STUDIES IN ALKALOID BIOSYNTHESIS

a thesis submitted by

EDWIN JAMES HERBERT

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Chemistry Department,
Imperial College,
London, S.W.7.

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ABSTRACTS

In part I the biosynthesis of diterpenoids is discussed in terms of the acetate-mevalonate pathway. Emphasis is placed on tracer studies with diterpenoid mould metabolites. The work leading to the elucidation of the structure of diterpenoid alkaloid delpheline is reviewed. Little or no incorporation into delpheline produced by Delphinium elatum L. occurred with the following [^{14}C] labelled precursors: mevalonic acid, sodium acetate, sodium formate, and serine. In contrast, efficient incorporation into delphinium phytosterols was observed; the reasons for this difference are discussed. The degradation of delpheline derived from [methyl-C^{14}]methionine is described.

In part II the biosynthesis of indole alkaloids is reviewed. A short account of the elucidation of the structures of the Calycanthaceous alkaloids is given. Tryptophan was incorporated into chimonanthine elaborated by Chimonanthus fragrans Lindle; N-methyltryptamine was found to be a less efficient precursor. The preparation of [2-^{3}H]indole is described.

ACKNOWLEDGEMENTS

The author wishes to express his very sincere gratitude to Dr. G. W. Kirby for his encouragement and valued supervision during the course of this work. He would also like to thank Professor Barton, F.R.S., for the privilege of working in his laboratories. Grateful acknowledgement is made of financial support from the Department of Scientific and Industrial Research.

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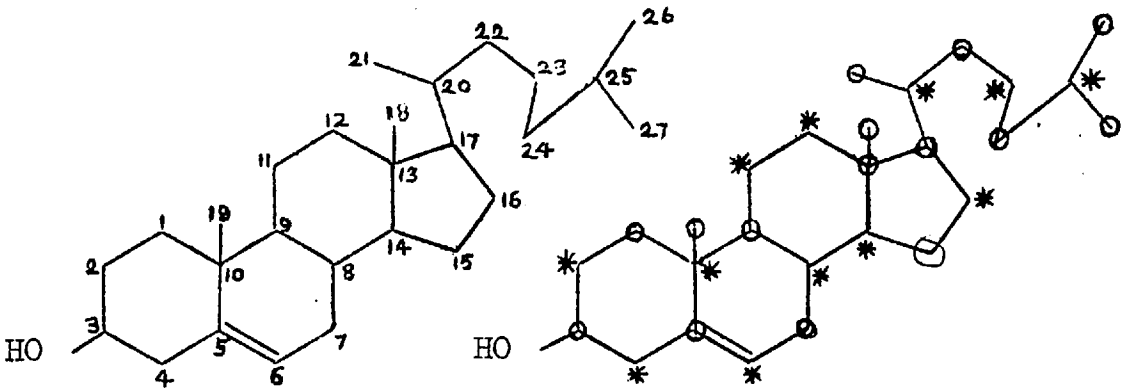
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CHAPTER ONE: BIOSYNTHESIS OF DITERPENOIDS

The isoprene rule, which was based on the observation that the carbon skeletons of many natural products consist of two or more isoprene units linked together, implied that there was an underlying unity in the biochemical mechanisms that led to the synthesis of these compounds. The biogenetic isoprene rule of Ruzicka[1,2] extended this idea and suggested that the terpenoids were formed from a few acyclic precursors such as geraniol, farnesol, and geranylgeraniol. In order to discuss the biosynthesis of the diterpenes it is necessary first to describe briefly studies on cholesterol (I), since much of our present knowledge of terpene biogenesis has come from work on this degraded triterpenoid. Current knowledge on the biosynthesis of terpenes and sterols is summarised in the publications of two symposia.[3,4]

The three main problems that have to be considered are: (a) the origin and nature of the fundamental isopentane unit, (b) the reactions by which the building units are combined, and (c) the secondary processes whereby the initially formed polyisopentane units are converted to the final terpenoid.

Cholesterol.



(I) (II)

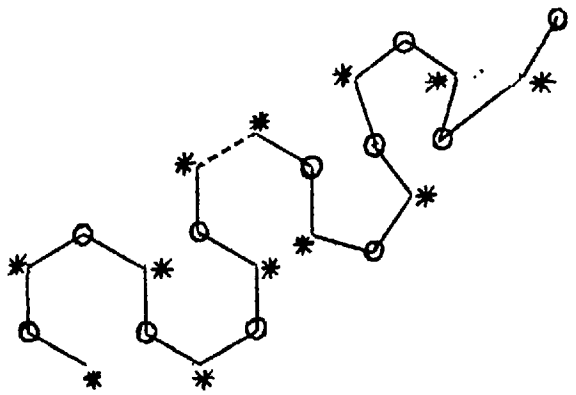
In 1937 Rittenberg and Schoenheimer[5] showed that mice fed with deuterated water produced cholesterol containing a considerable amount of the isotope. At about the same time, Sonderhoff and Thomas[6] found that deuterated sodium acetate was converted by yeast into ergosterol. Later, Bloch and Rittenberg[7] obtained a similar result for cholesterol synthesis in rats and mice. Further proof that acetate was a major carbon source for cholesterol came with the introduction of the radio-isotope ^{14}C in experimental quantities. Both methyl- and carboxyl-labelled acetate were found to give rise to radioactive cholesterol.[8] The task of degrading the cholesterol molecule to ascertain the labelling pattern was undertaking by two groups - Bloch and his

co-workers at Harvard and Chicago and Cornforth, Popják, and others in London. Leading references to the chemical degradative work are given by Fieser and Fieser[9]; it will suffice here merely to show the final result (II), where (*) represents a carboxyl-derived carbon atom and (°) a methyl-derived one.

It was noticed during these systematic degradations that, although the carboxyl group of acetate was incorporated exclusively into the atoms marked (*) in the cholesterol molecule, about 20% of "scrambling" occurred with methyl-labelled acetate. This was found to be a general phenomenon and is due to the passage of acetate through the tricarboxylic acid cycle.

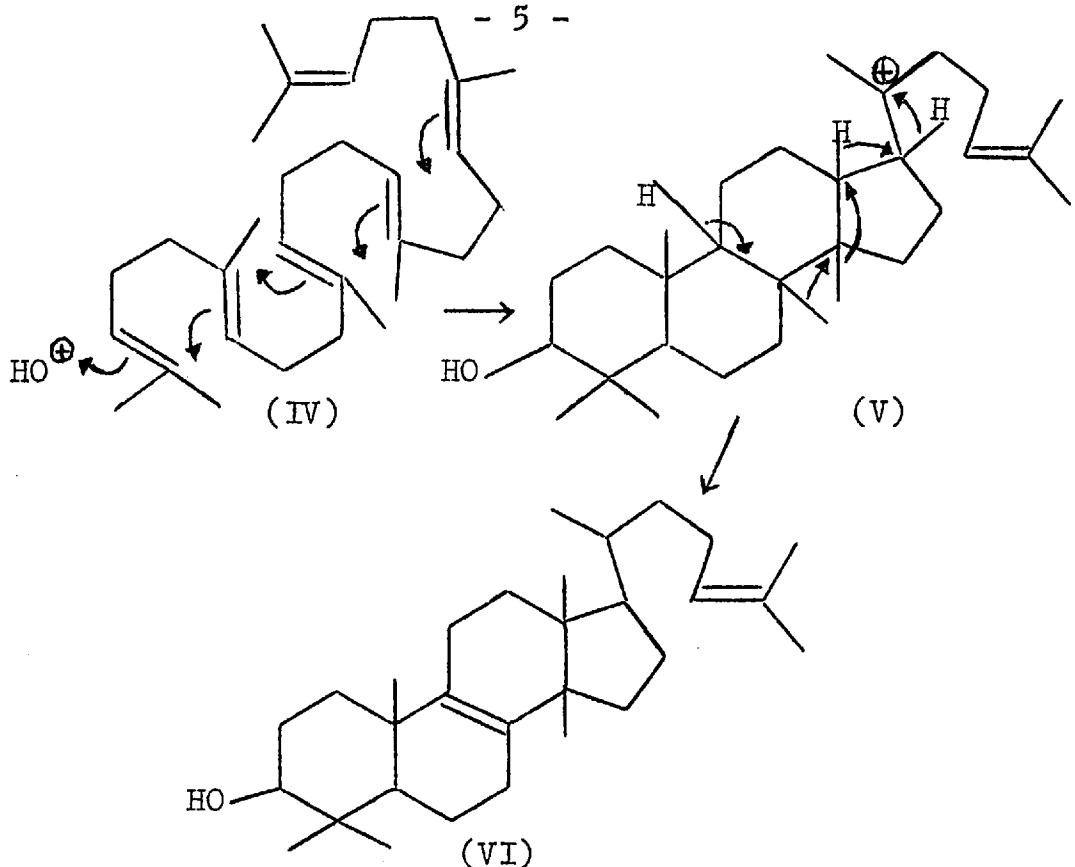
Squalene and lanosterol.

If angular methyl groups are ignored, two alternating methyl-carboxyl sequences can be traced in the labelled molecule (III)



(III)

This symmetry suggested at once that the molecule might be formed by tail-to-tail condensation of two acyclic precursors. As early as 1926 Heilbron, Kamm, and Owens[10] had suggested that the shark liver triterpenoid, squalene (IV), might be a precursor of cholesterol and balance studies had supported this suggestion.[11] Now Langdon and Bloch[12] provided definite experimental proof for the conversion by feeding labelled squalene to rats and obtaining radioactive cholesterol from the livers and intestinal tracts. The incorporation was 10-20 times greater than with acetate. The key to the squalene-cholesterol conversion was provided by Ruzicka and his co-workers[13] who established the structure of the wool fat triterpenoid, lanosterol (VI). This compound clearly has features common to both squalene and cholesterol and Woodward and Bloch[14] proposed the folding of squalene (IV) which, after cyclisation and two methyl migrations, should give lanosterol (VI).



The sequence - acetate ⟶ squalene ⟶ lanosterol ⟶ cholesterol - has now been rigorously established by the following experiments. Squalene, biosynthesised from $^{14}CH_3.CO_2H$ by liver mince, was degraded by ozonolysis to acetone, succinic acid, and laevulinic acid. The labelling pattern observed in the three fragments[15] was consistent with that found for cholesterol. The isolation of active lanosterol from rat liver homogenates incubated with $CH_3.^{14}CO_2H$, and the conversion of biosynthesised lanosterol to cholesterol in the same system, completed the proof.[16]

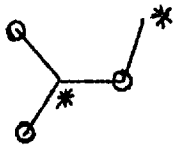
Attack by an oxidising species causes concerted cyclisation of squalene to the ion (V) and simultaneous methyl and hydrogen shifts give lanosterol. The migration of the methyl groups was found to occur by two 1,2-shifts rather than a single 1,3-shift by the work of Maudgal, Tchen and Bloch.[17] The biogenetically necessary oxygen for the cyclisation step was found[18] to come not from water but frommolecular oxygen, by use of the radioisotope ^{18}O. The stereochemistry of the product depends on the original folding of the squalene chain, presumably on an enzyme surface. Natural squalene is the all-trans isomer.

During the transformation of lanosterol to cholesterol, three methyl groups are lost, the 8,9-double bond migrates to the 5,6-position, and the 24,25-double bond becomes saturated. The demethylation of lanosterol was found to require molecular oxygen, the methyl carbon appearing as carbon dioxide.[19] The details of the conversion are still not completely understood and will not be discussed further here.

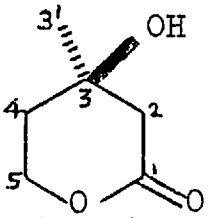
Mevalonic acid.

As the acetate labelling pattern of cholesterol began to emerge, a variety of branched chain acids

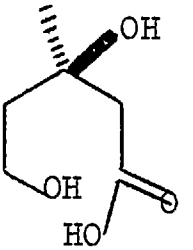
containing the structural unit (VII) were tested as possible precursors of cholesterol - without success. The nature of the isopentane unit and its mode of synthesis from acetate remained obscure until a biologically active, branched chain compound was isolated from distillers' solubles by Folkers and his colleagues.[20] This was mevalonic lactone (VIII), the cyclic form of 3,5-dihydroxy-3-methylvaleric acid; the functional groups of the acid are strategically placed for its conversion into an "isoprene" unit. Its structure was confirmed[21] by synthesis of the DL-acid.



(VII)

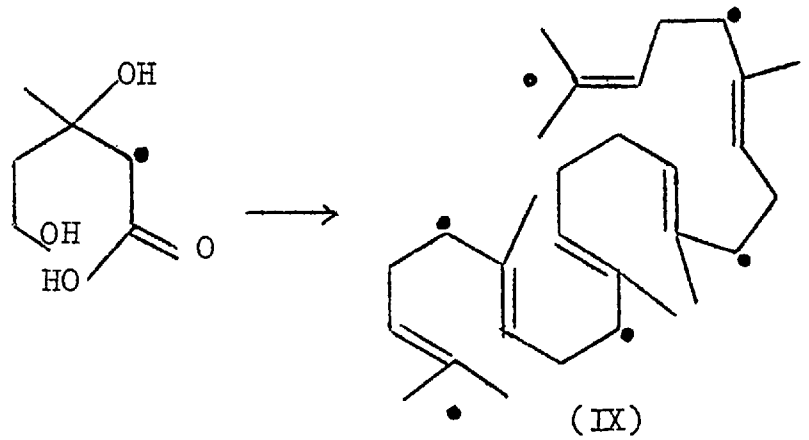


(VIII)



The critical feeding experiment was soon performed.[22] (±)-[2-^{14}C] Mevalonic acid gave 43% incorporation into cholesterol in a rat liver homogenate. Since only the (+)-isomer can be used biologically and one sixth of the activity (in a methyl group) is lost during conversion into cholesterol, the observed incorporation

was essentially quantitative. The carbon atom lost during transformation of mevalonic acid into the isopentane unit was shown to be that of the carboxyl group by incubating [1-^{14}C] mevalonate with cell-free homogenates of rat liver. The carbon dioxide evolved during the experiment was collected as barium carbonate and found to be radioactive; inactive cholesterol was obtained from the homogenates.[23] Further confirmation for the incorporation of mevalonate without prior breakdown to acetate was obtained by degradation of labelled squalene (IX) produced under anaerobic conditions when cyclisation of squalene to lanosterol does not occur.[24]



(IX)

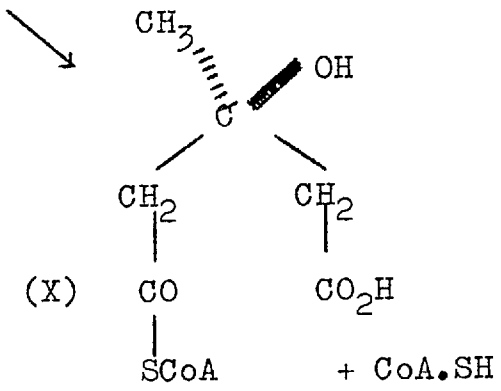
Acetate to mevalonic acid.

The biosynthesis of mevalonic acid involves reactions which have close chemical analogies. Acetate is

employed in the form of the reactive thiol ester, acetyl-coenzyme A, which condenses to give acetoacetyl-coenzyme A. This step is reversible and accounts for the failure of Blecher and Gurin to observe incorporation of intact acetoacetate into cholesterol in surviving rat liver slices.[25] The remaining stages have been studied mainly by Rudney[26] and Lynen[27] using yeast enzyme systems. Acetoacetyl-coenzyme A condenses with another mole of acetyl-coenzyme A to yield the immediate precursor of mevalonic acid, 3-hydroxy-3-methylglutaryl-coenzyme A (X). The final reductive step requires two moles of reduced triphosphopyridine nucleotide and is apparently catalysed by a single enzyme.[28] The reduction is irreversible in the systems so far studied and, once formed, mevalonic acid is committed to polyisoprenoid formation.

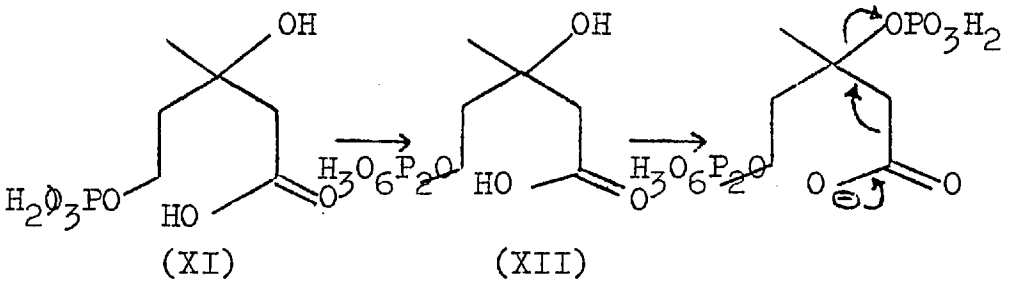
$$CH_3CO.SCoA + CH_3CO.SCoA \longrightarrow CH_3COCH_2CO.SCoA + CoA.SH$$

$$CH_3COCH_2CO.SCoA + CH_3CO.SCoA$$

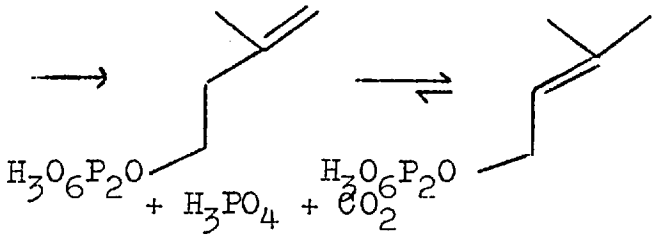


Mevalonic acid to squalene.

Studies with yeast extracts by Bloch and his co-workers[29] and with liver enzyme preparations by Popják and his colleagues[30] showed that, for the conversion of mevalonate into squalene, both systems required TPNH, Mg^{2+} or Mn^{2+} ions, and adenosine-5'-triphosphate (ATP) as coenzymes. The requirement for ATP, the familiar biological phosphorylating agent, suggested that phosphorylated intermediates might be involved in the condensation of mevalonate units. The first phosphate ester to be identified[31] was mevalonic acid-5-phosphate (XI), which was found[32] to be phosphorylated enzymatically to give the corresponding pyrophosphate (XII). A concerted decarboxylation and dehydration now takes place. This step also requires ATP and may involve preliminary phosphorylation of the tertiary hydroxyl group to provide a better leaving group. When ATP, labelled with ^{32}P in its terminal phosphate group, is employed, radioactive orthophosphate is liberated.[33] The product of this elimination is isopentenyl pyrophosphate (XIII), a masked isoprene which illustrates how closely the implications of the isoprene rule have been fulfilled.



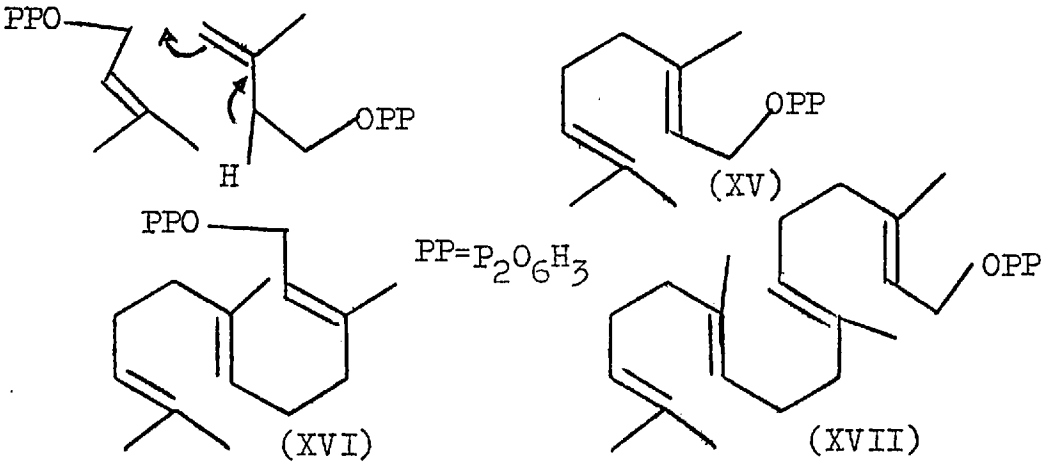
(XI) (XII)



(XIII) (XIV)

Lynen's group was able to show[34] that isopentenyl pyrophosphate was isomerised by yeast extracts to give 3,3-dimethylallyl pyrophosphate (XIV). They observed that isopentenyl pyrophosphate accumulated when squalene synthesis was inhibited by iodoacetamide. This suggests that the isomerisation step involves an enzyme containing a thiol group. They also found that iodoacetamide inhibited squalene formation from synthetic [1-^{14}C] isopentenyl pyrophosphate but that radioactive squalene was again produced when dimethylallyl pyrophosphate was added to the yeast extract. This clearly established that the isomerisation was necessary for the condensation of C_5 units. Dimethylallyl

pyrophosphate loses pyrophosphate by attack of isopentenyl pyrophosphate at the reactive allylic centre. The elimination of a proton leads to the homologous allyl pyrophosphate (XV) which can then enter in the next alkylation step to give a C_{15} compound (XVI).



Farnesyl pyrophosphate (XVI) was in fact isolated[35] from incubation mixtures under conditions inhibiting squalene formation. The biosynthesis of farnesyl pyrophosphate was shown to proceed, in experiments using purified enzymes, by the coupling of one mole of dimethylallyl pyrophosphate with two moles of isopentenyl pyrophosphate, geranyl pyrophosphate (XV) being an intermediate.[34] Synthesis of geranyl and farnesyl pyrophosphates from natural geraniol, which is known[36] to be the trans-isomer, showed that they had the

trans-double bonds expected of squalene precursors. Lynen and Kirschner[37] isolated the next homologue, geranylgeranyl pyrophosphate (XVII), from incubation of yeast extracts with [1-^{14}C]isopentenyl pyrophosphate, farnesyl pyrophosphate, and a polymerising enzyme. No additional source of energy is required for the synthesis of the polyisoprenoid carbon chain, sufficient being made available by the expulsion of pyrophosphate from allyl derivatives.

Only one more step remains to be discussed, namely the joining of two molecules of farnesyl pyrophosphate (XVI) to give squalene (IV). For this condensation, one mole of TPNH, Mg^{2+} ions, and a specific enzyme, located in the small particulate fractions of yeast or liver cells, are required. Popják et al.[38] showed that the process is asymmetric in the sense that one of the farnesyl pyrophosphate molecules condensing is subject to reactions different from those of the other. They found that the role of TPNH cannot be the reduction of a double bond in the dihydrotriterpene as in that case not only the hydride ion from the TPNH but also a proton from the water should have entered the squalene molecule.

Recent investigations by the Popják-Cornforth group have been concerned with the detailed mechanisms of the reactions leading from mevalonic acid to squalene. Ingenious use has been made of intermediates containing asymmetrically deuterated (- CHD -) methylene groups. However, a proper account of this work is beyond the scope of this short survey.

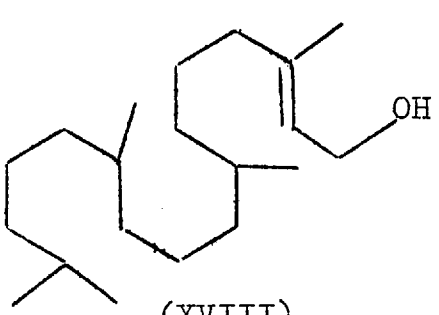
Polyisoprenoids.

