Indirect Involvement of Geissoschizine in the Biosynthesis of Ajmalicine and Related Alkaloids

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Summary Geissoschizine (**10**) is not a central intermediate in the biosynthesis of ajmalicine (**7**) and the related *Corynanthe* alkaloids (**8**) and (**9**), but rather enters the main pathway by a $NADP^+$ dependent reaction.

In vivo feeding experiments of labelled geissoschizine (**10**) to *Catharanthus roseus* (syn. *Vinca rosea*) plants have demonstrated its role as a biosynthetic precursor for the three major classes of indole alkaloids, the *Iboga*, *Aspidosperma*, and *Corynanthe* families.[1] Subsequent studies on the detailed structure of the pathway leading to the *Corynanthe*-type alkaloids, *e.g.* ajmalicine (**7**), 19-epiajmalicine (**8**), and tetrahydroalstonine (**9**), have been carried out with a cell free system isolated from *C. roseus* tissue cultures.[2] This pathway is initiated by the formation of strictosidine (**3**) from the biosynthetic precursors tryptamine (**1**) and secologanin (**2**), catalysed by the enzyme strictosidine synthase. Compound (**3**) is then converted *via* a number of intermediates (see ref. 2) into 4,21-dehydrocorynantheine aldehyde (**4**) and then into cathenamine (**6**), which in turn is reduced by the cathenamine reductase in the presence of NADPH to the three ajmalicine isomers (**7**)—(**9**).

An enzymatic system from *C. roseus* callus tissue was reported[3] to synthesize geissoschizine (**10**) and to convert it into ajmalicine (**7**); thus, the specific precursor role of (**10**) seemed to be confirmed. However, the cell free system from *C. roseus* cell suspension cultures,[2] which forms ten times more of the alkaloids (**7**)—(**9**) than the callus system, did not yield (**10**).[4] Therefore a re-investigation of the role of geissoschizine (**10**) in the biosynthesis of the *Corynanthe*-type alkaloids was necessary; this paper reports results which clarify this point.

Firstly the role of (**10**) as a precursor of (**7**) *in vivo* was re-investigated by feeding [Ar-^{3}H]geissoschizine (**10**) (0·52 μmol, 9·32 μCi) to *C. roseus* plants (age 4 weeks) for 48 h. After chromatographic isolation and dilution with unlabelled ajmalicine (**7**) (25 mg) the alkaloid was recrystallized three times to constant specific radioactivity (d.p.m./25 mg: $2{\cdot}95 \times 10^4$; $2{\cdot}50 \times 10^4$; $2{\cdot}44 \times 10^4$). The incorporation

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rate of (**10**) into (**7**) was found to be 0·12%. This confirms the published data[1] and biosynthetic precursor role of (**10**) *in vivo*.

