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Altered Glycine Decarboxylation Inhibition in Isonicotinic Acid Hydrazide-Resistant Mutant Callus Lines and in Regenerated Plants and Seed Progeny

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ISRAEL ZELITCH AND MARY B. BERLYN
Department of Biochemistry and Genetics, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504

ABSTRACT

Isonicotinic acid hydrazide (INH), an inhibitor of the photorespiratory pathway blocking the conversion of glycine to serine and CO_2, has been used as a selective agent to obtain INH-resistant tobacco (*Nicotiana tabacum*) callus cells. Of 22 cell lines that were INH-resistant, none were different from wild-type cells in their ability to take up [^{3}H]INH or to oxidize INH to isonicotinic acid. In 7 of the 22 cell lines, INH resistance was associated with decreased inhibition of NAD-dependent glycine decarboxylation activity in isolated mitochondrial preparations. In the cell line that was most extensively investigated (I 24), this biochemical phenotype (exhibiting a 3-fold higher K_i with INH) was observed in leaf mitochondria of regenerated plants and of plants produced from them by self-fertilization. After crosses between resistant and sensitive plants, the decreased inhibition of glycine decarboxylation was observed among F_2 and backcross progeny only in those plants previously identified as INH-resistant by callus growth tests. In contrast, in siblings identified as INH-sensitive, glycine decarboxylation was inhibited by INH at the wild-type level. This demonstration of the transfer of an altered enzyme property from callus to regenerated plants and through seed progeny fulfills an important requirement for the use of somatic cell genetics to produce biochemical mutants of higher plants.

The glycolate pathway leads to the decarboxylation of glycine to produce serine and CO_2, and a large part of the rapidly released photorespiratory CO_2 of C_3 species is attributable to this reaction (11, 15, 20, 23, 28). The glycolate pathway is subject to metabolic regulation by glutamate, aspartate, P-enolpyruvate, or glyoxylate (17, 27). Treatment of leaf discs with a solution of glutamate or glyoxylate blocked glycolate synthesis and photorespiration, increased net photosynthesis (15, 16), and reversed the inhibition of photosynthesis by oxygen (14). Berlyn (2, 3) suggested that mutations affecting the synthesis, utilization, or accumulation of specific compounds related to the glycolate pathway could be important in clarifying the control mechanisms operating in the pathway and in producing mutants with regulated photorespiration. One mutant phenotype that could reflect such changes is resistance to INH[1] inhibition.

The inhibitor INH blocks the conversion of glycine to serine and CO_2 in *Chlorella* (18) and tobacco leaf segments (1), and inhibits photorespiration (10) and glycolate synthesis (27) in tobacco leaf discs. The glycine decarboxylation reaction is localized in the inner membrane of leaf mitochondria (13, 25), is active only in intact mitochondria (26), and occurs by the following schematic sequence:

$$NH_2CH_2{}^{*}COOH + THF \xrightarrow[NAD \rightarrow NADH]{\text{Pyridoxal-P}} {}^{*}CO_2 + NH_3 + \text{Methylene-THF}$$

$$NH_2CH_2{}^{*}COOH + \text{Methylene-THF} \xrightleftharpoons{\text{Pyridoxal-P}} HOCH_2CHNH_2{}^{*}COOH + THF$$

$$\text{NET: } 2\ NH_2CH_2{}^{*}COOH \xrightarrow[NAD \rightarrow NADH]{} HOCH_2CHNH_2{}^{*}COOH + {}^{*}CO_2 + NH_3$$

Pyridoxal-P is an essential coenzyme for the above sequence (19). INH reacts with the coenzyme in other pyridoxal-P enzymes in a time-dependent reaction to form the hydrazone (8), and thereby inhibits the enzymic activity. The rather specific effect of INH on photorespiring tissues suggests that INH attacks the glycine decarboxylation complex in preference to other pyridoxal-P-containing enzymes.

Mutants affecting the glycine-serine conversion reactions were sought by isolating tobacco callus lines resistant to toxic concentrations of INH, and the isolation and genetic characterization of INH-resistant mutants have recently been described (3). Conditionally lethal seedling mutants of *Arabidopsis* lacking serine hydroxymethyltransferase activity have also recently been reported (22). For the INH-resistant mutants, growth tests of explanted tissue on INH demonstrated that the INH-resistant characteristic was transmitted from cultured cells to regenerated plants and to seed progeny of several lines. Among the various other types of plant mutants which have been isolated from somatic cell culture (*cf.* 6), the identification of an altered enzyme property in seed progeny of regenerated plants has not been described. It is shown here that in 7 of the 22 INH-resistant tobacco cell lines, resistance is associated with a decrease in the extent of INH inhibition of the glycine decarboxylation reaction in mitochondria from callus, and in one of these cell lines (I 24) this biochemical phenotype is observed in regenerated plants and in seed progeny. After crossing with wild type, this decreased inhibition of glycine decarboxylation is found among F_2 and backcross progeny only in individuals previously identified as resistant to INH by callus growth tests.

MATERIALS AND METHODS

Growth and Selection of Cultures and Regeneration of Plants. Callus was obtained from explants of haploid plants of *Nicotiana tabacum* L., var. John Williams Broadleaf, and was grown on the LS-2 agar medium as described by Berlyn (3). After UV irradiation, selections were made for growth and survival on medium containing 1 and 2 mM INH, and the 20 lines that survived retests

[1] Abbreviations: INH, isonicotinic acid hydrazide; INA, isonicotinic acid; THF, tetrahydrofolic acid.

and were judged stably resistant in comparison to wild type cells were maintained; the regeneration of plants and the testing of explanted tissue from regenerated plants for INH resistance has been reported previously (3). Callus lines examined in this study include two lines not included in these earlier quantitative growth tests. Wild-type plants used to prepare mitochondrial extracts originated from plants regenerated from untreated, INH-sensitive callus.

