8 ml of either 10 mM [1-^{14}C]glycine (specific radioactivity 3.2×10^5 dpm/μmol), 10 mM sodium [1-^{14}C]glyoxylate (1.6×10^5 dpm/μmol), or 10 mM potassium [1-^{14}C]glycolate (2.2×10^5 dpm/μmol). In [1-^{14}C]glycine feeding experiments, the cells were incubated for 1.75 h at 30 C and then washed with water and 10 mM nonradioactive glycine before analysis of the radioactive products as described above for [1-^{14}C]glycine hydroxamate incorporation. In [1-^{14}C]glycolate and [1-^{14}C]glyoxylate incorporation experiments, callus cells were incubated for 2.67 h and washed with water and either 10 mM nonradioactive sodium glyoxylate or 10 mM potassium glycolate. Metabolites present in the supernatant fluid after centrifuging the homogenates were separated by chromatography on columns of Dowex 1-acetate. The neutral and basic compounds were separated on Dowex 50-H^+ columns and the radioactivity in glycine and serine was determined as described above for [1-^{14}C]glycine hydroxamate incorporation.

Chemicals. Glycine ethyl ester-HCl, hydroxylamine-HCl, and glycinamide-HCl were purchased from Sigma Chemical Co. Glycine ethyl ester-HCl and hydroxylamine-HCl were desalted by adding equal molar amounts of KOH in absolute methanol at 0 C, centrifuging, and discarding the precipitated KCl (2). The hydroxylamine-HCl was assumed to contain 50% water by weight. [1-^{14}C]Glycine (Amersham Corp.) and [1-^{14}C]glyoxylate were prepared and further purified as described previously (11).

[1-^{14}C]Glycolate (Amersham Corp.) was purified on a column (0.7 × 6 cm) of Dowex 1-acetate anion exchange resin. After eluting the basic and neutral compounds with water, the glycolic acid was eluted between 4 and 14 ml of 4 N acetic acid (22). The volume was reduced almost to dryness with a stream of air at 40 C, and carrier glycolate solution was added.

Glycine hydroxamate was synthesized from glycine ethyl ester by slowly mixing the ethyl ester with hydroxylamine (in a molar ratio of 1.0:1.5) in absolute methanol in an ice bath (12). Crystals of glycine hydroxamate formed overnight. The methanol was decanted, and the crystals were dissolved in 40% (v/v) methanol at 60 C. Recrystallization occurred overnight upon refrigeration. The yield of glycine hydroxamate was approximately 50%.

The synthesis of [1-^{14}C]glycine hydroxamate of high specific radioactivity from [1-^{14}C]glycine via [1-^{14}C]glycine ethyl ester was carried out by modifying the methods of Fisher and Skita (8) and Ley and Männchen (12) and is described in detail elsewhere (10). Dried carrier-free [1-^{14}C]glycine (Amersham Corp.) and 200 mg (2.66 mmol) nonradioactive glycine were added to a 50-ml flat bottomed flask containing a boiling chip and 15 ml of 3 N HCl in absolute ethanol. The contents were refluxed for 1 h in the absence of moisture (8). The resulting [1-^{14}C]glycine ethyl ester-HCl solution was taken to dryness *in vacuo* and dried overnight in a vacuum desiccator over KOH. Approximately 100% yield of [1-^{14}C]glycine ethyl ester-HCl was obtained from [1-^{14}C]glycine. The crystalline [1-^{14}C]glycine ethyl ester-HCl was transferred with absolute methanol into a conical centrifuge tube and the volume reduced to less than 1 ml by blowing N_2 on the surface at 40 C. Then HCl-free [1-^{14}C]glycine ethyl ester was produced by slowly adding an equivalent of 5 N KOH in absolute methanol. The resulting mixture was centrifuged twice, suspending the pellet in 0.5 ml absolute methanol. The combined supernatants were decreased to 0.82 ml with N_2 at 45 C and cooled in an ice bath.

