# Biosynthesis of Hyoscyamine and Scopolamine in *Datura stramonium*

By E. W. UNDERHILL† and H. W. YOUNGKEN, Jr.

*Datura stramonium* L. plants were allowed to metabolize certain $C^{14}$-labeled compounds for 10 days following which hyoscyamine and scopolamine were isolated and purified. Of the compounds employed, phenylalanine-3-$C^{14}$, phenylacetic acid-1-$C^{14}$, and sodium acetate-2-$C^{14}$ were the most efficient precursors, followed by sodium propionate-2-$C^{14}$, sodium formate-$C^{14}$, and serine-3-$C^{14}$ in that order; zinc lactate-2-$C^{14}$ proved to be a comparatively inefficient precursor. The distribution of $C^{14}$ within the alkaloids was entirely in their tropic acid moiety when phenylalanine and phenylacetic acid were used as precursors, and almost entirely in their tropine or scopine portions when acetate, propionate, formate, and serine were employed. The samples of tropic acid obtained, following administration of phenylalanine and phenylacetic acid, were degraded and the location of the $C^{14}$ determined.

A NUMBER of theories on the biosynthesis of of the tropane moiety of hyoscyamine and scopolamine have been proposed and these have recently been reviewed (1). Experiments involving $C^{14}$-labeled compounds as precursors of this portion of the alkaloids have provided positive results in the case of ornithine-2-$C^{14}$ (2), methionine-$C^{14}$ (3), and acetate-$C^{14}$ (4, 5), while negative results have been obtained when putrescine-1,4-$C^{14}$ (6) and citric acid-3-$C^{14}$ (7) were employed. Recently attention has been focused upon the formation of the tropic acid moiety of these alkaloids. Trautner (8) has suggested that tropic acid, itself possessing an isopropylbenzene skeleton, may be derived from the the terpenes, limonene and terpinene. Shortly after this work was commenced, Wenkert (9) suggested that this aromatic branched-chain acid may be derived from an aldol product of formaldehyde and prephenic acid. A number of routes leading from such an aldol product to tropic acid are possible and these have been elaborated upon in the experimental work reported by Leete (10), who administered DL-phenylalanine-3-$C^{14}$, sodium formate-$C^{14}$, and formaldehyde-$C^{14}$ to *Datura stramonium*. Of the compounds employed by Leete, only phenylalanine was a precursor of tropic acid and in this instance the activity resided in the carbon adjacent to the aromatic group. Goodeve and Ramstad (11) have found that the administration of tryptophan-3-$C^{14}$ to *D. stramonium* yielded hyoscyamine with the totality of its activity in the carboxyl carbon of tropic acid.

In the present paper experiments are reported in which the validity of the following three hypotheses for tropic acid biosynthesis was determined: (*a*) Acetate, an effective precursor of isoprenoid compounds might also be expected to become incorporated into tropic acid via the terpene pathway suggested by Trautner (8). (*b*) The aromatic branched-chain structure of tropic acid may be formed through the condensation of shikimic acid with the alpha carbon of a 3-carbon acid such as lactate or propionate. (*c*) Tropic acid may be formed by the attack of such an "active" 1-carbon compound as formate or the beta carbon from serine at the alpha carbon of phenylacetic acid or its coenzyme derivative (derived by oxidative decarboxylation of the transaminated product of phenylalanine, *viz.*, phenylpyruvic acid).

## EXPERIMENTAL

**$C^{14}$-Sample Preparation and Counting.**—Radioactive samples were assayed by combustion of the material to carbon dioxide and converting the evolved gas to barium carbonate. The Van Slyke-Folch wet combustion mixture, prepared according to Calvin, *et al.* (12), was employed in an apparatus similar to that described by Claycomb, *et al.* (13). Barium carbonate samples were counted in a windowless flow counter at a finite thickness and the activity corrected to infinite thinness.

**Cultivation of Plants.**—*Datura stramonium* plants were grown in soil until the most mature plants bore flower buds (44 days). The roots of 24 plants of uniform size and appearance (no flower buds appearing) were washed free of soil and the plants placed in hydroponic culture. After the plants had remained in the hydroponic culture for 1 week, the roots of each plant were removed to within approximately $^1/_8$ inch of the crown. The purpose of the root excision was to provide a soil-free root system wherein a high rate of alkaloid synthesis might be expected to occur in the abundant quantity of young, actively metabolizing root tissue which formed during the following 10 days.

