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STUDIES ON THE BIOSYNTHETIC RELATIONSHIP

BETWEEN

LEUCINE AND THE INDOLE ALKALOIDS

by

Betty P. Wen, B.Sc., M.Sc.

A thesis submitted to the Faculty of Graduate Studies in
partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Department of Chemistry

Carleton University

Ottawa, Ontario

April 1977

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The undersigned recommend to the Faculty of
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ABSTRACT

Owing to the anomalies in the chemical literature concerning the behaviour of sodium acetate in the biosynthesis of the indole alkaloids, the possible rôle of leucine as an alternative precursor has been examined. The alkaloids examined were Catharanthine, having an Iboga skeleton, (Structure XI, page 4) and Vindoline, having an Aspidosperma skeleton (Structure X, page 4), in the plant Vinca rosea Linn. (Catharanthus roseus G. Don).

Incorporations of radioactive leucine into both catharanthine (0.006%) and vindoline (0.024%) were achieved. This level of incorporation is comparable with those previously achieved with acetate. However, further investigation revealed that the isotopic incorporation is non-specific. This result followed both from ^{13}C -leucine incorporations and direct examination of label distribution by n.m.r. spectroscopy, and also from double-labelled ^{14}C - ^3H incorporations, the results of which were inconsistent with any of a number of pathways considered. Leucine, therefore, in common with acetate, appears not to be a specific precursor of the indole alkaloids.

In the course of reaching these conclusions, two side issues were pursued. The first of these involved evaluating the factors governing the feasibility of using ^{13}C biosynthetic feedings in systems of very low incorporation. The second issue concerned the chemical degradation of vindoline, which proved to be extraordinary resistant to double bond oxidation.

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INTRODUCTION

Alkaloids have been known to men since ancient times as medicines and poisons without knowing anything about their chemical structures. Not until the beginning of last century, after the discovery and isolation of morphine (I), has man gained the interest and started the research in alkaloid chemistry.

(I)

Alkaloids, the natural occurring products of complex molecular structure, in a general sense, are basic, nitrogen-containing compounds mainly found among flowering plants and rarely occur in animals, simple vascular plants, mosses, ferns, fungi and algae. However a number of alkaloids have been isolated from animals^(1,2).

Among the alkaloids, one of the largest families are those containing the indole nucleus, and these so-called indole alkaloids, which number about 700⁽³⁾ have received special attention and speculation from the organic chemists as to structural elucidation, synthesis and biosynthesis. Interest in these natural products by pharmacologists, physiologists and physicians has also arisen since the

discovery of the remarkable physiological properties of LSD (II) (the diethyl amine of naturally occurring lysergic acid), the potent oncolytic agent of the dimeric alkaloids, vincalkeboblustine⁽⁴⁾ (VLB) (III) and leurocristine (IV)⁽⁵⁾ as well as the widely used sedative medicine reserpine (V) which is useful in treatment of hypertension and of various mental disorders.

(II)

(III) R = CH₃

(IV) R = CHO

(V)

The modern development of instrumentation has simplified the problem of structural elucidation, and in recent years more concentration has been placed on the total syntheses and biosyntheses of these indole alkaloids.

As early as the 1930's, the fascinating structures of indole alkaloids stimulated men to find out how these complex natural products are synthesized in the living system. Indole alkaloids, from the structural similarity, are presumably composed of tryptophan (VI) or its decarboxylation product, tryptamine (VII), and a "C₉-C₁₀" unit. Many ingenious postulates were made but not until the radioactive precursors became readily available, could the postulated biosynthetic pathways be tested.

(VI)

(VII)

Radioactive labelled tryptophan and later tryptamine were found to be good specific precursors in indole alkaloid biosynthesis by different laboratories⁽⁶⁻¹³⁾ and radioactive alkaloids, e.g. akuammicine (VIII), ajmalicine (IX), vindoline (X) and catharanthine (XI) which occur in V. rosea Linn. = Catharanthus roseus G. Don⁽¹⁴⁾, were isolated.

Unlike the obvious ~~tryptophan~~ tryptophan portion of the indole alkaloids, the remaining nine or ten skeletal carbon atoms of the indole alkaloids vary in different arrangements but close investigation brings

(VIII)

(IX)

(X)

(XI)

the vast majority of alkaloids of this large family into three main groups⁽¹⁵⁾. They are recognized as the Corynanthe-Strychnos type, the Aspidosperma type and the Iboga type. The Corynanthe-Strychnos type alkaloids possess the C₉₋₁₀ unit (XII), corynantheine (XIII) and strychnine (XIV) are examples of this group.

(XII)

(XIII)

(XIV)

The Aspidosperma alkaloids having the C_{9-10} unit (XV) are represented by tabersonine (XVI) and vindoline (X). The Iboga type of alkaloids have the C_{9-10} unit as (XVII), coronaridine (XVIII) and catharanthine (XI) belong to this group. The dotted line in these three fundamental skeletons (XII), (XV) and (XVII) represents the lost carbon where only nine skeletal carbons are present in the alkaloids besides the tryptamine residue (VII).

(XV)

(XVI)

(XVII)

(XVIII)

In contrast with the straightforward segment of the "tryptamine" portion of indole alkaloids, different hypotheses were postulated concerning the biosynthesis of the ubiquitous " C_{9-10} " unit. The earliest hypothesis derived from the combination of several workers, the Barger⁽¹⁶⁾-Hahn^(17,18)-Robinson⁽¹⁹⁾-Woodward⁽²⁰⁻²¹⁾ hypothesis was based on the structural similarity of the indole alkaloids. This hypothesis (Figure 1) involved 3,4-dihydroxyphenylpyruvic acid (XIX) or an equivalent compound and two C_1 units which were required at different stages during the biosynthesis, in the nontryptamine portion of alkaloids. Condensation of this portion through the α -

position of the tryptamine would give rise to the yohimbine (XX) type alkaloids while condensation at the β -position would give the strychnine (XIV) type of alkaloids.

Figure 1. Barger-Hahn-Robinson-Woodward hypothesis

In this hypothesis, cleavage of ring E is necessary in order to furnish the biosynthesis of strychnine (XIV) and further ring closure is necessary for the ajmaline (XXI) type of alkaloids.

The stereochemistry at C-15 of these alkaloids with the above

(XXI)

theory made Wenkert and Bringi^(22,23) propose an alternate hypothesis, that of a hydrated prephenic acid (XXII) to be the precursor (Figure 2). This hypothesis gave the correct absolute stereochemistry at

Figure 2. Wenkert and Bringi's hydrated prephenic acid hypothesis.

the C-15 position of the various indole alkaloids, the ring E of the intermediates never became aromatic and the carbomethoxy group was retained in the condensation, so that only one C₁ unit, usually formaldehyde (XXIII), was required. But later Wenkert⁽²⁴⁾ modified his hypothesis, prephenic acid (XXIV) itself was the direct precursor of the alkaloids since the hydrated prephenic acid was found^(25,26) to be not involved in the shikimic acid (XXV) to prephenic acid transformation. This hypothesis is shown in Figure 3 using corynantheine (XIII) and yohimbine (XX) as the examples.

Figure 3. Wenkert's prephenic acid hypothesis.

Acetate-origin of the C₉₋₁₀ non-tryptophan portion was proposed by Schlittler and Taylor⁽¹⁵⁾ in 1960 and later by Leete⁽²⁷⁻²⁹⁾ in 1961.

This hypothesis involved the linear condensation of three molecules of acetylcoenzyme A (XXVI) and then combination of this six carbon chain with malonyl coenzyme A (XXVII) and a C_1 unit (Figure 4), but later investigation⁽⁹⁾ has eliminated this "acetate hypothesis".

Figure 4. Acetate hypothesis.

Since then, evaluation of these postulates as well as new speculations on the biosynthetic pathway was attempted by different laboratories. The tracer experimental results⁽³⁰⁻³⁴⁾ disproved the above hypotheses, in particular that the C_1 unit was involved in the biosynthetic pathway.

Monoterpenoid origin for the non-tryptophan moiety of the indole alkaloids was suggested by Thomas⁽³⁵⁾ and Wenkert^(24,36) based on the structural relationships between them. This hypothesis, at one time, seemed to have languished due to initial tracer feeding experiments, but gained popularity again since 1965 when Scott and co-workers^(37,38,46,47) first reported the specific incorporation of mevalonate (XXVIII) into the C₁₀ unit of vindoline (X).

Workers from other laboratories⁽³⁹⁻⁴⁸⁾ independently confirmed this mevalonoid origin which was required by the monoterpenoid hypothesis. Two residues of mevalonate (XXVIII) are combined biologically in the normal head-to-tail way to give the geraniol intermediate (XXIX). A bond is formed between the two C-4 carbons to yield a cyclopentane system (XXX). Subsequently bond cleavage gives the secoversion of a cyclopentanoid monoterpene. This is in accordance with

the Thomas⁽³⁵⁾ and Wenkert⁽²⁴⁾ hypothesis that the Corynanthe skeleton (XII) is generated by fission of the cyclopentane ring of some

Figure 5. Thomas and Wenkert hypothesis.

monoterpene (XXX), the iridoid, and followed by rotation about the indicated single bond as shown in Figure 5. Loss of one carbon (indicated by the dotted line in (XII), (XV) and (XVII)) gives the C_9 units respectively.

Also shown in Figure 5, one will notice that the Aspidosperma and Iboga type of alkaloids are structurally related to the Corynanthe-Strychnos type of alkaloids and can be transformed mentally.

Cleavage of the bond between C_{15} - C_{16} ⁽⁴⁹⁾, structure (XII), and forming a bond between C_{17} and C_{20} , path A, will give the Aspidosperma alkaloids. Similarly, Iboga alkaloids are derived by fission the C_{15} - C_{16} bond and formation of the C_{17} and C_{14} bond, path B. The Yohimbe alkaloids (XXXI) are also formed from (XII) simply by ring closure between C_{17} and C_{18} .

In order to obtain evidence that a head-to-tail C_{10} unit is

by the evidence of feeding experiments from Battersby and coworkers (43,54-58) as well as others^(59,60) since 1965. These results led to the proposal (Figure 7) from mevalonate to loganin or secologanin as the early part of the pathway to the large and varied family of indole alkaloids by Battersby⁽⁶¹⁾.

Later, secologanin (XXXIII) was proved^(62,63) to be a good precursor of these three families of indole alkaloids. The transformation from loganin to secologanin was proposed⁽⁶⁴⁾. Two possible mechanisms were considered: a direct fragmentation initiated effectively by hydride abstraction by the enzyme system could be involved as in path A of Figure 8 or the hydroxyloganin (XXXVIII) could be involved in a 1,3-fragmentation reaction, path B; where X could be a phosphate residue to provide a good leaving group. Condensation of secologanin with tryptamine^(11,13,65) would then give vincoside (XXXIX) and its 3-~~isomer~~ isovincoside^(66,67) (XL), thus finishing the early stage of the biosynthetic pathway⁽⁶⁸⁾, Figure 9. Feeding experiments^(11,13) showed that vincoside was incorporated into all the three types of indole alkaloids in Vinca rosea plants but isovincoside was biologically inert and was not incorporated into the alkaloids.

Figure 7. Proposal of the early part of the biosynthetic pathway from mevalonate to indole alkaloids.

The second stage is the transformation of vincoside to the Corynanthe and Strychnos alkaloids. During this transformation, no skeletal rearrangement is required but enzymatic removal of the glucose is required as a first step, followed by cyclisation of the resultant

Figure 8. Possible mechanism for transformation of loganin to secologanin.

Figure 9. Biosynthetic pathway from secologanin to vincoside and isovincoside via condensation.

dialdehyde ester (XLI) to afford the Corynanthe alkaloids^(69,57), Figure 10. Experimental results have shown that the inversion of stereochemistry at C-3 of the Corynanthe alkaloids occurs after the condensation of vincoside with tryptamine since isovincoside is inert biologically. Also, tritium feeding experiments⁽⁶⁵⁾ showed that inversion at C-3 occurs without loss of tritium. Therefore it is very probable that epimerization occurs via cleavage of a carbon-carbon or carbon-nitrogen bond⁽⁷⁰⁾. The proposed mechanism involving fission of the C-N bond⁽⁶⁸⁾ is shown in Figure 11. Germinating seedling experiments of *Vinca rosea* seeds were carried out⁽⁷¹⁾ in order to get more information about the sequence of alkaloid transformations along

Figure 10. The second stage along the biosynthetic pathway of indole alkaloids.

Figure 11. Proposed mechanism for inversion of the configuration and retention of the hydrogen at C-3 along the biosynthetic pathway.

the biosynthetic route. Geissoschizine (XLIII) was found to be a precursor of the non-rearranged alkaloids^(72,73) such as ajmalicine (IX) and akuammicine (VIII). The transformation from vincoside to ajmalicine is shown in Figure 12. The mechanism connecting geissoschizine with akuammicine was postulated⁽⁷³⁾ based on several reported experimental results and is illustrated in Figure 13.

The original idea of structural rearrangement proposed by Wenkert⁽²²⁾ is supported by further evidence lately obtained from experimental results from the isolation of stemmadenine (XLVIII), tabersonine (XVI) and preakuammicine (XLVII) and the incorporation of the first two into alkaloids of vindoline (X) and catharanthine (XI) in *Vinca rosea* plants^(69,73). This biological transformation from stemmadenine to Iboga and Aspidosperma alkaloids can be rationalised

Figure 12. Vincoside to ajmalicine.

Figure 13. Plausible mechanism of the transformation of geissoschizine to akuammicine.

by involving a reactive dihydropyridine-acrylic ester, the dehydrosecodine (LI or LII)^(74,75). This dehydrosecodine is widely accepted as the key intermediate in indole alkaloid biosynthesis, although it has not yet been found in nature or been synthesized. The plausible mechanism of these conversions was proposed⁽⁷⁶⁻⁸¹⁾ as shown in Figure 14.

Experimental results⁽⁸¹⁾ in vitro showed that there was no evidence for the equilibration between the dehydrosecodine A (LI) and its isomer B (LII). However, the biochemical interconversion of the *Aspidosperma* alkaloids to Iboga alkaloids was possible as (-)-tabersonine (XVI) was transformed into pseudocatharanthine (LIII) and catharanthine (XI) under reflux in acetic acid via the dehydrosecodine A⁽⁸¹⁾ (Figure 15).

In summary, mevalonate can be derived from a common precursor, acetate⁽⁸²⁾ as shown in Figure 16, and by joining figures 16, 7, 12, 13 and 14 together, the biosynthetic pathway from acetate via mevalonate to the three large families of indole alkaloids can be seen.

Figure 14.

Stemmadenine(XLVIII) → Preakuammicine(XLVII) → akuammicine(VIII)

Figure 14. Biological transformation from stemmadenine to Iboga and Aspidosperma alkaloids.

Figure 15. Transformation of tabersonine to catharanthine via dehydrosecodine A.

Figure 16. Acetate to mevalonate.

REASONS FOR INVESTIGATING LEUCINE AS THE PRECURSOR

As mentioned in the introduction, the major portion of the biosynthetic pathway from mevalonic acid on to the final alkaloids has been delineated. Acetic acid, a possible common starting material for the biosynthesis of mevalonic acid has been considered as outlined in Figure 16. Acetate is activated for biosynthesis by combination with the thiocoenzyme A to give the thioester, acetyl coenzyme A. Carboxylation of the substance yields malonyl coenzyme A, which then undergoes a Claisen-type condensation with acetyl coenzyme A accompanied by decarboxylation to yield acetoacetyl coenzyme A. Aldol addition of the acetoacetyl coenzyme A at the keto carbonyl group with acetyl coenzyme A gives the glutaric acid derivatives. Upon reduction of one of the carboxyl groups of the glutaric acid derivatives, mevalonic acid is obtained. Decarboxylation and dehydration of mevalonic acid and phosphorylation yields the isopentenyl pyrophosphate which is the isoprene unit used in the normal head to tail combination to form the monoterpene C₁₀ unit. Carboxyl-labelled acetate, sodium acetate-1-¹⁴C labeled precursor yields the mevalonic acid labelled at C-1, C-3 and C-5 while methyl-labelled acetate, sodium acetate-2-¹⁴C labelled precursor gives the mevalonic acid labelled at C-2, C-4 and C-6 as shown in Figure 17.

Attempts have been made to incorporate acetate into the indole alkaloids. Both acetate-1-¹⁴C and 2-¹⁴C have been admitted to the *V. rosea* plants^(10,8) and the results showed that both of the acetate could be incorporated but in low yield. Partial degradations of the alkaloids, vindoline, catharanthine and ajmalicine, from acetate-1-¹⁴C feeding⁽¹⁰⁾ were carried out and the results indicated randomi-

Figure 17. Labelled mevalonic acid from different labeled acetate precursors.

zation of the ^{14}C label. For vindoline, uniform labeling was found at C-5, C-20 and C-21, about 4% total activity each at the ethyl side chain carbons and the adjacent carbon. Degradation of the catharanthine indicated uniform labelling at C-4, C-20 and C-21 (5-6% each). Degradation of the ajmalicine also showed uniformly labelled at the methyl side chain C-18 and the adjacent carbon C-19, Figure 18.

No degradation was made on the alkaloids isolated from *V. rosea* plants after sodium acetate-2- ^{14}C feeding⁽⁸⁾. Randomization of the ^{14}C label in the total alkaloid was reported⁽⁸³⁾ from sodium acetate-2- ^{14}C feeding to *Vinca minor* plants.

This low level of activity found at the individual carbons of vindoline, catharanthine and ajmalicine may indicate that the acetate-1- ^{14}C has entered the general metabolic pool involving carbohydrate biosynthesis. This may lead to the biosynthesis of shikimic acid which in turn is the precursor of anthranilic acid and tryptophan⁽⁸⁴⁾.

In contrast with this low level of incorporation of acetate to indole alkaloids, the biosynthetic pathway from mevalonic acid on to

Figure 18. Labelled vindoline, catharanthine and ajmalicine from sodium acetate-1-¹⁴C feeding.

the final alkaloids has been delineated clearly with ambiguities only of fine detail as mentioned in the introduction part of this thesis. Besides the obvious acetate precursor of mevalonic acid in living systems, leucine (LV) has also been shown to lead to mevalonic acid in some systems⁽⁸⁵⁾. The metabolic breakdown of leucine to mevalonic acid is shown in Figure 19.

Oxidation and deamination of leucine gives α -ketoisocaproate (LVI). Enzyme esterification of this yields isovaleryl-CoA (LVII). Oxidation of (LVII) gives β -methylcrotonyl-CoA (LVIII). Biotin-dependent

Figure 19. Metabolic breakdown of leucine to mevalonic acid.

carboxylation of (LVIII) gives β -methylglutaconyl-CoA (LIX) and hydroxylation of it affords β -hydroxy- β -methylglutaryl-CoA (LX). Reduction of (LX) yields mevalonic acid.

This demonstrated metabolic connection between leucine and mevalonic acid, coupled with the poor and non-specific incorporation of acetate, raises the question of whether leucine, rather than acetate, might not be the fundamental indole alkaloid precursor. This thesis is concerned with the investigation of this possibility.

In particular, if leucine is labelled at C-2, according to Figure 19, metabolic breakdown will give mevalonic acid which is labelled at C-5. Also, if this degradation of leucine into mevalonic acid is important during the biosynthetic pathway of indole alkaloids and the monoterpenoid hypothesis operates, leucine will be incorporated into the three major types of indole alkaloid. If the incorporation is specific, the labelled carbon of leucine will be expected to locate at C-14 and C-21 in the alkaloids. This relationship is

summarized in Figure 20.

Figure 20. Proposed biosynthetic pathway of leucine to the three types of indole alkaloid.

RESULTS and DISCUSSION

Methodology Used in the Investigations of Leucine being a Precursor of the Major Indole Alkaloids.

Before giving details and discussions of the methods used in this investigation, the general approaches are briefly outlined. First of all, similar to any other studies involving isotopic tracers, detection of the isotopes is primarily important; therefore the preparation of samples for liquid scintillation counting and the difficulties involved are discussed. Secondly, the cultivation and feeding of the plants are considered. Thirdly, harvesting of the plants which were fed with the radioactive precursor, and the isolation of the alkaloids needed is discussed.

Preparation of samples for liquid scintillation counting

The preparation of samples for liquid scintillation counting includes suitable choosing of the scintillators, the solvents and the solubilizing agents if necessary. Also the material being counted should be examined carefully.

Scintillators are compounds which produce light efficiently when bombarded by beta or alpha radiation. The light output should have a wavelength to which the photomultiplier is responsive. The spectral response of the photomultiplier tubes of our Beckman LS 150 Liquid Scintillation Counter covers the approximate range of from 2600 to 6000 Å. The maximum response wavelengths are between 3350 and 4350 Å. There are many scintillators in the market, for example,

p-Terphenyl, 2,5-Diphenyloxazole (PPO), 2,5-Bis-2-(5-t-Butylbenzoxazolyl)-Thiophene (BBOT) and others. The stable and relatively inexpensive PPO which is soluble in most of the solvents and with the fluorescence maximum range of 3400 to 3600 Å is used in our laboratory as the primary scintillator. Since PPO emits the light at a rather short wavelength compared to the response region of the photomultiplier tubes, a secondary scintillator is also used which is suitably chosen so that the absorption band overlaps the emission band of the primary scintillator. POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene fulfills this criterion and is thus used as a secondary scintillator.

Besides the scintillators, the bulk of a liquid scintillation solution is the solvent. A good solvent must transfer energy efficiently from the point of beta ray emission to a scintillator molecule. It also must be transparent to the photons emitted and should be able to dissolve the sample. The most common used solvents are the aromatic hydrocarbons. Toluene is the most common one. Dioxane is also useful as a liquid scintillation solvent for measuring aqueous solutions containing carbon-14 activity. Both of the solvents have been used in this study, but toluene is found superior to dioxane which showed a lower counting efficiency even with the addition of naphthalene. Therefore, a suitable solubilizer is required to prepare aqueous samples when toluene is used as the solvent. BBS-3*, one of the surface-active agent or detergents, has the ability to incorporate sizable volumes of water solutions into the aromatic solvent (toluene) with a minimum amount of quenching is employed in

*) A Beckman product

this study. These three or four foregoing materials are the fundamental constituents of a liquid scintillation solution, which is rather straightforward. More attention has to be placed on the material being counted. Adsorption of certain organic compound to the surface of the counting vial is a serious problem which may lead to false counting results⁽⁸⁶⁾. Two methods have been used to check the adsorption problems of the feeding precursor — leucine.

The tipping off method was employed first. This was done by preparing a sample and recording the apparent activity, then emptying the vial, refilling it with fresh scintillation solution and recording the residue activity. It was found that about 4 to 6.6 per cent of the activity remained in the vial. This result showed that adsorption was not a serious problem for leucine. This was checked with the Adsorption Shift method (or channels ratio method)^(87,38).

According to this adsorption shift method, an adsorption shift value A of 0.0 refers to non-adsorbed compounds while a value of 0.4 ± 0.05 is regarded as fully adsorbed compounds. The average value of the adsorption shift A of nine leucine samples was 0.073 which indicated a partial but not serious adsorption to the wall of the counting vial.

A well-established method of overcoming this adsorption is the carrier dilution method⁽⁸⁹⁾. The counting sample is diluted with the inactive carrier which may be used to saturate the adsorption sites, therefore the small quantity of the labelled material will be effectively present in the solution for counting. This experiment was carried out. Several counting solution were prepared with varying amounts of inactive leucine in it. The same amount of active leucine solution was then added to each vial and the activities were determined.

The results indicated that no significant change was observed in the apparent activity of the samples and when a large amount of inactive carrier was added, the counting efficiency decreased due to chemical quenching. Although addition of the 'cold' carrier made no significance in counting activity, it was added to the feeding container to minimize any loss of activity due to the adhesion of leucine molecules to the wall of the feeding vessel.

In contrast to the counting of leucine (and also sodium acetate, another precursor used), the isolated alkaloids were directly soluble in organic solvents. Thus the counting of these materials did not require solubilization with BBS-3.

Cultivation and feeding of plants

To investigate whether or not leucine can act as a precursor of the major indole alkaloids, vindoline, catharanthine and ajmalicine in Vinca rosea plants, the first important question to be solved is to find out the optimum condition for feeding leucine to the plants. The Vinca rosea plants are growing in the greenhouse of the university⁽⁹⁰⁾. Freshly cut shoots or whole plants were taken directly from the greenhouse and used for experiments. Leucine (LV), an amino acid, with solubility of 0.0744 and 0.1710 moles per liter in water for dl-isomer and l-isomer respectively⁽⁹¹⁾, can be dissolved in water easily and its aqueous solution is used for feeding. There are several methods of feeding the labelled precursor to the living plants⁽⁹²⁾. One of the common methods is the Cotton Wick technique⁽⁹³⁻⁹⁵⁾, in which, several strands of non-mercerized cotton are threaded through the plant stem and the ends are immersed in a small volume of solution in a convenient container which can be taped to

the stem. In such an arrangement, the radio active solution is transferred into the plant by capillary action. If the plant has a hollow stem or leaf, an excellent method is injection of considerable volume of substrate solution into the leaf vein or its interior^(96,97), where they are readily absorbed. There is one technique of immersing the whole plant, the roots of which are free of soil, into the feeding solution but unfortunately, plant roots occasionally appear to be impermeable to some organic compounds such as mevalonate and acetate^(98,99). The most popular technique is the one to administer the precursor through the cut ends of stems immersed in a solution⁽¹⁰⁰⁻¹⁰²⁾.