[1-^{14}C]Glycine hydroxamate was formed by slowly adding 1 ml of 5.34 mM HCl-free hydroxylamine to the solution of HCl-free [1-^{14}C]glycine ethyl ester while shaking at 30 C. After 30 min, the volume was reduced to 0.84 ml with a stream of N_2 at 45 C and the solution was refrigerated overnight. The glycine hydroxamate crystals were washed with absolute methanol and recrystallized after dissolving them in 40% (v/v) methanol at 60 C as described elsewhere (10). The recovery of crystalline material, based on 2.66 mmol of starting material, was 66%. The specific radioactivity was 245,000 dpm/μmol and was 99% pure by colorimetric determinations and by the distribution of radioactivity after TLC and paper electrophoresis as described below. Dilution of the initial reaction mixtures described above produced lower yields and higher impurities.

Glycine hydroxamate decomposes above 70 C hence it was always maintained below 65 C. Glycine hydroxamate is stable in alkali and is a strong chelator of metal ions. Contact with metals was therefore avoided during preparation whenever possible.

Glycine hydroxamate was assayed colorimetrically by utilizing its ability to chelate ferric ions (14). A 0.5-ml solution containing glycine hydroxamate was mixed with 1 ml of 5% (w/v) $FeCl_3$ in 0.1 N HCl. After 5 min, the *A* at 525 nm was measured in a colorimeter. Glycine hydroxamate was detected by its orange-brown color when a spray consisting of 5% (w/v) $FeCl_3$ in 95% ethanol with 0.1 N HCl was applied after TLC or paper electro-

phoresis. Glycine hydroxamate also gave a brown color when sprayed with ninhydrin in 1-butanol (Pierce Chemical Co.) and heated at 100 C for 3 to 5 min.

Glycine hydroxamate, glycine, and glycine ethyl ester were separated by TLC with phenol-water (3:1, w/w) using glass-backed silica plates (*e.g.*, Silica Gel 60, EM Laboratories Inc., Elmsford, N.Y.). In 6.5 to 7 h the solvent front moved 17 cm and glycine hydroxamate, glycine, and glycine ethyl ester migrated 0 to 1.2 cm, 1.6 to 3 cm, and 7 to 9.2 cm, respectively. Glycine was separated from glycine hydroxamate and glycine ethyl ester by electrophoresis on Whatman 3MM paper at 3,000 v for 45 min in a formic acid-acetic acid-water (12:48:340, v/v) system at pH 1.9.

RESULTS

Inhibition of Glycine Decarboxylase by Glycine Hydroxamate. Glycine hydroxamate was a competitive inhibitor of glycine decarboxylase activity in particulate preparations isolated from tobacco callus tissue. Kinetic constants calculated from Lineweaver-Burk double reciprocal plots using three different substrate concentrations revealed that glycine hydroxamate, at 2.1 mM, had no effect on the V_{max} (0.13 μmol CO_2/g fresh weight·h or 0.51 μmol CO_2/mg protein·h) but did increase the apparent K_m for the substrate. The K_i for glycine hydroxamate in glycine decarboxylase assays was 0.24 ± 0.03 mM while the K_m for glycine was 5.0 ± 0.5 mM. Glycine hydroxamate also competitively inhibited glycine decarboxylase activity in particulate preparations obtained from tobacco leaves.

The formation of 1 mol of $^{14}CO_2$ and 1 mol of [1-^{14}C]serine should be expected for every 2 mol of [1-^{14}C]glycine utilized if glycine synthase and serine hydroxymethyltransferase are coupled in plant mitochondria (6, 21). In experiments using particulate preparations obtained from callus tissue, 1.0 mol $^{14}CO_2$ to 1.12 mol [^{14}C]serine was obtained. Serine production was measured as described previously (11).

Glycine hydroxamate was chemically stable during the assay of glycine decarboxylase activity. To investigate this, reaction mixtures were prepared as above except that the radioactive label was present in [1-^{14}C]glycine hydroxamate (Table I). Essentially no breakdown of the hydroxamate was observed at the termination of the 1-h assaying period (Table I).