Following the synthesis of open chain terpenyl derivatives, secondary transformations such as cyclisation, rearrangement, and oxidation might lead to the cyclic, oxygenated terpenoids. Although the conversion - mevalonate ⟶ geranyl pyrophosphate ⟶ farnesyl pyrophosphate ⟶ squalene ⟶ cyclic triterpenes and sterols - has been proved, other schemes are hypothetical. Geranyl pyrophosphate might give the various monoterpenes, farnesyl pyrophosphate the sesquiterpenes, and geranylgeranyl pyrophosphate might be a precursor of phytol, the diterpenes, and the carotenoids. Feeding experiments with pyrophosphates would probably not be successful with intact plants or even crude homogenates. Phosphorylated compounds are usually

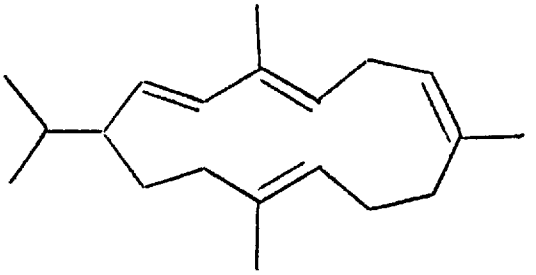
unable to pass through cell walls and the enzyme phosphotase present in crude homogenates would cause hydrolysis.

Diterpenoid biosynthesis.

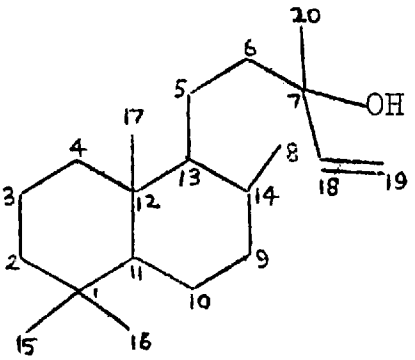
The diterpenoids themselves can now be considered. Many diterpenoids, particularly the resin acids, have been known for over a century but only fairly recently have the structures of the major types been established. Since reviews by de Mayo,[39] Barltrop and Rogers,[40] and Nicholas[41] summarise present knowledge on the biosynthesis of the higher terpenoids only a few aspects will be discussed here and emphasis will be placed on experimental results in the diterpenoid field. It was pointed out by Ruzicka[1] that, with the exception of the abietane type, all of the higher plant diterpenes could be constructed from a phytol skeleton (XVIII) built up of four isoprene units linked in head-to-tail fashion. The majority of the diterpenes possess a bicyclic structure (e.g. XX, sclareol) but tricyclic (XXI, abietic acid), tetracyclic (XXII, phyllocladene), and a few monocyclic (XIX, cembrene) and pentacyclic (XXIII, trachylobanic acid) forms are known.



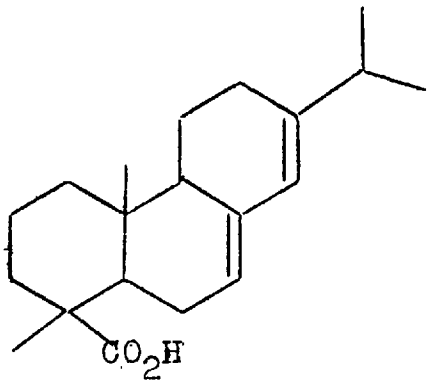
(XVIII)



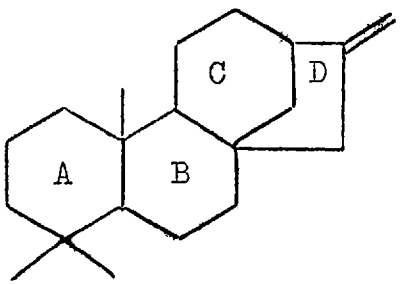
(XIX)



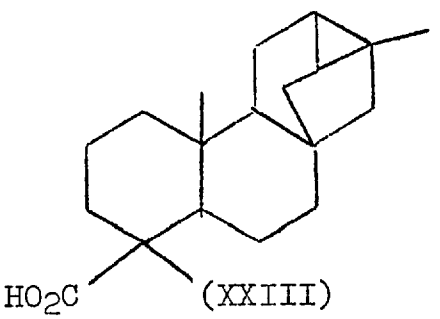
(XX)



(XXI)



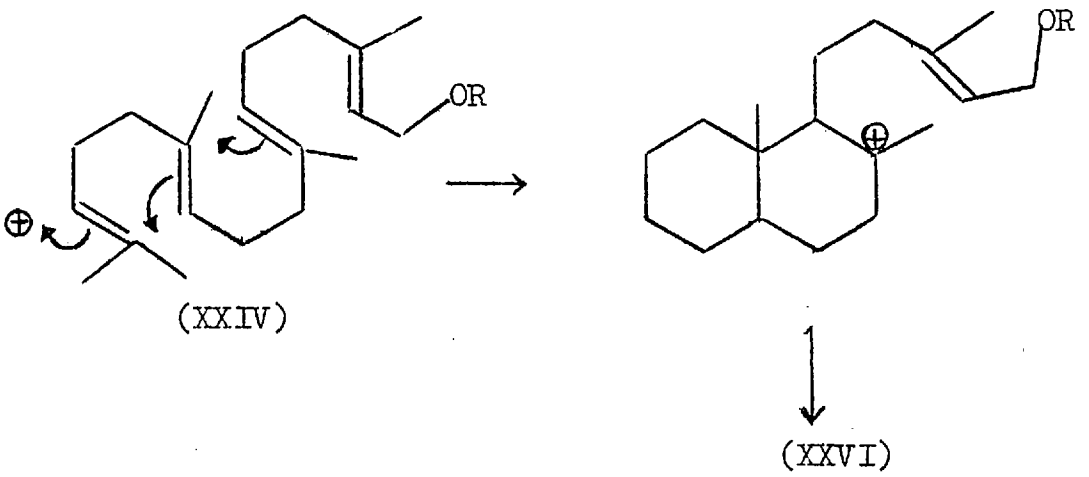
(XXII)



(XXIII)

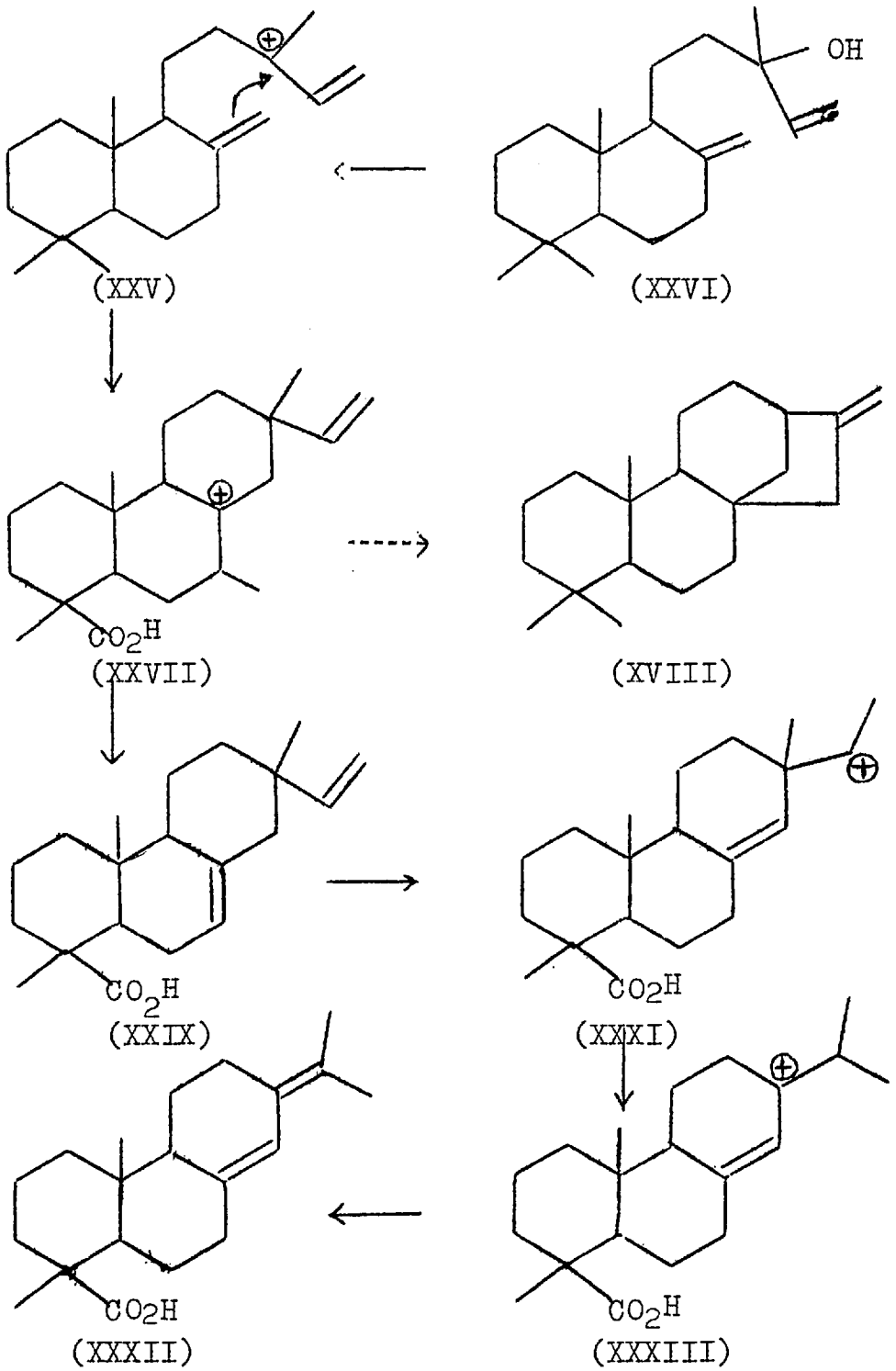
Ruzicka[1] postulated an acyclic precursor such as geranylgeraniol (XXIV; R = OH), geranylinalool, or geranylmyrcene. The recent work outlined above has

shown that geranylgeranyl phrophosphate (XXIV; R = $OP_2O_6H_3$) is a more plausible precursor. Ruzicka's scheme is shown below; for simplicity the stereochemistry of the various products has been omitted. Cyclisation, initiated by a cationoid entity, usually a proton, occurs at one end of the chain (XXIV), accompanied by an allylic rearrangement at the other end, to give the sclareol and manool (XXVI) types. Cyclisation via the ions (XXV) and (XXVII) gives pimaric acid (XXIX). A Wagner-Meerwein rearrangement then leads to neoabietic acid (XXXII), which readily yields the related diterpene abietic acid. Appropriate rearrangements and cyclisations give other forms e.g. phyllocladene (XXVIII).

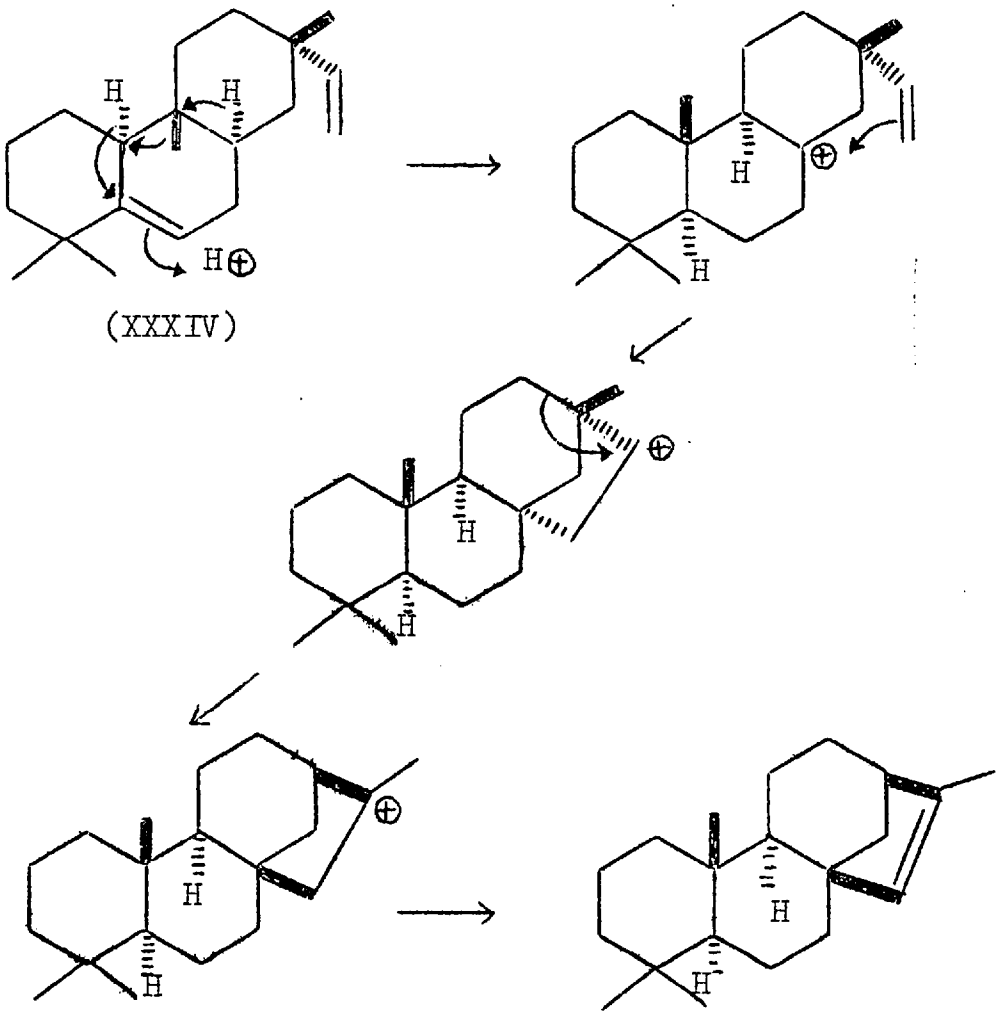


(XXIV)

(XXVI)


OH
(XXV)
(XXVI)
CO_2H
(XXVII)
(XVIII)
CO_2H
(XXIX)
CO_2H
(XXXI)
CO_2H
(XXXII)
CO_2H
(XXXIII)

The interesting rearrangement (XXVII) to XXVIII) was reported to occur during conversion of rimuene[42,43] (XXXIV) to isophyllocladene (XXXV) on vigorous treatment with formic acid.[44] However, this transformation could not be repeated, even in small amount, by Overton and his co-workers.[43]



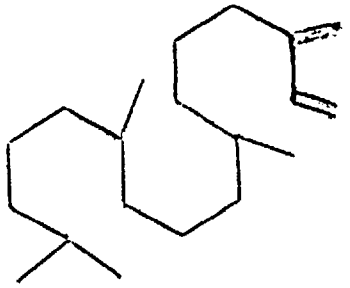
(XXXIV)

(XXXV)

The success of the biogenetic isoprene rule in interpreting the stereochemical transformations involved in steroid and triterpenoid biosynthesis was not at first repeated in the diterpene family. In particular, there appeared several apparent exceptions to the trans-anti-trans geometry of rings A, B, and C commonly found in the triterpenoids. However, the recent work of Scott and his colleagues[45] on diterpenoid stereochemistry has shown that almost all the known diterpenes have in fact the trans-anti-trans configuration or can be derived from a precursor with such an arrangement. The constant revision, during the last four years, of structure and especially stereochemistry in the diterpenoid field has rendered several ingenious attempts to predict configurations on biosynthetic grounds invalid (see for example refs.46 and 47). Fortunately, the derivation of structural types by cationic rearrangements of the Wagner-Meerwein type is unaffected and still makes good chemical sense.

The little that is known experimentally about the biosynthesis of diterpenoids in the higher plants can be briefly summarised. The acyclic phytol (XVIII) occurs throughout the higher plants as a side-chain of

chlorophyll and the vitamins K. The hydrocarbon neophytadiene (XXXVI) has been found in nature[48]; it may arise from dehydration of phytol or its allylic isomer. In etiolated maize and oats leaves, Goodwin observed high incorporation of $^{14}CO_2$ into phytol (and β-carotene) but very low incorporation of mevalonate, indicating that the chloroplastidic membrane may be impermeable to the latter precursor.[49] According to Goodwin,[50] the key "backbone" substrate of the chloroplast is geranylgeranyl pyrophosphate, which can undergo two characteristic reactions. The first of these involves tail-to-tail condensation of two molecules to form phytoene, the C_{40} precursor of the carotenoids. In the second, geranylgeranyl pyrophosphate is incorporated into the side-chains of the chlorophylls, tocopherols, and vitamins K, via an "active" phytol intermediate. The lack of mevalonate incorporation found by Goodwin into the chloroplast terpenoids and by Nicholas[51] into sclareol and marrubiin will be discussed later.



(XXXVI)

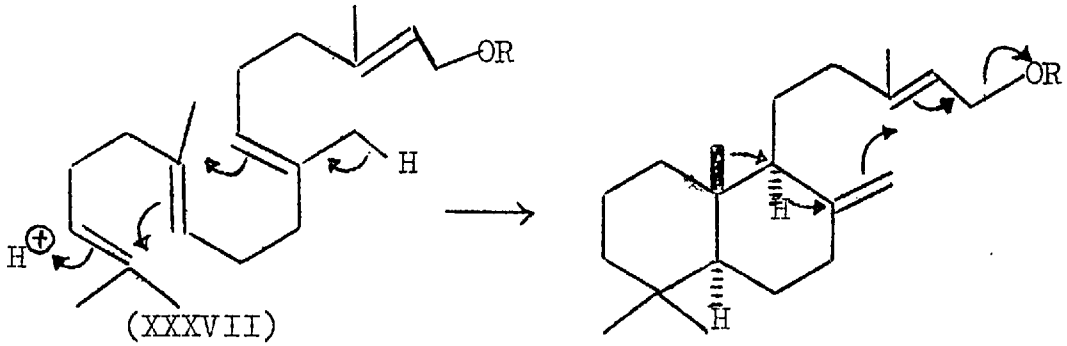
Sandermann and Stockmann[52] have reported incorporation of [carboxyl-^{14}C]-β-methylcrotonic acid into phytol, pimaric, and levopimaric acids in the pine tree. This precursor might in principle have been converted biologically into mevalonic acid, which was then incorporated into the resin acids. Alternatively, the precursor might have been degraded to acetate which was then re-incorporated. A reinvestigation of this result using labelled mevalonic acid seems desirable.

Thus there is, at present, no well-authenticated example of mevalonate incorporation into a diterpenoid in a higher plant. Our only detailed knowledge of diterpenoid biosynthesis has come from the study of mould metabolites. Micro-organisms are generally more easily fed with an organic substrate than are the higher plants and quite high incorporation of acetate and mevalonate have been observed. The few mould diterpenoids that have been studied so far are treated separately below.

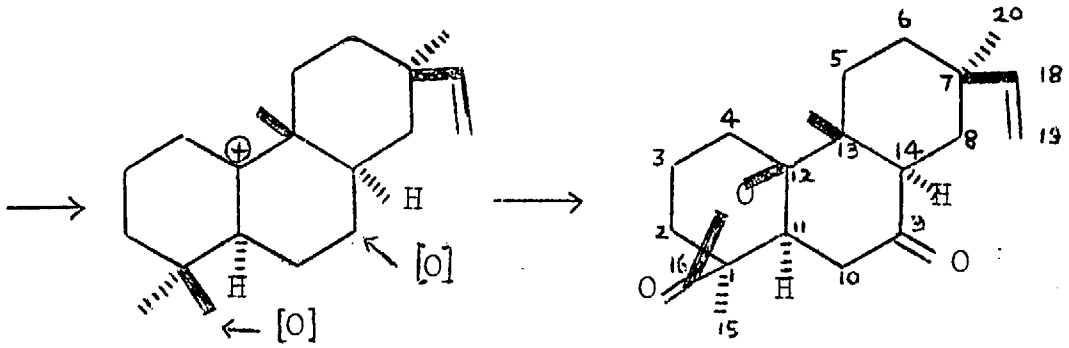
(a) Rosenonolactone.

The structure[53,54] of rosenonolactone (XXXVIII), a metabolite of Trichothecium roseum Link, does not obey the isoprene rule but can be derived from a tricyclic

isoprenoid precursor (XXXVII) by migration of an angular methyl group from the 12- to the 13-position as indicated below.



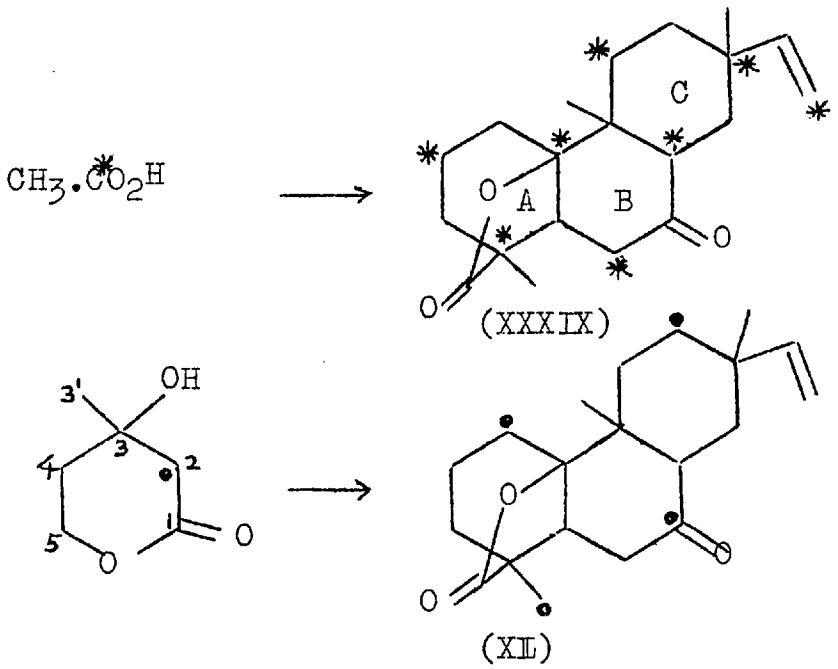
(XXXVII)



(XXXVIII)

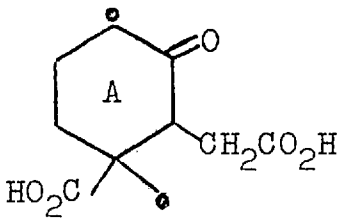
Birch and his co-workers grew the mould on media containing $CH_3.{}^{14}CO_2H$ and [2-^{14}C]mevalonic lactone and obtained labelled rosenonolactone.[55,56] Britt and Arigoni[57] also obtained mevalonate-labelled material.

Birch isolated carbon atoms 1, 13, 14, 15, 16, 17 and 19 individually in degradation products, and radioactive assays for a number of other fragments were also obtained.

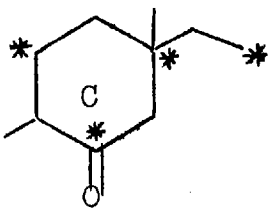


The results were in quantitative agreement with the patterns predicted from the biogenetic isoprene rule for acetate (XXXIX) and mevalonate (XL). Kuhn-Roth oxidation of the fragment (XLI) from ring A of mevalonate-derived rosenonolactone showed one-quarter of the total activity of the molecule in the methyl group at C - 1. Opening of the lactone ring and decarboxylation of the acid gave carbon dioxide which was collected as barium carbonate and found to be inactive.