The first set of experiments concerned the determination of the best method of feeding leucine showed that the latter method was superior to other methods. The only drawback of this method is that the amount of ajmalicine (IX) having the Corynanthe skeleton (XII) isolated is too small to be investigated since it is primarily present in the roots of the plant.

Several shoots were used for each feeding and they were usually taken from different plants. Each plant was cut diagonally across its stem and the basal part of the stem was placed in water immediately. After dead leaves were cut off from the stem, the total fresh weight of the shoots was determined. Just before feeding the solution of the radioactive precursor to the excised shoots, a second cut was made under the water⁽¹⁰³⁾ to remove the basal portion of the stem and immediately placed into the feeding solution contained in a big tube or a beaker, depending on the size of the shoots. The second cut of the shoots ensured that airlocks did not develop in the severed

end of the shoots, whereas a current of air over the plant tends to accelerate uptake of the feeding solution. Plants under such conditions ~~may~~ wilt. The flowering shoots were allowed to take up the solution of the labelled precursor and distilled water was added when it was necessary to keep the severed end under the water except towards the end of the feeding period, when the shoots were allowed to take in all the water left in the feeding container.

The feeding period for obtaining best results of incorporation was investigated and the results are summarized in Table I.

Table I. Incorporation of DL-Leucine-2-¹⁴C and Uniformly Labelled-¹⁴C-L-Leucine into V. rosea L. plant at Various Feeding Time Intervals.

Feeding Expt. No.	Precursor	Time(day)	Total per cent rate incorporation	
			Catharanthine-HCl	Vindoline
L-I	DL-Leucine-2- ¹⁴ C	2	0.002	0.005
L-III		2	0.001	0.007
L-IV	L-Leucine-UL- ¹⁴ C	2	0.002	0.002
L-V		7	0.003	0.007
L-VI		7	0.002	0.003
L-VII		14	0.003	0.006
L-X		14	0.004	0.008
L-XV	DL-Leucine-2- ¹⁴ C	14	0.004	0.009
L-XVII		14	0.007	0.012

This investigation involved feeding of the aqueous solution of the amino acid to the plants with the aid of fluorescence lighting to give a daylength of 12 hours at room temperature. After the appropriate feeding time, the alkaloids were isolated and the activities were determined. The results, as shown in Table I, indicated that

the longer feeding time, up to two weeks as in common with administration of other precursors, gave a better incorporation. Therefore, a feeding interval of two weeks was employed through the rest of the feeding experiments.*

The influence of the age of the plants on the incorporation was examined next. Feeding experiments with different ages of plants were carried out and the results are summarized in Table II.

Table II. Incorporation of L-Leucine-UL-¹⁴C into Different Ages of Vinca rosea L. plants.

Feeding Expt. No.	Plant Age	Total per cent rate incorporation	
		Catharanthine-HCl	Vindoline
L-v	6 months	0.003	0.007
L-VII	8 months	0.003	0.006
L-X	10 months	0.004	0.008
L-XIII	12 months	0.003	0.003
L-IX	3 years	0.004	0.005

The results indicated that the age of the plant did not seem to affect the level of incorporation greatly. However, the plants of the age between six months and eighteen months were chosen in most cases of investigation since the stems of the older plants were harder than the younger one and were very difficult to cut the second time under the water.

Harvesting of the plants and isolation of the alkaloids

After the plants had been maintained in the feeding solution for a period of two weeks, they were due for harvesting. The shoots were broken into small pieces and placed in a Waring blender (Osterizer blender was used later). Methanol was used as the extracting solvent

*) It has been pointed out that the two week feeding period raises questions concerning metabolism in dying plants. However, in the V. rosea system this is standard practice(10).

and plants were macerated immediately by using the blender. The mixture was filtered and the residue was washed with fresh methanol until the filtrate was free from the green color. The radioactivity of the methanol extract was measured and the solvent was evaporated to dryness. The dark residue was taken up into dilute hydrochloric acid to dissolve the alkaloids present in the V.rosea L. plant. The acidic solution was then filtered to remove any undissolved materials. Any soluble neutral components were removed by extracting the acidic solution with benzene and discarded. The alkaloids were precipitated by basifying the acidic solution with concentrated ammonia and extracted into chloroform solution. After drying, the chloroform was evaporated and gave a dark brown oil. This dark brown residue was chromatographed through a very short alumina column eluted with chloroform to remove the very polar components in the alkaloid mixture. The desired alkaloids of catharanthine and vindoline were isolated by a second chromatography eluted with a series of different solvents. The isolated crude catharanthine or vindoline was diluted with the corresponding inactive alkaloid and crystallized. Catharanthine hydrochloride was made before recrystallization because the free alkaloid itself was unstable and easily oxidized. Recrystallization was continued until a constant specific activity of the alkaloids was obtained, usually about five times. Conversion of vindoline into its hydrochloride salt was also made to check the constancy of the activity.

Successful Incorporation of Leucine into Major Indole Alkaloids

The initial feeding experiments concerned the determination of the best method of feeding, the most suitable feeding period and the age of the plants had indicated that a value of 0.001 or over was obtained (Table I and II) in all feeding experiments. A value of 0.001% has been considered as the lower limit of a positive incorporation⁽¹⁰⁾; therefore, from the results shown in Table I and II, despite the low incorporation, leucine is no doubt biosynthetically incorporated into the major indole alkaloids of vindoline and catharanthine by V. rosea plants. Interestingly, this level of incorporation is rather close to the published values obtained from the incorporation of acetate⁽¹⁰⁾. In order to compare with the published value, sodium acetate-1-¹⁴C as well as the 2-¹⁴C was administered to the V. rosea plants using the same conditions set up for the feeding of leucine. Vindoline and catharanthine were isolated and gave 0.007% and 0.003% of rate incorporation respectively. These levels of incorporations are comparable with the values of incorporation of leucine. However, sodium acetate-2-¹⁴C gave a higher level of incorporation and this will be discussed in the later part of this thesis.

Effect of Puromycin

The low incorporation of leucine into the major indole alkaloids may be possibly caused by the predominant use of it in the protein synthesis. In order to prevent this, the protein biosynthesis inhibitor puromycin⁽¹⁰⁵⁾ was considered and its effect was investigated. 1.5 mg of puromycin was dissolved in the feeding solution and was administered to the V. rosea plants at the same time as the uniformly labelled ^{14}C -L-leucine. Vindoline and catharanthine were isolated with 0.008% and 0.004% of total rate incorporation respectively (feeding experiment No. L-X). A measurable increase in the incorporation was observed although it was not dramatic. When puromycin was fed 24 hours preceeding the feeding of leucine, inactive alkaloids were obtained⁽¹⁰⁶⁾, (feeding experiment No. L-XI).

Approaches to Investigate Specificity

From the results discussed above, it was clear that leucine had been established as a possible candidate for precursor of the non-tryptophan portion of the indole alkaloids. The next step in the investigation of the rôle of leucine concerns (a) whether it is incorporated into the tryptophan or the monoterpene portion of the alkaloids and (b) whether incorporation is specific or randomized. For these purposes, radioactive tracer isotopes such as tritium and ^{14}C which are often used have a severe disadvantage. There is, at present, no method for directly determining their location within a molecule, and hence chemical degradation must be resorted to. This is often very difficult with the compounds of formidable structure or the presence of reactive carbons such as quaternary carbons or carbons in an aromatic framework; in such cases, most of the time, only a partial analysis is obtained. In addition, in many cases of structure elucidation of natural products, chemical degradations have no longer been necessary as proof of structure; thus the biosynthetic chemist has no known pathway to follow. On the other hand, researches based on the stable isotope labelling such as ^{13}C (nuclear spin $\frac{1}{2}$ and natural abundance 1.1%), combined with the use of ^{13}C n.m.r. have already been prove to be a very powerful and useful tool for the study of biosynthetic pathways recently^(107,108). ^{13}C n.m.r. itself is an integral component of structural elucidation, hence, its use in the biosynthetic studies is very attractive since it allows one to find out the location of the labelled atom(s) in a metabolite without recourse to the extensive time consuming chemical degradations. Both of the radio-isotope ^{14}C and the stable isotope

of ^{13}C were employed in the investigation of the specificity and are discussed below.

(I) Application of ^{13}C labelling combined with ^{13}C n.m.r. spectroscopy in the investigation of specificity.

The major advantage of the use of ^{13}C n.m.r. method is the practical elimination of time consuming chemical manipulation to determine the labelling patterns. At early stages the ^{13}C -proton satellite method was employed by most of the researchers. The recent availability of the ^{13}C enriched materials has made this method useful for location the labelled carbon(s) in the metabolites. The degree of enrichment of ^{13}C into the metabolite over the natural abundance of 1.1% is an important consideration in the study of the biosynthetic pathway. However, despite the feasibility of requiring only the readily available instrumentation, this technique has limitations of studying carbons with attached protons. Also this method is limited to simple molecules since complex proton spectra with many overlapping peaks may obscure the satellite signals. Besides this, traces of impurities and spinning side-bands sometimes overlap with satellite signals and this adds to the limitation of this technique as well. All these disadvantages and limitations of the indirect ^{13}C -proton satellite method can be overcome by the application of the direct ^{13}C n.m.r. to ^{13}C metabolites. The recent advances in techniques and the improvements in the instrumentation have not only made this method possible, but have also led to the rapid growth in ^{13}C studies and its application in the field of natural products. The recent application of Fourier-transform techniques to ^{13}C n.m.r. has given tremendous increases in the measurement of sensitivity of ^{13}C

n.m.r.; this enables much faster spectral determination with the reduction in sample size than the conventional swept-mode time-averaging method. Researches by using the ^{13}C labelled compounds combined with ^{13}C n.m.r. have shown that this is a very useful and powerful method for the study of biosynthetic pathways.

Idea of boosting up amount fed

With all these advantages of ^{13}C labelling and the availability of Fourier transform ^{13}C n.m.r. in the university, in view of the predicted difficulties of degrading vindoline and catharanthine, it was decided to study this project by using ^{13}C enriched precursors to establish the labelled patterns by ^{13}C n.m.r. spectroscopy.

However, in the literature, most of the ^{13}C studies have been used only in partially purified enzyme system or in micro-organisms (109, 110). Only a few applications of the ^{13}C technique to the higher plants have been published (108, 111). The main problems in applying ^{13}C labelling with higher plants are from the low incorporations usually observed and the dilution of labelled metabolite by unlabelled endogenous materials which can be so large that the ^{13}C content of the isolated substance does not differ significantly from natural ^{13}C abundance despite a good incorporation. In order to apply this elegant ^{13}C method to this study, one has to find out the necessary conditions for suitable incorporations of precursors into the plants. To obtain unequivocal results in ^{13}C n.m.r. studies, a minimum of 0.5% increment in the ^{13}C n.m.r. resonance intensities would be desirable to ascertain the enrichment has occurred (112). In order to achieve this, a relatively large amount of precursor has to be used to feed the plants. This may cause interference of a normal metabolism

in the plants. Toxicity has been reported in the literature for using concentrated feeding solution^(113,114). Hence, different concentrations of feeding solutions were prepared and used to feed the plants to find out the actual situation in this study. The results using sodium acetate as the precursor are shown in Table III. The results indicated that the plants were poisoned in high concentrations but a concentration of the sodium acetate feeding solution around 0.15% were suitable and gave reasonable amounts of the alkaloids with satisfactory incorporations.

Table III: Investigation of the concentration of the feeding solution (sodium acetate) in which the plants can survive.

Feeding Expt. No.	Concentration of Feeding solution	Wt. of plant used for feeding	Days plants surviving
	1.00%	86 g.	3
	0.57%	57	3-7
	0.33%	65	10
A-XXIV	0.16%	80	14 ^a
A-XIX	0.13%	110	14 ^b

a) 13.8 mg. of catharanthine and 22.2 mg. of vindoline were isolated

b) Sodium acetate 1-¹³C and 1-¹⁴C were used as the precursor.

22.6 mg. of catharanthine·HCl were isolated with 0.0020% rate incorporation and 38.1 mg. of vindoline were isolated with 0.028% rate incorporation.

Following the establishment of suitable concentration of feeding solution for the plants, a question arises as to the incorporation, whether it is dependant or independant of amount of precursor fed. Feeding experiments using sodium acetate were therefore carried out

in which variable amounts of inactive sodium acetate were fed together with the small quantity of radioactive material. The incorporations obtained in these experiments are summarized in Table IV.

Table IV. Relation between percent rate incorporation and amount of precursor fed (sodium acetate-2- ^{14}C as precursor).

Feeding Expt. No.	Activity fed (dpm)	Amount of 'cold' material added (mg)	% Rate Incorporation	
			Catharanthine-HCl	Vindoline-HCl
A-VIII* A-XIII A-XIV	1.3-1.5 $\times 10^8$	10	0.017	0.049
A-XV	2.064 $\times 10^8$	46.6	0.024	0.050
A-XVI	5.927 $\times 10^8$	132.8	0.022	0.052

*) Average of three experiments

From the results shown in Table IV, the answer to that question is clear: the percent incorporation is independent of the weight fed.

After the success of feeding this high concentration of the precursor to the plant, the next problem needed to be solved was to find out the minimum amount of ^{13}C precursor required to feed the plants in order to obtain the satisfactory enriched ^{13}C n.m.r. spectrum in which the peak intensity of the labelled carbon can be distinguished from its natural abundance reference. By taking the advantage of the known information from ^{14}C feeding, the minimum amount of ^{13}C precursor required can be calculated.

From the ^{14}C feeding experiments, vindoline showed a higher incorporation than catharanthine, therefore the calculation was done on the information of vindoline and the following factors are taken into account:

- (1) The value of 0.040 is used as the average per cent incorporation of leucine into vindoline, i.e. 1 m mole of leucine will give 4×10^{-4} m mole of vindoline.
- (2) In the experiments with ^{14}C , the alkaloids are isolated and diluted with inactive material so that several recrystallizations can be performed to produce pure compound. This dilution would not be suitable when working with ^{13}C precursor since this will reduce the peak intensity in the ^{13}C n.m.r. spectra. In order to overcome this, sufficient alkaloid, about 30-40 mg., would need to be isolated to allow several recrystallizations performed. In other words, sufficient plants need to be fed to provide the direct isolation of the alkaloids which without any further dilution with 'cold' material could be crystallized to pure state. The relations between the weights of plants and the amounts of alkaloids isolated from previous ^{14}C feeding experiments are summarized in Table V:

Table V. Relation between amount of plant used and amount of alkaloids isolated

Feeding Expt. No.	Precursor	Wt. of fresh plants	Amount of alkaloids isolated	
			Catharanthine-HCl	Vindoline
L-XXIV	DL-Leucine-	69.2 g.	22.09 mg	26.99 mg
L-XXXIII	2- ^{14}C	82.2	16.74	21.02
L-XXXII	L-Leucine-	68.3	16.70	17.77
L-XXXVI	UL- ^{14}C	60.0	18.56	17.37
A-XVI	NaOAC- ^{14}C	71.2	22.04	23.36
A-XXI		112.0	31.10	35.10

From the results shown in Table V, about 130-140 grams of fresh cut shoots of V. rosea were required to yield 30-40 mg. of alkaloids.

- (3) 0.5% enrichment is considered as the objective in this calculation.

In order to achieve the set requirement stated above, the amount of ^{13}C labelled leucine needed to feed the plant can be calculated as follows:

The actual incorporation is defined as

$$\text{Incorporation} = \frac{(\text{Total activity})_{\text{alkaloid}}}{(\text{Total activity})_{\text{precursor}}} \quad (1)$$

Equation may be multiplied by 100 to obtain the usual percent incorporation. The total activity is the sum of activities at each carbon atom; thus equation may be modified to

$$\text{Incorporation} = \frac{\Sigma(\text{Total activity at each carbon atom})_{\text{alkaloid}}}{\Sigma(\text{Total activity at each carbon atom})_{\text{precursor}}} \quad (2)$$

This equation is true for ^{14}C , but not for ^{13}C because, while there is no significant ^{14}C natural abundance, there is significant ^{13}C natural abundance. Thus a modification of equation (2), valid for both ^{13}C and ^{14}C is

$$\text{Incorporation} = \frac{\Sigma(\text{Total enrichment at each carbon atom})_{\text{alkaloid}}}{\Sigma(\text{Total enrichment at each carbon atom})_{\text{precursor}}} \quad (3)$$

Experimentally, the ^{13}C enrichment of alkaloid is determined by increase in the intensity of the n.m.r. signal. This relative parameter needs to be multiplied by the number of moles of material in order to obtain total "activity". Thus

$$\text{Incorporation} = \frac{\sum (\text{Spectral enhancement})_{\text{alkaloid}}}{\sum (\text{Spectral enhancement})_{\text{precursor}}} \times \frac{\text{No. of moles alkaloid isolated}}{\text{No. of moles precursor fed}} \quad (4)$$

Defining R_i as the relative peak size of atom i in the isolated alkaloid, relative to peak size in the natural abundance spectrum, the percentage of ^{13}C will be equal to $(1.1\% \times R_i)$. This corresponds to an enrichment of $(R_i \times 1.1\%) - 1.1\% = 1.1(R_i - 1)\%$. Thus the $\sum (\text{Spectral enhancement})_{\text{alkaloid}}$ term of equation (4) becomes $\sum (R_i - 1) \times 1.1\%$

If precursors that are 90% ^{13}C are fed, the enrichment is $90 - 1.1\% = 88.9\%$. In all cases only one carbon of its precursor is labelled.

Equation (4) may therefore be modified

$$\text{Incorporation} = \frac{\sum (R_i - 1) \times 1.1}{88.9} \times \frac{\text{No. of moles of alkaloid isolated}}{\text{No. of moles of precursor fed}} \quad (5)$$

Multiplying by 100 to obtain per cent incorporation, one obtains

$$\% \text{ Incorporation} = 1.24 \sum (R_i - 1) \frac{W_{\text{alk}} \times \text{M. Wt.}_{\text{pre}}}{\text{M. Wt.}_{\text{alk}} \times W_{\text{pre}}} \quad (6)$$

where W is the weight of alkaloid or precursor in mg. For the incorporation of leucine, $\% \text{ Incorporation} = 0.04$; $R_i = 1.5$ at two carbons; therefore $\sum (R_i - 1) = 1.0$; $W_{\text{alk}} = 40 \text{ mg}$; $\text{M. Wt.}_{\text{pre}} = 132^*$; $\text{M. Wt.}_{\text{alk}} = 456$. Thus from equation (6) the weight of leucine required is calculated to be 359 mg. The actual experiment was performed using slightly more than this amount.

* Atomic weight of 13 was used for one carbon in the leucine molecule in calculating of the molecular weight.

Calculation of incorporation from peak enhancements

After the ^{13}C enriched alkaloids — vindoline and catharanthine-HCl were isolated and purified, ^{13}C n.m.r. spectra were taken. Emphasis was placed on vindoline because of its higher incorporation and the ^{13}C chemical shifts of all carbon atoms were already assigned⁽¹¹⁵⁾. Unlike the proton n.m.r., the line intensities of the ^{13}C n.m.r. are non-integral due to the variable nuclear Overhauser effect and different relaxation times for individual carbons, therefore it is difficult or unreliable to calculate the incorporation by obtaining the integral ^{13}C n.m.r. spectra. The direct comparison of the respective line intensities in the natural-abundance and enriched spectra is still the only reliable method⁽¹¹⁶⁾ provided both sets of the spectra obtained are subject to identical nuclear Overhauser effects and relaxation time. This can be carried on by using identical concentrations and instrument parameters. Practically, it is very difficult to prepare samples in identical concentrations when only small quantities of the labelled metabolites are isolated. In order to overcome this, a convenient technique is employed by normalizing both of the spectra to a reliable standard. This can be achieved by using all the unlabelled peaks in the spectrum for normalization⁽¹¹⁷⁾.

In this study, the sets of ^{13}C n.m.r. spectra were taken using identical instrumental parameters, the direct comparison of peak intensities was made and incorporation was calculated. In doing this, two methods were tried. The classic weighing method was used. The homogeneity of the weighing paper was checked by weighing eight weighing papers of the size of four inches by four inches. A weigh-

ing difference of 3.1% was obtained. Several smaller strips of the size of 1.25 inches by 0.125 inches from four different weighing papers were weighed and found a 2.4% difference between weighing. The incorporations from sodium acetate-1- ^{13}C and 2- ^{13}C feeding (feeding expt. no. A-XIX and A-XX respectively) based on ^{13}C spectra were calculated by using the weighing method. Each peak was traced using the weighing paper, cut carefully and weighed. The results are tabulated in Table VI. The weight of individual signals of the acetate fed vindoline was normalized with the solvent peak and compared with the weight of the corresponding signal of the natural abundance. The ratio of signals or the enrichment at individual position of vindoline derived from the 1- ^{13}C and 2- ^{13}C sodium acetate are summarized in Table VII. Due to the intensity problem in the ^{13}C n.m.r. spectra⁽¹¹⁸⁾ a peak ratio of 1.4 : 1 is taken as the lower limit of an enrichment, to make certain a positive enhancement occurs. Five carbons in the vindoline derived from acetate-1- ^{13}C are enriched, C-3, C-9, C-13, C-26 and C-27. Similarly, eight carbons in vindoline derived from acetate-2- ^{13}C are enriched. They are C-3, C-6, C-7, C-9, C-13, C-22, C-23 and C-26. From the ratios of the signals, the average intensity of the labelled sites of the acetate-1- ^{13}C derived vindoline can be calculated and found to be 1.77. The average intensity of the unlabelled carbon sites (the remaining 20 carbons) is 1.04. Therefore, the enhancement of the intensity on the labelled carbon is 0.73% in average. Similar calculation can be obtained from the acetate-2- ^{13}C derived spectrum. The average peak intensity of the labelled carbons is 1.65, and the average peak intensity of the remaining sixteen unlabelled carbons is 1.05. Therefore, the

Table VI. Relative weights of individual signals in the ^{13}C n.m.r. spectra of vindoline.

Carbon	Chemical shift (ppm)		Weights of the signals $\times 10^3$		
	published(a)	observed(b)	Natural	1-C-13	2-C-13
2	83.2	83.5	1.40	1.70	1.35
3	50.9	51.2	0.90	1.25	1.35
5	51.9	52.1	1.68*	1.38*	1.62*
6	43.9	44.2	1.01	0.95	1.40
7	52.6	52.9	1.05	1.26	1.40
8	124.9	125.2	1.36	1.37	1.44
9	122.4	122.7	0.90	1.21	1.22
10	104.5	104.9	1.05	1.11	1.30
11	161.1	161.4	0.65	0.61	0.58
12	95.6	96.0	1.21	1.17	1.43
13	153.6	153.9	0.39	0.94	0.74
14	123.9	124.0	1.24	1.47	1.49
15	130.2	130.7	1.05	1.26	1.21
16	79.5	79.7	1.39	1.33	1.30
17	76.2	76.5	1.51	1.44	1.47
18	7.5	7.8	0.60	0.60	0.74
19	30.6	30.9	0.89	0.79	1.15
20	42.8	43.1	0.64	0.56	0.77
21	67.0	67.3	1.49	1.32	1.29
22	171.7	171.9	0.67	0.64	0.91
23	20.8	21.0	0.64	0.59	1.37
24	170.4	170.5	0.97	0.93	0.66
25	51.9	52.1	1.68*	1.38*	1.62*
26	38.0	38.2	0.61	0.82	1.03
27	55.1	55.1	0.66	1.01	0.79
CDCl_3			4.38	3.97	4.20

(a) See Ref. (115)

(b) In deuterio chloroform solution

*) overall enrichment for C-5 and C-25

Table VII. Ratio of peaks at individual positions for vindoline derived from 1- ^{13}C and 2- ^{13}C sodium acetate to the natural abundance.

Carbon	1-Carbon-13 fed	2-Carbon-13 fed
2	1.34	1.01
3	1.53	1.56
5	0.91*	1.01*
6	1.03	1.45
7	1.32	1.39
8	1.11	1.10
9	1.48	1.41
10	1.17	1.29
11	1.03	0.93
12	1.07	1.23
13	2.66	1.98
14	1.31	1.25
15	1.32	1.20
16	1.06	0.98
17	1.05	1.02
18	1.10	1.29
19	0.98	1.35
20	0.96	1.25
21	0.98	0.90
22	1.05	1.42
23	1.02	2.23
24	1.06	0.71
25	0.91*	1.01*
26	1.48	1.76
27	1.69	1.25

*) overall for C-5 and C-25

average enhancement of the intensity of the labelled carbons is 0.60%.

