STUDIES ON INDOLE ALKALOID BIOSYNTHESIS. PART I. ALKALOIDS CONTAINING THE NORMAL TRYPTOPHAN SIDE CHAIN. THE LATER STAGES.

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A summary of the experiments relating to the biosynthesis of various indole alklaoids and performed in the author's laboratory is presented. The results as they relate to the later stages of the biosynthetic pathways in the Aspidosperma and Iboga bases are discussed in terms of the experiments performed in Catharanthus roseus G. Don (Vinca rosea L.) with the alkaloids vindoline and catharanthine. The plant, Vinca minor L., has been employed for obtaining some information about the eburnamine-vincamine series of alkaloids. The implications of these results and the remaining questions to be answered before definitive pathways can be proposed are also discussed.

The area of indole alkaloid biosynthesis has received a great deal of attention particularly during the last fifteen years. Numerous reviews[1-17] have appeared and these summarize the various experiments which have been carried out in the different laboratories. It is therefore

not the purpose of this article to provide yet another review on the subject but rather to focus attention on particular aspects of the biosynthetic pathway for which much definitive information is unavailable and which have continued to maintain an interest in our most recent investigations. This article will deal specifically with the later stages of various alkaloids which retain the two carbon side chain of tryptophan, now normally considered as the indolic building unit while Part II will concern itself with systems in which these carbon atoms are not evident.

As the above reviews indicate much information is now available on the earlier phases of the biosynthetic pathway but an objective analysis of published data reveals that most aspects of the later stages still remain in the realm of either pure speculation or, at best, approximations of the true in vivo processes.

With respect to the later stages our initial investigations[18,19,11,17] were stimulated by our synthetic studies in the indole alkaloid area wherein we had demonstrated that the transannular cyclization of appropriate nine-membered ring systems in the Aspidosperma and Iboga families could provide a particularly versatile and general approach to a considerable number of these bases[20-25]. Thus in vitro conversations of quebrachamine (I) → aspidospermidine (II), vincadine (III, R = H) → vincadifformine (IV, R = H) and carbomethoxydihydrocleavamine (V) → coronaridine (VI) could be accomplished via the iminium salts of I, III and V generated by means of mercuric acetate (Figure 1). It was thus logical to inquire whether this type of cyclization may play a role in the in vivo processes. Wenkert[26]

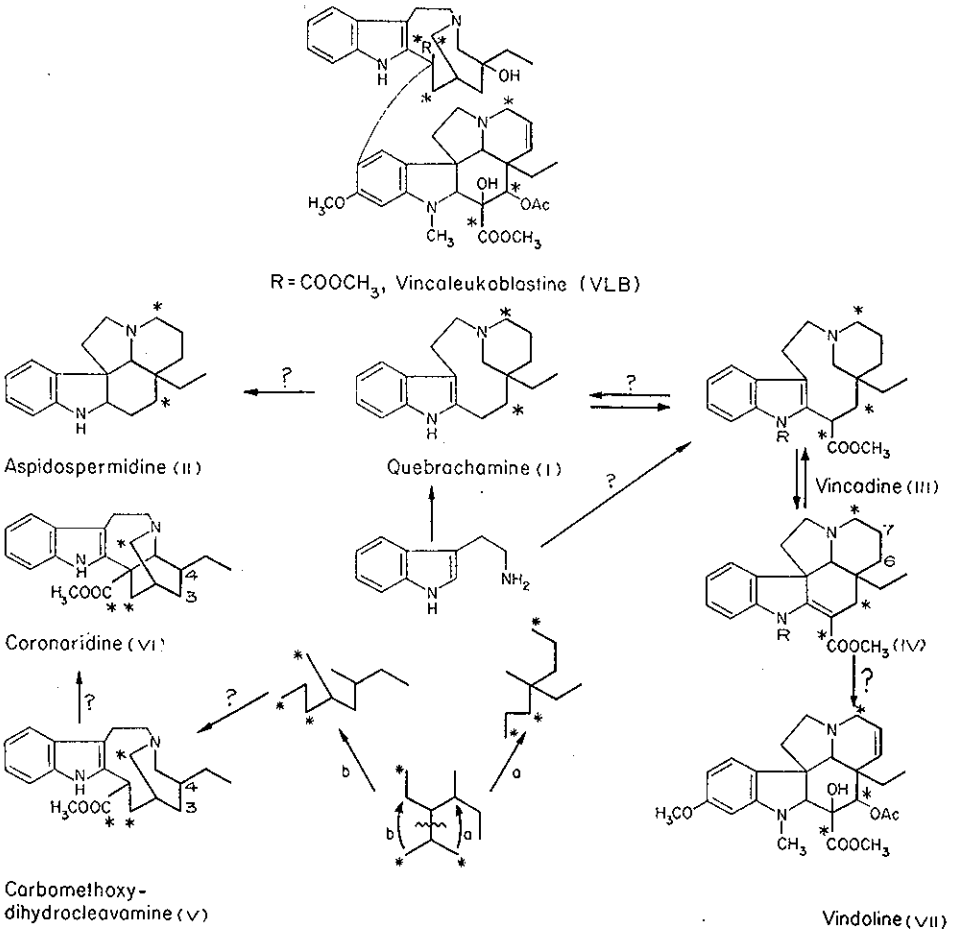


Figure 1. Schematic outline of our initial objectives as they pertain to later stages of Aspidosperma and Iboga alkaloid biosynthesis.

had already provided a postulate which invoked such a reaction in the late stages of the biosynthetic pathway (Figure 2).