**Administration of $C^{14}$-Labeled Compounds to Plants.**—At the end of the 10-day root regeneration

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period, the $C^{14}$-labeled compounds were administered to the plants. Eight groups of three plants each were employed as follows: one group was selected as a control and as such received no radioactive compounds; the remaining seven groups were separately fed the following radioactive compounds, purchased from commercial sources: *dl*-phenylalanine-3-$C^{14}$, phenylacetic acid-1-$C^{14}$, sodium acetate-2-$C^{14}$, sodium propionate-2-$C^{14}$, sodium formate-$C^{14}$, *dl*-serine-2-$C^{14}$, and *dl*-zinc lactate-2-$C^{14}$.

The plants were individually placed in small beakers containing equal quantities (0.07 m*M* per plant) of the $C^{14}$-labeled compounds dissolved in 2 to 3 ml. of water. The illuminated plants absorbed their respective solutions within 20 minutes; the last traces were washed into the plant by addition of at least 12 successive 2-ml. portions of water to the beaker as it approached dryness. The plants were then transferred to hydroponic culture and were allowed to metabolize in a greenhouse under ordinary conditions of sunlight for a further period of 10 days.

**Isolation and Purification of Alkaloids.**—The plant material was dried at 65° for 36 hours and then ground in a semimicro Wiley mill to a No. 40 powder. The powdered material was extracted with ether in a Soxhlet apparatus according to the method described for this plant in the N.F. X (14). Further purification of the combined alkaloids was effected by extraction of the bases from the ethereal solution into acidulated water and then into chloroform. Hyoscyamine was separated from scopolamine by countercurrent liquid-liquid extraction using a phosphate buffer (0.2 *M*, pH 6.8) and chloroform as the two phases (15). Final purification of the individual alkaloids was carried out on Celite[1] columns after the method of Evans and Partridge (16). The amount of column-purified alkaloid was determined by colorimetric assay (17).

**Hydrolysis of Alkaloids.**—Those alkaloids possessing sufficient radioactivity to warrant further study were hydrolyzed to tropine or scopine and tropic acid in order to determine the distribution of $C^{14}$ between these two portions of the alkaloids.

Sufficient nonradioactive alkaloid was added to the column-purified alkaloids to provide a sample of known weight between 100 and 150 mg. Complete hydrolysis was accomplished by dissolving the alkaloid in 35 ml. of water to which was added 10 ml. of 2 *N* sodium hydroxide and heating the mixture on a water bath for 2 hours. Tropine or scopine was extracted from the hydrolysate with five 25-ml. portions of chloroform. Following acidification of the hydrolysate, tropic acid was recovered by extraction with several portions of a mixture of isopropanol (1 part) and chloroform (3 parts).

Evaporation of the isopropanol-chloroform mixture yielded a light brown residue from which tropic acid was recovered by sublimation at 114–115° and 1–2 mm. Hg pressure. Crystallization of the sublimed acid gave pure tropic acid, m.p. 117–118°; yield based on the alkaloid was 80% or more.

Tropine was purified by formation of its picrate derivative and recrystallization of tropine picrate from water, m.p. 290–293°; yield 30 to 40% based on hyoscyamine. Repeated attempts to form the picrate of scopine failed to provide a derivative with a sharp melting point.

**Degradation of Tropic Acid.**—Two methods of degradation were employed. The first involved permanganate oxidation of tropic acid to benzoic acid while the second involved the decarboxylation of the branched-chain acid and recovery of the liberated carbon dioxide.

Tropic acid (30 to 50 mg.) was refluxed with 250 mg. of potassium permanganate and 50 mg. of sodium hydroxide in 5 ml. of water for 2 hours. The hot solution was filtered, acidified, and then extracted with successive 20-ml. portions of ether. Removal of the solvent left a light brown residue which, on sublimation, yielded 10 to 15 mg. of long, colorless crystals of benzoic acid, m.p. 122°.