The second method tried was the method of comparison of the peak

intensity in height. This method was found not as satisfactory as the weighing method.

From the results shown above, a few points are clear. Firstly, it shows the success of this method of feeding certain (calculated) amount of ^{13}C enriched precursor which lead to the isolation of labelled compounds with detectable enhancement of peak intensities in their ^{13}C n.m.r. spectra. Secondly, since limited amount of enriched precursor was used in this feeding, small enhancement in peak intensities is observed. Larger enhancement in the peak intensities is possible to obtain if larger amount of precursor is used. Thirdly, from the pattern of the peak enhancement in the molecule, one can conclude that the incorporation of both the acetate into vindoline molecule is not specific but randomized among the monoterpene- C_{10} unit as well as the tryptophan unit. The same result of randomization of sodium acetate $1\text{-}^{14}\text{C}$ incorporated into vindoline by V. rosea plant was found by Leete and co-workers⁽¹⁰⁾.

Synthesis of DL-Leucine-2- ^{13}C

Having established the technique with commercial available sodium acetate- ^{13}C , investigation with leucine was undertaken. ^{13}C labelled leucine is not available commercially, it needs to be synthesized from a commercial available labelled precursor. Different routes or reaction sequences may be used to synthesize 2- ^{13}C labelled leucine; the particular one used here is summarized in Figure 21.

Acetic acid which labelled at C-2 position was considered to be an appropriate precursor since it is commercially available and this labelled position will be eventually become the C-2 position of leucine.

Figure 21. Synthetic scheme of DL-Leucine-2- ^{13}C from acetic acid 2- ^{13}C .

The labelled acetic acid-2- ^{13}C purchased (2 g.) was contained in a vacuum sealed tube. Modification was done on this tubing by lengthening it at top and a bended side arm was attached. A magnetic bar was placed carefully near the fragile tip of the vacuum tubing and the top was then closed. The purpose of enclosing a magnetic bar is that, when it is lifted and dropped freely, it will break the brittle end of the sealed tube and acetic acid can then be transferred. The apparatus used for transferring the acetic acid into the reaction flask was arranged as in Figure 22. The side arm of the modified tubing was connected to a two neck reaction flask which was immersed into liquid nitrogen. One neck of the flask was connected to a vacuum pump. A trap and a stop cock were placed in between the flask and the pump. The whole system was evacuated first and the stop cock was closed. The fragile tip was broken by the magnetic bar, acetic acid was vaporized when the tube was placed in a warm

Figure 22. Device used to transfer acetic acid into the reaction flask.

water bath or a heating gun was used (b.p. of acetic acid is 117.9° at 760 mm and 17° at 10 mm⁽⁹¹⁾) and condensed in the reaction flask. In this way, acetic acid can be transferred to the reaction flask easily and completely. Some impurity was found in this acetic acid since a trace of grey solid residue (0.0407 g) remained inside the tube after the acetic acid was transferred to the reaction flask.

This flask protected with drying tubes was warmed up to room temperature and bromination of acetic acid with calculated amount of bromine was performed using Vogel's method⁽¹¹⁹⁾. Before working on

the labelled compound, bromination of ordinary acetic acid was attempted. To the acetic acid, acetic anhydride and pyridine were added. Bromine was added to this stirred mixture and monobromoacetic acid was formed and a low yield was found (literature yield, 36.2%). After repeating this reaction several times, the yield was improved to over 50% by using freshly distilled acetic anhydride and pyridine. When the labelled acetic acid was brominated in such condition, bromoacetic acid-2- ^{13}C was obtained in 58.8% yield, m.p. 44.0° (sharp).

The fairly pure bromoacetic acid obtained was used to prepare cyano acetic acid directly without recrystallization, thus avoiding loss of material. Bromoacetic acid can be converted to cyanoacetic acid when it is treated with sodium cyanide, the bromine group is replaced by the cyano group and gives cyanoacetic acid upon acidification. This cyano acetic acid is very easy to convert into its ethyl ester by reacting with absolute ethyl alcohol. The procedure of preparing ethyl cyanoacetate from chloroacetic acid was followed (120). When the aqueous solution of sodium cyanide and the aqueous solution of sodium bromoacetate were mixed together, a vigorous exothermic reaction occurred. Care should be taken in order to control the reaction, but when working in small quantities as in this case, the reaction was very easy to control. This acid, cyanoacetic acid, was freed when hydrochloric acid was added to its sodium salt. This acid usually solidified on standing overnight in the refrigerator but sometimes took longer to crystallize. Since the cyanoacetic acid was difficult to solidify, immediately after preparation, this product was checked with ir and n.m.r., these were comparable with

the spectra of authentic sample, therefore, esterification of this crude cyanoacetic acid was performed following the procedure. Ethyl cyanoacetate was obtained after cyanoacetic acid was refluxed with absolute alcohol, the product was brown in color. The color impurity was removed by eluting the compound through a small column containing 3 g of alumina (activity grade III) with ether. Thus ethyl cyanoacetate-2-¹³C obtained after purification from bromoacetic acid was pale yellow in color and the yield (from bromoacetic acid) was 69.0%.

The reaction of ethyl cyanoacetate with nitrous acid will yield ethyl nitroso cyanoacetate; this secondary nitoso compound is unstable and rearranges to the isonitroso cyanoacetate (oxime). This nitrosation of ethyl cyanoacetate was followed by modifying Snyder and Smith's method of preparation of ethyl acetaminomalonate⁽¹²¹⁾. Ethyl cyanoacetate was dissolved in glacial acetic acid and an aqueous solution of sodium nitrite was added below 20° with stirring. Although not mentioned by the author, the mixture gradually emulsified and in some cases, the mixture even solidified during the addition of nitrite solution. Stirring of this mixture has to be continued for an additional one and half hour in order to maximize the yield. Ethyl cyanoacetate-2-¹³C was converted to ethyl isonitrosocyanoacetate-2-¹³C by reacting with sodium nitrite in 86.6% yield.

One advantage of using this synthetic sequence is that the C=N double bond of this isonitroso ester can be reduced with zinc dust in acetic acid and acetylation of the amino group will proceed in the presence of acetic anhydride without isolation of any of the intermediates. Therefore, ethyl isonitroso cyanoacetate was dissolved in glacial acetic acid and zinc dust was added⁽¹²²⁾, in this stage,

the reaction apparatus should be protected with a calcium chloride drying tube, since trace of moisture would reduce the yield. The resulting amine was acetylated with acetic anhydride which was present in the reaction mixture. Ethyl acetamidocyanoacetate-2-¹³C was produced from ethyl isonitrosocyanoacetate-2-¹³C and recrystallized from anhydrous ether, gave 63.6% yield.

Alkylation of ethyl acetamidocyanoacetate with isobutylbromide was followed by modifying Albertson and Archer's method⁽¹²³⁾. After repeating the reaction several times, sodium isopropoxide was found to be superior than the sodium ethoxide as a base to remove the α -proton of ethyl acetamidocyanoacetate. Therefore, newly cut sodium was dissolved in isopropyl alcohol with gentle heating, a drying tube was placed on top of the condenser during the whole reaction to prevent moisture, hence to maximize the yield. After sodium was dissolved in isopropyl alcohol, ethyl acetamidocyanoacetate was introduced and the mixture was brought to boiling. Excess of isobutyl bromide was added and the mixture was refluxed for six hours. Excess isobutyl bromide was used since it was quite volatile, the loss during the addition to a boiling solution might be compensated by the excess amount added and any unreacted isobutyl bromide could be removed when the reaction mixture was concentrated under reduced pressure during work up. It was also found that, the residue after removing the solvent was much easier to dissolve in a mixture of chloroform and dilute hydrochloric acid (0.7 N) than a mixture of chloroform and water. This dilute acid could also be used to neutralize the base present in the reaction mixture. When the chloroform solution after drying was concentrated to dryness, an oily

residue of the adduct was formed which was very difficult to crystallize. This was probably due to the formation of a mixture of ethyl and isopropyl esters instead of only ethyl ester which would be the product when absolute ethanol was used during the alkylation. Evidence that the isopropyl ester had formed during the reaction came from the n.m.r. spectrum of the reaction product. The mixture of esters does not need to be separated since the ethyl or isopropyl group will be removed during the hydrolysis in the next step. Ethyl acetamidocyanoacetate-2-¹³C was converted to isopropyl (or ethyl)-2-acetamino-2-cyano-4-methyl-pentanoate-2-¹³C in 79.2% yield (calculation was based on formation of isopropyl ester).

A one step conversion of the ester of 2-acetamino-2-cyano-4-methyl-pentanoate to the amino acid was established by Albertson and Tullar⁽¹²⁴⁾ and was followed. Hydrolysis of the ester and the acetylated amino group as well as the cyano group of the adduct will take place when refluxed with concentrated hydrobromic acid and followed by decarboxylation will give the corresponding amino acid. The highest yield was obtained when the alkyl acetamino ester was refluxed with concentrated hydrobromic acid for seven and a half hours. After the excess hydrobromic acid was removed, the solution was made to pH 6 with concentrated ammonia solution and leucine was precipitated. A fairly pure second crop of product was obtained when the mother liquid was concentrated. DL-Leucine-2-¹³C was obtained from the hydrolysis of isopropyl (or ethyl)-2-acetamino-2-cyano-4-methyl-pentanoate-2-¹³C with 48% hydrobromic acid in 64.7% yield. Recrystallization from water gave white shiny plate crystals which melted at 281-283° (sub.).

An overall yield of 11.5% was obtained from acetic acid to leucine in an eight steps synthesis. A total of 374.6 mg of dl-leucine was available for feeding after spectra were taken. ^{13}C n.m.r. spectrum of the synthesized leucine was also taken. Deuterated water was used as the solvent and pure methanol was used as external standard. Only two peaks were observed after 2,875 scans. Off-resonance decoupling technique was applied to distinguish these two peaks. A quartet pattern was observed at upper field which was corresponding to the carbon in methanol. This absorption should be at 49.3 ppm⁽¹²⁵⁾. The peak at lower field which split into a doublet in the off-resonance decoupling spectrum is at 53.1 ppm relative to the absorption of methanol and this matches the value published in the Handbook of ^{13}C n.m.r. spectrum⁽¹²⁶⁾ as 52.5 ppm for the C-2 of leucine. A full ^{13}C n.m.r. spectrum of this leucine has been tried. After 21,355 scans, five peaks at 22.1, 22.9, 25.3, 39.7 and 53.5 ppm were observed besides the methanol peak (at 49.3 ppm). These absorptions are parallel to the published values for leucine carbons except the carbonyl carbon which was not observed due to its long relaxation time.

Results of feeding DL-leucine-2- ^{13}C

The synthesized DL-leucine-2- ^{13}C was fed to the plant as with the ^{14}C precursor (feeding expt. No. XXXIX). The plants were harvested when it was due and the alkaloids were isolated. ^{13}C n.m.r. spectrum was taken after the alkaloids were purified by recrystallization. The peak intensities of all carbon atoms in the n.m.r. spectrum were measured and compared to the peak intensities of carbon of natural abundance. The data of peak intensities of both the

Table VIII. Peak intensities of vindoline of natural abundance and from the labelled precursor (2-¹³C-leucine) and the results of comparison of the peak intensities.

Carbon	Peak intensity I of N.A. $\times 10^3$	Peak intensity II from labelled precursor $\times 10^3$	Ratio of the peak intensities II/I
2	3.37	3.71	1.101
3	2.55	2.71	1.063
5	4.53	4.69	1.035
6	2.15	2.29	1.065
7	2.95	3.57	1.210
8	4.12	3.72	0.903
9	2.54	3.11	1.224
10	3.61	3.64	1.008
11	2.49	3.01	1.209
12	3.96	3.68	0.929
13	2.73	3.41	1.249
14	4.24	4.13	0.974
15	4.73	4.31	0.911
16	3.24	3.43	1.059
17	4.52	4.22	0.934
18	2.32	2.46	1.060
19	2.45	2.36	0.963
20	3.06	3.52	1.150
21	2.94	2.92	0.993
22	2.68	2.41	0.899
23	2.35	2.74	1.166
24	2.72	3.63	1.335
25	4.53	4.69	1.035
26	3.07	2.91	0.948
27	3.58	3.19	0.891

natural abundance and the labelled compound, and the results of comparison are summarized in Table VIII.

Table IX. Increment in peak intensity of vindoline derived from DL-leucine-2- ^{13}C .

Carbon No.	Increment of peak intensity	Enriched ?
2	+16.0%	possible
3	+12.0%	possible
5	+ 9.1%	no
6	+12.2%	possible
7	+27.5%	yes
8	- 4.8%	no
9	+29.0%	yes
10	+ 6.2%	no
11	+27.4%	yes
12	- 2.1%	no
13	+31.6%	yes
14	+ 2.6%	no
15	- 4.0%	no
16	+11.6%	possible
17	- 1.1%	no
18	+11.7%	possible
19	+ 1.5%	no
20	+21.2%	yes
21	+ 4.6%	no
22	- 5.3%	no
23	+22.9%	yes
24	+40.7%	yes
25	+ 9.1%	no
26	0.0	no
27	- 6.1%	no

Analysis of these results indicates that about one half of the carbon atoms had shown increments in their peak intensity. Therefore, the same conclusion can be achieved as from the results of ^{14}C feeding

that, leucine is no doubt incorporated into the indole alkaloids of vindoline. The ratios of the peak intensity of the labelled compound to natural abundance at individual carbon atoms were calculated and are also tabulated in the last column of Table VIII. The ratios are normalized with all the unlabelled carbon atoms in the labelled compound and the per cent increment of the peak intensity at the individual carbon was calculated and outlined in Table IX. The results were analyzed and, at first sight, it appeared that seven carbons, namely, C-7, C-9, C-11, C-13, C-20, C-23 and C-24 are enriched while other five carbon atoms, C-2, C-3, C-6, C-16 and C-18 are possibly labelled. The remaining thirteen carbons are not enriched.

From the ratio of the peak intensities of the labelled and unlabelled vindoline, Table VIII, the incorporation may be calculated by using Eq. (6). However, using the seven apparently labelled carbon atoms, the calculated percent incorporation based on ^{13}C n.m.r. spectra was significantly higher than that determined from ^{14}C data. The average peak size relative to the natural abundance spectrum of the seven atoms is 1.22; the average of the remaining eighteen atoms is 0.95. The enrichment is thus $0.27/0.95 = 0.28$ and therefore $R_f = 1.28$ at seven atoms. Using Eq. (6), since 374.6 mg of dl-leucine-2- ^{13}C precursor was used to feed the plant and 64.5 mg of vindoline was isolated, the percent incorporation is calculated to be 0.12%. The percent incorporation from ^{14}C data is, however, only 0.037%.

The ^{14}C data and the ^{13}C data may only be mutually accommodated if it is assumed that only C-13 and C-24, the two peaks with the highest relative intensities, are in fact enriched, and that the other

five atoms, at first sight apparently labelled, have higher relative intensities simply due to instrument irreproducibility. If this assumption is made, the average of C-13 and C-24 relative peak sizes is 1.29; the average of the remaining twenty three atoms is 1.00 (i.e. the high values are exactly balanced by the low ones). Thus the value of $R_i = 1.29$ and $\sum(R_i - 1) = 0.58$. Use of Eq. (6) gives the percent incorporation as 0.036%. This compares well with that from ^{14}C data of 0.037%.

Regardless of which interpretation is considered, these results are clearly inconsistent with leucine being a specific precursor of the non-tryptophan portion of vindoline. Of the seven carbons originally considered labelled, only two of these atoms (C-20, C-24) are in the monoterpene portion of the alkaloid, and neither of these corresponds to either of the two atoms expected to be labelled from the proposed biosynthetic pathway. Of the remaining five atoms, four are in the indole nucleus and one in the acetyl group. This overall result is clearly randomization. On the other hand, if the result of only two atoms being labelled is considered, then there is one labelled atom in the indole portion (C-13), and one in the monoterpene portion (C-24). Considering (a) that there is no known direct metabolic process connecting leucine with tryptophan, and (b) that the monoterpene portion is known to arise from two identical C_5 fragments, this is a result that does not appear to represent any specific biosynthetic route.* These results indicate that although

*) Furthermore, the integration at C-13 appears to be erratic on the basis of a number of spectra we have obtained. Its integration in the case of the C-1-labelled acetate feeding (Table VII) was apparently so high that the calculation of percent incorporation is meaningless.

activity is transmitted from leucine to vindoline in Vinca rosea, the specific route suggested by simple degradation to mevalonic acid is not a significant pathway followed.

(II) Attempted degradation on vindoline in order to establish the way for investigating the specificity using ^{14}C labelled compound.

As the ^{13}C n.m.r. method in the study of biosynthesis for higher plants is still in its infancy, a parallel way of investigation of the specificity was also considered. Despite the foregoing statements about the difficulties involved in the degradation of ^{14}C labelled compounds, this is still the only alternative method of determining the labelling pattern within a molecule. Hence, degradation of vindoline was planned. Unfortunately, since the structure of vindoline was determined without the use of chemical degradation⁽¹⁴⁵⁾, little is known of the chemistry of vindoline. The biosynthetic pathway from 2- ^{14}C -leucine to the alkaloids shown in Figure 20, corresponds to C-14 and C-21 becoming labelled. Comparing the reactivity of these two carbons, C-21, being a tertiary carbon at a bridgehead, will be less reactive than C-14, an olefinic carbon, which is susceptible to electrophilic attack. Thus reactions of the double bond were considered in order to cleave the C-14 atom. Oxidative cleavage of this isolated olefin double bond becomes the main target. There are several methods for oxidizing and cleaving the olefin double bond. A one step cleavage of an alkene double bond to dialdehydes by using osmium tetroxide and sodium periodate as the oxidant⁽¹²⁷⁾ is one. Generally, in this reaction osmium tetroxide adds to the double bond to afford an osmate ester which is oxidatively

cleaved by sodium periodate to give two carbonyl compounds. The advantage of this method is that, only a catalytic amount of the expensive osmium tetroxide is needed since it is regenerated in the presence of periodate. In addition to this, although the reaction intermediate, the osmate ester, does not need to be separated, it is sufficiently stable to be isolated and can be hydrolyzed easily to the corresponding diol when desired. In the case of vindoline, if reaction proceeds similarly, addition of osmium tetroxide to the C-14, C-15 double bond gives the osmate ester and cleavage of it by the periodate will yield a dialdehyde. This one step conversion reaction has been tried unsuccessfully⁽¹²⁸⁾; apparently, no reaction occurred.

Since this oxidation can be stopped at the stage of osmate ester formation, in order to find out whether there is any reaction of the vindoline double bond with osmium tetroxide or not, hydrolysis of the osmate ester was followed instead of the oxidative cleavage of the C-C bond by sodium periodate. In this case, the product would be a cis-glycol.

Practically, the reaction is generally conducted by dissolving the compound as well as the osmium tetroxide in a dry solvent since osmium tetroxide is very sensitive to moisture, reducing its oxidative power. Osmium tetroxide is also sensitive to light; therefore, the reaction needs to be performed in the dark. Pyridine is usually added to the reaction mixture or even used as a solvent for the reaction; it facilitates the reaction greatly by complexing with the osmate ester and causing it to precipitate out from the reaction mixture.

The procedure of osmium tetroxide reaction in the conversion of corynantheine to its diol by Brown and coworker⁽¹²⁹⁾ was followed. Vindoline was dissolved in dry pyridine and anhydrous tetrahydrofuran, osmium tetroxide in tetrahydrofuran was added at -70° . The reaction was continued for three hours and hydrolyzed of the osmate complex by passing gaseous hydrogen sulphide. After work up, only about 5% of the vindoline had reacted, the product was a mixture of about three major components. This suggested that, vindoline was less reactive than corynantheine since 95% of the vindoline was recovered under this reaction condition. Presumably either a longer reaction time or a higher reaction temperature might be necessary. In the next trial, the reaction mixture was kept at -70° for two hours; since there was no precipitate formed as it should have, the reaction temperature was raised to between -15° and -25° and the reaction was continued for 5 days. Decomposing the osmate complex with gaseous hydrogen sulphide, a mixture of products (about 20%) was obtained. This mixture was composed of one slightly predominant component C* with a R_f value of 0.35; three other main products A, R_f 0.52; D, R_f 0.26 and E, R_f 0.13 plus traces of others. As seen from this result, increasing the reaction temperature increased the yield but also increased the number of side products. The reaction was repeated to maintain the reaction at -70° for a longer time in hopes of getting a cleaner product. The mixture of vindoline and osmium tetroxide solution was kept at -70° for 12 hours, but no

*) For clarity, the reaction products are designated A, B, C, D and E in order of increasing polarity on silica gel chromatoplates, where unreacted vindoline is the component B.

precipitate was observed. However, the reaction was continued at about -20° for 9 days. Same work up gave a mixture of products similar to the previous experiment but without any component in the mixture being a major product. Although the yield of the reaction at low temperature was very low, it could still be worthwhile if only one major product was obtained since by repetition of the reaction a certain amount of product could be accumulated, and the time for separation of a mixture saved. Therefore, the reaction was tried at low temperature for even longer times. This idea was abandoned after the reaction was conducted at this temperature (-70°) for one week. No apparent improvement was observed. The reaction took place at about -20° and two main products, C and D, were obtained in addition to the small amount of A and E, the yield was about 20%.

On the other hand, reaction may occur in a much better yield at higher temperature, although more side products may also formed. If some products are predominant than the others, separation of the mixture might give the desired product(s) provided the reaction gives good yield. Hence, the reaction was tried at room temperature using same solvent and same method of hydrolysis. When this reaction was tried for two days, one product (16.4 mg, 15% yield) with a R_f value of 0.64 (R_f of vindoline: 0.27) was obtained. This was most probably a side product since the expected hydroxy compound should be more polar than vindoline with a smaller R_f -value. A small percent of compound C, D and E and mainly unreacted vindoline (B) were found in the filtrate.

When this reaction was repeated for seven days, similar components were found in both the filtrate and the precipitate. In this

case, half of vindoline was reacted and gave mainly product C and D. From this result, it indicated that, the yield was improved with increase in the reaction temperature, but still a poor result.

Besides the temperature factor, the solvent used for the reaction is another factor that can be varied. Pyridine was used as the only solvent in the next trial since in repeating the reaction of converting 3 β -hydroxy-androst-5-en-17-one (LXI) to the corresponding triol (LXII) with osmium tetroxide⁽¹³⁰⁾ in which pyridine was used as the solvent, a yield of 86.4% was obtained. But in the case of vindoline, when pyridine was used alone, less than 10% of vindoline had reacted and gave a similar mixture of reaction products.

Aqueous dioxane was used as the solvent in the next attempt. The reaction was carried out at room temperature for more than two weeks and tlc was checked periodically. After one week the proportion of the reaction mixture remained constant. The osmate ester was cleaved by saturating with hydrogen sulphide and the component with R_f value of 0.51, product A, was found to be slightly predominant over the other products. Although estimation* of about 60 to 70% of vindoline was recovered, this reaction condition did not produce the desired product.

*) Estimation was based on the size and color intensity of the spot on the tlc plate.

Different methods of hydrolysis were tried. Aqueous sodium sulphite was used instead of hydrogen sulphide and the whole reaction mixture was subject to hydrolysis. About 25 to 30% of vindoline had reacted and one of the five reaction products, product D, seemed to be a major one.

Since the oxidation of the double bond with osmium tetroxide went slightly better at room temperature than at around -20° , another trial was performed at higher temperature. Anhydrous diethyl ether (b.p. 35°) was used as the solvent and the reaction mixture was refluxed for one week. The osmate ester complex was decomposed by aqueous sodium sulphite solution. Increasing the reaction temperature from room temperature to boiling ether temperature did not seem to increase the yield very much since about 60 to 70% of vindoline was still recovered. Among the four reaction products, D seemed to predominate.

The different conditions for the reaction of vindoline and osmium tetroxide were summarized in Table X.

After all these trials, despite the advantage of using osmium tetroxide as the oxidant in conjugation with sodium periodate, this oxidant reacted with vindoline in the extent of 30 to 40% and gave a reaction product as a mixture of four or five components. The reaction mixture of all these reaction were separated by thin layer chromatography. Fairly pure product A and D but with very small quantities (about 10 mg each) were obtained after chromatography twice on the silica gel prep-tlc plates. Pure vindoline was recovered by this method. It was not possible to isolate pure product C on silica gel plates; a mixture of product A, B and C was obtained

Table X. Reactions of vindoline and osmium tetroxide under various conditions.

