Biosynthesis of Tropic Acid in *Datura innoxia*

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Phenylalanine-UL-^{14}C, phenylalanine-1,3-^{14}C, and shikimic acid-UL-^{14}C were fed to mature *Datura innoxia* Mill. plants, and the radioactive hyoscyamine and scopolamine were extracted and degraded to determine the position of the radioactive carbon of the tropic acid moiety. The results indicated that all the carbons of tropic acid are derived from the carbons of phenylalanine and the carboxyl carbon of tropic acid is derived from the carboxyl carbon of phenylalanine by way of some intramolecular shift mechanism.

IN THE PAST, several compounds have been studied as possible precursors of the branched chain aromatic acid, tropic acid. Among these phenylalanine, sodium acetate, phenylacetic acid, propionic acid, and tryptophan were employed. Although phenylacetic acid (1) and tryptophan (2) can be incorporated into tropic acid, more significant results have been obtained with phenylalanine as the labeled precursor (3–7).

When phenylalanine-3-^{14}C was fed to mature *Datura stramonium* plants, the hyoscyamine and scopolamine were labeled in carbon 2 of the tropic acid moiety (1, 3, 7). The hydroxymethyl group (carbon 3) was labeled when phenylalanine-2-^{14}C (4) or phenylacetic acid-1-^{14}C (1) was the radioactive precursor. The above labeling patterns have led to the formulation of a proposed pathway of biosynthesis in which an active 1-C unit serves as the precursor of the carboxyl group of tropic acid (1). Since ^{14}C-labeled carbonate, formate, formaldehyde, methionine, and serine do not significantly label the carboxyl carbon of tropic acid (1, 6), considerable interest has been focused upon the origin of this carbon atom.

Louden and Leete (5, 6) reported that tropic acid was labeled in the carboxyl carbon after *D. stramonium* plants were fed with L-phenylalanine-1-^{14}C. These results indicate the involvement of the carboxyl carbon of phenylalanine as a precursor of the carboxyl group of tropic acid. These workers have proposed a pathway of biosynthesis involving the equivalent of an intramolecular shift of the carboxyl carbon of phenylalanine to the position occupied in the tropic acid molecule. The work reported here was accomplished in order to add to the evidence in support of the latter pathway.

If a phenylpropanoid molecule, such as phenylalanine or phenylpyruvic acid, is actually involved in the biosynthesis of tropic acid, then labeled shikimic acid should label only the aromatic ring portion of this acid. Also, generally labeled phenylalanine should label tropic acid in such a way that benzoic acid, obtained by oxidation of tropic acid, would contain 7/9 the specific activity of its parent tropic acid. A third precursor, phenylalanine-1,3-^{14}C in which the ratio of the specific activities of the two radioactive carbon atoms is known, should label the carbons 1 and 2 tropic acid in the same ratio of specific activities as that of the parent compound if the "carboxyl shift" pathway is functioning.

EXPERIMENTAL

Growth and Processing of Plant Material—*D. innoxia* Mill. plants were grown from seeds selected from plants previously grown in the Drug Plant Garden, University of Rhode Island. The seedlings were potted in sandy loam and were grown in a Hotpack environment control room, model 1288-13, using the following conditions: light period, 16 hr. at a temperature of 32° ± 1° and a relative humidity of 40% ± 5%; dark period, 8 hr. at a temperature of 23° ± 1° and a relative humidity of 60% ± 5%.

The plants were maintained under the above conditions exposed to approximately 1800 f.-c. of light during the "light" period until the plants were 3 months old. The radioactive compounds were administered to the 3-month-old plants by means of cotton wicks pulled through their stems according to a modification of the procedure given by Comar (8). Details of the amount of tracer fed and the activities of the alkaloids are shown in Table I. The alkaloids were diluted prior to degradation.

