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FORMATION OF ACETYLTROPINE IN *DATURA* CALLUS CULTURES

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Key Word Index—*Datura innoxia*; Solanaceae; tropane alkaloids; tropine; acetyltropine; tissue culture.

Abstract—The production of tropoylesters in suspension cultures of *Datura innoxia* stem callus was significantly stimulated by DL-tropic acid, phenylpyruvate or tropine, but was little affected by L-phenylalanine or L-ornithine. Analyses have shown that acetyltropine is synthesized in large quantities by cultured cells when tropine has been supplied to various cultures of *D. innoxia* and *D. tatula*. Acetyltropine has been isolated from either the culture medium or cells supplied with tropine. These results indicate that tropine absorbed by the cultured cells of *Datura* is esterified predominantly by acetic acid to form acetyltropine, instead of other tropane alkaloids.

INTRODUCTION

ALTHOUGH tropane alkaloids have been detected in callus tissues from plants of *Atropa*,[1,2] *Scopolia*[3–5] and *Datura*,[5–12] the alkaloid contents are generally much less than those of the original plants. However, we[12] found that the addition of tropic acid, the acid moiety of the ester alkaloids hyoscyamine and scopolamine, to the callus cultures of *Datura tatula* was highly effective in increasing the amounts of alkaloids in cells, whereas tropine, the base moiety of the alkaloids, was proved to be ineffective. In order to ascertain whether or not this interesting effect might be observed in a different species of *Datura*, we have examined the relationship between the precursor supply and the alkaloid production in the callus cultures of *D. innoxia*. In the course of this work, it has been found that a large amount of acetyltropine is synthesized by the cultured cells which have been supplied with tropine. Results of these experiments are described in this paper.

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RESULTS

Responses of Cultured Cells to Alkaloid Precursors

Two experiments were conducted to examine the effects of the probable precursors of tropane alkaloids on growth and alkaloid formation in the suspension cultures of *D. innoxia* stem callus. In the first experiment, suspended cells were inoculated in the basal medium containing an alkaloid precursor (10^{-7}–10^{-2} M), and then cultured for 4 weeks. The precursors added were somewhat inhibitory to cell growth; 50% inhibition of growth was observed at 5×10^{-5} M of DL-tropic acid, 5×10^{-4} M of L-phenylalanine, 10^{-3} M of L-ornithine–HCl or Na–phenylpyruvate, and 10^{-2} M of tropine, respectively. Interestingly, tropic acid and tropine differ widely in toxicity. Alkaloid contents on the basis of dry weight of cells, however, were little affected by the precursors, excepting tropine, in a wide range of concentrations tested. Table 1 shows results obtained from cultures which were grown at a precursor concentration of 10^{-3} M. Analyses have shown that alkaloids were mostly found in the cells, only 2–18% of the total alkaloids accumulating in the culture medium.

TABLE 1. EFFECTS OF PRECURSORS ON THE PRODUCTION OF VITALI–MORIN POSITIVE ALKALOID IN SUSPENSION CULTURES OF *Datura innoxia* CALLUS GROWN IN BASAL MEDIUM (250 ml) AT 25° IN THE DARK

Precursor added to the medium (10^{-3} M)	Experiment I*			Experiment II†		
	Dry wt g/flask	Alkaloid content		Dry wt g/flask	Alkaloid content	
		μg/flask	μg/g dry wt		μg/flask	μg/g dry wt
None	2·67	115	43	2·80	115	41
L-Ornithine–HCl	1·95	172	88	3·00	159	53
Tropine	3·20	349	109	3·00	117	39
L-Phenylalanine	0·25	15	60	2·90	58	20
Na–Phenylpyruvate	2·06	171	83	3·17	673	212
DL-Tropic acid	0·43	20	47	2·82	569	202
Tropine plus DL-Tropic acid	0·18	19	107	2·85	473	166

* The cultures were supplied with precursors at the initiation of culture and incubated for 4 weeks.
† The cultures were supplied with precursors after 2 weeks, for a period of 1 week.

In the second experiment, each precursor (10^{-3} M) was supplied to suspension culture 2 weeks after cell inoculation so that growth inhibition at the early culture stage would be avoided, and the cells were harvested after an additional week of incubation. The results indicate that the alkaloid content was significantly increased by the delayed feeding of tropic acid or phenylpyruvate, although the other precursors including tropine were still ineffective in increasing the production of Vitali–Morin-positive alkaloids (Table 1).