Hence the gem-dimethyl groups in the acyclic precursor (XXXVII) were asymmetrically labelled and at no stage in the biosynthesis do the C - 2 and C - 3' atoms become equivalent. The asymmetry closely parallels the findings reported for pentacyclic triterpenes in higher plants.[58] Although the cyclisation initiators in the two types of compound may be different, the mechanism of ring closure appears to be essentially the same. The migration of the methyl group from the 12- to the 13-position was confirmed by assay of the acetate-derived ketone (XLII) whose molar activity was calculated on a formula $C_{10}H_{18}O$. The full degradative scheme is given by Birch.[56] Similar verification of the terpenoid character of rosenonolactone was obtained by the degradative work of Arigoni and his co-workers.[59]



(XLI)

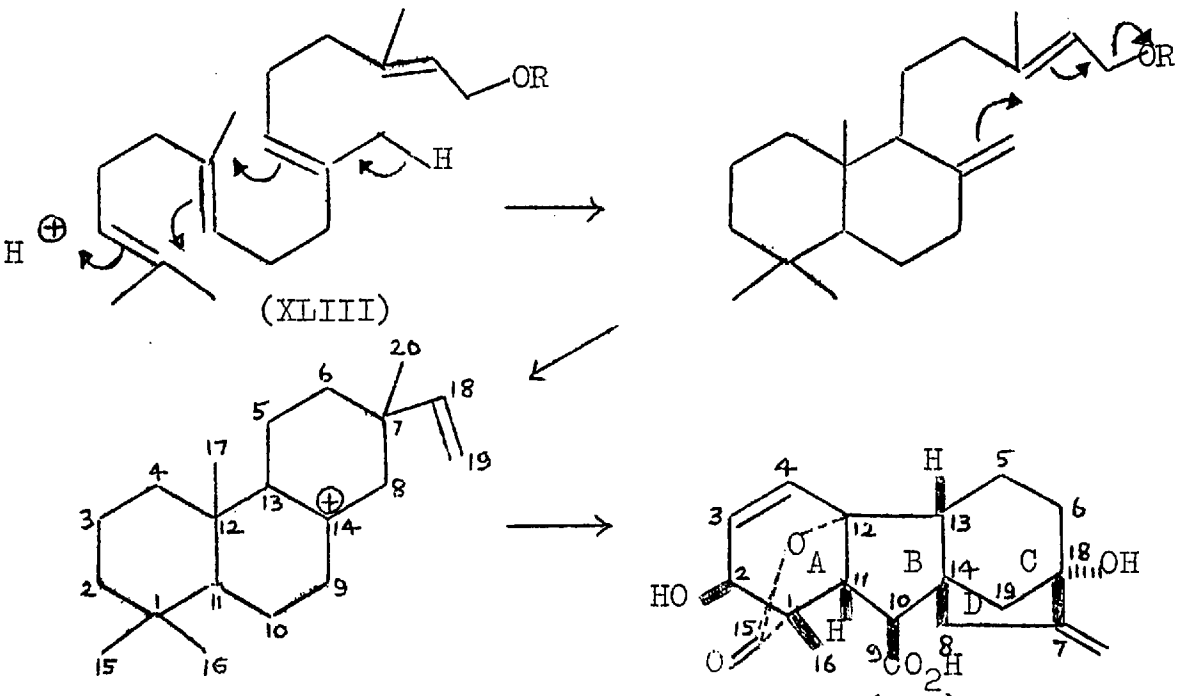


(XLII)

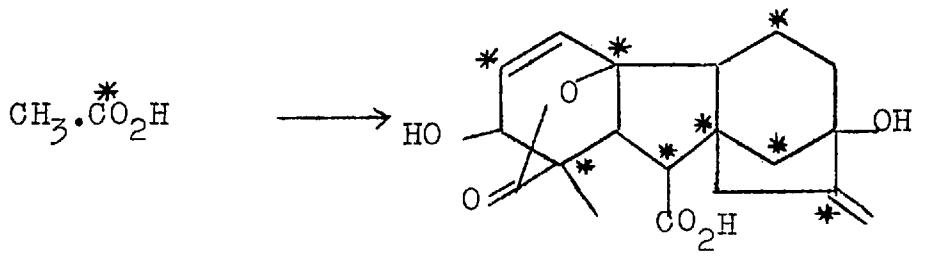
(b) Giberellic acid.

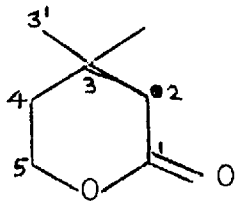
The biosynthesis of the plant growth hormone, giberellic acid,[60] was investigated by Birch, Rickards

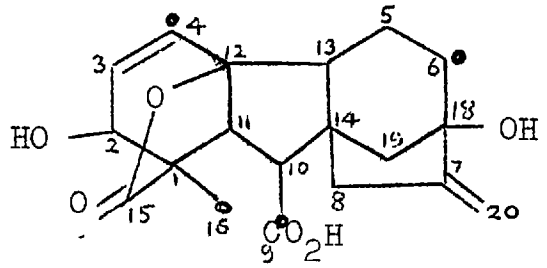
and Smith.[56,61] This compound (XLV) superficially bears little resemblance to the cyclic diterpenes already discussed but it can be derived from the usual precursor (XLIII) by the following scheme. Oxidative loss of the 17-methyl group, contraction of ring B, and rearrangement of ring C gives a phyllocladene-type of bridged-ring structure. In view of the occurrence of diterpenes with oxygen substituents in the 9- or 10-positions the contraction of ring B could proceed through a 9, 10-dioxygenated derivative.



Birch and his co-workers obtained information on these rearrangements by degradation of giberellic acid derived from $CH_3.{}^{14}CO_2H$ and [2-^{14}C]mevalonic lactone added to a fermentation of Giberella fujikuroi. The results were in quantitative agreement with the predicted labelling patterns (XLVI) and (XLVII) respectively.





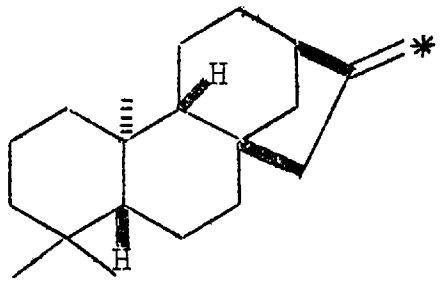


Carbon atoms 1, 7, 9, 15, 16, and 20 were isolated[56] and assay of other fragments supported the proposed patterns. As for rosenonolactone, the methyl-carbon attached to ring A was found to be derived specifically from the C - 2 of mevalonic acid. The carboxyl group is derived exclusively from the C - 9 of the diterpene

precursor. Formation of the phyllocladene ring has occurred by migration of C - 6 rather than of C - 20 to the 18-position, as had been predicted by Wenkert.[46] This was shown by a Kuhn-Roth oxidation on acetate-derived tetrahydrogiberellic acid. The isotope distribution suggested that the path followed in the formation of the bridged-ring was similar to that proposed for the acid-catalysed cyclisation of rimuene to isophyllocladene described earlier. The order and manner of these transformations, and those necessary to introduce the two hydroxyl groups, the 3,4-double bond, and the γ-lactone ring, remained uncertain.

Cross, Galt, and Hanson[62] extended this work in order to elucidate some of the steps between the pimaradiene carbonium ion (XLIV) and giberellic acid. (-)-Kaurene[63] (XLIX), which has also been found[64] in G. fujikuroi fermentations, is derivable from the carbonium ion (XLIV) by the Wenkert rearrangement[46] and might therefore be a precursor of giberellic acid. This was found to be the case and incorporation of (-)-[20-^{14}C]-kaurene (XLIX) into the acid was observed, indicating that rearrangement of the ion (XLIV) takes place to form a tetracyclic intermediate before oxidative attack and contraction of ring B. Ozonolysis of the

methyl ester of giberellic acid and isolation of the liberated formaldehyde as its dimedone derivative showed that 99% of the activity resided in the terminal methylene group. The authors do, however, qualify their results by stating that (-)-kaurene may not be an obligatory precursor and that the giberellins may be derived from the ion (XLIV) by alternative pathways.

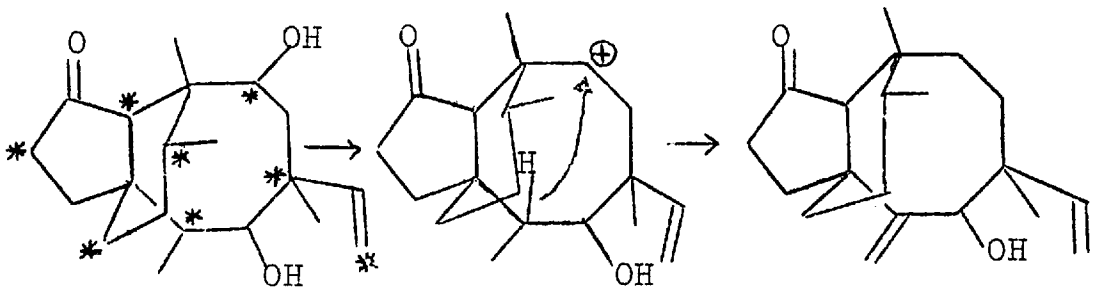


(XLIX)

(c) Pleuromutilin.

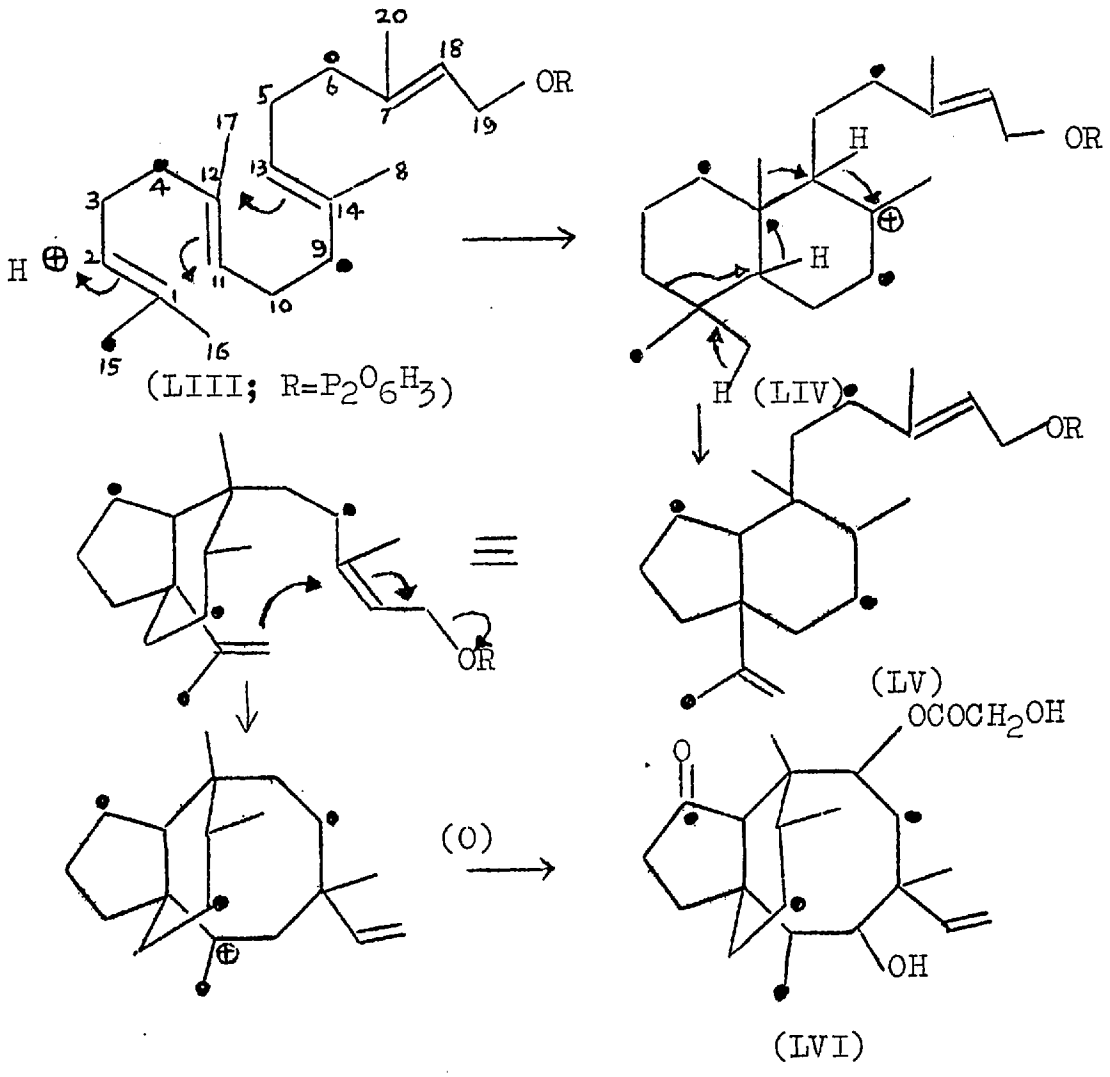
Evidence for the biosynthesis of the last mould metabolite to be described has come from the work of Birch and Arigoni and their respective co-workers. Growth of Pleuromutilus mutilus in the presence of [2-^{14}C] mevalonic lactone and of [1-^{14}C] acetate gave respective incorporations of 5.5% and 1.7% into pleuromutilin (LVI). Birch and his co-workers[65] performed

Kuhn-Roth oxidations on acetate-derived pleuromutenol (L), the alkaline hydrolysis product of pleuromutilin, and on the corresponding triketone. They claimed that the results supported the biosynthetic pathway for pleuromutilin proposed by Arigoni.[66] They stated that the acetic acid from the oxidations was unlabelled in its methyl group but carried $\frac{3}{24}$ths of the total molar activity in its carboxyl group. Since there are four C-methyl groups in the molecule and the carboxyl group should therefore contain $\frac{4}{24}$ths of the activity, it was claimed that this proved that one methyl group had migrated during the cyclisation from its original position to an inactive carbon derived from acetate-methyl. However, since the yields from the two oxidations were only 50% and 62% respectively, the results were not conclusive.



(L) (LI) (LII)

Arigoni obtained more evidence for his proposed biosynthetic pathway of pleuromutilin by degradation of mevalonate-derived metabolite.[59] Cyclisation of geranylgeranyl pyrophosphate (LIII) gives the carbonium ion (LIV) which undergoes a concerted series of 1,2 shifts leading to the bicyclic precursor (LV) which in turn rearranges to give pleuromutilin (LVI).



As in the previous work of Birch and his colleagues, carbon atoms 8, 15, 17 and 20 were obtained by Kuhn-Roth oxidation as the methyl group of acetic acid and carbon atoms 1, 7, 13 and 14 as the carboxyl group. Anomalous results were obtained by Kuhn-Roth oxidation of pleuromutenol (L) which gave a 62% yield of acetic acid; after allowing for the dilution factor of the three inactive methyl groups only 30% of the calculated molar activity was found in the methyl group. However, the corresponding triketone gave a better yield of acetic acid (84%) and 84% of the required activity was found in the methyl group. Arigoni accounts for these results by postulating a trans-annular effect as in (L) to (LII) which would not be possible in the triketone. The assay of other fragments was in agreement with pattern (LVI).

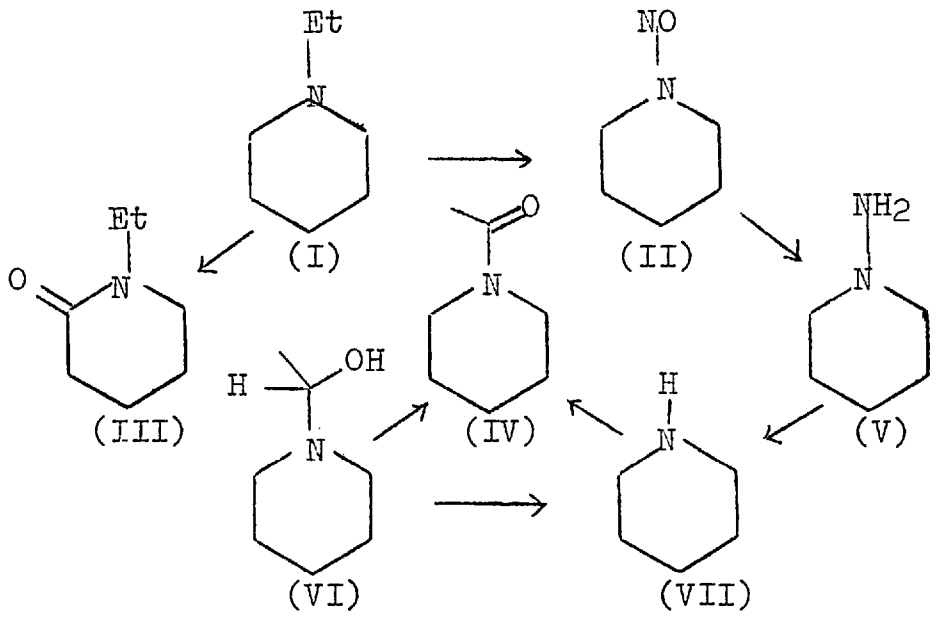
CHAPTER TWO: DELPHELINE

Structure.

From the seeds of the hybrid Delphinium elatum L., Goodson isolated a crystalline alkaloid which he called delpheline.[67a] Analysis[67b] gave the empirical formula $C_{25}H_{39}NO_6$ and potentiometric titration showed that the equivalent weight was 450. Since the alkaloid formed a basic monoacetate with acetyl chloride, there was one hydroxyl group in the molecule. Treatment with hyriodic acid showed the presence of three methoxyl groups and further heating with hydriodic acid and ammonium iodide gave ethyl iodide, indicating an N-ethyl group. The base liberated formaldehyde on treatment with strong acid and Goodson suggested that the remaining two oxygen atoms were accounted for by a methylenedioxy-group.

Cookson and Trevett[68a] confirmed the empirical formula and showed that delpheline contains an N-ethyl-piperidine ring with a methylene group adjacent to the nitrogen atom. Oxidation of the alkaloid with potassium permanganate in acetone gave a neutral compound, oxodelpheline, the carbonyl absorption of which at

1648 cm^{-1} indicated a six(or more)-membered lactam ring as in partial formula (III). Nitrous acid treatment of delpheline (I) gave N-nitrosodesethyldelpheline (II) which could be reduced by lithium in ammonia to N-aminodesethyldelpheline (V). Hydrogenolysis of the reduction product with zinc and hydrochloric acid gave desethyldelpheline (VII) which on acetylation to the neutral monoacetyl derivative (IV) and reduction with lithium aluminium hydride regenerated delpheline. Oxidation of delpheline with mercuric acetate yielded α-hydroxy-delpheline (VI) as one of the products. Oxidation of this derivative with potassium permanganate gave the N-acetyl derivative (IV) and cleavage with dilute acid gave desethyldelpheline (VII) again.

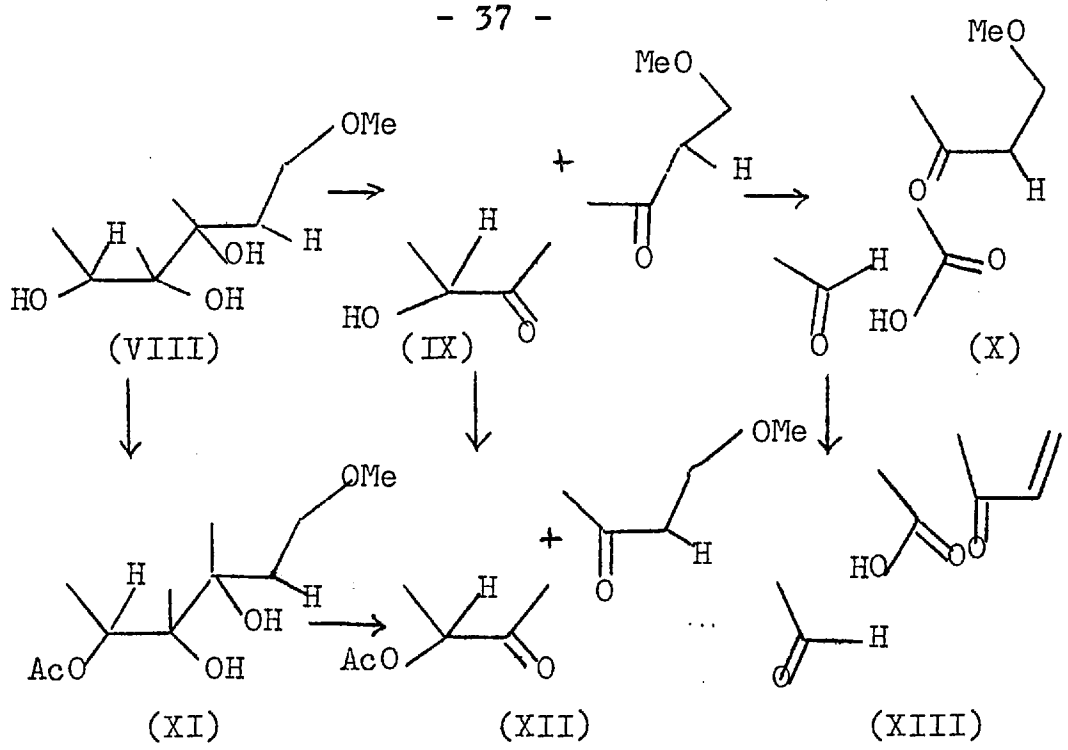


As delpheline is acetylated by acetyl chloride but only very slowly by acetic anhydride and pyridine, and as oxidation of the alkaloid with N-bromosuccinimide gave dehydrodelpheline with the loss of two hydrogen atoms, the hydroxyl group in delpheline is secondary. A band at 1748 cm^{-1} in the infrared spectra of the dehydro-compound indicated that the hydroxyl is on a five-membered ring. The absence of a band in the O - H stretching region showed that the two inert oxygen atoms were both ethereal. Since delpheline has a very low extinction coefficient at 210 mμ, and oxodelpheline is inert to ozone and perbenzoic acid, the molecule is saturated and contains seven rings.

Delpheline, oxodelpheline, dehydrodelpheline, and dehydro-oxodelpheline all liberated one mole of formaldehyde on treatment with strong acid but did not react with lead tetra-acetate in acetic acid.[68b] The demethylene-compounds obtained by acidic hydrolysis, however, took up one mole of lead tetra-acetate immediately and a second mole more slowly. Demethylene-oxodelpheline also took up two moles of periodic acid in a day. Demethylenedelpheline is therefore a 1,2,3-triol and since dehydrodemethyleneoxodelpheline could

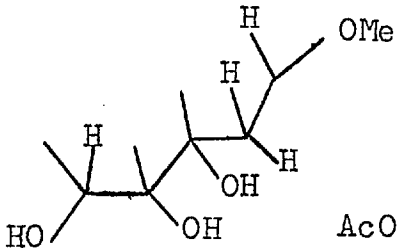
be reduced by lithium aluminium hydride to demethylene-oxodelpheline, no ketol rearrangement had occurred in cleavage of formaldehyde. As chromic oxide in pyridine destroyed demethyleneoxodelpheline but did not affect its acetate, the acetylated hydroxyl group was probably the one present in delpheline and the two new hydroxyl groups were probably both tertiary

Treatment of demethyleneoxodelpheline (partial formula VIII) with one mole of periodic acid gave a crystalline secodiketone (IX) with one carbonyl group in a five-membered ring and the other in a six(or more)-membered ring. The more readily split glycol is ditertiary and the original secondary hydroxyl group is not involved as the secodiketone could be acetylated to a substance (XII) identical with that obtained by oxidation of demethyleneoxodelpheline acetate (XI) with lead tetra-acetate. Further cleavage of the secodi-ketone with lead tetra-acetate gave a crystalline diseco-acid (X) which underwent elimination of methanol on treatment with dilute acid to give the αβ-unsaturated keto-acid (XIII).

