206. Biosynthesis of the Indole Alkaloids. Cell-free Systems from *Catharanthus roseus* Plants[1])

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Summary

Cell-free systems from *Catharanthus roseus* plants are utilized for various studies relating to the biosynthesis of indole alkaloids. Tryptamine (**5**) and secologanin (**6**), two fundamental building units, are shown to be incorporated into the alkaloid vindoline (**7**). In another study, catharanthine (**18**) and vindoline (**7**) are utilized by this enzyme system and coupled to the important bisindole biointermediate 3′,4′-anhydrovinblastine[3]) (**17**). The latter substance is, in turn, incorporated and converted to the natural alkaloids leurosine (**8**), catharine (**9**) and vinblastine (**10**), thereby providing information about the biosynthesis of these complex molecules. High pressure liquid chromatography assay of the enzymic mixture sheds light on the enzymes involved in the coupling of **18** and **7**.

The utilization of cell-free systems from *Catharanthus roseus* seedlings, plants or tissue cultures in indole alkaloid biosynthesis has been the subject of a number of recent publications from several laboratories. *Scott & Lee* [5] were the first to describe a cell-free system capable of synthesizing ajmalicine (**1**) and geissoschizine (**4**) from tryptamine (**5**) and secologanin (**6**; s. below). Subsequent studies in their or other laboratories [6–18] have provided information on some of the stages relating to the biosynthesis of such *Corynanthé* type alkaloids. For example, the condensation of tryptamine and secologanin for the biosynthesis of **1**, **4** and the related alkaloids 19-epiajmalicine (**2**) and tetrahydroalstonine (**3**) occurs in the presence of NADPH as a co-factor. More recent studies, particularly by *Scott, Zenk, Stöckigt* and their co-workers, have provided detailed information on the later-stage biointermediates [18]. While these investigations were underway we addressed ourselves to the biosyntheses of *Aspidosperma* and *Iboga* alkaloids. We wish to describe our experiments concerning the use of cell-free systems to demonstrate certain aspects of the biosynthesis of the alkaloid vindoline (**7**; s. *Scheme 2,*

[1]) For preliminary reports of this work, see [1–4].

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[3]) The previously [20] used name for **17**, 3′,4′-dehydrovinblastine, is incorrect.

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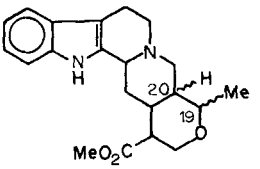
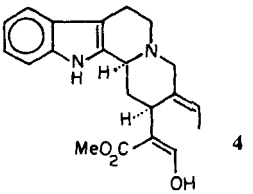
below) [1] and the more complex bisindole alkaloids leurosine (**8**), catharine (**9**) and vinblastine (**10**; s. *Scheme 3*, below) [2–4].

	Configuration at C(19)	C(20)
1	β	β
2	α	β
3	β	α





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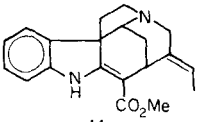
Cell-free extracts from *C. roseus*. – Leaves from mature, and in some cases flowering plants, were homogenized in tris-maleate buffer (0.05 M, pH 7.0) or phosphate buffer (0.1 M, pH 6.3), with or without added β-mercaptoethanol, in the presence of an equal weight of *Polyclar AT*. The supernatant produced (crude enzyme, *Scheme 1*) by centrifuging the extract at 30000 *g* was used in the experiments as described below. *Scheme 1* outlines the general procedure involved.

Scheme 1. *Outline of the procedure for the preparation of cell free extracts from* C. roseus

C. roseus leaves $\xrightarrow{a)}\ \xrightarrow{b)}$ crude enzyme $\xrightarrow{c)}\ \xrightarrow{d)}\ \xrightarrow{e)}$ partially purified enzyme.

a) Homogenization in 0.1 M potassium phosphate buffer, pH 6.3.
b) Centrifugation at 30,000 *g* for 20 min.
c) Ammonium sulfate precipitation (70% saturation), dialysis.
d) DEAE-cellulose chromatography.
e) *Sephadex G-200* chromatography.

Incorporations into vindoline (7). – The above cell-free preparation was incubated with [2-^{14}C]tryptamine hydrosuccinate and secologanin for 2 h at 34° in the presence of NADPH and FAD. Alkaloid products were extracted with chloroform after adjusting the solution to pH 9. Two-dimensional thin layer chromatography with reference alkaloids as standards showed, with the aid of autoradiography, that radioactive vindoline (**7**), tetrahydroalstonine (**3**), ajmalicine (**1**), akuammicine (**11**) and several other unidentified alkaloids were present. By preparative TLC. ^{14}C-labeled vindoline was isolated from the extract after dilution with inactive material. Vindoline hydrochloride was then formed and recrystallized to constant activity (at least 5 times). Radioisotope purity was confirmed by conversion to



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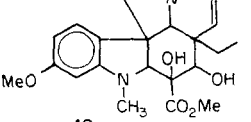

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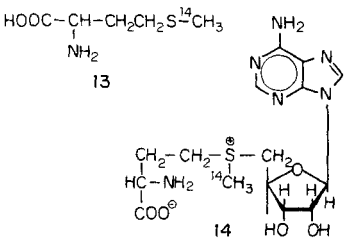

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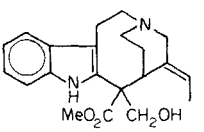

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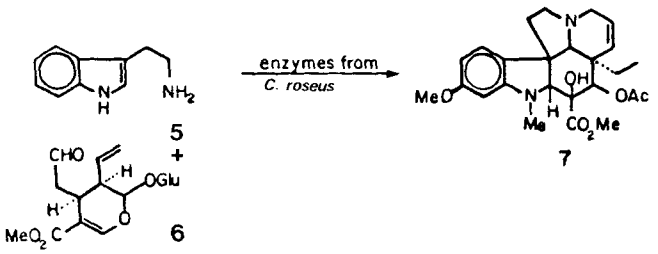
desacetylvindoline (**12**; purified by TLC. and recrystallized to constant activity) having the same molar radioactivity as vindoline hydrochloride.