Extraction and Separation of the Alkaloids—The plants were harvested 14 days after the administration of the ^{14}C-labeled compounds and the alkaloids extracted according to the procedure reported by Leete (9). Further purification of the total alkaloid extract was accomplished by immiscible solvent extraction in a separator. The hyoscyamine and scopolamine were separated by way of the column chromatography method reported by Leete (10) using diatomaceous earth[1] as the stationary phase. Final purification of the individual alkaloids was carried out by means of thin-layer chromatography. Glass thin-layer chromatography plates

Received March 13, 1967, from the †Department of Pharmacognosy, College of Pharmacy, University of Rhode Island, Kingston, RI 02881

Accepted for publication April 19, 1967.

Abstracted in part from a thesis submitted by Coy A. Gibson to the College of Pharmacy, University of Rhode Island, in partial fulfillment of Doctor of Philosophy degree requirements.

Supported in part by funds from the American Foundation for Pharmaceutical Education, Washington, D. C., and in part by grant NH-06511 from the National Institutes of Mental Health, U. S. Public Health Service, Bethesda, Md.

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[1] Marketed as Celite 545 by the Johns-Manville Corp., New York, N. Y.

TABLE I—*Datura innoxia* MILL.: ^{14}C-LABELED COMPOUNDS AND ALKALOIDS

	L-Phenylalanine-UL-^{14}C	DL-Phenylalanine-1,3-^{14}C[a]	Shikimic Acid-UL-^{14}C[b]
Wt., mg.	0.009	2.01	1.2
Activity, μc.	20.	60.5	2.82
Fresh wt. of plants, Gm.	70.5	289.0	73.4
Specific act., μc./mM	3.59×10^5	2.7×10^3	4.12×10^2
Hyoscyamine and Its Degradation Products (Act. in μc./mM)			
Incorp., %	0.1	0.28	0.3[c]
Hyoscyamine	0.437	1.66	0.101[c]
Tropine[d]	0.02[d]	0.04[d]	0.00[cd]
Tropic acid (TA)	0.416	1.62	0.1[c]
Benzoic acid (BA)	0.365	0.889	0.091[c]
Ratio, $\frac{\text{sp. act. TA}}{\text{sp. act. BA}}$	1.22	1.82	1.09[c]
Scopolamine and Its Degradation Products			
Incorp., %	0.07	0.08	...
Scopolamine	0.058	0.43	...
Scopine[d]	0.001[d]	0.03[d]	...
Tropic acid	0.058	0.397	...
Benzoic acid	0.047	0.159	...
Ratio, $\frac{\text{sp. act. TA}}{\text{sp. act. BA}}$	1.21	2.4	...

[a] Obtained by mixing known quantities of DL-phenylalanine-1-^{14}C and DL-phenylalanine-3-^{14}C. [b] Biosynthesized by Dr. B. A. Bohm, College of Pharmacy, University of Rhode Island. [c] Total alkaloids calculated as hyoscyamine. [d] Calculated by difference.

(20 cm. × 20 cm.) were prepared with a spreader[2] using Silica Gel G[2] as the adsorbent (25 Gm. Silica Gel G in 50 ml. water). The alkaloids were applied streakwise to the plates and chromatographed in sealed glass chambers using dimethylformamide–diethylamine–ethanol–ethyl acetate (1:1:6:12) as the solvent (11). The thin-layer chromatograms were air dried and kept in a vacuum desiccator for 24 hr. in order to remove the volatile solvents, particularly diethylamine and dimethylformamide. Dragendorff spray reagent, as modified by Munier and Macheboeuf (12), was used to locate the alkaloid zones by spraying a small strip across the plate perpendicular to the solvent front. Since dimethylformamide interferes with the Dragendorff color reaction (13), the strip had to be sprayed first with a 0.25% solution of sodium nitrate in 0.5% hydrochloric acid and dried before spraying with the Dragendorff reagent.