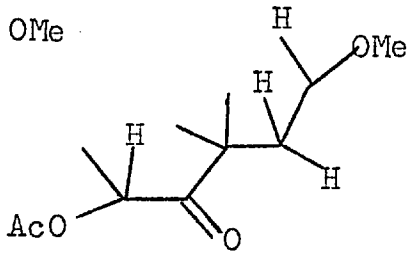


Treatment of demethyleneoxodelpheline (XIV) with acetyl chloride[68c] caused dehydration as well as acetylation to give a secondary α-acetoxy-ketone (XV). Hydrolysis of this product and re-acetylation gave an isomeric acetate (XVII). Hydrolysis and oxidation with chromic oxide in pyridine gave an α-diketone (XVI), the spectra of which suggested it was a bridged cyclohexadione. On treatment with strong acid, the diketone rearranged with elimination of methanol and hydrolysis of a second methoxyl group to form an αβ-unsaturated ketone. Cookson and Trevett[68c] called this compound delphelamone in comparison with lycoctamone[69,70] formed by an analogous transformation of lycoctonine,

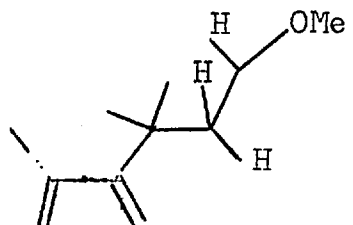
an alkaloid found in both Aconitum and Delphinium species. Cleavage of the diketone with alkaline hydrogen peroxide gave the di-acid (XVIII) which formed a dimethyl ester with diazomethane. However, it titrated as a strong monobasic acid due to the strong intramolecular hydrogen bonding in the anion-acid.



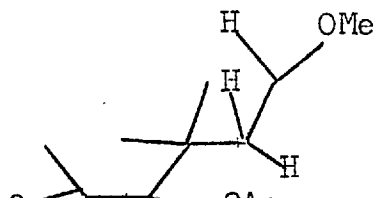
(XIV)



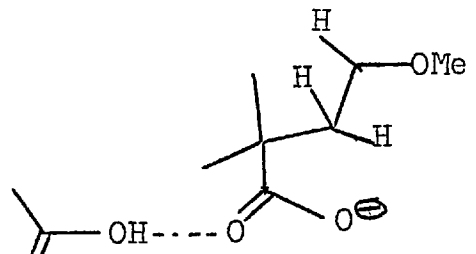
(XV)



(XVI)



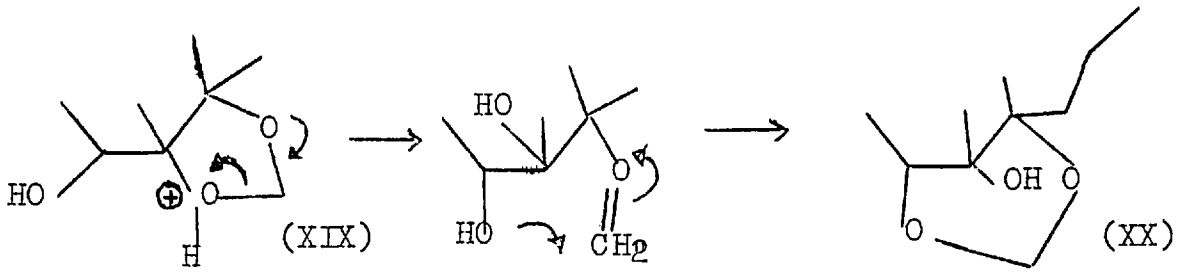
(XVII)



(XVIII)

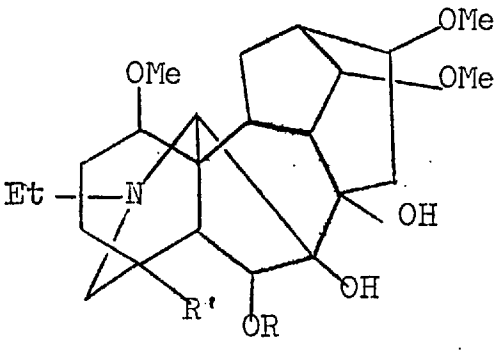
To end this brief summary of the reactions of delpheline, it is noted that oxodelpheline (XIX) could be converted by acid into an isoner iso-oxodelpheline tentatively assigned the structure (XX), which was inert to chromic oxide in pyridine, and thus contained a tertiary hydroxyl group instead of the original

secondary one. The physical properties of delpheline and its derivatives are tabulated by Manske.[71]



The reactions of the secodiketone (IX) from demethylene-oxodelpheline not involving the secondary hydroxyl group are closely analogous to those of the secodiketones from lycoctonam and de(hydroxymethyl) lycoctonine.[72] Also the acid-catalysed rearrangements of lycoctonam[70] and demethyleneoxodelpheline are similar and show that both alkaloids contain the same ditertiary glycol group and that the secondary hydroxyl group in demethylenedelpheline replaces a secondary methoxyl group in lycoctonine. X-ray crystallography[73] had enabled the structure (XXI) to be assigned to de(hydroxymethyl) lycoctonine and from chemical evidence[69] the hydroxymethyl group can be placed at the 1-position as indicated. Cookson and Trevett[68b] reasoned that the simplest way in which the two bases could be related was that demethylene-

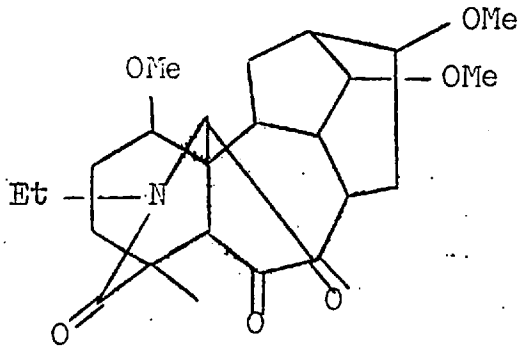
delpheline should have structure (XXII). All the above reactions can be reconciled with this structure. For instance, the acetylation and dehydration of demethyleneoxodelpheline can be explained, as for lycoctonam, by a pinacol-pinacone rearrangement.



(XXI; R=Me,R'=H)

(XXII; R=H, R'=Me)

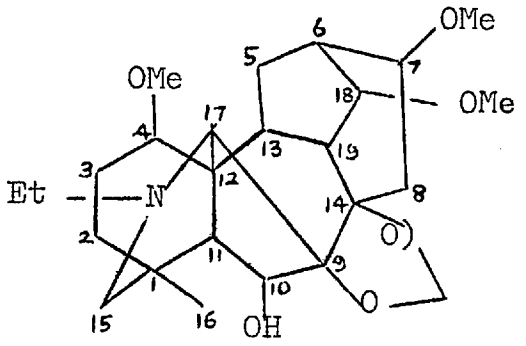
(XXIII; R=Me, R'=Me)



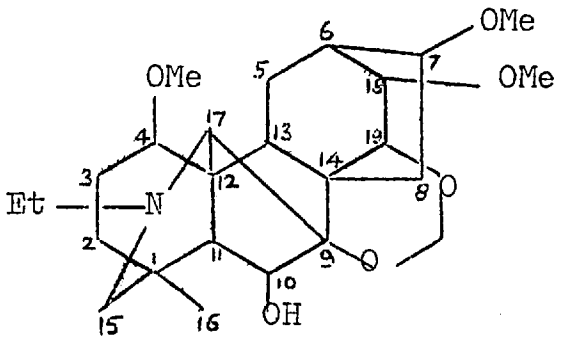
(XXIV)

Two correlations between demethylenedelpheline and lycoctonine confirmed the assignment. Carmack and his co-workers[74] converted demethylenedelpheline to desoxylycoctonine (XXIII) by methylation and Edwards, Marion, and Palmer[75] converted desoxylycoctonam to a diketone (XXIV) also obtainable from delpheline. The pinacone resulting from acid treatment of desoxylycoctonam

was treated with sodium amalgam to remove the methoxyl group α to the carbonyl and the product was oxidised with selenium dioxide to give the diketone (XXIV). These correlations establish definitely the structure (XXII) for demethylenedelpheline but the possibility of rearrangement of the carbon skeleton of delpheline during the hydrolysis of the methylenedioxy-group is still not excluded. Cookson and Trevett[68b] favoured the structure (XXVI) for delpheline rather than the structure (XXV) as demethyleneoxodelpheline, in low yield, was obtained from permanganate oxidation of delpheline and chromatography on alumina. They state that the removal of the methylene group in the absence of strong acid tends to discount the possibility of skeletal rearrangement.



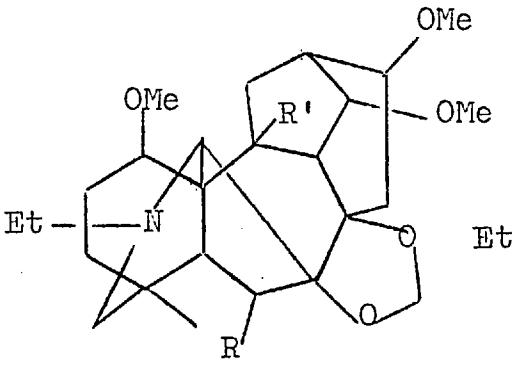
(XXVI)

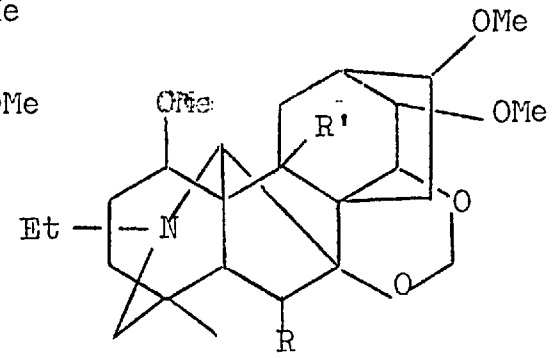


(XXV)

This point was pursued by Carmack and his co-workers, who found that delpheline, lycoctonine, and a further alkaloid, deltaline, occur together in Delphinium barbeyi Huth.[74] They established[76] that deltaline was a hydroxyacetyldelpheline, from which the free hydroxyl could be removed by chlorination with thionyl chloride, followed by refluxing in collidine to give dehydrodesoxydeltaline. The conversion of chlorodeltaline to delpheline by hydrogenolysis with lithium aluminium hydride established that delpheline and deltaline had the same skeleton. Carmack, Ferris, and Mayo[76] also found that demethylenedeltamine (demethylenedesacetyldeltaline) consumed two moles of periodic acid to give a disecodiketo-acid, which spontaneously formed a γ-lactone. This fact, together with the knowledge that elimination of acetic acid from diacetyldeltamine yielded an olefin that gave delpheline acetate on hydrogenation, enabled the additional hydroxyl group in deltaline and deltamine to be placed as in structures (XXVII) and (XXVIII) respectively. Kuzovkov and Platonova[77] confirmed the location of the hydroxyl group at position 13 in parallel reactions with demethyleneoxoeldeline and proposed structure (XXVII)

for eldeline, identical with that of deltaline.





(XXVII; R=OAc,R'=OH)

(XXVIII; R=OH,R'=OH)

(XXIX; R=OAc,R'=Cl)

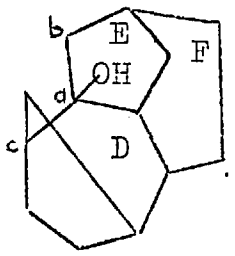
(XXX; R=OH,R'=H)

(XXXI; R=OAc,R'=OH)

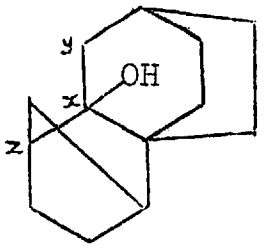
Carmack and his co-workers[76] emphasised that the additional hydroxyl group in deltaline is readily removed to give the olefin dehydrodesoxydeltaline. They claim that the resulting formation of a double bond cannot be accommodated in formula (XXVII) without violating Bredt's rule. They also point out that the reactivity of the halogen atom in chloroeldeline towards nucleophilic attack cannot be explained by structure (XXIX) as this would place the halogen atom at a "bridgehead". They further state that the pattern of reactivity of the hydroxyl group is quite different from that of the hydroxyl group in β-carophyllene alcohol, which also contains a bicyclo [4,3,1] decane system.

Their conclusion was that delpheline should be represented by the perhydrophenanthrene formula (XXX) discussed by Cookson and Trevett[68b] and deltamine by (XXXI). These formulae can be considered as that of possible biogenetic precursors of the lycoctonine skeleton.

On the other hand, Marion[78] considered that it is misleading to regard the lycoctonine skeleton in (XXVII) as containing a bicyclo [4,3,1] decane system as this involves including three rings (D,E,F) in it. However, in both structures (XXVII) and (XXXI) there is a bicyclo [4,3,0] nonane system in which C-13 is part of a fused-ring system rather than a bridgehead and therefore Bredt's rule does not apply. Elimination could thus occur between a and b (XXXII) or x and y (XXXIII)



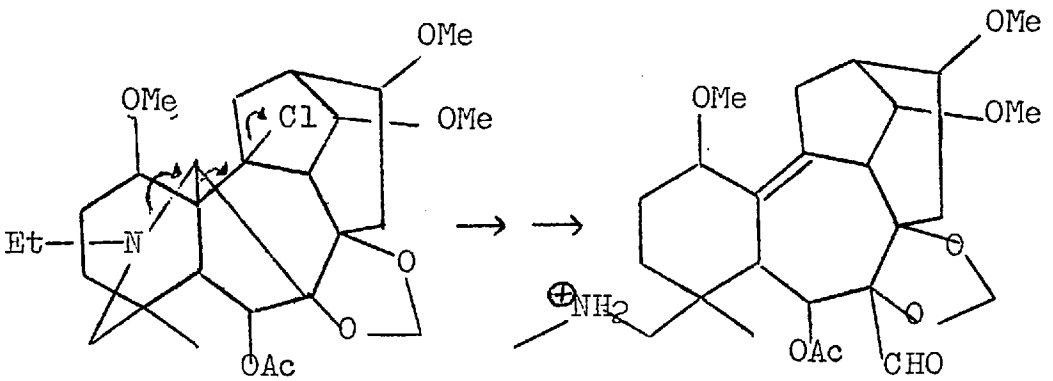
(XXXII)



(XXXIII)

Vizner, Kuzovkov, and Platonova[79] have now shown that iso-eldeline hydrochloride (XXXV), the product of

solvolysis of chloroeldeline (XXXIV) with aqueous ethanol, is a rearranged product. Therefore, since elimination of hydrogen chloride could occur after rearrangement, Carmack's objection to placing the hydroxyl group at a "bridgehead" is no longer valid. However, they propose the following mechanism by which a double bond is formed between a and c (C-13 and C-12).

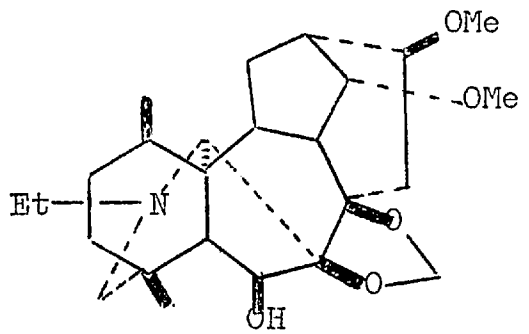


(XXXIV) (XXXV)

Recently, Crabb and Cookson[80] have examined the spin splitting of the non-equivalent methylene protons in the methylenedioxy-group of delpheline and its derivatives. They state that the low coupling constant for the protons, by comparison with model compounds, shows that they are on a five-membered ring whereas the larger coupling constant in iso-oxodelpheline indicates a six-membered ring as formulated (XX). Also evidence

presented by Montgomery,[81] working under Carmack, discounts the perhydrophenanthrene structure (XXXI). He claims to have ruled out the possibility of re-arrangement during hydrolysis of the methylenedioxy-group in deltaline by reversing the hydrolysis and recovering the alkaloid in good yield. He states that models with a moderate amount of flexibility in the bonds (e.g. Dreiding stereomodels) showed that dihydro-desoxydeltaline (presumably built with the 5,13-double bond) possessed surprisingly little more strain than the corresponding saturated compound.

The above evidence, coupled with determination of the absolute configuration of the lycoctonine skeleton by refinement of the X-ray data,[82] enables the full formula (XXXVI) to be written for delpheline.



(XXXVI)

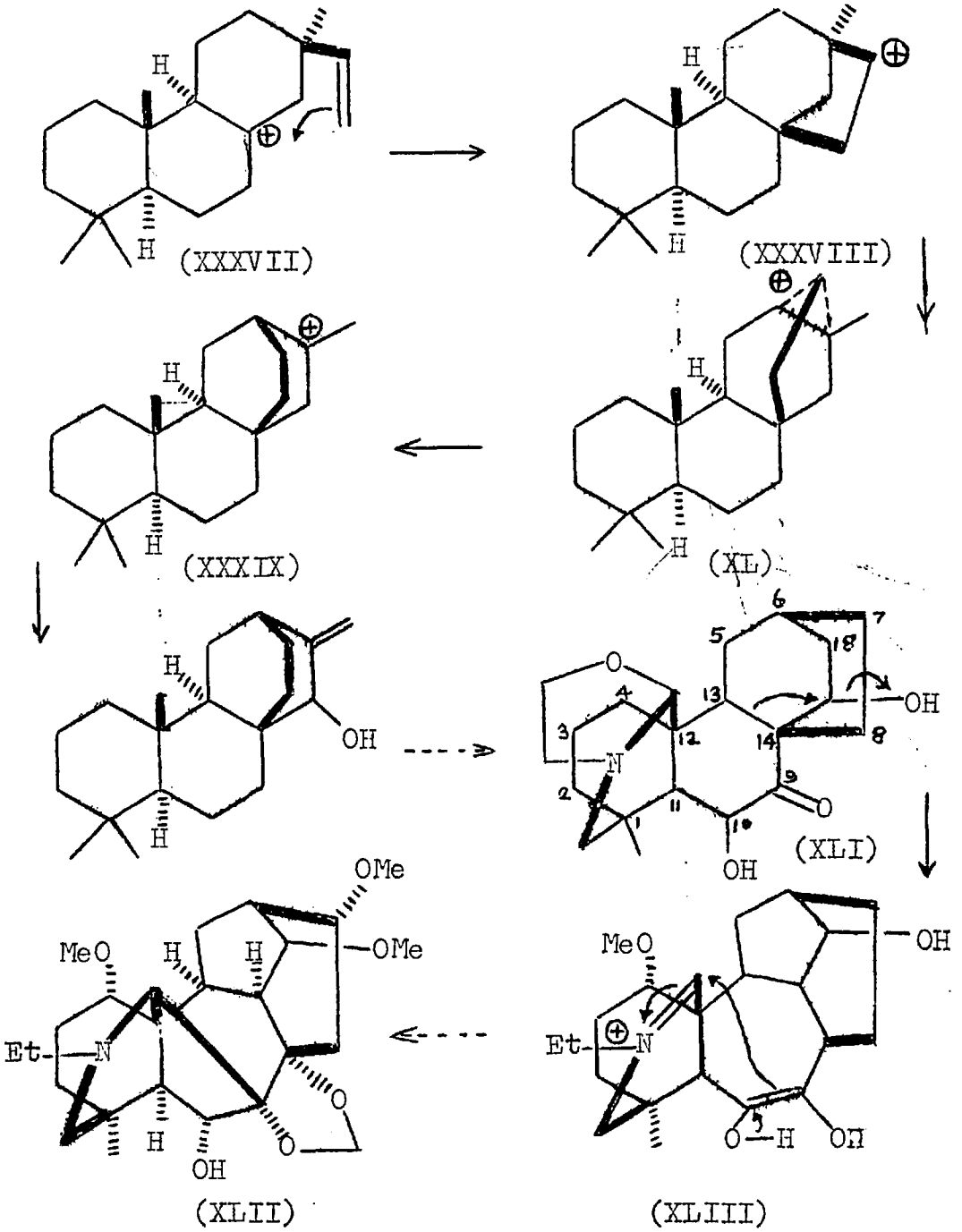
Biogenesis.

Wenkert[46] suggested that the diterpenoid alkaloids such as atisine could be derived from a simple pimaradiene precursor (XXXVII) with a quasi-axial vinyl substituent via the non-classical bridged-ring ion (XL). Cookson and Trevett[68b] envisaged the biosynthesis of lycoctonine and delpheline as arising from an atisine-type precursor (XLI). Wiesner and Valenta[83] also pointed out the plausible biogenetic relationship of atisine to lycoctonine. The stereochemistry and biosynthesis of some diterpenoid alkaloids were discussed by Whalley[47] but much of his argument has now been invalidated by structural re-assignments.

The hypothetical derivation of delpheline is outlined below. For simplicity of representation the mirror images of the structures are used. Cyclisation of the cation (XXXVII) gives the isomeric ion (XXXVIII) which can rearrange to, or already exists as, the non-classical ion (XL). This can collapse to give the new ring system (XXXIX) which leads to the atisine-type precursor (XLI). Loss of a proton, allylic hydroxylation, and another acid-catalysed rearrangement, can give delpheline (XLII). Formation of the final ring (XLIII)

to (XLII) is indicated by a form of the Mannich reaction involving attack on the quaternary Schiff's base (XLIII) as described by Cookson and Trevett.[68b] At some stage the C-20 exocyclic methylene group must be lost, presumably by biological oxidation, and a hydroxyl group introduced at the same position (C-18).

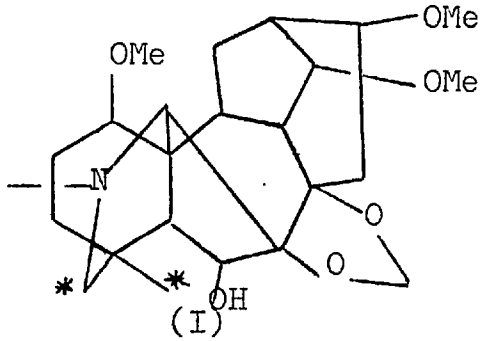
Clearly, variations of this scheme are possible and the order and manner of introduction of the oxygen functions cannot be predicted with certainty. Also the origin of the N-ethyl and methylenedioxy-functions is obscure. These questions can only be settled by appropriate tracer experiments with the living plant.


(XXXVII)
(XXXVIII)
(XXXIX)
(XL)
(XLI)
(XLII)
(XLIII)
MeO
OMe
Et
OH
N
O
H

CHAPTER THREE: DISCUSSION OF BIOSYNTHESIS OF DELPHELINE

Introduction.

At the time that work began, no investigation of the biosynthesis of diterpenoids in the higher plants had been carried out. Although it seemed very likely that the diterpenoid skeleton was derived from mevalonate, as indicated in the previous chapter, the later stages in the metabolic pathway were not clearly defined. For example, geranylgeranyl pyrophosphate, a plausible intermediate, had not been detected in any plant.



(I)

Delpheline (I) was chosen as a suitable diterpenoid to study for various reasons. The large range of transformations that had been worked out during elucidation of its structure would be useful for degradative procedures. The crystalline alkaloid can be isolated

in a pure state and in comparatively good yield from delphinium seeds and from growing plants.[67a,68a] Moreover, delpheline has several features of biogenetic interest. The aliphatic methylenedioxy-group is found only in delpheline and in a few closely related alkaloids and the N-ethyl group is also uncommon. The starred atoms in (1), which are presumably derived from the gem-dimethyl group of a simple diterpenoid precursor, are chemically distinguishable from each other. One of them could be isolated, for instance, by a Kuhn-Roth oxidation of desethyldelpheline.

In cases where it has been possible to distinguish between the two terminal methyl groups, usually by specific biological oxidation, activity has been found in only one of the relevant carbon atoms when the molecule was labelled with [2-^{14}C]mevalonate. This stereospecificity has been demonstrated for rosenonolactone,[56,57] giberellic acid,[56] and soyasapogenol-A.[58] Non-randomisation of the label is also indicated by work on agroclamine and elymoclavine,[84] methylgeraniolene,[85] trichothecin,[86] and fusidic acid.[59]

Ontogeny of delpheline.

Non-viable seeds of D.elatum L. (Carter's New Giant Flowered Mixed) were extracted by the procedure of Cookson and Trevett[68a] to give delpheline in 0.3 - 0.45% yield, m.p. 216-217°, $[\alpha]_D$ - 25° [lit.[67a,68a] m.p. 216-219°, 227°, $[\alpha]_D$ - 26° ($CHCl_3$), - 27° (EtOH)]. The alkaloid was rigorously purified by chromatography on alumina and repeated recrystallisation from acetone. It gave the required analytical values and its n.m.r. spectra was consistent with the structure assigned to it. The identity of the alkaloid with a sample provided by Professor R. C. Cookson was shown by mixed m.p. and by correspondence of the infrared spectra. Gradient elution with benzene-ethyl acetate on an alumina column of the basic fraction (1.6%) from the seeds (Fig.1) showed the presence of five other alkaloids, the major one being methyl-lycaconitine. For the small scale work involved in the detection of alkaloid in young plates, thin-layer chromatography offered obvious advantages, the six alkaloids being well separated.

