36. Disproof of the Overall Enzymatic Biosynthesis of Vindoline from Tryptamine and Secologanin by Cell-Free Extracts from the Leaves of *Catharanthus roseus* (L.) G. DON

by **Joachim Stöckigt*, Heidrun Gundlach,** and **Brigitte Deus-Neumann**

Institut für Pharmazeutische Biologie der Universität München, Karlstr. 29, D-8000 München 2

(14.XII.84)

A cell-free extract, which was isolated from the leaves of mature *Catharanthus roseus* plants by a previously published procedure, *does not* convert a mixture of secologanin and radiolabelled tryptamine to vindoline, as was recently claimed. The radioactivity in the purified alkaloid extract determined by earlier workers is certainly due to 'impurities' in the presumed 'vindoline'. This was shown by extensive purification of the alkaloid extract (which contained added unlabelled vindoline as a carrier) followed by chemical conversion of vindoline to two derivatives and subsequent purification, ultimately giving unlabelled deacetylvindoline.

Introduction. – The monoterpenoid indole alkaloid vindoline has attracted much interest over the last 20 years. It is this monomer used in the chemical synthesis of the clinically important antitumor agent vinblastine by the modified *Polonovski* reaction [1]. The biosynthesis of vindoline has been extensively studied by *in vivo* feeding experiments with labelled precursors [2–4]. Unfortunately, the knowledge obtained from these experiments using intact plants provides only a very general description of the vindoline pathway. Vindoline is one of the *Aspidosperma*-type alkaloids which are synthesized by very complex biochemical routes. The results of feeding studies along with a structural comparison of vindoline and its biogenetic precursors tryptamine and secologanin, suggest that as many as 14 enzymatic reactions may be involved in the whole biosynthetic process.

A publication by *Kutney et al.* [5], claiming that vindoline could be produced from tryptamine and secologanin by a *single* crude enzyme extract, naturally generated much interest and surprise. Their results suggested to the first time the possibility of examining the vindoline pathway in detail at the cell-free level. A vindoline-specific radioimmunoassay (RIA) was subsequently developed and also applied to the study of such a cell-free system [6]. In this later report, it was stated that 'no trace of the formation of vindoline was observed' in an incubation mixture containing the crude enzyme extract, tryptamine, secologanin and co-factors. In [5], however, essential experimental data obtained had not yet been included[1]); so the direct comparison of the two sets of results was not possible. The Canadian group subsequently published a complete description of their work [7]. This permitted us to reinvestigate their cell-free system to resolve the conflicting conclusions.

[1]) Personal communication of Prof. *J. P. Kutney* to one of us *(J. S.)* at the '28th Congress of the International Union of Pure and Applied Chemistry', Vancouver 1981.

Results and Discussion. – A crude mixture of enzymes was isolated from the leaves of mature *Catharanthus roseus* plants and incubated in the presence of the known vindoline precursors [2-^{14}C]tryptamine and secologanin, together with FAD and NADPH as previously described (2 h at 34 °C) [7]. We modified the existing procedure by adding 10 mg unlabelled vindoline *immediately* after the incubation to insure the extraction of all of the ^{14}C-labelled vindoline which may have formed. The alkaloids from this incubation mixture were then extracted as described in [7], however, they were extensively purified by TLC and derivatisation. Vindoline, which had been isolated by TLC was converted to deacetylvindolinic acid. The later compound was subsequently converted to deacetylvindoline after chromatographic purification.

Previous workers attempted to determine the incorporation of [2-^{14}C]tryptamine into [^{14}C]vindoline by precipitation and recrystallization of the hydrochloride of vindoline. However, this procedure is quite ambiguous, because any of the formed alkaloids would also precipitate and would not lead to the correct specific radioactivity of vindoline. For example, we found in a quite different case [8] that six crystallization steps with a 'well-behaved' crystalline compound were needed to ensure an incorporation rate, because of co-crystallization of one or more other radioactive product(s). Clearly the above methodology of crystallization is not adequate for purification of alkaloids in any metabolic study with labelled substances. In particular, data obtained here concerning vindoline biosynthesis support and serve to this conclusion.

After extraction of the alkaloids from the above mentioned enzyme incubation mixture, we purified the residue on TLC and isolated the alkaloid vindoline. After rechromatography of the alkaloid, the radioscanner-trace *(Fig. 1)* showed that most of the label was associated with the vindoline-containing band. At this first stage of purification, the specific activity of the sample was 1.17×10^4 dpm/μmol. This value corresponds to a 1.16% specific incorporation of [2-^{14}C]tryptamine into [^{14}C]vindoline, which can satisfactorily be compared with that previously reported (1.1–1.36%) [5] [7]. After isolation and conversion of the alkaloid to deacetylvindolinic acid using an excess of EtONa, [^{14}C]-analysis of the purified acid *(Fig. 2)* showed only about 20% of the original 'vindoline' activity at the origin of the chromatogram. Based on the new determined specific activity of this acid (8.3×10^2 dpm/μmol), the incorporation of the progenitor tryptamine into vindoline would be only 0.08%. Deacetylvindolinic acid which was then rechroma-