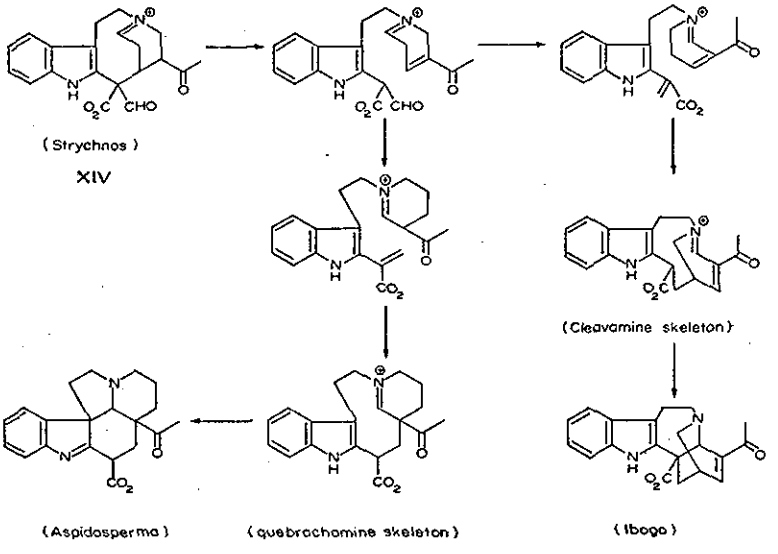
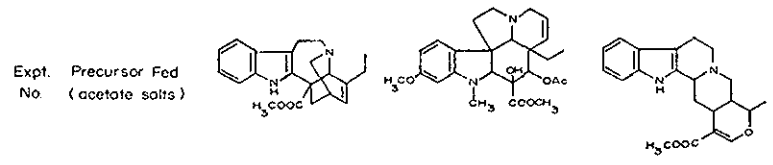


Figure 2. Wenkert's postulates as they relate to later stages of Aspidosperma and Iboga alkaloid biosynthesis.

In spite of numerous experiments (more than thirty) involving the incorporation of tritium-labelled derivatives of quebrachamine (experiment 9 in Figure 3) and cleavamine (experiment 11 in Figure 3) into the alkaloids of Vinca rosea L. under varying conditions, the results were not definitive. Figure 3 provides a brief summary of these various experiments.



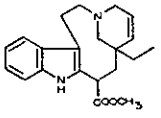
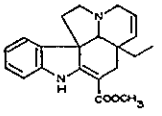
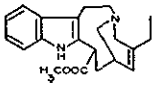
Expt. No.	Precursor Fed (acetate salts)	% Incorporation		
7	DL-Tryptophan [$3^{-14}C$]	0.05	0.15	0.8
8	[$ar^{3}H$]-tryptamine	0.01	0.003	0.4
9		<0.001	<0.001	<0.001
10		0.05	0.03	<0.001
11		0.03	<0.001	inactive
12	Blank	0.04	—	—

Figure 3. Results of incorporation of various intermediates into Vinca rosea L.

Although these experiments did not allow definitive evaluation of the importance of the transannular cyclization reaction in the biosynthetic pathway one set of important data did emerge from such investigations. Experiment 10 (Figure 3) performed in order to determine the plant's ability to utilize higher molecular weight substances in the biosynthesis provided a surprising result. The pentacyclic Vinca alkaloid tabersonine, labelled with tritium in the aromatic ring, was observed to incorporate into the Iboga system portrayed by catharanthine to the extent of about 0.05%. This experiment was repeated several times to ensure its authenticity. In an independent investigation with V. rosea seedlings Scott[27-29] observed a similar transformation and subsequently confirmed our findings in V. rosea plants. This bioconversion demands a considerable number of transformations since the structural features of the two alkaloid families are substantially different. In a formal sense the rearrangement requires the breaking of several bonds (see dotted lines in Figure 4) to provide an intermediate VIII which would cyclize to the

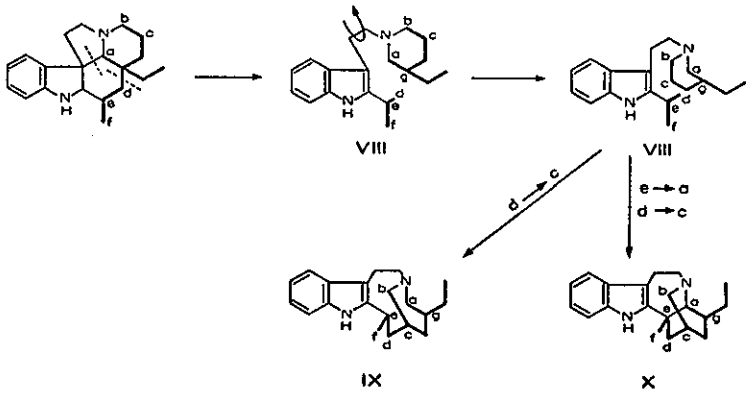


Figure 4. Outline of formal bond-breaking and bond-making process in the skeletal rearrangement of Aspidosperma to Iboga systems.

