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Keyphrases

Aminophylline suppositories—*in vivo* absorption rate
Nonionic surfactant effect—*in vivo* absorption rate
Blood levels—aminophylline from suppositories
UV spectrophotometry—analysis

Datura Tissue Cultures: Production of Minor Alkaloids from Chlorophyllous and Nonchlorophyllous Strains

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The highest medium concentration of manganese used (14 p.p m.) appeared to stimulate the production of cuscohygrine, choline, and pseudotropine from *Datura stramonium* L., strain 5450, suspension cultures. Amino acid precursors, or chlorophyllous tissue had no significant effect upon the production of these alkaloids. No hyoscyamine or scopolamine was detected. Significant amounts of *Datura* suspension tissues were grown in multiliter fermentors.

RECENTLY, ALKALOIDS have been reported produced by *Catharanthus* (1), *Conium* (2), *Datura* (3), *Ipomoea* (4), *Nicotiana* (5), and *Rauwolfia* (6) tissue cultures. In the past a number of investigators have studied alkaloid production by plant tissue or organ cultures, and their results are comprehensively discussed in review articles (7–9). Plant tissue cultures have been used to biochemically alter alkaloids (10) and cardenolides (11).

The tropane alkaloids are reportedly produced by *Datura* (3, 12, 13) and *Hyoscyamus* (14) callus tissue cultures, and to be potentiated by the addition of certain amino acids to the medium (13). The tropane alkaloids may also be degraded and metabolized by microorganisms (15). Manganese ion supplements stimulated alkaloid production and arginase activity in both sand and field cultures of *Datura stramonium* plants (16).

The principal objective of this study was to determine the nature of the alkaloids present in *Datura* suspension cultures and if they might be increased or modified by altering the manganese ion concentration in the medium, by adding tropane alkaloid precursors to the medium, or by inducing chloroplast formation.

EXPERIMENTAL

Tissue Cultures—The *Datura* tissue culture principally studied was 36 month-old seed callus of *Datura stramonium* L., strain 5450. This culture was established in June 1963 (13) from plants believed to be high in alkaloid content and subcultured to the liquid medium in June 1966. The long-term experiment shown in Table I represents seven continuous subcultures of this strain which were grown as previously described (13, 17).

Forty-one month-old seed callus of *D. stramonium* (13); 36 month-old seed callus of *D. quercifolia* L., strain 52146 and *D. innoxia* Mill. (13); and 4 month-old seed callus of *D. stramonium* (seed origin: Drug Plant Greenhouse, Univ. of Nebraska) were grown as suspension cultures for approximately 3 months, and subsequently the suspension cultures were analyzed for their alkaloid content. A continuous light period was provided (150 ftc. from 40-w. cool-white, fluorescent tubes; Amplex Corp.) to establish and maintain chlorophyllous suspension cultures (*D. stramonium* L., strain 5450).

Three 7.5-L. fermentors (model FS-300, New Brunswick Sci. Co., New Brunswick, N. J.) containing 3.4 L. of medium with 14 p.p.m. of manganese were each inoculated with 12 day-old suspension

Received April 28, 1967, from the College of Pharmacy, University of Nebraska, Lincoln NB 68508
Accepted for publication December 21, 1967.
Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by a National Science Foundation senior foreign scientist fellowship (FY-1965) to Dr. A. Jindra, and by grant GM 13440-01 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.
The authors are indebted to Mr. Keith W. Johnson for technical assistance.
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cells (approx. 5 Gm. dry wt.). The tissues were grown in the fermentors for 21 days at 28°, and each fermentor received an aeration rate of 1 L. of sterile air per min. and an agitation rate of 80 r.p.m.

Alkaloid Analysis—Approximately 2 Gm. of dry tissue was finely ground and mixed in a porcelain, dish with 1.5 ml. of ammonium hydroxide (28%), 2.0 ml. of ethanol (95%), and 4.0 ml. of ethyl ether. After 12 hr. the dried material was extracted with chloroform in a Soxhlet apparatus for 24 hr. The chloroform extract was evaporated *in vacuo* and the residue redissolved in chloroform. The resultant solution was placed in a glass tube and the solvent removed using infrared heat. The residue was then mixed with 0.50 ml. of 0.1 *N* HCl (in 90% ethanol) and the mixture centrifuged.