Decarboxylation of tropic acid was accomplished by refluxing 10 to 20 mg. of the acid with 10 mg. of cupric oxide in 3 ml. of quinoline for 1 hour. The reaction was carried out in an atmosphere of nitrogen and the evolved carbon dioxide was collected in carbon dioxide-free sodium hydroxide. The yield of carbonate ranged from 95 to 110% of the theoretical after allowance was made for the carbonate which formed when appropriate blanks were employed.

The styrene and/or polystyrene, which would be expected to be formed as a result of tropic acid decarboxylation, was recovered from the reaction mixture in the following manner. The quinoline solution was acidified and then extracted with seven successive 10-ml. portions of ether. The combined ether extract was washed several times, first with acidulated water and then with dilute sodium hydroxide. Evaporation of the washed ether extract left a brown residue.

## RESULTS

The relative effectiveness of the administered compounds as precursors of hyoscyamine and scopolamine can be seen by comparing the dilution values given in Table I. Phenylalanine, phenylacetic acid, and sodium acetate, which gave hyoscyamine and scopolamine showing the least dilution of $C^{14}$, were the best alkaloid precursors of the seven compounds tested. Sodium propionate, sodium formate, and serine were less efficient precursors, while the radioactive carbon from zinc lactate was only slightly incorporated into the alkaloids. The similarity in dilution values for the two alkaloids, upon administration of any one precursor, suggests that both hyoscyamine and scopolamine were being formed at the same time within the plants.

Tables II and III show the distribution of $C^{14}$ within the alkaloids as determined by the specific activities of the hydrolysis products, tropic acid and tropine. The specific activity for scopine could not be determined due to the questionable composition of its picrate derivative and the specific activity values listed for this base have been derived by calculation. It is apparent from the results that, of the seven compounds administered to the plants, only phenylalanine and phenylacetic acid were precursors of tropic acid specifically. The radioactivity derived from sodium propionate also occurred to a limited extent in the acid but the majority of its activity, like that of acetate, formate, and serine, was located in tropine or scopine.

[1] A product of Johns-Manville, Celite Division, New York, N. Y.

TABLE I.—COMPARISON OF $C^{14}$-LABELED COMPOUNDS AS PRECURSORS OF ALKALOIDS IN *Datura Stramonium*

| Compound Administered | Amount Used, $\mu M$/Gm. Dried Plant | Specific Activity, c.p.m. × $10^5$/m$M$ | Specific Activity of Alkaloids, c.p.m. × $10^5$/m$M$ Hyoscyamine | Specific Activity of Alkaloids, c.p.m. × $10^5$/m$M$ Scopolamine | Dilution Values Hyoscyamine | Dilution Values Scopolamine |
|---|---|---|---|---|---|---|
| *dl*-Phenylalanine-3-$C^{14}$ | 7.33 | 1,380 | 9.73 | 7.30 | 142 | 189 |
| Phenylacetic acid-1-$C^{14}$ | 9.90 | 292 | 1.24 | 1.43 | 235 | 204 |
| Sodium acetate-2-$C^{14}$ | 8.70 | 1,160 | 12.22 | 9.68 | 97 | 120 |
| Sodium propionate-2-$C^{14}$ | 9.23 | 358 | 0.99 | 0.78 | 361 | 459 |
| Sodium formate-$C^{14}$ | 6.58 | 750 | 1.45 | 0.97 | 517 | 772 |
| *dl*-Serine-3-$C^{14}$ | 7.32 | 1,525 | 1.30 | 1.08 | 1,173 | 1,413 |
| *dl*-Zinc lactate-2-$C^{14}$ | 12.00 | 352 | 0.03 | 0.01 | 12,000 | 25,500 |

TABLE II.—DISTRIBUTION OF $C^{14}$ IN HYOSCYAMINE

| Compound Administered | Specific Activity of Hydrolysis Products, c.p.m./m$M$ × $10^5$ Tropic Acid | Tropine | % Activity[a] Found in Hydrolysis Products Tropic Acid | Tropine |
|---|---|---|---|---|
| *dl*-Phenylalanine-3-$C^{14}$ | 9.60 | 0.00 | 100 | 0 |
| Phenylacetic acid-1-$C^{14}$ | 1.02 | 0.00 | 100 | 0 |
| Sodium acetate-2-$C^{14}$ | 0.40 | 8.55 | 5 | 95 |
| Sodium propionate-2-$C^{14}$ | 0.14 | 0.85 | 14 | 86 |
| Sodium formate-$C^{14}$ | 0.05 | 1.35 | 4 | 96 |
| *dl*-Serine-3-$C^{14}$ | 0.06 | 1.40 | 4 | 96 |
| *dl*-Zinc lactate-2-$C^{14}$ | [b] | [b] | [b] | [b] |

[a] % Activity = $\frac{\text{specific activity of hydrolysis product}}{\text{sum of specific activities of both hydrolysis products}} \times 100$.