Isolation and Identification of Acetyltropine

TLC of alkaloid extracts from various cultures supplied with precursors usually showed 2–4 Dragendorff-positive spots (red), including those of hyoscyamine and scopolamine. However, a striking difference in alkaloid constitution was revealed in the cultures supplied with tropine, where a Dragendorff-positive (orange) but Vitali–Morin-negative alkaloid was present in large quantities in both cells and culture media. For isolation of this compound, the stem callus of *D. innoxia* was cultured in the basal medium containing 10^{-3} M

tropine for 27 days, and the specific alkaloid was isolated from either the cells or the medium by preparative TLC. The picrate of this alkaloid was identified as the picrate of 3α-acetyltropine by elemental analysis, m.m.p., IR and NMR spectra with authentic material.

Contents of Acetyltropine in Callus Cultures

Since acetyltropine cannot be detected by the Vitali–Morin reaction used for quantitative estimation of tropane alkaloids, a titration method was adopted for its assay. All the cultures to be assayed were grown in the basal medium containing 10^{-3} M tropine. Table 2 shows that acetyltropine contents in the suspension culture of 19-month-old *D. innoxia* stem callus were 528 μg/g dry wt in the cells and 16·7 μg/ml in the medium. This means that *ca.* 8% of tropine added to the medium was converted to acetyltropine during the period of culturing. There were considerable differences in acetyltropine content among *D. innoxia* cultures; the reason for this variation is not clear.

TABLE 2. ACETYLTROPINE CONTENTS IN *Datura innoxia* SUSPENSION CULTURES GROWN IN 250 ml-BASAL MEDIUM CONTAINING 10^{-3} M TROPINE IN THE DARK FOR 35 DAYS AT 25°

Origin of callus	Age of callus (months)	Acetyltropine in the cells μg/flask	Acetyltropine in the cells μg/dry wt	Acetyltropine in the medium μg/flask	Acetyltropine in the medium μg/ml
Stem	19	362	528	3 130	16·7
Stem	21	458	153	92	0·49
Embryo	4	148	167	305	3·05

Formation of acetyltropine has been found also in callus tissues derived from *D. innoxia* root and *D. tatula* stem when they were grown on the basal agar medium containing 10^{-3} M tropine. The acetyltropine contents in the tissues were 1870 μg/g dry wt of the *D. innoxia* root callus and 425 μg/g dry wt of the *D. tatula* stem callus, in comparison with 608 μg/g dry wt of the *D. innoxia* stem callus. As expected, no acetyltropine was detectable in the control cultures grown without tropine.

DISCUSSION

From screening tests conducted for a variety of compounds in an attempt to find effective precursors for alkaloid production in *Datura* cultures, quite different results have been obtained by various workers regarding the effectiveness of L-phenylalanine, the probable precursor of tropic acid.[13,14] Chan and Staba[7] reported that phenylalanine stimulated alkaloid production in *Datura stramonium* leaf suspension cultures. Sairam and Khanna[11] also reported that the alkaloid content in the *D. tatula* seed callus was greatly increased by phenylalanine. On the other hand, Yatazawa *et al.*[6] observed that the root callus tissue of *D. stramonium* developed an alkaloid reaction when supplied simultaneously with ornithine and tropic acid, but the reaction was negative when the callus was cultured with ornithine and phenylalanine. We[4,5] showed that tropic acid was the most effective precursor for stimulating alkaloid production in the callus cultures of *D. tatula*, *Scopolia*

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japonica and *S. parviflora*, whereas phenylalanine was ineffective in each case. A similar result has been obtained from the present study with *D. innoxia* cultures. Our studies suggest, therefore, that biosynthesis of tropic acid from phenylalanine might be strongly repressed in callus tissues, unless organization[4] is induced from the undifferentiated tissues. This would explain why tropine added to callus cultures failed to increase the amounts of hyoscyamine and scopolamine, the tropic esters. In fact, tropine was found to be used mostly for synthesis of acetyltropine in the cells.

Acetyltropine was first isolated from the leaves of *Datura sanguinea*[15] and later from the root and hypocotyl of *D. innoxia* seedlings.[16] Romeike and Aurich[16] demonstrated further that the radioactivity of either tropine-[$^{14}CH_3$] or sodium acetate-[^{14}C] was incorporated at a high rate into acetyltropine synthesized by the excised root culture of *D. innoxia*, although acetyltropine formed might be readily destroyed or converted to other metabolites.

