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Isolation and Characterization of Glycine Hydroxamate-resistant Cell Lines of *Nicotiana tabacum*[1]

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ABSTRACT

Seven lines of haploid *Nicotiana tabacum* tissue culture selected for resistance to normally toxic levels of the glycine analog glycine hydroxamate, a competitive inhibitor of the glycine decarboxylase reaction, were investigated. The presence of glycine hydroxamate greatly increased the intracellular concentration of both glycine and alanine in wild type and resistant cell lines, suggesting that the inhibitor blocks both glycine- and alanine-utilizing reactions. All the resistant cell lines, whether grown in the presence or absence of glycine hydroxamate, had high intracellular concentrations of the 12 free amino acids which were analyzed, including glycine and serine. (These lines averaged 3.6 times the total amino acid content of wild-type cells in the absence of the inhibitor). The resistant cell lines were indistinguishable from wild-type cell lines in their metabolism of radioactively labeled glycine hydroxamate and glycine. Comparison of the metabolism of radioactively labeled alanine, glycolate, and glyoxylate in wild-type and α resistant line also revealed no distinctive differences. Glycine decarboxylase activities were unaltered in the resistant cell lines. The cellular toxicity of glycine hydroxamate is considered in relation to (1) the competitive inhibition by glycine hydroxamate of the glycine- and alanine-utilizing enzymes and (2) the resultant imbalances caused by high intracellular concentrations of these amino acids. The significance of elevation of total free amino acid concentration in effecting resistance to the inhibitor is discussed.

Plants were regenerated from 5 of these lines and callus cultures of explants were tested for glycine hydroxamate resistance. Plants from seedlings of two lines which retained the resistant characteristic in explanted callus did not have high amino acid levels in leaves.

Amino acid analogs have been used as selective agents in plant tissue cultures by several laboratories (6, 7, 19, 20). In these cases, analog toxicity was ascribed to false feedback inhibition of synthesis of a metabolically related amino acid (7, 17) or loss of enzymic activities of proteins which have incorporated the analog (15). Analogs could also interfere with utilization of the amino acid in metabolically important reactions or create imbalances due to accumulation of individual amino acids (cf. Ref. 2) as a result of analog-blocked utilization reactions.

The false feedback inhibition caused by some amino acid analogs is inoperative in cells with decreased regulation at the site of feedback inhibition (and therefore increased synthesis of the amino acid) (17). Incorporation of an amino acid analog into proteins and competition of an analog in essential enzymic reactions are less effective in cells with increased concentrations of the analogous amino acid. Thus selection of plant cell lines that are resistant to an amino acid analog has resulted in lines with increased intracellular concentrations of the analogous amino acid (6, 7, 18-20).

Glycine hydroxamate is an analog which reversibly inhibits the photorespiratory conversion of glycine to CO_2 and serine in tobacco and causes accumulations of glycine in the callus cells (13). Little is known about the effect of glycine levels on photorespiration. High glycine concentrations could result in feedback inhibition of glycolate synthesis. Blocking the conversion of glycine to serine could favor a possible alternate pathway for glycine and serine metabolism with lower rates of CO_2 production. To analyze such possibilities, tobacco cell lines resistant to the toxicity of glycine hydroxamate were selected, and glycine levels and other factors involved in glycine hydroxamate resistance in tobacco cell lines were studied.

MATERIALS AND METHODS

Tissue Culture and Selection. Tobacco callus tissues were maintained on solid medium containing Murashige and Skoog plant salts mixture (Flow Laboratories, Rockville, MD) with 1% agar, 0.3 mg/l naphthaleneacetic acid, 0.3 mg/l isopentenylaminopurine, 100 mg/l inositol, 1 mg/l thiamine, and 2% sucrose (14). The nitrogen sources in the salt mixture were NH_4NO_3 (1650 mg/l) and KNO_3 (1900 mg/l). For suspension cultures, the agar was eliminated and the concentration of naphthaleneacetic acid was increased to 3.0 mg/l. Photoautotrophic medium (standard medium eliminating sucrose), (4) was also used.

The cultures originated from anthers of *Nicotiana tabacum* var. John Williams Broadleaf *aurea*, as previously described by Berlyn and Zelitch (3). Suspensions of these haploid cells were irradiated with UV light for 3 min at 5.5 $J/m^2 \cdot s$, plated on medium with 3.0 mg/l naphthalene acetic acid for 2 weeks, and then transferred to Petri dishes containing standard growth medium supplemented with 5 or 10 mM glycine hydroxamate. Callus isolates growing on these media were transferred and repeatedly tested for their resistance to 2 mM glycine hydroxamate-containing culture medium.

Long Term Incorporation and Metabolism of [1-^{14}C]Glycine Hydroxamate by Callus Tissue. For each cell line tested, a sample of more than 1 g of callus tissue was placed on an agar plate containing standard growth medium supplemented with 1 mM [1-^{14}C]glycine hydroxamate (filter sterilized, 2.45×10^5 dpm/μmol) and incubated for 4, 6, 7, and 9 days at 29 C under an irradiance of 100 to 115 $\mu E/m^2 \cdot s$ (400-700 nm) in a ventilated hood. After the incubation, 1 g of callus was homogenized in a 15-ml Ten

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Broeck tissue grinder with 1 ml of 95% ethanol:1 N HCl (4:1 v/v). The homogenate was made to a final volume of 25 ml with water, and 0.2-ml aliquots were removed for determination of radioactivity. The homogenates were then centrifuged twice, and the pellets resuspended in 10 ml water. The combined supernatants were fractionated on Dowex-1-acetate and Dowex-50-H^+ ion exchange columns, and glycine, serine, and glycine hydroxamate were separated by paper electrophoresis (13).

