THE BIOGENESIS OF ALKALOIDS

XIV. A STUDY OF THE BIOSYNTHESIS OF DAMASCENINE AND TRIGONELLINE

By Edward Leete,[2] Léo Marion, and Ian D. Spenser[2]

ABSTRACT

Tryptophan-3-C^{14} was prepared from methyl labelled sodium acetate and fed to mature *Nigella damascena* L. plants. Radioactivity was translocated throughout the plant, but no activity was detected in the damascenine isolated from it. Similarly the radioactive tryptophan was fed to pea seedlings and trigonelline detected in the plant extracts. This was also inactive, and these results are discussed.

It has been suggested (25) that trigonelline (the methyl betaine of nicotinic acid) arises from proline by ring opening to δ-aminovaleric acid and thence to nicotinic acid by reaction with a one carbon fragment such as formic acid. Klein and Linser (18, 19) injected various amino acids into the hollow stems of *Dahlia variabilis* and other plants which produce trigonelline and they claimed that the amount of trigonelline increased, relative to that in control plants, after the feeding of ornithine, proline, glutamic acid, or δ-aminovaleric acid. No increase was observed after feeding arginine, tyrosine, aspartic acid, or other amino acids. Hexamethylene tetramine produced increases in the amount of alkaloid, possibly by acting as a source of formic acid. These results have been critically examined by James (15) who raised doubts about the supposed increases in the amount of alkaloid.

Barger (3) was the first to place damascenine (the methyl ester of 2-methylamino-3-methoxybenzoic acid) amongst the alkaloids derived from tryptophan by the oxidation of indole to anthranilic acid followed by hydroxylation and methylation. In animals and molds it has been conclusively proved that 3-hydroxyanthranilic acid and nicotinic acid arise in the course of tryptophan metabolism (4, 6, 14, 20). Many biological reactions which occur in plants have been shown to be similar to if not identical with those occurring in animals and molds. It was thus conceivable that tryptophan might be the source not only of damascenine, but also of trigonelline. It was therefore decided to feed tryptophan labelled with C^{14} in the 3-position of the indole nucleus to plants producing damascenine and trigonelline. If tryptophan were the precursor of these alkaloids radioactivity would be expected in the carboxyl group of both trigonelline and damascenine.

The preparation of tryptophan-3-C^{14} in 5% yield from carboxyl labelled benzoic acid in 11 steps has been reported (14). In the present work methyl labelled sodium acetate was converted to pyruvamide-3-C^{14} by the method described in the literature (2, 24) for the preparation of pyruvamide-2-C^{14} from carboxyl labelled sodium acetate. This was converted to the phenylhydrazone and quantitatively hydrolyzed to pyruvic acid phenylhydrazone

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[2]*National Research Council of Canada Postdoctorate Fellow.*

which was esterified with diazomethane and cyclized to methyl indole-2-carboxylate (7). The ester was hydrolyzed and the indole-2-carboxylic acid decarboxylated in quinoline in the presence of cupric oxide. The indole-3-C^{14} was converted via gramine to tryptophan-3-C^{14} (1, 13, 23). The over-all radiochemical yield from pyruvamide to tryptophan was 6%. Direct ring closure of pyruvic acid phenylhydrazone to indole-2-carboxylic acid failed completely (11) while cyclization of pyruvamide phenylhydrazone to indole-3-carboxamide was accomplished in 12% yield only. Attempts to synthesize pyruvic acid phenylhydrazone according to Japp and Klingemann (16) in two steps from methyl iodide and ethyl methylmalonate (10) or from methyl iodide and ethylacetoacetate (9) led to the desired product in poor yields only.

The labelled tryptophan was fed to mature five-month-old *Nigella damascena* plants via the roots and after eight days the damascenine isolated from the whole plant by ether extraction. The amino acid was also fed to five-week-old pea seedlings and after seven days trigonelline was detected in the extracts of the aerial parts of the plant. No trigonelline was found in the roots. Neither alkaloid, however, was radioactive.

EXPERIMENTAL[3]

Synthesis of Tryptophan-3-C^{14}

Pyruvamide-3-C^{14}.—This was prepared from methyl labelled sodium acetate by the same method as that described for the synthesis of pyruvamide-2-C^{14} (2, 24).

Pyruvic acid-3-C^{14} phenylhydrazone.—Pyruvamide-3-C^{14} (0.834 gm., 0.0096 mole) of activity 2.78×10^8 disintegrations per minute was dissolved in water (30 ml.) and a solution of freshly distilled phenylhydrazine (0.95 ml., 0.0096 mole) in 2 *N* hydrochloric acid (5 ml.) was added. The mixture was kept at 0° for one hour and the product filtered off and washed with water (100 ml.). It was wetted with a little methanol, dissolved in boiling water (100 ml.), 2 *N* hydrochloric acid (150 ml.) added, and the solution kept on the steam bath for two hours. The solution was cooled and the crystalline pyruvic acid-3-C^{14} phenylhydrazone filtered, m.p. 173–174°, wt. 1.382 gm., yield 81%.