Reactivity of Glycine Hydroxamate with Glyoxylate. Glycine hydroxamate also appeared to be a competitive inhibitor in assays of glutamate:glyoxylate and serine:glyoxylate aminotransferase activities (giving pseudo-K_i values of about 2 mM). However, under these reaction conditions with the radioactive label present in [1-^{14}C]glycine hydroxamate (Table I), 98% of the glycine hydroxamate broke down after 1 h and was recovered as acidic compounds (74%), glycine, and neutral compounds.

Glycine hydroxamate reacted stoichiometrically and nonenzymically with glyoxylate. Reaction mixtures (1.2 ml) were prepared that contained assay medium at pH 7.2, and 7.5 μmol glyoxylate and/or 2.5 μmol glycine hydroxamate with the radioactive label present in either [1-^{14}C]glycine hydroxamate or [1-^{14}C]glyoxylate. After 1 h at 30 C the reaction mixtures were subjected to electrophoresis. When glycine hydroxamate and glyoxylate were incubated together, a new product formed which chelated ferric ions and co-migrated with neutral and acidic compounds. On anion exchange columns the acidic product eluted in the later portion of the glycolic acid fraction. Radioactivity was found in the product regardless of whether the initial label was in [1-^{14}C]glycine hydroxamate or [1-^{14}C]glyoxylate. When 8.5 μmol of [1-^{14}C]glyoxylate and 2.5 μmol of glycine hydroxamate were mixed, 2.8 μmol of [1-^{14}C]glyoxylate was removed by the reaction, indicating that the reaction involved 1 mol of glyoxylate with 1 mol of glycine hydroxamate and went to completion in 1 h at 30 C. This reaction between glycine hydroxamate and glyoxylate during the assays of glutamate:glyoxylate and serine:glyoxylate aminotransferase activities can easily account for the apparent competitive inhibition of the enzymes by glycine hydroxamate.

Table I. *Stability of [1-^{14}C]Glycine Hydroxamate during the Assays for Glycine Decarboxylase and Glutamate:Glyoxylate Aminotransferase Activity from Tobacco Callus*

The reaction mixtures contained particulate preparations obtained from 0.48 g of callus in 1 ml assay medium (containing 0.4 M sucrose, 33 mM KH_2PO_4, 20 mM sodium citrate, and 4 mM $MgCl_2$ at pH 7.2) and 1.4 mM [1-^{14}C]glycine hydroxamate (1.9×10^6 dpm/μmol) where indicated in a final volume of 1.2 ml. The glycine decarboxylase reaction mixtures contained 1 μmol NAD^+, 0.1 μmol pyridoxal 5-phosphate, and either 8.3 mM glycine or [1-^{14}C]glycine (when assaying enzymic activity). The glutamate: glyoxylate aminotransferase reaction mixtures contained 16.7 mM glutamate, and either 12.9 mM glyoxylate or [1-^{14}C]glyoxylate. After 1 h at 30 C, the control enzymic assays were analyzed as usual, and the mixtures containing [1-^{14}C]glycine hydroxamate were analyzed for $^{14}CO_2$ production, and the distribution of radioactivity in other products by ion exchange chromatography and paper electrophoresis as described under "Materials and Methods" ("Enzyme Assays"). The data shown are the averages of duplicate determinations. The distribution observed in the glycine decarboxylase reaction can be accounted for by nonspecific fractionation on cation exchange columns and electrophoresis paper.

