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ACKNOWLEDGMENTS AND ADDRESSES

Received May 22, 1968, from the *Departments of Materia Medica and Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication October 1, 1968.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported in part by a grant from the University of Kentucky Research Foundation and by grant 1R01 AM12971-01 from the National Institute of Health, U. S. Public Health Service, Bethesda, Md. for which the author is grateful.

The receipt of a Lederle Pharmacy Faculty Award is gratefully acknowledged.

Thanks are extended to Mr. Bahjat Faraj for technical assistance at an early stage of the study and to Dr. L. Dittert for useful discussions.

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

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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

were then dried at 50° for 24 hr., cut into small pieces, and defatted with *n*-hexane for 48 hr. The hexane extract was found to contain very little radioactivity and was discarded.

Isolation and Identification of Indole-3-Carbinol (III) and Indole-3-Carboxylic Acid (IV)—The dried and defatted herb was shaken with ammoniacal chloroform in a stoppered conical flask for 2 days and filtered through glass wool. The marc was then thoroughly washed with chloroform, and the combined filtrates were evaporated to dryness at 40° under reduced pressure. The residue, taken up in 3 ml. of chloroform, was spotted on twelve 20 × 20-cm. Silica Gel H[1] TLC plates (0.25 mm.). The plates were immediately subjected under nitrogen and in the dark to two-dimensional chromatography,[2] according to the method of Glombitza (3). The plates were developed with benzene–dioxane (65:35, v/v)[3] and dried under a stream of nitrogen. They were then turned 90° and developed with dimethylformamide (DMF)–isopropyl ether (20:80, v/v).[4] The indole-3-carbinol (III) and indole-3-carboxylic acid (IV) spots were located on TLC chromatograms by comparison with authentic samples[5] treated similarly and visualized by spraying the known plates with *p*-dimethylaminobenzaldehyde reagent.[6]

Compounds (III) and (IV) were eluted with methanol from the TLC plates carrying the metabolites. The eluates were evaporated to dryness under reduced pressure, and the residues (III and IV) were taken up in methanol and chromatographed on separate 20 × 20-cm. silica gel plates[7] (0.1 mm.) using chloroform–acetic acid (95:5, v/v)[8] as the mobile phase. The spots corresponding to alcohol (III) and acid (IV) were again located by comparison with authentic samples which were treated similarly. The spots were eluted from the plates in two separate 10.0-ml. volumes of methanol to form Solutions A and B, respectively. The solutions were immediately placed in a vacuum desiccator and stored under nitrogen in the dark at 5°.[9] Five milliliters of each of the Solutions A and B were diluted with 5 ml. of methanol to constitute Solutions C (carbinol III) and D (acid IV), respectively. Solutions C and D were then treated as follows.

A 1.0-ml. aliquot of Solution C (containing III) was assayed for ^{14}C and tritium and another from D (containing IV) for ^{14}C. Two- and four-milliliter aliquots of Solutions C and D, respectively, were assayed colorimetrically for their indole-3-carbinol and indole-3-carboxylic acid contents, according to the method of Correale (6).[10] To a 3.0-ml. aliquot of Solution C, 50 mg. of pure indole-3-carbinol[5] was added followed by the addition of 7 ml. of methanol. The clear solution was evaporated to dryness under reduced pressure, and the residue was recrystallized five times to constant activity from benzene and hexane (7:3, v/v). A sample (3.0 mg.) was taken after each recrystallization and assayed for its ^{14}C and tritium content. Similarly, to a 3.0-ml. aliquot of solution D, 50 mg. of authentic indole-3-carboxylic acid dissolved in 10 ml. of methanol was added and the resulting solution evaporated to dryness under reduced pressure. The residue was recrystallized to constant activity with benzene and assayed in the manner described for its corresponding alcohol (III).

Conversion of Indole-3-carbinol (III) to 3,3′-Di-indolylmethane (V)—The method followed was similar to that of Leete and Marion (5). Fifty milligrams of authentic indole-3-carbinol in 10 ml. of methanol was added to 5.0 ml. of Solution A (containing carbinol III), and the solution was evaporated to dryness under reduced pressure. The residue was refluxed in 25 ml. of distilled water under nitrogen for 6 hr. On cooling, a brown crystalline product was deposited, which after two recrystallizations from benzene, afforded 20 mg. of 3-3′-di-indolylmethane (m.p. 162–163°). A 5-mg. sample of this material was used for scintillation counting.