[b] Insufficient material or radioactivity to permit hydrolysis.

TABLE III.—DISTRIBUTION OF $C^{14}$ IN SCOPOLAMINE

| Compound Administered | Specific Activity of Hydrolysis Products, c.p.m./m$M$ × $10^5$ Tropic Acid | Scopine[a] | % Activity[b] Found in Hydrolysis Products Tropic Acid | Scopine[a] |
|---|---|---|---|---|
| *dl*-Phenylalanine-3-$C^{14}$ | 7.32 | 0.00 | 100 | 0 |
| Phenylacetic acid-1-$C^{14}$ | 1.61 | 0.00 | 100 | 0 |
| Sodium acetate-2-$C^{14}$ | 0.24 | 9.43 | 2 | 98 |
| Sodium propionate-2-$C^{14}$ | [c] | [c] | [c] | [c] |
| Sodium formate-$C^{14}$ | 0.06 | 0.91 | 7 | 93 |
| *dl*-Serine-3-$C^{14}$ | 0.04 | 1.04 | 4 | 96 |
| *dl*-Zinc lactate-2-$C^{14}$ | [c] | [c] | [c] | [c] |

[a] Calculated by difference of specific activities of alkaloid and tropic acid.

[b] % Activity = $\frac{\text{specific activity of hydrolysis product}}{\text{sum of specific activities of both hydrolysis products}} \times 100$

[c] Insufficient material or radioactivity to permit hydrolysis.

While all of the tropic acid obtained following hydrolysis of the alkaloids possessed radioactivity, only four samples were sufficiently active to be considered for degradation; these were obtained when phenylalanine and phenylacetic acid were used as precursors. The original hypothesis concerning the biosynthesis of tropic acid from phenylalanine suggested that the carbon skeleton of this amino acid would go to form the phenyl, alpha, and carboxyl carbons of tropic acid. On this premise, the activity within tropic acid should be located in the carbon adjacent to the benzene ring since phenylalanine-3-$C^{14}$ was employed as the precursor. This hypothesis is supported by the results of permanganate oxidation of tropic acid to benzoic acid (Table IV) wherein essentially all of the activity present in the tropic acid obtained from both alkaloids was contained in the benzoic acid.

The tropic acid recovered following administration of phenylacetic acid was decarboxylated since, according to the starting hypothesis, the $C^{14}$ from

TABLE IV.—DEGRADATION OF TROPIC ACID

| Compounds Administered | Alkaloid Yielding Tropic Acid | Specific Activity, c.p.m./m$M$ Tropic Acid | Specific Activity, c.p.m./m$M$ Benzoic Acid |
|---|---|---|---|
| *dl*-Phenylalanine-3-$C^{14}$ | Hyoscyamine | 9.60 × $10^5$ | 9.23 × $10^5$ |
| *dl*-Phenylalanine-3-$C^{14}$ | Scopolamine | 7.32 × $10^5$ | 7.05 × $10^5$ |

TABLE V.—RADIOACTIVITY PRESENT IN DECARBOXYLATED PRODUCTS OF TROPIC ACID FROM PHENYLACETIC ACID-1-$C^{14}$

| Alkaloid Containing Tropic Acid | Radioactivity Present c.p.m. Tropic Acid Prior to Decarboxylation | Radioactivity Present c.p.m. Decarboxylated Products | % Radioactivity Present in Decarboxylated Products |
|---|---|---|---|
| Hyoscyamine | 6,740 | 5,470 | 81 |
| Scopolamine | 11,060 | 8,400 | 76 |

phenylacetic acid-1-$C^{14}$ should be present in the carboxyl carbon of tropic acid. However, the liberated carbon dioxide, collected and counted as barium carbonate, proved to be nonradioactive. The decarboxylation products, believed to be styrene and polystyrene, were recovered from the reaction mixture and their total activity determined. Table V shows the radioactivity recovered in these styrene products expressed as a percentage of the total radioactivity present in the tropic acid samples prior to decarboxylation. While most of the radioactivity was recovered, failure to obtain complete recovery is believed to be due to incomplete extraction of the degradation products into ether and also incomplete decarboxylation.