Uptake and Metabolism of [^{3}H]INH by Callus Tissues. In short-term experiments, no longer than 120 min, 0.8 ml of 2.0 mM [^{3}H]INH was placed in large (75-ml) Warburg flasks fitted with a Miracloth disc on the bottom of the flask. Callus tissue, about 1 g, was fragmented into 5-mm pieces and spread over the moistened Miracloth. Incubation was carried out at 30°C with shaking, and at the end of the experiment the tissue was transferred with water to a Büchner funnel fitted with a Whatman 42 filter paper. The tissue was washed twice with a 10-ml portion of water, which was removed after 15 s by applying a vacuum. The tissue was then washed four times in a similar manner with 10-ml portions of nonradioactive 2 mM INH, killed by adding it to boiling 20% ethanol, and boiling for several min. The cells were homogenized and the homogenate was brought to a volume of 25 ml. After removal of a sample for determination of radioactivity, the homogenate was centrifuged and the residue washed twice with water to obtain the soluble extract used for the determination of the radioactive products.

[^{3}H]INH and [^{3}H]INA, its only detectable metabolite, were separated and isolated by three different methods. Most frequently the extract was passed through a column (0.7 × 6 cm) of Dowex-1 acetate (Bio-Rad), and INH was recovered in the water eluate; INA was then obtained by elution with 10 ml 0.1 N HCl. A second method involved use of high voltage electrophoresis on Whatman 3MM paper for 30 min at 3,000 v and 1°C in a formic acid:acetic acid:water (12:48:340, v/v/v) system at pH 1.9. Samples of 0.1 μmol nonradioactive INH and INA were added to the origin, and after electrophoresis and drying, their location could be detected as dark spots (about 14 and 7.5 cm from the origin, respectively) under UV light (254 nm). INH and INA were also separated by descending paper chromatography for 18 h using 1-propanol: water (4:1, v/v) as solvent (7). The R_F for INH was 0.69 and for INA was 0.39 by this method. Radioactivity was determined by placing sections of the paper into vials containing 10 ml of scintillation counting solution and 0.2 ml water.

In long-term experiments lasting 14 d, 0.5 mM [^{3}H]INH was filter-sterilized and added to the normal agar growth medium in small Petri plates containing about 10 ml medium. The inoculum contained 0.8 to 1.5 g fresh weight of callus tissue. After 14 d, the increase in fresh weight was determined, the cells were killed in boiling 20% ethanol, and the radioactivity in INH and INA was determined in extracts by the paper electrophoresis method described above. All appreciable radioactivity on the paper was found in the regions containing INH and INA.

Mitochondrial Preparations from Callus Tissue and Leaves. The method used for obtaining mitochondrial preparations for the assay of glycine decarboxylation activity was based on those used previously (4, 12). Callus tissue (2–3 g) grown for 3 to 6 weeks was ground for 75 s in cold grinding medium containing mercaptoethanol at pH 7.0 or 7.7, and after filtration through Miracloth and collection by centrifugation for 10 min at 38,000*g*, the mitochondria-containing pellet was suspended in 4 ml assay medium at pH 7.2. Grinding wild type cells for 105 s instead of the usual 75 s had little effect on the final specific activity of the mitochondrial activity.

For leaf mitochondrial preparations, washed tobacco leaves of 1.5 to 4.0 g fresh weight were used. The leaves were excised from the plants and stored for 1 h in darkness with their bases in water. The laminae (totaling 1.5–3.0 g/preparation) were ground for 75 s in an ice-cold mortar with 1.5 g sand, using 30 ml grinding medium at pH 7.7. As with callus, increasing the time of grinding of the leaf during the preparation of the extracts had little effect on the specific activity. However, a ratio of grinding solution to lamina fresh weight of at least 10:1 was essential for obtaining stable preparations. The ground tissue was passed through eight layers of cheesecloth on a Büchner funnel, and the filtrate was centrifuged at 1,000*g* for 10 min to remove cell debris and chloroplasts. The mitochondrial fraction was collected by centrifugation at 38,000*g* for 10 min. After allowing the tube to drain for 30 s and wiping the walls of the tube, the residue was converted to a homogeneous paste by gentle stirring with a glass rod, and was suspended in 7.5 ml assay medium. Protein content was determined by the coomassie blue staining method (5) using bovine serum albumin as a standard.

Enzyme Assays. Mitochondrial preparations from callus tissue and leaves were used in the assay of glycine decarboxylation activity (4, 12). The reactions were carried out in Warburg flasks with sidearms and center wells. The main compartment contained mitochondria and additional assay medium to make the total 0.80 ml (containing about 100 μg protein with callus preparations and 200 μg protein with leaf preparations), INH, and NAD (1.0 μmol). Exogenous pyridoxal-P was not added in any experiments because of its reactivity with INH; hence, only levels endogenous to the enzyme complex were present. The sidearm contained [1-^{14}C]-glycine (20 μmol), about 800,000 dpm, and in some experiments with leaf mitochondria the NAD was also placed in the sidearm. The final volume was 1.0 ml. The flasks were placed in an ice bath. At 30-s intervals, the flasks were transferred to a 30°C water bath and preincubated for the times indicated in each experiment. The contents of the sidearm were then mixed with those in the main compartment to initiate the reaction. The $^{14}CO_2$ released was trapped on a paper wick in the center well moistened with 125 μmol ethanolamine. The reactions were terminated after 10, 30, or 60 min by addition of 0.15 ml of 0.8 N H_2SO_4. Thirty min after the reaction was completed the wicks were transferred to scintillation vials containing 10 ml counting solution and 0.1 ml Protosol (New England Nuclear). Addition of Protosol to the scintillation counting fluid increased the recovery of known quantities of $^{14}CO_2$ from 81 to 97% and the radioactivity in the vials remained constant for at least 48 h.

