Feeding Experiments with Precursors of Tropane Alkaloids Using Suspension Cultures of *Atropa belladonna*

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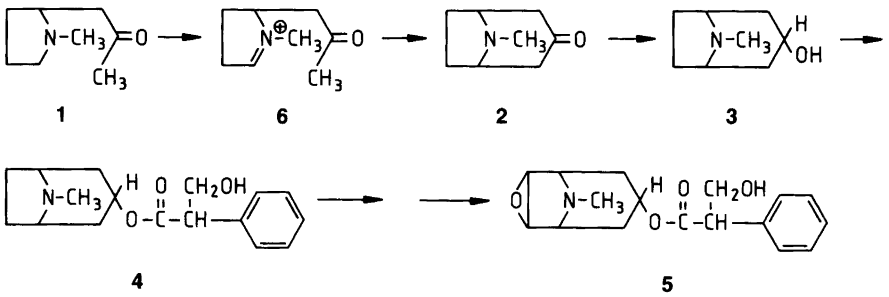
Feeding experiments have been carried out with alkaloid precursors (hygrine, tropinone and tropic acid) using suspension cultures of *Atropa belladonna* to study the transport of these compounds into the cells, stimulation of alkaloid synthesis, and possible repressed steps in their biosynthesis. Both hygrine and tropinone are transported into the cells and the turnover of hygrine is very high. The turnover rate of tropinone is considerably enhanced in cultures fed with both compounds simultaneously, but tropanol, hyoscyamine and scopolamine were consistently undetected in the cell material. Biosynthesis of precursors and alkaloids was repressed in the cultures studied, and the repression could not be reversed by increased intracellular substrate levels.

The biotransformation of tropane alkaloids and the uptake of their precursors in plant tissue cultures is not well understood,[1–4] even though such knowledge might one day allow increased production of valuable alkaloid components through the addition of less expensive precursors. Whether or not these precursors are really transported into the cells has not yet been established, and until that time biotechnological applications are hardly worth planning.

In this investigation, suspension cultures of *Atropa belladonna* L. (Solanaceae) were used for alkaloid precursor feeding experiments aiming at an induction or at possibly repressed steps of tropane alkaloid biosynthesis.[1,5–7] Our experiments were undertaken with hygrine (**1**), tropinone (**2**) and tropic acid. Whereas hygrine and tropinone [and tropanol (**3**)] (themselves alkaloids) are established precursors of the amino alcohol moieties in the ester alkaloids hyoscyamine (**4**) and scopolamine (**5**) (Scheme 1), the acyl moiety in the esters is provided by tropic acid.[1,5–7] Owing to the chemical lability of hygrine and in order to increase the intracellular level of alkaloid precursors to a reasonable substrate concentration for enzymatic reactions, experiments were carried out with unlabelled compounds and relatively short feeding times.

Results and discussion

In suspension cultures of *A. belladonna* the turnover rate of exogenously added hygrine is considerably higher than that of tropinone. Even in the 4 h feeding experiments, hygrine was not detected in the nutrient media nor in the peduncle-derived cells, though it was still present at a reasonable concentration in 1 h and 4 h feeding experiments of stem-derived cells (Table 1). It is further significant that no tropinone was found in any of the hygrine-fed cultures.



Scheme 1. Biosynthetic relationship between the tropane alkaloids **1–5** (and the intermediate **6**).

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Table 1. Feeding experiments with alkaloid precursors using suspension cultures of *A. belladonna.* The concentrations, given as millimolar intracellular concentrations (fwt) and as mg/culture, are expressed as mean values for two or three parallel samples.