Short Term Incorporation and Metabolism of ^{14}C-Metabolites by Callus Cell Lines. The procedures for investigating short-term incorporation of [1-^{14}C]glycine hydroxamate or [1-^{14}C]glycine into callus tissue follow those described by Lawyer and Zelitch (13) except that 8 mM [1-^{14}C]glycine hydroxamate (1.6 to 1.8 × 10^5 dpm/μmol) was used, and the specific radioactivities of [1-^{14}C]-glycine ranged from 1.3 to 3.2 × 10^5 dpm/μmol in three separate experiments. The experimental methods used in studying the effect of glycine hydroxamate on [U-^{14}C]alanine incorporation and metabolism in tobacco cells were similar to those used in the [1-^{14}C]glycine experiments with the following exceptions: 10 mM [U-^{14}C]alanine with a specific radioactivity of 1.53 × 10^5 dpm/μmol was used, and after incubation for 2 h at 30 C, the cells were washed twice with 10 ml water and twice with 10 ml nonradioactive 10 mM alanine. Electrophoretic separation was carried out for 80 min (formic acid:acetic acid:water, 12:48:340 v/v, 3,000 v) with 4,000 dpm/sample and 0.25 μmol carrier glycine, alanine, and serine.

Enzyme Assays. Assays of glycine decarboxylase activity in particulate preparations of tobacco callus were carried out with [1-^{14}C]glycine as described by Lawyer and Zelitch, (12, 13).

Determination of Amino Acid Concentrations. The preparation of tobacco callus cells for amino acid analysis used 1 g fresh weight of callus tissue. The tissue was homogenized in 1 ml of ethanol: 1 N HCl (4:1 v/v), the suspension made to 20 ml with water and centrifuged twice at 38,000*g* for 10 min, and the pellet resuspended in 10 ml water between centrifugations. The combined supernatant fractions were transferred with 6 ml H_2O into a 125-ml separatory funnel. The lipids and Chl were extracted with 30 ml, and then 20 ml of chloroform. The remaining aqueous phase was flushed with N_2 gas to remove some of the dissolved chloroform, and passed through columns (0.7 x 6 cm) of Dowex-1-acetate which had about 0.5 cm of activated charcoal (Darco G-60, ICI America, Inc., Wilmington, Del) at the bottom of the anion exchange resin. The aqueous eluate from the columns was passed through a Dowex-50-H^+ column (0.7 × 6 cm), washed with water, and the amino acid fraction then eluted with 10 ml of 2 N NH_4OH. Aliquots, 200 μl, of the amino acid fractions were transferred to 1.2 ml ampules (Wheaton, Millville, NJ), taken to dryness by blowing a stream of N_2 gas on the surface, redissolved in 100 μl water, and taken to dryness again. Then 200 μl of 6 N HCl were added, the ampules were sealed *in vacuo* and incubated at 100 C for 24 h to hydrolyze the asparagine and glutamine. The ampules were opened, the contents dried, dissolved in 100 μl water and taken to dryness again. The samples were analyzed using a Beckman 121M Automatic Amino Acid Analyzer.

The acid hydrolysis was necessary because the quantities of asparagine and glutamine present in the callus tissue (over 10,000 nmol/g fresh weight) interfered with the quantitative estimation of serine and threonine in the amino acid analyzer. The high asparagine and glutamine concentrations are probably due to growth of the callus cells on ammonium salts as sources of nitrogen. Leaves of plants regenerated from callus tissue had normal levels of asparagine and glutamine, less than 10 nmol/g fresh weight. Methionine and cysteic acid concentrations were too low and glutamic and aspartic acid concentrations were too high to estimate accurately. Tryptophan was degraded during sample preparation and proline determinations were inconsistent due to the interference by the large glutamate-glutamine peak in the analyzer. These amino acids are therefore not listed in Tables I, II, III, and VI. Almost all the results of amino acid analysis shown were obtained from two or more separate determinations. The variation between duplicate samples was rarely over 30% and was commonly under 20%.

The effects of 2 mM glycine hydroxamate, 2 mM hydroxylamine, 10 mM glycine, or 10 mM alanine on the free amino acid concentrations in tobacco callus were determined by analyzing tissue which had been incubated for 48 h on growth medium modified accordingly. Amino acid analysis of dark-grown cells was carried out on tissue grown (from a small inoculum) in the dark for a month prior to extraction of the tissue.

A quantitative method for determination of concentrations of glycine and alanine after separation by paper electrophoresis was occasionally used. Two ml of the amino acid fraction, eluted from the Dowex-50 columns as described above, were taken to dryness with a stream of N_2. After redissolving the samples in 100 μl of water, 50-μl portions were spotted on Whatman 3 MM paper and electrophoresis was carried out for 80 min. The amino acid concentrations were determined by treating the dried paper with a cadmium-ninhydrin reagent as described by Atfield and Morris (1) (0.1 g cadmium acetate, 2 ml acetic acid, 10 mM H_2O, 100 ml acetone, 1.0 g ninhydrin). The red-purple spots were cut out and soaked in 50% ethanol for 2 h in the dark. The *A* at 500 nm was determined in a colorimeter.

