SYNTHESIS OF AJMALICINE AND RELATED INDOLE ALKALOIDS BY CELL FREE EXTRACTS OF *CATHARANTHUS ROSEUS* CELL SUSPENSION CULTURES

J. STÖCKIGT, J. TREIMER and M. H. ZENK
Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D 4630 Bochum, FGR

Received 30 August 1976

1. Introduction

On the basis of in vivo incorporation experiments, using radioactively labelled precursors, it has been demonstrated in several laboratories [1–4], that *Corynanthe*-type indole alkaloids are formed by condensation of tryptamine with a secoiridoid glucoside to yield vincoside. The latter indole glycoside is subsequently transformed into the diverse structures of the indole and dihydro-indole alkaloid series. In order to elucidate the exact mechanism by which these alkaloids are formed and their synthesis regulated, enzymatic studies were necessary. A break through in this aspect occurred recently when Scott and Lee [5] succeeded in obtaining from callus tissue of *Catharanthus roseus*, crude cell free system, capable of synthesizing geissoschizine and ajmalicine.

For the past two years we have been involved in setting up a cell suspension culture system to investigate the conditions for the production of indole alkaloids by cells of *C. roseus* [6]. We now wish to report the enzymatic synthesis of the indole alkaloids; ajmalicine, 19-epiajmalicine and tetrahydroalstonine, and some characteristics of the conditions for the overall enzymatic reaction using this cell culture system.

2. Materials and methods

Cell suspension cultures of a high-alkaloid producing strain of *C. roseus* cells were grown in a modified MS medium [7] at 30°C in 30 liter airlift fermenters with 0.3 vvm aeration [6]. After 20 days of growth, the cells were harvested, immediately frozen with liquid nitrogen and used as an enzyme source.

A crude enzyme preparation was obtained by crushing the frozen cells in a BIO X-press and subsequent treatment of the extract as described [8]. The enzyme preparation was freed of low molecular contaminants, especially nucleotides, by the addition of 5 mg/ml of dextran coated charcoal. The solution was stirred for 10 min in an ice bath and the charcoal removed by centrifugation. The supernatant crude enzyme preparation had an average protein concentration of 3 mg/ml. Incubation of this preparation with labelled tryptamine and secologanine plus various cofactors was carried out at 31°C for 120 min. The reaction was terminated by evaporation of an aliquot of the incubation mixture on a TLC GF 254 plate. The chromatograms were developed in I. (acetone/light petrol (40–60°)/diethylamine = 20 : 70 : 10) and after elution of the alkaloid containing zone five different solvent systems were used for chromatographic identification; II. ethylether/ethylacetate/*n*-hexane = 20 : 20 : 8; III. $CHCl_3$/acetone/*n*-hexane = 25 : 20 : 38; IV. ethylacetate/iso-propanol/NH_3/*n*-hexane = 50 : 1 : 0.5 : 31; V. xylene/*n*-hexane/ethylacetate/ethylether = 15 : 45 : 5 : 40; VI. acetone/light petrol (40–60°)/CCl_4/*n*-hexane = 35 : 30 : 20 : 30. Labelled ajmalicine and tetrahydroalstonine were further identified by oxidation to serpentine and alstonine as follows: labelled material was diluted with 0.5 mg of unlabelled carrier alkaloid, dissolved in 50% aqueous methanol and 0.85 mg maleic acid and 2.5 mg Pd on charcoal (10%) were added. The mixture was heated for 2.5 h under reflux. Then chromatographed in VII: acetone/methanol/diethyl-

amine = 7 : 2 : 1. The zones containing the alkaloids were eluted and rechromatographed in VIII: ethanol/ethylacetate/diethylamine/dimethyl formamide = 60 : 30 : 5 : 20 (serpentine: R_F = 0.46; alstonine: R_F 0.52).

The labelled alkaloids isolated from reaction mixtures were further characterized by crystallization. Each was diluted with carrier compound and recrystallized several times from methanol, ethanol and ethylacetate successively until a constant specific activity was reached.

For large scale preparation of the alkaloids the isolation procedure was modified as follows. The enzyme-incubation mixture was added to a column (1.5 X 6 cm) of amberlite XAD-4 (200–300 μ) [9] previously washed with water. The column was washed with 160 ml distilled water then with 20 ml MeOH and eluted with methanol containing 1% glacial acetic acid. Recovery of radioactivity in the above fractions was 95%. The alkaloids in question were quantitatively eluted with 100 ml of the acidified methanol. The solvent was evaporated and the residue chromatographed on silical gel plates in solvent system I. The alkaloid containing zones were scraped off and rechromatographed in solvent V.

The isolated alkaloids were subsequently subjected to masspectroscopy direct inlet using a Varian MAT 111 at an electron energy of 80 eV. [2-^{14}C]Tryptamine was purchased from Amersham, secologanin was isolated from *Lonicera* sp. [10], all biochemicals were from Boehringer, Mannheim.

