Metabolic Fates of Gramine in Barley I: Mechanism of Incorporation of Gramine into Tryptophan in Barley Shoots

GEORGE A. DIGENIS

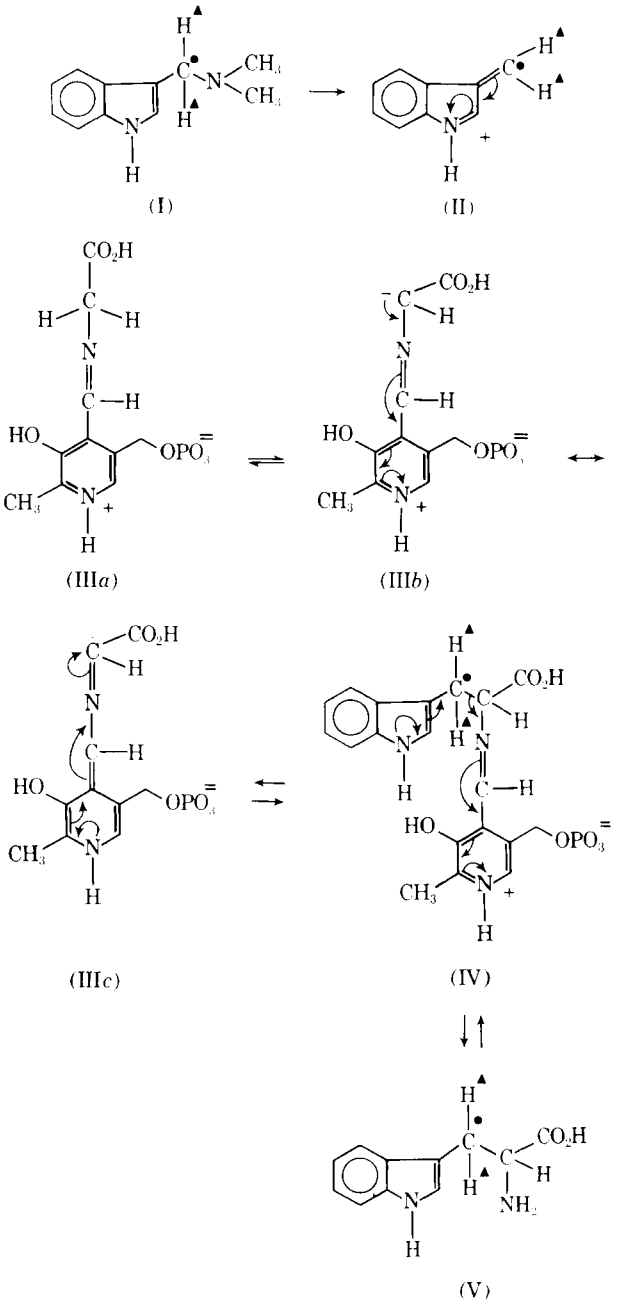
Abstract □ Gramine tagged on the methylene group of its side chain with ^{14}C and tritium was administered to 60-day-old barley shoots and the alkaloid allowed to be metabolized for 7 days. It was found that 0.84% of the radioactivity was incorporated into plant tryptophan, and the ratio of ^{14}C to tritium remained the same as that of the administered gramine. A mechanistic rationalization is proposed for the biotransformation.

Keyphrases □ Gramine in barley—metabolism □ Biotransformation—radiolabeled gramine □ Tryptophan—gramine metabolite □ Column chromatography—separation □ TLC—separation, identity □ UV spectrophotometry—identity □ Radioautography—analysis □ Liquid scintillation counting—radioactivity determination

Gramine (I) is one of the most extensively studied of all known alkaloids because of its indolealkylamine character and its extensive application in preparative indole chemistry. From the tracer work of Leete and Marion (1–4), it is now established that the alkaloid gramine (I) arises from tryptophan (V) in germinating barley (Scheme I). Based on other tracer experiments and the fact that tryptophan was formed in *Neurospora* by a condensation reaction between indole and L-serine (5, 6), Bowden and Marion (2, 3) suggested that a reversal of the above-mentioned tryptophan biosynthesis could possibly lead to indole and L-serine in barley. This suggested that the indole formed could subsequently react in a Mannich-type reaction with formaldehyde and dimethylamine to produce gramine (I). A mechanistic rationalization for the above reversal of tryptophan to indole was proposed by Kosower (7) and was apparently substantiated by the *in vitro* work of McEvoy-Bowe (8). However, Leete and Marion (4) were able to show that the bond between the 3-position of the indole nucleus and the side chain of tryptophan remained intact during the biosynthesis of gramine in barley, thus disproving the hypothesis of Bowden and Marion described above.