Extracts (100–200 μl.) were applied to Whatman paper No. 3 and developed by descending chromatography using the organic phase of the mixture, *n*-butanol–glacial acetic acid–water (50:3:25) (18). The alkaloids were detected by dipping the chromatograms in modified Dragendorff's reagent.[1] The R_f values obtained for the standards were: cuscohygrine[2] 0.08, choline 1.17, pseudotropine[2] 0.29, tropine 0.36, scopolamine 0.50, hyoscyamine 0.69. The alkaloids in the sample were identified by co-chromatography with the standard alkaloids.

Some extracts were applied to Whatman paper No. 3 impregnated with 0.5 *M* KCl and developed in *n*-butanol–concentrated hydrochloric acid (98:2) saturated with water (19). Alkaloid detection was achieved as described above.

Concentrations of alkaloids were estimated either by eluting the Dragendorff-positive zone from the chromatogram with acetic anhydride and photometrically comparing the resultant color with standard hyoscyamine concentrations (20), or by comparing the Dragendorff-positive zone with known concentrations of alkaloids on the chromatogram.

Amino Acid Analysis—The tissue (10 Gm. wet wt.) was ground with washed sand and extracted with 70% ethanol in a conical flask for several hours. This extraction process was repeated five times. The combined extracts (approximately 150 ml.) were filtered and evaporated to dryness *in vacuo*. The residue was dissolved in 5.0 ml. of 70% ethanol.

The amino acid extracts (200 μl.) were analyzed on Whatman paper No. 1 by two-dimensional chromatography[3] (21). The first solvent system was a mixture of phenol–ethanol–chloroform (2:1:1) with 0.1% of oxime. To each liter of this mixture was added 100 ml. of 25% ammonium hydroxide solution. Tropeolin 000 was added to the amino acid mixture to indicate the extent of amino acid resolution. The paper was allowed to develop for approximately 48 hr. After drying at normal room temperature, the chromatograms were developed twice in the organic phase of the second solvent system, *n*-butanol–glacial acetic–water (10:1:3).

The analysis of basic amino acids, especially arginine and ornithine, was achieved by purifying and concentrating a sample through a system of ion exchangers. An Amberlite IR 120 (H^+) column (23.8 Gm. wet wt.) was prepared, and after applying the extract, it was eluted with 25–30 ml. of 1 *N* NH_4OH. The eluate was evaporated to dryness at 50–55°, and the resultant residue dissolved in 5.0 ml. of water. This solution was applied to an Amberlite IRA 400 (CO_3^{-2}) column (11.8 Gm. wet wt.) and the effluent collected and evaporated to dryness at 50–55°. The residue was dissolved in 2.0 ml. of water and either 100 or 200 μl. applied to Whatman paper No. 1 for one-dimensional chromatography using a mixture of amyl alcohol–pyridine–water (35:30:30) (22). The amino acids were detected by dipping the chromatograms in a 0.5% ninhydrin solution (acetone–pyridine; 9:1). After drying at normal room temperature for 10 min. the chromatograms were heated at 60° for 2–3 min. The zones were fixed by dipping in a solution of cupric nitrate.[4] The chromatograms were allowed to dry in the dark for 30 min.

RESULTS AND DISCUSSION

The principal alkaloids detected in the tissue cultures were cuscohygrine (CC), choline (CH), and pseudotropine (PT). Their identification was based upon reported R_f values (23) in two chromatographic procedures previously described (18, 20), and by co-chromatography with reference compounds. At no time was hyoscyamine or scopolamine detected in the tissue cultures. These minor alkaloids are present in *Datura* (24), *Withania* (25, 26), and *Scopolia* (23). No relationship could be established between the minor alkaloid content and callus, suspension, or chlorophyllous tissue cultures. These minor alkaloids were sometimes not found, and also could not be related to tissue age, or tissue morphology (creamy-white suspension cells or vesicular brown tissue). The *D. stramonium*, strain 5450, tissue culture used for this study was previously reported to contain scopolamine and a total alkaloid content of 15 mg.% (13). West and Mika (12) reported *Datura* root callus to contain 47–53 mg. % total alkaloids, and Netien and Combet (3) reported *D. metel* L. stem callus to contain approximately 15.5 mg.% of hyoscyamine and scopolamine.