The absence of NAD in the reaction mixture decreased the activity at least 50% in both callus and leaf preparations (12). When NAD was added with glycine to initiate the reaction in leaf preparations, the activity was 30% higher than in assays which included NAD in the preincubation mixture. However, the location and timing of the added NAD had little effect on the extent of INH inhibition. For callus preparations, the activity was the same whether NAD was added to the main compartment during preincubation or was tipped in with glycine to initiate the reaction.

The specific activities of leaf preparations were comparable to those obtained with purified mitochondria from spinach (9, 13, 26) or from tobacco mitochondria purified by sucrose gradient centrifugation (preparation courtesy of R. B. Sparks). Any assay in which the specific activity of the preparation was anomolously low (less than 1.0 μmol CO_2/mg protein·h for leaves and less than 0.10 μmol CO_2/mg protein·h for callus) was not included in the inhibition studies, and experiments in which abnormally low levels of inhibition by INH were observed for all preparations (including wild types) were discounted in their entirety.

Cyt *c* oxidase was at times used as a standard for mitochondrial activity in our particulate preparations and was assayed by the method of Wharton and Tzagaloff (24). Ten and twenty μl of particulate preparation were added to 0.5 ml 100 mM phosphate buffer (pH 7.0) in 1 ml cuvettes at 30°C. After 1 min equilibration, 20 μl 1 mM reduced Cyt *c* (gift of A. L. Lawyer) was added and the rate of oxidation was followed for 4 min as change in *A* at 550

nm.

Chemicals. The [1-^{14}C]glycine (Amersham) was purified by electrophoresis to remove a contaminant that produced troublesome quantities of $^{14}CO_2$ nonenzymically (12). The [^{3}H]INH (Amersham-Searle) was eluted with water through a column of Dowex-1 acetate (0.7 × 6 cm) prior to use. INH and INA were obtained from Eastman Organic Chem. Co. and Aldrich Chemical Co., respectively.

RESULTS

Uptake and Metabolism of [^{3}H]INH by Callus Tissue. The kinetics of 2 mM INH uptake and metabolism by wild-type and INH-resistant (cell line I 1) callus tissue are demonstrated in Figure 1. The rates of INH uptake and INA formation on a fresh weight basis were similar in both types of cells. In a series of experiments in which 2 mM [^{3}H]INH was supplied for 60 min, in nine determinations the mean (± SD) rate of uptake into wild-type cells was 0.22 (± 0.04) μmol/g fresh weight and the range was 0.16 to 0.30. The uptake rate for the 22 INH-resistant cell lines ranged from 0.16 to 0.31 μmol/g fresh weight, indicating that uptake in these resistant cells was similar to that of wild type. In these experiments the percentage of the ^{3}H found in INA in wild-type callus averaged 18 (SD 2.4; range 13 to 21) while the values for INH-resistant callus ranged from 10 to 20%. Thus, in short-term experiments, neither the rates of INH uptake nor metabolism to INA in INH-resistant cell lines varied greatly from wild type values.

In long-term experiments in which 0.5 mM [^{3}H]INH was added to growth medium for 14 d, the ratio of the final fresh weight to initial fresh weight in five wild type cultures averaged 1.21 (SD 0.32) and the ratio for INH-resistant cultures was generally greater, ranging from 1.30 to 2.55. The total [^{3}H]INH uptake for the 14 d ranged from 1.60 to 2.33 (mean 2.07; SD 0.30) μmol/g final fresh weight for the wild-type cells and from 0.93 to 2.05 for resistant cell lines. At the end of 14 d, 78% (SD 1.8; range 76 to 79) of the ^{3}H was present as INA in the wild-type tissue, while this value ranged from 63 to 84% in the resistant cells. Thus, long-term as well as short-term experiments with [^{3}H]INH supplied to callus tissue indicated that INH resistance in these cell lines was not caused by decreased ability of resistant cells to take up INH or by accelerated breakdown of INH once it was present.

Assay of Glycine Decarboxylation Activity and INH Inhibition in Callus Mitochondria. Since mitochondrial preparations from different callus tissue were to be compared, a number of experiments were carried out to evaluate the limitations and sources of variability attributable to the assay and to standardize conditions for measuring activity and inhibition (see also "Materials and Methods"). Since resistance to INH inhibition of the reaction, in contrast to growth inhibition, could be caused by the breakdown of INH during the reaction, recoveries of [^{3}H]INH following glycine decarboxylation assays of callus mitochondrial preparations were determined. At the conclusion of an experiment with 0.25 mM INH, recovery of [^{3}H]INH was the same for INH-resistant (I 18) mitochondria inhibited 31% as for wild-type mitochondria inhibited 50% or for an incubation mixture lacking mitochondria.