From the fact that the microbiological synthesis of tryptophan involves a pyridoxal (vitamin B6)-aided Michael process (9), Wenkert suggested that the biosynthesis of the alkaloid gramine probably involves a pyridoxal-induced retro-Michael degradation of tryptophan (10). Wenkert's hypothesis has been supported by the *in vitro* results of O'Donovan and Leete (1) who demonstrated that when a mixture of DL-tryptophan-β-^{14}C and DL-tryptophan-β-^{3}H (V) (Scheme I) was fed to barley seedlings, the gramine (I) formed was solely labeled on the methylene group of its side chain with ^{14}C and tritium in the same ratio as that of the administered tryptophan. Thus, they suggested that the methylene group of tryptophan's side chain retains its integrity and that a key intermediate in the biotransformation is a protonated 3-methyleneindolenine species (II) which could arise (along with IIIc) by a reverse Michael degradation of the Schiff base (IV) (Scheme I).

The final steps in gramine biosynthesis are believed to involve an attack of the electrophilic intermediate



Scheme I

(II) by ammonia[1] with subsequent *N*-methylation from methyl groups provided by methionine (11). This hypothesis has recently received considerable experimental support from the work of Mudd (12) and Gower and Leete (13). It is noteworthy to observe that the Wenkert hypothesis for the biological transformation of tryptophan to gramine involves a breakage of the bond connecting the α and β carbons of the tryptophan side chain which can be viewed as an oxidative amine fragmentation employing pyridoxal as an electron abstractor. A similar carbon-carbon bond breakage has been proposed by Daly and Witkop (15) for the presumed biosynthesis of *p*-hydroxybenzylamine from tyrosine in humans. The author has recently reported (14) an *in vitro* oxidative amine fragmentation with vitamin B6 which can be considered as a model in support of Wenkert's mechanism for the biosynthesis of gramine.

When gramine and its methiodide salt is heated or treated with base *in vitro*, it loses its amino function, yielding products that are believed to arise *via* the methyleneindolenine species (II) (16, 17). The lability of the dimethylamino group of gramine is one of the chief reasons for making the alkaloid chemically interesting. Since gramine appears in barley shoots on the third day after germination and remains in detectable quantities until the fiftieth day (18, 19), it appeared that this alkaloid could possibly lose its dimethylamino function to revert back to indolenine (II). The resulting methyleneindolenine (II), which is electrophilic in nature, could then be attacked by a nucleophile such as III*b* or III*c* to yield tryptophan (Scheme I). It is interesting to note that species III*a* is the pyridoxylidene derivative of glycine and that the mobility of the hydrogen atoms attached to the α-carbon of this amino acid is greatly increased when in its azomethine form (III*a*). Such species could lose a proton, and the resulting nucleophile could subsequently attack an electrophile (20). In this context, it has been shown that species III acts as a nucleophile in the biosynthesis of serine from glycine and formaldehyde (21, 22).

In view of this information it seemed probable that the alkaloid gramine could be metabolized in barley by following a pathway similar to that depicted in Scheme I. The author has tested this hypothesis by administering gramine labeled with ^{14}C in the carbon attached to its ring to 60- and 120-day-old barley which was shown to be free of the alkaloid. It was found that 0.84% of the alkaloid was converted into tryptophan (23). The present communication describes the experiments performed to obtain more information about the mechanism of the biotransformation of gramine into tryptophan in barley shoots.

EXPERIMENTAL

Preparation of Labeled Gramines—Gramine, tagged in the carbon attached to its indole ring, was synthesized from indole, dimethylamine, and ^{14}C-paraformaldehyde[2] by a procedure similar to that of Kuhn and Stein (24). The ^{14}C-gramine was purified by two sublimations at 90–95° and 1.0 mm. followed by one crystallization from *n*-hexane. The gramine obtained was shown to be pure by mixed melting point (131–132°) with authentic gramine, by UV spectroscopy, and by TLC in two solvents. Subsequent radioautography of the chromatograms exhibited only one radioactive spot.