Reaction No.	Amount of Vindoline mg (m mole) (R_f)	Amount of OsO ₄ mg (m mole)	Solvent	Time of Reaction	Temperature	Method of Hydrolysis	Residue obtained after Reaction mg (R_f)
1	207.5(0.455)(0.38)	116.0 (0.457)	THF/PY	3 hrs	-70°	H ₂ S 5 min	196.5 (0.57;0.23;0.11. + 90% 0.38)
2	210.0(0.461)(0.40)	78.5 (0.309)	THF/PY	2 hrs 5 days	-70° -20°	H ₂ S 10 min	209.9 (0.52;0.35*; <u>0.26</u> ;0.13 + 80% 0.40)
3	207.7(0.455)(0.40)	100.4 (0.395)	THF/PY	12 hrs 9 days	-70° -20°	H ₂ S 1 hr	213.4 (0.49;0.36;0.31; <u>0.25</u> ; <u>0.12</u> + 90% 0.40)
4	211.7(0.464)(0.43)	78.0 (0.307)	THF/PY	7 days 2 days	-70° -20°	H ₂ S 1 hr	220.9 (0.52;0.38;0.30;0.13 + 90% 0.43)
5	111.1(0.244)(0.27)	65.8 (0.259)	THF/PY	2 days	r.t.	H ₂ S ½ hr	16.4 (0.64) fr. ppt. 102.5 (0.19; <u>0.15</u> ;0.07 + 75% 0.27) fr. filtrate
6	107.8(0.236)(0.37)	65.7 (0.258)	THF/PY	7 days	r.t.	H ₂ S ½ hr	21.2(0.55;0.45;0.31;0.22;0.065; <u>0.04</u> + 0.37) fr. ppt. <u>96.8(0.31</u> ; <u>0.22</u> + 50% 0.37) fr. filtrate
7	102.8(0.225)(0.38)	63.7 (0.251)	PY	7 days	r.t.	H ₂ S ½ hr	7.0(0.54;0.44;0.29;0.23 + 0.38) 86.4(0.44;0.29;0.23 + 90% 0.38)
8	95.1(0.209)(0.40)	69.2 (0.272)	aq. dioxane	16 days	r.t.	H ₂ S 20 min	81.6(<u>0.51</u> ;0.34;0.25;0.03 + 60% 0.40)
9	107.3(0.235)(0.39)	61.8 (0.243)	THF/PY	4 days	r.t.	NaHSO ₃	135.2(0.48;0.31; <u>0.22</u> ;0.18 + 70% 0.39)
10	102.7(0.225)(0.42)	75.6 (0.298)	Et ₂ O/PY	7 days	35°	NaHSO ₃	100.9(0.50;0.32; <u>0.24</u> ;0.11 + 60% 0.42)

*) R_f value underlined indicated that was the main component in the product mixture.

each time even after four separations. The small amount of product E was not obtainable in pure form either. A mixture of D and E was obtained each time. It appears, therefore, that both C and E are unstable on silica gel and are convertible to the other components.

Due to the small amount of product obtained, it seemed that this reaction of osmium tetroxide was not a suitable one for degrading the labelled vindoline.

For this hindered reaction between vindoline and osmium tetroxide, there is a possibility of basicity correlation of the compound with the oxidant. Vindoline is a basic compound, the acidic osmium tetroxide is most probably complexing with the N(b) nitrogen rather than the double bond in the reaction mixture. A similar result has been reported of no appreciable reaction with sitsirikine or its derivatives with osmium tetroxide⁽¹³¹⁾.

Oxidation of a double bond with permanganate ion is also very common and the reaction condition is mild; this is important for vindoline since it contains other sensitive functionalities. Permanganate ion reacts with double bonds but the reaction is more versatile than the osmium tetroxide reagent since the product obtained is a function of pH. If the reaction is conducted in neutral media, α -ketols are formed while if the reaction is performed in alkaline conditions, cis-glycols will be the products. In the case of α -ketol formation, if both of the olefinic carbons are monosubstituted, the C-C bond will cleave to give the carbonyl compound after basic work up. A typical procedure of permanganate oxidation was followed⁽¹³²⁾.

Vindoline was dissolved in acetone and an aqueous solution of potassium permanganate and magnesium sulphate was added at -15° to

-20°. Basic work-up gave a mixture consisting of three almost equal portion main products plus the unreacted vindoline (about 80% estimated from tlc). The low yield of this reaction was considered to be probably due to the low solubility of permanganate in the organic solvents. This would possibly be improved by using crown ether together with the permanganate as the oxidant. In this case, a complex ion (LXIV) is formed between the crown ether and the potassium ion increasing the solubility of the oxidizing agent and hence the oxidizing power. This method was tried using cyclohexene, a good result was obtained and this procedure⁽¹³³⁾ was used to oxidize vindoline.

Finely ground potassium permanganate was dissolved in dicyclohexyl-18-crown-6 (LXIII) and benzene. Vindoline in benzene solution was added slowly into the vigorously stirred oxidant and reaction was allowed to proceed overnight. Before work up, the reaction mixture was checked with tlc and was found to be a mixture of nine components. Among these, one was found to be the major component in about 90% yield. None of these components gave same R_f value as the vindoline reference. But this major component (R_f 0.38) in the reaction mixture was soon found to be in fact the unreacted vindoline since it gave the purple vindoline color when the plate was stained with antimony pentachloride spraying reagent. The smaller R_f value of this component compared to the vindoline reference was probably due to the crown ether in the reaction mixture, this tended to hold the components from eluting due to its high viscosity. Two main reaction products were observed on the tlc plate but the low yield most likely was due to insufficient stirring (12.3 mg; 10.5%) discouraged further consideration of this method.

The Lemieux-von Rudloff reagent was considered next. This reagent consists of a catalytic amount of potassium permanganate and excess sodium periodate. This reagent was employed first by Lemieux and von Rudloff⁽¹³⁴⁾ to oxidize the C-C double bond at room temperature in a slightly alkaline medium. In this oxidation, similar to the oxidation with osmium tetroxide, the olefin double bond is attacked by permanganate ion, which in turn is reduced to the manganate ion. The manganate ion is continuously oxidized back to permanganate by the periodate between pH 5 to 10, therefore, the reaction medium is usually maintained neutral or slightly basic by adding potassium carbonate. This reagent was used to oxidize vindoline⁽¹³⁵⁾.

In this reaction, t-butanol was used as the solvent and the reaction was performed at around 26° since t-butanol would freeze below that temperature. The sodium periodate and potassium permanganate stock solution was added dropwise, the purple permanganate ion color disappeared immediately after addition, indicating that oxidation was occurring. On the first trial of this reagent, only 0.7 mg, corresponding to 11% yield, of the reaction product was obtained. On the second trial, the amount of vindoline used was doubled and excess of the stock oxidant was used; this gave a 16% yield.

UV of this reaction mixture showed only absorption at 202.6 nm, while the uv spectrum of vindoline has absorptions at 210, 254 and 306 nm and this indicated that the indole ring had been attacked by this oxidizing reagent.

The reactions of vindoline with potassium permanganate are summarized in Table XI.

Ozonolysis is another common and useful method for cleavage of an olefin to afford two carbonyl compounds or a compound with two additional carbonyl groups. In general, ozonolysis followed by oxidative cleavage will afford a ketone or a carboxylic acid while reductive cleavage will generally give an aldehyde or a ketone. An additional advantage of performing ozonolysis on vindoline could be observed if only the C_{14} - C_{15} double bond was attacked by ozone. This is because the nitrogen (b) is allylic with respect to the double bond; therefore, ozonolysis of vindoline followed by oxidative work up will give an abnormal reaction, besides cleaving of the C_{14} - C_{15} double bond, the bond between C_{14} and C_3 might also be cleaved, degrading C_{14} out as carbon monoxide. The question of whether the 50% activity is located at C_{14} position could be answered after the evolved gas is collected and subjected to radioactivity measurement.

An ozonator was set up. After establishing its reliability with the ozonolysis of cyclohexene⁽¹³⁶⁾ (yielding 91.7% adipic acid) and 4-cholesten-3-one⁽¹³⁷⁾ (which gave a 79.2% yield of the keto acid) and progest-4-ene-3,11,20-trione⁽¹³⁷⁾, ozonolysis of vindoline was performed. Although low yield was obtained in the reaction of progest-4-en-3,11,20-trione with ozone, one predominant product was obtained.

Table XI. Summary of reactions of vindoline and potassium permanganate

Reaction No.	Amount of vindoline mg (m mole) (R_f)	Oxidant		Solvent	Reaction Time	Temperature	Products mg(R_f)
		KMnO ₄ mg (m mole)	Other Cpd				
1	114.0(0.250)(0.46)	32.0 (0.203)	MgSO ₄	acetone		-15 - -20°	145.9(0.63;0.54;0.36;0.29 0.19 + 80% 0.46)
2	116.7(0.256)(0.45)	140.7 (0.891)	Crown ether	benzene	over night	r.t.	12.3(0.25;0.18;0.13 + trace of other cpd)
3	6.13(0.013)	6.246 (0.0016)	NaIO ₄	t-BuOH	17 hrs	26°	0.7
4	13.6(0.030)	3.28 (0.021)	NaIO ₄	t-BuOH	16 hrs	26°	2.3

In the reaction of vindoline and ozone, ethyl acetate was used as the solvent and the reaction was conducted at -70° . The reaction conditions and the results of all trials are tabulated in Table XII.

In the first trial, 0.262 mmole of vindoline was dissolved in ethyl acetate and the solution was subjected to ozonolysis. According to the previous setting of the ozonator (Welsbach Model T-816), the concentration of ozone produced was 0.474 milliequivalent per minute or 11.38 milligram per minute; therefore the ozonized oxygen was required to pass into the reaction mixture for about 1.1 minutes theoretically. In this experiment, the potassium iodide trap turned into yellow color in less than 1 minute; this was probably due to the rate of passing ozone into the reaction mixture was faster than the rate of taking up ozone by vindoline molecules, the unreacted ozone escaping into the trap before the reaction was complete. In this case, it was necessary to experiment passing ozone into the solution for a longer time. Five minutes was tried on the first trial. After work-up with hydrogen peroxide, about 20% reaction products were obtained and it contained mainly two components of R_f value 0.53 and 0.33 (vindoline R_f 0.44). The residue from the aqueous layer was checked with tlc as well and found to be a mixture of seven components. None of these compounds gave a purple color when stained with antimony pentachloride solution. Attempts to separate this mixture by using column chromatography (alumina) were unsuccessful. A mixture was found in most of the fractions. The low recovery yield, 25%, from the column made it difficult for further separation. UV of the first two chromatographic fractions were taken and the

Table XII. Summary of reactions of vindoline and ozone.

Trail No.	Amount of vindoline mg (m mole) (R_f)	Solvent	Time of passing O_3	Temperature	Work up	Products-mg (R_f)
1	119.5(0.262)(0.44)	EtOAc	5 min	-70°	30% H_2O_2 reflux 1hr	24(0.53;0.33) 97.9 fr. aq. layer(0.50;0.42; 0.35;0.31;0.27;0.22;0.17 + 0.44)
2	111.6(0.245)(0.77)	EtOAc	3 min	-70°	30% H_2O_2 reflux 1hr	11.8(0.77;0.69 + trace other) 100.1 fr. aq. layer(smeared: 0.36;0.27;0.20;0.09)
3	111.9(0.245)	EtOAc	6 min	-70°	30% H_2O_2 18hrs r.t.	15.5 from CH_2Cl_2 126.2 from aq. layer
4	119.4(0.262)	EtOAc	6 min	-70°	30% H_2O_2 18hrs r.t.	Total 136.1
5	115.7(0.254)(0.54)	EtOAc	15 min	-70°	Zn/HOAc 1hr	8.7(0.61;0.51;0.26) 4.2(0.65 + others, smeared)
6	117.7(0.258)(0.59)	EtOAc	6½ min	-70°	Zn/HOAc overnight	98.7(0.59;0.56;0.50;0.40 + other traces)
7	111.7(0.245)(0.51)	MeOH	7 min	-60°	$(CH_3)_2S$	28.1 fr. org. layer(0.49;0.41; 0.34) 99.6 fr. aq. layer
8	113.0(0.248)(0.71)	MeOH	6 min	-60°	$(CH_3)_2S/HC(OEt)_3$ H ⁺ /MeOH	153.3(0.72;0.71;0.65;0.58;0.51; 0.44;0.35;0.15)

had also been attacked by ozone.

On the second trial, for similar quantities of vindoline, ozone was passed into the reaction mixture for three minutes in the hope that the ozone was just enough to oxidize the double bond. After ozonolysis, spectra of the ozonide were taken before hydrogen peroxide work-up. This ozonide had similar uv and ir absorptions as vindoline except the carbonyl group which was shifted about 20 cm^{-1} to higher frequency in the ozonide spectrum. After work up, about 10% reaction products were obtained in which, one product with the R_f value of 0.69 (R_f of vindoline was 0.77, the plate had been developed twice) was found to be the major component. The number of compounds presented in the mixture was not clearly shown up on the tlc plate since it smeared during developing. The low yield might be due to incomplete ozonolysis; therefore, the time of passing ozone in the solution was extended to 6 minutes and the reaction was performed twice using the same reaction condition in order to obtain enough material to perform separation of the reaction mixture into its pure components. The reason for not scaling up the quantities for ozonolysis was due to the unknown explosive properties of the vindoline ozonide.

Over 250 mg of the product mixture was obtained and the separation was carried out by using an alumina column. Ethyl acetate was used as the first eluting solvent. The solvent polarity was increased by adding methanol gradually. Difficulty arose again during the separation, only very small amounts (less than 1 mg in most 60 ml fraction) were found in each fraction. About 25% material was recovered after washing the column with methanol. This suggested that,

the products were very polar and acidic, thus binding with the alumina in the column. Large amount of the reaction products were found in the aqueous layer and the smearing phenomenon shown on the tlc plate were also indicative of polar and acidic components in the product mixture.

It was thought that this problem might be alleviated by a reductive work up after ozonolysis. In this case, dialdehyde would be the product instead of keto acid with reduced polarity.

In the next experiment, ozone was passed into the reaction mixture continuously in the hope of finding the saturation point, i.e., at this reaction temperature, the solution should turn to blue color when saturated with ozone, but after 15 minutes, while the solution was still yellow in color, a precipitate formed. The reaction mixture was work up by using glacial acetic acid and zinc dust, 8.7 mg or 7.5% reaction products were obtained. Two main components with R_f of 0.61 and 0.51 (vindoline 0.54) were found in the product mixtures.

In trial 6, vindoline was reacted with ozone for 6 minutes at -70° . Dry nitrogen was passed into the reaction vessel in order to remove excess ozone in the reaction mixture. After removing solvent, reductive work-up was performed by using zinc dust and glacial acetic acid as the reducing agent as the previous trial except that the reduction was continued overnight. The residue after the reaction was also a mixture of several compounds which included the unreacted vindoline. Ir of this reaction mixture was taken and no aldehyde C-H stretching was shown. Also, when the tlc plate was developed, smearing was observed. In order to find out the presence of the al-

solution. Only a trace of material was isolated as the sodium bisulphite reaction product. The result indicated that no aldehyde (or if any, only trace amounts) was formed after ozonolysis and reductive work up.

From the above experiments, the reducing agent used might not be efficient enough to reduce the ozonide. Different methods⁽¹³⁸⁾ for reductive work up were tried. In this method, dimethyl sulphide was used as the reducing agent which in turn was oxidized to dimethyl sulfoxide. In this experiment, methanol was used as the solvent and ozone was passed in for 7 minutes at -60° . The reaction was monitored by tlc to find out the amount of ozone required for the reaction. After 6 minutes, vindoline seemed to be consumed. Dimethyl sulphide was added and worked up as usual. When the methylene chloride containing the reaction products was washed with water to remove dimethyl sulfoxide, it seemed that, part of the products in the organic layer were also soluble in aqueous layer since the yellow color in the organic solution was continuously passed into the aqueous solution. The residue from both layers showed the same mixture of compounds on tlc plate. Two main compounds (R_f 0.49 and 0.34; vindoline 0.51) were found in the mixture. Attempts to separate this mixture into its pure components by using silica gel column chromatography were also unsuccessful. Changing the slightly basic absorbent alumina to slightly acidic silica gel did not help in the separation. Only one component of a small quantity of 1.7 mg (R_f 0.70, vindoline 0.62) was isolated pure; all the other fractions were still a mixture. No aldehyde C-H absorption was shown on the spectrum. The great solubility of these compounds in water and

in base suggested that even after reductive work up, carboxylic acid products were still formed. If carboxylic acid was one of the compounds in the mixture, it should form an ester with diazomethane and be able to be separated from the mixture. Methylation of the partially separated mixture was performed by using diazomethane. No reaction occurred as judged from the tlc and ir evidences. The uv of the reaction products did not give indole pattern of absorption, and this indicated that ozone had attacked the indole part of the molecule besides the olefin double bond. Ionic compound(s) was(were) formed after the reaction.

Another procedure of work up by using dimethyl sulphide, triethyl orthoformate and methanol containing acid as the reducing agent was followed⁽¹³⁹⁾. The reducing agent was stirred with the reaction mixture overnight; removing dimethylsulfoxide gave a eight component mixture. One of the components with R_f 0.35 (vindoline R_f 0.71) appeared to be the main product. Separation of this mixture by preparative tlc plate isolated this component in a fairly pure state yielding 16.9 mg, m.p. 143-148^o. Ir of this compound gave a very intense absorption at 1647 and 1571 cm^{-1} ; in addition to this, a broad OH absorption. No obvious aldehyde C-H absorption was shown on the ir unless it was hidden under the OH absorption. UV spectrum showed absorption at 216 and 363 nm with a weak shoulder at 253 nm. The yield of less than 15% was still too low for this method to be useful in the degradation of vindoline.

Other methods of oxidation such as ruthenium tetroxide oxidation was tried⁽¹⁴⁰⁾. Ruthenium tetroxide is a more vigorous oxidant than

xide it might be useful. This reagent converts olefins directly to carbonyl compounds without formation of an isolable intermediate.

An unsatisfactory result was obtained after the reaction was performed. In addition to the 90% unreacted vindoline, four products of equal proportion were obtained.

Hofmann degradation to cleave the C-N bond was also tried⁽¹⁴¹⁾. No precipitate was found after the reaction of vindoline and methyl iodide was performed in a sealed tube at 56°. When the reaction was performed at room temperature and atmosphere pressure, less than 10% of vindoline had reacted. Low yield of the reaction caused abandonment of this method.

After all these experiments, no effective and suitable method has been found for the degradation of labelled vindoline. Osmium tetroxide or ruthenium tetroxide was not an effective oxidant for vindoline due to low yield and complicated products. Hofmann degradation would also lead to the complicity of the reaction products since there were more than one way of cleaving the C-N bond. Ozone was a too vigorous oxidant to vindoline since ozonolysis seemed to occur not only on the olefinic double bond and at the same time to give very polar products which caused trouble in the separation of the mixture. Lemieux-von Rudloff reagent was also apparently too strong for vindoline. Of the degradation methods of vindoline, the mild oxidation method of potassium permanganate could possibly be pursued using more of the oxidant. The amount of potassium permanganate could be increased to a ratio of 4 to 3 compared to the olefinic compound. In the experiment performed, the ratio of the oxi-

reacted. As the amount of oxidant increased, the yield of the reaction might also increase. The two major products in the product mixture were different in polarity (R_f value), and therefore separation of them into their pure form might be possible.

At this stage work on the oxidative degradation of vindoline was discontinued. Considerable effort had been expended and a variety of methods investigated. None of these were found satisfactory. Since the methods were confirmed to work satisfactorily on other compounds, it is clear that the difficulty lies in the vindoline molecule itself, reagents probably either being diverted by the basic nitrogen, or else attacking the indole nucleus. Since the objective of degradation was only to confirm the results of the ^{13}C approach, further effort in this direction did not appear justified, and the non-specific incorporation of leucine result must therefore rest solely on the ^{13}C n.m.r. experiments.

Further Information on the Fate of Acetate and Leucine

Double label experiments

In an early part of this discussion, the incorporation of DL-leucine-2- ^{14}C and L-leucine-UL- ^{14}C into vindoline and catharanthine hydrochloride under different feeding conditions were summarized (Tables I and II). After establishing the suitable condition for feeding leucine into V. rosea plants, besides the above mentioned leucine, other commercial available radioactive leucines were purchased and fed to the plants. In so doing, more information of the incorporation of leucine into the alkaloids with the isotopes in different positions could be obtained and give information on the probable mechanism of incorporation. This included the feeding of L-leucine-1- ^{14}C , L-leucine-4,5- ^3H and DL-leucine-4,5- ^3H . Similarly, besides sodium acetate-1- ^{14}C and 2- ^{14}C , sodium acetate- ^3H was also fed to the plants in order to obtain more information about the fate of acetate. The incorporations of these precursors into vindoline and catharanthine hydrochloride are summarized in Table XIII.

The results indicated that L-leucine-1- ^{14}C was incorporated into both catharanthine and vindoline but with low rate of incorporation. Both L-leucine-4,5- ^3H and DL-leucine-4,5- ^3H were incorporated into vindoline with reasonable percent rate incorporation of 0.01% but L-leucine-4,5- ^3H was incorporated into catharanthine hydrochloride with very low rate of incorporation while DL-leucine-4,5- ^3H was not incorporated into catharanthine hydrochloride (less than 0.001%). From the data shown in Table XIII, the value of incorporation of DL-leucine-4,5- ^3H into catharanthine hydrochloride was about half of the value of incorporation of L-leucine-4,5- ^3H , this might suggest that

Table XIII. Results of incorporation of leucines and acetates into vindoline and catharanthine in *V. rosea* L. plants.

Feeding Expt. No.	Precursor	Activity fed (dpm)	Percent rate incorporation	
			Catharanthine-HCl	Vindoline-HCl
L-XIX	L-Leucine-1- ¹⁴ C	1.110×10^7	0.004	0.003
L-XXII		1.110×10^7	0.006	0.004
L-XX	L-Leucine-4,5- ³ H	1.110×10^8	0.0016	0.012
L-XXI		1.110×10^8	0.0013	0.013
L-XXVII	DL-Leucine-4,5- ³ H	0.938×10^8	0.0008	0.011
L-XXVIII		0.938×10^8	0.0007	0.014
A-V	NaOAc-1- ¹⁴ C	1.403×10^8	0.0029	0.0087
A-VII		1.322×10^8	0.0018	0.0050
A-XV	NaOAc-2- ¹⁴ C	2.064×10^8	0.024	0.050
A-XVI		5.927×10^8	0.022	0.052
A-IX	NaOAc- ³ H	1.110×10^8	0.0024	0.010
A-X		1.110×10^8	0.0012	0.0055

only the L-enantiomer was incorporated during the biosynthetic pathway. Both sodium acetate-1-¹⁴C and 2-¹⁴C were incorporated into the alkaloids in question but the results were different from the work of Groger et al⁽⁸⁾. In this study, sodium acetate-2-¹⁴C gave a much higher rate incorporation than the 1-¹⁴C precursor in both vindoline hydrochloride and catharanthine hydrochloride. According to the biosynthetic route of acetate to mevalonic acid (Figure 16) and thence to the alkaloids, some of the acetate molecules will lose C-1 during the pathway. On the other hand, all C-2 of acetate will remain. Therefore, it is reasonable to have higher incorporation from acetate-2-¹⁴C precursor than 1-¹⁴C precursor.

Sodium acetate-³H was also incorporated into both vindoline and

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into catharanthine.

Although valuable and interesting results may be obtained from this type of ratio of incorporations in which different atoms in a precursor have been labelled, such ratios are subject to intolerable error. This is due to the fact that in each feeding experiment, a different biological sample was used; therefore, biological variability would cause discrepancies in the percent incorporation or the dilution values, and any conclusions drawn from such comparison of these individual data might not be correct. More reliable results will be obtained from the results of experiments using the double labelling technique. This was done by preparing a known isotope ratio precursor or a mixture of precursors and feeding this to the plants. In this case, any error due to biological and experimental deviation is removed.

In this study, mixtures of feeding precursor of DL-leucine-2- ^{14}C and L-leucine-4,5- ^3H ; L-leucine-UL- ^{14}C and L-leucine-4,5- ^3H ; L-leucine-UL- ^{14}C and DL-leucine-4,5- ^3H ; L-leucine-1- ^{14}C and L-leucine-4,5- ^3H ; sodium acetate-1- ^{14}C and sodium acetate- ^3H as well as sodium acetate-2- ^{14}C and sodium acetate- ^3H were prepared with known (measured) isotope ratio and used to feed the plants. After two weeks feeding period, the plants were harvested, the alkaloids were isolated and radioactivities determined. These results are summarized in Table XIV. The isotope ratio of the precursors and of the alkaloids isolated were calculated and tabulated in Table XV. The consistency of these results with the proposed mechanism of incorporation can be examined.

*) It has been pointed out that any difference between enantiomers of leucine must arise prior to any symmetrical intermediates.

Table XIV. Percent incorporations of double labelled experiments.