The Decarboxylation of Indole-3-carboxylic Acid (IV)—The procedure followed for the decarboxylation of (IV) was similar to that of Bowden and Marion (7). A solution of 50 mg. of indole-3-carboxylic acid in 10 ml. of methanol was added to a 5-ml. aliquot from Solution B. The resulting solution was transferred into a vertical tube fitted with a nitrogen inlet leading almost to its bottom and subsequently evaporated to dryness under a nitrogen stream. The apparatus was flushed with CO_2-free nitrogen and heated in a sand bath at 190°. At this temperature, CO_2 began to evolve and was swept *via* the nitrogen stream through a solution of $Ba(OH)_2$. After 1 hr., the temperature was raised and maintained at 220° for 3 hr. Finally, the temperature was raised to 240° causing indole (VI) to sublime into the upper part of the reaction tube.

The precipitated $BaCO_3$ was filtered off and converted to CO_2 which was trapped and counted in a methanolic solution of hyamine hydroxide by a procedure similar to that of Passmann *et al.* (8). The sublimed indole was extracted with chloroform and chromatographed on a 20 × 20-cm. silica gel (0.1 mm.) TLC plate[7] with chloroform–acetic acid (95:5, v/v). It was then recovered (R_f = 0.87) from the plate by elution with methanol and assayed for its ^{14}C content.

Radioactivity Measurements—All radioactivity measurements were performed in a liquid scintillation counter.[11] All labeled compounds were assayed in 18 ml. of a scintillation "cocktail" containing 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. ^{14}C and ^{3}H-toluene were used as standards to establish counting efficiency for the two isotopes (1).

RESULTS AND DISCUSSION

When gramine (I) labeled with ^{14}C and tritium in the side chain methylene group was administered to 60-day-old excised shoots of barley, it was found that 10.1% of the initial alkaloid radioactivity was incorporated into indole-3-carbinol (III) and 6.2% into indole-3-carboxylic acid (IV). Compounds III and IV were isolated by two-dimensional TLC, and their identities were confirmed by isotope dilution analysis with authentic samples and by conversion to 3,3′-di-indolylmethane (V) and indole (VI), respectively (Scheme I).

Table I shows that the ratio of $^{14}C/^{3}H$ found in indole-3-carbinol was the same as that of the fed gramine. Furthermore, the specific activity of the alcohol dropped to half of its value when it was converted to 3,3′-di-indolylmethane (V). This observation strongly supports the hypothesis that the side chain carbon of the carbinol is derived from the methylenic group of gramine. When indole-3-carboxylic acid (IV) was decarboxylated, the label was also shown to reside in the carboxyl group and not in its indole moiety (VI).

The above results show that the methylene group of gramine remained intact during its biotransformation to III and IV. The results also tend to support the intermediacy of indolenine (II) in this biotransformation. It has been suggested previously that this species is important in the biosynthesis of gramine from tryptophan (9) and in hydrogen-transfer reactions catalyzed by yeast alcohol dehydrogenase (10). The author has recently obtained data which suggest that species II is also a probable intermediate in the biotransformation of gramine to tryptophan in 60-day-old non-excised growing barley (1).

Indole-3-carboxylic acid arises from indoleacetonitrile in plants which do not form indoleacetic acid from the nitrile, and the existing evidence tends to indicate that indolealdehyde is the intermediate for this biotransformation (11). In the light of the author's findings, it could be postulated that a possible pathway for the production of indole-3-carboxylic acid from indole acetonitrile might be through the loss of a cyanide moiety from the latter to produce the indolenine species (II) which subsequently would be attacked by

[1] Merck & Co., Rahway, N. J.

[2] The classic fractionation of the different classes of compounds was avoided because very little radioactive gramine could be recovered (1, 2), and it was necessary to keep the number of manipulations at a minimum.

[3] In this solvent pair indole-3-carbinol (R_f = 0.65) was separated from indole-3-carboxylic acid (R_f = 0.20).

[4] Further resolution of indole-3-carbinol (R_f = 0.35) and indole-3-carboxylic acid (R_f = 0.08) was achieved in this solvent pair.

[5] Purchased from Aldrich Chemical Co., Milwaukee, Wis., and purified further by sublimination.

[6] Prepared according to Waldi (4).

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

[8] In such solvent gramine (R_f = 0.0) was well separated from indolecarbinol (R_f = 0.63) and indolecarboxylic acid (R_f = 0.45).

[9] Indole-3-carbinol (III) is known to be unstable to air, light, and heat (5).