In the present study, analyses by TLC have indicated that a large portion of tropine absorbed by the suspension cultures of *D. innoxia* stem callus remains unchanged in the cells, only a relatively small portion having been converted to acetyltropine. It is interesting that tropine that has accumulated in the cell is not esterified substantially by organic acids other than acetic acid to yield different tropane alkaloids. It may be noted in this connection that in our experiment in which *D. innoxia* suspension cultures were supplied with tropine and l-mandelic acid, formation of the synthetic ester alkaloid homatropine from the two compounds could not be detected. These findings suggest that synthesis of acetyltropine might be catalyzed by a specific esterase.

EXPERIMENTAL

Plant material and culture method. Callus tissues of *Datura innoxia* Mill. were derived from the stem and embryo in July 1969 and in October 1970, respectively. The stock cultures have been maintained in the liquid basal medium (250 ml) in 500-ml Sakaguchi flasks agitated by a reciprocal shaker at a speed of 100 rpm. The root callus of *D. innoxia* (induced July 1969) and the stem callus of *D. tatula* L. (induced July 1966) have been maintained in test tubes on the basal medium solidified with 0·9% agar. The basal medium used for both suspension and static cultures was the Linsmaier–Skoog solution[17] supplemented with 10^{-6} M 2,4-dichlorophenoxyacetic acid. Suspension cultures were transferred to fresh media every 2–3 weeks, while static cultures every 4–5 weeks. For precursor experiments, similar culture methods as mentioned above were used, except that static cultures were grown in 100-ml Erlenmeyer flasks containing 40 ml agar medium. All the cultures were incubated in the dark at 25°.

Isolation and identification of acetyltropine. D. innoxia stem callus cultures (19-month-old) were grown in the basal liquid medium supplied with 10^{-3} M tropine for 27 days. The medium (1 l.) was made alkaline with 3 N NaOH and extracted with $CHCl_3$. From the concentrated $CHCl_3$ extract, the alkaloid was separated by preparative TLC on Silica gel G with $CHCl_3$–EtOH–28% NH_4OH (85:14:1). The alkaloid was extracted from the gel with MeOH, and the solution was evaporated to dryness. Treatment of the residue with an ethanolic solution of picric acid gave the picrate of the alkaloid, which was recrystallized twice from EtOH to yield 14·3 mg of yellow needles, m.p. 214° (Found: C, 46·79; H, 4·82; N, 13·61. Calc. for $C_{10}H_{17}NO_2$. $C_6H_3N_3O_7$: C, 46·60; H, 4·89; N, 13·59%). This compound was identified with authentic sample of 3α-acetyltropine picrate by m.m.p., IR and NMR spectra. Acetyltropine was isolated also from the $CHCl_3$ extract of the harvested cells.

Quantitative analysis of acetyltropine. The dry powdered sample was extracted with MeOH 2×, each for 1 hr. After evaporation of MeOH, the residue was dissolved and shaken in a mixture of 0·1 N HCl (20 ml) and $CHCl_3$ (30 ml). The aqueous layer was made alkaline with 3 N NaOH and then extracted with $CHCl_3$ (30 ml × 3). The $CHCl_3$ extract was dried with anhydrous K_2CO_3, and then evaporated to dryness. Alkaloids were separated by TLC as described previously. The alkaloid corresponding to acetyltropine was extracted from the silica gel with MeOH (5 ml) in a stoppered glass tube, which was vigorously shaken for 30 min.

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The MeOH solution was filtered through a glass filter and evaporated to dryness. The residue was dissolved in 0·01 N H_2SO_4 (10 ml) and titrated with 0·01 N NaOH using methyl red as indicator. 1 ml of 0·01 N H_2SO_4 = 1·832 mg acetyltropine. Recovery of known quantities of acetyltropine taken through the above procedure was 95%.

Quantitative estimation of tropane alkaloids. Amounts of tropane alkaloids were assayed spectrophotometrically at 545 nm after the Vitali–Morin reaction,[18] and alkaloid contents were calculated as the amounts of hyoscyamine based on dry wt of the samples. Details of the procedure are described elsewhere.[4,5]

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