Cookson and Trevett[68a] showed that the amount of delpheline present varied from 0.44% in young shoots to none in the green leaves and stalks of a mature plant

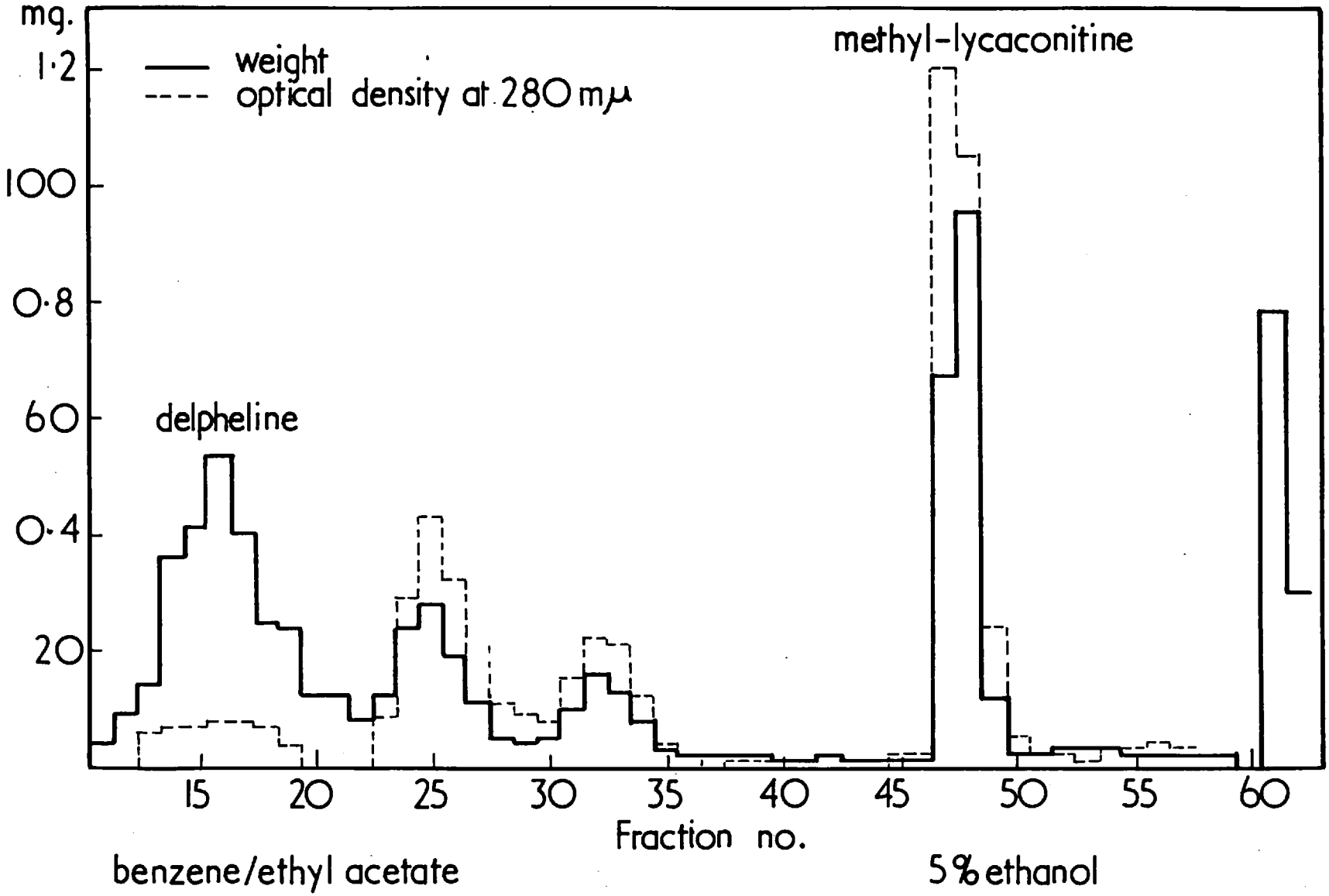


Fig.1 Gradient elution of basic fraction from D *elatum* seeds.

collected after flowering. In preliminary feeding experiments with [2-^{14}C] acetate small and variable incorporations were obtained. Clearly, it was important to establish when the plant was actively synthesising alkaloid. In the assay procedure used young plants were uprooted at various periods of growth, crushed under alcohol, dried, and extracted with 2½% acetic acid. The basic fraction was run on a chromatoplate and the delpheline band eluted and estimated colorimetrically by hydrolysis of the methylenedioxy group to give formaldehyde which was condensed with chromotropic acid.[8] To save time, some feeding experiments were performed before the production of delpheline had been examined; most of these were later found to have been performed when the amount of delpheline in the plant was increasing.

The results from young plants of the first season, sown in October 1961, are given in Fig.2. During the first two months delpheline content dropped from 0.3% in the seeds to zero. When the plants reached a dry weight of about 0.4 g., after five months of growth, the production of delpheline rose sharply. This increase corresponded with rapid growth of the plants in spring and was a suitable feeding point. In Fig.3 the

Fig. 2. Wt. of delpheline against dry wt. of plant

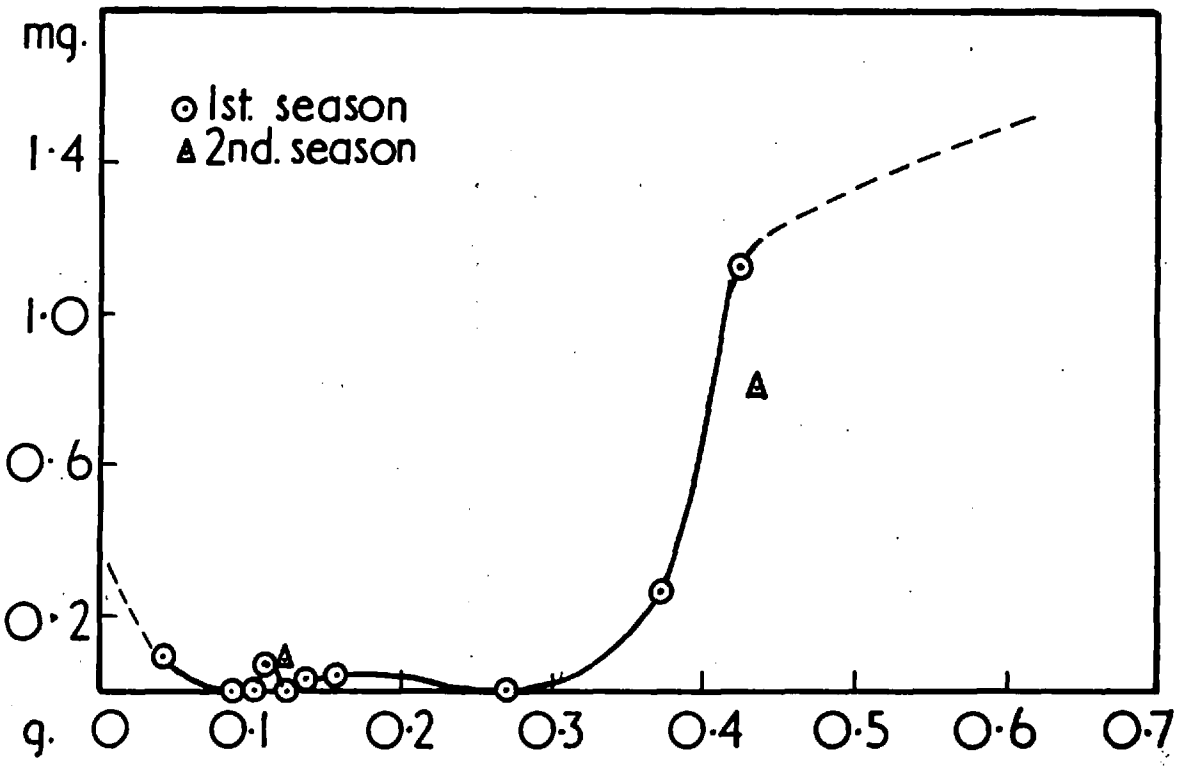
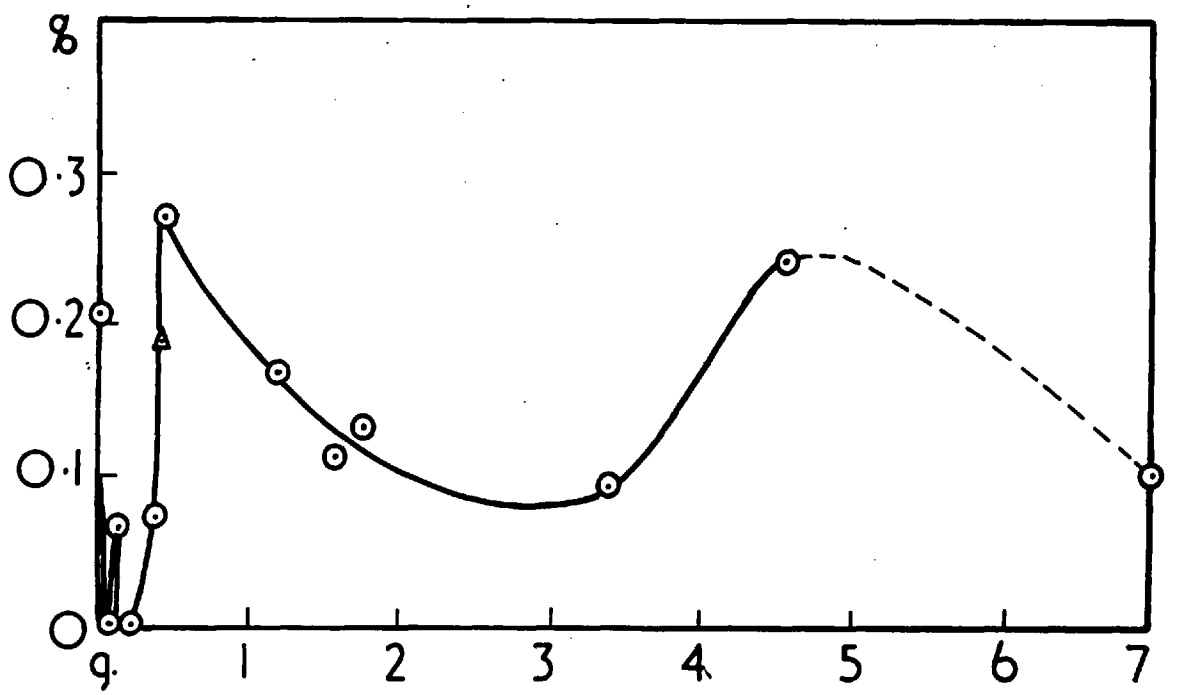


Fig. 3 % Delpheline against dry wt. of plant.



curve of delpheline content per gram of plant is shown. During the range 2-4 g. the production of delpheline is maintained although not at the high rate observed in the earlier stage. The general shape of the curve was followed by a few points obtained from plants grown in the second season. It is interesting that the production of methyl-lycaconitine, as judged qualitatively by thin-layer chromatography, did not correspond with that of delpheline.

Feeding methods.

In most of the feedings performed, a leaf was excised and both cut ends of the stem were inserted into a "poly-top" tube containing the radioactive substrate in distilled water at pH 5. In all cases over 99% of the activity was taken up by the plant. To increase the chance of incorporation several plants were fed in the same experiment. The first [2-^{14}C) acetate feeding, on a young plant, gave 0.004% incorporation but a second feeding in which the precursor was injected into the hollow stalk of a flowering plant gave negligible incorporation. The wick-feeding method used by Leete[87] was later used for administration of methionine but was slower than the above procedure and was not generally

adopted. The plant was extracted after several days growth and the basic fraction separated by thin-layer chromatography or by gradient elution of diluted material. A Zeisel determination on active delpheline from a preliminary acetate feeding showed no activity in the N-ethyl or methoxyl groups. This accorded with the expectation that the acetate was incorporated into the terpenoid skeleton. Unfortunately there was insufficient material for an accurate Kuhn-Roth determination.

[2-^{14}C] Mevalonate feedings.

For the first mevalonate feeding a flowering plant was employed but two later ones were performed with plants near the optimum point for delpheline synthesis. No significant incorporation was found. It was desirable to present mevalonate to the tissue without slicing or otherwise damaging it. Since the alkaloid is concentrated in the seeds, a fourth feeding was attempted by allowing freshly excised seed-pods containing ripening seeds to soak up the radioactive solution. Again no incorporation was observed.

The lipid fraction (3.15% incorporation) from a mevalonate feeding was examined to see if the non-

incorporation could be accounted for by the plant not properly metabolising the precursor. The specific activity increased on separation of the non-saponifiable fraction (1.15% incorporation) and chromatography on alumina to give a crude sterol fraction, which had an activity at least 10^4 times that of delpheline. Since Cookson and Trevett[68a] had described the isolation of β-sitosterol from D.elatum, the active material was diluted with this compound. Crystallisation from methanol gave radiochemically pure β-sitosterol (0.017% incorporation) which retained its activity when converted into the corresponding acetate. Clearly, β-sitosterol was present only as one component of a phytosterol mixture. A more detailed examination of the sterols was achieved as follows. The non-saponifiable fraction from a separate feeding was subjected to the procedure worked out by Professor T. W. Goodwin and Dr. E. I. Mercer at Aberystwyth. The following table (1) shows the percentage weight and activity of fractions eluted from a short alumina column by mixtures (20 ml) of diethyl ether (E) in light petroleum (P).

Solvent	% of Total wt. (8 mg.)	% of Total activity
P	6.25	0.01
2% E/P	0	2.85
4% E/P	22.5	0.38
6% E/P	18.75	1.23
8% E/P	11.25	1.72
10% E/P	6.25	2.10
12% E/P	2.5	7.10
20% E/P	2.5	9.89
E	0	0
Total	70.0	25.1
Insoluble	30.0	74.9
Absolute total	100	100

Again the presence of active β-sitosterol or a closely related compound (β-sitosterol is eluted by 10 or 12% E/P) was indicated. Dilution experiments with β-sitosterol and stigmasterol showed incorporations of 0.017% and 0.004% respectively. No squalene (2% E/P), lanosterol or β-amyrin (4 or 6% E/P) were detected on radioautography of the fractions. The

insoluble portion of the material was interesting in that it was 30% by weight of the total fraction yet contained 75% of the activity; this may represent incorporation into polar triterpenoids. Possible reasons for the failure to obtain incorporation of mevalonate into delpheline will be discussed later.

Methionine.

As mentioned above, there was evidence to show that alkaloid synthesis was taking place during the feeding experiments. This could be confirmed by observing incorporation of a different precursor under the same conditions. L-[Methyl-^{14}C]methionine was chosen for this purpose. The structures of the delphinium alkaloids suggest that O-methylation is one of the last stages in biosynthesis and it was hoped that incorporation would be readily obtained. Also the origin of the N-ethyl and the methylenedioxy groups in delpheline and of the ethyl side-chain in β-sitosterol could be investigated.

A typical gradient elution of the basic fraction from a methionine feeding is shown in Fig.4. The histogram indicates activity in both delpheline and

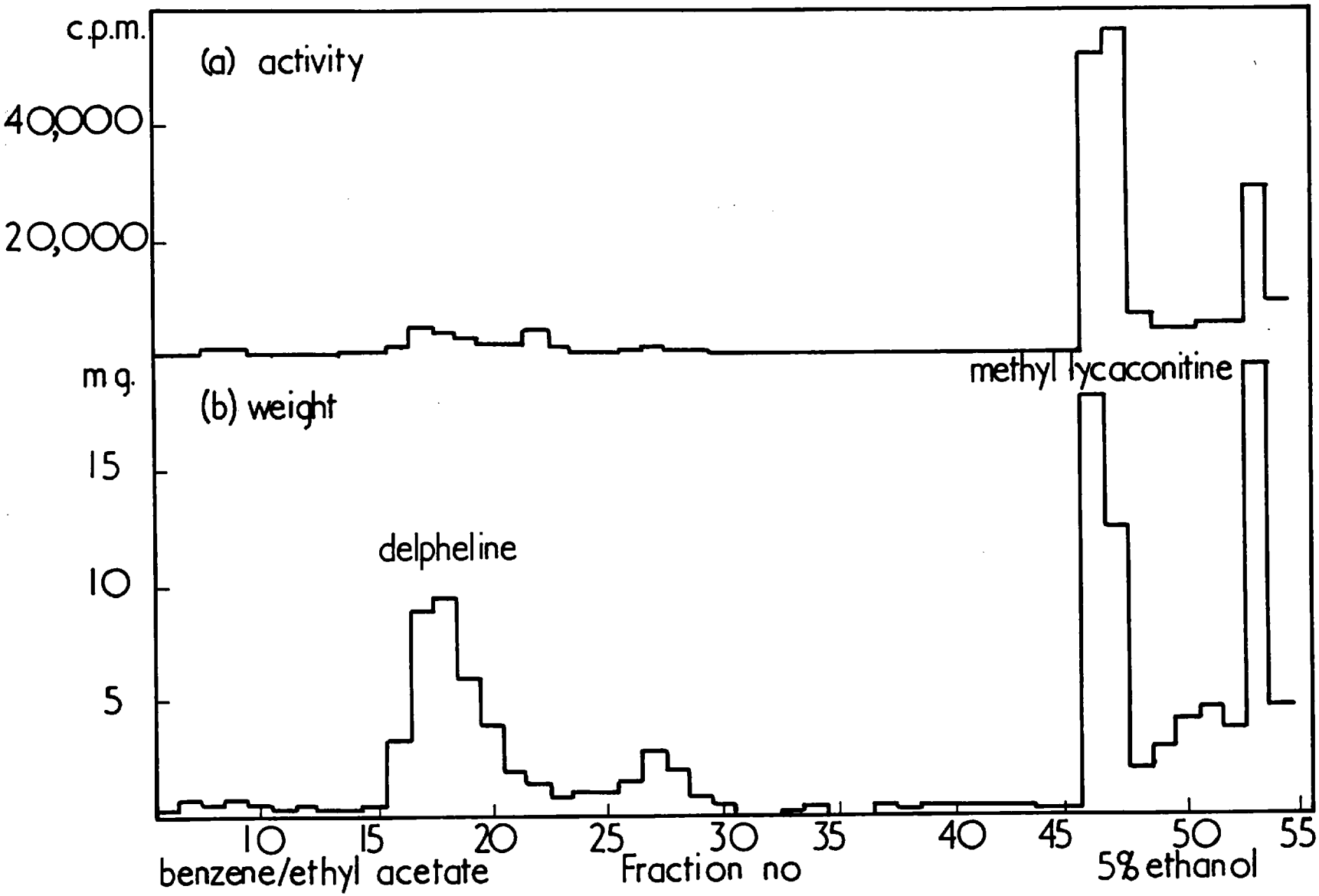


Fig. 4 Gradient elution of basic fraction from L methionine feeding

methyl-lycaconitine but as ethanolic solvents were needed to bring the latter off the column, the activity may have been present in the impurities. Only delpheline was examined further. The delpheline fractions were combined and rechromatographed (Fig.5). The specific activity curve from fractions 14-19 approximates well to the ideal rectangular shape (i.e. constant specific activity) and this representation shows clearly the presence of a small amount of impurity at fraction 21 in the tail of the curve. There is close correspondence between the weight and total activity curves. The impurity was cut off by taking fractions 13-20 and recrystallising the residues to constant activity from acetone. In fact, only a small drop in activity was observed after the first crystallisation, the chromatographed material already being radiochemically pure. As a further check, a sample of the active delpheline was oxidised to dehydrodelpheline, which was found to have the same molar activity as the parent alkaloid. These precautions are necessary to establish radiochemical purity since methionine is likely to be incorporated into minor compounds containing methoxyl groups.

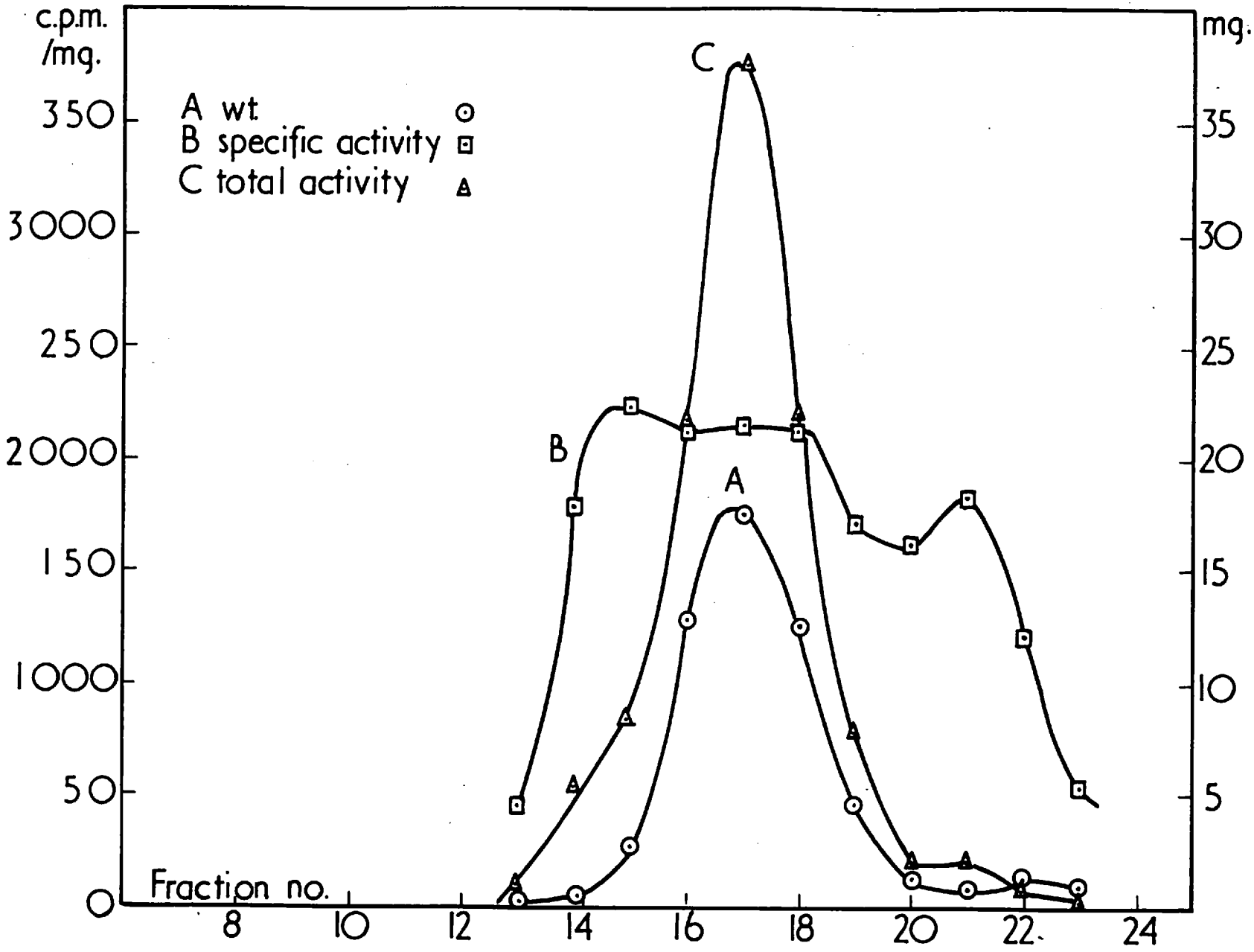


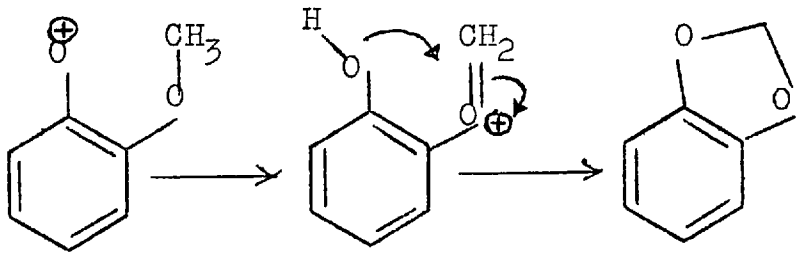
Fig.5 Rechromatography of delpheline from L methionine feeding

Degradative results are shown in Table 2. Dealkylation with hydrogen iodide revealed 88% of the total activity in the three methoxyls. Cleavage of the residue under more vigorous conditions with hydrogen iodide gave tetraethylammonium iodide containing only 11% of the total activity; some of this may be due to contamination from methoxyl. The activity of the methylenedioxy-group, as determined by hydrolysis to formaldehyde isolated as its dimedone derivative, was very low (< 5%).