Iboga skeleton X. In consideration of our previous results involving the transannular cyclization it was obviously attractive to consider intermediate VIII for the later stages of both Aspidosperma and Iboga bases. Thus cyclization of VIII could proceed to the nine-membered cleavamine system (IX) and the pentacyclic skeleton X as shown in Figure 4, while Figure 5 reveals the possible pathways to the nine-membered quebrachamine (XI) and pentacyclic Aspidosperma alkaloids (XII).

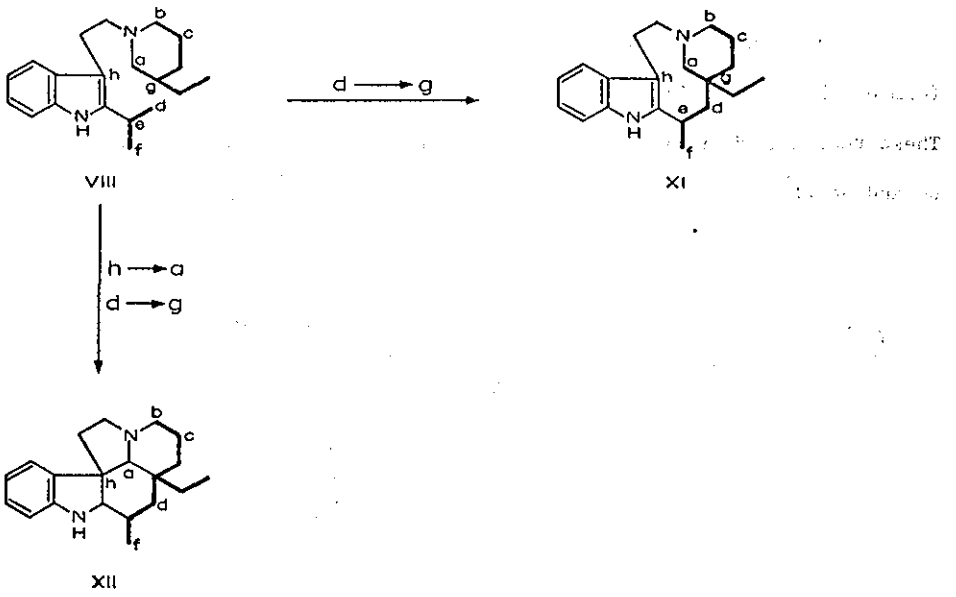


Figure 5. Outline of formal bond-making process in the biosynthesis of Aspidosperma alkaloids from intermediate VIII.

Thus the most important consideration which arose from the above results was the possibility that a common late stage intermediate bearing the structural features portrayed in VIII could be employed by the plant enzymes for the biosynthesis of all the alkaloid systems mentioned above.

It was now of interest to consider the nature of the functional groups which intermediate VIII may possess. Such considerations bring us back to the original Wenkert postulate (Figure 2) in which he suggested that the Strychnos alkaloids (for example, XIV) could serve as the precursors to the Aspidosperma and Iboga bases. Support for this postulate is now available from Scott's experiments on germinated V. rosea seedlings mentioned earlier in which it was shown that stemmadenine (XIII), merely a reduced form of the Wenkert intermediate XIV, does incorporate into these alkaloids.[28] These results, when considered with our investigations noted above allowed us and Scott[19,27] to postulate a rationale which is summarized in Figure 6.

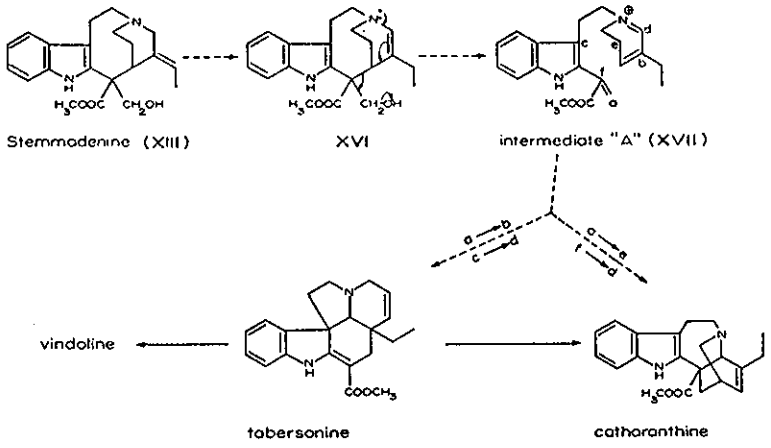


Figure 6. A possible rationale for the implication of intermediate "A" in the biosynthesis of Aspidosperma and Iboga alkaloids.

The rearrangement of stemmadenine to the "isostemmadenine" system is purely speculative and simply allows a convenient mechanism for the ring fission to intermediate "A" (XVII). The close similarity of these latter stages with those proposed by Wenkert (Figure 2) is of interest.

It was now clear that some experimental support for intermediates bearing the structural features portrayed in VIII and XVII was required. Unfortunately suitable compounds of this type are not readily available from natural sources so a suitable laboratory pathway was essential. The most desirable synthetic route should possess sufficient versatility to allow introduction of label at various positions in the molecule and, simultaneously, take account of the well-known instability of dihydropyridine systems and the high reactivity of acrylic esters, both features of which were present in intermediate "A". Consideration of these various factors led to the development of the pathway summarized in Figure 7.

