CADAVERINE–PYRUVATE TRANSAMINATION: THE PRINCIPAL STEP OF ENZYMATIC QUINOLIZIDINE ALKALOID BIOSYNTHESIS IN *LUPINUS POLYPHYLLUS* CELL SUSPENSION CULTURES

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Received 12 March 1979

1. Introduction

In vivo incorporation experiments with labelled precursors have established that the quinolizidine alkaloids are derived from lysine, via its decarboxylation product cadaverine [1–3]. Free intermediates between cadaverine and the tetracyclic alkaloids have not been detected so far. Several hypothetical mechanisms have been proposed for the pathway leading from cadaverine to lupine alkaloids [1,3,4]. Some authors have suggested that diamine oxidase, which is known for alkaloid producing plants, also might be involved in the synthesis of lupine alkaloids [3,5–7].

Here we report the transamination of cadaverine as the principal step in the enzymatic synthesis of tetracyclic alkaloids, catalyzed by a crude enzyme system prepared from *Lupinus polyphyllus* cell suspension cultures.

2. Materials and methods

Cell suspension cultures of *L. polyphyllus* were grown in a modified MS medium at 25°C under continuous illumination as shake cultures or as semi-continuous fermenter cultures. Cells were harvested in the stationary growth phase and homogenized in cold acetone (–20°C). Dried acetone powders were stored at –20°C.

Acetone powder (5 g) was suspended in 100 ml 0.05 M sodium-phosphate buffer (pH 7.8) containing 1 mM dithioerythritol and stirred in an ice bath for 30 min. The pellet, recovered by centrifugation, was resuspended in buffer. The standard reaction mixture (total vol. 5 ml; radioactive assays 2 ml) consisted of: the enzyme–buffer suspension (corresponding to ~200 mg acetone powder), 5 mM cadaverine, 10 mM pyruvate, 10 mM diethyldithiocarbamate (DIECA). The reaction vessels were gassed with N_2 for 1 min, thoroughly sealed and shaken for 3–5 h in the dark. The reaction was terminated with trichloroacetic acid and the precipitate was removed by centrifugation.

The total alkaloid content of the enzymatic assays was determined photometrically with modified Reifer's reagent [8] at 830 nm. The extinction was linear over 1–10 μg alkaloid/ml. Sparteine was chosen as a standard.

Thin-layer chromatographic separation of alkaloid mixtures and cadaverine was on silica-gel HF-256 (Merck, Darmstadt) with the solvents: I, cyclohexane/diethylamine (7/3); II, methylene chloride/methanol/NH_3 (80/20/1); detection, Dragendorff's reagent.

Gas–liquid chromatographic separation of lupine alkaloids was with a Perkin-Elmer gas chromatograph (F 22) equipped with capillary columns and flame ionisation and nitrogen detectors. Gas–liquid chromatography/mass spectroscopy was carried out using a Perkin-Elmer GC/AEI MS 30 combination. Identification of reference alkaloids isolated by preparative layer chromatography from plant extracts was performed by mass spectroscopy (AEI MS 9).

[^{14}C]alanine and [^{14}C]pyruvate were isolated from enzyme assays by ion-exchange chromatography

(Amberlite-IR-120) according to [9]. [2,5-^{14}C]cadaverine was purchased from NEN and [U-^{14}C]pyruvate from Buchler/Amersham.

3. Results

The alkaloid content of *L. polyphyllus* cell suspension cultures (~5 μg/g fresh wt) is ~500-times lower than in the differentiated plant. It can be raised however, when cadaverine is fed to the cultures. Thus the presence of an alkaloid synthesizing enzyme system is indicated. Enzyme preparations of cell cultures offer the advantage that background alkaloid content cannot interfere with the quantification of enzymatically produced alkaloids.

Crude insoluble enzyme preparations synthesize alkaloids from cadaverine in the presence of pyruvate (table 1). The activity could be increased by the exclusion of oxygen during incubation. Therefore a participation of diamine oxidase in alkaloid biosynthesis is unlikely. This could be proven in experiments with the diamine oxidase inhibitor DIECA [10]. Diamine oxidase activity in enzyme preparations of *L. polyphyllus* and *Pisum sativum* (as a reference) is totally inhibited in the presence of 10 mM DIECA (table 2) whereas alkaloid synthesis is even increased (table 1).