Chemicals. Glycine hydroxamate and [1-^{14}C]glycine hydroxamate were prepared from glycine ethyl ester and [1-^{14}C]glycine, respectively, as described elsewhere (13). The [U-^{14}C]alanine and [1-^{14}C]glycine were purchased from New England Nuclear. DL-serine hydroxamate, DL-alanine hydroxamate, and L-lysine hydroxamate were purchased from Sigma Chemical Co.

RESULTS

Isolation of Glycine Hydroxamate-Resistant Cell Lines. From 30 isolates initially selected for growth on glycine hydroxamate-containing medium, seven cell lines retained their resistance to glycine hydroxamate and satisfactory growth characteristics during the course of repeated tests. These seven cell lines were given laboratory designations UGH-11, UGH-20, UGH-21, UGH-101, UGH-102, UGH-103 and UGH-105.

Wild-type tobacco callus tissue turned brown after 1 week on culture medium supplemented with 2 mM glycine hydroxamate. After 2 weeks, growth totally ceased. Glycine hydroxamate-resistant cell lines usually had decreased growth rates after 2 weeks on 2 mM glycine hydroxamate-supplemented medium but often appeared fully recovered after 4 weeks. The seven resistant cell lines varied in their morphology, growth rates, color, texture, and friability. Occasionally one of several subcultures showed poor growth on selective medium, usually associated with simultaneous poor growth on standard medium, and this subculture was not maintained in subsequent passages.

Preliminary tests using diphenylamine determinations of DNA content indicated that considerable variation exists among the different cell lines, but resistance was not correlated with changes in the ploidy level of the tissue.

Comparison of [1-^{14}C]Glycine Hydroxamate Uptake and Metabolism by Wild-type and Glycine Hydroxamate-Resistant Cell Lines. Glycine hydroxamate resistance was not associated with decreased rates of uptake or increased rates of glycine hydroxamate breakdown in the glycine hydroxamate-resistant cell lines. When wild-type and glycine hydroxamate-resistant cell lines were allowed to incorporate [1-^{14}C]glycine hydroxamate for 2 h, the rate of uptake of inhibitor by the resistant cell lines was not substantially different from that of the wild-type cell lines. If anything, in glycine hydroxamate-resistant cell lines the hydroxamate was taken up at faster rates. Measurements of the breakdown and metabolism of [1-^{14}C]glycine hydroxamate into $^{14}CO_2$

and other compounds indicated that wild-type and resistant cell lines metabolize the hydroxamate at similar rates, about 10 nmol CO_2/g fresh weight·h. Cell lines which had high rates of [1-^{14}C]-glycine hydroxamate uptake did not necessarily have correspondingly high rates of inhibitor breakdown and metabolism during these short term incorporation experiments. For example, UHG-105 had 1.6 times the average rate of incorporation but only 0.9 times the average rate of breakdown and metabolism.

In long-term experiments, when wild type and glycine hydroxamate-resistant cell lines were grown for 4 to 9 days on agar medium supplemented with 1 mM [1-^{14}C]glycine hydroxamate, the cellular levels of glycine hydroxamate and its metabolic products were constant throughout the period, indicating steady relative rates of uptake and breakdown during long-term incubations with glycine hydroxamate. Total radioactivity recovered in soluble extracts of the cellular homogenates showed no systematic pattern of change during the 4 to 9 day period of incorporation. Glycine hydroxamate concentrations of all cell lines at all sampling times ranged from 0.31 to 0.57 μmol/g fresh weight. The radioactivity in the insoluble fraction of the homogenate accounted for almost one-half of the radioactivity recorded, suggesting that much of the [^{14}C]glycine produced by hydrolysis of the inhibitor was being metabolized and incorporated into proteins. Total radioactivity in the insoluble fraction did not increase significantly during the 5 days of observations.

Comparison of Glycine Decarboxylase Activities in Wild-type and Glycine Hydroxamate-Resistant Cell Lines. As previously reported (13), glycine hydroxamate competitively inhibits glycine decarboxylation and serine formation in tobacco preparations. The kinetic constants (K_m, K_i, and V_{max}) of the glycine decarboxylase activities determined in particulate preparations from callus tissue of resistant and wild-type cell lines were not consistently different between lines. When standardized on the basis of either protein concentration or Cyt oxidase activity, the maximal velocities of the glycine hydroxamate-resistant lines were not significantly different from the average V_{max} for wild-type cell lines, (0.5 μmol CO_2/mg protein·h). Comparison of the K_m values for glycine and of the K_i values for glycine hydroxamate determined in several experiments for the different cell lines did not identify any resistant line with lower K_m or higher K_i than the wild-type lines.

Effect of Glycine Hydroxamate on Amino Acid Concentrations in Wild-type Callus Tissue. The intracellular concentrations of some free amino acids in the wild-type cell lines, 1-M6 and 1-J6, are presented in Table I. The most dramatic effect of incubating wild-type callus on medium containing 2 mM glycine hydroxamate for 48 h was an increase in the intracellular concentrations of glycine and alanine (Table I). These concentrations increased by 6.3- and 3.0-fold, an absolute increase of 1,100 and 420 nmol/g fresh weight, respectively. Changes in other free amino acid concentrations were small, when calculated either on an absolute basis or as a percentage change. Proline pool sizes (data not shown) were also unaffected by glycine hydroxamate. The total free amino acid concentration (of the amino acids analyzed) increased only 78% in the presence of the inhibitor and most of this increase was caused by the change in the glycine and alanine concentrations.