Enzyme Activity in Control Assays with Labeled Substrates	Glycine Decarboxylase	Glutamate:Glyoxylate Aminotransferase
	μmol/g fresh weight·h	
	0.039	0.563
Incubation with [1-^{14}C]glycine hydroxamate: % of recovered ^{14}C in	%	
Glycine hydroxamate	94.0	2.1
Glycine	2.2	7.7
Serine	1.1	1.0
Acidic compounds	2.6	74.0
Neutral compounds		15.2

Effect of Other Inhibitors. Hydroxylamine, a possible product of glycine hydroxamate hydrolysis, strongly inhibited glycine decarboxylase activity in particulate preparations from leaf tissues, completely eliminating activity at 0.2 mM. However, the chemical stability of glycine hydroxamate during the 1-h glycine decarboxylase assays (Table I) indicates that the inhibition by hydroxylamine is not important *in vitro*. Glycinamide, at 2.07 mM, weakly inhibited glycine decarboxylase activity.

Incorporation and Metabolism of [1-^{14}C]Glycine Hydroxamate by Callus Tissue. Glycine hydroxamate was slowly hydroxlyzed to glycine when tobacco callus tissue was allowed to incorporate 5.3 mM [1-^{14}C]glycine hydroxamate for 2 h at 30 C. The rate of [1-^{14}C]glycine hydroxamate uptake under these conditions was about 1.2 μmol/g fresh weight·h. Zero time controls were carried out to correct for the radioactivity that migrated with carrier glycine and serine during electrophoresis. Corrections were also made for nonspecific fractionation on columns of cation exchange resin. With these corrections, the rate of glycine hydroxamate metabolism in tobacco callus tissue was 0.011 μmol/g fresh weight·h. The distribution of products recovered from the breakdown of glycine hydroxamate was 74% CO_2, 15% glycine, and 12% serine. The large recovery in CO_2 compared to the recovery in serine suggests that the glycine produced by hydrolysis of glycine hydroxamate was decarboxylated to CO_2 and serine, and that the serine was then further metabolized. The rates of glycine hydroxamate incorporation and metabolism observed in callus tissue were much slower than the rates observed with common metabolites such as glycine, glycolate, and glyoxylate.

Effect of Glycine Hydroxamate on the Metabolism of Glycolate Pathway Intermediates in Callus Tissue. The presence of glycine hydroxamate had a small effect on the uptake of [1-^{14}C]glycine, but severely decreased glycine metabolism by tobacco callus tissue.

Table II. *Effect of Glycine Hydroxamate on [1-^{14}C]Glycine Incorporation and Metabolism in Tobacco Callus Tissue*

Callus tissue was allowed to incorporate 0.8 ml of 10 mM [1-^{14}C]glycine (specific radioactivity 3.2 × 10^5 dpm/μmol) on a Miracloth filter in 75-ml Warburg flasks for 1.75 h at 30 C. The cells were then washed, homogenized, fractionated on columns of Dowex 1-acetate and Dowex 50-H^+, and the basic compound fractions further separated by paper electrophoresis. Half of the callus samples had been incubated for 24 h on culture medium supplemented with 2 mM glycine hydroxamate. Data shown are the average of five determinations.

Amount Recovered in:	[1-^{14}C]Glycine Incorporation Without Glycine Hydroxamate	[1-^{14}C]Glycine Incorporation With Glycine Hydroxamate	Effect of Glycine Hydroxamate
	μmol/g fresh weight·h		*% change*
CO_2	0.184	0.040	−78
Serine	0.220	0.039	−82
Glycine	0.260	0.432	+66
Acids and neutrals	0.020	0.011	−45
Uptake	0.684	0.522	−24
Metabolism	0.424	0.090	−79

Table II illustrates the results obtained with callus cells allowed to incorporate 10 mM [1-^{14}C]glycine for 1.75 h at 30 C after they had been incubated for 24 h prior to the experiment on culture medium supplemented with 2 mM glycine hydroxamate. In the absence of glycine hydroxamate, [1-^{14}C]glycine was metabolized at a rate of 0.42 μmol/g fresh weight·h, primarily into CO_2 and serine at 0.18 and 0.22 μmol/g fresh weight·h, respectively (Table II). These results support the expected stoichiometry from the *in vitro* characterization of glycine decarboxylase activity. The rate of glycine incorporation by callus cells, 0.68 μmol/g fresh weight·h, was not rate-limiting since the rate of glycine metabolism was almost independent of the glycine concentration in the range used (10 mM).