The activation of the enzymatic alkaloid formation by pyruvate suggests that a transamination of cadaverine is involved. To prove this an enzyme preparation was incubated with 3 mM [^{14}C]pyruvate (1 μCi) and 3 mM cadaverine. The only labelled compound which could be identified in the amino acid fraction was alanine. The correlation between alanine formation and cadaverine transformation was followed in experiments in which either pyruvate or cadaverine served as labelled precursor. A positive correlation between the rates of net transamination and cadaverine transformation can be established (fig.1). Both rates are of the same order, since no significant difference could be calculated using the Wilcoxon matched pairs signed rank test of the Dixon-Mood test.

Furthermore carbonyl reagents (10 mM each) as semicarbazid, cyanide or hydroxylamine, which are known to interact with pyridoxal phosphate-dependent enzymes cause 85–95% inhibition of both pyruvate–cadaverine transamination and alkaloid production.

Under physiological conditions 5-aminopentanal, the deamination product of cadaverine, undergoes spontaneous cyclization to Δ^1-piperideine. Piperideine forms an orange-coloured adduct with *o*-aminobenzaldehyde which can be measured photometrically at 435 nm [11]. In fact this method was employed to determine diamine oxidase activity (table 2). However, when *o*-aminobenzaldehyde was added to actively transaminating assays the formation of the coloured adduct could never be detected.

The pH optimum for the alkaloid production was 7.8. The reaction was found to be linear with time for ⩾ 5 h. An app. K_m 0.3–0.8 mM has been calculated for cadaverine. Addition of pyridoxal phosphate did

Table 1
Conditions of alkaloid production from cadaverine by an enzyme preparation from *L. polyphyllus* cell cultures

Assay	Total alkaloid produced (%)
Complete (standard assay)	100
minus pyruvate	10
minus pyruvate plus 2-oxoglutarate	20
minus DIECA	58
in presence of air	42
boiled enzyme	< 5

Enzymatic incubation was performed under standard assay conditions. Total alkaloids were determined with modified Reifer's reagent. At least 2 trials were performed for each assay. All values were corrected by blank activity (without added cadaverine). Complete assays produced ⩽ 14 μg alkaloids/5 h and 100 mg acetone powder

Table 2
Diamine oxidase activity in acetone powder preparations from *L. polyphyllus* plants and *P. sativum* seedlings

Assay	Diamine oxidase act. (% control)	
	L. polyphyllus	*P. sativum*
Complete (control)	100	100
N_2-atmosphere	28	30
plus 1 mM DIECA	14	48
plus 10 mM DIECA	0	0

Enzyme activity was determined according to [11]

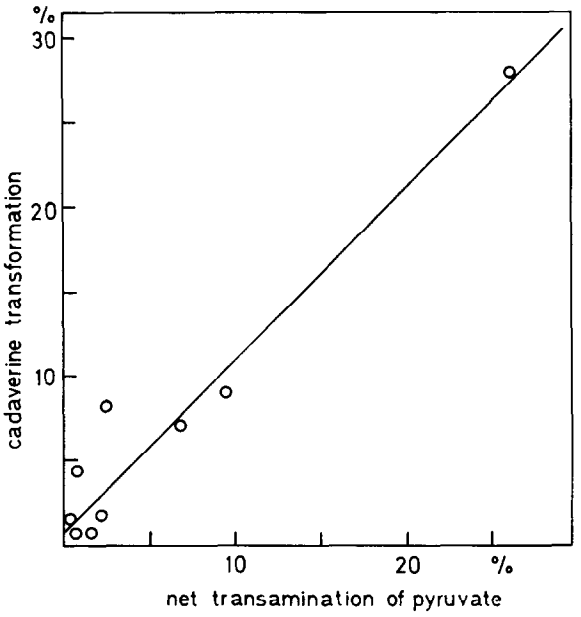

Fig.1. Correlation between the rates of pyruvate transamination and cadaverine transformation. Enzymatic incubations were performed under standard assay conditions with 3 mM cadaverine and 3 mM pyruvate using various enzyme preparations of different age and origin. In each experimental set one sample contained 1 μCi [^{14}C]pyruvate (total transamination) and a corresponding sample 1 μCi [^{14}C]cadaverine (cadaverine transformation). Net transamination was calculated from the produced [^{14}C]alanine minus background activity ([^{14}C]alanine produced in controls without added cadaverine; ~5% total activity). For the calculation of cadaverine transformation the reaction mixture (300 μl) was separated by thin-layer chromatography. The cadaverine and alkaloid zones were localized by radioscanning, scraped off the plates, eluted with acidified 70% methanol and measured by scintillation counting. The regression follows the equation: $Y = 0.99 + 1.017\ (x); p < 0.001$.

not influence enzyme activity. During storage of acetone powders at −20°C, ~80% of enzyme activity is lost within 3 months.