The increases in the intracellular glycine concentration, 1,100 nmol/g fresh weight when grown on medium containing 2 mM glycine hydroxamate (Table I), were greater than could be accounted for by the acid hydrolysis of the intracellular glycine hydroxamate during the extraction procedures. Long term incubation of callus tissue on culture medium supplemented with 1 mM [1-^{14}C]glycine hydroxamate resulted in a calculated steady state concentration of glycine hydroxamate of approximately 100 nmol/g fresh weight. If growth on 2 mM glycine hydroxamate-containing medium were to double these intracellular concentrations of the hydroxamate (up to 200 nmol/g fresh weight), the complete hydrolysis of the hydroxamate to glycine during sample preparation would account for less than one-fifth of the increases in intracellular glycine observed after growth in the presence of glycine hydroxamate.

Because of the concurrent increases in glycine and alanine in these experiments, the effects of supplementation of the growth medium with either glycine or alanine were examined. When wild type callus was incubated for 48 h on culture medium supplemented with 10 mM glycine, the intracellular glycine concentration increased over 13-fold and the alanine pool size in these cells remained unchanged. Growing wild type callus on medium containing 10 mM alanine increased the alanine content 34%, while the concentration of glycine decreased by 10%. Therefore, under these conditions, free alanine and glycine concentrations vary independently in wild-type cells.

Comparison of Amino Acid Concentrations in Wild-type and Glycine Hydroxamate-Resistant Cell Lines. The concentrations of all the amino acids determined were substantially greater in the seven glycine hydroxamate-resistant cell lines than in the wild-type cell lines. The total concentration of the free amino acids analyzed was larger than in the wild-type callus by a factor of 4.4 for UGH-11, 4.4 for UGH-20, 3.4 for UGH-21, 3.1 for UGH-101, 3.2 for UGH-102, 2.8 for UGH-103, and 4.1 for UGH-105 (Table II). On the average, resistant cell lines had 3.6 times the total amino acid content of the wild type cells. Glycine concentrations ranged from 1.9 to 5.8 times larger than wild-type concentrations, equivalent to a 190 to 1,020 nmol/g fresh weight increase, respectively. Alanine concentrations were also larger than those observed in wild-type cell lines by a factor of 1.2 to 5.6 (Table II).

The concentrations of all the other amino acids analyzed were also greater in the resistant cell lines. Increased concentrations were found in Thr, Ser, Val, Ile, Leu, Tyr, Phe, His, Lys, and Arg (Table II), and in Pro (data not shown). The relative intracellular concentrations of glycine and alanine were fairly well coordinated. For example, UGH-20 had the largest concentrations of both amino acids, and UGH-21 had the smallest of both. Although percentage increases larger than those of glycine and alanine were observed for other amino acids, notably the aromatic amino acids or arginine in most cell lines, these changes represent absolute increases of small magnitude. The pattern of increase appeared to be somewhat distinctive for each cell line.

The effect of glycine hydroxamate on the amino acid concentrations of the glycine hydroxamate-resistant cell lines was similar to that observed in wild-type cells: the intracellular concentrations of glycine and alanine increased in all resistant cell lines (Table III). Glycine hydroxamate increased the glycine concentrations 1.8- to 8.4-fold, while it increased alanine 1.4- to 6.3-fold. The effect of the hydroxamate on the pool sizes of the other amino acids analyzed in glycine hydroxamate-resistant cell lines was small.

Comparison of the Incorporation and Metabolism of [^{14}C]Glycine, [^{14}C]Alanine, [^{14}C]Glycolate, and [^{14}C]Glyoxylate in Wild-type and Glycine Hydroxamate-resistant Cell Lines. The uptake of [1-^{14}C]glycine by glycine hydroxamate-resistant cell lines was greater than the uptake in wild-type cells, but the subsequent rate of metabolism was similar in all cell lines tested. After incubation of callus on 10 mM [1-^{14}C]glycine for 2 h at 30 C glycine uptake (the total soluble radioactivity extracted from the cellular homogenate plus the $^{14}CO_2$ production) in the glycine hydroxamate-resistant cell lines was 20 to 230% larger than the wild-type value of 330 nmol/g fresh weight (Table IV). The amounts of radioactivity recovered in the [^{14}C]glycine fractions were in all cases larger in the resistant cell lines than in wild-type cells (Table IV).

The rates of [1-^{14}C]glycine metabolism into other compounds varied from 160 nmol/g fresh weight·h for UGH-105 to 780 nmol/g fresh weight·h for UGH-102, compared to 200 nmol/g fresh weight for wild-type (Table IV). These rates were somewhat

Table I. *Effect of Glycine Hydroxamate on Free Amino Acid Concentrations in Wild-Type Callus*

Two wild-type cell lines, 1-M6 and 1-J6, were analyzed. The effects of glycine hydroxamate shown are the averages of the two cell lines. The variation between duplicate samples was 30% or less. Where indicated, callus was incubated in 2 mM glycine hydroxamate-containing medium for 48 h prior to analysis. Determinations of the total amino acid concentrations are the summation of the 12 amino acids analyzed (see "Materials and Methods" for details of the analysis of these amino acids and those omitted from the Table).