In order to demonstrate the reproducibility of the enzymatic activity in the cell-free system, the above experiment was repeated twice using leaves from different *C. roseus* plants. Although no attempts were made to optimize the conditions, it was clear that the tryptamine was incorporated into vindoline to the extent of 1.10–1.36%. Parallel experiments with boiled cell-free extracts failed to give radio-labeled vindoline, indicating no incorporation. Furthermore, attempts to remove preformed alkaloids in the cell-free system with the aid of activated charcoal, prior to incubation, also resulted in the lack of incorporation of [2-^{14}C]tryptamine hydrosuccinate into vindoline. The removal of one or more unknown co-factors by the activated charcoal could be one possible explanation. The results of these experiments are summarized in *Scheme 2.*

Scheme 2. *The enzyme catalyzed synthesis of vindoline* (**7**) *from tryptamine* (**5**) *and secologanin* (**6**)



Incubation experiments using the crude cell-free extracts were also carried out with [*methyl*-^{14}C]methionine (**13**) and *S*-adenosyl[*methyl*-^{14}C]methionine (**14**). Whereas **14** was incorporated into vindoline (0.04%), **13** was not utilized.

Stemmadenine (**15**) has been postulated as a possible intermediate on the biosynthetic pathway to the *Aspidosperma* and *Iboga* alkaloids from the *Corynanthé-Strychnos* unit [19]. However, incubation of the cell-free preparation with [*Ar*-^{3}H]stemmadenine ([*Ar*-^{3}H]-**15**), under similar conditions as above, did not show significant incorporation into vindoline when compared to a boiled enzyme experiment. It is interesting to note that *Scott & Lee* did not detect the formation of stemmadenine (**15**) in their cell-free experiments [5]. Thus the exact role of **15** in the biosynthesis of *Aspidosperma* and *Iboga* alkaloids remained a challenge for further investigation. The above results concerning various substrate incorporations into vindoline with our cell-free extracts are summarized in *Table 1.*

Table 1. *Incorporations into vindoline* (**7**) *with cell free extracts*

Substrates	Incorporation [%]
[2-^{14}C]Tryptamine (**5**)	1.10
[2-^{14}C]Tryptamine (**5**)	1.36
[2-^{14}C]Tryptamine (**5**; boiled enzyme)	0.00
[*methyl*-^{14}C]Methionine (**13**)	0.00
S-Adenosyl[*methyl*-^{14}C]methionine (**14**)	0.04
S-Adenosyl[*methyl*-^{14}C]methionine (**14**)	0.04
[*Ar*-^{3}H]Stemmadenine ([*Ar*-^{3}H]-**15**)	0.006
[*Ar*-^{3}H]Stemmadenine ([*Ar*-^{3}H]-**15**; boiled enzyme)	0.007

Incubations of labeled 3′,4′-anhydrovinblastine[3] (17) and leurosine (8). – One of the major objectives in recent indole alkaloid research has been to improve the yield of vinblastine (**10**) and vincristine (**16**), the two clinically important antineoplastic agents isolated from *C. roseus* plants as very minor components (generally about $3 \cdot 10^{-4}$% based on dried plant). Questions concerning the biosynthetic pathway(s) to these bisindole alkaloids and related compounds have also provided a challenging experimental problem. In general, however, the majority of the biosynthetic experiments have been plagued by very low levels of precursors utilized and, in turn, inconclusive results.

The development of the 'biogenetic' approach in our [20] and other [21] laboratories, involving the coupling of catharanthine *N*-oxide with vindoline, afforded an important synthetic route to the bisindole system. Under our optimum conditions, 3′,4′-anhydrovinblastine[3] (**17**) could be obtained in respectable yield (>60%), and its role as a synthetic intermediate toward a variety of bisindole alkaloids and derivatives is also established from the extensive studies completed in our laboratories [22–26]. The question as to the relationship of the synthetic experiments and the enzymatic transformations involving **17** became therefore of considerable interest.

Our success with the cell-free extracts from *C. roseus* leaves in the vindoline area as described above immediately prompted an investigation into the possible role of **17** as the pivotal intermediate in the final stages of the biosynthesis of vinblastine (**10**), vincristine (**16**) and related compounds. To this end 3′,4′-anhydro-[*Ar*-^{3}H]vinblastine ([*Ar*-^{3}H]-**17**) was incubated at room temperature in solutions of the cell-free extracts at pH 6.3. Dilution with appropriate inactive alkaloids and rigorous purification to constant specific radioactivity gave the results listed in *Table 2*.

Direct incorporation of **17** into leurosine (**8**) and catharine (**9**) was high (8.15 and 15.15%, respectively). Significant incorporation (1.84%) of **17** into vinblastine (**10**) was also observed. These results support the theory that **17** is the key biointermediate of the vinblastine-type dimeric alkaloids. Incubation of [*Ar*-^{3}H]-

Table 2. *Incubation*[a] *of 3′,4′-anhydro*[Ar-^{3}*H*]*vinblastine* ([Ar-^{3}*H*]-**17**) *and* [Ar-^{3}*H*]*leurosine* ([Ar-^{3}*H*]-**8**) *with cell-free extracts*[b]

Expt.	Substrate	Alkaloid	Time [h]	Specific Incorporation [%][c]
1	[*Ar*-^{3}H]-**17**[d]	**16**	3	<0.0047[e]
2	[*Ar*-^{3}H]-**17**[d]	**16**	50	<0.03[f]
3	[*Ar*-^{3}H]-**17**[d]	**10**	3	1.84
4	[*Ar*-^{3}H]-**17**[d]	**8**	2	8.15
5	[*Ar*-^{3}H]-**17**[d]	**9**	3	15.15
6	[*Ar*-^{3}H]-**8**[g]	**9**	3	12.08
7[h]	[*Ar*-^{3}H]-**8**[g]	**9**	3	1.17