Indole-3-C^{14}-2-carboxylic acid.—The pyruvic acid phenylhydrazone was suspended in ether and treated with excess diazomethane. The solvent and excess reagent were removed by distillation, the residual ester dissolved in glacial acetic acid (6 ml.), and after cooling concentrated sulphuric acid (0.7 ml.) was added. The resulting solution was kept at 80° for one hour when crystallization of ammonium acetate was complete. It was filtered, the filtrate cooled, water (250 ml.) and 2 *N* ammonium hydroxide (50 ml.) added, and the mixture kept at 0° for 48 hr. The supernatant liquor was sucked off and the crude methyl indole-2-carboxylate transferred to a sublimation tube and distilled at 120–140° at 0.001 mm. The white sublimate together with a small amount of yellow oil was washed out with methanol, taken to dryness, and hydrolyzed with hot *N* sodium hydroxide (10 ml.). 2 *N*-Hydrochloric acid (12 ml.) was added, the mixture cooled for 36 hr., and the acid filtered. After

[3]*All melting points are corrected.*

drying over concentrated sulphuric acid the crude indole-2-carboxylic acid, m.p. 176–179°, wt. 0.371 gm., was obtained in 29.7% yield based on pyruvic acid phenylhydrazone.

Indole-3-C^{14}.—The active indole-2-carboxylic acid was refluxed with cupric oxide (20 mgm.) in quinoline (6 ml.) in an atmosphere of nitrogen for 16 hr. The mixture was cooled, diluted with ether (200 ml.), filtered, and the filtrate washed with aqueous sodium hydroxide and then with dilute hydrochloric acid. The ether solution was dried over sodium sulphate and evaporated *in vacuo* at room temperature. It left a residue consisting of crude indole-3-C^{14} (0.290 gm.).

DL-Tryptophan-3-C^{14}.—The crude active indole was converted to gramine by reaction with formaldehyde and dimethylamine. The gramine (0.214 gm.) was diluted with inactive gramine to give a weight of 1.59 gm. which in a Mannich reaction yielded tryptophan-3-C^{14} acetate (with 1 mole of acetic acid of crystallization), wt. 1.342 gm., with a specific activity of 1.24×10^4 disintegrations per min. per mgm., or 4.02×10^6 disintegrations per min. per millimole, representing an over-all radiochemical yield of 6% from pyruvamide.

Pyruwamide Phenylhydrazone

In the course of the radioactive synthesis this compound was not isolated. Pyruvamide (1.09 gm., 0.0125 mole) in water (10 ml.) was added to a warm solution of phenylhydrazine (1.35 gm., 0.0125 mole) in 2 *N* hydrochloric acid (6.3 ml.). A bulky crop of crystals separated almost immediately. After being allowed to stand for 10 min. the product was filtered off and recrystallized from hot water containing a little sodium hydroxide. Pyruvamide phenylhydrazone separated as shiny plates (1.95 gm., 88% yield), m.p. 143–144°. The literature reports m.p. 144° for this compound prepared by another method (12). Calc. for $C_9H_{11}ON_3$: C, 61.00; H, 6.26; N, 23.72. Found: C, 61.21; H, 6.33; N, 23.34%. The substance is only very slowly hydrolyzed by 2 *N* aqueous sodium hydroxide, but is readily converted to pyruvic acid phenylhydrazone by dilute aqueous hydrochloric acid.

Indole-2-carboxamide.—Ring closure of pyruvamide phenylhydrazone could not be effected by means of a mixture of acetic and sulphuric acids, nor by dry ethanolic hydrogen chloride. Boron trifluoride gave the product in poor yield. Pyruvamide phenylhydrazone (0.89 gm., 0.005 mole) in glacial acetic acid (3 ml.) was treated with boron trifluoride dietherate (1 ml.). The mixture was heated for 20 min. on the water bath, cooled, poured into water and the suspension extracted with ether. The dried ether extract was distilled at 0.01 mm., yielding two products. The first fraction was an oil (0.31 gm.) which on standing for two months set to a glass and was not further investigated. The second fraction, a crystalline sublimate (0.10 gm., 12% yield), m.p. 234.5–235.5°, was the desired product. Calc. for $C_9H_8ON_2$: C, 67.48; H, 5.03. Found: C, 67.53; H, 5.09%.

Administration of the Tryptophan-3-C^{14} *to Nigella damascena L.*

Nigella damascena L. seeds were germinated in soil and allowed to grow for five months. Eighteen mature plants were transferred to a hydroponics set up

with the roots dipping into the nutrient solution. The nutrient solution contained, per liter, potassium nitrate (505 mgm.), calcium nitrate tetrahydrate (1180 mgm.), magnesium sulphate heptahydrate (495 mgm.), potassium dihydrogen phosphate (272 mgm.), ferrous sulphate heptahydrate (2 mgm.), plus traces of micronutrients (B, Mn, Zn, Mo, Cu). After two weeks in the nutrient solution new roots were being produced and some of the plants were flowering. At this stage the tryptophan-3-C^{14} acetate (580.1 mgm., with a specific activity of 4.02×10^6 disintegrations per min. per millimole, and a total activity of 7.21×10^6 disintegrations per min.) was equally divided between the plants. The tryptophan was taken up by the plant as shown by the day to day decreasing activity in the nutrient solution. The first day after feeding the total activity remaining in the nutrient solution was 5.9×10^6, second day 4.4×10^6, third day 2.9×10^6, fourth day 2.0×10^6, fifth day 0.8×10^6, sixth day 0.2×10^6, seventh day 0.1×10^6 disintegrations per min.