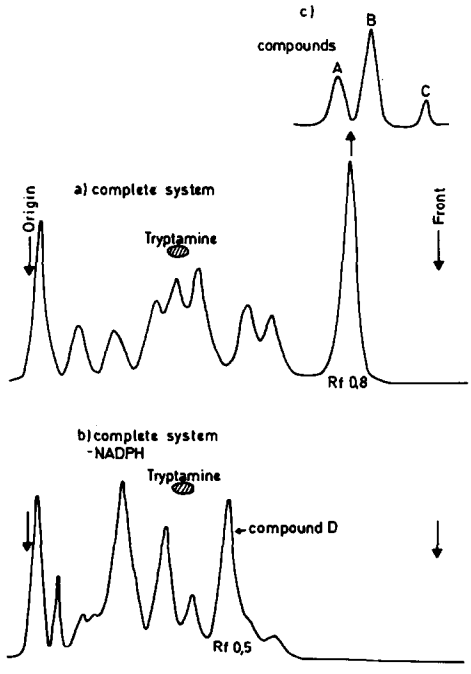

Fig.1. Metabolism of [2-^{14}C]tryptamine by cell free extracts of *C. roseus*. TL chromatogram of (a) complete incubation mixture, as given in table 1. (b) complete incubation mixture minus NADPH. Development in solvent system acetone/light petrol (40–50°)/diethylamine 2 : 7 : 1. (c) TL rechromatography of peak R_F 0.8 in chloroform/acetone/*n*-hexane 2.5 : 2 : 3.8. Radioactivity was recorded with the thinlayer Scanner II (Berthold, Wildbad).

3. Results and discussion

Cell suspension cultures of selected strains of *C. roseus* produce substantial amounts of the indole alkaloids, ajmalicine and serpentine in vivo [6]. Crude cell free extracts of this material were incubated with tryptamine and secologanin, which are known to serve as precursors of these alkaloids [1–4]. As shown in figure 1a, under these conditions and in the presence of NADPH, labelled tryptamine is almost completely metabolized. Characteristic for this conversion is a radioactive peak at R_F 0.8, which when isolated and rechromatographed in solvent system(s) V (or II–VI) splits into 3 distinct and well separated labelled compounds. In the absence of NADPH (fig.1b) tryptamine was not converted to this material, however, a new radioactivity peak at an R_F value of 0.5 was produced. In order to characterize the products which were formed in the presence of NADPH, a large scale incubation was performed. In a total volume of 100 ml were incubated: 10^4 μmol KPO_4^-, pH 7.0, 50 μmol NADPH, 25 μmol [2-^{14}C]-tryptamine (10 μCi), 125 μmol secologanin and 225 mg *C. roseus* protein. For the regeneration of NADPH 100 μmol D-isocitrate, 400 μmol $MgCl_2$, and 50 mg isocitrate dehydrogenase (100 U) were also added. Incubation was performed at 31°C for 135 min and under these conditions 26% of the labelled tryptamine was converted to the substance with an R_F of 0.8. The incubation mixture was worked up as described under materials and methods using reverse phase adsorption chromatography of the alkaloids with the macroreticular polyacrylic ester Amberlite XAD-4

followed by thinlayer chromatographic separation. After purification the three unknown compounds were subsequently subjected to mass-spectrometry. Compounds A, B, C yielded an identical fragmentation pattern with m/e 352, 351, 337, 225, 223, 209, 184, 169, 156. The mass-peak and the typical fragmentation pattern of all 3 compounds suggested the occurrence of ajmalicine and two of its isomers.

The radioactive unknown compounds were therefore cochromatographed with authentic isomers of ajmalicine in 5 different solvent systems (II–VI).

The three unknowns had in all chromatographic systems exactly the same R_F values as: ajmalicine, 19-epiajmalicine and tetrahydroalstonine. Subsequently, the labelled unknown compounds were each diluted with the suspected unlabelled carrier material and recrystallized (5 times) from different solvents. Constant specific activity was reached in each case after the second crystallization step. Furthermore, compounds A and C were dehydrogenated by use of Pd. Under these conditions compound A was converted to serpentine and compound C to alstonine. The 3 compounds which were synthesized from tryptamine and secologanin under the catalysis of *C. roseus* enzymes can therefore unequivocally be assigned the following structure. A = ajmalicine, B = 19-epi-ajmalicine, C = tetrahydroalstonine (see fig.2). The compounds were formed during several incubations

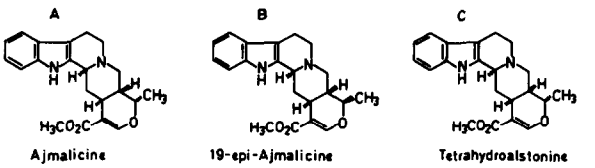

Fig.2. Structures of *Corynanthe*-type alkaloids synthetised from tryptamine, secologanin and reduced pyridine nucleotides by cell free extracts of *C. roseus*.

in an approximate ratio of A : B : C = 1 : 2 : 0.5. It is of interest to note that 19-epiajmalicine has up to now not been isolated from the differentiated plants while the other two compounds do occur in *Catharanthus* [11]. Formation of rauniticine and geissoschizine which are present in the intact plant was not detected under these cell free conditions. Compound D which accumulates in the absence of NADPH is a close precursor of ajmalicine. When this compound is isolated and subsequently incubated with NADPH in the presence of a crude homogenate of *C. roseus* cells, it is transformed predominantly into ajmalicine. The structure of this unknown metabolite (which is neither vincoside nor isovincoside), which is now under investigation should give a clue to the enzymatic mechanism involved in the biosynthesis of *Corynanthe* type alkaloids.