The presence of glycine hydroxamate severely decreased the rate of [1-^{14}C]glycine metabolism in callus tissue. [1-^{14}C]Glycine metabolism decreased 79%, from 0.42 to 0.09 μmol/g fresh weight·h (Table II). The amount of radioactivity recovered as [^{14}C]glycine increased 66% while the radioactivity recovered in $^{14}CO_2$ and [^{14}C]serine decreased 78 and 82% (both to about 0.04 μmol/g fresh weight·h), respectively. The rate of [1-^{14}C]glycine uptake into callus cells only decreased 24% under these conditions. When the ^{14}C-pool sizes were calculated as a percentage of the [1-^{14}C]glycine incorporated, the results were similar.

Feeding experiments using either [1-^{14}C]glycolate or [1-^{14}C]glyoxylate were carried out to determine whether the nonenzymic reaction between glycine hydroxamate and glyoxylate was important in the over-all effect of glycine hydroxamate on cellular metabolism and whether glycine hydroxamate had any effect on other enzymes of the glycolate pathway such as the glyoxylate aminotransferases. These aminotransferases could not be assayed by the usual procedure due to the reactivity between glycine hydroxamate and the substrate, glyoxylate. Callus tissues, which were incubated with or without glycine hydroxamate for 24 h, were allowed to incorporate and metabolize 10 mM potassium [1-^{14}C]glycolate or 10 mM sodium [1-^{14}C]glyoxylate for over 2 h at 30 C. Fractionation of the resulting ^{14}C-metabolites by ion exchange chromatography was modified to separate glycolate from the radioactive adduct between glycine hydroxamate and [^{14}C]-glyoxylate by subdividing the glycolic acid fraction.

Little or none of the addition product was found after feeding either [1-^{14}C]glycolate or [1-^{14}C]glyoxylate to callus tissue in the presence of glycine hydroxamate. The small changes in fractions from the anion exchange columns could not account for any blockage of [1-^{14}C]glycolate or [1-^{14}C]glyoxylate metabolism *in vivo*.

The main effect of glycine hydroxamate on [1-^{14}C]glycolate metabolism in tobacco callus tissue was to block the conversion of glycine to CO_2 and serine. Table III shows that the [^{14}C]glycine pool size increased approximately 7-fold in the presence of glycine hydroxamate, an increase from about 6.8 to 51.6% of the glycolate metabolized. Serine and CO_2 formation were decreased by 43 and 48%, respectively. Further metabolism of serine, as estimated by the radioactivities in the neutral and strong acid fractions, also decreased (Table III). Whether calculated on the basis of the total radioactivity in the extract or as a percentage of glycolate metabolism, the [^{14}C]glyoxylate recovered in the [1-^{14}C]glycolate incor-

Table III. *Effect of Glycine Hydroxamate on [1-^{14}C]Glycolate Incorporation and Metabolism in Tobacco Callus Tissue*

Callus cells which had been incubated for 24 h either on standard culture medium or on 2 mM glycine hydroxamate-containing medium were allowed to incorporate 0.8 ml of 10 mM potassium [1-^{14}C]glycolate (2.2 × 10^5 dpm/μmol) on Miracloth filters in 75-ml Warburg flasks. After 2.67 h at 30 C the cells were washed, homogenized, centrifuged, and the metabolites present in the supernatant fluid were separated on columns (0.7 × 6 cm) of Dowex 1-acetate. After the basic and neutral compounds were collected by elution with water, the following fractions were eluted with 4 N acetic acid: the glutamic and aspartic acid fraction with 4 ml, the glycolic acid fraction with two 5-ml elutions, a discard fraction with 6 ml, and the glyoxylic acid fraction with 30 ml. The strong acids were then eluted with 10 ml of 1 N HCl. The neutral and basic compounds were separated on Dowex 50-H^+ columns followed by paper electrophoresis of the basic fraction.