Amino Acid	Amino Acid Concentrations				Effect of Glycine Hydroxamate	
	Cell Line 1-M6		Cell Line 1-J6			
	Without Glycine Hydroxamate	With Glycine Hydroxamate	Without Glycine Hydroxamate	With Glycine Hydroxamate	Ratio With/ Without	Absolute Change
	nmol/g fresh wt					*nmol/g fresh wt*
Gly	170	1200	260	1400	6.3	+1,100
Ala	210	510	220	760	3.0	+420
Thr	300	600	330	390	1.6	+180
Ser	390	510	350	560	1.5	+170
Val	80	30	100	90	0.7	−20
Ile	50	20	90	80	0.6	−20
Leu	80	50	100	80	0.7	−20
Tyr	20	—	60	90	1.6	+30
Phe	40	30	120	120	0.9	−10
His	570	540	390	410	1.0	0
Lys	120	220	200	220	1.5	+60
Arg	30	50	60	70	1.5	+20
Total amino acids	2040	3720	2170	4290	1.8	+1,900

Table II. *Comparison of the Free Amino Acid Content of Wild-Type and Glycine Hydroxamate-Resistant Callus Cell Lines*

Values shown are the ratios of the amino acid concentrations of glycine hydroxamate-resistant (UGH) cell lines compared to the average concentrations in wild-type cell lines, 1-M6 and 1-J6 (Table I).

Amino Acid	Ratio of Free Amino Acid Concentrations Relative to Wild-Type Concentrations						
	UGH 11	UGH 20	UGH 21	UGH 101	UGH 102	UGH 103	UGH 105
Gly	1.9	5.8	1.9	2.1	2.4	4.0	4.3
Ala	2.8	5.4	1.3	2.4	5.6	2.4	4.0
Thr	7.2	4.8	3.6	2.4	3.7	4.0	4.6
Ser	4.4	7.6	1.4	2.4	3.8	3.7	4.9
Val	2.9	2.6	4.1	3.4	4.4	3.1	4.3
Ile	7.0	3.9	6.1	5.5	3.2	4.0	4.8
Leu	7.2	3.6	4.6	9.1	3.0	2.9	4.4
Tyr	7.7	5.0	11.2	9.0	2.8	2.8	10.5
Phe	8.2	4.2	21.6	9.9	3.0	3.2	7.4
His	1.9	2.5	1.6	1.7	2.3	0.9	2.2
Lys	3.1	4.9	2.9	2.1	2.9	1.5	2.3
Arg	10.0	8.4	4.3	6.1	8.7	1.1	4.1
Total amino acids	4.3	4.4	3.4	3.1	3.2	2.8	4.1

correlated with total uptake; for example, the two cell lines with the most rapid metabolism of [1-^{14}C]glycine had the largest uptake of [1-^{14}C]glycine, suggesting that the rate of metabolism was at least partially dependent on the rate of uptake.

The presence of glycine hydroxamate had little effect on the rate of [1-^{14}C]glycine uptake by either wild-type or resistant callus. However, as observed with wild-type cells (13), glycine hydroxamate greatly decreased the rate of [1-^{14}C]glycine metabolism in glycine hydroxamate-resistant cell lines. For all cell lines preincubated for 24 h on growth media with and without glycine hydroxamate, the amount of inhibition observed in the hydroxamate-treated tissue was approximately 70%.

Since intracellular alanine pool sizes increased markedly in the presence of glycine hydroxamate (Table I), experiments were undertaken to compare the effect of glycine hydroxamate on alanine uptake and metabolism in a wild-type cell line, 1-M6, and a resistant cell line, UGH-11. Cells were allowed to incorporate 10 mM [U-^{14}C]alanine for 2 h at 30 C. Table V shows that [U-

Table III. *Effect of Glycine Hydroxamate on Free Glycine and Alanine Concentrations in Wild-Type and Glycine Hydroxamate-Resistant Cell Lines*

The procedures for preparing and analyzing the amino acid fractions are described under "Materials and Methods." Hydroxamate denotes glycine hydroxamate; calculations for wild-type are derived from the average of the two wild-type cell lines analyzed in Table 1.

	Wild Type	Glycine Hydroxamate-resistant Cell Line						
		UGH 11	UGH 20	UGH 21	UGH 101	UGH 102	UGH 103	UGH 105
		nmol/g fresh wt						
Glycine concentration								
Without hydroxamate	200	390	1200	380	410	470	800	840
With hydroxamate	1300	1200	2100	690	3500	1900	1600	3200
Alanine concentration								
Without hydroxamate	210	580	1100	260	490	580	500	840
With hydroxamate	630	3600	1600	780	1200	1700	840	3100
Ratio of change in concentration								
Glycine	6.6	3.2	1.8	1.8	8.4	4.2	2.0	3.8
Alanine	3.0	6.3	1.4	3.0	2.5	3.0	1.7	3.7
Total amino acids	1.9	1.1	1.1	0.7	1.6	1.3	0.8	1.3

Table IV. *Comparison of [1-^{14}C]Glycine Uptake and Metabolism by Wild-Type and Glycine Hydroxamate-Resistant Cell Lines*

Callus tissue samples (about 1.0 g) were placed on Miracloth filters wet with a solution of 10 mM [1-^{14}C]glycine (specific radioactivity from 1.3 to 3.2 × 10^5 dpm/μmol) in Warburg flasks and incubated for 2 h at 30 C. The cells were then removed and washed with water and nonradioactive glycine, homogenized, and fractionated by ion exchange chromatography and electrophoresis. Production of $^{14}CO_2$ was also determined and is included in the calculation of the uptake and metabolism. The wild type values are averages of determinations made with 1-M6 and 1-J6 cell lines; the standard errors are shown in parentheses.