The zones of the thin-layer chromatograms containing the respective alkaloids were carefully scraped from the plates, and the alkaloids were eluted with absolute ethanol and rechromatographed repeating the above procedure. Each alkaloid, hyoscyamine and scopolamine, purified by this procedure, showed only a single spot on thin-layer chromatograms using either the system given above or with chloroform–diethylamine (9:1) as the solvent. The amount of the purified alkaloid was determined by colorimetric assay (14).

Hydrolysis of Alkaloids—Sufficient nonradioactive alkaloid was added to the purified alkaloids to provide a sample of known weight between 125 and 150 mg. Complete hydrolysis was accomplished by dissolving the alkaloid in 35 ml. of warm water and adding 10 ml. of 2 N sodium hydroxide. The mixture was evaporated on a water bath to 10 to 15 ml. The aminoalcohol, tropine or scopine, was extracted with five 25-ml. portions of chloroform, each chloroform portion being washed with 10 ml. of water. No further purification of the aminoalcohol was attempted.

Following acidification with dilute hydrochloric acid, the aqueous hydrolysate was extracted with several portions of isopropanol–chloroform (1:3). Crude tropic acid was obtained by evaporation of the isopropanol–chloroform solvent. Purification of the tropic acid was accomplished by repeated recrystallization from a mixture of hot benzene and petroleum ether yielding glistening white plates, m.p. 116.5–118°.

Degradation of Tropic Acid—Several workers (1, 3, 7) have shown that phenylalanine-3-^{14}C yields tropic acid labeled only in the carbon adjacent to the ring, and Leete (4, 6) has shown that L-phenylalanine-1-^{14}C labels only the carboxyl carbon of tropic acid. In no case has it been reported that either phenylalanine-3-^{14}C or phenylalanine-1-^{14}C can label the hydroxymethyl carbon of tropic acid. It was therefore determined that the specific radioactivity of tropic acid, along with that of the benzoic acid obtained from tropic acid, would yield the relative specific radioactivities of the two labeled carbons of tropic acid.

Tropic acid (40 to 50 mg.) was refluxed with 250 mg. of potassium permanganate in 5 ml. of water for 2 hr. The hot solution was filtered and, after cooling, acidified with hydrochloric acid. The acidic solution was then extracted with ether for 10 hr. using continuous liquid–liquid extraction. Removal of the ether yielded a light brown residue which, upon sublimation at 100–105° for 1 hr., yielded 10–12 mg. of colorless needles of benzoic acid, m.p. 121–122°.

Preparation of ^{14}C Samples and Determination of Radioactivity—Radioactive samples were assayed by combustion of the material to carbon dioxide and collecting the evolved gas in an ion chamber. The radioactivity of the gas was then determined on the Dynacon[3] model 6000 vibrating capacitor elec-

[2] Thin-layer chromatography kit, supplied by Research Specialties Co., Richmond, Calif.

[3] Nuclear Chicago Corp., Des Plaines Ill.

trometer using the rate of charge method of determining radioactivity.

The apparatus used for combustion of the organic material was the van Slyke wet combustion apparatus designed for use with the Dynacon model 6000. The reagents were prepared according to van Slyke *et al.* (15).

RESULTS

While all the alkaloid samples obtained after feeding with the three radioactive compounds were radioactive, the samples obtained from shikimic acid-fed plants were not sufficiently active to permit separation of the hyoscyamine and scopolamine. The low activity was due to the relatively very low specific activity of the shikimic acid sample. The alkaloids obtained from the plants fed L-phenylalanine-UL-^{14}C and DL-phenylalanine-1,3-^{14}C, respectively, were separated and the hyoscyamine and scopolamine purified before degradation.

If all the carbon atoms of L-phenylalanine-UL-^{14}C are incorporated in tropic acid, then the tropic acid should possess 1.28 times the activity present in the benzoic acid derived from it by oxidation. The results reported here support this theory since the tropic acid/benzoic acid specific activity ratios were 1.22 and 1.21, respectively (Table I). Shikimic acid-UL-^{14}C labels only the aromatic ring of tropic acid, as evidenced by the fact that almost all (91%) of the specific activity of the tropic acid obtained from the total alkaloid fraction was recovered in the benzoic acid obtained by oxidation.