Gramine, having both methylenic hydrogens of its side chain labeled with tritium, was synthesized and purified as described above, using tritiated paraformaldehyde.[3]

Administration of Tracers to Plants—A strain of Lebanese barley (Baladi 25, Telamara, attributed to *Hordeum distichon* L.) was grown in a botanical garden exposed to normal atmospheric conditions. The temperature ranged from 18–20° during the day and 14–16° at night in the months of March and April. It was previously shown that the shoots of the above strain of barley contain 0.37% w/w of gramine (on dried-herb basis) 17 days after germination (25), when analyzed by the method of Gower and Leete (13). A mixture of ^{14}C-tagged gramine and ^{3}H-tagged gramine was dissolved in 6 ml. of 0.05 *N* acetic acid and subsequently supplied equally to twelve 60-day-old barley shoots[4] in 5-ml. beakers by the wick-feeding technique of Leete (26). Water was added periodically to the beakers and wick assembly for the next 3 days to ensure complete absorption of the alkaloids by the plants. The shoots were harvested 7 days after the administration of the tracers by cutting them very close to the grain. The cuttings were dried at 50° for 24 hr. and subsequently defatted with *n*-hexane for 48 hr. The hexane extract was shown to contain very little radioactivity and was consequently discarded.

Hydrolysis of Plant Proteins and Isolation of Tryptophan—The defatted plant material (4.0 g.) was subjected to alkaline hydrolysis in 20% w/v NaOH (30 ml.) at 110° in a tube sealed under vacuum for 24 hr. The digested material was filtered, and the filtrate, after adjustment to pH = 6.0, was centrifuged. The brown supernatant solution was separated, reduced to 40 ml. (60°, reduced pressure), and filtered. The filtrate was further concentrated to 20 ml. *in vacuo*. This concentrate, containing the total plant amino acids, was adsorbed on a 1.5 × 40 cm. column of ion-exchange resin.[5] The column was washed with 350 ml. of distilled water, and the adsorbed amino acids were eluted with 300 ml. of 1.5 *N* ammonium hydroxide.[6] The eluate was subsequently evaporated to dryness under reduced pressure at 60°. The residue containing the total plant amino acids was taken up in 3 ml. of 0.5 *N* acetic acid and chromatographed through a column of ion-exchange resin[7] (2 × 60 cm.), using the method of Hirs *et al.* (27). The effluent was collected in 1-ml. fractions on an automatic fraction collector at a rate of 8 ml./hr. The chromatogram was performed at ambient room temperature (about 25°). Fractions 165–210 were combined[8] and evaporated to dryness under reduced pressure to yield 0.0112 g. of tryptophan[9] which was purified further by chromatography on five 20 × 20-cm. silica gel plates[10] (0.1 mm.) with *n*-butanol–acetic acid–water (80:20:20, v/v) as eluent. The purified tryptophan was dissolved in 10.0 ml. of 0.5 *N* acetic acid and subsequently counted for radioactivity by taking appropriate aliquots.

As a control when a sample of labeled gramine was mixed with

[1] Gross *et al.* (32) have recently shown that the amino nitrogen of gramine is not derived from the general nitrogen pool but rather from the amino nitrogen of tryptophan. This indicates that perhaps a more complex reaction mechanism takes place for the biosynthesis of gramine than that outlined in the earlier hypothesis.

[2] Supplied by The Radiochemical Centre, Amersham, Bucks, England.

[3] Purchased from The Radiochemical Centre, Bucks, England.

[4] Sixty-day-old barley was shown to contain no gramine when analyzed according to the procedure of Gower and Leete (13).

[5] Amberlite IR-120 (H^+ form), Rohm and Haas, Philadelphia, Pa.

[6] When a mixture of ^{14}C- and ^{3}H-tagged gramines was digested with 20% w/v NaOH, and chromatographed through ion-exchange resin, no radioactivity was detected in the ammoniacal washings of the resin.

[7] Dowex-1-acetate ×8 (200-mesh) purchased from Dow Chemical Co., Midland, Mich., in its chloride form, was converted to the 1-acetate form by the procedure of Hirs *et al.* (27).

[8] When a sample of known tryptophan (10 mg.) was chromatographed in a manner described above, the amino acid was recovered almost quantitatively in Fractions 185–200.

[9] The identity of tryptophan obtained from a duplicate determination was unequivocally shown by mixed melting point determination, recrystallization, to constant activity with carrier tryptophan, in three different solvents, and by two-dimensional paper chromatography, according to the author's previously published method (23).

[10] Chromagram, Eastman Kodak Co., Inc., Rochester, N. Y.

4.0 g. of inactive plant material and subjected to the same alkaline treatment as that described above, no radioactive tryptophan was isolated.