	[1-^{14}C]Glycolate Incorporation Without Glycine Hydroxamate	[1-^{14}C]Glycolate Incorporation With Glycine Hydroxamate	Effect of Glycine Hydroxamate
	% of metabolism		*% change*
Fraction:			
CO_2	56.6	29.5	−48
Asp and Glu	2.1	2.6	+24
Glyoxylate	3.4	2.5	−26
Strong acids	3.7	2.0	−46
Neutrals	10.4	2.1	−80
Serine	16.4	9.4	−43
Glycine	6.8	51.6	+659
	μmol/g fresh weight·h		
Rate of:			
Uptake	0.306	0.195	
Metabolism	0.145	0.091	

Table IV. *Effect of Glycine Hydroxamate on [1-^{14}C]Glyoxylate Incorporation and Metabolism in Tobacco Callus Tissue*

The experimental procedures used are described under "Materials and Methods" and followed the procedures outlined in Table III except that 10 mM sodium [1-^{14}C]glyoxylate (specific radioactivity 1.6 × 10^5 dpm/μmol) was used.

	[1-^{14}C]Glyoxylate Incorporation Without Glycine Hydroxamate	[1-^{14}C]Glyoxylate Incorporation With Glycine Hydroxamate	Effect of Glycine Hydroxamate
	% of metabolism		*% change*
Fraction:			
CO_2	51.3	39.5	−23
Asp and Glu	1.8	2.7	+50
Glycolate	8.4	8.6	+2
Strong Acids	7.8	7.8	0
Neutrals	6.4	1.3	−80
Serine	15.3	6.0	−61
Glycine	5.8	28.2	+386
	μmol/g fresh weight·h		
Rate of:			
Uptake	0.257	0.232	
Metabolism	0.167	0.134	

poration experiments did not increase in the cells preincubated with glycine hydroxamate. Hence, the glyoxylate aminotransferases were not the sites of glycine hydroxamate inhibition *in vivo* (Table III).

Glycine hydroxamate also inhibited the conversion of glycine to CO_2 and serine in callus cells fed [1-^{14}C]glyoxylate. The [^{14}C]glycine fraction increased almost 4-fold in the presence of glycine hydroxamate, whereas CO_2 and serine decreased by 23 and 61%, respectively, as a per cent of the [1-^{14}C]glyoxylate metabolized (Table IV) decreases in the formation of ^{14}C-neutral compounds were also observed.

DISCUSSION

Glycine hydroxamate was stable during the course of the glycine decarboxylase assay (Table I). However, the hydroxamate reacted quickly and completely with glyoxylate during assays of either glutamate:glyoxylate or serine:glyoxylate aminotransferase activities (Table I), resulting in an apparent competitive inhibition of these enzymes due to the depletion of the substrate. The distribution of products observed in experiments feeding [1-^{14}C]glycolate and [1-^{14}C]glyoxylate to callus tissue in the presence of glycine hydroxamate indicated that the nonenzymic reaction between glyoxylate and glycine hydroxamate is not metabolically important *in vivo*. The feeding experiments also show that glycine hydroxamate does not greatly inhibit either of the glyoxylate aminotransferase activities *in vivo*.

The product formed upon the reaction of glycine hydroxamate with glyoxylate is a weak acid, chelates ferric ions as does the hydroxamic acid moiety of glycine hydroxamate, and contains the carbon atoms derived from the carboxyl groups of both glycine hydroxamate and glyoxylate. The simplest chemical structure consistent with the experimental observations would be a Schiff base formed between the carbonyl moiety of glyoxylate and the amino group of glycine hydroxamate.