Feeding Expt. No.	Precursor	Percent incorporation			
		Catharanthine-HCl		Vindoline-HCl	
		^{14}C	^3H	^{14}C	^3H
L-XXIII	DL-Leucine-2- ^{14}C	0.009	0.001	0.030	0.010
L-XXIV	L-Leucine-4,5- ^3H	0.007	0.0008	0.026	0.010
L-XXXIII	"	0.005	0.0006	0.016	0.005
L-XXV	L-Leucine-UL- ^{14}C	0.008	0.0009	0.042	0.007
L-XXVI	L-Leucine-4,5- ^3H	0.018	0.0012	0.073	0.010
L-XXIX	"	0.012	0.0014	0.055	0.010
L-XXXII	"	0.010	0.0010	0.041	0.007
L-XXXV	"	0.006	0.0003	0.037	0.007
L-XXVII	L-Leucine-UL- ^{14}C	0.012	0.0003	0.040	0.011
L-XXVIII	DL-Leucine-4,5- ^3H	0.008	0.0007	0.042	0.014
L-XXXVI	"	0.008	0.004	0.042	0.013
L-XXX	L-Leucine-1- ^{14}C	0.005	0.0008	0.006	0.008
L-XXXI	L-Leucine-4,5- ^3H	0.004	0.0011	0.006	0.009
L-XXXIV	"	0.0026	0.0007	0.0023	0.006
A-XI	NaOAc-1- ^{14}C	0.0058	0.0048	0.027	0.026
A-XII	NaOAc- ^3H	0.0053	0.0045	0.0203	0.017
A-XIII	NaOAc-2- ^{14}C	0.034	0.0055	0.080	0.032
A-XIV	NaOAc- ^3H	0.008	0.0014	0.050	0.028

Counting procedure

Before discussing the results obtained from the double labelled feeding experiments, the counting procedure for double labelling experiments deserves mention.

The typical tritium and carbon-14 Beta spectra are shown in Figure 23. Practically, the activity of a sample is measured by using different discriminator window settings and the counting rate in the discriminator window is proportional to the area under the

Table XV. Isotope ratio of ^{14}C and ^3H of precursors and the ratio of incorporation of ^{14}C and ^3H of the alkaloids.

Feeding Expt. No.	Precursor	Ratio of activity of ^{14}C and ^3H	Ratio of incorp. of ^{14}C & ^3H	
			Catharanthine·HCl	Vindoline·HCl
L-XXIII	DL-Leucine-2- ^{14}C	0.201	8.54	2.98
L-XXIV	L-Leucine-4,5- ^3H	0.115	8.98	2.46
L-XXXIII		0.185	8.69	3.23
L-XXV	L-Leucine-UL- ^{14}C	0.126	9.72	6.05
L-XXVI	L-Leucine-4,5- ^3H	0.0998	15.0	7.30
L-XXIX		0.0424	8.7	5.5
L-XXXII		0.117	10.4	6.1
L-XXXV		0.0427	7.5	5.3
L-XXVII	L-Leucine-UL- ^{14}C	0.091	14.7	3.39
L-XXVIII	DL-Leucine-4,5- ^3H	0.109	11.5	3.01
L-XXX	L-Leucine-1- ^{14}C	0.0693	6.20	0.86
L-XXXI	L-Leucine-4,5- ^3H	0.0787	3.95	0.69
L-XXXIV		0.233	3.71	0.40
A-XI	NaOAc-1- ^{14}C	0.345	1.21	1.06
A-XII	NaOAc- ^3H	0.509	1.16	1.14
A-XIII	NaOAc-2- ^{14}C	0.556	6.19	2.48
A-XIV	NaOAc- ^3H	0.578	5.86	1.70

spectrum included between the window limits. The discriminator window setting for measuring tritium or the wide tritium window is usually set from 0 to 300 and for measuring ^{14}C or the wide ^{14}C channel is usually set from 0 to 500. In this study, the lower limit of the discriminator window for the wide ^{14}C channel is set at 150 in order to avoid the fluorescence. In this setting, it gives 54% counting efficiency for tritium samples and 86% counting efficiency for ^{14}C samples.

In single isotope feeding experiments, no complication is in-

Figure 23. Typical tritium and carbon-14 beta spectra

- I wide tritium window
- II wide carbon-14 window
- III narrow tritium window
- IV narrow carbon-14 window

involved in this discriminator window setting. In counting a sample containing both tritium and ^{14}C isotopes, higher counting rates will be obtained when the wide tritium window is used, due to the overlap of ^{14}C spectrum in this area. Similarly, the wide ^{14}C window to count the ^{14}C activity will include the activity due to tritium. Therefore, different discriminator windows need to be used when counting double label (tritium and ^{14}C) samples. The narrow tritium discriminator window is set to give 50% counting efficiency, in this setting the majority of ^{14}C activity spillover is eliminated. Since it is not possible to eliminate all of the ^{14}C activity from the tritium window, a calibration curve is set up for ^{14}C activity spillover. The true tritium activities due to the sample will be obtained by subtracting the spillover activity from the total activity. Similarly, a narrow ^{14}C discriminator window is set up in such a way, that about 0.5% of ^{14}C activity is left out on the upper limit side and only about 0.5% of tritium activity is allowed to extend over this lower window limit. No calibration of tritium spillover into the ^{14}C window is required since this spillover has been eliminated.

When counting double labelled samples, the narrow windows are used and all samples are quenched to some degree, a correction to the counting efficiency of every sample is made by using AQC* (Automatic quench compensation). AQC, which adjusts the Gain value to restore the spectra to the unquenched position, eliminates the problem of very high carbon spillover at high quench values.

The calibration for wide tritium and ^{14}C windows and for the

*) This instrumental development is on Beckmann instruments only.

narrow windows are included in Appendix I and II.

Mechanisms and expected result ratios

As shown in Figure 16, acetate can be converted into mevalonate; thus the first possible route is from acetate to mevalonate to indole alkaloids. Mevalonate is also a metabolic product of leucine as shown in Figure 19; therefore, the second possible mechanism of incorporation is from leucine to mevalonate and then to indole alkaloids.

On the other hand, leucine can be biosynthesized from acetate (84) as shown in Figure 24, this gives another possible route of incorporation as acetate to indole alkaloids via leucine and mevalonic acid. α -Acetolactate (LXV) is formed from acetaldehyde and pyruvate. Pinacol type isomerization (or methyl migration) followed by dehydration gives 2-oxoisovalerate (LXVI). Combination of this and acetyl coenzyme A yields 3-carboxy-3-hydroxyisocaproic acid (LXVIII) after the coenzyme A intermediate (LXVII) has been reduced (84). Dehydration of (LXVIII) gives an intermediate (LXIX) which is converted into 3-carboxy-2-hydroxyisocaproic acid (LXX) upon hydration. Reaction of the dehydrogenase upon (LXX) affords 2-oxoisocaproic acid (LXXI) and, upon transamination, leucine (LV).

The possibility of metabolic breakdown of leucine to acetate (85) leads to another proposal of the biosynthetic pathway: this is the route of leucine to acetate to mevalonic acid and then to indole alkaloids. This route is shown in Figure 25. The metabolic connection between leucine (LV) and β -hydroxy- β -methyl glutaryl coenzyme (A) (LX), one of the intermediate product in the conversion of leucine to mevalonate, is shown in Figure 19. This intermediate (LX)

Figure 24. Biosynthetic connection between acetate and leucine.

can proceed in either way: reduction of it will lead to the formation of mevalonic acid; or cleavage of $\text{C}_3\text{-C}_4$ bond will give acetyl coenzyme A and acetoacetic acid.

Figure 25. Metabolic conversion of leucine to acetate.

Based on all these transformations, the four possible biosynthetic pathways are summarized as follows:

- (1) Acetate $\longrightarrow$ mevalonate $\longrightarrow$ alkaloids. (The overall transformation can be followed by joining Figures 16, 7, 12, 13 and 14 together.)
- (2) Leucine $\longrightarrow$ mevalonate $\longrightarrow$ alkaloids. (This pathway can be followed by Figures 19, 7, 12, 13 and 14.)
- (3) Acetate $\longrightarrow$ leucine $\longrightarrow$ mevalonate $\longrightarrow$ alkaloids. (This route can be followed by joining Figures 24, 19, 7, 12, 13 and 14 together.)
- (4) Leucine $\longrightarrow$ acetate $\longrightarrow$ mevalonate $\longrightarrow$ alkaloids. (This is followed by Figures 25, 16, 7, 12, 13 and 14.)

By analysis of the above proposed biosynthetic pathways, the expected result ratio by using precursors labelled in different positions can be calculated, and compared to experimental results.

If route (1) operates, according to Figure 16, all C_2 of acetate is retained but one third of C_1 is lost during the conversion to

isopentenyl pyrophosphate. Since mevalonate has been confirmed to be a specific precursor of the C_{10} unit in the indole alkaloids, all carbons in isopentenyl pyrophosphate are converted into the corresponding carbons in the alkaloids; therefore, relations between acetate and indole alkaloids can be revealed by the relations between acetate and isopentenyl pyrophosphate. An incorporation ratio of sodium acetate-1- ^{14}C to 2- ^{14}C , of

$$\frac{2/3}{3/3} \quad \text{or } 0.67$$

should be obtained.

If route (2) operates, according to Figure 19, all carbons in leucine except C_1 are converted into the carbons of mevalonate, i.e., when uniformly labelled leucine is used as the feeding precursor, 5/6 of the activity will remain. The ratio of the incorporation between uniformly labelled leucine and 2- ^{14}C -leucine should be

$$\frac{5/6}{1/1} \quad \text{or } 0.83.$$

If route (3) operates, according to Figure 25, C_1 and C_2 of acetate will convert to C_1 and C_2 of leucine respectively and C_1 will eventually be lost during the conversion from leucine to mevalonate but C_2 will remain. In this case, the ratio of the incorporation of C_1 and C_2 acetate should be

$$\frac{0/1}{1/1} \quad \text{or } 0.$$

Similarly, if route (4) operates, according to Figure 24, C_2 and C_3 of leucine will transform into C_1 and C_2 of acetate and C_4 , C_5 and C_6 of leucine will become the three carbons of acetoacetate (C_2 , C_3 and C_4). One third of the C_1 of acetate (that is the C_2 of leucine) will be lost but C_2 of acetate (C_3 of leucine) will remain

during the conversion to mevalonate. Therefore, the ratio of incorporation of uniformly labelled leucine to 2-labelled leucine should be

$$\frac{2/6 \times 5/6}{1/1 \times 2/3} \quad \text{or } 0.42.$$

This calculation is based on the assumption that the acetoacetate part is also not incorporated in the later stage. If acetoacetate residue is however also incorporated during the later part of biosynthesis, the maximum value of the ratio of incorporation will

$$\text{be } \frac{3/6 + 2/3 \times 5/6}{1/1 \times 2/3} \quad \text{or } 1.17.$$

Results and discussions

The experimental results from double labelled experiments are shown in Table XV. For clarity, the average values of these individual experiments from different precursors were calculated and tabulated in Table XVI. Comparison can be made by using the data from this table. The results obtained after comparison are summarized in Table XVII.

By comparing entry 2 and 1 from Table XVI, the ratio of incorporation of the uniformly labelled and 2-labelled leucine was obtained and shown in row A of Table XVII, a ratio of 1.03 was found for catharanthine hydrochloride and 1.93 was found for vindoline hydrochloride. Both values were found higher than the expected value for route (2). Also, a value of non-zero was found when comparison was made to obtain the ratio of 1-labelled and uniformly labelled leucine, row B. Hence, the values of incorporation of leucine to indole alkaloids are not exactly consistent with route (2). From the experimental results, L-leucine-1-¹⁴C was substantially incorporated into catharanthine hydrochloride but little into vindoline hydrochloride.

Table XVI. Average values of Incorporation of ^{14}C / Incorporation of ^3H from various precursor.

Entry	Precursor	Ratio of Incorporation for	
		Catharanthine-HCl	Vindoline-HCl
1	DL-Leucine-2- ^{14}C L-Leucine-4,5- ^3H	8.74	2.89
2	L-Leucine-UL- ^{14}C L-Leucine-4,5- ^3H	9.08*	5.74*
3	L-Leucine-UL- ^{14}C DL-Leucine-4,5- ^3H	13.10	3.18
4	L-Leucine-1- ^{14}C L-Leucine-4,5- ^3H	4.60	0.65
5	NaOAc-1- ^{14}C NaOAc- ^3H	1.18	1.10
6	NaOAc-2- ^{14}C NaOAc- ^3H	6.03	2.09

*) Average was taken from four feedings, the extremely high value was not included.

ride. Since route (2) is proposed for only C_2 to C_6 of leucine being incorporated, the experimental results need to be corrected by the incorporation of 1- ^{14}C -leucine. This can be done by using the value of row B in Table XVII. The correction for catharanthine hydrochloride was made as follows:

$$\text{since } \frac{\text{L-1-}^{14}\text{C}}{\text{L-UL-}^{14}\text{C}} = 0.51, \text{ i.e. } \text{L-1-}^{14}\text{C} = 0.51 \times \text{L-UL-}^{14}\text{C}$$

therefore L-(2-6)- ^{14}C should be

$$1 - (\text{L-1-}^{14}\text{C}) \text{ or } (1 - 0.51) \times \text{L-UL-}^{14}\text{C}$$

$$\text{or } 0.49 \times \text{L-UL-}^{14}\text{C}$$

Table XVII. Expected and experimental ratio of incorporation from different precursors.

Row	Comparison made by	Ratio	Expected value from various rout	Experimental ratio	
				Cath·HCl	Vind·HCl
A	2/1*	L-Leucine-UL- ^{14}C	a) 0.83 Route (2)	1.03	1.93
		DL-Leucine-2- ^{14}C	b) 0.42 or Route (4) 1.17		
B	4/2	L-Leucine-1- ^{14}C	0 Route (2)	0.51	0.11
		L-Leucine-UL- ^{14}C			
C	2/3	DL-Leucine-4,5- ^3H	a) 0.5 if only L-enantiomer incorporated	0.69	1.80
		L-Leucine-4,5- ^3H	b) 1.0 if both D & L-enantiomer incorporated c) >1.0 if D-enantiomer incorporated preferentially		
D	5/6	NaOAc-1- ^{14}C	a) 0 Route (3)	0.20	0.52
		NaOAc-2- ^{14}C	b) 0.67 Route (1)		

*) These numbers are the corresponding numbers of entry from Table XVI.

$$\text{and } \frac{\text{L-(2-6)-}^{14}\text{C}}{\text{DL-2-}^{14}\text{C}} = 0.49 \times 1.03 = 0.505$$

This result indicates that about two or three carbons but not the five carbons of leucine are incorporated provided no optical specificity is involved in the incorporation. From the results shown in row C of Table XVII, it indicated that, the optical activity of precursor might take a part during the biosynthesis, i.e., one enantiomer might incorporate preferentially over the other one in

a racemic mixture. When row C in Table XVII is considered, the ratio of incorporation of the racemic leucine-4,5-¹⁴C and the L-enantiomer, $\frac{DL}{L}$, for catharanthine hydrochloride was found to be 0.69, this indicated that L-isomer was preferentially incorporated. This was used to correct the value of

$$\frac{L-(2-6)-^{14}C}{DL-2-^{14}C}$$

a value of 0.25 should be obtained instead of 0.505. This indicates that only one of the carbon in the uniformly labelled leucine is incorporated into catharanthine hydrochloride.

Similar correction can be done for vindoline hydrochloride:

$$\text{since } \frac{L-1-^{14}C}{L-UL-^{14}C} = 0.11 \quad \text{i.e.} \quad L-1-^{14}C = 0.11 \times L-UL-^{14}C$$

$$\begin{aligned} \text{then } L-(2-6)-^{14}C &= [1 - (L-1-^{14}C)] \times L-UL-^{14}C \\ &\text{or } (1 - 0.11) \times L-UL-^{14}C \\ &\text{or } 0.89 \times L-UL-^{14}C. \end{aligned}$$

$$\begin{aligned} \text{Therefore } \frac{L-(2-6)-^{14}C}{DL-2-^{14}C} &= 0.89 \times 1.93 \\ &= 1.72 \end{aligned}$$

When the incorporation of preferential optical isomer from row C in Table XVII is considered, it shows that, the D-isomer was preferentially incorporated. As seen in the introduction part of this thesis, in the early stage along the biosynthetic pathway, i.e., from mevalonic acid to stemmadenine, both of the catharanthine and vindoline had the same intermediates, therefore, the preferential incorporation of different enantiomer into them would be only accounted in the later part of the biosynthesis. If this is true, when the value of

$$\frac{L-(2-6)-^{14}C}{DL-2-^{14}C}$$

was corrected by the enantiomer factor, a value of 0.86 was obtained. This indicates that, most of the carbons in the uniformly labelled leucine is incorporated into vindoline.

From these results, one can conclude that, neither route (2) nor route (4) is followed during the biosynthesis from leucine to catharanthine and vindoline. These results are consistent with the indication of randomization from the carbon-13 approach and the incorporation of activity partially into the aromatic portion of the alkaloid.

As for the biosynthesis of acetate to indole alkaloids in V. rosea, when entry 5 was divided by entry 6, the ratio of incorporation of sodium acetate-1-¹⁴C to 2-¹⁴C was obtained and as shown in row D of Table XVII. These values are different from the expected values, none of the proposed route has been followed. This is consistent with the indication of randomization of the precursors during the incorporation.

SUMMARY OF CONCLUSIONS

The following conclusions can be reached from the experimental results:

- (1) It has been demonstrated that leucine is incorporated into both catharanthine and vindoline, in comparable levels of incorporation to sodium acetate.
- (2) The protein biosynthesis inhibitor puromycin is found to cause a measurable, but not dramatic, increase in the incorporation.
- (3) The use of carbon-13 in investigating biosynthesis in higher plants has been studied. The difficulty is the lack of sufficient alkaloid of sufficient enrichment to obtain a carbon-13 n.m.r. spectrum. It has been shown that this difficulty can be overcome by the expedient of feeding large quantities of carbon-13 enriched precursor to a sufficient amount of plant material to enable an n.m.r. spectrum of isolated alkaloid to be measured. A formula for determining the amount of precursor required has been developed. Vinca rosea plants survived this massive dose of both acetate and leucine.
- (4) Results from the carbon-13 n.m.r. indicate that the incorporation of leucine is ~~not~~ specific. Randomization was found in the monoterpene unit as well as the tryptophan unit of the alkaloids. This may suggest that leucine has been degraded during the biosynthesis and has entered the general metabolic pool involving

carbohydrate synthesis in the plant.

- (5) Vindoline is extremely sensitive to certain reagents. Osmium tetroxide, ruthenium tetroxide, potassium permanganate, Lemieux-von Rudloff reagent as well as ozone were used to cleave the double bond of vindoline. No satisfactory results were obtained.
- (6) Results from double labelled experiments again confirm the randomization of incorporation of acetate and leucine into vindoline and catharanthine. The experimental data are consistent with none of the specific biosynthetic pathways considered.
- (7) Significantly differing results for vindoline and catharanthine were observed in the double-labelled feedings. This does not appear consistent with the recognized biosynthetic pathway from mevalonic acid which differ only at the final step. This may suggest that the biosynthesis of vindoline and catharanthine is not as simple as has been thought.
- (8) The problem remains of what is the ultimate precursor of the monoterpene portion of the indole alkaloids.

EXPERIMENTAL

General

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. The infrared (ir) spectra were recorded on a Perkin-Elmer model 237 B Grating Infrared Spectrophotometer. Electronic absorption spectra (uv) were taken in ethanol on a Perkin-Elmer 202 Ultraviolet Spectrophotometer. The proton nuclear magnetic resonance (nmr) spectra were recorded in deuteriochloroform (unless otherwise indicated) on a Varian T60 NMR Spectrometer. Carbon-13 nmr spectra were obtained on a Varian XL-100 Spectrometer and deuteriochloroform was used as the solvent (except in the case of DL-leucine-2-¹³C sample). Chemical shifts are expressed in the δ scale with reference to tetramethylsilane as internal standard. Precoated Silica Gel 60 F-254 analytical and preparative chromatoplates supplied by E. Merckag, Darmstadt, Germany were employed for thin-layer chromatography (tlc). Chromatoplates were developed with 2:1 carbon tetrachloride-antimony pentachloride spray reagent. Woelm neutral alumina (activity III) was used for column chromatography (unless otherwise indicated). All solvents for chromatography were redistilled before use. Refractive indices were measured on a Officine Galileo refractometer No. 50532.) Ozonolysis was performed by using Welsbach Model T-816 ozonator. In all experiments, the pressure was set at 3 psig, A.C. volt at 50 and both sample flow and ozone flow at 0.1 scale. The concentration of ozone in oxygen gas

per minute was determined by passing the ozonated oxygen into a potassium iodide solution (2%). This oxidized potassium iodide solution was titrated with standardized sodium thiosulphate solution using starch solution as the indicator. The concentration of ozone thus determined at the set condition was 11.38 mg per minute.

Radioactivity was measured on a Beckman LS-150 Liquid Scintillation counter in counts per minute (cpm). The radioactivity of a sample in disintegrations per minute (dpm) was calculated using the counting efficiency which was determined for each sample by the external standard ratio; the appropriate calibration curves are included in Appendices I and II. Radioactivity of the double labelled samples was measured with AQC on. Glass low background spectravials supplied by Amersham/Searle corporation, Illinois, were employed and the background of the vials was determined by filling the vial with scintillator solution and counting (6 X 500 min) to determine the background cpm. The difference in cpm between the background count and the sample count was used for subsequent calculations. The scintillator solution used for determining the radioactivity of the samples was prepared by diluting spectrafluor* PPO-POPPOP concentrated liquid scintillator (42 ml) with freshly distilled spectral grade toluene (to 1 l.). In practice, a sample of an alkaloid as its free base was dissolved in spectral grade benzene (1 ml) in a counting-vial, or in the case of the salt of an alkaloids, the sample was dissolved in spectral grade methanol (1 ml) in a counting-vial, and then in both cases, a constant volume (15 ml) of the above scintillator solution was added by means of an automatic tilting

*). Trademark of Amersham-Searle Corporation.

dispenser. Each sample was counted at least three times. In case of counting aqueous samples, a 5% (v/v) toluene/BBS-3 cocktail which was prepared by adding Beckman Bio-SolvTM solubilizer BBS-3 (5 ml) into the above scintillator solution to make up a total volume of 100 ml, was used. In a few cases, 1,4-dioxane scintillator solution was used. This was made up by dissolving naphthalene (5.0 g), 2,5-diphenyloxazole (PPO, 0.7 g) and 1,4-bis[2-(5-phenyloxazole)] benzene (POPOP, 0.005 g) in redistilled 1,4-dioxane (A.C.S. grade, 100 ml).

General method of extraction of alkaloids from *V. rosea* L.

The following procedure was developed in order to extract and purify the alkaloids of *Vinca rosea* L. plants. This procedure was used for all extractions of *V. rosea* L. plants and was scaled according to the wet weight of plants used.

Shoots of *V. rosea* L. plants were obtained from the greenhouse of the university. The plants (69.2 g wet weight) were broken into small pieces, soaked in methanol for 10 to 15 minutes in a Waring blender and then mascerated in this blender (or a Osterizer blender), filtered and re-mascerated and refiltered, the residue was washed with methanol until the filtrate was colorless. This combined green filtrate (about 1500 ml) was evaporated under gentle heating on a water bath to dryness using a rotary evaporator, the residue was taken up in 2N hydrochloric acid (100 ml) and filtered. An additional portion of hydrochloric acid (2N, 25 ml) was used to rinse the flask and wash the residue. This aqueous solution was washed with benzene (2 X 70 ml) and was made basic with 15N ammonium hydroxide and extracted with chloroform (5 X 50 ml). The combined chloroform extracts were washed with water (75 ml), dried over anhydrous sodium sulfate and evaporated to give a brown oil (206.3 mg). Radioactivity

of the alkaloid mixture was monitored after methanol extraction and after chloroform extraction.

This crude alkaloid mixture was partially purified by dissolving in chloroform and run through a short alumina column (5 cm high) with chloroform (150 ml) to remove the very polar components. The chloroform solution was concentrated again on a rotary evaporator. The yellow residue was dissolved in the minimum amount of benzene and chromatographed on alumina (15 g). The column was eluted successively with petroleum ether 30°/60°, petroleum ether/benzene mixture, benzene, benzene/chloroform mixture, chloroform and methanol; fractions of 35 ml were taken. Every other fraction was checked by tlc with the authentic samples of catharanthine and vindoline as the reference. The later fractions of petroleum ether/benzene (1/1) were combined and crystallized from methanol affording catharanthine (XI, 22.09 mg), the middle fractions of benzene/chloroform (9/1) were combined and crystallized from ether giving vindoline (X, 26.99 mg). The hydrochloride salt of catharanthine was formed by blowing hydrogen chloride gas on the surface of a methanol solution of the alkaloid and was recrystallized from methanol. The hydrochloride salt of vindoline was also made by blowing hydrogen chloride gas on the surface of an ethereal solution of the alkaloid and was recrystallized from acetone.