When DL-phenylalanine-1,3-^{14}C, prepared by mixing known quantities of DL-phenylalanine-1-^{14}C and DL-phenylalanine-3-^{14}C, was fed and the values were determined by the following formula:

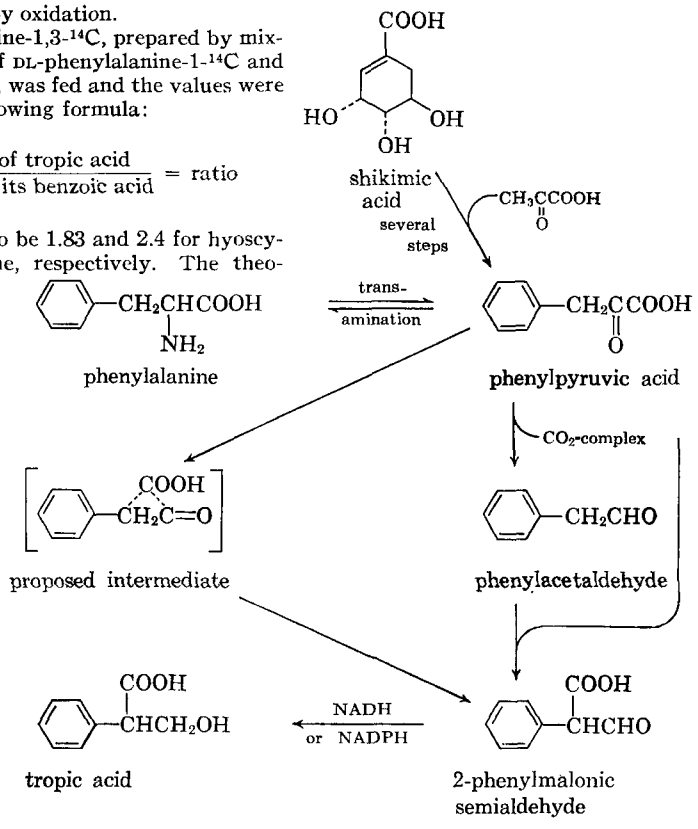
$$\frac{\text{specific activity of tropic acid}}{\text{specific activity of its benzoic acid}} = \text{ratio}$$

the ratios were found to be 1.83 and 2.4 for hyoscyamine and scopolamine, respectively. The theoretical value based upon the DL-phenylalanine-1,3-^{14}C is 1.74. The value found for hyoscyamine is a little higher than expected, but this may be explained by a more rapid hyoscyamine turnover or by losses incurred due to chemical manipulation. However, the ratio value for scopolamine (2.4) is significantly high and cannot be reasonably explained without further study.

DISCUSSION

Two general pathways have been proposed for the biosynthesis of tropic acid in *Datura*. Briefly, these may be stated as follows: (*a*) phenylalanine, after transamination to phenylpyruvic acid, undergoes decarboxylation to phenylacetic acid (or possibly its coenzyme A derivative); recarboxylation at the α-carbon of this acid followed by reduction of the original carbonyl group yields tropic acid; (*b*) phenylalanine, after transamination to phenylpyruvic acid, undergoes an intramolecular shift of the carboxyl group to the carbon adjacent to the ring; reduction at the carbonyl carbon yields tropic acid. The results of this research support the second pathway.