Table 2.

Zeisel I	c.p.m./mg	c.p.m./mMole	Activity (%)	Yield (%)
Delpheline	92 ± 3	4.1×10^4	100	
O-Me	42 ± 2	3.1×10^4	76 ± 6	94
N-Et	7 ± 2	2.2×10^3	6 ± 2	86
Zeisel II				
Delpheline	92 ± 3	4.1×10^4	100	
O-Me	50 ± 4	3.6×10^4	88 ± 9	90
N-Et	14 ± 4	4.4×10^3	11 ± 3	78
Dimedone				
Delpheline	245 ± 8	1.1×10^5	100	
Formaldehyde-dimedone	17 ± 3	5.0×10^3	4.5 ± 1	33
Derivative				
Delpheline	63 ± 5	2.8×10^4	100	
Dehydrodelpheline	66 ± 9	2.9×10^4	106 ± 22	50

In contrast to the latter result, methionine is an efficient precursor for aromatic methylenedioxy-groups,[88] which are known to be derived by cyclisation of O-methoxy-phenols.[89] Indeed, methionine is incorporated into methoxyl and aromatic methylenedioxy-groups, contained in the same alkaloid, with comparable efficiency. One possible mechanism[89] is that an oxidation process applied to the phenolic hydroxyl of an O-methoxyphenol gives the cation (II) which is destablished relative to the oxonium ion (III). The intermediates could also be radical in nature. The generation of an oxonium ion or oxygen radical is much less likely from an aliphatic hydroxyl group and this may account for the difference observed in the present work concerning the formation of the aliphatic methylenedioxy-group. It seemed that the C_1 unit might be introduced already at a higher oxidation level than methionine. However, administration of [^{14}C] formate in an experiment carried out at the same time as the methionine feeding, gave no incorporation into delpheline. It is not, of course, unexpected that formate should prove a less efficient precursor than methionine for methoxyl groups.



(II) (III)

Attempts at estimating the activity in the N-ethyl group by preparation of desethyldelpheline in high yield were not successful. Acid hydrolysis of α-hydroxydelpheline would enable the fragment to be isolated as an acetaldehyde derivative but mercuric acetate oxidation of delpheline unexpectedly gave variable results. In one attempt the major crystalline products were dehydrodelpheline and oxodelpheline. Nitrous acid treatment of delpheline gave, as reported,[68a] desethyl-N-nitrosodelpheline but in low yield. Since the amounts of labelled material were small, the simpler though less clear-cut Zeisel determination was used. The low activity in the N-ethyl group indicates that it is unlikely to be derived from two C_1 units and it may possibly arise from β-ethanolamine, a metabolic product of serine. This would also account for the

> N - CH_2 - CH_2 - O - system present in related alkaloids of the atisine type. However, again no incorporation was observed in a L-[3-^{14}C]serine feeding.

A start was made in examining the ethyl side-chain at C-24 in methionine-derived β-sitosterol. However, recent work has clarified this point. Nicholas[90] reported an increased percentage of labelling in the side-chain as compared to the nucleus when methionine was fed after mevalonate. Castle, Blondin, and Nes[91] obtained β-sitosterol labelled with methionine but gave no direct evidence for the position of the label. Then Arigoni[92] established that the ethyl group in the related sterol, spinasterol, is derived from two methionine C_1 units by a double alkylation process. This result was confirmed by Lederer[93] for the ethylidene side-chain of fucosterol and Battersby[94] has shown that the ethyl group in β-sitosterol is not acetate-derived.

Table 3.

Precursor	Average plant wt. (mg.)	Activity fed (mc.)	Incorporation into delpheline (%)
[2-^{14}C] Sodium acetate	-	0.10	0.004
" " "	500	0.09	0.001
" " "	5000	0.10	0.001
" " "	6000	0.10	0.000
DL-[2-^{14}C]-mevalonic acid	9000	0.03	0.000
" " "	2330	0.07	0.000
" " "	.575	0.01	0.001
" " "	2100	0.04	0.000
L-[methyl-^{14}C]-methionine	1575	0.01	0.023
" " "	1900	0.01	0.028
" " "	19800	0.01	0.002
" " "	700	0.05	0.025
L-[3-^{14}C]serine	1690	0.01	0.001
[^{14}C]sodium formate	1050	0.10	0.000

Conclusions.

As explained earlier, mevalonate was efficiently incorporated into delphinium lipids but not to any detectable extent into delpheline. Moreover, it has been shown that delpheline is being synthesised at the time of feeding. Not only was the delpheline content of the plants increasing at this time but parallel experiments with methionine gave radioactive alkaloid. The incorporation of mevalonate into β-sitosterol, which has been shown by Battersby[99] to follow the normal acetate/mevalonate pathway in Rauwolfia sempentina, shows that the plant is metabolising the precursor. Thus either the mevalonate is being intercepted and used for sterol and triterpene synthesis before reaching the site of alkaloid biosynthesis, or else the delpheline is elaborated in a sub-cellular fraction of the plant impermeable to mevalonate.

Some parallel with these findings can be obtained from other work on diterpenoids. With flowering sections of Salvia sclarea, Nicholas[95] observed incorporation into β-sitosterol and into oleanolic acid but not into the diterpene alcohol sclareol. In a more detailed study[51] using periods of administration of from 4 hrs.

to a week he obtained no activity in the sclareol with either acetate or mevalonate. Cell-free preparations also gave negative results. He obtained comparable findings from M.vulgare plants containing the diterpene marrubiin. He suggests that there is a high degree of compartmentalisation restricting access of the lebelled substrate to the synthetic site and also states that fractions containing crude sclareol were highly active so that there appeared to be competition for the precursor by other unknown terpenoid substances in the plant.

Similar observations in a different context were made by Goodwin. He found that when etiolated maize seedlings were presented with [2^{14}C] mevalonate and illuminated for 24 hours, neither β-carotene[96] nor the phytyl residue of chlorophyll[97] were significantly labelled although both are rapidly synthesised during this period. Activity was found in the phytosterols and the triterpenoid fraction. Goodwin[50] suggests that the plant cell regulates the production of terpenoid materials to suit its requirements at various stages of its development and that in the early stages of germination regulation of sterol, carotenoid, and phytol

biosynthesis is by a process of compartmentalisation. The lack of incorporation into the carotenoids and diterpenes means either that the precursor is used in the cytoplasm for sterol and triterpenoid synthesis before it reaches the plastid or else it cannot penetrate the membrane surrounding the plastid. The plastidic and extraplastidic sites are two distinct regions of terpenoid biosynthesis in the plant cell.

Thus it seemed worthwhile to fractionate delphinium plant material to see if delpheline was associated with any specific subcellular component. Professor Goodwin kindly allowed the present author to perform a preparative differential centrifugation in his laboratories and, with the assistance of Dr. E. I. Mercer and Mr. K. J. Treharne, the results given in Table 4 were obtained.

Table 4.

Fraction (g.)	Chlorophyll (%)	Delpheline (%)	Composition
600	58	1	"chloroplasts"
1000	11	2	"chloroplasts"
10000	19	1	"chloroplast fragments"
20000	9	5	"mitochondria"
105000	3	8	"microsomes"
Supernatant	0	83	cell sap

Delpheline was found almost entirely in the cell sap. The possibility that the alkaloid is biosynthesed in the chloroplast and then rapidly transferred to an extrachloroplastidic site for storage is not eliminated. Also the conditions used, though mild, may have been sufficient to rupture the bodies producing delpheline. Chloroplasts purified by density gradient showed a small amount of delpheline present but this was probably due to incomplete separation or to external matter adsorbed by the large surface area of the lamellar system of the chloroplast. There was no correlation between the percentages of chlorophyll and delpheline in the various fractions.

In a recent paper Benn and May[98] reported a significant incorporation (0.010%) into esterified brownliine and methyl-lycaconitine when [2-^{14}C] mevalonate was fed to D.brownii Rydb. However, hydrolysis of the ester alkaloids caused a drop in activity of 40%. No comment is made on this result, but it must be assumed that if the methylsuccinylanthranilic acid esterifying group contained 40% of the total activity then the mevalonate must have been degraded and reincorporated in another form. The only other interpretation is that their alkaloids were not radiochemically pure; no

degradation is described.

The lack of incorporation into delpheline leads to the conclusion that extreme caution should be used in interpreting negative results from mevalonate feedings. Mevalonate has come to be regarded almost as a "reagent" for testing whether a natural product is of terpenoid origin. For example, mevalonate was not incorporated into cephaeline, or into ajmaline, although β-sitosterol isolated from the same plant was highly active.[99] In the light of the above results this does not disprove the hypotheses of Thomas[100] and Wenkert[101] that the C_9 or C_{10} unit of the indole and Ipecacuanha alkaloids is derived from two isoprenoid units.

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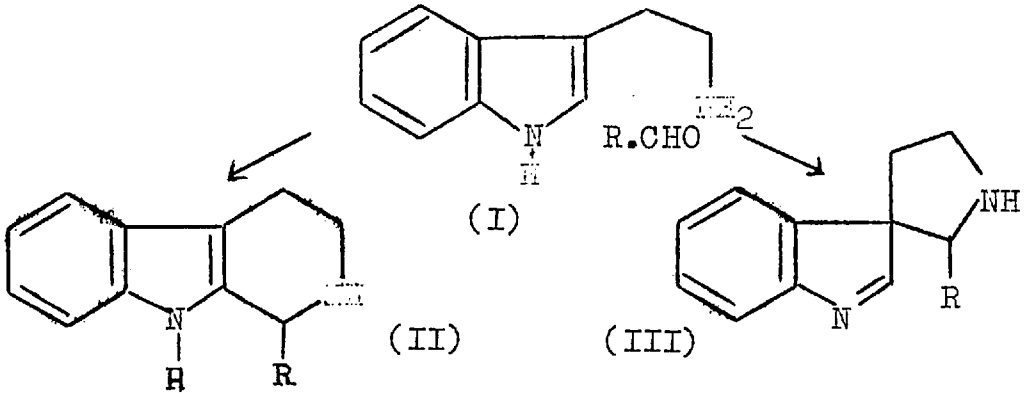
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CHAPTER FOUR: BIOSYNTHESIS OF INDOLE ALKALOIDS

The group of alkaloids containing an indole nucleus has a wide phytochemical distribution and includes such diverse forms as the simple base gramine and the complex polycyclic base strychnine. In the sense that they are probably derived from an indolylic precursor, the Cinchona alkaloids can also be considered to be members of this group. It is noticeable that many of the indole alkaloids contain, besides the indolic nitrogen atom, a second nitrogen atom separated from the β-position of the indole nucleus by a two-carbon unit. This suggests that they may be formed by a type of Mannich reaction involving the α- or β-position of the indole nucleus of tryptamine (I), an aldehyde or α-keto-acid, and the primary amino-group of the tryptamine side-chain. α-Condensation would give a β-carboline (II), whereas β-condensation would lead to an indolenine (III).



Although there have been many papers published in the last decade on theoretical aspects of the biosynthesis of indole alkaloids (see, for example, ref.1 and references cited therein), the actual experimental work that has been reported is relatively limited. In order to keep this account concise, hypotheses will not in general be considered. Since most of our present knowledge on the biosynthesis of indole alkaloids has come from tracer studies on gramine, the Rauwolfia alkaloids, quinine, the ergot alkaloids, echinulin, and the Iboga alkaloids, discussion will be confined to these few types.

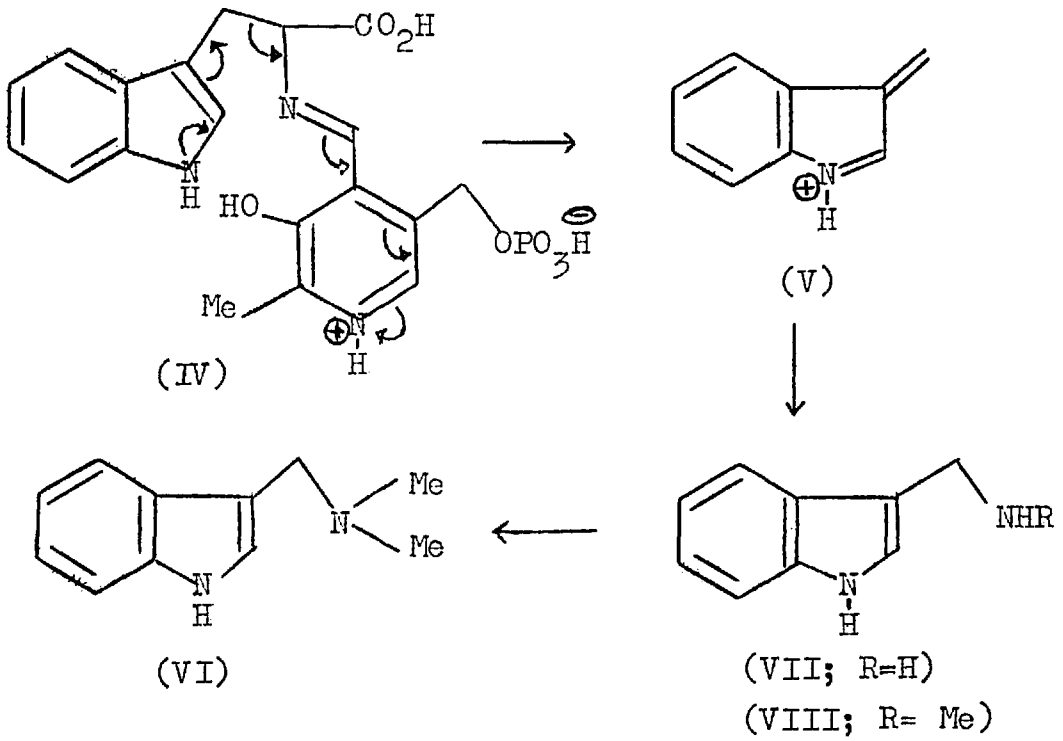
Gramine.

Gramine (VI), the main alkaloid found in the leaves of germinating barley, was one of the first alkaloids to be investigated by the use of radioactive tracer techniques. It was soon established[2] that tryptophan is a precursor of this compound. However, the mechanism of the shortening of the alanine side-chain and the nature of the intermediates in the biosynthetic pathway have remained obscure. Wightman et al.[3] fed a variety of compounds to excised barley shoots; one result they

obtained was that indole-β-[2-^{14}C]acetic acid was incorporated (0.48%) into gramine. This is contrary to recent findings.[4] Breccia and Marion[5] found that the administration of indole-β-[3-^{14}C]pyruvic acid and indole-β-[3-^{14}C]acrylic acid to barley yielded radioactive gramine (0.66 and 0.8% incorporation respectively). The authors suggested that these two acids were intermediates in the conversion of tryptophan to gramine.

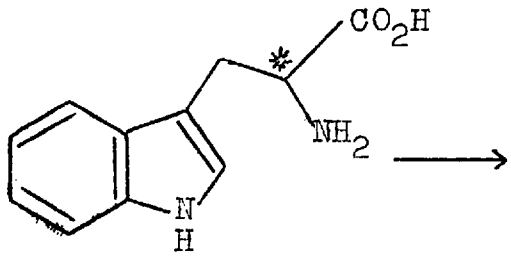
Leete and O'Donovan[4] have reported that a mixture of DL-[alanine-3-^{14}C]tryptophan and DL-[alanine-3-^{3}H]-tryptophan fed to barley seedlings gave gramine in which the radiocarbon/tritium ratio for the methylene-group was the same as in the precursor. The authors claim that this result strongly suggests that the bond between the β-position of the indole nucleus and the 3-position of the side-chain is not broken during the conversion. Assuming that there is no isotope effect, compounds such as indole-β-acrylic acid are eliminated as precursors because in this case part of the tritium would be lost. Furthermore, compounds such as indole-β-acetaldehyde are unlikely to be involved as they would contain labile tritium.

Further studies, by Leete and Gower[6], have shown that β-aminomethyl-[α-^{14}C]indole (VII) and β-methylaminomethyl-[α-^{14}C]indole (VIII) are incorporated into gramine. The high values observed (14.2 and 24.5% respectively) are claimed to be a strong indication that the two compounds are the immediate precursors of gramine. It had earlier been found that the methyl groups of gramine could be derived from the S-methyl group of methionine.[7] The above results are not in disagreement with Wenkert's suggestion[8] that the degradation of tryptophan involves a pyridoxal-induced retro-Michael reaction followed by amination of the resulting indolenine (V).

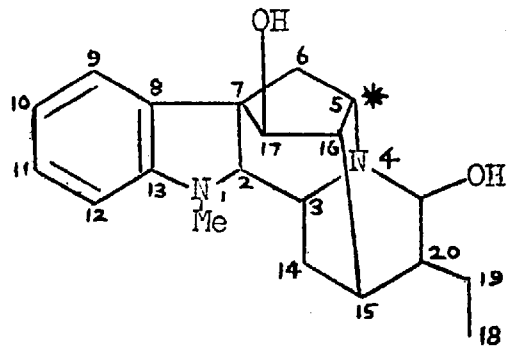


Rauwolfia alkaloids.

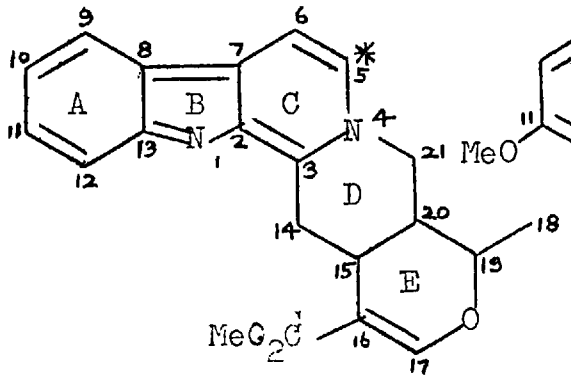
The tryptamine residue in these alkaloids has been shown to be derived from tryptophan (or a closely related metabolite) by the work of Leete. He fed DL-[alanine-2-^{14}C]tryptophan (IX) to R.serpentina plants and obtained radioactive ajmaline (X), serpentine (XI), and reserpine (XII). Degradation of the active ajmaline[9] and serpentine[10] showed that the alkaloids were specifically labelled in the 5-position. Breakdown of the tryptophan before incorporation is thus unlikely to have occurred. The higher "dilution" (i.e. ratio of specific activities of precursor to isolated alkaloid) found for reserpine may be due to the effect of the methoxyl group at C - 11.



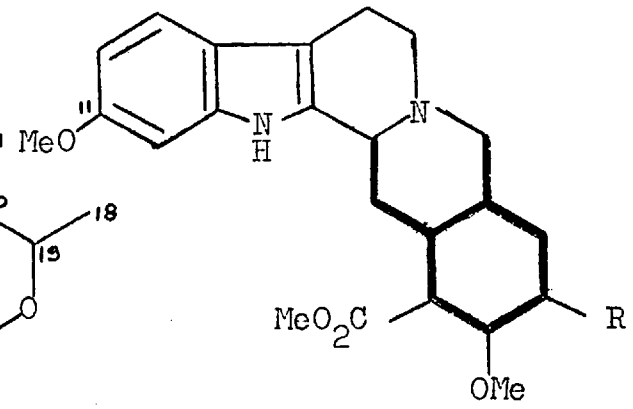
(IX)



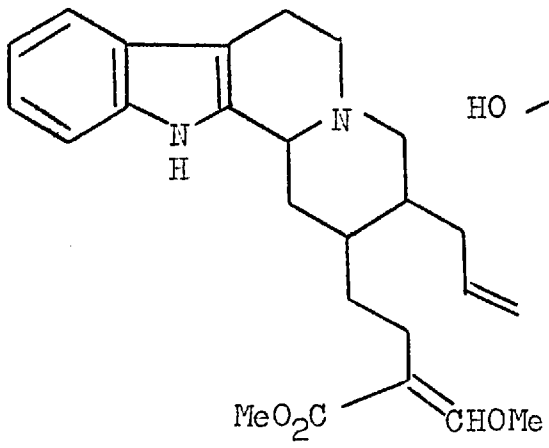
(X)



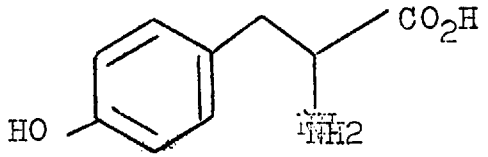
(XI)



(XII; R= $OCOC_6H_2(OMe)_3$



(XIII)



(XIV)

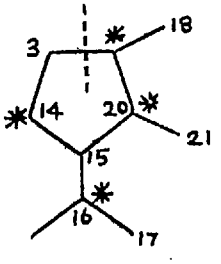
However, the origin of the non-tryptophan derived carbon atoms - the C_9-C_{10} unit indicated in structural formula (XII) - in this type of alkaloid is still not clear despite much recent interest. One suggested scheme[11] envisaged ring E, together with two of its attached carbon atoms, as being formed from an aromatic amino-acid of the dihydroxyphenylalanine type. After

the introduction of two C_1 units at C-16 and C-20 to give the serpentine (XII) skeleton, Woodward-Fission[12] could lead to structures such as that of coryantheine (XIII) which in turn could give rise to ajmaline (X) by two further ring-closures. Leete and his co-workers[13] fed [2-^{14}C]tyrosine, a known precursor of 3,4-dihydroxyphenylalanine, to R.serpentina plants and isolated inactive ajmaline. Whilst this result in itself is not conclusive in invalidating the hypothesis, parallel studies[14] on the Ipecacuanha alkaloid, cephaeline, have strongly suggested that tyrosine is not used to form the C_9-C_{10} fragment.

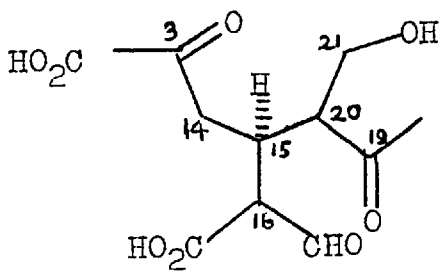
Wenkert[8,15] considered that a precursor of the secoprephanate-formaldehyde type (XV) would account for several of the structural and stereochemical features of the Rauwolfia alkaloids. Since alanine is metabolised to pyruvate, which can be incorporated into the side-chain of prephenic acid, administration of DL-[2-^{14}C]-alanine should give ajmaline labelled in the 3-position if this hypothesis is correct. However, Leete, Ghosal, and Edwards[13] obtained ajmaline of low specific activity from plants fed with this precursor and, moreover, only 2% of the total activity of the molecule was

present at C-3.