## DISCUSSION

In view of Leete's success (10), as well as that reported here, in isolating both radioactive hyoscyamine and scopolamine, it would now appear that the failure of earlier workers (2, 3) to obtain radioactive scopolamine in the presence of $C^{14}$-labeled hyoscyamine was a function of the maturity of the plants in relation to the time of precursor administration. Preliminary experiments to those reported in this paper have shown that hyoscyamine contained most, if not all, of the $C^{14}$ within the two alkaloids when these same seven radioactive compounds were administered to mature *D. stramonium* (plants bearing fruit). The failure to obtain radioactive scopolamine in the presence of $C^{14}$-labeled hyoscyamine using mature *D. stramonium* plants has been demonstrated by Romeike and Fodor (18).

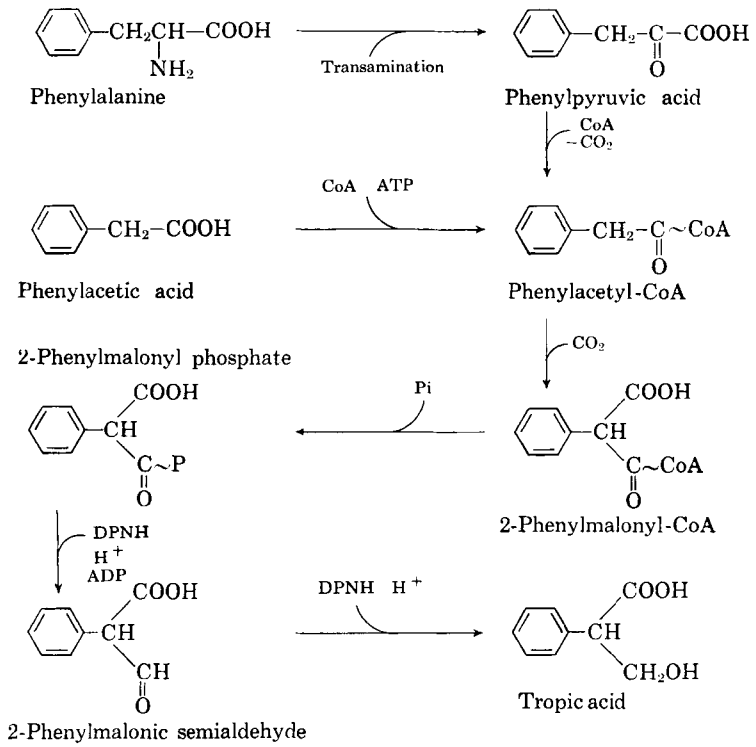


Fig. 1.—Proposed pathway for tropic acid biosynthesis.

The foregoing results provide no evidence which would support two of the proposed hypotheses for tropic acid biosynthesis, namely, the participation of acetate via the isoprene pathway suggested by Trautner (8) and the condensation of shikimic acid with lactate or propionate. The third hypothesis involved the formation of phenylacetic acid or its coenzyme derivative from phenylalanine followed by a 1-carbon attack at its alpha carbon by formate or the beta carbon of serine. This hypothesis has been substantiated in part by the results obtained in that both phenylalanine-3-$C^{14}$ and phenylacetic acid-1-$C^{14}$ were efficient precursors of tropic acid.

Two major discrepancies in the experimental results and the third hypothesis were met. First, the activity derived from formate-$C^{14}$ and serine-3-$C^{14}$ was not found in tropic acid but rather in the tropane moiety of the alkaloids. The second discrepancy, and the one which has provided the greatest interest and speculation, was that the radioactivity present in tropic acid derived from phenylacetic acid-1-$C^{14}$ was not in the carboxyl group as was expected but is believed to have been present in the hydroxymethyl carbon.