The total number of carbon atoms present in the molecule is the same for both phenylalanine and tropic acid. Several experiments (1, 3–7) have shown that phenylalanine can serve as the precursor of at least carbons 2 and 3 of tropic acid, *i. e.*, DL-



Proposed Biosynthetic Pathway for Tropic Acid

Scheme I

phenylalanine-3-^{14}C labels carbon 2 of tropic acid whereas DL-phenylalanine-2-^{14}C labels carbon 3 of this acid. One experiment (6) has indicated that L-phenylalanine-1-^{14}C can label the carboxyl carbon (carbon 1) of this aromatic branched-chain acid. Since no variations in this labeling pattern have been observed, the incorporation of phenylalanine-1,-3-^{14}C into tropic acid, wherein the ratio of the specific activity of carbons 1 and 2 of tropic acid equals that of carbons 1 and 3 of the phenylalanine precursor, strongly supports the hypothesis that the carboxyl carbon of phenylalanine is a precursor of the carboxyl carbon of tropic acid.

Since benzoic acid, which was obtained by oxidation of the tropic acid from *D. innoxia* plants that were fed phenylalanine-UL-^{14}C, possesses a specific activity equal to 80% of that of its parent tropic acid, phenylalanine appears to be a precursor of all the carbon atoms of the tropic acid. The incorporation of shikimic acid-UL-^{14}C in only the aromatic ring structure of tropic acid further supports the involvement of a C_6-C_3 compound. Phenylalanine is a C_6-C_3 compound biosynthesized by way of the so-called shikimic acid pathway (16). The incorporation of shikimic acid into tropic acid does not directly implicate phenylalanine in the biosynthesis of tropic acid, but it does implicate the involvement of the shikimic acid pathway by which phenylalanine is formed.

Scheme I shows a biosynthetic pathway for tropic acid [essentially the pathway proposed by Leete (6)] based upon the results given above and previous reports in the literature. It may best be described in the following three major steps.

Synthesis of Phenylalanine—This compound is formed by transamination of phenylpyruvic acid (16) which is formed by way of the shikimic acid pathway. [An excellent review on shikimic acid has been published by Bohm (17).] In the plant, tropic acid may well be formed from phenylpyruvic acid without the actual conversion of this acid to phenylalanine. However, the ease with which these two compounds undergo interconversion makes this point very difficult to confirm.

Formation of 2-Phenylmalonic Semialdehyde—The carboxyl group of this compound is derived from the carboxyl group of phenylpyruvic acid (phenylalanine). This transfer or shift of the carboxyl group may occur *via* an intramolecular shift, analogous to the methyl malonyl coenzyme A to succinyl coenzyme A isomerization (18). A second possible route by which this shift may occur is *via* an enzyme-bound decarboxylation–recarboxylation sequence. These two possible pathways for the carboxyl shift have also been suggested by Leete (6). Since no attempts have been made to determine the form in which the 2-phenylmalonic aldehyde occurs, *i. e.*, coenzyme A derivative, phosphate derivative, *etc.*, no suggestions are proposed for this pathway.

Formation of Tropic Acid—Reduction of the aldehyde group of 2-phenylmalonic semialdehyde would yield tropic acid. This reaction could be accomplished by NADH or NADPH mediated by an enzyme analogous to alcohol dehydrogenase.

The results of this work support the pathway given in Scheme I for the following reasons.

(*a*) The incorporation of shikimic acid into the ring-carbons of phenylalanine is in agreement with the known pathway of phenylalanine biosynthesis (16). (*b*) The results are in agreement with the results of other experiments (1, 3, 7) with respect to the incorporation of carbon-1 of the amino acid. (*c*) The ratio of the radioactivity of carbons 1 and 2 of tropic acid equals that of carbons 1 and 3 of phenylalanine as shown by degradation of the tropic acids obtained from plants fed DL-phenylalanine-1,3-^{14}C, as well as those fed L-phenyalanine-UL-^{14}C.

These results do not necessarily contradict the entire pathway proposed by Underhill and Youngken (1), but do contradict a sequence of decarboxylation of phenylpyruvic acid (from phenylalanine) followed by a separate recarboxylation step. However, a similar sequence during which all the reactants remain enzyme-bound so that the carbon dioxide removed in the first step is utilized in the recarboxylation step cannot be ruled out.

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