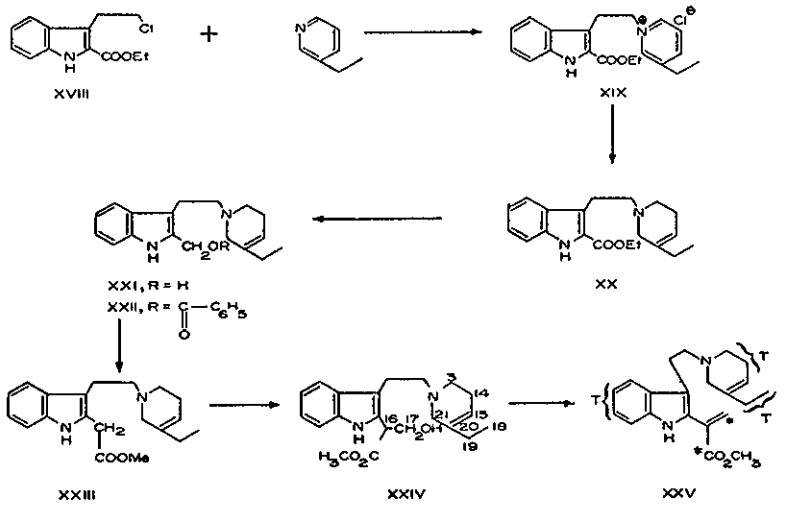


Figure 7. Synthesis of 16,17-dihydrosecodin-17-ol (XXIV) and secodine (XXV).

The positions of tritium and carbon-14 (asterisk carbons) which could be achieved in this manner are shown in XXV.

Initially our synthetic investigations led to 16,17-dihydrosecodin-17-ol (XXIV). Since the latter is a stable compound its utilization in the biosynthetic experiments was considered. It was hoped that the required dehydration of XXIV to the acrylic ester side chain and oxidation of the tetrahydropyridine unit to the dihydro stage, as required in XVII, could be readily achieved by the plant enzymes. Unfortunately all attempts to incorporate this substance into various alkaloids present in V. rosea, V. minor and Aspidosperma pyricollum met with failure (Table I). Whether

TABLE 1

Results of Incorporation of [ar^{3}H]-16,17-Dihydrosecodin-17-ol into Different Plant Systems

Plant Species	Feeding Period	Feeding Method	Activity Fed	% Incorporation	
V. minor				Minovine	Vincamine
1	24 hrs*	Hydroponic (acetate)	1.86 x 10^{7} dpm	0	<0.001
2	96 hrs*	"	2.40x 10^{7} dpm	<0.001	<0.001
V. roseo	216 hrs	Wick	1.89 x 10^{7} dpm	Vindoline	Catharanthine
				<0.001	<0.001
A. pyricollum	5 days	Hydroponic (roots)	9.20x10^{7} dpm	Apparicine	Uleine
				<0.001	<0.001

*Marked plant deterioration after 24 hrs.

these negative results were an indication that the plant systems employed were incapable of the required conversions, XXIV → XVII, for example, remained an open question at this point in time. It was thus decided that a chemical conversion of the alcohol XXIV to secodine (XXV) was an obvious requirement.

The dehydration of XXIV to XXV proved extremely difficult. The usual reagents normally employed for such a transformation simply led to intractable tars. The anticipated instability of secodine was clearly apparent from these investigations and it became obvious that its isolation would require non-acidic conditions and an inert atmosphere. After many frustrations it was observed that treatment of XXIV with sodium hydride under carefully controlled conditions provided secodine and thus a pathway to variously labelled forms of this compound became available. It was now appropriate to evaluate the role of this substance in the later stages of the biosynthetic pathway.

The initial experiments with radioactive secodine were performed in V. rosea and V. minor. Various details concerning these results are already published elsewhere [30,31] so only the most pertinent results are summarized here. As Table 2 reveals, our first observations with [ar^3 H]-secodine showed that incorporation were generally low. The reasons for this are, at least in part, associated with the instability of the secodine molecule. We could show in blank experiments that this substance undergoes dimerization in the manner already described by Smith [32] to the extent of about 40% in the time period (usually 2 - 4 hours) required for the precursor to be absorbed by the Vinca plants. Clearly unless the plant enzymes are

TABLE 2

Results of Incorporation of [ar^{3}H]-Secodine into V. rosea L. and V. minor L.

Plant Species	Feeding Period	Feeding Method	Activity Fed	% Incorporation		
V. minor				Minovine	Vincamine	
1	24 hrs	Hydroponic (acetate)	3.40×10^6 dpm	< 0.001	< 0.001	
2	96 hrs	"	2.65×10^6 dpm		0.0027	
3	96 hrs	Tween 20 Suspension	2.51×10^6 dpm	< 0.001	< 0.001	
V. rosea				Ajmalicine	Catharantine	Vindoline
1	216 hrs	Wick (acetate)	3.31×10^6 dpm	< 0.001	< 0.001	0.01

capable of converting these dimers to the secodine system, at least this portion of the substance is lost for the biosynthetic purposes. The results as quoted in the various Tables are not corrected for such losses and thus the levels of incorporation are actually significantly higher than reported. A low but definite incorporation of secodine was clearly established by repeated experiments with various plants at different stages of development. Subsequent experiments employed doubly labelled secodine to determine if the entire secodine molecule is being utilized by the plant and Table 3 provides a brief summary of some of the pertinent results.