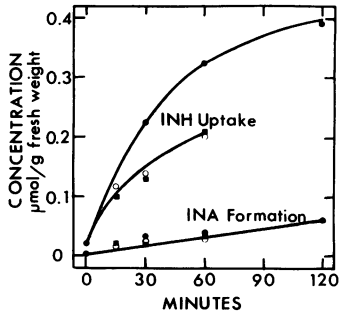

FIG. 1. Rate of [^{3}H]INH uptake and INA formation by wild type and INH-resistant tobacco callus tissue. The [^{3}H]INH, 2 mM containing 14.7 × 10^6 dpm/μmol, was supplied as described under "Materials and Methods"; (○), wild type M6; (●), wild type M5; (■) resistant I 1. The INA concentration was determined on the assumption that its specific radioactivity was the same as that of the [^{3}H]INH supplied. At the end of 60 min, the amount of [^{3}H]INA, as determined following electrophoresis, paper chromatography, or anion exchange chromatography, was 15.6, 14.4, and 14.9%, respectively, of the total ^{3}H taken up in M6 callus and 18.3, 15.5, and 16.7%, respectively, of the total ^{3}H in I 1 callus.

The metabolic product of INH in callus tissue, INA, at a concentration of 1 mM, inhibited the glycine decarboxylation reaction less than 10%. In mitochondrial preparations from callus stored for 3 h as a suspension in assay medium in an ice bath, there was no detectable change in activity or INH inhibition.

Because of the relatively low activity of callus mitochondrial preparations, a 60-min reaction time was required. The preincubation time affected the activity as well as the extent of INH inhibition. For example, extending preincubation from 15 to 45 min with 0.25 mM INH increased inhibition from 34 to 44%. The standard assay consisted of 15 min preincubation and 60 min reaction. Under these conditions, with 100 μg mitochondrial protein, the reaction was constant with time for at least 60 min, the K_m (glycine) was about 5 mM (12), and INH acted as a classical noncompetitive inhibitor in wild-type and INH-resistant preparations.

A comparison of the activities and INH inhibition of mitochondrial preparations from cultures after 17- or 41-d passages failed to reveal any differences in either specific activity or INH sensitivity due to age of culture. To establish that an observed decrease in inhibition of glycine decarboxylation by INH in a preparation was not caused by a soluble inhibitor in the extract or by instability of the activity, samples from preparations with different levels of inhibition were combined and assayed together. The mitochondrial preparation from INH-sensitive cell line I 22 had a specific activity (100 μg protein) of 0.36 μmol, CO_2/mg protein·h and was inhibited 64% by 0.25 mM INH. Under the same conditions, a mitochondrial preparation from line I 24 had a specific activity of 0.68 and was inhibited only 36% by INH. Assay of a mixture of 50 μg protein of each of the preparations gave a specific activity of 0.56 μmol $^{14}CO_2$/mg protein·h and an INH inhibition of 43%, in agreement with expected values of 0.52 μmol/mg·h and 46% inhibition.

Comparison of INH Inhibition of Glycine Decarboxylation by Mitochondria from Wild-Type and INH-Resistant Callus. A number of assays were carried out on preparations from callus of wild type and of the 22 INH-resistant cell lines to determine whether resistance in a number of the lines could be attributed to greater INH resistance of the glycine decarboxylation activity (Table I).

Although the specific activities for eight of the INH resistant lines in Table I were significantly different from wild type, the range for all values was only 3-fold. When the specific activities of glycine decarboxylation were compared to cytochrome oxidase activity (as a marker of general mitochondrial activity) in the same preparations, no significant differences were observed in the ratio of decarboxylation/cytochrome oxidase-activity between wild type and any of the resistant lines (data not shown). Thus, it is unlikely that the differences in specific activity shown in Table I can account for resistance to INH in any of these lines.

Under the standard assay conditions given in Table I, wild-type preparations showed a mean inhibition in the presence of 0.25 mM INH of 55%, and a t test showed that seven of the resistant cell lines had significantly less inhibition. Thus, in these lines (I 5, I 7, I 12, I 18, I 21, I24, and I 40) INH resistance is observed not only as a growth response, but also as an alteration in the properties of the enzymes responsible for glycine decarboxylation. Figure 2

Table I. *Effect of INH on Glycine Decarboxylation Activity in Mitochondrial Preparations from Cell Lines of Wild Type and INH-Resistant Tobacco Callus*

INH was present with the mitochondrial preparations (about 100 μg protein) for 15 min at 30°C before the [1-^{14}C]glycine was added to the reaction mixture for 60 min. In each assay, preparations from wild type and INH-resistant callus were assayed in duplicate flasks. The means ± SD of activities in the absence of INH and of the inhibition percentage are shown and the number of preparations assayed indicated in parentheses. The mean for each INH-resistant cell line was compared with the wild types by a *t* test and significant differences are indicated by asterisks.

Cell Line	Mean Glycine Decarboxylation Activity ± SD without INH	Number of Preparations Assayed	Mean Inhibition ± SD by 0.25 mM INH
	μmol/mg protein·h		%
Wild types	0.28 ± 0.09	(17)	55 ± 6.4
I 1	0.55 ± 0.05**	(2)	55 ± 4.2
I 4	0.26 ± 0.02	(2)	54 ± 0.7
I 5	0.52 ± 0.01**	(2)	35 ± 12**
I 6	0.64 ± 0.22**	(2)	50 ± 0
I 7	0.27 ± 0.07	(3)	40 ± 2.5**
I 9	0.21 ± 0.09	(2)	49 ± 7.1
I 10	0.32 ± 0.08	(2)	68 ± 3.5
I 11	0.23 ± 0.05	(3)	49 ± 18
I 12	0.40 ± 0.14	(2)	45 ± 2.8*
I 15	0.23	(1)	59
I 16	0.17 ± 0.08	(3)	48 ± 7.4
I 18	0.63 ± 0.16**	(6)	40 ± 2.1**
I 19	0.25 ± 0.09	(3)	51 ± 15
I 21	0.33 ± 0.13	(6)	40 ± 15*
I 22	0.46 ± 0.11**	(3)	53 ± 9.5
I 24	0.42 ± 0.21*	(7)	40 ± 9.6**
I 27	0.25 ± 0.06	(3)	49 ± 14
I 29	0.26 ± 0.02	(2)	47 ± 12
I 32	0.46 ± 0.08*	(2)	57 ± 2.8
I 35	0.39 ± 0.06	(2)	48 ± 7.8
I 39	0.36 ± 0.05	(2)	49 ± 11
I 40	0.19 ± 0.05*	(6)	40 ± 12**