Added compound(s)	Concentration		Feeding time/h	Concentrations at the end of the experiment							
				Nutrient medium				Cell cultures			
				Hygrine		Tropinone		Hygrine		Tropinone	
	mM	mg/10 ml medium		mM	mg/10 ml medium	mM	mg/10 ml medium	mM	mg/culture	mM	mg/culture
A. Stem suspension											
Control	–	–		–	–	–	–	–	–	–	–
Hygrine	0.5	0.705	1	–	–	–	–	0.335	0.100	–	–
Hygrine	0.5	0.705	4	–	–	–	–	0.325	0.070	–	–
Hygrine	0.5	0.705	24	–	–	–	–	–	–	–	–
Tropinone	0.25	0.350	24	–	–	0.035	0.050	–	–	0.385	0.040
B. Peduncle suspension											
Control	–	–		–	–	–	–	–	–	–	–
Hygrine	0.5	0.705	4	–	–	–	–	–	–	–	–
Hygrine	0.5	0.705	24	–	–	–	–	–	–	–	–
Tropinone	0.25	0.350	24	–	–	0.030	0.045	–	–	0.610	0.025
Hygrine + tropinone	0.5 0.25	0.705 0.350	24	–	–	0.020	0.025	–	–	–	–
Tropinone + tropic acid	0.25 0.25	0.350 0.415	24	–	–	0.065	0.090	–	–	0.580	0.030
Hygrine + tropinone + tropic acid	0.5 0.25 0.25	0.705 0.350 0.415	24	–	–	0.060	0.080	–	–	–	–

The first rinsing water (*vide infra*) of the cultures incubated for 4 h was analyzed to check for the possible adsorption of hygrine onto the cell walls. No hygrine was detected.

In association with the present work, preliminary screening investigations were undertaken with a number of *A. belladonna* tissue cultures. Of 36 callus cultures of stem cortex or pith origin, four cultures were found to be hygrine positive, but they all were tropinone negative. Hygrine was not detected in suspension cultures of *A. belladonna*,[8] though it is known to occur in root cultures of this species.[9] Moreover, hygrine did not stimulate alkaloid production in suspension cultures of *Hyoscyamus niger.*[10] Unfortunately, the intracellular level of hygrine was not investigated.

It has been reported that both enantiomers of hygrine function as tropane alkaloid precursors in *A. belladonna.*[11,12] However, as the claim is in need of further confirmation,[13] we applied racemic hygrine at initial 0.5 mM concentrations instead of the 0.25 mM concentrations used for tropinone and (±)-tropic acid. (±)-Tropic acid was used at 0.25 mM instead of 0.5 mM concentrations because of the known growth-retarding effect of this acid on *Scopolia* and *Datura.*[14] Owing to the facile racemization,[15] we rejected the use of the expensive (−)-tropic acid.

Exogenously added tropinone underwent considerably slower turnover than hygrine in the cultures, but was nevertheless effectively transported into the cells (Table 1). Although tropinone was still present in reasonable concentrations in the liquid media at the end of the 24 h feeding period, substantial concentrations were also present in peduncle-derived cells: here the corresponding liquid-media levels were exceeded by about one order of magnitude. A similar though less pronounced pattern was observed in the experiments involving stem-derived cultures. The sums of extra- and intra-cellular amounts of alkaloid precursors after the feeding experiments are lower than the total amounts added to the nutrient media (Table 1). This may indicate conversion of these compounds to unknown conjugates [pectic derivatives, phosphates (*vide infra*) and/or glucosides]. Such storage forms of primary and/or secondary products have been reported, for example, in connection with the metabolism of nicotinic acid (glucosides) in several plant cell cultures and of morphinan alkaloids (pectic derivatives) in callus cultures of *Papaver somniferum.*[16,17]

An interesting change in the cell-concentration pattern of tropinone was effected by the concomitant application of hygrine: tropinone disappeared from the peduncle-derived cells at the 24 h stage (Table 1), while the associated liquid-media levels remained similar or even slightly lower than the corresponding levels observed without hygrine as co-additive. This means that the transport of tropinone into the cells was unaffected by hygrine or its metabolites, but the intracellular presence of the latter compound(s) ef-

fected a metabolic activation of tropinone. It is possible that tropinone enters the biosynthetic pathway established for tropane alkaloids.[1,5–7]

Considering the biosynthetic steps between hygrine and tropanol (Scheme 1), the observed hygrine-enhanced tropinone turnover (*vide supra*) may indicate that a multienzyme complex or possibly even an allosteric enzyme system is involved in the process. Starting with the oxidation of hygrine to dehydrohygrine (**6**), practically simultaneous catalysis of a number of successive biosynthetic steps could conceivably ensue. Some of the intermediates in this reaction series, such as dehydrohygrine and possible phosphorylated compounds (*vide supra*), are not covered by the present analytical procedure. A few samples were treated with alkaline phosphatase for the cleavage of possible phosphate ester bonds. With regard to the compounds of interest, however, the analytical data were similar to those from the corresponding control samples. Neither the biosynthetic end products hyoscyamine and scopolamine, nor the intermediate tropanol, could be detected in any of the cultures. This indicates that the enzyme catalyzing the synthesis of tropanol from tropinone is not a constitutive one and cannot be induced by the alkaloid precursors tested.