As Table 3 shows experiments were performed in three different plant systems and the indicated alkaloids were isolated and evaluated for their tritium and carbon-14 levels. It is clear that there is an insignificant exchange of tritium in the aromatic ring, a result consistent with that observed previously in the tryptophan incorporations.[33] It was also obvious that the carbomethoxy group of the secodine molecule was being retained and subsequent degradation experiments as discussed below established the position of the carbon-14 label.

TABLE 3

Results of Incorporation of [ar^{3}H,^{14}COOCH$_3$]-Secodine into Different Plant Systems

Expt	Plant fed	Ratio of activity fed (^{3}H/^{14}C)	Ratio of activity isolated (^{3}H/^{14}C)
1	V. rosea	8.8	Vindoline 8.3
2	V. minor	8.4	Vincamine 8.6
3	A.pyricollum	8.7	Apparicine 8.4

Since the levels of incorporation of secodine into the alkaloid vindoline were consistently higher than the other alkaloids isolated from V. rosea, this alkaloid was first investigated in terms of further incorporation experiments and degradation of the isolated alkaloid.

Additional incorporation experiments with different labelled forms of secodine included samples in which the tritium label was present in the piperideine unit (Figure 8) and the ethyl side chain (Table 4). In the series of experiments summarized in Figure 8 it was observed that 60% loss of tritium occurs when this label is situated in the piperideine unit of the secodine molecule. The degradation of vindoline revealed that the ester group of the acrylic ester side chain is retained in the ester function of the isolated alkaloid. These results, in summary, indicated that the indole unit of the secodine system was being utilized by the plant with little or no alteration while the piperideine unit was being transformed in some fashion so as to account for the observed loss of tritium. The most attractive postulate to explain the loss of the latter label involves some oxidative processes in which a higher oxidized form, perhaps

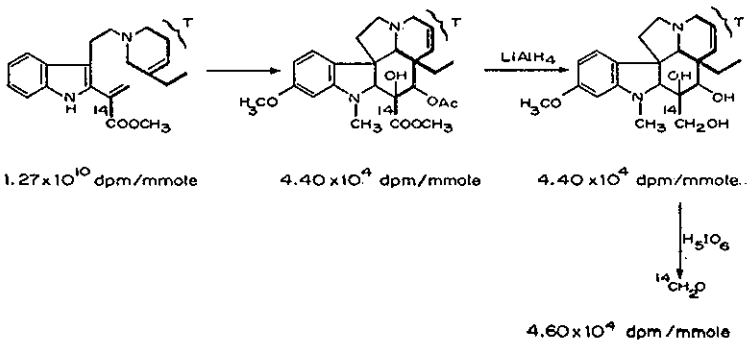


Figure 8. Degradation of vindoline from [^{14}COOCH$_3$,3,14,15,21-^{3}H]-secodine.

a dehydrosecodine as shown in intermediate "A", is produced. At this point in time no more definitive conclusions could be made.

TABLE 4

Results of Incorporation of [$^{14}COOCH_3$, 19-^{3}H]-Secodine into Different Plant Systems

Expt	Plant fed	Ratio of activity fed ($^3H/^{14}C$)	Ratio of activity isolated ($^3H/^{14}C$)
1	V. rosea	1.54	Vindoline 1.35
2	V. minor	1.82, 1.84	Vincamine 1.80, 2.06
3	A. pyricollum	1.91	Apparicine 2.60 Uleine

The experiments summarized in Table 4 provide important information about the incorporation of the secodine system into the various alkaloids isolated. Thus the obtained results are consistent only with the intact incorporation of the entire secodine molecule into vindoline, vincamine and apparicine. The latter alkaloid will be discussed in greater detail in Part II while investigations in the vincamine series are presented here.

Vinca minor was the plant selected for biosynthetic investigations in the eburnamine-vincamine family. Interestingly, although considerable attention had been given to the structural and synthetic chemistry within this series of alkaloids, no experimental data relating to the biosynthetic pathway were available when our experiments were initiated. Wenkert[34] had proposed (Figure 9) that an Aspidosperma intermediate (XXVI) was a likely precursor which via rearrangements to XXVII, XXVIII

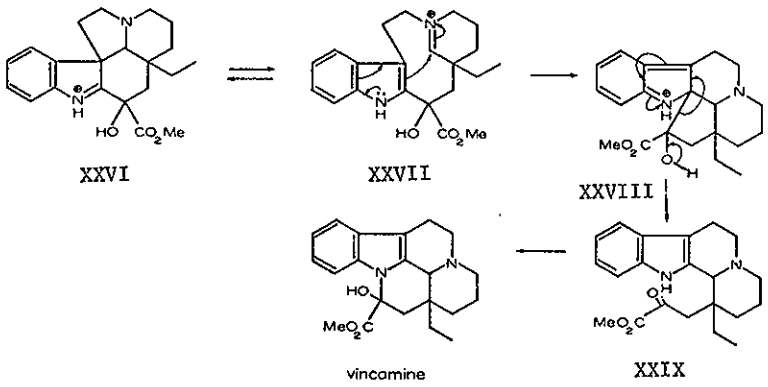


Figure 9. Wenkert's postulate as it relates to the eburnamine-vincamine family of alkaloids.

and XXIX eventually leads to vincamine. Obviously this postulate in view of previous results[8,11] implicates the Corynantheinoid (geissoschizine), Strychnos (stemmadenine) and secodine systems as precursors in the biosynthesis of vincamine (Figure 10).

