Production of Scopolamine and Hyoscyamine by *Datura stramonium* L. Suspension Cultures

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Abstract □ The production of radioactive scopolamine and hyoscyamine in tissue suspension cultures of *D. stramonium*, *D. tatula*, and *Nicotiana tabacum* to which tropine and α-^{14}C-tropic acid was administered is described. The highest yields of incorporation of radioactive tropic acid into scopolamine and hyoscyamine were obtained in 3-month old suspension cultures of *D. stramonium* and *N. tabacum*. Older cultures of *D. stramonium* appear to lose much of their ability to synthesize these two alkaloids. The amount of ^{14}C-scopolamine produced exceeded the formation of ^{14}C-hyoscyamine. Several other metabolic products of α-^{14}C-tropic acid were also detected.

Keyphrases □ Tropane alkaloid production—*Datura Stramonium* tissue suspension cultures □ Scopolamine, hyoscyamine—tropine, ^{14}C-tropic acid incorporation □ Paper chromatography—separation, identification □ TLC—separation, identification □ Scintillometry, liquid—radioactivity analysis

Tropane alkaloids have been reported to be produced in tissue cultures of *Atropa* (1), *Datura* (1–6), *Hyoscyamus* (7), and *Scopolia* (8). Although *Datura stramonium* callus and suspension cultures have been produced and examined for alkaloids, no one has reported the presence of scopolamine (hyoscine) or hyoscyamine. Only total alkaloids assayed spectrophotometrically (2), or the minor alkaloids choline, pseudotropine, cuscohygrine (6) have been reported.

The most common procedures for the detection and quantitation of tropane alkaloids have been the methods of Colby and Beal (9), Vitali-Morine as modified by Freeman (10), and the use of modified Dragendorff's reagent (6). The application of radioisotopically labeled precursors to follow and verify the biosynthesis of tropine alkaloids by plant tissues in aseptic culture has largely been neglected. Romeike and Aurich (4, 5) have incubated *Datura innoxia* and *D. stramonium* root cultures with tropine (N-$^{14}CH_3$), obtaining approximately 15% incorporation of the radioactivity into acetyltropine, with less than 1% each into scopolamine and hyoscyamine. The administration of ^{14}C-acetate to sterile root cultures of *D. metel* led to the formation of ^{14}C-hyoscyamine (11).

The reaction involving the esterification of tropine and tropic acid to hyoscyamine has been demonstrated in intact plants (12–14). The enzymatic synthesis and hydrolysis of hyoscyamine *in vitro* has also been shown employing homogenates and partially purified fractions of young leaves, pericarps, and flowers of *D. stramonium* (15).

The purpose of this study was to investigate the ability of *Datura* suspension cultures to produce alkaloids following the addition of tropine and α-^{14}C-tropic acid.

EXPERIMENTAL

Tissue Cultures—The principal culture studied was *Datura stramonium* L., strain 5450, which was established in June 1963, subcultured to liquid medium in June 1966, and maintained as such (6). In addition, 3-month old seed callus cultures of *Datura tatula*, *Nicotiana tabacum*, and *D. stramonium* strain 312 were transferred to liquid medium and maintained at least 3 months as suspension cultures prior to their use. The tissues were grown in 250-ml. conical flasks which contained 50 ml. of revised tobacco medium (16) and 0.1 p.p.m. of 2,4-dichlorophenoxyacetic acid (2,4-D). They were maintained by transferring at 4-week intervals.

Incubations—Ten fourteen-day old cultures, initially inoculated with tissue corresponding to approximately 70 mg. dry weight, were each given 20 mg. tropine and 1 mcg. α-^{14}C-tropic acid[1] in 0.1 ml. 70% ethanol. The isotope had a specific activity of 1.06 mc./m*M*. All cultures were incubated for 2 weeks at room temperature on a reciprocating shaker in a hood, except one *Datura stramonium* experiment, the growth and incubation of which was continued for 4 weeks.

Alkaloid Extraction and Analysis—The dried tissues were extracted as previously described (6). Extracts (100–250 μl.) were co-chromatographed with a standard mixture of scopolamine, tropine, hyoscyamine, and choline on 2 × 58 cm. strips of Whatman No. 3 paper. The chromatograms were developed for 30 hr. by descending chromatography using the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25) (17). The chromatograms were either dipped or sprayed with modified Dragendorff's reagent (6), allowing the determination of R_f values for standard and unknown alkaloids. The developed 2-cm. wide chromatographic strips were then cut into twenty-nine 2-cm. lengths, and each 2-cm. square was placed in a glass liquid scintillation counting vial. Bray's counting solution (18) was added to each vial (15 ml.), and the radioactivity was measured by liquid scintillation spectrometry[2] after allowing the samples to stand overnight. Preliminary studies revealed that Bray's counting solution extracted 100% of the radioactivity from the paper chromatograms while toluene and dioxane counting solutions were less efficient. Each sample was counted for 20 min., and the results were calculated as counts per minute above a background control.