Feeding Experimental No. L-I.

DL-Leucine-2-¹⁴C (1.4 mg, 0.5 mc/75 mg) was dissolved in distilled water in a test tube (25 X 115 mm). Two shoots of V. rosea L. plants (wet weight, 15 g) were placed in this solution of the tracer. After the plants had taken up the solution until there was just

enough solution to keep the cut end moist, additional distilled water was added to give about one inch high solution. This procedure was repeated several times to ensure that essentially all the tracer had been taken up by the plants. The plants were placed under intermittent fluorescent lamp illumination for two days and were extracted to afford the alkaloids as a brown gum (41.45 mg). The alkaloid extract was purified to afford catharanthine (6.29 mg) and vindoline (9.99 mg) as oily residues. Catharanthine hydrochloride was prepared after isolation of the free base. Inactive catharanthine hydrochloride (27.0 mg) was added and the catharanthine hydrochloride was crystallized to constant activity. The total activity in the catharanthine hydrochloride was 293 dpm after three recrystallizations representing a maximum incorporation of 0.002%. Similarly for vindoline, inactive vindoline (25.48 mg) was added and the vindoline was crystallized to constant activity. The total activity in vindoline was 945.8 dpm after recrystallized three times and this represented a maximum incorporation of 0.005%. Vindoline hydrochloride was made to check the constancy of activity. The total activity in the vindoline hydrochloride after recrystallization from acetone was 899.7 dpm representing a incorporation of 0.005%.

Feeding Experimental No. L-III.

DL-Leucine-2-¹⁴C (1.08 mg, 0.5 mc/75 mg) was dissolved in distilled water and this solution was administered to shoots of V. rosea L. plant (wet weight 16 g) as in the previous experiment. Mixture of alkaloids (90.02 mg) was obtained after extraction. After purification, catharanthine (6.51 mg) and vindoline (9.47 mg) were isolated. Inactive catharanthine hydrochloride (22.94 mg) was added and

recrystallized twice to constant activity of 220.2 dpm representing an incorporation of 0.0013%. Inactive vindoline (26.94 mg) was added and crystallized twice before making vindoline hydrochloride. After recrystallization of the vindoline hydrochloride, constant activity of 1396 dpm was obtained and this represented an incorporation of 0.007%.

Feeding Experiment No. L-IV.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in distilled water and this solution was administered to shoots of V. rosea L. plant (wet weight, 14 g) as in the previous experiment. After extraction, crude alkaloids (36.64 mg) were isolated. The mixture was purified to yield catharanthine (6.58 mg) and vindoline (4.46 mg). Catharanthine hydrochloride was made and inactive material (24.18 mg) was added. Constant activity was obtained after two recrystallization, total activity in catharanthine hydrochloride was 217.1 dpm representing a maximum incorporation of 0.002%. Inactive vindoline (26.25 mg) was added and recrystallized twice and then was converted to vindoline hydrochloride. Total activity in vindoline hydrochloride was 257.7 dpm and this represented a maximum incorporation of 0.0023%.

Feeding Experiment No. L-V.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in water and this solution was administered to shoots of V. rosea L. plant (wet weight 12 g, 6 months old). After seven days under intermittent fluorescent lamp illumination, the plant was extracted to give a mixture of alkaloids (42.95 mg). After purification, catharanthine (7.32 mg) and vindoline (7.7 mg) were isolated.

Catharanthine was converted into its hydrochloride salt and inactive catharanthine hydrochloride (23.52 mg) and vindoline (26.01 mg) were added into the active materials respectively. Crystallization was performed twice and vindoline was converted to vindoline hydrochloride later. Total activities in catharanthine hydrochloride and vindoline hydrochloride were 391.3 and 768.8 dpm which represented the maximum incorporation of 0.003% and 0.007% respectively.

Feeding Experiment No. L-VI.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in water and administered to shoots of V. rosea L. plants (wet weight 18 g, 8 months old) as in the previous experiment for seven days. After the feeding period, the cuttings were extracted to afford the alkaloids as brown gum (91.33 mg, 1.75×10^4 dpm). Catharanthine (10.62 mg) and vindoline (12.08 mg) were isolated after purification. Crystallization as in the previous experiment was performed on both alkaloids with inactive catharanthine hydrochloride (25.26 mg) and vindoline (25.35 mg) three times. Total activity in catharanthine hydrochloride was 218 dpm representing an incorporation of 0.002%. Total activity in vindoline hydrochloride was 361.7 dpm, an incorporation of 0.003%.

Feeding Experiment No. L-VII.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in water and used to feed shoots of V. rosea L. plants (wet weight 22 g, 8 months old) for two weeks. The plants were extracted to afford crude mixture of alkaloids (99.07 mg, 1.294×10^4 dpm). Purification of this crude material gave catharanthine (14.96 mg) and vindoline (15.67 mg). Crystallization with the inactive catha-

ranthine hydrochloride (25.42 mg) and vindoline (25.96 mg) respectively yielded pure catharanthine hydrochloride of total activity 323.6 dpm representing an incorporation of 0.003% and pure vindoline hydrochloride of total activity 642.8 dpm representing an incorporation of 0.006%.

Feeding Experiment No. L-IX.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in water and administered to the shoots of V. rosea L. plant (wet weight, 22 g, 3 years old) under intermittent fluorescent lamp illumination and watered as required. After two weeks, the plants were extracted to yield a brown residue (165.46 mg, 1.402×10^4 dpm). Purification of this mixture afforded catharanthine (16.43 mg) and vindoline (10.89 mg). Crystallization with inactive catharanthine hydrochloride (23.90 mg) gave catharanthine hydrochloride of constant activity of 474.2 dpm, an incorporation of 0.004%. Crystallization with inactive vindoline (26.82 mg) gave, vindoline hydrochloride obtained with a constant activity of 667.9 dpm, an incorporation of 0.005%.

Feeding Experiment No. L-X.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in distilled water and administered to the plants (wet weight 40 g, 10 months old). Puromycin dihydrochloride (1.66 mg) was also dissolved in this feeding solution. After two weeks, plants were extracted to give a brown residue (161.67 mg, 2.362×10^4 dpm). Catharanthine (18.36 mg) and vindoline (23.24 mg) were isolated after separation of the mixture. Inactive catharanthine hydrochloride (23.84 mg) and vindoline (25.97 mg) were used for recrystallization.

Total activity in catharanthine hydrochloride was 398.9 dpm representing a maximum incorporation of 0.004%. Total activity in vindoline hydrochloride was 398.9 dpm, an incorporation of 0.003%.

Feeding Experiment No. L-XI.

Puromycin dihydrochloride (1.55 mg) was dissolved in water and fed to the plants (wet weight 40 g, 10 months old). L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in water and added to the puromycin dihydrochloride solution after 24 hours. After two weeks, the plants were extracted to afford a brown gum (165.05 mg, 0.091% incorporation).

Feeding Experiment No. L-XIII.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) and puromycin dihydrochloride (1.26 mg) were dissolved in distilled water and this solution was administered to shoots of V. rosea L. plants (wet weight 30 g, 1 year old) for two weeks. After extraction, a mixture of alkaloids (224.52 mg) was obtained. Separation of this mixture gave catharanthine (22.71 mg) and vindoline (10.12 mg). After crystallization with inactive catharanthine hydrochloride (26.31 mg) and vindoline (25.78 mg) respectively, total activity in catharanthine hydrochloride was 374.3 dpm, an incorporation of 0.003%, and total activity in vindoline hydrochloride was 322.1 dpm, an incorporation of 0.003%.

Feeding Experiment No. L-XV.

DL-Leucine-2- ^{14}C (5.46 mg, 0.5 mc/75 mg) was dissolved in distilled water and this solution was administered into shoots of V. rosea L. plants (wet weight 56 g, 13 months old). Alkaloid mixture (234.57 mg) was obtained after extraction. Catharanthine (19.82 mg)

and vindoline (20.75 mg) were obtained after separation. Inactive catharanthine hydrochloride (26.22 mg) and vindoline (26.03 mg) were added to the crude alkaloids for crystallization. Total activity of 2985.9 dpm, an incorporation of 0.004% was found for catharanthine hydrochloride. Total activity of 7100.1 dpm, an incorporation of 0.009% was found for vindoline hydrochloride.

Feeding Experiment No. L-XVII.

DL-Leucine-2-¹⁴C (5.39 mg, 0.5 mc/75 mg) was dissolved in distilled water and used to feed shoots of *V. rosea* L. plants (69.5 g, 14 months old) for two weeks. Extraction of the plants gave a residue (195.06 mg, 5.48×10^5 dpm). Separation of this mixture afforded catharanthine (24.77 mg) and vindoline (18.25 mg). Recrystallization of these crude materials, diluted with inactive catharanthine hydrochloride (23.72 mg) and vindoline (26.58 mg) respectively, gave total activity in catharanthine hydrochloride of 5908 dpm, an incorporation of 0.007% and 9289.7 dpm, an incorporation of 0.012% for vindoline hydrochloride.

Feeding Experiment No. L-XIX.

L-Leucine (7.59 mg) was dissolved in distilled water in the feeding container. L-Leucine-1-¹⁴C (in 0.01N HCl, 0.1 ml, 50 μ C/ml) was added to the above solution. This solution was administered to the plants (wet weight 86.5 g) for two weeks. Extraction of these shoots gave a mixture (206.18 mg, 8496.2 dpm). After separation, catharanthine (23.19 mg) and vindoline (26.03 mg) were obtained. Crystallization of them with the inactive materials, catharanthine hydrochloride (24.63 mg) and vindoline (25.22 mg), gave the radioactive pure materials. Total activity in catharanthine hydrochloride was 497.5 dpm, an incorporation of 0.004%. Total activity in vindoline

line hydrochloride was 375.03 dpm, an incorporation of 0.003%.

Feeding Experiment No. L-XX.

L-Leucine (14.35 mg) was dissolved in distilled water in a big test tube of size 25 X 115 mm. L-Leucine-4,5-³H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) was added to the above solution. Shoots of V. rosea L. plants (71.5 g) were placed in the above solution for two weeks. Distilled water was added as required. A mixture of alkaloids (183.63 mg, 2.574×10^5 dpm) was obtained after the plants were extracted. The alkaloid extract was purified to give catharanthine (27.12 mg) and vindoline (22.81 mg). Inactive catharanthine hydrochloride (25.60 mg) and vindoline (26.42 mg) were used in the crystallization of crude materials. After recrystallized five times, total activity in catharanthine hydrochloride was 1767.6 dpm representing a maximum incorporation of 0.0016% and the total activity in vindoline was 1.305×10^4 dpm, an incorporation of 0.012%.

Feeding Experiment No. L-XXI.

L-Leucine (6.54 mg) was dissolved in distilled water and L-leucine-4,5-³H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) was added. This solution was administered to the plants (65.2 g). After two weeks, the plants were extracted to afford a brown gum (127.82 mg, 3.029×10^5 dpm). Separation of this mixture gave catharanthine (20.05 mg) and vindoline (25.29 mg). Crystallization of the crude catharanthine hydrochloride with inactive material (24.23 mg) five times, total activity in catharanthine hydrochloride of 1480.3 dpm resulted. This represented a maximum incorporation of 0.0013%. Crystallization of crude vindoline with inactive material (24.73 mg), total activity in vindoline hydrochloride of 1.434×10^4 dpm resulted.

This represented a maximum incorporation of 0.013%.

Feeding Experiment No. L-XXII.

L-Leucine (6.20 mg) was dissolved in distilled water and L-leucine-1-¹⁴C (in 0.01N HCl, 0.1 ml, 50 µc/ml) was added. This solution was used to feed shoots of *V. rosea* L. plants (69 g) for two weeks. A brown residue (182.09 mg, 7677 dpm) resulted after extraction. Separation of this mixture afforded catharanthine (24.93 mg) and vindoline (20.51 mg). Inactive catharanthine hydrochloride (24.05 mg) was added for crystallization. Total activity in catharanthine hydrochloride was found to be 702.5 dpm, an incorporation of 0.006%. Inactive vindoline (27.51 mg) was added for crystallization of the crude material. Total activity in vindoline hydrochloride was found to be 424.7 dpm, an incorporation of 0.004%.

Feeding Experiment No. L-XXIII.

L-Leucine (7.29 mg) was dissolved in distilled water in the feeding container. DL-Leucine-2-¹⁴C (1.16 mg, 0.5 mc/75 mg) was also dissolved in distilled water and added to the above solution. L-Leucine-4,5-³H (in 0.01N HCl, 0.05 ml, 100 µc/ml) was added to the above mixture. This solution after checking the activity was administered to the plants (75.5 g) for two weeks. After the feeding period, the plants were extracted to give a mixture of alkaloids (159.91 mg, 5.4169×10^4 dpm as ¹⁴C activity and 2.4415×10^5 dpm as ³H activity). This mixture was separated to give catharanthine (23.53 mg) and vindoline (30.32 mg). Inactive catharanthine hydrochloride (25.63 mg) was added and recrystallized to constant activity. Total ¹⁴C activity in catharanthine hydrochloride was 1506 dpm representing an incorporation of 0.0088%. Total tritium activity in

catharanthine hydrochloride was 1139 dpm representing an incorporation of 0.0010%. Inactive vindoline (26.04 mg) was added to crystallization. Total activity of ^{14}C and tritium in vindoline was 4932 dpm and 1.0686×10^4 dpm which represented an incorporation of 0.030% and 0.0096% respectively.

Feeding Experiment No. L-XXIV.

L-Leucine (6.11 mg) was dissolved in distilled water. DL-Leucine-2- ^{14}C (1.06 mg, 0.5 mc/75 mg) was dissolved in the above solution and L-leucine-4,5- ^3H (in 0.01N HCl, 0.05 ml, 0.25 mc/ 0.25 ml) was added. This solution after checking the activity was used to feed the plants (69.2 g). After two weeks, the plants were harvested and the mixture of alkaloids (206.34 mg, 1.1786×10^5 dpm in ^{14}C and 3.735×10^5 dpm in tritium). This mixture was separated to afford catharanthine (22.09 mg) and vindoline (26.99 mg). After crystallization with inactive catharanthine hydrochloride (23.46 mg) and vindoline (26.30 mg) to constant activity respectively, the following results obtained: total activities of ^{14}C and tritium for catharanthine hydrochloride were 1143.6 dpm and 1037 dpm which represented an incorporation of 0.007% and 0.0008% respectively; total activities of ^{14}C and tritium in vindoline hydrochloride were 3966.4 dpm and 1.3112×10^4 dpm which represented an incorporation of 0.026% and 0.010% respectively.

Feeding Experiment No. L-XXV.

L-Leucine (6.13 mg) was dissolved in distilled water in the feeding container. L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) and L-leucine-4,5- ^3H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) were added to the above solution. The activity of this mixture was

checked and was administered to the plants (49.1 g). The plants were extracted as usual to give a mixture of alkaloids (133.15 mg, 3.8869×10^4 dpm and 2.2538×10^5 dpm in ^{14}C and ^3H respectively). After separation, catharanthine (16.95 mg) and vindoline (22.67 mg) were obtained. Inactive catharanthine hydrochloride (25.23 mg) was added and crystallized five times, total activity of 1009.2 dpm in ^{14}C and 849.9 dpm in ^3H were obtained. This represented a maximum incorporation of 0.008% in ^{14}C and 0.0009% in ^3H . Inactive vindoline (24.64 mg) was added and crystallized to constant activities. Total activity of ^{14}C in vindoline was found to be 5165 dpm and total activity of tritium was 6977 dpm. These represented the maximum incorporation of 0.042% and 0.007% respectively.

Feeding Experiment No. L-XXVI.

L-Leucine (6.86 mg) was dissolved in distilled water. L-Leucine- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) and L-leucine-4,5- ^3H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) were added to the above solution. The activity in ^{14}C and in ^3H of this mixture was checked and this solution was used to feed shoots of *V. rosea* L. plants (61.1 g). After extraction, the alkaloids were isolated (171.66 mg, 3.2293×10^4 dpm in ^{14}C and 1.962×10^5 in ^3H). Separation of the alkaloid mixture gave catharanthine (20.93 mg) and vindoline (25.67 mg) as yellow oils. Crystallization with inactive catharanthine hydrochloride (27.19 mg) and inactive vindoline (25.17 mg) to constant activity, the following results were obtained: total ^{14}C activity and tritium activity in catharanthine hydrochloride were 2023.4 dpm and 1021.2 dpm representing incorporations of 0.018% and 0.0010% respectively; total ^{14}C activity and tritium activity in vindoline hydrochloride were 8170.6 dpm and 9773.6 dpm representing incorporations

of 0.073% and 0.010% respectively.

Feeding Experiment No. L-XXVII.

L-Leucine (5.33 mg) was dissolved in distilled water in the feeding container. L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) and DL-leucine-4,5- ^3H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) were also dissolved in the solution. This mixture after checking the activities was administered to the plants (61.5 g) for two weeks. After extraction with chloroform, a brown residue (131.81 mg, 1.9789×10^4 dpm in ^{14}C and 2.7336×10^5 dpm in tritium) resulted. Separation of this mixture yielded catharanthine (22.53 mg) and vindoline (26.00 mg). Crystallization with inactive catharanthine hydrochloride (25.38 mg) and vindoline (26.68 mg) individually to constant activity, the total ^{14}C activity and tritium activity in catharanthine hydrochloride and vindoline hydrochloride were 1176.5 dpm, an incorporation of 0.012% and 767.6 dpm, an incorporation of 0.0008%; 3993.6 dpm, an incorporation of 0.040% and 1.066×10^4 dpm, an incorporation of 0.011% respectively.

Feeding Experiment No. L-XXVIII.

L-Leucine (5.42 mg) was dissolved in distilled water. L-leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) and DL-leucine (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) were dissolved in the above solution. The ^{14}C and tritium activities of this mixture were checked and used to feed the plants (64.8 g). After two weeks, the plants were extracted to give a brown oil (166.28 mg, 1.7075×10^4 dpm in ^{14}C and 3.4333×10^5 dpm in tritium). Separation of this mixture afforded catharanthine (24.55 mg) and vindoline (28.84 mg). Crystallization of the crude catharanthine hydrochloride with inactive

material (25.22 mg) to a constant activity of 890.4 dpm in ^{14}C and 672.04 dpm in tritium, this represented an incorporation of 0.008% in ^{14}C and 0.0007% in tritium. Crystallization of the crude vindoline with inactive material (26.84 mg) to a constant activity and vindoline hydrochloride was made. Total activity in the vindoline hydrochloride was 4495.7 dpm in ^{14}C and 1.3048×10^4 dpm in tritium, this represented an incorporation of 0.042% in ^{14}C and 0.014% in tritium.

Feeding Experiment No. L-XXIX.

L-Leucine (6.48 mg) was dissolved in distilled water, L-leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) and L-leucine-4,5- ^3H (in 0.01N HCl, 0.15 ml, 0.25 mc/0.25 ml) were also dissolved in the above solution. Total ^{14}C and tritium activities were checked and the solution was used to administered to the plants (59.4 g). After two weeks, the plants were extracted to give a brown gum (118.25 mg, 2.2847×10^4 dpm in ^{14}C and 5.977×10^5 dpm in tritium). Separation of this mixture gave catharanthine (23.61 mg) and vindoline (28.32 mg) as yellow oils. Crystallization of the crude catharanthine hydrochloride with inactive material (25.20 mg) to a constant activity of 1340.4 dpm in ^{14}C and 4108.7 dpm in tritium, this represented an incorporation of 0.012% and 0.0014% respectively. Crystallization of the crude vindoline with inactive material (25.41 mg) to a constant activity and its hydrochloride salt was made. Total activities of ^{14}C and tritium in vindoline hydrochloride were 6174.7 dpm and 29800.2 dpm which represented an incorporation of 0.055% and 0.010% respectively.

Feeding Experiment No. L-XXX.

L-Leucine (9.11 mg) was dissolved in distilled water, L-leucine-

$1-^{14}\text{C}$ (in 0.01N HCl, 0.20 ml, 50 $\mu\text{C}/\text{ml}$) and L-leucine-4,5- ^3H (in 0.01 N HCl, 0.15 ml, 0.25 mc/0.25 ml) were dissolved in distilled water. The activity of this mixture was checked and was used to feed the plants (77.3 g) for two weeks. Extraction of the plants with chloroform gave a brown oil (146.47 mg, 2.6978×10^4 dpm in ^{14}C and 7.1634×10^5 dpm in tritium). Separation of this mixture gave catharanthine (22.72 mg) and vindoline (30.78 mg). Crystallization of the crude catharanthine hydrochloride with inactive material (25.20 mg) to give radioactive pure catharanthine hydrochloride (1039.8 dpm, 0.005% in ^{14}C and 2306.9 dpm which was 0.0008% incorporation in tritium). Crystallization of the crude vindoline with inactive material (23.77 mg) to give a radioactive pure vindoline which was converted into its hydrochloride salt later. The total activity in vindoline hydrochloride was 1420.1 in ^{14}C which represented an incorporation of 0.006% and 2.2858×10^4 dpm in tritium which represented an incorporation of 0.008%.

Feeding Experiment No. L-XXXI.

L-Leucine (6.60 mg) was dissolved in distilled water, L-leucine- $1-^{14}\text{C}$ (in 0.01N HCl, 0.20 ml, 50 $\mu\text{C}/\text{ml}$) and L-leucine-4,5- ^3H (in 0.01N HCl, 0.15 ml, 1 mc/ml) were also dissolved in the distilled water and mixed. The activity of this mixture was checked and administered to the plants (78.3 g). After two weeks, the plants were extracted to afford a brown oil (192.98 mg, 4.4750×10^4 dpm in ^{14}C , 2.5156×10^6 dpm in tritium). Separation of this mixture gave catharanthine (22.89 mg) and vindoline (28.90 mg) as yellow oils. Inactive catharanthine hydrochloride (25.57 mg) was added to the crude material and crystallized to constant activities: 922.2 dpm in ^{14}C

and 3190.8 dpm in tritium which represented an incorporation of 0.004% and 0.0011% respectively. Inactive vindoline (25.41 mg) was added to the crude material and crystallized to constant activities: 1279.3 dpm was found in ^{14}C and 25773 dpm in tritium which represented an incorporation of 0.006% and 0.009% respectively.

Feeding Experiment No. L-XXXII.

L-Lecine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was added to a volumetric flask (5 ml) containing inactive aqueous L-leucine solution and diluted to the mark. L-Leucine-4,5- ^3H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) was added to another volumetric flask (5 ml) containing inactive aqueous L-leucine solution. Activities of these solutions were checked as well as after mixing. This mixture was administered to the plants (68.3 g) for two weeks. A brown residue of the alkaloid mixtures was obtained (131.13 mg, 3.4992×10^4 dpm in ^{14}C and 1.2621×10^6 dpm in tritium) after the plants were extracted. Separation of this mixture afforded catharanthine (16.70 mg) and vindoline (17.77 mg). Inactive catharanthine hydrochloride (25.00 mg) was added for crystallization to constant activity. Total activity in catharanthine hydrochloride was found to be 1106.6 dpm in ^{14}C and 1337.7 dpm in tritium, this represented an incorporation of 0.010% and 0.0014% respectively. Inactive vindoline (26.76 mg) was added for crystallization to constant activity. Total activity in vindoline hydrochloride was 4400.7 dpm in ^{14}C and 6214.2 dpm in ^3H , this represented an incorporation of 0.041% and 0.007% respectively.

Feeding Experiment No. L-XXXIII.

DL-Leucine-2- ^{14}C (1.13 mg, 0.5 mc/75 mg) was dissolved in distilled water in a volumetric flask (5 ml) containing inactive L-

leucine. L-Leucine-4,5-³H (in 0.01N HCl, 0.05 ml, 1 mc/ml) was added to another volumetric flask containing inactive L-leucine. Distilled water was added into both flasks to the mark. Activities of these solutions were checked as well as after mixing. This mixture was administered to the plants (82.2 g) for two weeks. Extraction of the plants afforded a brown residue (208.0 mg, 1.4866×10^5 dpm in ¹⁴C and 1.4983×10^6 dpm in tritium). Separation of this mixture gave crude catharanthine (16.72 mg) and vindoline (21.02 mg). Inactive catharanthine hydrochloride (24.32 mg) was added for crystallization. After recrystallized five times, total activity in catharanthine hydrochloride was 900.5 dpm, an incorporation of 0.005% in ¹⁴C and 581.2 dpm, an incorporation of 0.0006% in tritium. Inactive vindoline (26.14 mg) was added for crystallization. Total activity in vindoline hydrochloride was 2748.9 dpm, an incorporation of 0.016% in ¹⁴C and 4759.3 dpm, an incorporation of 0.005% in ³H.

Feeding Experiment No. L-XXXIV.