Cell Line	[1-^{14}C]Glycine Uptake	[1-^{14}C]Glycine Metabolized	[^{14}C]Glycine Remaining
	nmol/g fresh wt·h		
Wild-type	330 (SE 90)	200 (SE 30)	130 (SE 50)
UGH-11	910	660	250
UGH-20	540	170	370
UGH-21	530	270	260
UGH-101	400	220	180
UGH-102	1100	780	320
UGH-103	470	300	180
UGH-105	620	160	460

^{14}C]alanine was taken up by the UGH-11 cells at a rate of 1.34 μmol/g fresh weight·h, more than three times the rate of uptake into the wild-type tissue. As shown in Table II, the difference between the alanine concentrations found in the wild-type and UGH-11 tissues was also about 3-fold. Incubation of the callus tissue on culture medium supplemented with 2 mM glycine hydroxamate had little effect on the rate of [U-^{14}C]alanine uptake in 1-M6 or UGH-11 tissues (Table V).

Glycine hydroxamate had no striking effect on the metabolism of [U-^{14}C]alanine or on the distribution of its metabolic products (Table V). After 2 h, about 70% of the alanine incorporated was metabolized, probably by deamination of alanine to pyruvate, as indicated by the high percentage of acidic products found. The lack of effect of glycine hydroxamate on the metabolism of [U-^{14}C]alanine (Table V) contrasts with the inhibitor's strong effect on [^{14}C]glycine metabolism (13).

In both wild type and the UGH-103 cell lines, glycine hydroxamate had little effect on the uptake of [1-^{14}C]glycolate or [1-^{14}C] glyoxylate, but it did affect the metabolism of these substrates. Increases in [^{14}C]glycine accumulation and decreases in $^{14}CO_2$, [^{14}C]serine, and ^{14}C-neutral compounds were observed in the presence of glycine hydroxamate (13), changes typical of inhibitors which block glycine metabolism in the glycolate pathway. In these experiments there were no large differences between the wild-type and a resistant cell line (UGH-103) in their rates of uptake or metabolism of these ^{14}C-substrates or in their response to glycine hydroxamate. Significantly more radioactivity, however, was recovered in the basic compounds of the glycine hydroxamate-resistant line than in those of wild-type. The [^{14}C]serine fractions

Table V. *Incorporation and Metabolism of [U-^{14}C]Alanine by Tobacco Callus Cell Lines*

Callus tissue (1.0 g) which had been incubated in the light for 24 h on either standard culture medium or medium containing 2 mM glycine hydroxamate was placed on Miracloth filters wet with 0.8 ml of 10 mM [U-^{14}C]alanine (specific radioactivity 1.53 × 10^5 dpm/μmol) in large Warburg flasks. After 2 h at 30 C, the cells were removed, washed, homogenized, and fractionated. Portions of the basic compound fraction eluted from Dowex-50-H^+ columns were subjected to electrophoresis to determine the [^{14}C]alanine content. The remaining bases are termed "other bases". The cell lines used were the wild type 1-M6 and the resistant cell line UGH-11.

	Wild-Type Cell Line 1-M6		Resistant Cell Line UGH-11	
	Without Glycine Hydroxamate	With Glycine Hydroxamate	Without Glycine Hydroxamate	With Glycine Hydroxamate
	nmol/g fresh wt·h			
Uptake of [1-^{14}C]-alanine	390	320	1340	1220
	%			
Uptake present in:				
CO_2	23.7	33.1	17.5	24.4
Acids	22.9	18.6	13.1	17.3
Neutrals	1.0	1.9	1.3	1.9
Bases	52.4	46.4	68.1	56.3
Alanine	38.4	26.0		
Other Bases	14.0	20.4		

were two to three times larger than those of wild-type cells in both the [1-^{14}C]glycolate and [1-^{14}C]glyoxylate feeding experiments, and the [^{14}C]glycine pool in UGH-103 cells was about twice as large as in the wild-type cells in the [1-^{14}C]glycolate experiment.

Studies of Growth on Supplemented Media. Since the presence of glycine hydroxamate increases the intracellular pool sizes of glycine and alanine in all cell lines studied (Table II), the effect of growing these cell lines on culture medium supplemented with either 10 mM glycine or 10 mM L-alanine was studied. The presence of either 10 mM glycine or 10 mM alanine in the medium was inhibitory to wild-type callus. In contrast, all but one of the glycine hydroxamate-resistant cell lines grew on both media. The observed growth rates of the resistant lines on media supplemented with the amino acids ranged from marginal to strong normal growth (with the exception of UGH-103, which consistently did not grow on 10 mM glycine). In contrast, wild-type and resistant cell lines (except UGH-101) were sensitive to the presence of 10 mM L-serine or 10 mM L-lysine in the medium. These observations imply the specificity of the glycine and alanine effects.