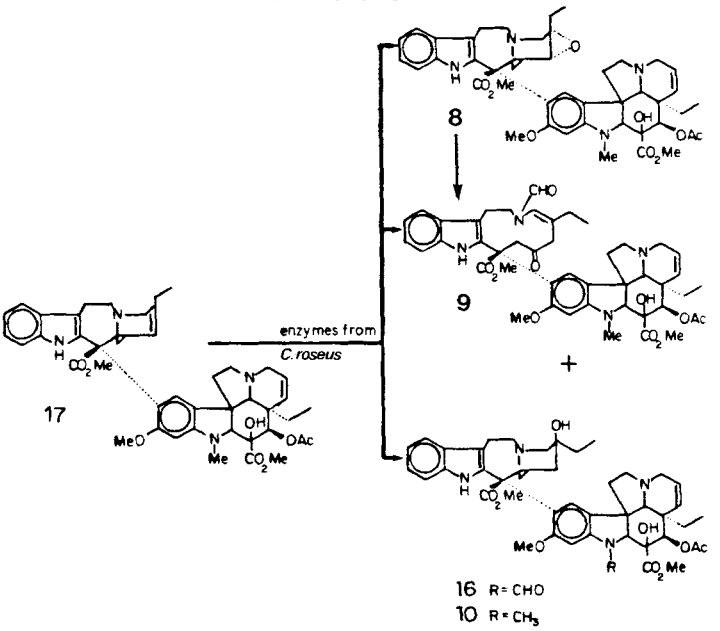
[a]) At room temperature. [b]) Oxidants (*e.g.*, H_2O_2) and antioxidants (*e.g.*, β-mercaptoethanol) were *not* added to these preparations. [c]) Factors accounting for the diversity of possible biochemical and dilution changes have *not* been used to adjust these values [27]. [d]) $1.40 \cdot 10^{11}$ dpm/mmol. [e]) Effectively zero. [f]) Insufficient material prevented purification to constant activity. [g]) $1.15 \cdot 10^9$ dpm/mmol. [h]) Blank reaction, *i.e.* identical to expt. 6, but without enzymes.

leurosine ([*Ar*-^{3}H]-**8**) afforded a 12% incorporation into **9**, while in a blank experiment only 1% conversion was noted (*Table 2*, expt. 6 and 7). These data provide substantial evidence that catharine (**9**) is indeed a 'natural product' and not an artefact [2] [3].

An independent and simultaneous study by *Scott et al.* [28] provided results which closely corroborate our above findings. Previously they observed that administration of labeled **17** to *C. roseus* shoots did not result in any measurable incorporation into vinblastine (**10**). However, utilizing cell-free preparations of *C. roseus* plants, they obtained radiochemical incorporation of **17** into **10** to the extent of 1.87%.

As shown in *Table 2* (expts. 1 and 2), even after a 50 h incubation negligible activity was transferred into vincristine (**16**). The very low incorporation values for vincristine clearly indicate that a more extensive stepwise approach will be necessary to determine the biosynthesis of this important dimeric alkaloid. A summary of these results is shown in *Scheme 3*.

Scheme 3. *Enzyme-catalyzed conversion of 3′,4′-anhydrovinblastine* (**17**) *to leurosine* (**8**), *catharine* (**9**) *and vinblastine* (**10**) *employing cell-free extracts*



Comparison of enzyme-catalyzed conversions of 17 to 8 with cell-free extracts and horseradish peroxidase. – Prior to the above-mentioned experiments with cell-free systems, we had already completed a chemical transformation of 3′,4′-anhydrovinblastine (**17**) into the alkaloids leurosine (**8**) and catharine (**9**) employing oxidative processes [25] [26]. In these latter studies, the most effective reagent was *t*-butylhydroperoxide although oxygen was also employed. The yield of leurosine in these chemical investigations was 52% while catharine could be obtained in 30% yield.

The closest 'enzymic analogy' to *t*-butylhydroperoxide is the commercial enzyme horseradish peroxidase (HRP), and it was therefore selected for the present studies. This choice was also influenced by the fact that peroxidase-type activity was detected in our cell-free extracts from *C. roseus* mature leaves, using two independent assay methods [29] [30]. As shown by expt. 2 in *Table 3*, addition of HRP, without activation, did not catalyze the formation of leurosine (**8**) from 3′,4′-anhydrovinblastine (**17**). However, on addition of a small excess of hydrogen peroxide, **8** was formed at a rate 12 times that observed for the blank experiment (expts. 3 and 4). Assuming a molecular weight of $4 \cdot 10^4$ for HRP [31] and a maximum of two oxidizing sites [32], this transformation was possible with a substrate/catalyst ratio of *ca.* 100.

Table 3. *Comparison of enzyme-catalyzed conversions of 3′,4′-anhydrovinblastine* (**17**)[a]) *to leurosine* (**8**) *with cell-free extracts and horseradish peroxidase*

Expt.	Enzyme[b])	H_2O_2 [mol-equiv.][c])	Time [h]	Yield[d]) [%]	
				17	**8**
1	None	0	100	> 98	–
2	A	0	100	> 98	–
3	None	1.3	1.5	87	5
4	A	1.3	1.5	28	65
5	None	0	3	> 98	–
6	B	0	3	46	22
7	None	1.3	3	49	26
8	B	1.3	3	5	25
9	B	1.3	1.5	5	27

[a]) At 23°, pH 6.3, *ca.* $5 \cdot 10^{-4}$ M. [b]) A: horseradish peroxidase, 17% by weight with respect to substrate. B: cell-free extracts from *C. roseus*. [c]) With respect to the substrate. [d]) As measured by HPLC. on the isolated product.

The reactions with the cell-free enzyme preparations from *C. roseus* plants were more complicated, not surprisingly, in view of the number of different enzymes expected to be present in the cell-free extracts. In fact, after a 3 h incubation and without added H_2O_2, only 46% of the substrate remained, and a 22% conversion to **8** was observed (expt. 6). When H_2O_2 was added (expt. 8), almost complete depletion of **17** was observed after 3 h, with a 25% yield of **8**. However, when compared to the corresponding blank experiment (expt. 7), this conversion is meaningless. More definitive results were obtained with a 1.5 h incubation (expt. 9), after which a 27% yield of **8** was obtained, compared with 5% for the blank experiment (expt. 3). Here also a 95% depletion of the substrate **17** was observed. Thus within the conditions of the experiment (expt. 9), a rate of formation of **8** approximately fivefold that of the corresponding blank was observed.