* Significantly different from wild type at the 5% level.
** Significant at the 1% level.

compares the INH inhibition at several INH concentrations for preparations from wild type callus and those from I 18 and I 24 callus assayed simultaneously. The wild type preparations had an estimated K_i of 0.20 mM INH, while the K_i was 0.38 mM for I 18 and 0.75 for I 24 mitochondria.

Mature plants had been regenerated from 9 lines of INH-resistant callus (3), including cell lines I 5, I 21, and I 24, listed above. Because of the level of significance of the difference in mitochondrial inhibition and the availability of seed progeny, line I 24 was selected for further investigation to determine whether the enzymic phenotype was transmitted and could be observed as altered sensitivity of mitochondrial activity in leaves from regenerated plants and seed progeny.

Assay of Glycine Decarboxylation Activity and INH Inhibition in Leaf Mitochondria. As with callus preparations, a number of experiments were carried out to determine the most suitable conditions for the assay of glycine decarboxylation and its inhibition by INH in isolated leaf mitochondria. Leaf mitochondria are known to be unstable especially at low protein concentration (13). Levels of 50 μg protein frequently failed to show linear reaction rates with time, whereas concentrations above 100 μg maintained linear rates for at least 30 min and the activity was proportional to mitochondrial concentration. A concentration of 200 μg protein was usually employed in assays with leaf preparations. The specific activity of glycine decarboxylation was the same in experiments utilizing 100 or 200 μg protein, and was not changed by increasing the grinding time to 2 min. The K_m (glycine) was about 3.0 mM with leaf mitochondria.

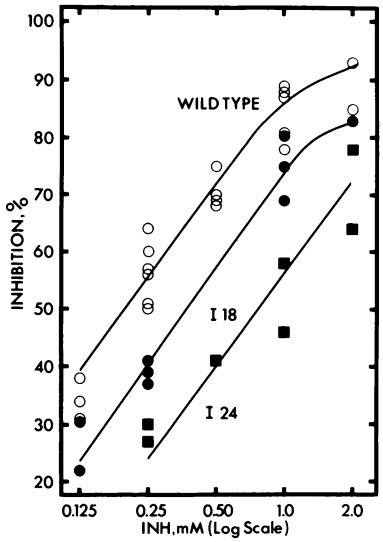

FIG. 2. Effect of INH concentration on glycine decarboxylation by mitochondrial preparations from wild type, I 18, and I 24 tobacco callus tissue. The reactions were carried out for 60 min at 30°C following 15 min preincubation of the mitochondrial preparations with INH.

Two different assay conditions were used with respect to preincubation and reaction time: 15 min preincubation with 10 min reaction, and 30 min preincubation with 30 min reaction. Preincubation times of 45 min greatly decreased the activity, while there was little difference between 15 and 30 min. The 30 min preincubation and reaction time increased the extent of INH inhibition and showed clearly noncompetitive kinetics.

To ensure that any observed INH resistance was not caused by the breakdown of INH during the reaction, recovery of INH after glycine decarboxylation assays of leaf mitochondrial preparations was determined for wild type and INH resistant (I 24) leaves supplied with 1.0 mM [^{3}H]INH. As with callus preparations, the recovery of [^{3}H]INH at the end of the reaction was the same for I 24 mitochondria as for wild type.

The size of the leaf taken had little influence on the glycine decarboxylation activity of isolated mitochondria when the leaf fresh weight was at least 1.5 g, but preparations from larger leaves, above 5 g fresh weight, gave preparations with somewhat less INH inhibition.

Comparison of INH Inhibition of Glycine Decarboxylation by Mitochondria from Leaves of Regenerated Plants, of Wild Type, and of I 24 Seed Progeny. Eight plants regenerated from callus of cell line I 24 were tested for ability of explanted tissue to grow in the presence of INH and all were classified as INH-resistant (3). Glycine decarboxylation activity of mitochondrial preparations was examined in callus from three of these plants and from plants regenerated from wild-type cell lines. Despite large standard deviations for the mean inhibition values in these assays, the lower level of inhibition by INH for the I 24-derived plant tissue was significantly different from wild-type inhibition for two of the three plants (Table II).

Callus resistance tests also demonstrated that in seedlings from selfed I 24 plants, the tissue was resistant, whereas wild-type seedling tissue was sensitive (3). Figure 3 shows the effect of INH concentration on the activity of mitochondrial preparations from leaves of plants obtained from selfing plant 40 (seed lot 78-64) and another I 24-derived plant, plant 78 (seed lot 77-44). In these experiments, carried out using a 15-min preincubation and 10-min

Table II. *Effect of INH Concentration on Glycine Decarboxylation Activity of Mitochondrial Preparations from Callus of Plants Regenerated from Wild Type and INH-Resistant (I 24) Cell Lines*

The mitochondria were isolated and assayed as in Table I. The means ± SD are indicated, and significance of the differences from wild type was determined by a *t* test.