Radioactivity Measurements—All radioactivity measurements for both isotopes were performed in a liquid scintillation counter.[11] Tritiated compounds were assayed in the presence of ^{14}C-tagged substances by using the discriminator-ratio method[12] of Okita *et al.* (28). The labeled gramine samples were assayed in 18 ml. of a scintillation "cocktail" consisting of 0.4% w/v of 2,5-diphenyloxazole (PPO) as a primary scintillator and 0.005% w/v of dimethyl-1,4-bis-2-(5-phenyloxazole)-benzene (POPOP) as a secondary scintillator in toluene. The tryptophan samples were first dissolved in 20% tetramethylammonium hydroxide in methanol. After the scintillator liquid[13] was added, thioglycol 0.02 ml. was added to prevent phosphorescence.[14] All samples were counted twice for at least 5 min. Brand new glassware was used almost exclusively in the study with doubly labeled compounds. Radioautographs were taken with X-ray film.[15]

Recovery of Labeled Gramine from Barley—The isolation of tagged alkaloid was performed by a procedure similar to that of Gower and Leete (13). The identity of the recovered gramine was proved by TLC (silica gel[10] plates) in two solvents: (*a*) butanol–acetic acid–water (2:1:1, v/v), (*b*) methylacetate–isopropanol–25% v/v ammonia (45:35:20, v/v). The spots were visualized by spraying the chromatograms with Dragendorff's reagent.

Tritium Exchange Studies—^{3}H-gramine tagged at its methylenic hydrogens was stirred in 0.5 *N* acetic acid (at 25°) for 3 days. The solution was made alkaline (pH = 10) and extracted with chloroform. The aqueous layer was acidified and distilled, and the distillate assayed for tritium.

^{3}H-tryptophan was stirred in 0.5 *N* acetic acid for 3 days. The acetic acid was distilled and assayed for tritium.

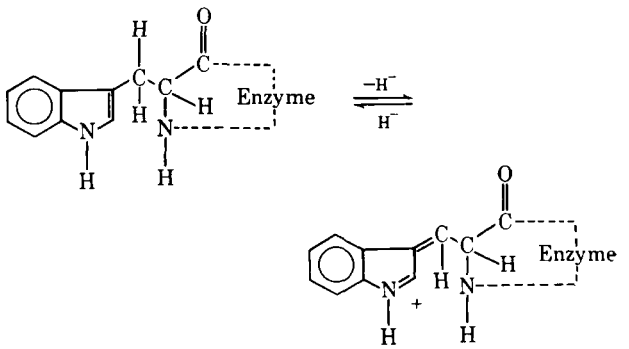
RESULTS AND DISCUSSION

Radioautographs of barley shoots taken every 24 hr. for 7 days after the administration of ^{14}C- and ^{3}H-gramine revealed that the alkaloid was well absorbed by the plants. It was also observed that the tracer was distributed throughout the entire length of the shoots. Very little radioactive gramine (3,350 d.p.m.)[16] was recovered from the beakers and threads that constituted the wicks by which the plants absorbed the labeled alkaloid. A negligible amount of radioactive gramine was recovered from twelve 60-day-old barley shoots which were administered gramine and harvested in a similar fashion. This observation suggested that the alkaloid was rapidly metabolized by 60-day-old plants.

In a representative experiment, 3.0 mg. of ^{14}C-gramine (7.16×10^5 d.p.m./mg.) and 50.0 mg. of ^{3}H-gramine (2.15×10^4 d.p.m./mg.) (ratio $^{14}C/^{3}H$ = 2/1) was administered to twelve 60-day-old barley shoots by the wick-feeding technique and the alkaloid allowed to be metabolized for 7 days. The defatted plant material (4.0 g.) was subjected to alkaline hydrolysis. The tryptophan fraction (0.0112 g.) was separated from the rest of the amino acids by column chromatography and found to contain 1.8×10^4 d.p.m. It was calculated that 0.84% gramine was incorporated into the plant's tryptophan fraction (on the basis of ^{14}C-incorporation), and the ratio of ^{14}C (1.8×10^4 d.p.m.) to tritium (9.0×10^3 d.p.m.) in the amino acid remained the same as that of the administered gramine (2:1).

The fact that the ratio of the two labels remained the same during the biotransformation of gramine to tryptophan, and the observation that none of the isolated amino acids was found to be radioactive, strongly support the intermediacy of methyleneindolenine (II) (Scheme I). It is interesting to note that if gramine had been fragmented to indole, formaldehyde, and dimethylamine, one would have expected incorporation of the label into the plant's serine.[17] Thus, it does not appear that the pathway by which gramine incorporates tryptophan in 60-day-old barley is the same as the well-known biogenetic route to this amino acid from indole and serine (9). Interestingly enough, however, tryptophan has been shown to be biosynthesized from indole and serine in 10-day-old barley seedlings (29).

The intermediacy of the methyleneindolenine species (II) is of particular interest since it has recently been found (30, 31) that a similar species is involved at the active site of yeast alcohol dehydrogenase during hydrogen transfer reactions as shown in Scheme II.