Selected extracts were also examined by TLC on cellulose (19) and Silica Gel G (8). The developing solvents were the organic phase of isobutanol–concentrated HCl–water (7:1:2), and 70% ethanol–25% ammonia (99:1), respectively. The alkaloids were detected by spraying with modified Dragendorff's reagent, the zones were removed from the plates, and radioactivity again determined by liquid scintillation. Bromophenol blue (0.04%) in ethanol was used to determine the location of tropic acid on both paper and thin-layer chromatograms.

RESULTS AND DISCUSSION

The minor alkaloids choline, cuscohygrine, and pseudotropine have previously been reported in *D. stramonium* strain 5450 (6). The presence of scopolamine and hyoscyamine have now been detected in this strain using α-^{14}C-tropic acid (Table I). The values given in Table I are expressed as percents of the total extractable ^{14}C with the standard deviations. Both the ^{14}C-hyoscyamine and ^{14}C-scopolamine are metabolized with time to other products. The subsequent metabolism of these tropane alkaloids is not surprising in light of the recent work of Fairbairn and Wassel (20) who found evidence for a rapid turnover of atropine in *Atropa belladonna*. Changes in the alkaloid content of *D. innoxia* have also been recently reported (21). These latter authors noted a gradual increase in hyoscyamine with a pronounced decrease in scopolamine with increased age of the plants (21).

[1] Tracer Labs, Waltham, Mass.
[2] Beckman model LS 100.

Table I—α-^{14}C-Tropic Acid Incorporation by *Datura Stramonium* 5450 in Suspension Culture after 2 and 4 Weeks

	R_f[a] Value	% of Extractable ^{14}C Given as α-^{14}C-Tropic Acid			
		Tissue		Medium	
		2 weeks	4 weeks	2 weeks	4 weeks
Hyoscyamine	0.81	0.20 ± 0.08%[b]	0.08 ± 0.02%	11.0 ± 2.1%	25.7 ± 12.4%
Scopolamine	0.65	0.34 ± 0.14%	0.05 ± 0.01%	8.9 ± 3.9%	2.7 ± 0.9%
Unknown	0.51	0.04 ± 0.01%	0.02 ± 0.02%	9.9 ± 3.9%	3.9 ± 2.3%
^{14}C Extracted/g. of tissue		(5.5 ± 0.85) × 10^5 c.p.m.	(8.0 ± 0.61) × 10^4 c.p.m.	—	—
^{14}C Extracted/50 ml. of medium		—	—	5750 ± 960 c.p.m.	2165 ± 435 c.p.m.
Tissue dry weight/flask		2.36 ± 0.33 g.	1.68 ± 0.41 g.	—	—

[a] The extracts were chromatographed on Whatman No. 3 paper, and developed by descending chromatography employing the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25). The radioactivity was determined by liquid scintillation counting as described under *Experimental*. [b] Each value represents the average of 12 determinations with the *SD*. The percentage values correspond to the percent of the total ^{14}C present in each fraction.

With a counting efficiency of approximately 50%, and assuming the specific activity of the ^{14}C-scopolamine to be identical to that of the α-^{14}C-tropic acid, the 0.34% production of labeled scopolamine at 2 weeks corresponds to 4.85 mcg./g. tissue.

The percentages of extractable ^{14}C in the medium present as hyoscyamine and scopolamine are quite high, being 11.0 and 8.9%, respectively, after 2 weeks of incubation of the α-^{14}C-tropic acid with *D. stramonium* strain 5450. However, the amount of medium/flask after 2 or 4 weeks incubation was less than the original 50 ml.