L-Leucine-1-¹⁴C (in 0.01N HCl, 0.20 ml, 50 μ c/ml) was dissolved in distilled water in a volumetric flask (5 ml) which contained inactive L-leucine. L-Leucine-4,5-³H (in 0.01N HCl, 0.05 ml, 1 mc/ml) was dissolved in distilled water in another volumetric flask (5 ml) which also contained inactive L-leucine. The activities of these solutions were checked as well as after they were mixed. This mixture was administered to the plants (67.8 g) for two weeks. The plants were extracted to give a brown residue (147.26 mg, 2.6528×10^4 dpm in ¹⁴C and 1.0802×10^6 dpm in tritium). Separation of this mixture gave catharanthine (21.01 mg) and vindoline (19.02 mg).

Crystallization of this crude material with inactive catharanthine.

hydrochloride (24.96 mg) and vindoline (25.64 mg) to constant activities, the following results obtained: total ^{14}C and tritium activities in catharanthine hydrochloride were 582.6 dpm and 599.6 dpm which represented an incorporation of 0.0026% and 0.0006% respectively; total ^{14}C and tritium activities in vindoline hydrochloride were 522.5 dpm and 5644.9 dpm which represented an incorporation of 0.0023% and 0.006% respectively.

Feeding Experiment No. L-XXXV.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in distilled water in a volumetric flask (5 ml) which contained inactive L-leucine. L-Leucine-4,5- ^3H (in 0.01N HCl, 0.15 ml, 0.25 mc/0.25 ml) was dissolved in distilled water in another volumetric flask (5 ml) also contained inactive L-leucine. Activities of these solutions were checked as well as after mixing. This mixture was used to feed the plants (65 g). After two weeks, the plants were extracted and yield a brown residue (120.02 mg, 3.2769×10^4 dpm in ^{14}C and 2.9946×10^6 dpm in tritium). Separation of this mixture gave crude catharanthine (14.92 mg) and vindoline (17.46 mg). Inactive catharanthine hydrochloride (25.02 mg) was added for crystallization. Total activities in catharanthine hydrochloride was 630.7 dpm in ^{14}C and 2265.3 dpm in tritium which represented an incorporation of 0.006% and 0.0008% respectively. Inactive vindoline (26.85 mg) was added for crystallization. Total activity in vindoline hydrochloride was 4090.1 dpm in ^{14}C and 20740.2 dpm in tritium, which represented an incorporation of 0.037% and 0.007% respectively.

Feeding Experiment No. L-XXXVI.

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.05 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved

in distilled water in a volumetric flask (5 ml) which contained inactive L-leucine. DL-Leucine-4,5-³H (in 0.01N HCl, 0.05 ml, 0.25 mc/0.25 ml) was also dissolved in distilled water in another volumetric flask (5 ml) contained inactive L-leucine. Activities of these solutions were measured and also determined after mixing. This solution was administered to the plants (60 g) for two weeks. Extraction of the plants gave a mixture of alkaloids (92.10 mg, 1.1497×10^4 dpm in ¹⁴C and 4.053×10^5 dpm in tritium). Separation of the mixture yielded crude catharanthine (18.56 mg) and vindoline (17.37 mg). Crystallization of the crude catharanthine hydrochloride with inactive material (26.76 mg) to constant activity. Total activity in this pure material was 910.0 dpm in ¹⁴C, an incorporation of 0.008% and 4398.0 dpm in tritium, an incorporation of 0.0037%. Crystallization of crude vindoline with inactive material (25.39 mg) to constant activity. Vindoline hydrochloride was made and total activity in it was 4711.9 dpm in ¹⁴C, an incorporation of 0.042% and 1.5798×10^4 dpm, an incorporation of 0.013%.

Feeding Experiment No. L-XXXIX.

Synthesized DL-leucine-2-¹³C (374.6 mg) was dissolved in distilled water in a beaker. DL-Leucine-2-¹⁴C (total activity 1.0137×10^8 dpm) was added to the above solution and this mixed solution was administered to shoots of V. rosea L. plants (145 g) for two weeks. The plants were then extracted and gave a brown residue (449.3 mg, 3.0782×10^5 dpm). Separation of this mixture yielded catharanthine (39.3 mg) and vindoline (64.5 mg) as pale yellow oils. Catharanthine hydrochloride was prepared and crystallized once, total activity was 8805.3 dpm representing an incorporation of 0.0087%.

Vindoline was also crystallized and total activity was 3.5543×10^4 dpm which represented an incorporation of 0.035%. The crystallized vindoline was used in the determination of carbon-13 n.m.r..

Feeding Experiment No. A-V.

Sodium acetate-1- ^{14}C (2.59 mg, 1mc/41 mg) was dissolved in distilled water. This solution was used to feed shoots of *V. rosea* L. plants (36.2 g) for two weeks. The plants were extracted and the mixture of alkaloids was separated to afford catharanthine (22.56 mg) and vindoline (24.10 mg). Inactive catharanthine hydrochloride (22.50 mg) and vindoline (24.29 mg) were used for crystallization individually. Total activity in pure catharanthine hydrochloride was 4018.4 dpm, an incorporation of 0.0029%. Total activity in vindoline hydrochloride was 1.2224×10^4 dpm, an incorporation of 0.0087%.

Feeding Experiment No. A-VII.

Same feeding procedure as the previous one was followed. For simplicity, only the figure was mentioned. Sodium acetate-1- ^{14}C (2.44 mg, 1 mc/41 mg) was used as the precursor to feed the plants (90.4 g). Crude catharanthine (23.85 mg) and vindoline (24.60 mg) were isolated. Inactive catharanthine hydrochloride (24.07 mg) and vindoline (24.41 mg) were employed. Total activity in catharanthine hydrochloride was 2370.5 dpm, 0.0018% incorporation. Total activity in vindoline hydrochloride was 6646.9 dpm, an incorporation of 0.005%.

Feeding Experiment No. A-VIII.

Sodium acetate-2- ^{14}C (2.47 mg, 1 mc/41 mg) was used as the precursor to feed the plants (77 g). Crude catharanthine (22.79 mg) and vindoline (23.61 mg) were isolated. Inactive catharanthine

hydrochloride (23.34 mg) and vindoline (24.97 mg) were used for crystallization. Total activity in catharanthine hydrochloride was 1.4235×10^4 dpm, 0.010%. Total activity in vindoline hydrochloride was 2.3787×10^4 dpm, 0.018%.

Feeding Experiment No. A-IX.

Sodium acetate- ^3H (in EtOH, 0.0125 ml, 1mc/0.25 ml) was used as the precursor to administered to the plants (45.4 g). Crude catharanthine (14.55 mg) and vindoline (15.29 mg) were isolated. Inactive catharanthine hydrochloride (24.67 mg) and vindoline (27.33 mg) were added for crystallizations. Total activity in catharanthine hydrochloride was 2.666×10^6 dpm, 0.0023%. Total activity in vindoline hydrochloride was 1.133×10^4 dpm, a 0.010% incorporation.

Feeding Experiment No. A-X.

Sodium acetate- ^3H (in EtOH, 0.0125 ml, 1 mc/0.25 ml) was used to feed the plants (75.2 g). Crude catharanthine (23.41 mg) and vindoline (20.89 mg) were isolated. Inactive catharanthine hydrochloride (25.74 mg) and vindoline (25.47 mg) were used for crystallization. Total activity in catharanthine hydrochloride was 1371 dpm, 0.0012% and total activity in vindoline hydrochloride was 6081 dpm, 0.0055%.

Feeding Experiment No. A-XI.

Inactive sodium acetate (anhydrous, 9.75 mg) was dissolved first in distilled water. Sodium acetate- $1\text{-}^{14}\text{C}$ (3.29 mg, 1 mc/41 mg) and sodium acetate- ^3H (in EtOH, 0.040 ml, 1 mc/0.25 ml) were used as the precursors to feed the plants (76.7 g). Activities of this feeding solution was checked. Crude catharanthine (24.30 mg) and vindoline (33.52 mg) were isolated. Inactive catharanthine hydrochloride

(23.10 mg) and vindoline (25.22 mg) were added. Total activity in catharanthine hydrochloride was 10355 dpm, an incorporation of 0.0058% in ^{14}C and 17954 dpm, an incorporation of 0.0048% in tritium. Total activity in vindoline hydrochloride was 48754 dpm, an incorporation of 0.0274% in ^{14}C and 96829 dpm, an incorporation of 0.026% in ^3H .

Feeding Experiment No. A-XII.

Anhydrous sodium acetate (5.89 mg), sodium acetate-1- ^{14}C (4.53 mg, 1 mc/41 mg) and sodium acetate- ^3H (in EtOH, 0.040 ml, 1mc/0.25 ml) were used as the precursors to feed the plants (85.4 g). Crude catharanthine (31.1 mg) and vindoline (28.99 mg) were isolated. Inactive catharanthine hydrochloride (28.85 mg) and vindoline (24.29 mg) were added. Total activity in catharanthine hydrochloride was 13076 dpm, an incorporation of 0.0053% in ^{14}C and 17104 dpm, an incorporation of 0.0045% in tritium. Total activity in vindoline hydrochloride was 49558 dpm, an incorporation of 0.020% in ^{14}C and 65962 dpm, an incorporation of 0.017% in tritium.

Feeding Experiment No. A-XIII.

Sodium acetate-2- ^{14}C (4.49 mg, 1 mc/41 mg) and sodium acetate- ^3H (in EtOH, 0.040 ml, 1 mc/0.25 ml) were used as the precursors to administer to the plants (78.2 g). Crude catharanthine (25.87 mg) and vindoline (28.37 mg) were isolated. Inactive catharanthine hydrochloride (25.66 mg) and vindoline (26.17 mg) were added. Total activity in catharanthine hydrochloride was 50916 dpm, an incorporation of 0.034% in ^{14}C , 14985 dpm, an incorporation of 0.0055% in tritium. Total ^{14}C and tritium activities in vindoline hydrochloride were 1.1957×10^5 dpm and 8.9535×10^4 dpm, an incorporation of 0.080% and 0.032% respectively.

Feeding Experiment No. A-XIV.

Sodium acetate-2- ^{14}C (4.46 mg, 1 mc/41 mg) and sodium acetate- ^3H (in EtOH, 0.040 ml, 1 mc/0.25 ml) were used to feed the plants (39.0 g). Crude catharanthine (10.87 mg) and vindoline (15.80 mg) were isolated. Inactive catharanthine hydrochloride (24.04 mg) and vindoline (27.05 mg) were added. Total ^{14}C and tritium activities in catharanthine hydrochloride were 12489 dpm and 3816.5 dpm, these represented an incorporation of 0.008% and 0.0014% respectively. Total activities in ^{14}C and tritium were 78466 dpm and 79691 dpm in vindoline hydrochloride, these represented an incorporation of 0.050% and 0.028% respectively.

Feeding Experiment No. A-XV.

Inactive sodium acetate (46.6 mg) and sodium acetate-2- ^{14}C (5.81 mg, 1 mc/41 mg) were used to feed the plants (75.5 g). Crude catharanthine (23.06 mg) and vindoline (25.91 mg) were isolated. Inactive catharanthine hydrochloride (25.26 mg) and vindoline (26.73 mg) were added. Total activity in catharanthine hydrochloride was 49049 dpm, an incorporation of 0.024%. Total activity in vindoline hydrochloride was 98941 dpm, an incorporation of 0.050%.

Feeding Experiment No. A-XVI.

Inactive sodium acetate (132.8 mg) and sodium acetate-2- ^{14}C (17.05 mg, 1 mc/41 mg) was used to feed the plants (71.2 g). Crude catharanthine (22.04 mg) and vindoline (23.36 mg) were isolated. Total activity in catharanthine hydrochloride and vindoline hydrochloride was 1.3649×10^5 dpm and 3.2138×10^5 dpm which represented an incorporation of 0.022% and 0.052% respectively.

Feeding Experiment No. A-XVIIc.

Anhydrous sodium acetate (202.4 mg) was dissolved in distilled

water (130 ml). This solution (0.156% in concentration) was administered to plants (80 g). After two weeks, the plants were extracted to afford catharanthine (23.1 mg) and vindoline (38.1 mg). Crystallization was performed to give pure catharanthine hydrochloride (95 - 97%) and vindoline (99%).

Feeding Experiment No. A-XIX.

Sodium acetate-1- ^{13}C (215.7 mg) and sodium acetate-1- ^{14}C (2.2 mg 1 mc/41 mg) were used to prepare a feeding solution of 0.13% in concentration. This solution was administered to the plants (110 g). Crude catharanthine (35.6 mg) and vindoline (33.1 mg) were isolated. Total activity in catharanthine hydrochloride was 2380.8 dpm, an incorporation of 0.002%. Total activity in vindoline was 33497 dpm, an incorporation of 0.028%. These pure alkaloids were used for carbon-13 n.m.r. measurement.

Feeding Experiment No. A-XX.

Sodium acetate-2- ^{13}C (222.6 mg) and sodium acetate-2- ^{14}C (2.4 mg 1 mc/41 mg) were used to prepare a feeding solution (0.15%) which was administered to plants (93 g). Crude catharanthine (28.9 mg) and vindoline (43.0 mg) were obtained. Total activity in catharanthine hydrochloride was 2432.7 dpm, an incorporation of 0.003%. Total activity in vindoline was 3.5058×10^5 dpm, an incorporation of 0.044%. The crystalline vindoline was used in the determination of carbon-13 n.m.r. spectrum.

Feeding Experiment No. A-XXI.

Sodium acetate-2- ^{13}C (208.1 mg) and sodium acetate-2- ^{14}C (2.45 mg 1 mc/41 mg) were dissolved in distilled water to give a 0.155% feeding solution which was administered to plants (112 g). Crude

catharanthine (31.1 mg) and vindoline (35.1 mg) were isolated. Total activity in catharanthine hydrochloride and vindoline was 1.6471×10^4 dpm and 1.0304×10^5 dpm, an incorporation of 0.018% and 0.110% respectively.

Tests for Adsorption of leucine on glass.

a) Vial Emptying Experiment

(1) L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.001 ml, 100 $\mu\text{C}/\text{ml}$) was dissolved in methanol (1 ml) in a spectravial and scintillator solution was added. The activity of this sample was determined (1.9837×10^5 dpm). The contents of this vial was poured off, drained and then refilled with scintillator solution and the activity redetermined (8292.7 dpm). 4.1% of activity was remained in the vial.

(2) L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.002 ml, 100 $\mu\text{C}/\text{ml}$) was used. Activity of this sample was 3.4539×10^5 dpm. After decanting and refilling, the activity was 2.2799×10^4 dpm. 6.6% of activity was remained in the vial.

b) Adsorption Shift Experiment ⁽⁸⁷⁾

Nine samples of L-leucine-UL- ^{14}C (in 0.01 N HCl, 0.01 ml, 100 $\mu\text{C}/\text{ml}$) were prepared and subjected to activity determination in channel I (440 - 500)* and channel II (150 - 390)*. The results are tabulated in Table XVIII.

The average adsorption shift A for these samples was found by using the equation ⁽⁸⁸⁾

$$\log I/II = 11.48 \log (\text{ESR}) + 1.158$$

to be 0.073.

*) Variable discriminator module assembly settings

Table XVIII. Data of adsorption shift experiment.

Sample No.	ESR	Activity in channel II	Activity in channel I
I	0.715	90410.0 dpm	23752.4 dpm
II	0.714	92353.0	23260.3
III	0.714	93730.9	23431.2
IV	0.716	92482.2	24283.7
V	0.713	93628.8	23635.2
VI	0.715	92851.0	24039.4
VII	0.719	93702.5	25101.2
VIII	0.717	92381.0	24763.5
IX	0.715	92981.2	24362.3

c) Carrier Dilution Experiment

L-Leucine-UL- ^{14}C (in 0.01N HCl, 0.001 ml, 100 $\mu\text{C}/\text{ml}$)

counting solution with different amount of inactive leucine were prepared. 5% BBS scintillator cocktail solution was used for sample I. 10% BBS scintillator cocktail solution was used for sample II, III and IV. 20% BBS solution was used for sample V. The activities of these samples were determined. Activity found in each sample is tabulated in Table XIX.

Table XIX. Data of carrier dilution experiment.

Sample No.	Amount of inactive leucine	Activity
I	0.86 mg	2.0099×10^5 dpm
II	2.29	2.0345×10^5
III	4.92	2.0064×10^5
IV	7.76	2.0189×10^5
V	16.70	1.9747×10^5

Synthesis of DL-leucine-2-¹³C.

The reactions for the synthesis of leucine are literature procedures from references (119) to (124).

(1) Monobromoacetic acid⁽¹¹⁹⁾ (LXXII).

Acetic acid-2-¹³C (90 atom % C-13, 2.0 g, purchased from Merck Sharp and Dohme Canada Ltd.) was transferred to a 2-neck 25 ml round bottom flask by using the device shown in Figure 22. To this transferred acid (1.9593 g; 1.87 ml, 0.032 mole) in the flask equipped with a reflux condenser (carrying a calcium chloride drying tube) and a dropping funnel with long stem which reached below the level of the liquid, acetic anhydride (freshly distilled, 0.412 g; 0.38 ml, 0.004 mole) and one drop of dry pyridine were added. The mixture was stirred and heated to boiling. The flame was removed, one drop of dry bromine was added and the reaction was allowed to proceed until the liquid became colorless. The remainder of the bromine (2.66 g; 0.91 ml, 0.0167 mole) was added as rapidly as it would react avoiding loss through the condenser, a small flame being used during this time to keep the solution boiling gently. The solution was then heated until it became colorless, cooled, and water (0.15 ml) was added slowly to destroy acetic anhydride. This acid and water were removed by rotary evaporation and the residue (2.6695 g, 58.7%) crystallized on cooling, m.p. 44° sharp (lit⁽¹¹⁹⁾ m.p. 50°); δ 3.9 (2H, s, -CH₂-) and 10.7 (1H, s, acid H).

(2) From bromoacetic acid to ethyl cyanoacetate (LXXIV) without isolating cyanoacetic acid (LXXIII)⁽¹²⁰⁾.

Bromoacetic acid-2-¹³C (2.6695 g, 0.019 mole) was dissolved in water (2.6 ml) in a 25 ml round bottom flask. This solution was

warmed to 50° and neutralized with anhydrous sodium carbonate (1.06 g, 0.01 mole), then cooled to room temperature.

Meanwhile, sodium cyanide (98.6%, 1.029 g, 0.0210 mole) was dissolved in water (3 ml) and warmed to 55°. This solution was then added to the sodium bromoacetate solution with rapid mixing. The mixture was cooled under the cold water tap when necessary to avoid the temperature reaching 95°. The mixture was then heated to boiling for five minutes and cooled with running water for one-half hour. The solution was filtered and the cyanoacetic acid was set free by adding with stirring concentrated hydrochloric acid (sp. gr. 1.1878, 37%; 1.74 ml; 0.021 mole). The solution was evaporated in vacuo on a water bath at 60 - 70° until practically no more distillate came out. To the residue, absolute ethanol (2.8 ml) was added. The solution was filtered from sodium chloride and the residue was washed with another portion of absolute ethanol (1.4 ml). Ethanol was removed in vacuo on a water bath held at 50 - 60° and yielded a brown residue, $\nu_{\text{max}}^{\text{KBr}}$ 2270 cm^{-1} (-CN). This crude cyanoacetic acid was used to prepare the ethyl ester without further purification.

Freshly distilled absolute ethanol (3.0 ml) and 2 drops of concentrated sulphuric acid were added to the crude cyanoacetic acid and the mixture was refluxed on a water bath for three hours. Excess ethanol and water which formed during the reaction were removed in vacuo. The residue again was refluxed with another portion of freshly distilled absolute ethanol (1.5 ml) and 1 drop of concentrated sulphuric acid for two hours. At the end of refluxing, the excess ethanol was removed. The ester mixture was allowed to cool to room temperature, the sulphuric acid was neutralized with saturated

sodium carbonate solution; the ester (upper layer) was separated and the aqueous solution was extracted several times with benzene. The benzene extracts were combined with the ester and was dried (anhydrous sodium sulphate). Benzene was removed to afford a light brown liquid residue (1.551 g). Chromatography of this crude product on alumina (3.0 g), eluted with anhydrous ether, pale yellow ester obtained (1.467 g, 67.6%) after removing the solvent, δ 1.31 (3H, t, $-\text{CH}_3$), 3.52 (2H, s, $-\text{CH}_2-$) and 4.29 (2H, q, $-\text{OCH}_2-$), $n_D^{20} = 1.4181$ (lit⁽⁹¹⁾ $n_D^{20} = 1.4175$).

(3) Ethyl isonitroso cyanoacetate (LXXV)⁽¹²¹⁾.

Ethyl cyanoacetate-2-¹³C (1.467 g, 0.0130 mole) was dissolved in glacial acetic acid (2.337 g, 2.23 ml) and cooled in an ice bath. Meanwhile, sodium nitrite (2.686 g, 0.0389 mole) was dissolved in water (3.7 ml) and also cooled in the ice bath. With stirring, the sodium nitrite solution was added into the ethyl cyanoacetate solution over a period of one hour and the temperature of the mixture was kept below 20°. The mixture was stirred for an additional hour after the addition of sodium nitrite solution was completed. This mixture was then extracted with ether several times. The combined ether extracts (40 ml) were washed with sodium carbonate solution (10%, 5 X 1.2 ml) and then with water (2 X 1.2 ml). The ether extract was dried (anhydrous calcium sulphate). Ethyl isonitrosocyanoacetate-2-¹³C (1.596 g, 86.6%) was obtained after removing the ether, m.p. 128-130° (lit⁽¹⁴²⁾ m.p. 129-130°); $\delta_{(\text{CD}_3)_2\text{CO}}$ 1.33 (3H, t, $-\text{CH}_3$), 2.05 (1H, s, broad, N-OH) and 4.38 (2H, q, $-\text{O}-\text{CH}_2-$).

(4) Ethyl acetamidocyanoacetate (LXXVI)⁽¹²²⁾.

Ethyl isonitrosocyanoacetate-2-¹³C (1.596 g, 0.0112 mole) was

dissolved in glacial acetic acid (8.88 g, 8.44 ml) and dry acetic anhydride (3.041 g, 2.82 ml) in a 50 ml erlynmeyer flask. With vigorous stirring, zinc dust (1.688 g, 0.0259 mole) was added portion-wise at 30-40° over a period of half an hour. Stirred for an additional 15 minutes and warm water (about 12 ml) was added to dissolve the zinc salt, and the excess zinc dust was removed by filtration. The filtrate was saturated with solid sodium chloride and cooled to 0°. Ethyl acetamidocyanoacetate crystallized out upon cooling.

Recrystallization from anhydrous ether gave the product as white needles (1.1207 g, 58.66%), m.p. 125-127° (lit⁽¹²²⁾ m.p. 127-129°).

δ , 1.37 (3H, t, -CH₃), 2.12 (3H, s, -COCH₃), 4.38 (2H, q, -OCH₂-), 5.53 (1H, d, -CH) and 6.47 (1H, broad, -NH).

(5) Alkylation of acetamidocyanoacetate⁽¹²³⁾.

Newly cut sodium metal (0.1516 g, 0.0066 mole) was dissolved in isopropanol (17 ml) with gentle heating in a 50 ml round bottom flask which was fitted with refluxing condenser and protected with a calcium chloride drying tube. After all sodium has dissolved, ethyl acetamidocyanoacetate-2-¹³C (1.120 g, 0.0066 mole) was added and the mixture was heated to boiling. To this stirred solution, isobutyl bromide (2.408 g, 0.0198 mole) was added and this mixture was refluxed for six hours. After refluxing, the mixture was concentrated in vacuo. The residue was taken up in a mixture of chloroform and hydrochloric acid (0.7N, 2.5 ml each). The aqueous layer was extracted thoroughly with chloroform (5 X 3 ml). The combined chloroform extract was decolorized with Norit and dried (anhydrous sodium sulphate). Removing of the solvent, a brown residue (1.2538 g, 79.23%) was obtained. This was a mixture of ethyl and isopropyl esters, mixture

of ethyl 2-acetamino-2-cyano-4-methyl-pentanoate-2- ^{13}C (LXXVIII) and isopropyl 2-acetamino-2-cyano-4-methyl-pentanoate-2- ^{13}C (LXXIX). This crude mixture was used for hydrolysis without further purification.

(6) Formation of DL-leucine-2- ^{13}C (LV)⁽¹²⁴⁾.