Callus from Plant Type	Glycine Decarboxylation Activity ± SD	No. Preparations Assayed	Inhibition of Activity by INH		
			0.125 mM	0.25 mM	0.50 mM
	μmol CO_2/mg protein·h		%	%	%
Wild type	0.27 ± 0.09	9	42 ± 6.7	57 ± 8.2	71 ± 6.7
I 24 (plant 39)	0.26 ± 0.04	4	27 ± 4.7**	40 ± 7.1**	51 ± 9.7**
I 24 (plant 40)	0.31 ± 0.10	6	33 ± 8.8*	43 ± 7.8**	58 ± 7.9**
I 24 (plant 41)	0.31 ± 0.19	3	39 ± 5.0	52 ± 2.6	66 ± 4.9

* Significantly different from the mean of wild-type inhibition at the 5% level.
** At the 1% level.

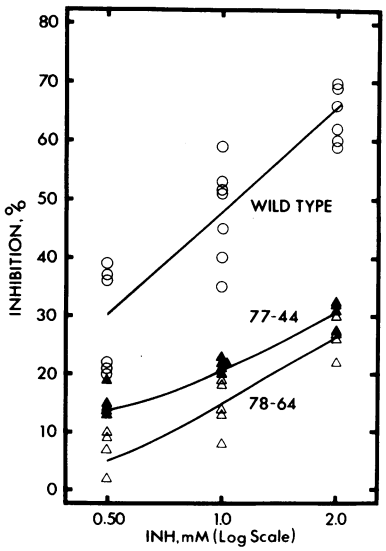

FIG. 3. Effect of INH concentration on glycine decarboxylation by mitochondrial preparations from leaves of wild type and progeny of I 24 plants. The reactions were carried out for 10 min at 30°C following 15 min preincubation of the mitochondrial preparations with INH. The INH-resistant I 24-derived plants are from progeny of plant 78 selfed (3 plants from seed lot 77-44) and of plant 40 selfed (4 plants from seed lot 78-64), which are further described in (3).

reaction time, the K_i estimated from the theoretical dissociation curve (K. R. Hanson, private communication) was 1.1 mM for wild-type leaves and 3.8 and 5.6 mM, for leaves from the plant 78 and plant 40 progenies, respectively. Thus, the transmission of the INH-resistant characteristic to seed progeny, previously demonstrated by callus growth tests, can be observed more directly as resistance to INH inhibition of glycine decarboxylation activity in mitochondrial preparations isolated from leaves of the plant.

Comparison of INH Inhibition of Glycine Decarboxylation and Callus Growth Tests in Plants of Wild Type, Hybrid, and Backcross Progenies with I 24. Although callus from F_1 hybrids between I 24 and wild type showed unusual growth responses, in that these cultures achieved resistant growth rates only after two full passages on INH (3), progeny of reciprocal backcrosses with wild type included segregants which showed clearly resistant callus growth responses, in numbers sufficient to suggest a dominant nuclear gene (M. Berlyn, unpublished data).

Assay of 13 preparations from four F_1 individuals from the cross between the I 24-derived plant 78 and wild type resulted in mean values for inhibition in the presence of 1 and 2 mM INH of 32 and 40%, respectively. Although the variability in the inhibitions observed was high (SD 21 and 24, respectively), differences from the mean wild-type inhibitions of 50% (SD 9.1) for 1 mM INH and 63% (SD 10.3) for 2 mM INH in this set of experiments were statistically significant. In four assays of leaf mitochondria from hybrids between wild type and another I 24 plant, plant 83, differences from wild-type levels of inhibition were not statistically significant, and progenies from these plants have not been examined further by the mitochondrial decarboxylation assays. The F_2 and backcross progenies which we have examined (see below) originate from hybrids between plant 78 and wild type.

Table III compares inhibition of glycine decarboxylation activity in leaves from representative resistant and sensitive individuals from F_2 (of plant 78/wild type hybrids) and backcross progeny with that of wild type individuals. For those individuals which scored as INH-sensitive in the callus growth tests, the INH inhibition of glycine decarboxylation activity was not significantly different from inhibition of wild type. Those individuals which were resistant in callus growth tests were also resistant to inhibition in these tests. At 1 mM INH, inhibition was less for all of the resistant plants and was in the range previously observed for resistant callus and leaves. Although the difference was not statistically significant for two of the four resistant plants at 1 mM INH (including one case of small sample size), the result was clearcut at the higher INH level. At 2 mM INH all inhibition values for the INH-resistant plants were lower than wild-type values and all differences were statistically significant. It seems clear that the alteration in mitochondrial enzyme activity that imparts INH resistance to glycine decarboxylation in callus tissue is transmitted to seed progeny of I 24 and of hybrids between I 24 and wild type.

DISCUSSION

The observed INH inhibition of the mitochondrial glycine decarboxylation activity in tobacco callus and leaves shows similarities to inhibition in other systems which results from formation of the hydrazone of pyridoxal-P. The inhibition is time-dependent, as is typical of such a reaction (8), and classical noncompetitive inhibition, which would be expected for this inhibitory mechanism, was observed in our standard assay when at least 60 min for the total preincubation and reaction times was used. INH is well known as an inhibitor of the tubercle bacillus, and there is some evidence that the formation of INA, an analog of NAD, may be responsible for toxicity in this organism (21). The main product of INH metabolism in tobacco callus was INA, but it was produced at similar rates by INH-resistant and INH-sensitive cell lines and added INA did not inhibit glycine decarboxylation in mitochondria. Therefore, it is unlikely that formation of INA is an important factor in inhibition of glycine decarboxylation and photorespiration in the plant systems investigated.