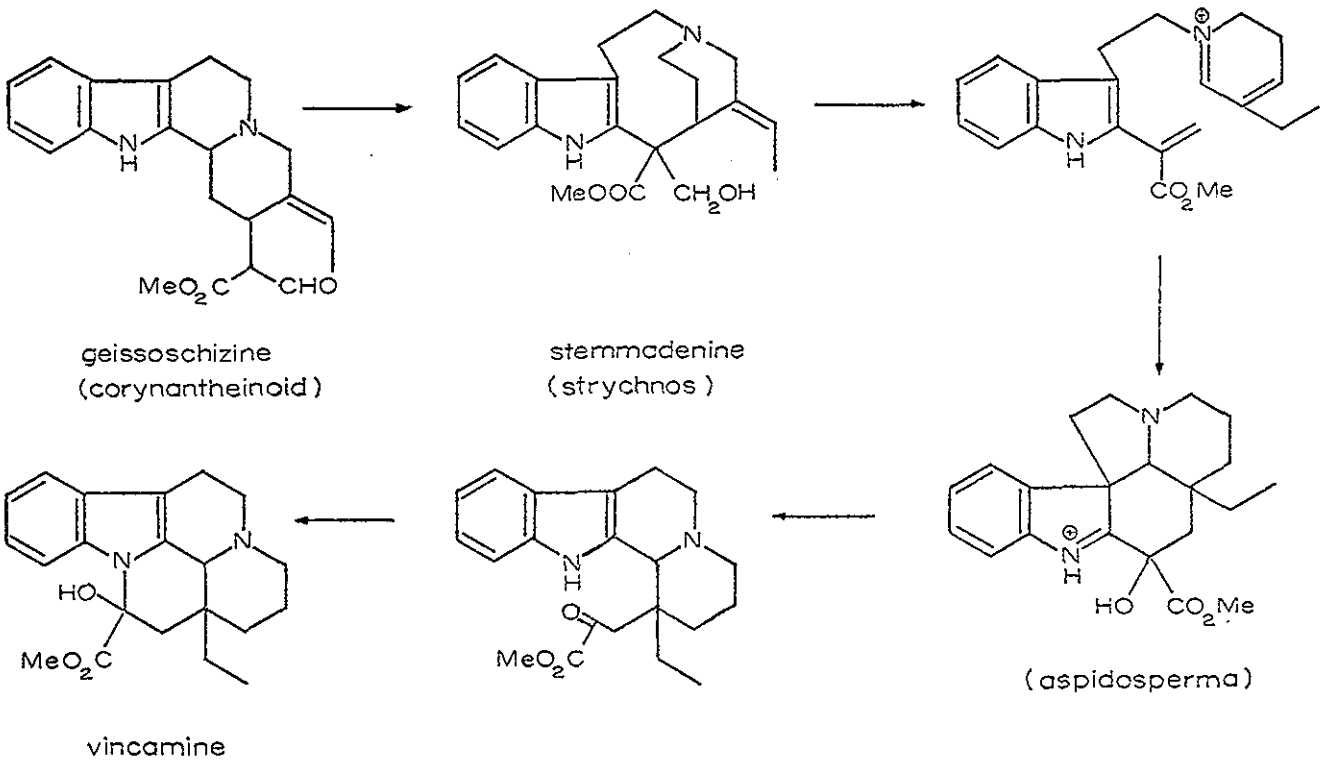


Figure 10. Summary of postulates which relate to the biosynthesis of vincamine.

Supporting evidence for the above proposals was provided in our initial experiments with various aromatically labelled compounds which were incorporated into vincamine in V. minor (Figure 11).[30] These investigations demonstrated that the interrelationships outlined in Figure 10 may indeed be correct and it was now appropriate to consider a more detailed evaluation of secodine in the late stages of the biosynthetic pathway.

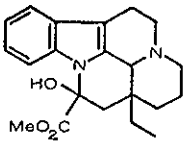
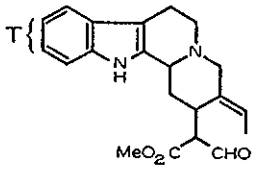
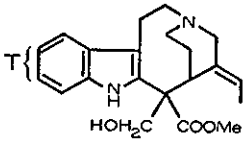
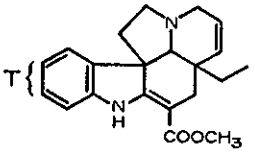
Expt	Compound fed (acetate salts)	% Incorporation
	 vincamine	
1	[ar^3–H]–Tryptophan	0.089
2	 (corynantheinoid)	0.005
3	 (strychnos)	0.076
4	 (aspidosperma)	0.07

Figure 11. Results of incorporation of various intermediates into V. minor.