Scheme II

In an elegant study, Schellenberg was able to show that a hydride can be abstracted from the β-carbon of the side chain of a tryptophan moiety found at the active site of yeast alcohol dehydrogenase (30, 31). These data are indirectly supported by the findings of Leete (1) and by the author's exchange studies in which neither ^{3}H-gramine nor ^{3}H-tryptophan were found to exchange their tag with the medium. It is therefore concluded that the hydrogen atoms residing on the carbon attached to the indole nucleus of gramine or tryptophan cannot be removed easily as protons.

Experiments are currently in progress to establish unequivocally the position of the label in the isolated tryptophan. However, since the ratio of $^{14}C/^{3}H$ did not change during the biotransformation of gramine to tryptophan, one can safely postulate that the position of the tags remained unaltered. The author has recently demonstrated that another pathway followed by gramine in barley involves the oxidation of its side chain to indole-3-carbinol and indole-3-carboxylic acid. In both the above metabolites the label was found to be residing at the carbon attached to the indole nucleus. These findings, described in detail in the following paper, tend to support the suggested position of the tag in tryptophan arising from gramine.

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[11] Tri-Carb, model 314E, Packard Instrument Co., La Grange, Ill.

[12] ^{14}C-toluene and ^{3}H-toluene were used as standards to obtain the optimum settings of the photomultiplier voltage, discriminator window, and the amplifier gains, for the maximum counting efficiency of the two isotopes, respectively.

[13] This consisted of a 1:1 toluene–methanol mixture containing 0.4% diphenyloxazole (PPO) and 0.5% dimethyl-1,4-bis-2-(5-phenyloxazole) benzene (POPOP).

[14] Controls containing equal amounts of nonradioactive substances were prepared and used to obtain background counts. Similar controls to which a known quantity of standard ^{3}H-toluene had been added served as external standard.

[15] Royal Blue X-ray film, Eastman Kodak Co., Inc., Rochester, N. Y.

[16] This abbreviation will be used throughout the paper to denote the number of decompositions per minute.

[17] Serine has been shown to be biosynthesized from glycine and formaldehyde (21, 22).

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Metabolic Fates of Gramine in Barley II: Biotransformation of Gramine into Indole-3-carbinol and Indole-3-carboxylic Acid in Barley

GEORGE A. DIGENIS

Abstract □ When gramine, labeled in the methylenic side chain with both ^{14}C and tritium, was administered to 60-day-old barley shoots, it was biotransformed to indole-3-carbinol and indole-3-carboxylic acid. A mechanism involving an indolenine intermediate is proposed. The mechanism is reminiscent of the stepwise degradation of serotonin to 5-hydroxyindoleacetic acid in man and suggests that enzymes capable of biodegrading the side chain of indoles may be common to both the animal and plant kingdoms.

Keyphrases □ Gramine in barley—metabolism □ Biotransformation—radiolabeled gramine □ Metabolites, gramine—isolated, identified □ TLC—separation, identity □ Liquid scintillation counting—radioactivity determination

In a previous paper (1) the author has reported that when gramine, labeled in the methylenic side chain with both ^{14}C and tritium, was administered to growing 60-day-old barley shoots, 0.84% of the alkaloid was incorporated into the tryptophan fraction of the plant. The ratio of $^{14}C/^{3}H$ in the isolated tryptophan was found to be the same as that in the administered gramine suggesting that an indolenine intermediate was involved.

In another series of experiments it was found that when ^{14}C-gramine was administered to 60-day-old excised shoots of barley in the dark, 10% of the radioactivity passed into the plant's expired CO_2 and 0.4% into its tryptophan fraction (2). These results suggested that in excised barley shoots the methylene carbon of the alkaloid side chain undergoes biodegradation to carbon dioxide. The present communication describes the techniques used to isolate and identify the intermediates in this degradative pathway.

EXPERIMENTAL

Administration of Tracers to Barley Shoots—Twelve shoots of a barley strain attributed to *Hordeum distichon* L. were allowed to grow under normal atmospheric conditions as described previously (1). When the seedlings were 60 days old, they were cut very close to the ground, washed, and placed in a beaker containing 10 ml. of a solution consisting of 3.0 mg. of ^{14}C-gramine (7.16×10^5 d.p.m./mg.) and 50.0 mg. of ^{3}H-gramine (2.15×10^4 d.p.m./mg.). The ^{14}C and tritium labels were introduced at the methylenic carbon and protons of the side chain of the alkaloid (I), using methods which have been previously reported (1). The shoots were covered with a black cloth and kept in the dark at ambient room temperature (25°) for 8 days. During this time 5 ml. of tap water was added every 12 hr. to the beaker containing the plants. The seedlings