Both wild-type and glycine hydroxamate-resistant cell lines grow in the dark. In the presence of 2 mM glycine hydroxamate, neither wild-type nor resistant cell lines grew in the dark. Comparison of the free amino acid concentrations of 1-M6 wild-type and UGH-20 cells grown in the dark for 1 month with the values obtained for light-grown tissue (Table I) is shown in Table VI. Wild type (1-M6) amino acid concentrations were relatively unaffected by growth in the dark or light (Table VI), but a very large decrease in free amino acids was observed when UGH-20 tissue was grown in the dark rather than in the light. The difference between amino acid levels in wild-type and UGH-20 was 4.9-fold in light-grown cells and only 1.5-fold in dark-grown cells.

Table VI. *Comparisons of Free Amino Acid Concentrations in Callus Cells Grown in the Light and in the Dark, and in the Presence of Hydroxylamine*

Where indicated, cells were grown in the dark for 1 month before being analyzed. Incubation of wild-type cell line 1-M6 on culture medium supplemented with 2 mM hydroxylamine was carried out for 48 h in the light. Preparation and analysis of the amino acid fraction was carried out. The amino acid concentration average used for comparisons to wild-type cell lines (2105 nmol/g fresh wt) is from Table I.

	Growth Conditions				
	Resistant Cell Line UGH-20		Wild-type Cell Line 1-M6		
	Normal	Dark	Normal	Dark	Hydroxylamine
	nmol/g fresh wt				
Amino acid concentration					
Gly	1,200	490	170	330	680
Ala	1,100	230	210	640	470
Thr	1,500	470	300	220	260
Ser	2,800	650	390	290	240
Val	220	120	80	100	50
Ile	270	80	50	40	20
Leu	310	100	80	140	70
Tyr	200	40	20		40
Phe	340	100	40	10	20
His	1,200	660	570	570	440
Lys	770	160	120	70	40
Arg	360	120	30	40	
Total amino acids	10,200	3,200	2,000	2,400	2,300
Ratio of increase over wild-type average	4.9	1.5	1.0	1.2	1.1

Both wild-type and glycine hydroxamate-resistant cell lines are capable of photoautotrophic growth in the absence but not in the presence of glycine hydroxamate.

To test whether hydroxylamine, a possible breakdown product of glycine hydroxamate, was the actual inhibitor to wild-type callus cells, both 1-M6 wild-type and UGH-101 callus were grown on culture medium containing 2 mM hydroxylamine. Both cell lines grew fairly well on hydroxylamine suggesting that the specific toxicity of glycine hydroxamate toward wild type cell lines was not due to hydroxylamine formation. When wild-type callus (1-M6) was grown on medium containing 2 mM hydroxylamine for 48 h, increases were observed in the intracellular glycine and alanine concentrations (Table VI), but the increases were not as large as those observed with 2 mM glycine hydroxamate (Table I).

To test whether glycine hydroxamate toxicity was due to a nonspecific hydroxamic acid effect, growth tests were made on media containing DL-alanine hydroxamate, DL-serine hydroxamate, or L-lysine hydroxamate. These analogs were for the most part nontoxic at a concentration of 2 mM.

Plants Regenerated from Glycine Hydroxamate-Resistant Cell Lines. Five of the seven glycine hydroxamate-resistant cell lines (UGH-20, UGH-21 UGH-102, UGH-103, UGH-105) have been regenerated into plants. At least one plant from each of these groups was fertile, and seeds resulting from self-pollination were planted. In the plants regenerated directly from resistant callus cells and in their seedling progeny, the free amino acid concentrations were not higher than those of plants regenerated from wild-type tissue or their seedlings (data not shown). Therefore, the high intracellular amino acid concentrations of the resistant callus tissue were not maintained upon regeneration of plants from these tissues.

Explants were made from most of the plants regenerated from callus tissue and more recently from seedlings of plants which were fertile. After growth for 2 months, callus produced from explants was tested for resistance to glycine hydroxamate toxicity. Some of the callus lines tested to date retained their resistance to the inhibitor; others were not resistant. Nine plants regenerated from UGH-21 as well as two seedlings from a UGH-21 plant yielded only sensitive callus. Callus cultures from three of four plants regenerated from UGH-20 were sensitive. Of the four regenerated plants of UGH-105 which were tested, three were sensitive and one was resistant. Two seedlings from the resistant plant were tested and callus from these plants also was resistant to glycine hydroxamate. One of the four regenerated plants of UGH-102 was resistant in callus tests, but callus from three seedlings from this plant grew only slowly on glycine hydroxamate-containing medium. These seedlings were scored as sensitive, but must be further tested. In callus tests of four seedlings derived from an untested UGH-103 plant, two were sensitive, one resistant, and one intermediate in response. Further tests with larger numbers of seedlings are necessary before the degree of stability and inheritance of this trait can be discerned.

DISCUSSION

A number of factors have been ruled out as effectors of the resistance of the UGH cell lines to glycine hydroxamate. In none of the lines is access of the inhibitor to cellular sites of inhibition limited, either by decreasing the rate of uptake or by increasing the rate of glycine hydroxamate breakdown. Our evidence also indicates that production of hydroxylamine is not an important factor in glycine hydroxamate resistance. Hydroxylamine is presumably formed during the slow hydrolysis of glycine hydroxamate to glycine under standard growth conditions (13), and is

known to act as a carbonyl reagent, inhibiting pyridoxal phosphate-containing enzymes (8). The possibility that hydroxylamine was the inhibitory compound was considered, but the evidence does not support this hypothesis. Glutamate:glyoxylate aminotransferase, serine:glyoxylate aminotransferase, and glycine decarboxylase are all pyridoxal phosphate-containing enzymes susceptible to carbonyl reagents, but only the decarboxylase is inhibited when cells are incubated on 2 mM glycine hydroxamate and fed radioactive glycolate or glyoxylate (13). There was no difference in the response of wild-type and resistant cell lines to growth in the presence of 2 mM hydroxylamine, and supplementation of the medium with 2 mM alanine hydroxamate, serine hydroxamate, or lysine hydroxamate, which are all potential sources of hydroxylamine, was not as inhibitory to callus growth as glycine hydroxamate supplementation. Thus, for these lines we have eliminated from consideration the most obvious factors involving the metabolism of the inhibitor itself rather than with the metabolism of glycine.