Experimental

General. (±)-Hygrine was synthesized according to the procedure described in Ref. 18. Tropinone and (±)-tropic acid were commercial compounds (Aldrich). Tropanol (tropine) was prepared by catalytic hydrogenation (PtO_2) of tropinone.[19] All four compounds were carefully purified before use.

Plant material. Two suspension cultures of *A. belladonna* (originating from stem and peduncle callus) were transferred into modified Wood and Braun's solution[4] supplemented with $CuSO_4$ (0.1 μM) and Na_2MoO_4 (1.0 μM). 1-Naphthylacetic acid (2 mg l^{-1}) and kinetin (0.1 mg l^{-1}) were used as growth regulators. The flasks were incubated in a rotary shaker (100 rpm) at 22 ± 1 °C in the dark. In appearance the cultures were slightly aggregated and white.

The feeding experiments were carried out when the suspensions were at the early stationary phase (day 14). Equally well grown cultures in liquid medium (10 ml) in Erlenmeyer flasks (100 ml) were supplemented with small volumes of filter-sterilized alkaloid precursors (hygrine, tropinone and tropic acid; 0.25 or 0.5 mM final concentration). The pH was adjusted to 5.2. After 1, 4 or 24 h incubation, the material was rinsed several times with distilled water and freeze-dried (to avoid artefacts).

The cell material and the corresponding cell-free media were submitted to gas chromatographic analysis (for hyoscyamine and scopolamine, see Ref. 20 and for hygrine and tropinone, see below). Recovery tests for the amines were performed with spiked callus samples which were cultured as described in Ref. 4.

Extraction and purification. The amines were extracted from 1 ml aliquots of methanolic standard solutions (containing a standard amount of cultured cell material devoid of the compounds in question) and from the freeze-dried callus or suspension culture samples (50 mg dwt) from the experiments, which had been equilibrated overnight in 1 ml of methanol at 4 °C (a modification of our earlier procedure[20]). The methanol solutions were mixed with 2 ml of carbonate buffer solution (pH 10) (0.025 M/0.025 M, $Na_2CO_3/NaHCO_3$), shaken for 30 min and centrifuged before chloroform extraction (*vide infra*). The cell precipitate thus obtained was thoroughly mixed with methanolic carbonate buffer (cf. above, 30 % MeOH), shaken for 15 min and centrifuged. The supernatants from both treatments were combined and extracted three times with 1 ml of $CHCl_3$. A 0.5 ml aliquot of the combined chloroform layers was mixed with 0.5 ml of a 150 μg ml^{-1} solution of the internal standard ketamine [2-(*o*-chlorophenyl)-2-(methylamino)cyclohexanone, Parke-Davis]. This solution (1 μl) was used for the analysis by GLC.

Enzymatic hydrolysis. For the hydrolysis of possibly occurring phosphate esters of alkaloid precursors (undertaken for a few samples), lyophilized cells (50 mg) were homogenized (ultrasonic bath for 30 min) in 1.5 ml of carbonate buffer solution (pH 9.5). 50 μl of the enzyme solution (alkaline phosphatase 0.5 U/ml, Sigma) were then added and the tubes were incubated for 1 h at 37 °C. After the addition of 3 ml of carbonate buffer solution (pH 10) containing 1 ml of MeOH, the samples were processed as described above.

GLC chromatography. The GLC analysis was carried out using a 25 m fused silica capillary column (Nordibond OV-1701, i.d. 0.32 mm, coating 0.25 μm) in a Dani HRGC 3200 gas chromatograph equipped with FID and PTV (programmed temperature vaporizer). Hydrogen was the carrier gas (3 ml min^{-1}) and the temperature program for the column oven was 80 °C (1 min), 12 °C min^{-1}, 255 °C (2 min). The PTV temperature range was 70–250 °C (splitless operation).

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