The measured yields of **8** seem affected by at least two factors. Firstly, the rapid consumption of **17** possibly by the peroxidase-type enzyme(s) as well as by other enzymes in the cell-free extracts, and secondly, further transformation of **8** may have occurred. The results suggest that leurosine (**8**) could possibly be formed from **17** by a peroxidase-type enzyme present in the cell-free preparation, although there is no direct proof at this stage as to the mechanism of this enzymatic conversion.

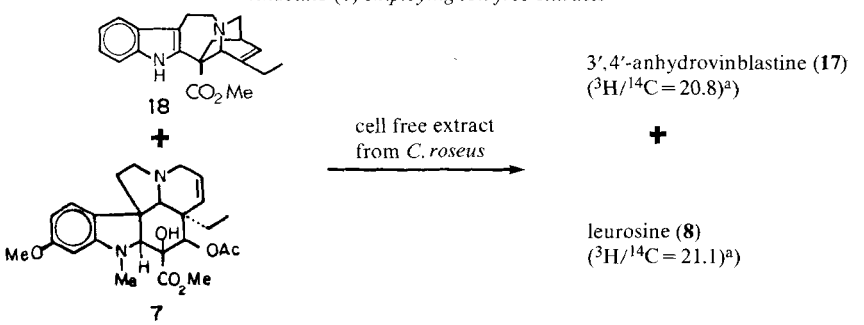
Incubations of labeled catharanthine (18) and vindoline (7). – Having demonstrated the enzymatic transformation of 3′,4′-anhydrovinblastine (**17**) to vinblastine (**10**), leurosine (**8**) and catharine (**9**) by the cell-free extracts, we next turned our attention to the biosynthesis of the important intermediate **17**. Earlier reports [33] have shown that while vindoline (**7**) appears to give satisfactory incorporation (0.05%) and served as a precursor for the *Aspidosperma* segment of **10**, the derivation of the '*Iboga*-half' of these bisindole molecules remained inconclusive. The use of apical cuttings of *C. roseus* resulted in an absolute incorporation of catharanthine (**18**) and **7** into **10** of only 0.006% [27]. Similar low incorporations were also observed with intact plants [34] [35].

Table 4. *Evaluation of catharanthine* (**18**) *and vindoline* (**7**) *as biosynthetic precursors of bisindole alkaloids employing cell-free extracts*

Expt.[a])	Substrate		Labeled products		Incorporation [%]	
	[*Ar*-^{3}H]-Catharanthine ([*Ar*-^{3}H]-**18**)	[*Ac*-^{14}C]-Vindoline ([*Ac*-^{14}C]-**7**)[b])	3′,4′-Anhydro-vinblastine (**17**)	Leurosine (**8**)	3′,4′-Anhydro-vinblastine (**17**)	Leurosine (**8**)
A	$1.667 \cdot 10^{10}$[c]) ($1.791 \cdot 10^{8}$[d])		$8.563 \cdot 10^{5}$[d])	$1.857 \cdot 10^{5}$[d])	0.48[e])	0.10[e])
B	$1.667 \cdot 10^{10}$[c]) ($1.671 \cdot 10^{8}$[d])		$8.955 \cdot 10^{5}$[d])	$5.925 \cdot 10^{5}$[d])	0.54[e])	0.36[e])
C	$1.667 \cdot 10^{10}$[c]) ($1.748 \cdot 10^{8}$[d])	$7.077 \cdot 10^{8}$[f]) ($9.451 \cdot 10^{6}$[g])	$6.201 \cdot 10^{5}$[d]) $2.979 \cdot 10^{4}$[g])	$5.936 \cdot 10^{5}$[d]) $2.812 \cdot 10^{4}$[g])	0.36[e]) 0.32[h])	0.34[e]) 0.30[h])

[a]) Expt. A: 3 h; Expt. B: 8 h; Expt. C: 6 h.
[b]) [*Ac*-^{14}C]means $CH_3{}^{14}CO$.
[c]) dpm ^{3}H/mmol.
[d]) dpm ^{3}H.
[e]) % Incorporation for ^{3}H.
[f]) dpm ^{14}C/mmol.
[g]) dpm ^{14}C.
[h]) % Incorporation for ^{14}C.

Scheme 4. *The biosynthesis of 3′,4′-anhydrovinblastine* (**17**) *and leurosine* (**8**) *from catharanthine* (**18**) *and vindoline* (**7**) *employing cell-free extracts*



[a]) See text and *Table 4*.

Using the present cell-free system we were able to simplify the methodology and obtained satisfying results. These are summarized in *Table 4*. [*Ar*-^{3}H]Catharanthine ([*Ar*-^{3}H]-**18**) was transformed to labeled **17** to the extent of 0.54%, and to **8** in 0.36% yield (expt. 3). Furthermore, simultaneous incubation of [*Ar*-^{3}H]-**18** and [*Ac*-^{14}C]vindoline ([*Ac*-^{14}C]-**7**) afforded doubly labeled **17** and **8** (expt. C). The ^{3}H/^{14}C ratio of an equimolar ratio of the substrates was 23.6 while that of the products was 20.8 for **17** and 21.1 for **8**, in good agreement with the obvious stoichiometry of the reaction. The results of this study are summarized in *Scheme 4*.

These results, together with the transformations of **17** to the alkaloids **10**, **8** and **9** strongly support the biosynthesis of the bisindole alkaloids as:

$$7 + 18 \rightarrow 17 \begin{matrix} \nearrow \mathbf{8} \\ \rightarrow \mathbf{9} \\ \searrow \mathbf{10} \end{matrix}$$

Enzymes involved in the coupling reaction. – It is obvious from the results described above that the cell-free extracts from the leaves of mature *C. roseus* plants contained a variety of enzyme systems responsible for the biosynthesis of various indole alkaloids. Of particular interest to us is the enzyme(s) that performed the coupling reaction of catharanthine (**18**) and vindoline (**7**) to the bisindole alkaloid **17**, and the subsequent transformation of the latter to other dimeric alkaloids. Thus we have initiated a study directed at the recognition and purification of the relevant enzyme(s) involved in this coupling reaction.