Of 22 INH-resistant cell lines isolated (3), an alteration in

Table III. *Comparison of Glycine Decarboxylation and INH Inhibition in Mitochondrial Preparations from Leaves of Wild Type and Representative F_2 and Backcross Progeny of I 24 Plants*

Preparations were obtained from leaves (1.5–3.0 g fresh wt) excised from shoots with flower buds. Control determinations on each preparation were made with 200 μg protein in triplicate, and assays of INH inhibition in duplicate. After 30 min preincubation at 30°C, the reaction was begun by addition of NAD and [1-^{14}C]glycine, and the reaction was terminated after 30 min. The means ± SD are shown, and the number of preparations assayed are given in parentheses. The mean inhibition for each plant was compared with the combined mean of the wild types (WT) by a *t* test and significant differences are indicated by asterisks.

Progeny Origin	Selected Individual from Progeny	Growth Response of Explant to INH	Glycine Decarboxylation Activity	No. of Preparations Assayed	Inhibition by INH	
					1.0 mM	2.0 mM
	plant no.		*μmol/mg protein·h*		%	
WT selfed	377	Sensitive	3.6 ± 1.0	(4)	48 ± 12	64 ± 7.5
	378	Sensitive	1.5 ± 0.4	(4)	57 ± 16	66 ± 17
F_2 (WT/I 24 selfed)	387	Sensitive	3.0 ± 1.1	(5)	48 ± 9.4	59 ± 8.2
	388	Resistant	5.0 ± 0.6	(2)	34 ± 0.7	42 ± 2.1*
	389	Resistant	3.5 ± 1.3	(4)	38 ± 2.4	49 ± 1.5*
Backcrosses (WT/I 24 × WT and reciprocal)	394	Resistant	4.4 ± 2.4	(4)	34 ± 3.9*	44 ± 6.8*
	397	Resistant	3.7 ± 2.1	(5)	34 ± 8.8*	43 ± 9.1**
	423	Sensitive	1.3 ± 0.2	(3)	62 ± 15	69 ± 11

* Significantly different from the mean of wild-type inhibition at the 5% level.
** At the 1% level.

inhibition of the glycine decarboxylation reaction was observed in seven of the lines. This change, and its mutational basis, has been documented in detail for one cell line, I 24. A number of control experiments have established that INH resistance of the enzyme activity was not a spurious effect of the growth conditions of callus or plants or an artifact of the enzyme assays. The activity described here is similar on a protein basis to that observed by others in tobacco (4), 12), or spinach mitochondria (9, 13, 26).

Our results suggest that the level of glycine decarboxylation inhibition by INH is sufficient to account for the observed inhibition of callus growth. The level of glycine decarboxylation activity observed in these callus studies was similar on a fresh weight basis to the amount of glycolate produced in the presence of glycolate oxidase inhibitor in previous work. On growth medium supplemented with 1 mM INH, wild-type callus growth is inhibited 70 to 90% during a 6-week passage (3). The experiments on INH uptake and metabolism indicated that the intracellular level of INH under these conditions is in the range of 0.4 mM, a concentration sufficient to inhibit callus glycine decarboxylation an estimated 55 to 65% (Table I and Fig. 2). At this INH concentration, glycine decarboxylation in resistant (I 24) mitochondria is inhibited about 25% and callus growth inhibition ranged from 0 to 50% (mean, 35%) for various I 24-derived plants. When the concentration is doubled, the inhibition of glycine decarboxylation in I 24 is greater than 50%, and large inhibition of callus growth is also observed in these resistant lines (unpublished data). These observations suggest that the reduction of that activity by 50% or more may be incompatible with vigorous callus growth. Accordingly, the decrease in INH sensitivity of the mitochondrial reaction in I 24 could account for the observed mitigation of callus growth inhibition by 1 mM INH in this line. The primary biochemical change in I 24 callus and plants that results in INH resistance is not known, but presumably a change has occurred that interferes with the interaction of INH with pyridoxal-P bound to the decarboxylation complex and results in the altered inhibition.

Differences in INH uptake or metabolism and differences in specific glycine decarboxylation activity were also examined as potential factors for conferring INH resistance to some cell lines. The results indicate that neither uptake nor metabolism of INH to INA could be responsible for INH resistance in any of the lines. Nor were large differences in specific activity between sensitive and resistant lines observed. Although some differences between lines were significant on a total protein basis, on a Cyt oxidase basis (as an approximation of enzymically active protein in the mitochondrial extract) differences were not statistically significant. Inhibition by INH could also be circumvented by high intracellular levels of pyridoxal-P, but preliminary examination of many of the INH-resistant cell lines has not clearly identified any lines with this phenotype (Berlyn, unpublished date).

If the basis for INH resistance in I 24 is, as postulated, a physical alteration in the glycine decarboxylation complex which does not allow INH to interfere effectively with the reaction, this type of mutation is unlikely to show the kinds of effects on the pathway that could result in regulation of photorespiration. This alteration should allow fairly normal functioning of the pathway in the presence of INH and would not be expected to result in altered patterns of accumulation or synthesis of pathway intermediates or products. If such mutants exist among the INH-resistant lines, it is more likely that they would be found among the remaining 15 lines, those which have overcome growth inhibition despite normal INH inhibition of the glycine decarboxylation reaction. It is in such lines that deleterious metabolic effects of INH inhibition may be overcome by altered regulation of glycolate or related pathways. Tests of alterations in pool sizes of metabolites of the glycolate pathway, assays for photorespiration and O_2 sensitivity of net photosynthesis, and measurements of rates of glycolate synthesis in INH-resistant and sensitive cell lines and plants have not yet been thoroughly carried out. Although the I 24-type mutants will not be excluded from our future examinations of glycolate accumulation and other photorespiratory indicators, we will look with special interest to plants derived from other cell lines.