The results obtained with suspension cultures are shown in Table I. The first two experiments of *Datura* suspension cultures grown in medium with 7 p.p.m. of manganese contained no Dragendorff-positive spots. Tissue grown in Experiment III with amino acid precursors grew very poorly and did not contain Dragendorff-positive spots. However, CC, CH, PT were present in some of the cultures without precursors. The tissue from Experiments IV *a*, *b*, *c* did not contain Dragendorff-positive spots, and was grown in media without precursors and with lower manganese concentrations (1 and 7 p.p.m.). Only 21 day-old tissue grown on medium with 14 p.p.m. of manganese contained the three alkaloids: CC, CH, and PT. Twenty-eight day-old tissue

[1] Modified Dragendorff's reagent: Mix together basic bismuth carbonate 2.6 Gm. and sodium iodide 7.0 Gm. Add 25 ml. glacial acetic acid and boil for 3–4 min., set overnight and filter. The filtrate (20 ml.) was added to ethyl acetate (80 ml.) to make the stock solution. Final detecting solution was formed by mixing and storing the following for at least 24 hr.: 20 ml. of stock solution, 4 ml. of glacial acetic acid, 120 ml. of ethyl acetate, and 10 ml. of water.

[2] Supplied through the courtesy of Dr. W. C. Evans, University of Nottingham, England.

[3] The two-dimensional chromatographic analysis was performed by Dr. V. Jiracek, Department of Biochemistry, Charles University, Praha, Czechoslovakia.

[4] Aqueous saturated solution of cupric nitrate (2 ml.) is mixed with concentrated nitric acid (0.04 ml.), and acetone added to 200 ml.

TABLE I—*D. stramonium* SUSPENSION CULTURE: INFLUENCE OF AGE, MANGANESE CONCENTRATION, AND AMINO ACIDS ON ALKALOID PRODUCTION

Experiment (Age of Culture: Months)	Age of Tissue Analyzed (Days)	Tissue Dry Wt./Flask[a] (Gm.)	Mean Concn. (p.p.m.)	Amino Acids Present[b] (Days)	Alkaloids Detected[c]		
					Cusco-hygrine	Choline	Pseudo-tropine
I (36)	23	...	7	0			
II (37)	27	...	7	0			
III (38)							
a	31	0.485	1	0	+	+	
b	31	0.270	7	0			
c	31	0.340	14	0	+		+
d	31	0.077	1	31			
e	31	0.120	7	31			
f	31	0.050	14	31			
IV (39)							
a	21	0.366	1	0			
b	21	0.474	7	0			
c	21	0.425	14	0	+	+	+
d	28	0.533	1	0		+	
e	28	0.200	7	0			
f	28	0.425	14	0		+	+
g	28	0.675	1	7		+	
h	28	0.725	7	7		+	+
i	28	0.40	14	7			+
V[d] (40)							
a	8	0.167	14	0			
b	20	0.267	14	0	+	+	+
c	34	0.792	14	0			
d	48	0.725	14	0			
e	20	0.244	7	0	+	+	
f	20	0.096	14	20	+	+	+
g	27	0.833	14	7	+	+	+
VI (42)							
a	16	0.765	7	0			
b	16	0.744	14	0			
c	16	0.808	7	8			
d	23	1.336	7	0			
e	23	1.340	14	0			
f	23	1.524	7	15			

[a] Average dry weight tissue obtained from 4–10 250-ml. conical flasks containing 75 ml. of medium. Tissue was dried at 90° for 24 hr. [b] Final concentration in medium: 0.02% of L-ornithine HCl and of L-arginine HCl; 0.01% of L-phenylalanine; 0.05% of L-asparagine (anhydrous). The amino acid stock solution used was sterilized by filtration and aseptically added to the medium. [c] Paper chromatographic procedure according to Trabert (18) and detection by modified Dragendorffs reagent. *Rf*: Cuscohygrine, 0.08; Choline, 0.17; Pseudotropine, 0.29. [d] The tissue from Experiment V*a–d* was combined, dried, and assayed as one sample.