Table 5. *HPLC. analysis of standard proteins* (molecular weight *vs.* retention time)

Protein	Molecular weight (Mol.-wt.)	log (Mol.-wt.)	Retention time [min]
Ferritin	450,000	5.65	12.67
Catalase	240,000	5.38	14.07
Aldolase	158,000	5.20	14.60
Albumin (bovin serum)	68,000	4.83	14.66
Albumin (hen egg)	45,000	4.65	15.33
Horseradish peroxidase	40,000	4.60	16.51
DN ase	31,000	4.49	16.91
Chymotrypsinogen A	25,000	4.40	20.74
Cytochrome C	12,500	4.09	28.35

We applied HPLC. methodology to analyze the protein contents of the cell-free enzyme mixtures. *Figure 1* shows the HPLC. profile of the crude enzyme from *C. roseus* leaves prepared according to the procedure outlined in *Scheme 1*. The HPLC. system employed two protein columns (*Waters Associates I-250* and *I-125*) which were calibrated with a number of standard proteins. *Table 5* lists the retention times for these standard proteins obtained under the conditions used for the analyses of the cell-free extracts. From this it can be seen that the crude enzyme is a mixture of proteins varying in molecular weights of approximately 15000–450000 *(Fig. 1)*.

In order to establish a relationship between the molecular size of the enzyme(s) involved in the coupling of catharanthine and vindoline to the bisindole system, we further separated the cell-free extract (crude enzyme) by precipitation, dialysis and chromatographic techniques to a 'partially purified' enzyme stage *(Scheme 1)*.

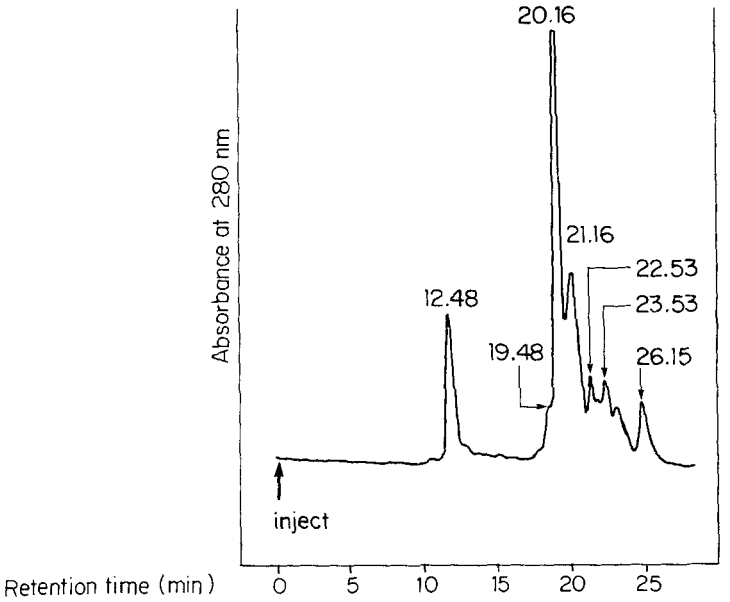


Fig. 1. *HPLC. analysis of cell-free extract prepared from* C. roseus *leaves*

The coupling enzyme activity was determined by monitoring the formation of **17** and **8** using radiolabeled tracer techniques with [*Ar*-^{3}H]catharanthine and vindoline as substrates.

For the further separation, the crude enzyme extract was brought to 70% saturation with ammonium sulfate. The precipitate thus formed was dialyzed against phosphate buffer (pH 6.8), and the dialyzate was applied on a DEAE-cellulose column equilibrated with potassium phosphate buffer (20 mM, pH 6.8). The elution

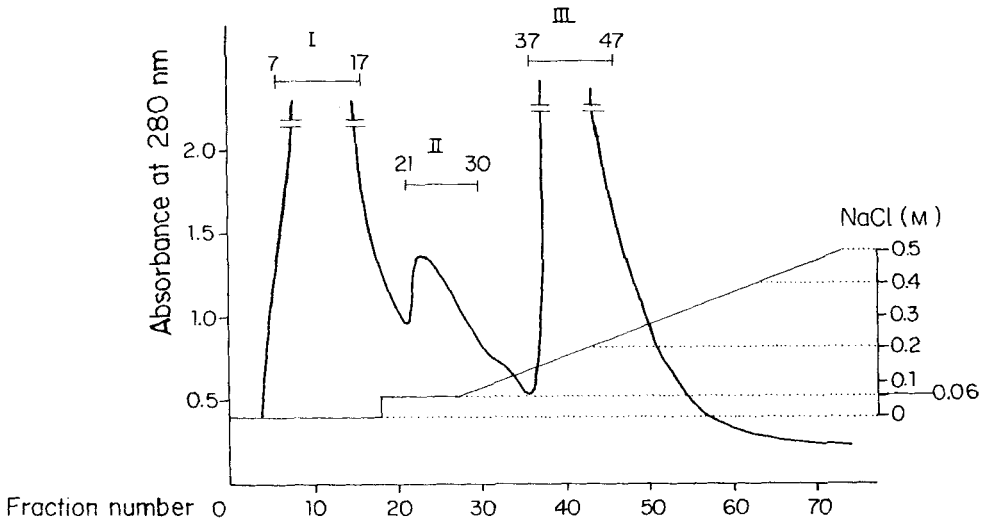


Fig. 2. *Elution profile of DEAE-cellulose chromatography of cell-free extract from* C. roseus *leaves*