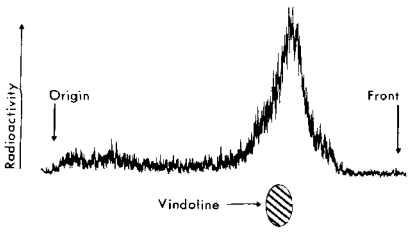


Fig. 1. *[^{14}C]-Analysis of the extracted enzyme incubation mixture after TLC (a) containing unlabelled vindoline as a carrier*

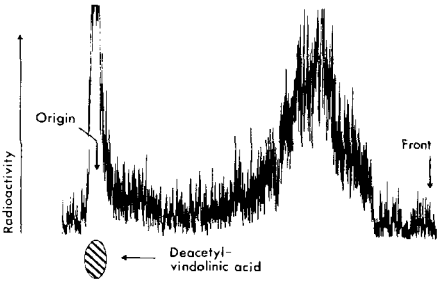


Fig. 2. *Separation of radioactivity from the vindoline-derived acid (treatment with EtONa).* Distribution of radioactivity in the chromatographed saponification mixture (a).

tographed in another solvent system (BuOH/acetone/H_2O/AcOH 3.5:3.5:2:1) contained extremly little of the [^{14}C]-activity which was originally present *(Fig. 3)*. Methylation of the reisolated deacetylvindolinic acid with CH_2N_2 yielded deacetylvindoline which was chromatographically purified twice. *Fig. 4* demonstrates the first chromatographic step and the distribution of radioactivity showed that deacetylvindoline is separated from any activity. The specific radioactivity of this once-purified vindoline derivative was determined to be 19 dpm/μmol (0.0019% yield of vindoline formation). A second purification gave, however, deacetylvindoline with no detectable radioactivity by szintillation counting *(Table)*.

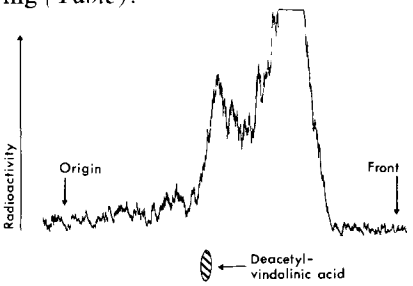


Fig. 3. *Rechromatography (b) of the extracted deacetylvindolinic acid from the chromatogram in* Fig. 2. The chromatogram shows that most of the radioactivity is separable from the vindoline derivative.

Fig. 4. *Distribution of radioactivity after esterification of deacetylvindolinic acid (excess CH_2N_2) and rechromatography (c) demonstrating that the vindoline derivative is unlabelled*

Table. *Rigorous Purification of [^{14}C]Vindoline by Derivatisation and the Determination of the Enzymatic Conversion of [2-^{14}C]Tryptamine to [^{14}C]Vindoline*

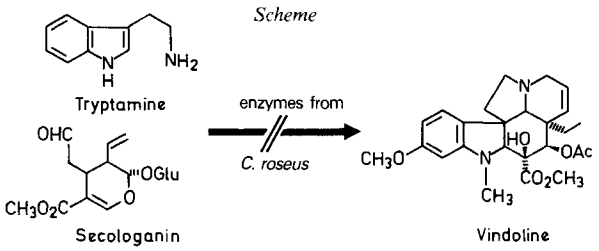
Vindoline and Derivatives	This work		Published data [5] [7]
	Specific Activity [dpm/μmol]	Conversion of [2-^{14}C]-tryptamine to [^{14}C]-vindoline [%]	Conversion of [2-^{14}C]-tryptamine to [^{14}C]-vindoline [%]
Vindoline	1.17×10^4	1.16	1.1–1.36
Deacetylvindolinic acid	8.3×10^2	8.2×10^{-2}	–
Deacetylvindoline	1.9×10^{1}[a])	1.9×10^{-3}[a])	1.1–1.36
Deacetylvindoline	0[b])	0[b])	–

[a]) After the first TLC (*c*). [b]) After the second TLC (*d*).

In summary, our data unambiguously demonstrate that a crude enzyme extract of *C. roseus* leaves described recently *does not* catalyze vindoline formation from tryptamine and secologanin *(Scheme)*. The results show that previous data [5] [7] on this subject are incorrect and that our results described here are in complete agreement with the earlier report in which vindoline biosynthesis could not be detected by the RIA method [6]. We also conclude that the complex vindoline pathway cannot be successfully investigated by the methodology employed in the past.

The authors would like to thank Mrs. *B. Krausch* for excellent technical assistance. Our thanks are also due to Dr. *W. R. Fields* (*Eli Lilly and Company*, Indianapolis) and to Prof. *H. G. Floss* (Ohio State University) for providing us with vindoline and to Dr. *J. Kilgore* (presently at Munich University) for his help in preparing the English version of the manuscript. The Deutsche Forschungsgemeinschaft is acknowledged for a *Finnigan MAT 44 S* instrument as well as for financial support *(SFB 145)*.