In a third suggestion put forward by Thomas[16] and by Wenkert[8] all the non-tryptophan derived carbon atoms are considered to arise from a monoterpene having the carbon skeleton (XVI). Cleavage of the cyclopentone ring (as shown by the dotted line) and rearrangement could give the required C_{10} fragment. The extra carbon at C-16 is absent in ajmaline but becomes the carbomethoxy group of serpentine, reserpine, and related alkaloids. Degradation of [1-^{14}C]acetate-labelled ajmaline by Leete and his co-workers[13,18] showed 26% of the total activity of the molecule to be present at C-3 and 26% at C-19; C-21 contained no activity. These results are incompatible with the labelling pattern (XVI) expected if the fragment is derived from mevalonate. Also, DL-[2-^{14}C]mevalonate gave completely inactive ajmaline, although, as was pointed out in Chapter 3, non-incorporation of mevalonate into a compound does not necessarily preclude part of the compound being terpenoid in nature.

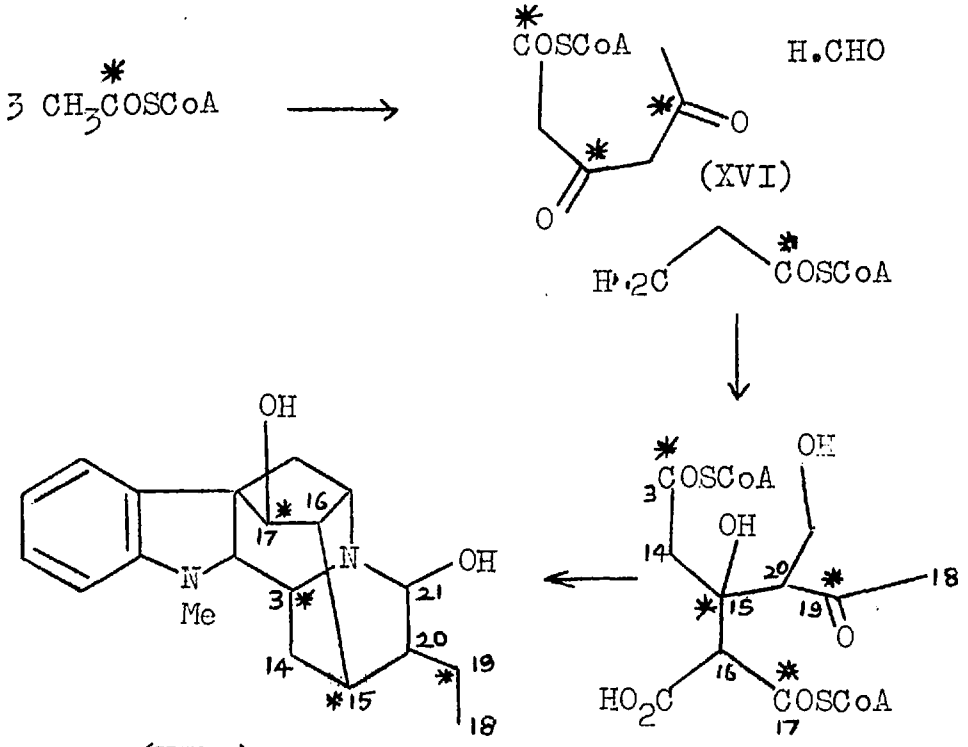


(XVI)



(XV)

However, the results are in exact accordance with a further scheme proposed by Leete and his co-workers[18] which initially involves the formation of the poly-β-keto-ester (XVI) by the linear condensation of three molecules of acetyl-coenzyme A. A C_1 unit is then attached at C-20 and condensation occurs with the methylene carbon of a molecule of malonyl-coenzyme A derived from acetyl-coenzyme A by carboxylation. The labelling pattern (XVII) would be expected for ajmaline if the alkaloid is formed in this way.



(XVII)

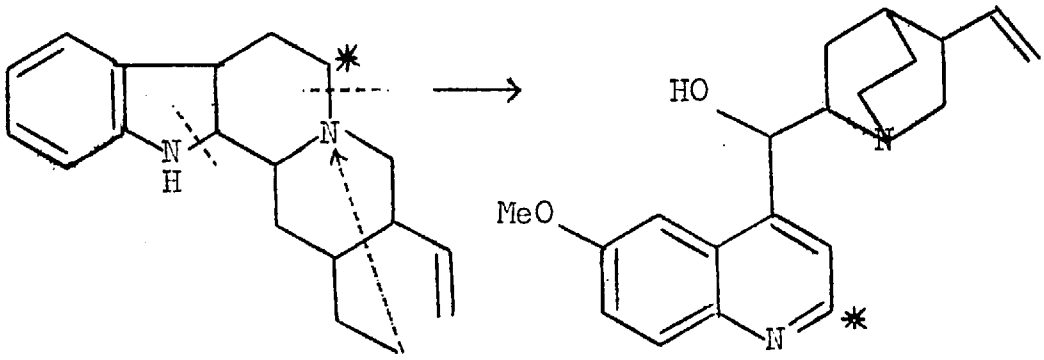
Corresponding results are described[18] for [1-^{14}C]-acetate-derived serpentine (XI). The carbomethoxy-group in the labelled alkaloid proved to be inactive, thus showing that no randomisation had occurred between the two carbonyls of the hypothetical intermediate, malonyl-coenzyme A. The carbon atom involved in the formation of the heterocyclic ring E is thus specifically the one carrying the thiol ester group in the intermediate. [1,3-^{14}C]Malonate-derived serpentine contained 48% of its activity in the carbomethoxy group, showing that no randomisation had occurred between C-16 and the carbon atom in this group. Additional work on ajmaline derived from [^{14}C]formate showed that the N-methyl group contained 48% of the total activity. A further 12% was found at C-21.

In surprising contrast, Battersby and his co-workers[20] reported that they had found little or no activity in the C-21 atom of ajmaline derived from [^{14}C]formate, whereas the N-methyl group carried 25% of the activity. Also the acetic and propionic acids obtained by Kuhn-Roth oxidation of [1-^{14}C]acetate-derived ajmaline had low and different levels of activity, thus showing scattering of the label.

Further negative evidence for the origin of C-21 in ajmaline has come from the feeding of [methyl-^{14}C]-methionine to R.serpentina.[21] The N-methyl group in the labelled alkaloid was found to contain 95% of the total activity; the carbon at position-21 was inactive. Thus it is unlikely that the C_1 unit is introduced by oxidative cyclisation of the N-methyl group of a tryptophan or tryptamine precursor.

Quinine.

It has been suggested[22] that the Cinchona alkaloids are likewise derived from an intermediate of type (XVIII) by fission of the 1,2-bond and appropriate cyclisations. Experimental proof of this suggestion was provided by Kowanko and Leete[23] who fed DL-[alanine-2-^{14}C]tryptophan to Cinchona succirubra plants and isolated quinine (XIX) labelled as predicted. Further work is being carried out by Leete on the quinuclidine portion of the molecule.



(XVIII) (XIX)

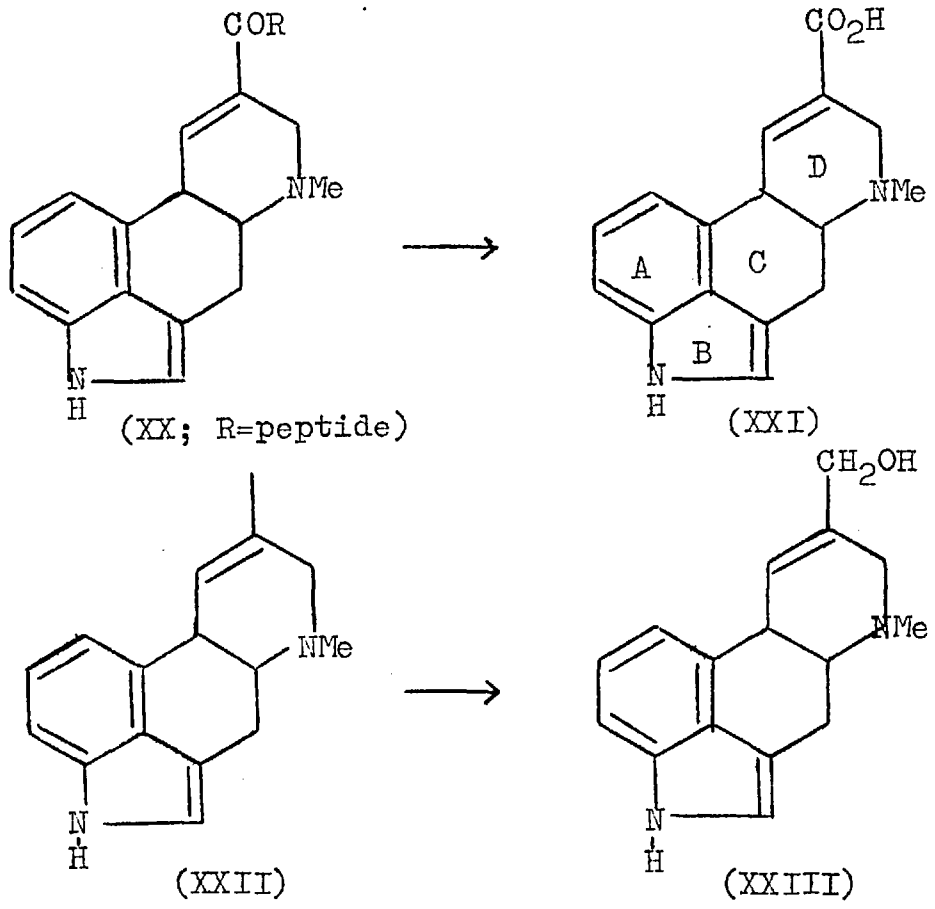
The ergot alkaloids.

Considerable attention has been paid to the biosynthesis of this group of alkaloids. The topic is reviewed by Tyler[24] up to 1961 and the present account will deal mainly with more recent work. Earlier investigators isolated peptide-type ergot alkaloids directly from the sclerotia of Claviceps purpurea (Fries) Tulasne, a rye parasite, but the saprophytic culture of certain strains of the fungus producing clavine-type alkaloids has proved more useful for comparative studies.

The first tracer evidence implicating tryptophan as a precursor of lysergic acid (XXI), the alkaline hydrolysis product of the peptide-type ergot alkaloids (XX), came from the work of Mothes et al.[25]; who obtained labelled lysergic acid from rye plants fed

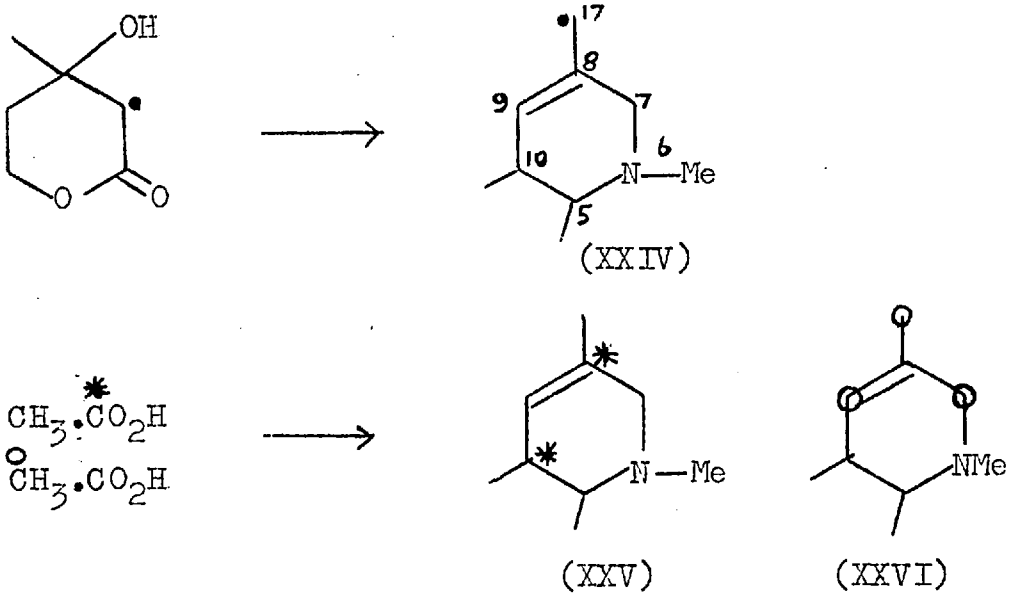
with DL-[alanine-3-^{14}C]tryptophan. A contradictory report by Suhadolnik and his co-workers[26] stated that DL-[alanine-2-^{14}C]tryptophan and tryptophan randomly labelled with tritium were incorporated into ergonovine (ergometrine) to such a small extent (0.003 to 0.014%) that the amino-acid was unlikely to be a direct precursor. The German workers[27] repeated their experiments on a quantitative basis and again found that DL-[alanine-3-^{14}C]tryptophan was incorporated (0.158%) into ergokryptinine, the principal alkaloid elaborated by the ergot strain used. They also obtained 10% incorporation into an alkaloid mixture, containing elymoclavine (XXIII) and agroclavine (XXII), produced by a different ergot strain cultured saprophytically. When pyridoxal phosphate was added, the incorporation could be increased to 40%. Taber and Vining[28] added a mixture of D- and L-[alanine-3-^{14}C]tryptophan, in which the D-isomer was more active than the L-isomer, to a saprophytic culture of the fungus. The specific activities of the isolated elymoclavine, agroclavine, ergosine, and ergometrinine indicated that they were derived in part from the D-isomer; the specific activity of the recovered precursor indicated preferential

use of the L-isomer overall. Baxter, Kandel, and Okany[29] found that DL-[alanine-3-^{14}C]tryptophan, but not DL-5-hydroxytryptophan, was incorporated into ergosine.



The origin of the remaining five carbon atoms of the ergoline nucleus was shown to be an isoprene unit by the work of several groups on the clavine alkaloids[30,31] and on the peptide alkaloids.[32] Weygand and his co-workers[30] obtained incorporation (9 to 23%) with

[2-^{14}C], [2-^{3}H], and [4-^{3}H]mevalonic acid. Birch, McLoughlin, and Smith[31] isolated labelled elymoclavine and agroclavine from cultures to which [2-^{14}C]mevalonic acid, [1-^{14}C]acetate, and [2-^{14}C]acetate had been added. The predicted labelling patterns in agroclavine derived from these precursors are as shown in the part structures (XXIV), (XXV) and (XXVI) respectively.



(XXIV)

(XXV)

(XXVI)

Preliminary degradations performed by these workers[31] gave results which, although they indicated the presence of a terpenoid fragment in agroclavine and elymoclavine, showed a surprising degree of randomisation and seemed to suggest different biosynthetic pathways for the two

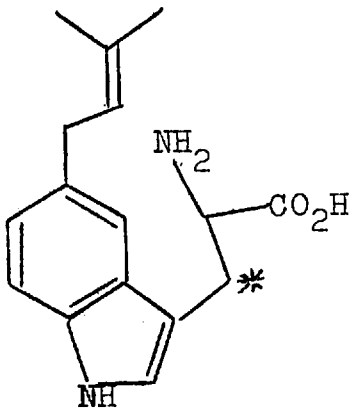
alkaloids. An improved procedure[33] was considered to give more reliable results. The three carbons 7, 8, and 17 were isolated as the 2,4-dinitrophenylhydrazone of acetone, which contained all the activity of mevalonate-derived material. In agreement with pattern (XXIV) there was no activity at C-7 whereas C-17 contained most of the activity (78% in agroclavine and 86% in elymoclavine). Slight randomisation between C-7 and C-17 had occurred. Similar work by Baxter, Kandel, and Okany[34] on ergosine derived from DL-[2-^{14}C]mevalonate showed that the C-2 of the precursor became the C-17 of lysergic acid. Again, some randomisation (6%) was found between C-7 and C-17. The same workers also investigated the origin of the N-methyl group in festuclavine and pyroclavine. They found[35] that methionine (1.35% incorporation) was a better precursor than formate (0.37%).

To summarise the above evidence, it appears that both the clavine- and peptide-type ergot alkaloids are formed by condensation of tryptophan, which is decarboxylated at some stage, with a C_5 unit derived from the acetate/mevalonate pathway. The condensation of the moieties is mediated by an enzyme system which requires pyridoxal phosphate as a cofactor. Methylation

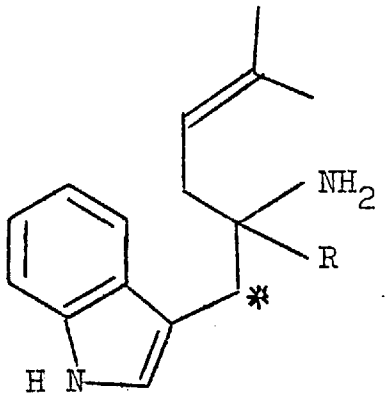
of the nitrogen atom at the 6-position is effected by transamination from methionine. It is now appropriate to consider the mode of condensation of the two units.

Plieninger and his co-workers[36] showed that loss of deuterium occurred when [4-^{2}H]tryptophan was incorporated into agroclavine and elymoclavine. Deuterium was retained when the tryptophan was specifically labelled at the 5- and at the 6-positions. Condensation therefore occurs at C-4 at some stage. Studies[37] with doubly-labelled precursors showed that DL-[2-^{14}C, 5-^{3}H]mevalonic acid suffered a loss of one hydrogen equivalent on incorporation into festuclavine and pyroclavine, such as would be observed on oxidation of a hydroxyl group to an aldehydic group. The ^{14}C/^{3}H ratio in clavine alkaloids derived from DL-[2-^{14}C, 2-^{3}H]mevalonate indicated no change in the oxidation level at the 2-position during the conversion.

Plieninger, Fischer, and Leide[38] suggest that the isoprene unit condenses in the 4-position of tryptophan, before the side-chain is involved in cyclisation, to give the intermediate (XXVII). Weygand and his co-workers[39] favour the alternative form (XXVIII).



(XXVII)



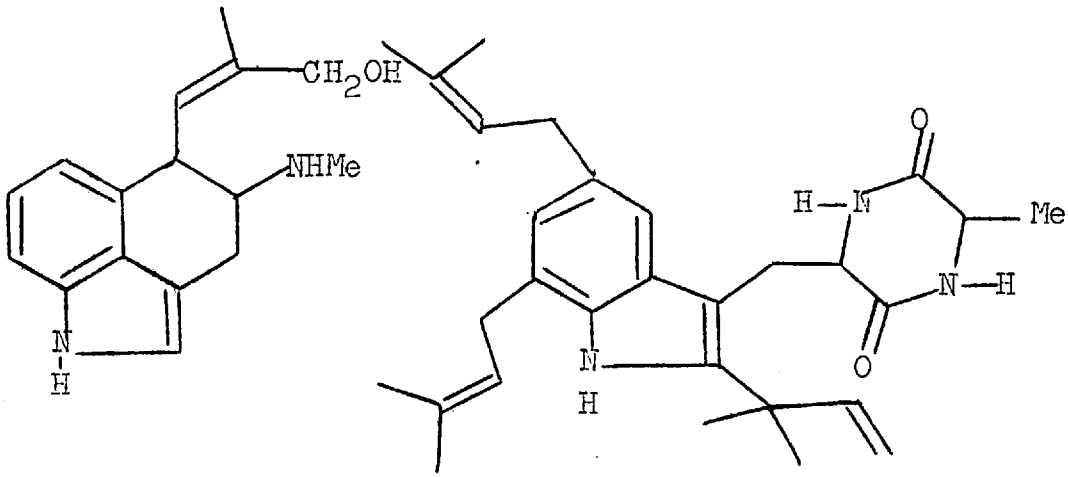
(XXVIII) R=H
(XXIX) R=CO_2H

Both groups have published experimental evidence to support their proposals. Plieninger and his co-workers[38] synthesised 4-dimethylallyl-[alanine-3-^{14}C]tryptophan (XXVII) and obtained "at least 5%" incorporation into elymoclavine. Weygand et al.[39] synthesised 1-(β-indolyl)-2-amine-5-methyhex-4-ene (XXVIII) labelled in the 1-position with ^{14}C and, separately, with ^{3}H in the benzenoid labelled ring. They also prepared 4-dimethylallyl-[alanine-3-^{14}C]tryptophan (XXIX). In general, the substituted tryptophan (XXIX) was a more efficient precursor for elymoclavine than the decarboxylated compound (XXVIII) but it was less efficient than DL-[alanine-3-^{14}C]-tryptophan itself. High incorporations (11.9 and 20.2%) were recorded for compound (XXIX) whereas the tritiated precursor (XXVIII) gave values of only 2.3 and 1.0% in the same feedings. This was, however, higher than

the incorporations obtained in parallel feedings with the same compound labelled with ^{14}C in the side-chain. It is thus a possibility that compound (XXVIII) is being degraded and the aromatic nucleus reincorporated as a fragment. The authors state that a clear distinction between the utilisation of the two precursors (XXVIII) and (XXIX) is not possible.

Both Weygand's and Plieninger's mechanisms could occur concurrently or, alternatively, inhibition of the normal biosynthetic pathway in a particular case might lead to the dominance of a minor metabolic pathway.[39] It was noticed in the early biosynthetic work on the ergot alkaloids that saprophytic cultures produced different alkaloid mixtures to those obtained under normal parasitic conditions. It has been suggested[40] that the fungus passes from an anaerobic stage, when the production of agroclavine is favoured, to a more developed aerobic stage, when mainly elymoclavine and the peptide-alkaloids are formed. Thus the biosynthetic pathway of the ergot alkaloids may depend on the physiological state of the fungus at that particular time and a fixed route is not obligatory.

Agurell and Ramstad[41] have studied the biogenetic relationships of the various ergot alkaloids and established that agroclavine is converted irreversibly into elymoclavine.in saprophytic culture. Chanoclavine (XXX) does not appear to be a precursor of the clavine alkaloids, as had been suggested, or indeed to be a metabolite of agroclavine or any alkaloid derived from it.[42] At present, it appears that in conversions involving peroxidases or oxygen transferases the following transformations may take place: agroclavine ⟶ elymoclavine ⟶ [lysergic aldehyde] ⟶ lysergic acid ⟶ peptide alkaloids.



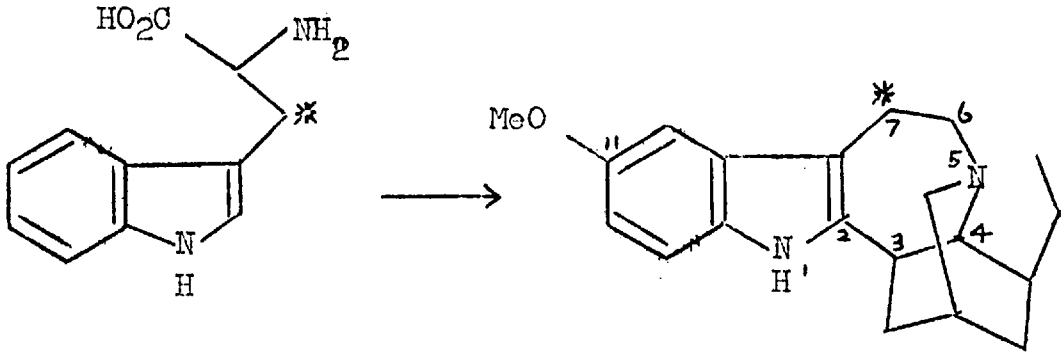
(XXX) (XXXI)

Echinulin.

Another fungal product, echinulin, isolated from Aspergillus amstelodami, has an interesting structure (XXXI) containing a reversed isoprene unit in the α-position of the indole nucleus. Birch and his co-workers[42] have performed a series of tracer experiments on this metabolite. Incorporation (4.25%) was observed with DL-[2-^{14}C] mevalonate and ozonolysis of labelled material gave acetone which had a specific molar activity 35% (i.e., one third) of that of the echinulin. It was thus deduced that there were three isoprenoid units in the molecule. This result was confirmed by degradation of [1-^{14}C]acetate-labelled material. Hydrolysis of [1-^{14}C]alanine-derived echinulin gave recovered alanine containing 68% of the total activity present in the molecule, showing that this amino-acid was a biosynthetic precursor of the dioxopiperazine ring. A further 19% of the activity was accounted for by random incorporation into the isoprene side-chains, presumably via pyruvate and acetyl-coenzyme A. Finally, DL-[3-^{14}C]tryptophan was found to be incorporated (1.36%) into echinulin.[44] Alanine isolated by hydrolysis of the labelled alkaloid with hydrobromic acid was inactive, as were the isoprene units.