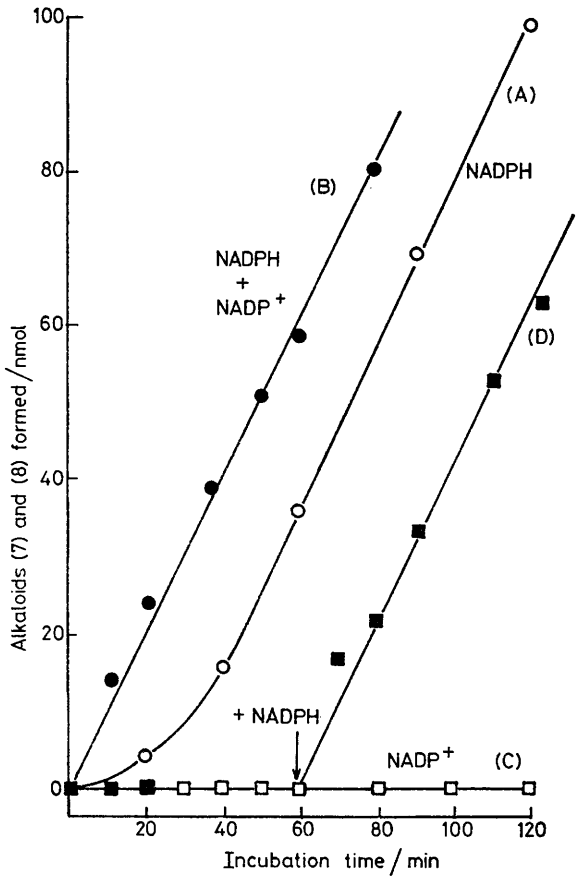
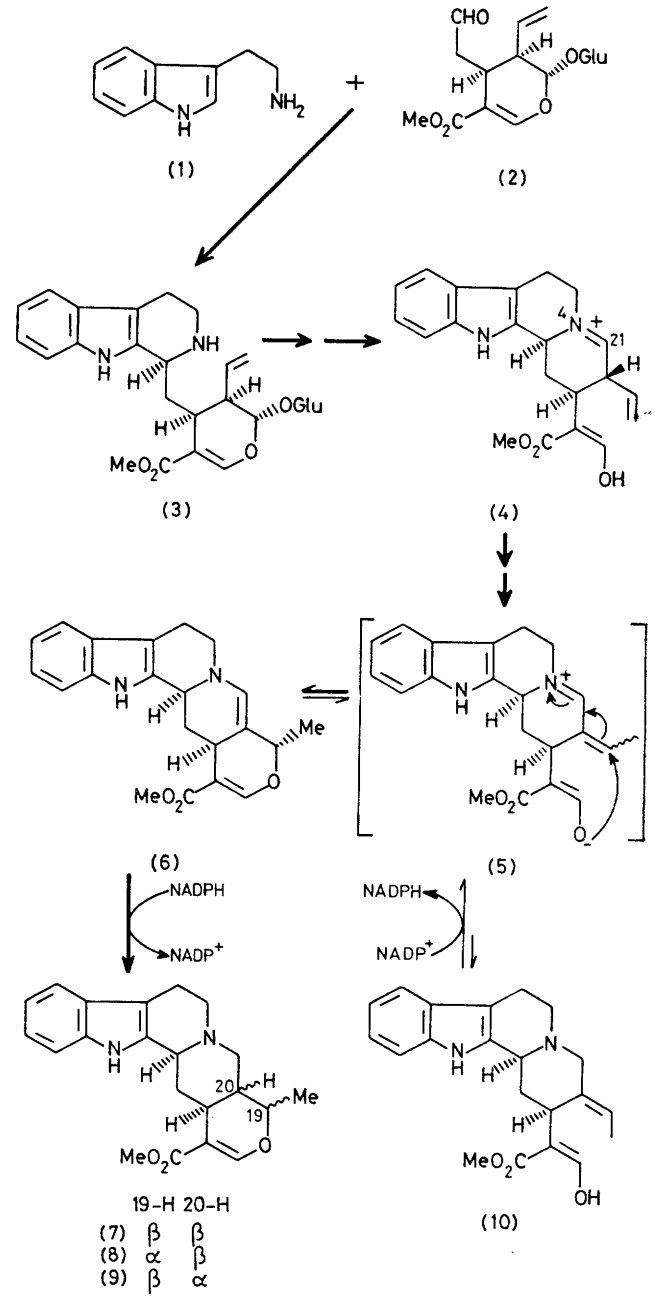


FIGURE. Enzymatic conversion of (**10**) into (**7**) and (**8**) and its dependence upon NADP+ and NADPH. Assays [4·0 ml, 0·4 mmol K–Pi buffer pH 7·0, 1·6 μmol (**10**), 29 °C] contained: A; 16 μmol NADPH, 14·4 mg protein. B; 8 μmol NADPH, 8 μmol NADP+, 17·9 mg protein. C; 16 μmol NADP+ 19·8 mg protein. D; 16 μmol NADP+, 19·8 mg protein (after 60 min 16 μmol NADPH was added).

Next the cell free conversion of (**10**) into (**7**) was re-investigated. An enzyme preparation obtained from *C. roseus* cell suspension cultures was incubated with [^{3}H]-(**10**) (56 nmol, 1 μCi) in the presence of NADPH (1 μmol) under previously described conditions.[4] T.l.c. isolation of the resulting *Corynanthe* alkaloids (**7**)—(**9**) (total conversion 7%) and isotope dilution analysis for (**7**) showed enzymatic formation of ajmalicine (**7**) in a yield of 1·6%. However, the same system under identical conditions synthesized 10% of (**7**) starting from [2-^{14}C]tryptamine (**1**) and secologanin (**2**) as precursors. This result and the high conversion rates (68%) of cathenamine (**6**) into (**7**)—(**9**)[5] indicate only indirect involvement of geissoschizine (**10**) in ajmalicine (**7**) synthesis.