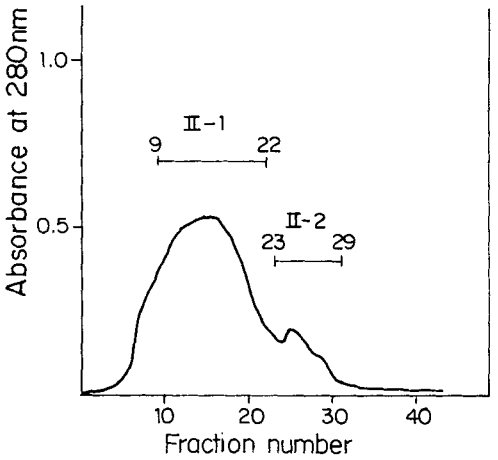


Fig. 3. *Elution profile of* Sephadex G-200 *chromatography of fraction II*

profile of the *DEAE-cellulose* chromatography is shown in *Figure 2*. Fractions 21–30 were found to possess the coupling enzyme activity and were therefore combined into fraction II, and concentrated to a small volume by ultrafiltration. This concentrate was then subjected to *Sephadex-G-200* chromatography which exhibited two peaks as monitored by the UV. absorbance at 280 nm *(Fig. 3)*. The fractions corresponding to the two peaks II-1 (fractions 9–22) and II-2 (fractions 23–29) were collected and analyzed by HPLC. (*Fig. 4* and *5*) as well as assayed for coupling enzyme activity. Fraction II-1 which possessed the desired coupling enzymes activity was further fractionated by HPLC. Three fractions (A, B and C;

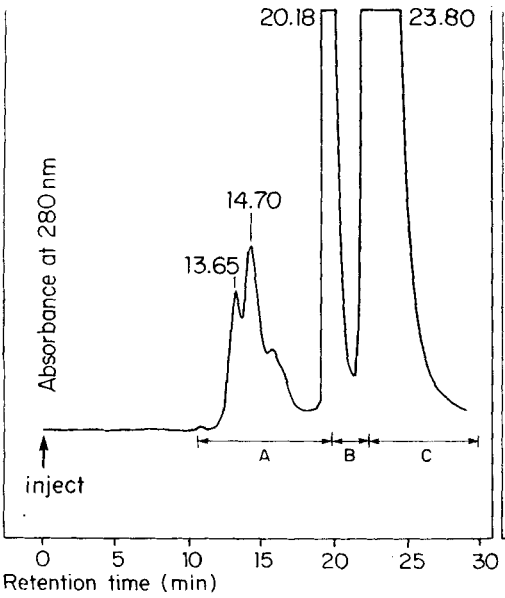


Fig. 4. *HPLC. analysis of fraction II-1*

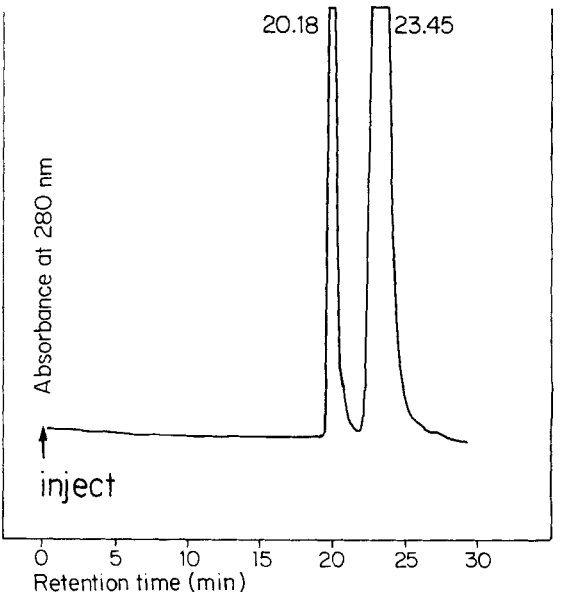


Fig. 5. *HPLC. analysis of fraction II-2*

Table 6. *Preparative HPLC. of the enzyme fraction II-1* (cf. Fig. 4) *and evaluation of coupling enzyme activity*

Fraction	Retention time [min]	Net activity [dpm] in labeled products	
		3′,4′-Anhydro-vinblastine (**17**)	Leurosine (**8**)
A	11–20	4137	3012
B	20–22.5	57	965
C	22.5–30	0	0

s. *Fig. 4*) corresponding to elution peaks of different retention times were collected and the results of their coupling enzyme activity determination are shown in *Table 6.*

It is clear from these investigations that the enzyme system(s) involved in the biosynthesis of **17** and **8** from the appropriate monomeric alkaloids are present in the short HPLC. retention time region of 11–20 min (Fraction A in *Fig. 4*). From the calibration standards *(Table 5)* this indicates proteins of molecular weight greater than 25 000.

All of the above studies were performed with cell-free systems obtained from *C. roseus* plants. We felt that a more preferable source would be tissue cultures in which a controlled and reproducible source of the enzymes could be available. Our extensive investigations with *C. roseus* cell cultures [37] have allowed us to pursue this direction, and indeed we have found similar enzyme systems in a number of the cell lines which we have developed. These results will form the subject of future publications.

We are grateful to the *Natural Sciences and Engineering Research Council of Canada* for financial support of these studies.

Experimental Part

Preparation of radioactive substrates. – *[Ar-^{3}H]Catharanthine ([Ar-^{3}H]-***18***).* [^{3}H]Trifluoroacetic acid, prepared by distillation of trifluoroacetic anhydride (0.9 ml) onto T_2O (0.1 ml, 500 mCi) using a vacuum system, was transferred by vacuum distillation to catharanthine (**18**; 220 mg, 0.65 mmol) at −78°. The resulting solution was maintained at −10° for 48 h. Excess [^{3}H]trifluoroacetic acid was removed by distillation after addition of CH_3OH (2 times 2 ml). The pH was adjusted to 8.5 with satd. $NaHCO_3$-solution at 0°, and then extraction with CH_2Cl_2 followed by drying (Na_2SO_4) and evaporation of the organic extracts afforded a crude product. This was purified by prep. TLC. (silica gel) and then recrystallization (5 times) from CH_3OH to give [*Ar*-^{3}H]-**18** of constant radioactivity ($1.667 \cdot 10^{10}$ dpm/mmol).