Isopropanyl and ethyl 2-acetamino-2-cyano-4-methyl pentanoate-2- ^{13}C mixture (1.2538 g, 0.0052 mole) were placed in a 10 ml round bottom flask. Hydrobromic acid (46-48%, 5 ml) was added. This solution was refluxed for seven and half hours and then concentrated on vacuo. The residue was neutralized with concentrated ammonia to pH 7. This mixture was chilled in the refrigerator overnight, crude leucine of beige color was formed. After filtration, the mother liquid was concentrated to yield a second crop of crude product (total 443.0 mg, 64.7%). Recrystallization from water afforded DL-leucine-2- ^{13}C as shiny white crystals (374.6 mg, 54.43%), m.p. 281-283° sub. (lit⁽¹²³⁾ 278-283°), mixed m.p. 289° sub., pmr $\delta_{\text{D}_2\text{O}}$ 1.20 (3H, s, -CH₃), 1.33 (3H, s, -CH₃), 1.98 (2H + 1H, >CH-CH₂-C) and 4.01 (1H, t, CH₂-CH-COOH). $^{13}\text{C}_{\text{nmr}}$: $\delta_{\text{D}_2\text{O}}^{\text{MeOH}}$ 22.1 and 22.9 ($\frac{\text{CH}_3}{\text{CH}_3} \text{C}$ -), 25.3 ($\frac{\text{Me}}{\text{Me}} \text{CH}$ -), 39.7 (>CH-CH₂-) and 53.5 (-CH-COOH), only one large peak was found at δ 53.5, the rest peaks were much smaller..

Reaction of vindoline and osmium tetroxide⁽¹²⁹⁾ (Rx. No. 1).

Vindoline (207.5 mg, 0.455 m mole) was dissolved in dry pyridine (0.25 ml) and freshly distilled tetrahydrofuran (THF) (0.43 ml) in a 25 ml 2-neck round bottom flask closed with a serum cap in one neck. Dry nitrogen was passed into the flask via a distilling head and the side arm of which was guarded with a calcium chloride drying tube. The solution was cooled to -70° (dry ice-acetone) under magnetic

stirring. A solution of osmium tetroxide (116.0 mg, 0.457 m mole) in THF (1.75 ml) was added dropwise via the serum cap to the stirred solution over a period of three minutes. Stirring was continued at -70° for a further two hours, by which time, a dark brown mixture had formed. Sodium-dried ether (12.5 ml) was added dropwise over five minutes and the solution allowed to warm up to room temperature. The golden-brown suspension thus formed was rapidly centrifuged down and the supernatant liquid was removed to another flask. The dark precipitate was taken up in methylene chloride-ethanol (1:1, 6 ml) to give an black solution into which hydrogen sulphide was passed until saturation (5 min.). The black precipitate thus formed was removed by centrifuging. The pale yellow solution was removed to a flask and the residue was washed repeatedly with methylene chloride-ethanol (1:1, 3 X 6 ml). The combined supernatant was evaporated under reduced pressure to give a dark brown oil (21.1 mg). Processing of the ether solution in the same way gave recovered vindoline contaminated with reaction products (175.4 mg). TLC was run for both the reaction products and recovered starting material. Three components (R_f : 0.38, 0.23 and 0.11) were shown on tlc for the recovered material. Three major components plus two traces components were found in the reaction product mixture (R_f : 0.57, 0.38, 0.30, 0.23 and 0.11). 90% (estimated from tlc) of unreacted vindoline was recovered.

Reaction of vindoline and osmium tetroxide (Rx. No. 2).

Vindoline (210.0 mg, 0.461 m mole) was dissolved in dry pyridine (0.25 ml) and THF (0.43 ml). Osmium tetroxide (78.5 mg, 0.309 m mole) in THF (1.75 ml) was used and the reaction was performed as in

Reaction No. 1. After stirring at -70° for two hours, a reddish brown solution was formed without precipitate. The reaction mixture was placed in the freezer (-20°) overnight; a precipitate formed. In the hope of increasing the yield of product, the mixture was allowed to stand at -20° for five days. The same work up as in Rx. No. 1 was applied except the time of passing hydrogen sulphide gas was extended to ten minutes. Similar components were found in both the methylene chloride-ethanol solution and ether solution. Total residue (209.9 mg) obtained was found containing four components in addition to the unreacted vindoline (R_f : 0.52, 0.35, 0.26, 0.13 and 0.40, vindoline, 80%).

Reaction of vindoline and osmium tetroxide (Rx. No. 3).

Vindoline (207.7 mg, 0.455 m mole) and osmium tetroxide (100.4 mg, 0.395 m mole) were used in this reaction which was performed as the previous one except the reaction was kept at -70° for 12 hours and then at -20° for nine days and hydrogen sulphide gas was passed in for one hour. The residue obtained (213.4 mg) was found containing mainly unreacted vindoline (90%, R_f 0.40) and small amount of other five components (R_f : 0.49, 0.36, 0.31, 0.25 and 0.12).

Reaction of vindoline and osmium tetroxide (Rx. No. 4).

Vindoline (211.7 mg, 0.464 m mole) and osmium tetroxide (78.0 mg, 0.307 m mole) were used in this reaction. The same procedure was followed as Rx. No. 3 except the reaction was kept at -70° for seven days and at -20° for two days. The residue (220.9 mg) contained mainly unreacted vindoline (90%, R_f 0.43) and four other components (R_f : 0.52, 0.38, 0.30 and 0.13).

Reaction of vindoline and osmium tetroxide (Rx. No. 5).

Vindoline (111.1 mg, 0.244 m mole) was dissolved in THF (1.5 ml) and dry pyridine (1 ml). The flask containing the vindoline solution was wrapped with tin foil and placed in a nitrogen filled glove bag. A solution of osmium tetroxide (65.8 mg, 0.259 m mole) in THF (2 ml) was added dropwise inside the glove bag to the stirred solution at room temperature. This mixture was allowed to stir for two days in the dark. The precipitate formed was filtered and washed with anhydrous ether. This residue was taken up in methylene chloride-ethanol solution and was saturated with hydrogen sulphide gas (30 min.), and then stirred in the dark overnight. The black sulphide salt was filtered off and the filtrate was concentrated under reduced pressure at 40°. A yellow precipitate (16.4 mg, R_f 0.64 m.p. 113-116°) was obtained. The ethereal filtrate was also concentrated under reduced pressure at room temperature afforded a dark brown gum (102.5 mg) which was solidified upon addition of petroleum ether (30/60°). This precipitate contained mainly unreacted vindoline (75%, R_f 0.27) and three other components (R_f : 0.19, 0.15 and 0.07).

Reaction of vindoline and osmium tetroxide (Rx. No. 6).

Vindoline (107.8 mg, 0.236 m mole) and osmium tetroxide (65.7 mg, 0.258 m mole) were used and the procedure in Rx. No. 5 was followed except the reaction time was extended to seven days. The residue (21.2 mg) obtained from the osmate ester-pyridine complex was found to contain six components besides vindoline (R_f : 0.37 vindoline, 0.55, 0.45, 0.31, 0.22, 0.065 and 0.04). The residue (96.8 mg) obtained from the ethereal filtrate contained unreacted vindoline (R_f 0.37, 50%) and other two equal portion components (R_f 0.31 and 0.22).

Reaction of vindoline and osmium tetroxide (Rx. No. 7).

Vindoline (102.8 mg, 0.225 m mole) was dissolved in dry pyridine (1 ml) and osmium tetroxide (63.7 mg, 0.251 m mole) was dissolved in pyridine (1 ml) only. The osmium tetroxide solution was added to the vindoline solution dropwise and the reaction was continued for seven days with stirring in the dark at room temperature. The same procedure as in Rx. No. 6 was followed. A yellow oil (7 mg) was obtained from the methylene chloride-ethanol solution and a brown gum (86.4 mg) was obtained from the ethereal filtrate. Most of the vindoline (> 90%, R_f 0.38) was recovered after the reaction and traces of other products were formed (R_f : 0.54, 0.44, 0.29 and 0.23).

Reaction of vindoline and osmium tetroxide (Rx. No. 8).

Vindoline (95.1 mg, 0.209 m mole) was dissolved in aqueous dioxane (80%, 8 ml) solution. A solution of osmium tetroxide (69.2 mg, 0.272 m mole) in aqueous dioxane (80%, 2 ml) was added to the vindoline solution dropwise with stirring. This mixture was stirred in the dark at room temperature for 16 days. Hydrogen sulphide was passed into this black solution for 20 minutes and the solution was extracted with chloroform (1 X 10 ml + 2 X 5 ml). The chloroform extract was dried (anhydrous sodium sulphate) and concentrated under reduced pressure to give a brown oil (81.6 mg). Besides the unreacted vindoline (60%, R_f 0.40), four compounds were formed (R_f 0.51, 0.34, 0.25 and 0.03).

Reaction of vindoline and osmium tetroxide (Rx. No. 9).

Vindoline (107.3 mg, 0.235 m mole) was dissolved in THF (1 ml) and pyridine (1 ml). A solution of osmium tetroxide (61.8 mg, 0.243 m mole) in THF (a few drops, just to dissolve the compound) was added

to the vindoline solution dropwise. This reaction mixture was stirred in the dark at room temperature for four days in the nitrogen filled glove bag. A solution of sodium hydrogen sulphite (104.7 mg, 1.00 m mole) in water (1.7 ml) and pyridine (1.5 ml) was added to the osmate ester-pyridine complex slowly and stirred overnight. This mixture was extracted with chloroform (1 X 10 ml + 3 X 5 ml). The chloroform extract was dried (anhydrous potassium carbonate) and concentrated under reduced pressure to yield a yellow oil (135.2 mg) which contained unreacted vindoline (70% R_f 0.39) and four other components (R_f : 0.22, 0.18, 0.48 and 0.31).

Reaction of vindoline and osmium tetroxide (Rx. No. 10).

Vindoline (102.7 mg, 0.225 m mole) was dissolved in absolute diethyl ether (2 ml) and dry pyridine (1 ml). A solution of osmium tetroxide (75.6 mg, 0.298 m mole) in absolute diethyl ether (1 ml) was added to the above solution dropwise in a glove bag filled with nitrogen in the dark. This mixture was refluxed gently (32-38°) for seven days. Fresh ether was added when it was required. The osmate ester-pyridine complex was hydrolyzed with sodium hydrogen sulphite solution (106.1 mg in 1.7 ml of water and 1.5 ml of pyridine) overnight. The same procedure as in Rx. No. 9 was followed and a dark oil (100.9 mg) resulted which contained unreacted vindoline (60-70% R_f 0.42) and four other components (R_f : 0.50, 0.32, 0.24 and 0.11).

Reaction of 3 β -hydroxy-androst-5-en-17-one with osmium tetroxide (130).

The literature procedure for this reaction was followed. A brown residue (60.8 mg, from 58.3 mg, 0.1995 m mole of starting material) was obtained at the end of the reaction. This residue

contained three components, the unreacted starting material (R_f 0.64, 15%), main product (75%, R_f 0.46) and trace of others. Purification of this mixture by pre-tlc yield a major component (40.5 mg, 63%), m.p. 236-239° (lit⁽¹³⁰⁾ m.p. 240-243°). The peak at $\delta_{(CD_3)_2CO}$ 5.37 (2H, olefin H) of the starting material had disappeared, and a peak at 3.63 (3H, -OH, disappeared on addition of D_2O) was found in the pmr of the product, androst-3 β ,5 α ,6 α -trihydroxy-17-one.

Reaction of vindoline with potassium permanganate⁽¹³²⁾ (Rx. No.1).

Vindoline (114.0 mg, 0.250 m mole) was dissolved in acetone (0.5 ml) in a 5 ml round bottom flask. The solution was cooled to -15 - -20° (salt-ice bath). A solution of potassium permanganate (32.0 mg, 0.203 m mole) and magnesium sulphate (24.0 mg) in water (0.75 ml) was added dropwise to the stirred vindoline solution, the temperature being maintained at -15 - -20° during the addition. After the addition was complete, the reaction mixture was allowed to warm up to room temperature and was filtered. The filtrate was made basic by adding sodium hydroxide solution (40%, 1 drop) and continuously extracted with ether for four days. The ether layer was then dried (sodium sulphate) and concentrated under reduced pressure, a yellow residue (145.9 mg) was obtained. This residue contained mainly unreacted vindoline (80%, R_f 0.46) and five other components (R_f : 0.63, 0.54, 0.36, 0.29 and 0.19).

Oxidation of cyclohexene with potassium permanganate-crown ether⁽¹³³⁾.

The literature procedure for this reaction was followed. White crystals of adipic acid (58% yield) were obtained, m.p. 148-149° (lit⁽⁹¹⁾ 153°).

Oxidation of vindoline with potassium permanganate-crown ether⁽¹³³⁾ (Rx. No. 2).

Finely ground potassium permanganate powder (140.7 mg, 0.891 m mole) was placed in a 10 ml round bottom flask. To it, dicyclohexyl-18-crown-6 (5 drops) and benzene (2 ml) were added and the mixture was stirred vigorously. Vindoline (116.7 mg, 0.256 m mole) was dissolved in benzene (1 ml) and this solution was added dropwise to the permanganate solution. This mixture was allowed to stir overnight at room temperature. Manganese dioxide was filtered off, the solution was concentrated to give a thick residue. This dark brown residue was dissolved in sodium hydroxide solution (5%, 10 ml) and acidified after filtering off the residue of manganese dioxide. This aqueous solution was extracted with ether. A yellow oil (12.3 mg) was obtained after the ethereal solution was dried and concentrated, $\lambda_{\text{max}}^{\text{EtOH}}$ 212.9, 256.9 and 305.9 nm, R_f : 0.25, 0.28 and 0.13.

Reaction of vindoline with Lemieux-von Rudloff reagent⁽¹³⁵⁾ (Rx. No. 3).

A stock solution of sodium periodate-potassium permanganate was prepared by dissolving sodium periodate (2.0839 g, 9.77 m mole) and potassium permanganate (41.0 mg, 0.26 m mole) in distilled water (500 ml) with slight warming. Vindoline (6.13 mg, 0.0134 m mole) was dissolved in t-butyl alcohol (spectral grade, 1.8 ml) with stirring. The stock solution (3 ml) was added to the stirred solution, and the purple color of the stock solution disappeared. Water (1.5 ml) was then added followed by powdered potassium carbonate to make the solution of pH 8-9. The solution was stirred overnight. The mixture was acidified with sulphuric acid (10%) and excess oxidizing

agent was converted to iodide by adding powdered sodium bisulphite. The resultant solution was basified with sodium hydroxide solution (10%) and t-butyl alcohol was removed under reduced pressure. After the solvent was removed, the resultant yellow solution was acidified with sulphuric acid (10%) and continuously extracted with ether for 72 hours. Evaporation of the dried (sodium sulphate) ethereal solution gave a yellow residue (0.7 mg).

Reaction of vindoline and Lemieux-von Rudloff reagent (Rx. No.4).

The previous experiment was repeated by using double amount of starting material. Vindoline (13.6 mg, 0.0298 m mole) was dissolved in t-butyl alcohol (2 ml). Excess sodium periodate-potassium permanganate stock solution (40 ml) was added. The same work up yielded a brown oil, 2.3 mg, $\lambda_{95\% \text{ EtOH}}$ 202.6 nm, indicating destruction of the indole ring.

Reaction of cyclohexene and ozone⁽¹³⁶⁾.

The literature procedure for this reaction was followed. A white precipitate of adipic acid was formed (0.6957g, 91.7%, m.p. 148-149°, lit⁽⁹¹⁾ 153°).

Reaction of 4-cholesten-3-one with ozone⁽¹³⁷⁾.

The literature procedure for this reaction was followed. A white precipitate (88.0 mg, 79.2%, $\lambda_{\text{max}}^{\text{EtOH}}$ 206.4 nm; $\nu_{\text{max}}^{\text{mull}}$ 3350 (broad -OH), 1703 cm^{-1} ; R_f 0.50) of the dicarboxylic acid was obtained. The unreacted cholestenone was recovered from the ethereal layer after sodium hydroxide extraction (4.3 mg, $\lambda_{\text{max}}^{\text{EtOH}}$ 241.7 nm; $\nu_{\text{max}}^{\text{mull}}$ 1676, 1614 cm^{-1} ; R_f 0.68).

Reaction of progest-4-ene-3,11,20-trione with ozone⁽¹³⁷⁾.

The procedure of the reaction of 4-cholesten-3-one with ozone

was followed in this reaction. A yellow oily residue (36.6 mg, R_f of this residue mixture: 0.67, 0.53, 0.44 and traces near the baseline) instead of solid precipitate was obtained⁽¹³⁷⁾.

Reaction of vindoline with ozone⁽¹³⁶⁾ (Trial No. 1)

Vindoline (119.5 mg, 0.262 m mole) was dissolved in ethyl acetate (5 ml) and the solution was cooled to -70° (dry ice-acetone bath). Ozonized oxygen was passed in for 5 minutes. The reaction mixture was allowed to warm up to room temperature and solvent was removed with rotary evaporator, a brown residue resulted. Hydrogen peroxide (30%, 1.5 ml) was added and the mixture was warmed almost to boiling for one hour. This aqueous solution was extracted with methylene chloride which was dried (anhydrous sodium sulphate) and concentrated to afford a product (24 mg, R_f 0.53 and 0.33). The aqueous solution after methylene chloride extract was also concentrated and afforded a yellow residue (97.9 mg, R_f : 0.50, 0.42, 0.35, 0.31, 0.27, 0.22 and 0.17).

This mixture was subjected to column chromatography separation (Alumina, 8 g). One pure component (6.2 mg) was obtained from pure ethyl acetate elution, $\lambda_{\max}^{\text{EtOH}}$ 212.3 nm; $\nu_{\max}^{\text{CH}_2\text{Cl}_2}$ 1737.2, 1692.7 cm^{-1} . The polarity of the elution solvent was increased by adding methanol, mixtures were obtained in the following fractions.

Reaction of vindoline with ozone, trial Nos. 2, 3 and 4.

The reaction of vindoline with ozone in these reactions were conducted in a manner similar to trial No. 1. The data for these reactions are summarized in Table XII. The combined mixture (385.2 mg) from these experiments and the previous one was separated using column chromatography (alumina, 30 g). Pure ethyl acetate was used as the eluting solvent, small quantities (less than 1 mg) were

found in each fraction (130 ml). The solvent was changed to ethyl acetate/methanol (19/1) after pure ethyl acetate elution (1410 ml). A mixture was found in the ethyl acetate/methanol elution. This combined mixture (93.5 mg) was re-separated using another column (alumina, 15 g) with pure ethyl acetate only. The components of the mixture could not be separated in this manner.

Reaction of vindoline and ozone (Trial No. 5).

Vindoline (115.7 mg, 0.254 mmole) was dissolved in ethyl acetate (5 ml) and ozonized oxygen was passed in at -70° for 15 minutes. The ozonide (152.6 mg) was obtained after the solvent was removed. This residue was dissolved in glacial acetic acid (0.3 ml) and water (1.25 ml). Zinc dust (0.075 mg) was then added with stirring and stirred for additional one hour. The mixture was filtered and the filtrate was concentrated to a brown residue. This residue was dissolved in a minimum of water (a few drops) and extracted with ether (5 X 10 ml). The ethereal solution was washed with sodium carbonate solution (10%) and then dried (anhydrous sodium carbonate). A brown precipitate (8.7 mg) was obtained after ether was removed. Another portion of solid residue (4.2 mg) was obtained from the aqueous solution.

Reaction of vindoline and ozone (Trial No. 6).

This reaction was conducted in a similar manner as in trial No. 5. The quantities used for this reaction are shown in Table XII. The only exception in this reaction was that, the reaction mixture after adding zinc dust was stirred vigorously overnight. A dark brown residue (98.7 mg) was obtained after removing methylene chloride solution, $\lambda_{\text{max}}^{\text{EtOH}}$ 213.7, 304.7 nm; $\nu_{\text{max}}^{\text{CH}_2\text{Cl}_2}$ 3601, 3462, 1740 and

1615 cm^{-1} .

Reaction of the above residue with sodium bisulphite⁽¹⁴³⁾.

The above brown residue (98.7 mg) was dissolved in chloroform (2 ml) and sodium bisulphite solution (3 ml; 1.8 g of sodium bisulphite in 7.2 ml of water, filtered) was added with vigorous stirring for one hour. The aqueous solution was separated and washed with chloroform (2 X 1 ml). The combined chloroform solution was dried (anhydrous sodium sulphate) and concentrated to give a brown residue (54.2 mg). The sodium bisulphite solution containing the aldehyde was acidified with concentrated sulphuric acid/water (1/1) and sulphur dioxide evolved. Nitrogen gas was passed into the solution to expel the remaining sulphur dioxide. The solution was cooled and extracted with chloroform which was dried (sodium sulphate) and concentrated, a residue (3.5 mg) was formed. Similar components were found in both residues as in the starting mixture.

Reaction of vindoline and ozone (Trial No. 7).

Vindoline (111.7 mg, 0.245 m mole) was dissolved in methanol (2 ml) and cooled to -60° . Ozonized oxygen was passed in for seven minutes. This mixture was then flushed with nitrogen gas, and dimethyl sulphide (1 ml, 13.6 m mole) was added at -60° . This solution was then warmed to -10° and stirred for one hour, and subsequently at 0° and at room temperature for one hour each. Methanol was removed and the residue was dissolved in methylene chloride. The methylene chloride solution was washed with water (8 X 5 ml) to remove dimethyl sulphoxide. The organic solution was dried (sodium sulphate) and concentrated to give a brown residue (28.1 mg). A brown residue (99.6 mg) was obtained when the aqueous layer was

concentrated. Both of these two residue contained similar components (R_f : 0.49, 0.41, 0.34 plus other trace components).

An attempt was made to separate the mixture using column chromatography packed with silica gel-60 (21 g) eluted with various proportions of benzene and chloroform, 30 ml being collected in each fraction. Small amounts (less than 1 mg) were recovered in each fraction. The polarity of the eluting solvent was increased by adding methanol and a small amount (1.9 mg, R_f 0.70, R_f of vindoline: 0.62) of one pure component was obtained. Mixtures were found on the rest of the fractions. The fractions (68-86, 25.6 mg) obtained from chloroform/methanol (19/1) were subjected to methylation.

Methylation of fractions 68-86.

The mixture from fractions 68-86 (5 mg) was dissolved in methanol (a few drops). Excess diazomethane (prepared from diazald, 1.5 ml) was added and the reaction mixture was left overnight to let the excess diazomethane evaporate. The spectral data of the product were: $\nu_{\text{max}}^{\text{CH}_2\text{Cl}_2}$ 2500-3500 cm^{-1} , broad; 1737, 1650, 1586, 1431 cm^{-1} . These were similar to the ir of the starting compound.

Reaction of vindoline and ozone (Trial No.8) (139).

Vindoline (113.0 mg, 0.248 m mole) was dissolved in methanol (2 ml) and the solution was cooled to -60° (dry ice-acetone bath). Ozonized oxygen was passed in for six minutes. Nitrogen gas was then bubbled in to remove the excess ozone. To this cold solution, dimethyl sulphide (0.03 ml, 0.4 m mole), triethyl orthoformate (0.42 ml) and acidic methanol (0.035 ml containing 0.012 ml of concentrated hydrochloric acid) were added. The solution was stirred at room temperature overnight. Solvent was removed using rotary evaporator.

A brown residue (153.3 mg) was obtained after the dimethyl sulfoxide was removed under vacuum. This mixture gave the following spectral data: $\lambda_{\text{max}}^{\text{EtOH}}$: 216, 363 nm; $\nu_{\text{max}}^{\text{CH}_2\text{Cl}_2}$: 2500-3500, broad, 1748, 1643, 1537, 1229, 1069 cm^{-1} ; R_f values were listed in Table XII. An attempt was made to separate this mixture by pre-tlc; a small amount (16.9 mg) of one component was isolated, m.p. 143-148 $^{\circ}$, $\nu_{\text{max}}^{\text{CH}_2\text{Cl}_2}$: 2500-3500 broad, 1647, 1571 cm^{-1} ; $\lambda_{\text{max}}^{\text{EtOH}}$: 216 and 363 nm.

Preparation of Ruthenium tetroxide from ruthenium dioxide⁽¹⁴⁴⁾

The literature procedure was followed for preparing ruthenium tetroxide. A solution of ruthenium tetroxide in carbon tetrachloride (25 ml) was prepared. The concentration of ruthenium tetroxide solution was found to be 0.59 mg per ml.

Oxidation of vindoline with ruthenium tetroxide⁽¹⁴⁰⁾

Vindoline (50.0 mg, 0.1096 mmole) was dissolved in carbon tetrachloride (5 ml). The carbon tetrachloride solution of ruthenium tetroxide was added with stirring and stirred overnight at room temperature. A few drops of methanol was added to destroy excess ruthenium tetroxide. The black precipitate of ruthenium dioxide was centrifuged and was washed with carbon tetrachloride (2 X 5 ml). The combined carbon tetrachloride solution was concentrated to give a grey residue (55.1 mg) which contained 90% unreacted vindoline.

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Gain: 2.56
AOC: 0.01

Approximate calibration for conversion of carbon-14 & tritium channels

APPENDIX II. BRITUM BRITUM BRITUM CURVE.

Gain: 2.56
Dial: 0.736
AQC: 6.29

carbon-14 efficiency

carbon-14 efficiency

carbon-14 efficiency

Ext. Std. Ratio

END

FIN