Hydroxylamine, presumably a product of glycine hydroxamate breakdown, also inhibits glycine decarboxylase activity. Other carbonyl reagents have been reported to inhibit glycine decarboxylase activity and serine formation in tobacco, spinach, and peas (1, 15). Slow hydrolysis of glycine hydroxamate *in vivo* could provide a source of hydroxylamine intracellularly, causing the apparent inhibition of glycine decarboxylase by glycine hydroxamate. This is unlikely to be the case in the *in vitro* inhibition of glycine decarboxylase activity due to the stability of the hydroxamate under the assay condition used (Table I).

Feeding radioactively labeled glycine, glycolate, or glyoxylate to tobacco callus tissue *in vivo* showed that glycine hydroxamate primarily blocks the conversion of glycine to CO_2 and serine (Tables II, III, and IV), supporting the *in vitro* observation of glycine decarboxylase inhibition. However, small amounts of hydroxylamine formed from glycine hydroxamate in the cells could also affect the metabolism of glycine *in vivo*.

In the [^{14}C]glycine-feeding experiments (Table II), the rates of $^{14}CO_2$ and [^{14}C]serine production were equivalent. The rates were also equivalent in the presence of glycine hydroxamate. This is consistent with the stoichiometry of the conversion of 2 mol of glycine into 1 mol each of CO_2 and serine in plant mitochondria (6, 21). In the [1-^{14}C]glycolate and [1-^{14}C]glyoxylate feeding experiments, the $^{14}CO_2$ production was greater than the [^{14}C]serine recovered (Tables III and IV). For example, the ratios of $^{14}CO_2$ to [^{14}C]serine produced by callus supplied with [1-^{14}C]glycolate or [1-^{14}C]glyoxylate were about 3.4. In the presence of glycine hydroxamate, the ratios were 3.1 and 6.6, respectively. The differences are probably due to the further metabolism of [^{14}C]serine into ^{14}C-neutral and ^{14}C-acidic compounds via the glycolate pathway in intact callus tissues (5). When the CO_2 to serine ratios are recalculated as the $^{14}CO_2$ produced to the [^{14}C]serine plus ^{14}C-neutral and ^{14}C-acidic compounds produced, the ratios obtained for callus tissue fed [1-^{14}C]glycolate and [1-^{14}C]glyoxylate were 1.9 and 1.7, respectively. In the presence of glycine hydroxamate the similarly recalculated ratios were 2.2 and 2.6, respectively.

The ratios are still larger than unity, perhaps because [^{14}C]serine is recycled through the photorespiratory pathway resulting in increased $^{14}CO_2$ production, or because $^{14}CO_2$ is being produced by another reaction such as the nonenzymic decarboxylation of glyoxylate (9). Since glycine hydroxamate would probably not affect this nonenzymic CO_2 production, the ratio of $^{14}CO_2$ to [^{14}C]serine production would be expected to increase in the presence of the inhibitor. Such an increase was observed in the [1-^{14}C]glyoxylate feeding experiments but not in experiments where cells were fed [1-^{14}C]glycolate. This difference could be explained by the larger intracellular [^{14}C]glyoxylate concentration during the [1-^{14}C]glyoxylate feeding experiments, suggesting that the nonenzymic decarboxylation of glyoxylate occurs mainly when high concentrations of [1-^{14}C]glyoxylate are present.

Glycine hydroxamate, as an analog of a common metabolite, could theoretically inhibit any biochemical reaction involving glycine such as the activation of glycine by glycyl-tRNA synthetase or the synthesis of δ-aminolevulinic acid. It could also inhibit reactions involving structurally related compounds, such as alanine. Glycine hydroxamate is presently the only known reversible inhibitor of the photorespiratory conversion of glycine to serine and CO_2.

The toxicity of glycine hydroxamate to tobacco cells in tissue culture was used by Berlyn (3) as a selective force to produce cell lines resistant to the inhibitor. Obtaining resistance to glycine hydroxamate toxicity was of interest because of the possible effect of the inhibitor on glycine decarboxylase activity and intracellular glycine concentration, thus providing the possibility of altering photosynthetic carbon flow. The biochemical characterization of such glycine hydroxamate-resistant cell lines of tobacco will be the subject of a subsequent paper.

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