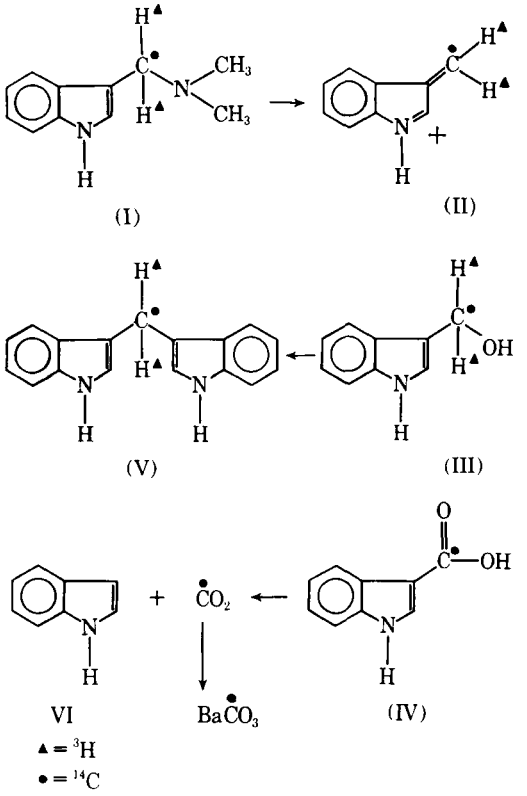
[10] The aliquots were evaporated to dryness under a stream of nitrogen, and the residue taken up in 2.0 ml. of distilled water and 1.0 ml. of 2% w/v of *p*-dimethylaminobenzaldehyde solution (6). These solutions were allowed to stand in a water bath at 58° for 12 hr. and were subsequently analyzed spectrophotometrically by reading the intensity of their colors at 600 mμ.

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

Table I—Activities of Gramine Administered to Barley and of Its Metabolites

	Amount Fed, mg.	Amount Isolated, mg.	Specific Activity,[a] d.p.m./mmole × 10^6 — ^{14}C Assay	^3H Assay	Ratio, $^{14}C/^3H$	Incorporation, %[b]
Gramine	53	—	7.05	3.5	2.0	—
Indole-3-carbinol	—	2.94	0.71	0.34	2.1	10.1
Indole-3-carboxylic acid	—	2.57	0.44	—	—	6.2

[a] All specific activities have been corrected for efficiency and self-absorption by using techniques which were previously described (1). [b] Calculated on the basis of ^{14}C incorporation.



Scheme I

water to produce indole-3-carbinol. The newly formed alcohol (III) could then be oxidized to indolealdehyde and finally to indole-3-carboxylic acid (IV).

The above metabolic pathways are reminiscent of the progressive oxidation of the side chain of serotonin to 5-hydroxyindoleacetaldehyde and 5-hydroxyindoleacetic acid (5-HIAA) which is the major metabolic product of serotonin in man (12). Thus, it appears that a degradative pathway involving the stepwise oxidation of the side chain of simple indole compounds is operative in both the animal and plant kingdoms. In the light of the foregoing considerations it could be proposed that biosynthetic studies with plants could be useful in drawing conclusions on the chemistry and biochemistry of biologically important compounds in animals and man.

The results obtained in the studies described above do not exclude the possibility of a biotransformation of gramine to products III and IV induced in the barley by these experimental conditions. In this context it can be argued that the elucidated metabolic pathway of gramine might not represent the normal pathway in live, uncut barley. It can be concluded with certainty, however, that barley shoots possess enzymes that are capable of biotransforming gramine to nonalkaloidal substances.[12] This is substantiated further by the fact that when tagged gramine was administered to the grains of 60-day-old barley shoots in a manner similar to that described for the administration to the shoots, no radioactive CO_2 was detected. Likewise, no radioactivity was observed in the plant's expired CO_2 when radioactive skatole (3-methylindole) was administered to excised shoots (2), suggesting that barley shoots lack the enzymes necessary to biodegrade a methyl group attached to the indole nucleus of skatole. However, the methylenic side chain carbon of gramine appears to be biodegradable due to the chemical mobility of its adjacent dimethylamino function.

Although these experiments demonstrated the metabolism of gramine to Compounds III and IV and the overall biodegradation of gramine's side chain to carbon dioxide, the ultimate evidence that III and IV are intermediates in the formation of CO_2 from the alkaloid's methylene carbon must await further investigation, due to the difficulties encountered in synthesizing tagged indole-3-carbinol and indole-3-carboxylic acid. However, the results from a preliminary experiment in which a small amount of metabolite (III), tagged at its methylenic carbon, was administered, indicated that its radioactivity passed into the plant's expired CO_2.

[12] Fairbairn and El-Masry (13) have recently shown that when labeled morphine was fed to *Papaver somniferum*, the alkaloid was rapidly metabolized in the capsule latex to form two nonalkaloidal polar substances.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 22, 1968, from the *Departments of Materia Medica and Pharmaceutical Chemistry, College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication October 1, 1968.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported in part by a grant from the University of Kentucky Research Foundation and by grant 1R01 AM 12971-01 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.

The receipt of a Lederle Pharmacy Faculty Award is gratefully acknowledged.

Thanks are extended to Mr. Bahjat Faraj and Mr. David Khaliel for technical assistance at an early stage of the study and to Dr. L. Dittert for useful discussions.

Previous paper: G. A. Digenis, *J. Pharm. Sci.*, **58**, 39(1969).