In order to analyse this enzymatic reaction sequence further, a radioimmunosassay (RIA) method was used for quantitative estimation of the main alkaloids (**7**) and (**8**).[6] The optimum concentration of geissoschizine (**10**) for conversion into (**7**) and (**8**) was determined in a standard enzyme assay [total volume 0·5 ml, 10 μM—0·8 mM of (**10**), 1 μmol NADPH, pH 7·0, 2·5 mg protein, 30 °C, 120 min] and was found to be 0·5 mM. Under these conditions about 10% of the incubated (**10**) was converted into (**7**) and (**8**); at higher precursor concentrations (0·5—1·0 mM) the formation of the alkaloids (**7**) and (**8**) reached a plateau. When



SCHEME

tryptamine (**1**) (125 μM) and secologanin (**2**) (625 μM) were incubated under the same conditions in the presence of geissoschizine (**10**) at different concentrations (0·1—0·4 mM) the formation of (**7**) and (**8**) from (**1**) and (**2**), and (**10**) was additive. This indicates that the conversion of (**10**) into (**7**) and (**8**) is at least partly independent of the synthesis of (**7**)

and (**8**) from (**1**) and (**2**) and that the limiting step in the sequence (**10**) → (**7**) + (**8**) is not part of the sequence (**1**) + (**2**) → (**7**) + (**8**). The time course of the conversion of (**10**) into (**7**) and (**8**) was studied (Figure, A) in a 4 ml incubation mixture containing 1·6 μmol (**10**), 16 μmol NADPH, and 14·4 mg protein. The reaction clearly shows a lag phase of about 40 min after which alkaloid synthesis proceeds linearly with time. A possible explanation for this observation is that the conversion of (**10**) into (**7**) and (**8**) is dependent on both NADPH and $NADP^+$ and that the lag is due to the time required to generate a sufficient concentration of $NADP^+$ from NADPH by unspecific oxidases† in the cell free extract. This explanation was proved to be correct by the following series of experiments. As shown in Figure, B, the lag phase is eliminated if the incubation mixture contains both $NADP^+$ and NADPH. No conversion occurs in the presence of only the oxidised form of the co-factor (Figure, C), but alkaloid synthesis without a lag phase can be initiated under these conditions by addition of NADPH (Figure, D). These results demonstrate that the conversion requires both $NADP^+$ and NADPH and also, since the overall pathway from (**1**) and (**2**) to (**7**) and (**8**) is independent[7] of $NADP^+$, that the $NADP^+$ requirement must be in a step of the sequence (**10**) → (**7**) + (**8**), which is not part of the sequence (**1**) + (**2**) → (**7**) + (**8**).

With only the oxidised form of the co-factor present (Figure, C) small amounts of cathenamine (**6**) accumulated which were identified by t.l.c. comparison with an authentic sample. Presumably under these conditions of very low conversion of geissoschizine (**10**), the traces of NADPH generated by the initial reaction are immediately oxidised to $NADP^+$ by NADPH-oxidases, blocking the last reduction step in the cell free synthesis of the *Corynanthe* system.

The results clearly indicate that geissoschizine (**10**) cannot be a true intermediate in the main pathway of the conversion of tryptamine (**1**) and secologanin (**2**) into ajmalicine (**7**) and its isomers (**8**) and (**9**). They show that (**10**) is most likely derived from and channelled into the pathway *via* interconversion with one of the pathway intermediates before cathenamine (**6**), most plausibly (**5**), and that channelling of (**10**) into the main sequence is $NADP^+$- dependent and rate limiting for its conversion into (**7**), (**8**), and (**9**). These relationships are summarized in the Scheme.

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† The presence of such oxidases was indirectly demonstrated. In the presence of dithiotreitol (5 mM), a known inhibitor of these enzymes (J. Feierabend, personal communication), the geissoschizine (**10**) conversion in the presence of NADPH was inhibited by more than 50% by blocking the initial unspecific reaction.

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