grown on similar medium contained a strong PT spot (estimated concentration 0.035%). Pseudotropine was also present (estimated concentration 0.015%) in 28 day-old tissue on similar medium with precursors (Experiment IV). The tissue appearance varied from a creamy-white suspension to a brown vesicular tissue. Supplemental analysis of these two forms indicated that they were similar with regard to their alkaloid content, and the creamy-white suspension tissue was selected for Experiments V and VI, and the multiliter experiment. The three alkaloids, CC, CH, and PT, were present in Experiment V tissues grown in media with high manganese levels (14 p.p.m.), and with or without amino acid precursors. The highest concentration of CC (estimated 0.01%) was in Experiment V*e* tissue, and the highest concentration of PT (estimated 0.017%) in Experiment V *a–d*. A growth curve for Experiment V *a–d* is shown in Fig. 1. At the time of maximum growth (34 days) the growth index value was 8.12; the growth efficiency index (G.E.I.)[5] value 3.18; and the Gm. of tissue (wet wt.) produced per liter per day was 6.2. The moisture content, determined by drying the tissue at 90° for approximately 24 hr. was 95.5%. The tissue grown for Experiment VI in medium with either 7 or 14 p.p.m. of manganese, with and without precursors, contained no Dragendorff-positive spots.

Chlorophyllous cultures grew very rapidly as a green, finely dispersed cellular culture. The cultures analyzed contained CH, but no other Dragendorff-positive spots.

The tissues obtained from the three fermentors contained no Dragendorff-positive spots. As much as 10 Gm. dry wt. of tissue was taken for an analysis, and yet the result was negative for alkaloids. The growth index values for the three fermentors are given in Fig. 1. The fermentor with the maximum growth had a growth index value of 9.34; a G.E.I. value of 0.13; and the Gm. of tissue (wet wt.) produced per liter per day was 14.1. These values were calculated for the growth yield obtained and do not necessarily represent the maximum growth yield possible. There is continued interest in the production of large amounts of plant cells (27, 28). The amount of fresh tissue obtained from the best multiliter fermentation was 14.1 Gm./L./day, and compares favorably with that reported for *Ammi visnaga* (19.0 Gm./L./day) (9).

The amino acids found in the two-dimensional chromatograms of tissue from Experiments III *a, b, c*

[5] G.E.I. = Growth index at maximum growth/medium (L.) × days required for maximum growth.

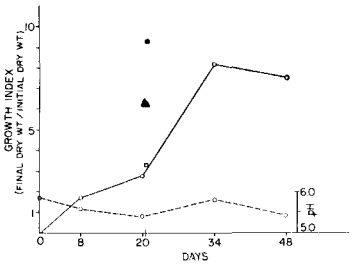

Fig. 1—Datura stramonium *L., strain 5450, growth in flasks (Experiment V), and from multiliter fermentors. Key:* O, *250-ml. conical flasks (75-ml. medium). Growth (average dry wt.) from 4 flasks. Inoculum dry weight: 0.098 Gm.;* □, *fermentor I;* Δ, *fermentor II;* ■, *fermentor III. Fermentors contained 3.4-L. medium and a tissue inoculum of approximately 5 Gm. dry weight. Final medium pH was 5.5.*

and IV *a*, *b*, *c* (Table I) in high concentrations were arginine, glutamic acid, and glutamine; in intermediate concentrations were alanine, γ-aminobutyric acid, and serine; and in low concentrations were aspartic acid, isoleucine, leucine, phenylalanine, proline, threonine, tyrosine, and valine. One-dimensional chromatograms of all samples had a high concentration of arginine and only traces of ornithine. The amino acids ornithine and phenylalanine, which are known to be the principal precursors of the tropane alkaloids (24), did not show any significant stimulating effects on the production of either minor or major alkaloids in the *Datura* tissue cultures studied. An analysis of the amino acids in *Datura* suspension cultures was qualitatively similar, except for the absence of lysine and tryptophan, to that reported for *D. stramonium* plants (16). Large amounts of arginine, and trace amounts of ornithine, are present in *Datura* tissue cultures.

Higher concentrations of manganese, a known activator of arginase (16), appeared to stimulate the production of the minor alkaloids present in *Datura* suspension cultures.

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Keyphrases

Datura tissue cultures
Alkaloid production—seed callus cultures
Chlorophyllous cultures—*Datura* tissue
Manganese—alkaloid growth in cultures
Ion exchange, column chromatography—separation, amino acids
Paper chromatography—analysis, identity