Table II—Chromatography of Tropane Alkaloids

	R_f Values in Three Chromatographic Systems		
	I Paper[a]	II Cellulose[b]	III Silica Gel G[c]
Choline	0.19	0.43	0.69
Tropine	0.43	0.65	0.76
Scopolamine	0.65	0.75	0.59
Hyoscyamine	0.81	0.89	0.17
Tropic acid	Solvent front	Solvent front	Origin

[a] Whatman No. 3 paper strips were developed by descending chromatography using the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25) (17). [b] Cellulose thin-layer plates were developed with the organic phase of isobutanol–concd. HCl–water (7:1:2) (19). [c] Silica Gel thin-layer plates were developed with 70% ethanol–25% ammonia (99:1) (8).

flask due to evaporation and tissue consumption. In addition, the amount of total radioactivity extractable from the medium was 1/150 to 1/800 of the quantity extracted from the tissues. Therefore, the amounts of hyoscyamine and scopolamine present in the medium were significantly lower than in the tissues.

It is apparent that the amount of ^{14}C which can be extracted from the tissues and medium decreases with time. The α-^{14}C-tropic acid is probably being metabolized to products which are nonextractable by the techniques employed. When flasks of cultures inoculated with α-^{14}C-tropic acid were flushed with oxygen for 2 weeks, and the expired CO_2 subsequently precipitated as the calcium salt, no $^{14}CO_2$ was detected. The lack of detectable $^{14}CO_2$ suggests that little or none of the side chain of α-^{14}C-tropic acid is being catabolized to CO_2.

Further evidence that the radioactive products isolated routinely by paper chromatography were indeed scopolamine and hyoscyamine was obtained by the use of two different thin-layer chromatographic systems (Table II). Cellulose plates developed with the organic phase of isobutanol–concentrated HCl–water (7:1:2) and Silica Gel plates developed with 70% ethanol–25% ammonia (99:1) were employed. The R_f values of choline, tropine, scopolamine, hyoscyamine, and tropic acid are given in Table II for the three systems. The R_f values of the extracted ^{14}C conversion products were identical to hyoscyamine and scopolamine in the three chromatographic systems.

Simazine (2-chloro-4,6-bis ethylamino-*s*-triazine) is an herbicide which at sublethal levels increases the growth and protein content of certain plant species (22). This increase in nitrogen content is associated with a marked increase in nitrate reductase activity (23). There have been no reports in the literature on the influence of simazine on the production of alkaloids, steroids, or other secondary plant products. It was found of interest to determine what effect simazine might have on alkaloid production by *D. stramonium* strain 5450 in suspension culture. As can be seen in Table III, the addition of 1 p.p.m. of simazine to the medium had no effect on the incorporation of α-^{14}C-tropic acid into the tropane alkaloids. A small inhibition in growth was noted as is indicated by the dry weights. Less radioactivity was extracted from both the tissues and medium of the simazine-treated cultures.

The *D. stramonium* tissue culture strain 5450 was started in 1963 and had grown continuously for 2 years in suspension culture. In order to determine whether this strain may have lost much of its ability to produce alkaloids or whether strain selection had occurred, a new strain of seed callus was initiated which was designated *D. stramonium* strain 312. This callus was transferred to suspension cultures after approximately 3 months. The results in

Table III—α-^{14}C-Tropic Acid Incorporation by *Datura Stramonium* 5450 Tissue in Suspension Culture in the Presence of Simazine

	R_f[a] Value	% of Extractable ^{14}C Given as α-^{14}C-Tropic Acid			
		Tissue		Medium	
		Control	Simazine (1 p.p.m.)	Control	Simazine (1 p.p.m.)
Hyoscyamine	0.81	0.18 ± 0.07%[b]	0.15 ± 0.08%	17.70 ± 12.5%	10.60 ± 6.8%
Scopolamine	0.65	0.30 ± 0.09%	0.20 ± 0.10%	9.10 ± 3.2%	5.45 ± 2.2%
Unknown	0.51	0.05 ± 0.02%	0.07 ± 0.03%	6.60 ± 4.3%	10.30 ± 2.6%
^{14}C Extracted/g. of tissue		(5.8 ± 0.88) × 10^5 c.p.m.	(3.4 ± 0.65) × 10^5 c.p.m.	—	—
^{14}C Extracted/50 ml. of medium		—	—	5270 ± 1080 c.p.m.	1860 ± 420 c.p.m.
Tissue dry weight/flask		2.51 ± 0.47 g.	2.06 ± 0.38 g.	—	—

[a] The extracts were chromatographed on Whatman No. 3 paper, and developed by descending chromatography employing the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25). The radioactivity was determined by the use of a liquid scintillation spectrometer as described under *Experimental*. [b] Each value represents the average of 12 determinations with the *SD*. The values correspond to the percent of total extractable ^{14}C present in each fraction.