These studies have followed a biochemical phenotype of a mutation produced in plant cells from culture to regenerated plants and selfed progeny and through hybrids and backcrosses

with wild-type plants. They have affirmed the potential for using somatic cell techniques to obtain characterizable biochemical mutants of higher plants.

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LITERATURE CITED

1. ASADA, K, K SAITO, S KITOH, Z KASAI 1956 Photosynthesis of glycine and serine in green plants. Plant Cell Physiol 6: 47–59
2. BERLYN MB 1978 A mutational approach to the study of photorespiration. *In* HW Siegelman, and G Hind, eds, Photosynthetic Carbon Assimilation. Plenum, New York, pp 153–164
3. BERLYN MB 1980 Isolation and characterization of isonicotinic acid hydrazide-resistant mutants of *Nicotiana tabacum*. Theor Appl Genet 58: 19–26
4. BIRD IF, MJ CORNELIUS, AJ KEYS, CP WHITTINGHAM 1972 Oxidation and phosphorylation associated with the conversion of glycine to serine. Phytochemistry 11: 1587–1594
5. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
6. CHALEFF RS 1981 Genetics of Higher Plants: Application of Cell Culture. Cambridge University Press, New York, pp 41–95
7. CUTHBERTSON WFJ, DM IRELAND, W WOLFF 1955 Detection and identification of some metabolites of isonicotinic acid hydrazide (isoniazid) in human urine. Biochem J 55: 699–671
8. DAVISON AN 1956 The mechanism of inhibition of decarboxylases by isonicotinyl hydrazide. Biochim Biophys Acta 19: 131–140
9. DOUCE R, AL MOORE, M NEUBERGER 1977 Isolation and oxidative properties of intact mitochondria isolated from spinach leaves. Plant Physiol 60: 625–628
10. GOLDSWORTHY A 1966 Experiments on the origin of CO_2 released by tobacco leaf segments in the light. Phytochemistry 5: 1013–1019
11. GRODZINSKI B 1978 Glyoxylate decarboxylation during photorespiration. Planta 144: 31–37
12. LAWYER AL, I ZELITCH 1978 Inhibition of glutamate:glyoxylate aminotransferase activity in tobacco leaves and callus by glycidate, an inhibitor of photorespiration. Plant Physiol 61: 242–247
13. MOORE AL, C JACKSON, B HALLIWELL, JE DENCH, DO HALL 1977 Intramitochondrial localisation of glycine decarboxylase in spinach leaves. Biochem Biophys Res Commun 78: 483–491
14. OLIVER DJ 1978 Effect of glyoxylate on the sensitivity of net photosynthesis to oxygen (the Warburg effect) in tobacco. Plant Physiol 62: 938–940
15. OLIVER DJ 1979 Mechanism of decarboxylation of glycine and glycolate by isolated soybean cells. Plant Physiol 64: 1048–1052
16. OLIVER DJ, I ZELITCH 1977 Increasing photosynthesis by inhibiting photorespiration with glyoxylate. Science 196: 1450–1451
17. OLIVER DJ, I ZELITCH 1977 Metabolic regulation of glycolate synthesis, photorespiration, and net photosynthesis in tobacco by L-glutamate. Plant Physiol 59: 688–694
18. PRITCHARD GG, CP WHITTINGHAM, WJ GRIFFIN 1963 The effect of isonicotinyl hydrazide on the photosynthetic incorporation of radioactive CO_2 into ethanol-soluble compounds of *Chlorella*. J Exp Bot 14: 281–289
19. RABINOWITZ JC 1959 Folic acid. *In* P Boyer, ed, The Enzymes, Vol 2. Academic Press, New York, pp 185–252
20. SERVAITES JC, WL OGREN 1977 Chemical inhibition of the glycolate pathway in soybean leaf cells. Plant Physiol 60: 461–466
21. SEYDEL J, K-J SCHAPER, E WEMPE, HP CORDES 1976 Mode of action and quantitative structure-activity correlations of tuberculostatic drugs of the isonicotinic acid hydrazide type. J Med Chem 19: 483–492
22. SOMERVILLE CR, WL OGREN 1981 Photorespiration-deficient mutants of *Arabidopsis thaliana* lacking mitochondrial serine transhydroxymethylase activity. Plant Physiol 67: 666–671
23. TOLBERT NE 1971 Microbodies-peroxisomes and glyoxysomes. Annu Rev Plant Physiol 22: 45–74
24. WHARTON DC, A TZAGALOFF 1967 Cytochrome oxidase from beef heart mitochondria. Methods Enzymol 10: 245–250
25. WOO KC 1979 Properties and intramitochondrial localization of serine hydroxymethyltransferase in leaves of higher plants. Plant Physiol 63: 783–787
26. WOO, KC, CB OSMOND 1976 Glycine decarboxylation in mitochondria isolated from spinach leaves. Aust J Plant Physiol 3: 771–785
27. ZELITCH I 1973 Alternate pathways of glycolate synthesis in tobacco and maize leaves in relation to rates of photorespiration. Plant Physiol 51: 299–305
28. ZELITCH I 1979 Photosynthesis and plant productivity. Chem Eng News 57: 28–48