Finally, the cofactor requirement for the alkaloid

Table 1
Cofactor requirement for alkaloid synthesis

System	Total alkaloid[a] formed nmol	[^{14}C]Tryptamine converted to alkaloids[a] %
Complete	81	32
minus NADPH	0	0
minus NADPH plus NADH	72	29
plus pyridoxalphosphate	58	23
plus FAD	35	14
Heat denatured enzyme	0	0

[a] The alkaloids in question were: ajmalicine, 19-epiajmalicine and tetrahydroalstonine.

The reaction mixture contained in a final volume of 1 ml: 100 μmol KPO_4^- pH 7.5, 2 μmol reduced pyridine nucleotide, 0.25 μmol [2-^{14}C]tryptamine (1.25 μCi), 1.25 μmol secologanin, 1.6 mg protein. To this system was added either 0.5 μmol pyridoxalphosphate or 0.5 μmol FAD. Incubation: 120 min at 31°C.

formation was investigated. In a preliminary report [5] it was shown that the conversion of tryptamine and secologanin to geissoschizine and ajmalicine was catalysed in the presence of FAD and NADPH. The essentiality for NADPH for the enzyme reactions was previously only suggested on the basis of a slight increase of incorporation of tryptamine into ajmalicine by addition of NADPH using undialysed enzyme solution. As shown in table 1, the formation of ajmalicine, its 19-epi-isomer and tetrahydroalstonine under our conditions is competely dependent up on the presence of a reduced pyridine nucleotide. Replacement of NADPH with NADH gives the same yields within experimental error, however, NADPH yields a greater percent of over all conversion than NADH on an equal molar basis. Pyridoxalphosphate did not increase the conversion of tryptamine to ajmalicine nor did FAD which, in fact, proved to be inhibitory in the enzymatic formation of the three alkaloids under investigation. The pH optimum for this conversion was between pH 7.0 and 7.5. Comparing the alkaloid yields which have been derived from extracts of cell suspension cultures used here with those obtained previously [5] using callus tissue of *C. roseus*, the suspension culture system proved superior by a factor of 10 over the callus culture system which had been found to be superior to intact seedlings [5].

Our results confirm the observation by Scott and Lee [5] that a complete system of soluble enzymes which catalyses the formation of *Corynanthe*-type alkaloids from tryptamine and secologanin can be isolated from *C. roseus* cells. Furthermore it was found that this reaction is absolutely dependent upon the presence of a reduced pyridine nucleotide (either NADPH or NADH) whereas FAD is dispensable. A total of three *Corynanthe*-type alkaloids have been isolated by large scale incubation, and chemically identified, these are: ajmalicine, 19-epiajmalicine and tetrahydroalstonine.

A precursor of ajmalicine accumulates in the incubation mixture in the absence of reduced pyridine nucleotide. The chemical structure of this metabolite, which is now under investigation, should give information as to the exact enzymatic mechanism of the formation of *Corynanthe*-type alkaloids.

Acknowledgements

Our thanks are due to Miss B. Ries for excellent technical assistance, to Dr Husson, Gif-sur-Yvette, Dr Lamine, Bruxelles, Professor Shamma, University Park, PA. and Professor Winterfeldt, Hannover, for generous gifts of 19-epiajmalicine, geissoschizine and rauniticine respectively, to the Bundesminister für Forschung und Technologie, Bonn, for financial support and to Professor R. L. Mansell, Tampa, for checking the English version of the manuscript.

References

[1] Escher, S., Loew, P. and Arigoni, D. (1970) Chem. Commun. 823.
[2] Battersby, A. R. (1972) Acc. Chem. Res. 5, 148.
[3] Scott, I., Reichardt, P., Slayton, M. B. and Sweeney, J. G. (1971) Bioorg. Chem. 1, 157.
[4] Inouye, H., Ueda, S. and Takeda, Y. (1971) Chem. Pharm. Bull. 19, 587.
[5] Scott, A. I. and Lee, S.-L. (1975) J. Amer. Chem. Soc. 97, 6906.
[6] Zenk, M. H., El-Shagi, H., Arens, H., Stöckigt, J., Weiler, E. W. and Deus, B. (1977) in: 'Plant cell cultures and its biotechnological application' Springer Verlag, Berlin, Heidelberg, New York, in press.
[7] Murashihe, T. and Skoog, F. (1962) Physiol. Plantarum 15, 473.
[8] Gross, G. G. and Zenk, M. H. (1974) Eur. J. Biochem. 42, 453.
[9] Vogelmann, H. and Wagner, F. (1973) J. Chromatogr. 76, 359.
[10] Taylor, W. I. and Farnsworth, N. R. (eds) (1975) in: The *Catharanthus* Alkaloids, Marcel Dekker, Inc. New York.