Isolation of Damascenine

On the eighth day the plants were harvested, dried at 50–60°, and ground in a Wiley mill. The ground material (18.8 gm.) was extracted with ether for three days in a Soxhlet extractor. This ether extract had a total activity of 1.07×10^4 disintegrations per min. The green solution was evaporated to dryness and a small sample of the residue chromatographed on Whatman No. 1 paper (buffered to pH 8 with phosphate – citric acid), with a mixture of *n*-butanol (80 ml.) and water (15 ml.) as the developing solvent. Authentic specimens of 3-hydroxyanthranilic acid, damascenine (obtained from *Nigella damascena* seeds according to the procedure of Ewins (8)), and damasceninic acid were run on the same chromatogram and had R_F values of 0.15, 0.88, and 0.76 respectively. The hydroxyanthranilic acid was detected by its pronounced fluorescence in ultraviolet light and the other two compounds with Dragendorff's reagent (21). Only damascenine was found in the plant extract. The residue from the ether extract of the plant was dissolved in *N* hydrochloric acid (100 ml.) and the solution extracted with ether. The aqueous layer was made alkaline with ammonia and the alkaloid extracted with ether. The fluorescent extract was dried and the ether removed by distillation under diminished pressure leaving a residue which was distilled *in vacuo*. Damascenine distilled as a colorless oil, b.p. 120° at 0.001 mm., wt. 5.6 mgm. This was completely non-radioactive.

The ground plant was further extracted with methanol, but no more alkaloid was obtained. The methanol extract was radioactive (1.6×10^5 disintegrations per min.) presumably owing to the extraction of uncombined radioactive tryptophan from the plant. After extraction with ether and methanol the plant had a total activity of 2.1×10^5 disintegrations per min.

Administration of Tryptophan-3-C^{14} to Garden Peas

Garden peas (Laxton's Progress) treated with fungicide (Semesan) were allowed to germinate in soil. After two weeks 48 plants were transferred to a hydroponic solution of the same composition as that used for the *Nigella*

damascena and the labelled tryptophan acetate (573.7 mgm. with a specific activity of 4.02×10^6 disintegrations per min. per millimole) was administered as in that case. The rate at which the amino acid was taken up by the plant was followed by determining the total activity of the nutrient solution every day. The plants were harvested on the seventh day and the roots and the aerial parts worked up separately. No trace of trigonelline was found in the roots. The aerial parts of the plant (64.9 gm. fresh weight) were extracted with water. The extract, having a total activity of 1.4×10^5 disintegrations per min., was treated with 20% lead acetate solution to precipitate the protein and then with hydrogen sulphide to remove the excess lead. The filtrate (having a total activity of 0.5×10^5 disintegrations per min.) was concentrated to 250 ml., made alkaline with ammonia, and treated with an excess of ammonium reineckate (3 gm. in 10 ml. of methanol) to precipitate the choline. The precipitate was centrifuged off after cooling overnight and the solution acidified with 1.0 *N* hydrochloric acid and kept cold for 24 hr. The precipitated reineckate was filtered off, washed with *n*-propanol, and dissolved in acetone. This acetone solution contained negligible radioactivity; it was chromatographed on Whatman No. 1 paper using the one phase system 95% ethanol–5% ammonia of d. 0.880 (5) which separates choline (R_F 0.45) from trigonelline (R_F 0.22), and the chromatogram developed with Dragendorff's reagent (21). The trigonelline spot on the chromatogram was not radioactive. Trigonelline reineckate was decomposed with silver sulphate and barium hydroxide (17), but only a trace of free base was obtained.

DISCUSSION

If the synthesis of damascenine and of trigonelline occurred in the plants while they were in contact with the radioactive tryptophan, the results would indicate that tryptophan was not utilized for the production of the bases. Since damascenine and trigonelline are methylated derivatives of 3-hydroxyanthranilic acid and of nicotinic acid respectively, both well established tryptophan metabolites in animals and in molds (4, 6, 14), and since methylation in plants has been shown to be a general and facile process, the present results would lead to the conclusion that tryptophan metabolism in the plants studied differs radically from that prevailing in animals and molds.

In feeding experiments with mature plants, there is the danger that alkaloids are no longer being actively produced at the time of the experiment. In our experiments, however, the labelled amino acid was fed while the plants were flowering and producing seeds. In both *Nigella damascena* (8) and peas (22) the alkaloid is present in the seeds, and while it is conceivable that it may be translocated there from other parts of the plant and that actual synthesis no longer takes place at the time, this seems unlikely.

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