In fig.2A thin-layer chromatographic separation of the reaction products of enzymatic [^{14}C]cadaverine transformation is illustrated. In both solvent systems identical R_F-values were found for some of the radioactive spots and authentic tetracyclic lupine alkaloids (fig.2B). For identification the enzymatic incubation mixtures were analyzed by gas–liquid chromatography and subjected to gas–liquid chromatography/mass spectroscopy. As the main product a tetracyclic alkaloid of the oxosparteine type (M^+ m/z 248) [12] could be identified, which presumably corresponds to peak I of fig.2A. The structural identification of all reaction products will be presented elsewhere. There is no indication so far, that tetrahydroanabasine or tripiperidine, which are known to be formed spontaneously from Δ^1-piperideine are present in the enzyme incubation mixtures.

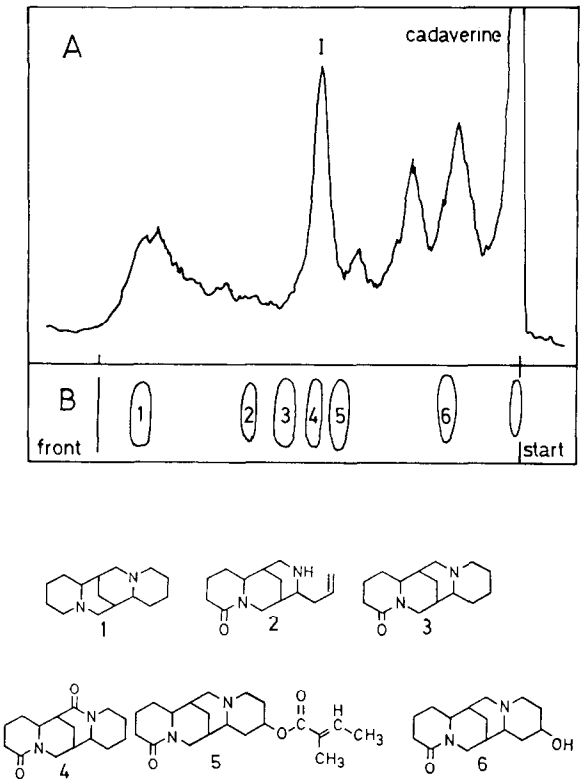

Fig.2. Thin-layer chromatographic separation of the enzymatically formed products from [^{14}C]cadaverine. Enzymatic incubation was performed under standard assay conditions with 1 mM [^{14}C]cadaverine (5 μCi) and 3 mM pyruvate. The alcalized reaction mixture was extracted with methylene chloride dried over Na_2SO_4, concentrated and co-chromatographed (solvent I) with the known alkaloid mixture obtained from *L. polyphyllus*. (A) Radioactive spots detected by radioscan. (B) Co-chromatographed alkaloids visualized with Dragendorff's reagent. The respective structures elucidated by mass spectroscopy: 1, sparteine; 2, angustifoline; 3, lupanine; 4, 17-oxolupanine; 5, 13-tigloyllupanine; 6, 13-hydroxylupanine.

4. Discussion

The existence of a cadaverine–pyruvate transaminating enzyme system which catalyzes the biosynthesis of lupine alkaloids could be established. The participation of diamine oxidase has been ruled out. In contrast to known diamine transaminases [13,14] the deamination product of cadaverine is not released from the enzyme, since neither Δ^1-piperideine nor any of its spontaneously formed condensation products could be found. Thus it appears reasonable that the enzyme system catalyzes the formation of tetracyclic alkaloids in a channeled manner without releasing free intermediates. This is in good accordance with the in vivo tracer studies [1–4]. Furthermore the identification of an oxosparteine as main product of the enzymatic process fits well to the hypothesis in [4]; there a compound closely related to 17-oxosparteine or 10-oxosparteine is postulated as the first tetracyclic product in the biosynthetic sequence.

Recent results (M. W., T. H., in preparation) confirmed that the cadaverine–pyruvate transaminating enzyme system is located in lupine chloroplasts. It seems to be membrane-bound or membrane-associated but it can be solubilized at least partially by digitonine treatment of isolated chloroplasts. Membrane association might be the reason for the inability to solubilize the enzyme system from acetone powders. Work on further characterization of the enzyme system is in progress.

Acknowledgements

We would like to thank Dr H.-M. Schiebel (Inst. f. Organische Chemie d. T. U. Braunschweig) and Dr L. Witte, D. Döring, H. Steinert (GBF, Braunschweig-Stöckheim) for collaboration in mass spectroscopy and gas–liquid chromatography/mass spectroscopy and the Studienstiftung des Deutschen Volkes for a grant to M.W. This work was supported by a research grant of the Land Niedersachsen.

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