Scheme



Experimental Part

General. Catharanthus roseus plants were grown under standard green house conditions. The crude enzyme extract was isolated from leaves of mature *C. roseus* plants and was incubated with [2-^{14}C]tryptamine, secologanin and co-factors exactly as described by *Kutney et al.* [7], only using a larger amount of radioactivity (10 μCi instead of 2.5 μCi [2-^{14}C]tryptamine). TLC was performed on 20 × 20 cm plates with 0.25 mm layers of silica gel *(Polygram, Sil G/UV$_{254}$, Macherey-Nagel)*. The plates were developed with the following solvent systems: *a* (AcOEt/Et$_2$O/MeOH 1:1:0.5), *b* (BuOH/acetone/H$_2$O/AcOH 3.5:3.5:2:1), *c* (petroleum ether (30–60°)/acetone/Et$_2$NH 7:2:1), *d* (hexane/Et$_2$O/MeOH 1:1:0.2). The distribution of radioactivity on TLC plates was measured qualitatively by using a *Berthold* TLC Scanner *LB 2722-2*. The radioactivity of purified compounds was determined by szintillation counting with [^{14}C]toluene as internal standard with a *Berthold BF 5000* szintillation counter. For calculating specific activities, the total amounts of alkaloids present in labelled samples were determined by measuring their UV absorption using a *Perkin Elmer 551 S* instrument.

Isolation of [^{14}C]Vindoline from the Enzyme Incubation Mixture. After 2 h incubation, 10 mg unlabelled vindoline was added to the total mixture (24 ml). This solution was brought to pH 9.0 with ammonia, and it was subsequently extracted with five 10 ml portions of $CHCl_3$. The org. extracts were combined and evaporated. The residue was chromatographed on two TLC plates (*a*), vindoline was isolated, rechromatographed in the same TLC system and then measured for radioactivity. After extraction of the vindoline-containing band with MeOH, the alkaloid revealed a specific activity of 1.17×10^4 dpm/μmol, corresponding to 1.16% transformation of [2-^{14}C]tryptamine into radioactive vindoline. The portion of the extracts which was not used was dissolved in 1 ml EtOH and hydrolysed by an excess of EtONa for 16 h at r.t. The crude mixture was directly applied to two TLC plates (*a*). The formed deacetylvindolinic acid was eluted with MeOH and showed a specific activity of 830 dpm/μmol. This value represents an 8.2×10^{-2}% specific incorporation of tryptamine into vindoline. This once-purified material was again chromatographed (*b*), the plate was scanned for radioactivity, and then extracted with MeOH. The isolated acid then was converted to deacetylvindoline with an excess of CH_2N_2. Deacetylvindoline thus obtained was purified by TLC (*c*). After monitoring the distribution of radioactivity this vindoline derivative was also isolated (specific activity 19 dpm/μmol; or 1.9×10^{-3}% conversion rate of [2-^{14}C]tryptamine to [^{14}C]vindoline). Rechromatography (*d*) of this material gave deacetylvindoline which was not radioactive. *Deacetylvindoline*, EI/MS: 414 (3, M^+), 298 (8), 240 (55), 216 (15), 189 (33), 188 (48), 174 (33), 162 (23), 161 (25), 135 (100), 122 (55), 121 (70), 111 (30), 107 (50).

REFERENCES

[1] P. Mangeney, R. Z. Andriamialisoa, N. Langlois, Y. Langlois, P. Potier, *J. Am. Chem. Soc.* **1979,** *101,* 2243; French Patent 78.34599.

[2] G. A. Cordell, *Lloydia* **1974,** *37,* 219.

[3] G. A. Cordell, in 'Introduction to Alkaloids: A Biogenetic Approach', John Wiley and Sons, New York–Chichester–Brisbane–Toronto, 1981, p. 823.

[4] A. U. Rahman, A. Basha, in 'Biosynthesis of Indole Alkaloids', Clarendon Press, Oxford, 1983, p. 110.

[5] K. L. Stuart, J. P. Kutney, T. Honda, N. G. Lewis, B. R. Worth, *Heterocycles* **1978,** *9,* 647.

[6] P. Westekemper, U. Wieczorek, F. Gueritte, N. Langlois, Y. Langlois, P. Potier, M. H. Zenk, *Planta Med.* **1980,** *39,* 24.

[7] J. P. Kutney, L. S. L. Choi, T. Honda, N. G. Lewis, T. Sato, K. L. Stuart, B. R. Worth, *Helv. Chim. Acta* **1982,** *65,* 2088.

[8] J. Stöckigt, B. Ries, in 'Plant Tissue Culture and its Biotechnological Application', Eds. W. Barz, E. Reinhard and M. H. Zenk, Springer Verlag, Berlin–Heidelberg–New York, 1977, p. 208.