These findings have led us to propose the following pathway for tropic acid biosynthesis (Fig. 1) which, in addition to accounting for the experimental findings, suggests the direct participation of carbon dioxide in the formation of the branched chain. The essential points in this proposal are as follows: (*a*) the addition of carbon dioxide to phenylacetyl-Co.A in a manner analogous to the formation of malonyl-Co.A from acetyl-Co.A in fat biosynthesis (19); (*b*) reduction in two stages of 2-phenylmalonyl-Co.A to tropic acid.

Degradative studies were not carried out to ascertain the location of the $C^{14}$ which was predominantly incorporated into tropine and scopine upon administration of formate, serine, acetate, and propionate; hence, the full significance of these results is not possible to determine. It was not surprising that the $C^{14}$ derived from formate-$C^{14}$ and serine-3-$C^{14}$ was incorporated into these bases since these compounds are known precursors of N-methyl groups in alkaloids. Leete (10) administered formaldehyde-$C^{14}$ and formate-$C^{14}$ to *D. stramonium* in addition to phenylalanine-3-$C^{14}$, and found that the activity from formate did in fact reside in the N-methyl of hyoscyamine. Acetate-2-$C^{14}$ and propionate-2-$C^{14}$ were very efficient precursors of tropine and scopine. Wenkert (9) and Birch (20), have suggested acetate as a possible precursor of the "acetone" moiety of tropine and scopine. Recently, Kaczkowski, Schütte, and Mothes (4, 5) have clearly demonstrated the participation of acetate in the formation of the tropine portion of hyoscyamine. Degradation of the alkaloid showed that the radioactivity, derived from acetate-1-$C^{14}$, was in carbon atom 3 while that derived from acetate-2-$C^{14}$ was in carbon atoms 2 and 4. A further possibility of the location of $C^{14}$ from both acetate-2-$C^{14}$ and propionate-2-$C^{14}$ stems from work on the biosynthesis of nicotine. Griffith and Byerrum (21) found that, following administration of these similarly labeled compounds to *Nicotiana rustica*, half of the $C^{14}$ within nicotine was located in the pyrrolidine ring.

Work is continuing to provide more definitive information concerning the participation of propionate and carbon dioxide in the biosynthesis of hyoscyamine and scopolamine.

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# Influence of Nonequilibrium Sample Temperature on Stability Predictions Extrapolated from Elevated Temperature Studies

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**Assay results for a single sample stored in an elevated temperature oven for a known period of time may be in error if instantaneous heating and cooling of the sample is not achieved. For usual kinetic studies however, the storage behavior of a group of samples which have been placed in the oven at the same time is followed. Theoretical and experimental considerations are presented to show that in the case of apparent first-order reactions the assay values for all samples in the group are subjected to the same degree of error. Rates of degradation calculated from the assay data are identical to those measured under isothermal conditions. Consequently, decomposition rates obtained under the usual oven storage conditions, where time lags in heating and cooling samples exist, give useful information for estimating product stability.**

CONSIDERABLE PROGRESS has been made in recent years in applying chemical kinetic principles for the prediction of the shelf life of pharmaceutical products. The techniques employed are gaining general acceptance in the pharmaceutical industry and have been the subject of an increasing number of literature reports.

In one early investigation, Garrett and Carper (1) predicted the color stability of a multisulfonamide preparation using colorimetric measurements of samples subjected to thermally accelerated degradation. Later, Swintosky, *et al.* (2), used chemical kinetics in predicting the shelf life of oral penicillin G procaine suspensions. In another report, Garrett (3) examined the thermal degradation of ascorbic acid, vitamin $B_{12}$, folic acid, and other components of a multivitamin preparation. The author was able to arrive at satisfactory estimates of room temperature stability from data obtained under accelerated test conditions. McLeod (4) used a similar approach to predict expiration dates for other multivitamin preparations.

These reports and others on the subject (5–7) have suggested a simple experimental procedure for the evaluation of product stability. The procedure generally followed involves storing the product, preferably packaged in its final market container, in three or four individual ovens maintained at constant elevated temperature. Periodically samples are removed from these ovens and assayed. The loss of active ingredient is calculated, and rates of decomposition for the

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