Particular attention was focused on the role of glycine hydroxamate as a competitive inhibitor of glycine decarboxylation (13). Metabolism of [^{14}C]glycine was strikingly altered in the presence of glycine hydroxamate both in wild-type and resistant cells. However, kinetic constants (V_{max}, K_m, and K_i) of glycine decarboxylase were not altered in any manner which could account for resistance in the UGH cell lines. Altered glycine decarboxylase is not the effector of resistance in these lines. Other glycine utilization enzymes, such as glycyl-tRNA synthetase (cf. Ref 16 for effect of serine hydroxamate on seryl-tRNA synthetase) or δ-aminolevulinic acid synthetase (10) were not examined.

By analogy with other amino acid analog-resistant cell lines, high levels of glycine were anticipated for at least some of the glycine hydroxamate-resistant lines. Therefore, intracellular amino acid concentrations were determined for wild-type and resistant lines in the presence and absence of glycine hydroxamate. The observed increase in the intracellular glycine concentration in the presence of the inhibitor (Table I) is consistent with the demonstration that glycine hydroxamate is a competitive inhibitor of the glycine decarboxylase reaction (13). The simultaneous increase in intracellular alanine concentration suggests that glycine hydroxamate functions as an alanine analog as well. The metabolism of [U-^{14}C]alanine in callus tissue was not altered by the presence of glycine hydroxamate (Table V). The inhibitor may act on a quantitatively minor use of alanine such as the activation of alanine by alanyl-tRNA synthetase, or it may increase alanine concentrations by increasing the rate of alanine synthesis in the cells.

The most striking difference observed between the glycine hydroxamate-resistant cell lines and unselected cell lines was the elevated intracellular concentrations of glycine, alanine, and other amino acids in the resistant cultures (Table II). Resistance to glycine hydroxamate may provide a special case requiring elevation of amino acids in general. If glycine hydroxamate acts as an analog of both glycine and alanine, and if glycine and alanine biosynthesis are not coordinately regulated, the simplest change which effects the concurrent increases in these amino acids may be one which results in increases in all amino acids. Alternatively, a more general mechanism may be involved. In selections for resistance to amino acid analogs in other plant cell systems elevation of the analogous amino acid has been reported (6, 7, 18–20). In instances where total amino acid content was reported, increases in some other (nonanalogous) amino acids were also observed (7, 20). The simultaneous elevation of other amino acid concentrations may be the only cellular response which avoids toxicity resulting from single amino acid increases. This toxicity is implicated in studies of single amino acid supplementation (2, 5, 9) and has been ascribed to interference with the regulation of nitrate assimilation in the cells (2). The resistant lines with elevated amino acid concentrations are resistant not only to glycine hydroxamate, but also to supplementation of the growth medium with 10 mM glycine or alanine.

The observations that resistant cell lines grown in the dark have free amino acid levels more nearly like those of wild-type cells and do not grow in the presence of glycine hydroxamate (Table VI) support the correlation between resistance and high amino acid concentration. These growth experiments imply that the observed amino acid increases are light-dependent.

Several aspects of our results may be explained by assuming multiple pools of glycine and alanine. Increases in glycine and alanine concentrations observed in the presence of glycine hydroxamate (Tables I and III) were not accompanied by increases in glycine (13) or alanine (Table V) uptake, whereas such increases observed as characteristic of resistant cell lines (Table II) were accompanied by increased uptake (Table V). Further, metabolism of exogenously supplied radioactive glycine or alanine was unaffected by the presence of these greatly increased intracellular concentrations. This suggests the existence of a metabolically active pool into which the radioactive glycine or alanine is incorporated and which may be large in the resistant cell lines, and also, an inactive pool which increases in the presence of glycine hydroxamate. The existence of multiple pools of glycine in wheat leaves has also been suggested by Kumarasinghe *et al.* (11).

Studies with regenerated plants from five lines and seedlings derived from them have not yet demonstrated the pattern of inheritance of the resistant trait. In two lines the resistance was not retained in the regenerated plant. In three lines some, but not all, of the regenerated plants yielded callus which was still resistant. Among the small number of seedlings tested, only in one case, a resistant plant of UGH-105, were all seedlings resistant. Thus, it is presently unclear whether the glycine hydroxamate-resistant trait under study is unstable during the course of differentiation, or the callus cultures represent a mixed population of cells which sort out during growth, differentiation, and reproduction. The high levels of amino acids found in callus were not found in leaves of regenerated plants or seedlings.

Although glycolate synthesis can be assayed in callus tissue (4), it would obviously be of greater interest to examine the glycolate pathway in leaves of regenerated plants. However, the similar amino acid concentrations found in both wild-type and resistant plants precluded the study of the effect of increased glycine levels on the magnitude and characteristics of the glycolate pathway of photorespiration in these plants.

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