Table IV—α-^{14}C-Tropic Acid Conversion Products by Tissue Suspension Cultures

Tissue	c.p.m. ^{14}C Extracted per g. Dry Weight	% of Extractable ^{14}C Given as α-^{14}C-Tropic Acid			
		R_f Values[a]			
		0.0	0.51	0.65 (Scopolamine)	0.81 (Hyoscyamine)
D. stramonium 312	$(2.5 \pm 0.31) \times 10^5$	5.3 ± 3.3%[b]	12.7 ± 11.0%	20.7 ± 16.1%	6.2 ± 3.0%
Datura tatula	$(2.05 \pm 0.15) \times 10^5$	3.6 ± 0.6%	—[c]	2.1 ± 1.1%	1.1 ± 0.5%
Nicotiana tabacum	$(5.6 \pm 1.0) \times 10^5$	9.2 ± 9.7%	—[c]	27.0 ± 7.7%	2.8 ± 1.5%

[a] The extracts were chromatographed on Whatman No. 3 paper, and developed by descending chromatography employing the organic phase of *n*-butanol–glacial acetic acid–water (50:3:25). The radioactivity was determined by liquid scintillation spectrometry as described under *Experimental*. [b] Each value represents the average percent conversion of the extracted ^{14}C with the *SD*. Each value is the average of six determinations. [c] No metabolite was detected.

Table IV indicate that *D. stramonium* strain 312 is capable of producing much higher levels of both hyoscyamine and scopolamine from α-^{14}C-tropic acid as compared to strain 5450. In addition to the unknown material with an R_f of 0.51 which is produced by both strains, strain 312 is able to convert the labeled tropic acid to a material(s) which remains at the origin of the paper chromatographic system. The amounts of all conversion products varied greatly among the six replicates, although the average values are 30 to 60 times larger for strain 312 as compared to strain 5450. These results suggest that it is possible for tissue cultures of *D. stramonium* to lose their alkaloid-producing ability with time. An alternate explanation is that further strain selection occurred during routine transfer of strain 5450 over the 4-yr. period.

The levels of conversion of α-^{14}C-tropic acid to scopolamine and hyoscyamine by *D. tatula* was 1–2%, much lower than by *D. stramonium* strain 312, but still five to six times higher than the level produced by *D. stramonium* strain 5450 (Table IV).

In order to determine the specificity of the esterase (synthetase) enzyme responsible for the formation of scopolamine and hyoscyamine, 3-month old suspension cultures of *Nicotiana tabacum* were incubated with 20 mg. tropine and 1 mcg. α-^{14}C-tropic acid. Surprisingly, 27% of the extracted ^{14}C is present as scopolamine with only 2.8% present as hyoscyamine. These results suggest that the esterase enzyme involved in the condensation of tropane and tropic acid to hyoscyamine is not specifically restricted to the genus *Datura*, nor apparently is the enzyme that is responsible for the formation of the ethylene oxide bridge on the tropane moiety giving rise to scopolamine from hyoscyamine.

In all experiments one observes that the amount of scopolamine exceeds the amount of hyoscyamine. Netien and Combet (3) have reported similar results from suspension cultures of *D. metel*, obtaining 4.1 times as much scopolamine as hyoscyamine. Similar results have been alluded to by other investigators (2, 24). One might not expect such results if one assumes that the biosynthesis of hyoscyamine precedes that of scopolamine as is believed to be the case (25). In the intact *D. stramonium* plant, the concentration of hyoscyamine is usually greater than the scopolamine concentration. For example, the hyoscyamine content of leaves, stems, and roots are approximately 0.4, 0.2, and 0.1%, respectively, while typical amounts of scopolamine in leaves, stems, and roots are 0.01, 0.05, and 0.1%, respectively (26). Very young plants have been reported to produce as much as 0.2% scopolamine (26).

The results which were obtained are not an artifact of the extraction procedure based on the addition of hyoscyamine to dried *D. stramonium* tissues prior to extraction, since no positive spots corresponding to scopolamine were detected following paper chromatography. Several possible explanations for these results exist: (*a*) if the enzymatic formation of the ester linkage between tropine and tropic acid to give hyoscyamine proceeds more slowly than does the formation of the ethylene oxide bridge on the tropine moiety of hyoscyamine, one might reasonably expect to observe a greater accumulation of scopolamine than hyoscyamine in the authors' cultures, and (*b*) the tissue cultures employed were derived from seeds, their exact morphological origin is not known, and as such they may be derived from roots or have the enzymatic composition of young plants, both of which may produce higher concentrations of scopolamine than hyoscyamine.

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