As Table 2 reveals, the experiments in V. minor were frustrated by low incorporations which made it considerably more difficult to interpret the obtained results. However numerous experiments in this plant with the different labelled forms of secodine were performed. These investigations also involved different time periods (4 days - 3 months) for the plants to perform the desired biosynthesis. The 3 month period was employed since Scott[35] had shown in the Strychnos series that long term feedings can provide interesting results. Unfortunately such was not the case in our studies. Various degradations on the isolated radioactive vincamine were carried out and it was clear that the entire secodine molecule was being incorporated into the alkaloid. The most pertinent results are summarized in Figure 12. Due to the frustrations associated with low levels of incorporation in V. minor plants, further experiments to derive additional biosynthetic information were discontinued.

Our interests now turned to the investigations concerning the late phases of Iboga alkaloid biosynthesis since, as already mentioned, only speculative mechanisms had been advanced. Catharanthine, one of the major alkaloids in V. rosea was the obvious candidate since as Table 2 reveals, we had already some data from earlier investigations. Experiment 11 in Table 2 revealed 0.03% incorporation of aromatically labelled carbomethoxycleavamine into catharanthine but subsequent blank experiments (experiment 12, Table 2) indicated that this result was simply due to aerial oxidation of precursor. In the latter experiment, the precursor was allowed to stand in solution for an identical period normally required for its uptake into the plants and then processed for alkaloids. It was clear that the level of activity in the isolated catharanthine was compar-

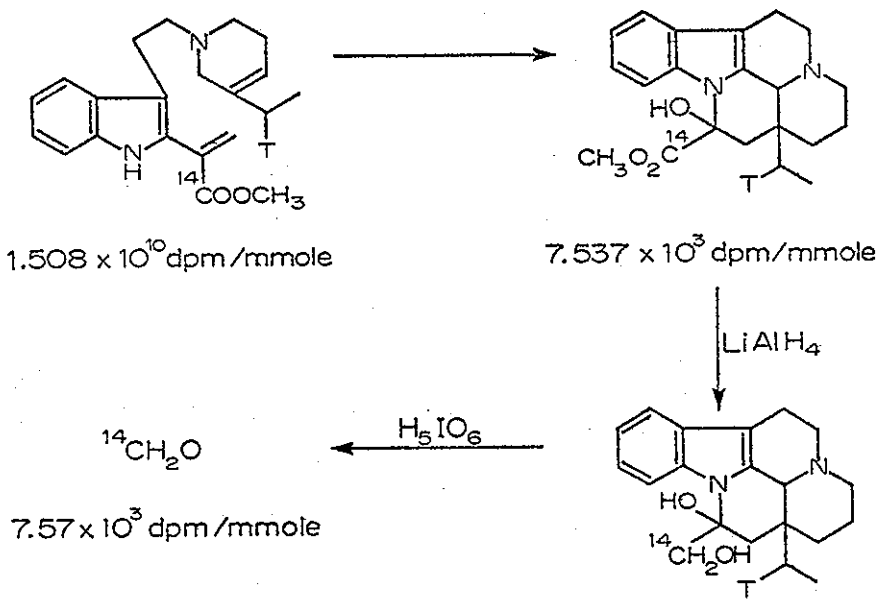


Figure 12. Degradation of vincamine from incorporation of [$^{14}COOCH_3$,19 ^3H]-secodine.

able with that obtained in the plant experiments. In summary, these experiments did not shed any information on the overall significance of the transannular cyclization reaction in the later stages of this Iboga alkaloid.

Due to the availability of variously labelled forms of secodine from the above-mentioned investigations it was appropriate to evaluate its role, if any, in the biosynthesis of catharanthine. A more detailed discussion of these experiments has already been published[36] so only a summary of the most pertinent data is provided in Table 5. A few comments concerning the data are in order.

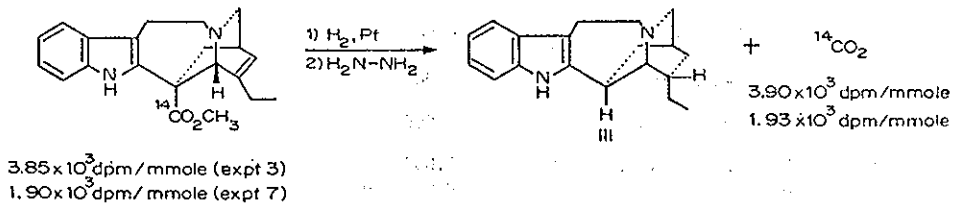
Table 5. Results of Incorporation of Secodine into Catharanthine.