*[Ar-^{3}H]Stemmadenine ([Ar-^{3}H]-***15***).* The procedure used was essentially similar to the one described above for **18** except that the tritium exchange period was performed at RT. for 63 h. Radioactivity of [*Ar*-^{3}H]-**15** was $7.1 \cdot 10^6$ dpm/mg ($2.51 \cdot 10^9$ dpm/mmol).

*3′,4′-Anhydro[Ar-^{3}H]vinblastine ([Ar-^{3}H]-***17***).* The 3′,4′-anhydrovinblastine (**17**) was radiolabeled by exchange with [^{3}H]trifluoroacetic acid using a special platinum catalyst [36]. [^{3}H]Trifluoroacetic acid, prepared from trifluoroacetic anhydride (2 ml) and T_2O (0.2 ml, 1 Ci), was vacuum distilled onto a mixture of **17** (200 mg) and the platinum catalyst (1.3 ml) at −78°. The mixture was stirred at RT. for 24 h and worked up in the usual manner. Purification by prep. TLC. (silica gel) and recrystallization (from CH_3OH) to constant activity afforded [*Ar*-^{3}H]-**17** ($1.4 \cdot 10^{11}$ dpm/mmol).

*[Acetyl-^{14}C]vindoline ([Ac-^{14}C]-***7***).* Desacetylvindoline (**12**; 42 mg) was acetylated with [1-^{14}C]-acetic anhydride (21 mg, 1 mCi) and sodium acetate (4.2 mg) in benzene (95 mg) for 2 days at RT. Purification by prep. TLC. (silica gel) and recrystallization (from Et_2O) to constant activity afforded [*Ac*-^{14}C]-**7** ($7.077 \cdot 10^8$ dpm/mmol).

Preparation of cell-free extracts. – Leaves (5 g) from mature *Catharanthus roseus* plants, grown under greenhouse conditions, were homogenized with 50 ml of tris-maleate buffer (0.05 M, pH 7.0) or phosphate buffer (0.1 M, pH 6.3) at 4°. *Polyclar* (5 g) was added portionwise during the homogenization process. The homogenate was filtered through cheesecloth and then centrifuged at 30000 *g* for 20 min. The supernatant (≈ 25 ml; crude enzyme extract in *Scheme 1*) was used for various incubation experiments described below.

Vindoline (7) formation. – *Incubation with [2-^{14}C]tryptamine* (**5**) *and secologanin* (**6**). Co-factors FAD (0.5 mg) and NADPH (0.5 mg) were added to the crude enzyme extract (24 ml) along with [2-^{14}C]tryptamine hydrosuccinate (2.5 μCi/0.53 μmol) and **6** (0.1 mg), and incubated for 2 h at 34°. β-Mercaptoethanol (100 mM) was added to some preparations, but its inclusion was found to be non essential. For each experiment, a boiled enzyme preparation was used to serve as control to demonstrate the net enzyme activity. All experiments were performed in triplicate. Results quoted *(Table 1)* are an average of these 3 experiments. Alkaloidal products were extracted by adjusting the solution to pH 9 using NH_3-solution, and extracting with $CHCl_3$. Two-dimension TLC. (*Eastman Kodak silica chromagram* sheets) with reference alkaloids (Solvent system 1: $CHCl_3/CH_3OH$ 9:1; solvent system 2: ethyl acetate/hexane 3:2) showed with the aid of autoradiography that radioactive **7**, **3**, **1**, **11** and several other unidentified alkaloids were present. ^{14}C-Labeled vindoline was isolated from the alkaloidal extracts by prep. TLC. after dilution with inactive **7** (10 mg). Vindoline hydrochloride was then formed and recrystallized (Et_2O/MeOH) to constant radioactivity (at least 5 times) and showed a 1.10–1.36% specific incorporation of [2-^{14}C]tryptamine hydrosuccinate into **7**. Radioisotope purity was confirmed by conversion to **12** and recrystallization of the latter to constant activity having the same molar activity as **7**.

The boiled enzyme preparation showed no incorporation of [2-^{14}C]tryptamine hydrosuccinate into **7** under the same conditions *(Table 1)*.

Attempts to remove preformed alkaloids from the cell free extracts with activated charcoal resulted in the lack of incorporation of **5** into **7**. The removal of unknown co-factors could be one possible reason.

*Incubation with [*methyl-^{14}*C]methionine* (**13**). Crude enzyme solution (24 ml) was incubated with FAD (0.86 mg), NADPH (1.05 mg), **6** (0.96 mg), tryptamine · HCl (1.22 mg), and **13** (6.98 μCi) for 2 h at 34°. Recrystallization of isolated **7** as before showed no incorporation of **13**.

Incubation with S-*adenosyl[*methyl-^{14}*C]methionine* (**14**). Crude enzyme extract (23 ml) was incubated with FAD (0.86 mg), NADPH (1.15 mg), **6** (0.84 mg), tryptamine · HCl (1.51 mg), and **14** (2 μCi) for 2 h at 34°. Vindoline (**7**), isolated and purified as described earlier, showed a 0.04% incorporation of **14** *(Table 1)*.

*Incubation with [*Ar-^{3}*H]stemmadenine ([*Ar-^{3}*H]*-**15**). [*Ar-*^{3}H]-**15** (1.407 μCi) was incubated with the crude enzyme extract for 2 h at 34°. Isolated vindoline showed a 0.006% incorporation while the boiled enzyme preparation had a 0.007% incorporation *(Table 1)*.

*Incubation with 3′,4′-anhydro[*Ar-^{3}*H]vinblastine ([*Ar-^{3}*H]*-**17**). The crude enzyme extract was incubated with [*Ar-*^{3}H]-**17** ($1.40 \cdot 10^{11}$ dpm/mmol) for various periods of time at RT. (*Table 2*, expt. 1–5). Oxidants (*e.g.* H_2O_2) and antioxidants (*e.g.* β-mercaptoethanol) were not added in these experiments. Radioactive products **16**, **10**, **8**, and **9** were isolated after dilution with the appropriate inactive compounds as described before. Specific incorporations of [*Ar-*^{3}H]-**17** to **16** (< 0.03%), **10** (1.84%), **8** (8.15%), and **9** (15.15%) were calculated after recrystallization of each product to constant activity (except for **16**).