Expt.	Compound Fed	% Incorporation	Ratio of Activity Fed ($^3H/^{14}C$)	Ratio of Activity Isolated ($^3H/^{14}C$)
1.	[ar-^{3}H]-secodine (I)	0.005		
2.	[ar^3-H]-secodine	0.002		
3.	[$^{14}COOCH_3$]-secodine	0.006		
4.	[3,14,15,21-^{3}H,$^{14}COOCH_3$]-secodine	0.003 (^{14}C) 0.001 (^{3}H)	5.02	1.93
5.	[ar^{3}H,$^{14}COOCH_3$]-secodine	0.0008 (^{14}C) 0.0007 (^{3}H)	3.93	3.39
6.	[ar^{3}H,$^{14}COOCH_3$]-secodine	0.001 (^{14}C) 0.0009 (^{3}H)	3.96	3.53
7.	[19-^{3}H,$^{14}COOCH_3$]-secodine	0.001 (^{14}C) 0.001 (^{3}H)	1.91	1.95
8.	[19-^{3}H,$^{14}COOCH_3$]-secodine	0.0006 (^{14}C) 0.0005 (^{3}H)	5.55	4.58

First of all, the incorporations of secodine into catharanthine are extremely low, a situation which has plagued other investigators as well.[37] In spite of numerous attempts to alleviate this problem in terms of different feeding methods, varying time periods for the biosynthesis, different ages of plants, etc., low incorporations not only

of secodine but other "precursors" continue to persist. In contrast to vindoline biosynthesis in V. rosea, where reasonable levels of incorporation are obtained with all the various precursors administered by various research groups, the problems associated with catharanthine still remain unsolved.

In spite of these difficulties, the numerous experiments which we have performed in terms of the various forms of secodine administered at various times coupled with numerous degradations as shown in Figure 13, have convinced us that a low but definite incorporation of secodine is observed.



3.85 x 10^3 dpm/mmole (expt 3)
1.90 x 10^3 dpm/mmole (expt 7)

Figure 13. Typical degradation of catharanthine isolated from the secodine incorporation experiments.

Accepting this situation, the data reveal the following important facts: 1) secodine is incorporated intact with little or no alteration to the indole portion of the molecule 2) the ester functionality of the acrylic ester side chain becomes the carbomethoxy group in catharanthine 3) the piperideine unit of secodine remains essentially unaffected when the tritium label is situated on the side chain (experiments 6 - 8)

but 61.5% loss of this label is observed when it is present in the ring (experiment 4). This latter result is remarkably similar with that observed in the corresponding experiment involving the biosynthesis of vindoline (60% loss of tritium) and lends support to the postulate portrayed in Figure 6 in which intermediate "A" is implicated for both series. The higher oxidation state of the latter intermediate would rationalize the observed losses of tritium in the secodine molecule. Obviously additional experiments are required for further clarification but unless methods can be found which will allow higher levels of incorporation of the administered compounds into the catharanthine systems, such experiments will be severely limited.

Overall Conclusions

The above discussion summarizes our experiments with secodine as a model for a late stage intermediate in the biosynthesis of Aspidosperma (vindoline), Iboga (catharanthine) and Hunteria (vincamine) alkaloids. The experiments presented are complicated by the high instability associated with secodine and the low levels of incorporations obtained particularly in the catharanthine and vincamine series. The data summarized do reveal that secodine is incorporated intact but that a higher oxidized form of secodine, perhaps a dehydrosecodine derivative, for example, XVII (Figure 6) provides a better candidate for the late stage intermediate.

Remaining Questions and Problems

As noted at the beginning of this article the late stages of the biosyntheses of all of the above families remain obscure and much information is required before a definitive move from speculative mechanisms can

be made. To be specific, information relating to the following questions is essential.

1) What is the mechanism involved in the rearrangement of the Corynanthe to the Strychnos alkaloids? Several speculative pathways have been proposed but none of these has received definitive experimental support.

2) What is the nature of the Strychnos intermediate that is involved in the steps leading to the Aspidosperma and Iboga bases? Is stemmadenine really the true intermediate as has been popularly accepted on the basis of a few experiments which merely suggest that it may be involved.

3) Relating to (2), what is the mechanism involved in the transformation of the Strychnos series to the Aspidosperma and Iboga bases? Is isostemmadenine (XVI, Figure 6) actually involved in the proposed fragmentation to the secodine series. No experimental data relating to this aspect are available.

4) What is the nature of the secodine intermediate involved in the required ring closures to the Aspidosperma and Iboga series? A "dehydrosecodine" has been popularly accepted by the various interested research groups but again definitive results are lacking. Our results, as summarized above, suggest but do not prove that such an intermediate may be important and they represent thus far the only experiments which relate directly to the in vivo processes.

5) It is clear that the dihydropyridine unit in the "dehydrosecodine" can be expressed in the form of two double bond isomers. Are both of these involved in the various pathways?

The above questions highlight the most important areas as they relate to the missing links in the late stages of the biosynthetic sequence. Considerable effort is obviously required to obtain the required data. In most instances there are substantial difficulties which must be overcome before meaningful biosynthetic data become available. Two areas, low incorporations and instability of the anticipated precursors, are major difficulties. The high instability associated with a dehydrosecodine system requires the development of appropriate chemical methods which hopefully will allow the utilization of such intermediates for biosynthetic purposes. We are presently investigating the role of chromium carbonyl complexes[38,39] with this objective in mind. More definitive information concerning stemmadenine, isostemmadenine and related systems must await the availability of such systems in variously labelled forms. The supplies of such alkaloids are limited and considerable development of chemistry is required for introduction of radiolabels.

In conclusion, the biosynthesis of the above-mentioned indole alkaloids still remains a challenging and fertile area of research in spite of many elegant investigations which have come forth from numerous laboratories all over the world. Unless the difficulties mentioned can be overcome progress will be slow.

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