*Incubation with [*Ar-^{3}*H]leurosine ([*Ar-^{3}*H]*-**8**). Incubation of [*Ar-*^{3}H]-**8** ($1.15 \cdot 10^{9}$ dpm/mmol) with the cell free extract showed a 12.08% incorporation into **9**, while a similar blank experiment without enzymes showed a 1.17% incorporation into **9** (*Table 2*, expt. 6 and 7).

Detection of peroxidase-type activity in the leaves of *C. roseus*. – a) See [29]. The test reagent consisted of solution A (0.5% benzidine · 2 HCl) and solution B (0.1% sodium nitroprusside). Three leaves from a mature *C. roseus* plant were macerated in phosphate buffer (2 ml, 0.1 M, pH 6.3), and the extract was filtered. A portion of the filtrate was absorbed on *Whatman* No. 1 paper and a drop of a 1:1 mixture of solution A and B added, followed by a drop of 0.012% H_2O_2-solution. After a few minutes, a blue colour developed, indicating the presence of peroxidase-type enzyme(s).

b) See [30]. The reagent consisted of a solution of 5% pyrogallol in a phosphate buffer (0.1 M, pH 6.3). When the crude enzyme preparation from *C. roseus* was added, along with a 0.5% (*v*/*v*) H_2O_2-solution, a yellow colour rapidly developed, indicating the presence of peroxidase-type enzyme(s).

Comparison of enzyme-catalyzed conversions of 17 to 8 with *C. roseus* cell-free extracts and horseradish peroxidase. – The experimental conditions and results obtained are shown in *Table 3.*

Incubation with [*Ar*-^{3}H]catharanthine ([*Ar*-^{3}H]-18). – [*Ar*-^{3}H]Catharanthine ([*Ar*-^{3}H]-**18**); 2.285 mg, $1.791 \cdot 10^8$ dpm) and vindoline (**7**; 1.965 mg) were incubated with the cell free extract at RT. for 3 h. Usual workup followed by dilution with inactive **17** and **8** and recrystallization to constant activity showed incorporation of [*Ar*-^{3}H]-**18** to **17** (0.48%) and **8** (0.10%) (expt. A, *Table 4*).

A second incubation (expt. B, *Table 4*) using [*Ar*-^{3}H]-**18** (2.132 mg, $1.671 \cdot 10^8$ dpm) and **7** 2.36 mg) for 8 h at RT. resulted in **17** (0.54% incorporation) and **8** (0.36%).

Incubation with [*Ar*-^{3}H]catharanthine ([*Ar*-^{3}H]-18) and [*acetyl*-^{14}C]vindoline ([*Ac*-^{14}C]-7). – The crude enzyme extract was incubated with [*Ar*-^{3}H]-**18** (2.23 mg, $1.748 \cdot 10^8$ dpm and [*Ac*-^{14}C]-7 (4.118 mg, $9.451 \cdot 10^6$ dpm) (^{3}H/^{14}C equimolar ratio 23.6:1) at RT. for 6 h (expt. C, *Table 4*). Doubly labeled **17** and **8** were isolated from the basic organic extracts by prep. TLC. and recrystallized to constant activity. Incorporation to **17** was 0.36% for ^{3}H and 0.32% for ^{14}C with a ^{3}H/^{14}C equimolar ratio of 20.8:1. Incorporation to **8** was 0.34% for ^{3}H and 0.30% for ^{14}C with a ^{3}H/^{14}C equimolar ratio of 21.1:1.

HPLC. analysis of cell-free extracts. – The HPLC. system used was a *Waters Associates* ALC 100 modified to incorporate a 440 UV detector and data module using detection at 280 nm. Separation was achieved employing two protein columns (*Waters Associates* I-250 and I-125) with potassium phosphate buffer (0.05 M, pH 6.3) as solvent at a flow rate of 1 ml/min. Standard proteins of various molecular weight were used for calibration, and the results are shown in *Table 5. Figure 1* showes the HPLC. profile of the crude enzyme preparation using the same conditions.

Ammonium sulfate precipitation. The crude enzyme preparation was brought to 70% saturation with solid ammonium sulfate. After stirring for 30 min, the mixture was centrifuged at 30000 *g* (20 min). The precipitate formed was dissolved in potassium phosphate buffer (20 mM, pH 6.8) and dialyzed column (2.5 × 10 cm) equilibrated with potassium phosphate buffer (20 mM, pH 6.8). Elution (flow rate 48 ml/h) with the same buffer (100 + 100 ml with 0.06 M NaCl) was followed by a linear solvent against the same buffer.

DEAE-Cellulose *chromatography.* The above dialyzate (30 ml) was applied on a *DEAE-cellulose* gradient from 0.06 M NaCl to 0.5 M NaCl in the same phosphate buffer. Fractions (75 times 7 ml) were collected and analyzed for coupling enzyme activity. The elution profile as monitored by the absorbance at 280 nm is shown in *Figure 2.* Fractions 21–30 (peak II) were found to possess the coupling enzyme activity and were concentrated by ultrafiltration *(Amicon PM-10).*

Sephadex-G-200 *chromatography.* The above concentrate (5 ml) was applied onto a *Sephadex G-200* column (1.5 × 4.0 cm) equilibrated with potassium phosphate buffer (50 mM, pH 6.3, containing 1 mM EDTA) and eluted with the same buffer at a flow rate of 7.5 ml/h. Fractions (40 times 2 ml) were collected and analyzed for coupling enzyme activity. The elution profile as monitored by the absorbance at 280 nm is shown in *Figure 3.* The HPLC. profiles of the two peaks II-1 (fractions 9–22) and II-2 (fractions 23–29) are shown in *Figures 4* and *5*, respectively. Fraction II-1 which possessed the coupling enzyme activity was further fractionated by HPLC. using the same system as described earlier. The three fractions A (11–20 min), B (20–22.5 min) and C (22.5–30 min) corresponding to elution peaks of different retention times *(Fig. 4)* were collected and the results of their coupling enzyme activity determinations are shown in *Table 6.*

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