The Iboga alkaloids.

To conclude this account, some recent work by Yamasaki and Leete[45] will be mentioned. They fed Tabernanthe iboga plants with DL-[alanine-3-^{14}C]-tryptophan (XXXII) and three weeks later isolated active ibogaine (XXXIII). The incorporation (6.7%) is stated by Leete to be the highest he has obtained into an alkaloid elaborated by a higher plant. Most of the activity was located at C-7, thus providing yet another instance of tryptophan being a direct precursor of an indole alkaloid. Work is proceeding on the origin of the non-tryptophan derived portion of ibogaine.



(XXXII) (XXXIII)

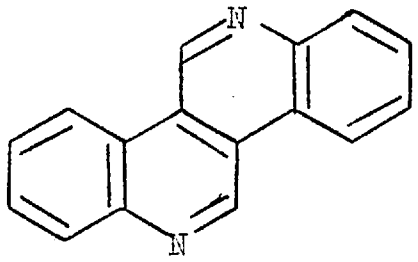
CHAPTER FIVE: THE CALYCANTHACEOUS ALKALOIDS

Calycanthine, the principal alkaloid of this family, was isolated in 1888 from the seeds of *Calycanthus glaucus* Willd.[46] It has since been found in several other species of the order Calycanthaceae and in addition two minor alkaloids, calycanthidine and folicanthine, have been isolated. A fourth alkaloid, chomonanthine, was extracted[47] from the leaves of *Chimonanthus fragrans* Lindle, apparently erroneously equated with *Meratia praecox* Rehd. and Wils. Little can be said here to supplement Robinson's recent review[48] on alkaloids containing the Ph - N - C - N system, but some discussion of calycanthine and related dimeric alkaloids will be useful as a background to the biosynthetic work described in the next chapter.

Calycanthine.

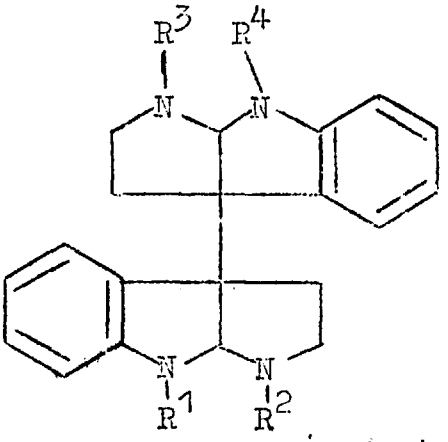
Early degradative procedures, reviewed[49] by Marion and Saxton, led to the production from this alkaloid of a variety of nitrogenous heterocycles, including tryptamines, indoles, β-carbolines, and quinolines. An important clue to the structure of calycanthine

($C_{22}H_{26}N_4$) came from the elucidation[50] of the structure of one of its dehydrogenation products, calycanine. X-Ray examination[51] of the compound showed the molecule to be symmetrical and favoured the molecular formula $C_{16}H_{10}N_2$. Calycanine was found[50] to be identical with synthetic quinolino (3',4':4,3) quinoline (I).

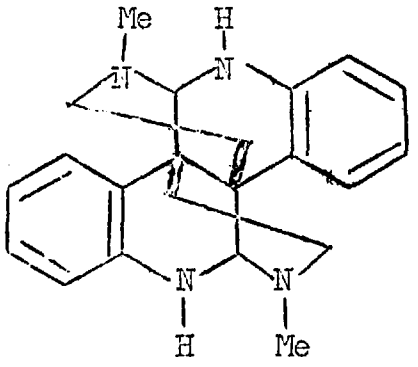


(I)

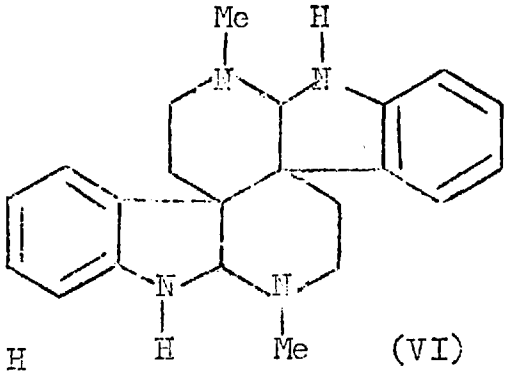
Biogenetic speculations,[50,52,53] taking into account the available chemical evidence, led to the consideration of five possible formulae, (II, V to VIII) for calycanthine. The facile production of N_b-methyltryptamine on pyrolysis suggested the obvious hypothesis that the alkaloid was formed by the oxidative dimerisation of two tryptamine units. Assuming the normal electron availability in indoles, ββ' coupling should take place preferentially.



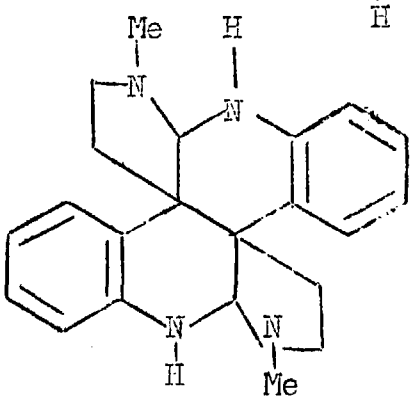
(II; $R^1=R^3=H$, $R^2=R^4=Me$)
(III; $R^1=R^2=R^3=R^4=Me$)
(IV; $R^1=R^2=R^3=Me$, $R^4=H$)



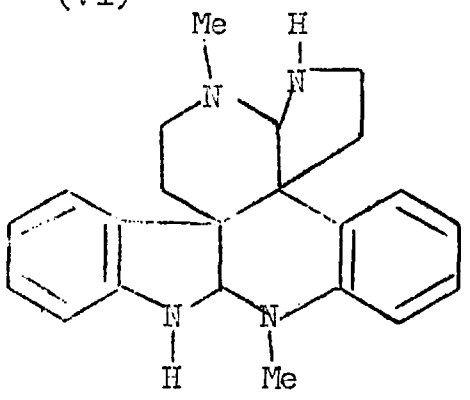
(V)



(VI)

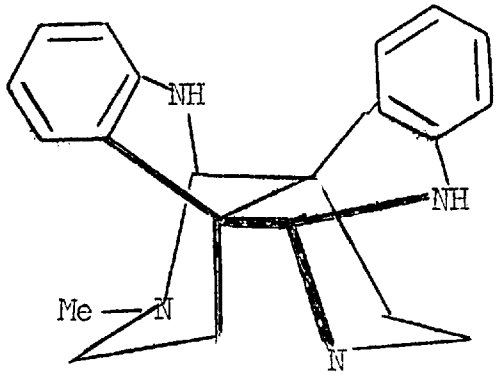


(VII)



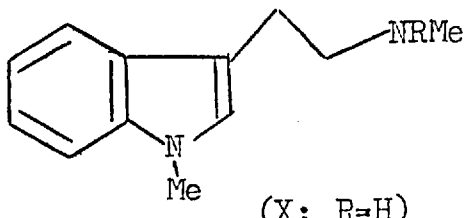
(VIII)

This structural problem was solved by two independent methods. Hamor and his co-workers[52] performed an X-ray crystallographic analysis on calycanthine dihydrobromide dihydrate and showed that structure (V) was the correct one. This conclusion was also reached by Woodward's group[50] who rationalised the chemical evidence on the basis of this structure. The stability of calycanthine to acid suggested that it had the configuration most favoured sterically and the structure and configuration (IX) was proposed for natural (+)-calycanthine. This was, in fact, found[54] to be the absolute configuration.

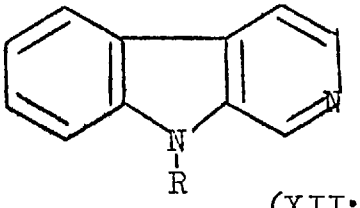


(IX)

Folicanthine.



(X; R=H)
(XI; R=Me)



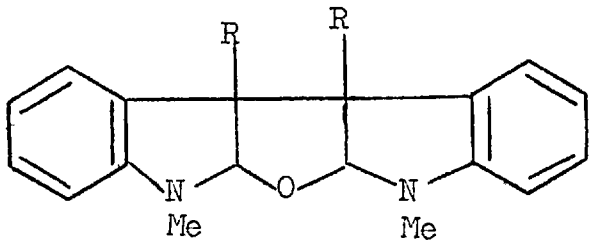
(XII; R=Me)
(XIII; R=H)

Initial studies on folicanthine gave three main degradation products, which were identified by Hodson and Smith.[55]

Treatment with acid, or zinc dust distillation, gave $\underline{N}_a$-methyl-β-(2-methylaminoethyl)indole (X). Methylation of this compound or Hofmann degradation of folicanthine methiodide gave $\underline{N}_a$-methyl-β-(2-dimethylamino)indole (XI). Treatment of folicanthine with silver acetate in 1% aqueous acetic acid gave crystalline 9-methylnorharman (XII), apparently by cyclisation of the $\underline{N}_b$-methyl group. The ultraviolet spectrum of folicanthine indicated an indoline and also a Ph - N - C - N system.[56] The presence in the molecule of two symmetrically dimerised $\underline{N}_a\underline{N}_b$-dimethyltryptamine residues was strongly indicated by reductive fission with zinc/hydrochloric acid to give $\underline{N}_a\underline{N}_b$-dimethyl-2,3-dihydrotryptamine.[56]

Basic treatment of folicanthine dimethiodide gave a product which was formulated[57] as (XIV). On treatment with acid this compound gave a mixture of $\underline{N}_a$-methyl-β(2-dimethylaminoethyl)indole (XI) and the corresponding oxindole. Structure (XIV) was later verified[58] by syntheses of the racemates (XIV) and (XV) both of which underwent acid decomposition to the required indole-

oxindole mixtures. Folicanthine was thus proposed[57] to have the structure (III), the mechanism of the indole-oxindole reaction proceeding by protonation of the $\underline{N}_b$-atoms and appropriate cleavage.



(XIV; R=$(CH_2)_2NMe_2$)
(XV; R=Me)

Chimonanthine.

The C_{22} formulation of this alkaloid was supported by potentiometric titration. The ultraviolet spectrum again indicated the presence of a Ph - N - C - N system in the molecule. The alkaloid contained two N-Me and two N-H groups and on reduction with zinc/hydrochloric acid afforded β-(2-methylaminoethyl)indoline. From these data and from comparison with the structures assigned to calycanthine and folicanthine, the structure (II) was proposed[47] for chimonanthine. $\underline{N}_a$-Dimethylation of chimonanthine gave folicanthine[59] in verification of

this proposal. The mass spectra of these two alkaloids indicated an easy symmetrical cleavage of the molecules,[60] and thus supported structures of type (II) rather than (VI). Final confirmation of formula (II) for chimonanthine and hence formula (III) for folicanthine was supplied by X-ray crystallographic analysis of chimonanthine dihydrobromide.[61]

Calycanthidine.

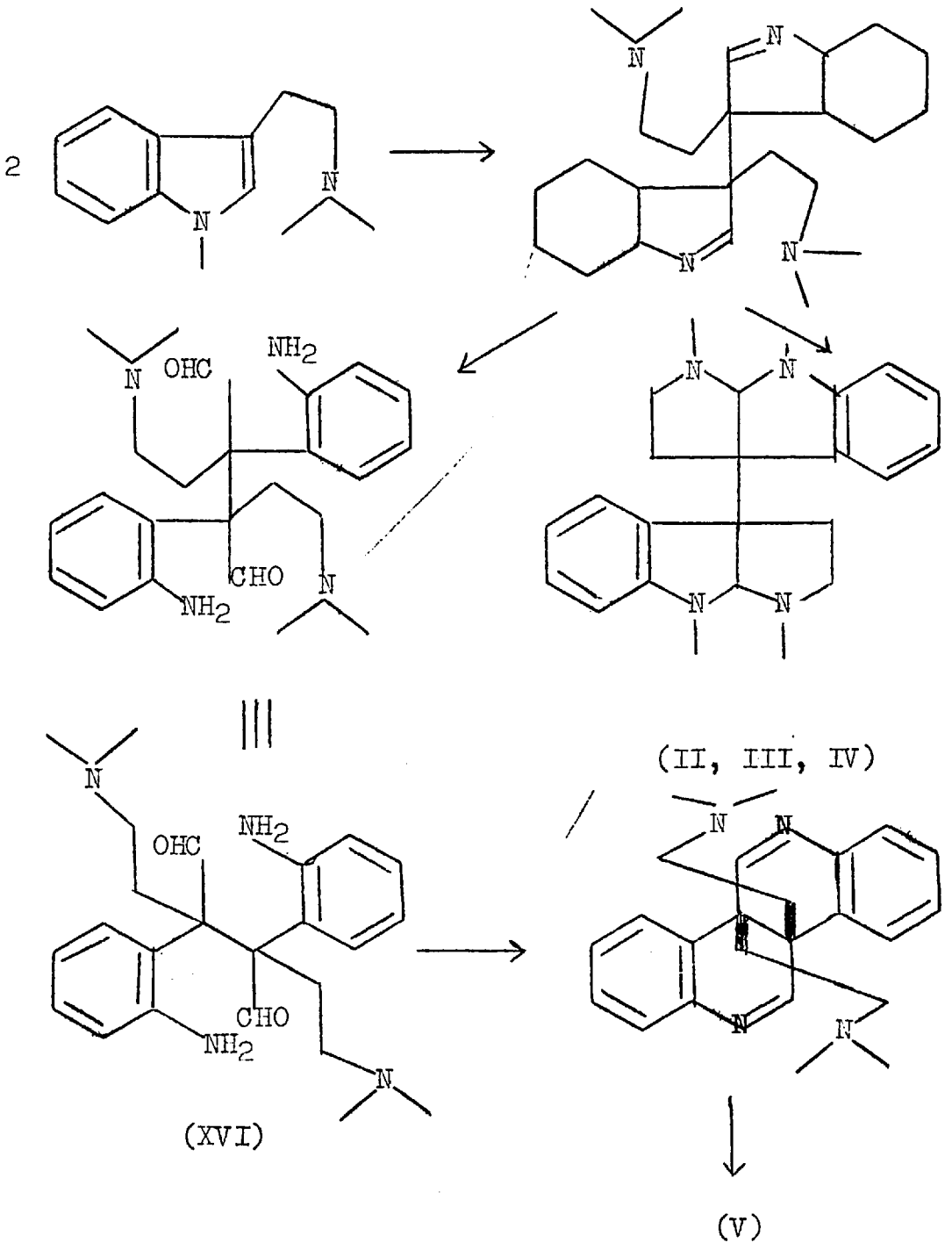
The correct molecular formula of this alkaloid was shown[62] to be $C_{23}H_{28}N_4$. The molecule contains one active hydrogen, no isolated double bond and no C-methyl group. Its ultraviolet spectrum indicated the presence of a Ph - N - C - N system. Zinc dust distillation gave[53] norharman (XIII) whilst reduction with zinc/hydrochloric acid gave a mixture of β-(2-methylaminoethyl)-indoline and N_a-methyl-β-(2-methylaminoethyl)indoline.[62] It was concluded[62] that calycanthidine is the intermediate stage in the N_a-methylation of chimonanthine to folicanthine. Comparison of the n.m.r. and mass spectra of the three alkaloids gave added support to this deduction, and the N_a-mononethylation of chimonanthine to calycanthidine and of the latter to

folicanthine provided adequate confirmation.[59] Calycanthidine therefore has the structure (IV).

Biogenesis.

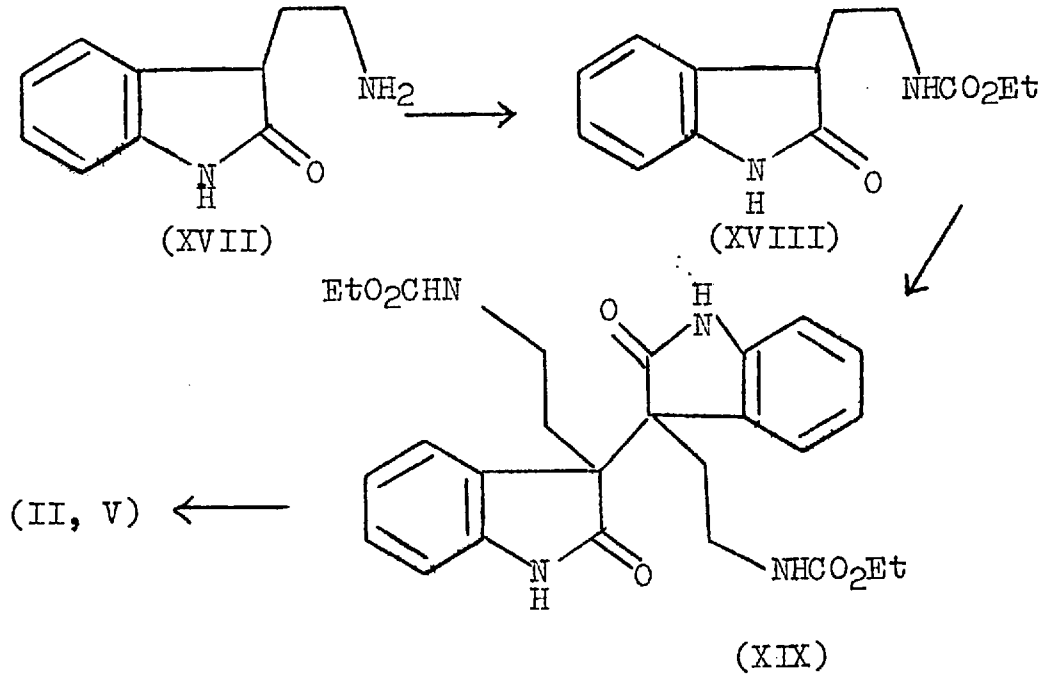
As has been indicated, the biogenesis of these alkaloids is readily explained by the ββ' oxidative dimerisation of two tryptamine units.[50,52,53] It has been stated[50] that the occurrence of calycanthine in species classified in two unrelated botanical orders (Calycanthus spp. and Meratia praecox) is indicative of a simple biogenesis. This, however, is not necessarily so. To cite just one example, the complex diterpenoid alkaloid, lycoctonine, has been found in both the orders Ranunculaceae and Compositae.[63] The production of N_a-methytryptamine on pyrolysis of calycanthine is a better indication of a simple biosynthetic pathway. The formation of indoles as well as quinolines on degradation of calycanthine can be envisaged from the scheme below as occurring by reversal of the final step and rearrangement of the parent tetra-aminodialdehyde (XVI). The isolation of all four alkaloids from Calycanthus floridus supports the idea of a common biosynthetic pathway.[59] No compounds resulting from the alternative cyclisations (VI), (VII) and (VIII) have yet

been detected, but it is suggested that eventually all five polycyclic systems will be found in nature.[64]



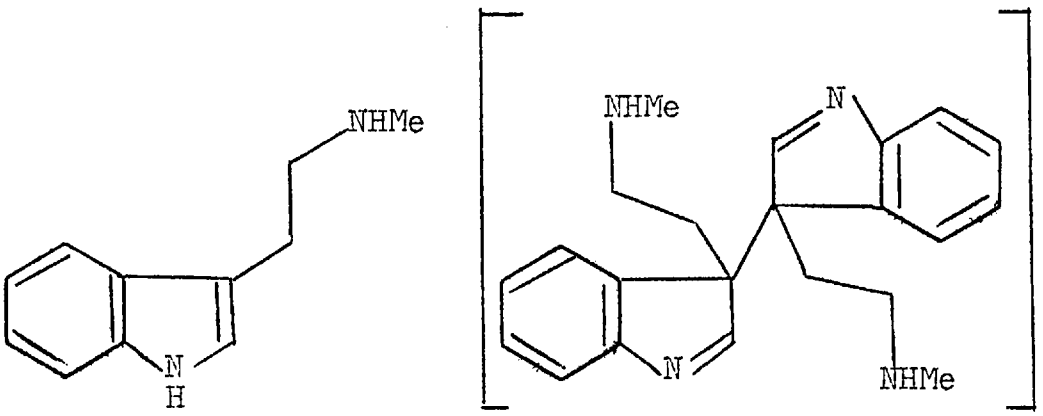
Total synthesis.

Two recent syntheses of (±)-chimonanthine have followed routes chemically analogous to the proposed biosynthetic pathway. Henrickson, Göschke and Rees[64] treated oxytryptamine (XVII) with ethyl chloroformate in chloroform to give N-carbethoxy-oxytryptamine (XVIII). The urethan grouping was utilised to prevent oxidation of the secondary amine. Treatment of the oxindole enolate with iodine in benzene yielded the two diastereoisomers represented by structure (XIX). In both cases, reduction of the diners with lithium aluminium hydride yielded a separable mixture of basic products. Two of the components were identified as (±)-chimonanthine (3% yield) and (±)-calycanthine (0.2%). Treatment of (±)-chimonanthine with 0.01 M hydrochloric acid gave N_b-methyltryptamine and (±)-calycanthine; similar treatment of natural (±)-calycanthine gave mostly unchanged alkaloid but some crude chimonanthine was isolated. This interconvertibility by acid-catalysed isomerisation is further proof for the existence of a biosynthetic intermediate such as the tetra-aminodialdehyde (XVI).



An elegant synthesis of (±)-chimonanthine was devised by Scott, McCapra, and Hall.[65] N-Methyltryptamine was dimerised directly to the alkaloid without hydrolytic cleavage of the indolenine or di-(N-acetal) groupings. Treatment of N-methyltryptamine (XX) with one equivalent of methylmagnesium iodide and reaction of the Grignard derivative with ferric chloride yielded (±)-chimonanthine in 20% yield. One of the synthetic fractions also proved to be identical with meso-chimonanthine, isolated from a crude sample of natural (-)-chimonanthine. The authors state that this direct

dimerisation may be a more relevant biosynthetic analogy than synthesis involving tryptamine units at a higher oxidation level.

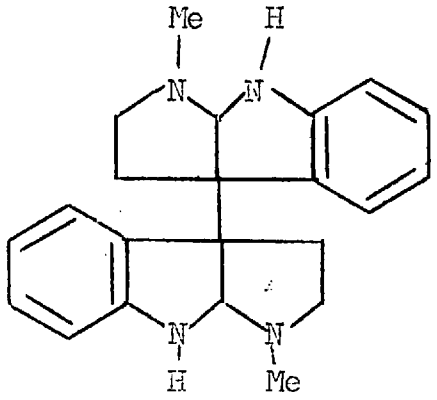


(XX)

(II)

CHAPTER SIX: DISCUSSION OF THE BIOSYNTHESIS OF CHIMONANTHINE

Introduction.



(I)

Since tryptophan has proved to be the biological precursor of the β-indolylethyl fragment in all the indole alkaloids so far examined, it seemed reasonable that this would be so for the simple dimeric alkaloid, chimonanthine (I). However, the steps leading from the amino-acid to the final alkaloid are not immediately obvious. In particular, the stage at which the N-methyl groups are introduced and the mechanism of the dimerisation reaction deserve study. At the onset of the present investigation it was necessary to devise suitable feeding methods for the shrub Chimonanthus fragrans.

An obvious choice for the precursor in a preliminary experiment was tryptophan.

Tryptophan feeding.

Since chimonanthine is found in the leaves of Chimonanthus fragrans, it was thought that the whole plant would not be necessary for the biosynthesis of the alkaloid. Accordingly, the stem of a detached leaf was placed in the radioactive solution (DL-[alanine-3-^{14}C]-tryptophan, 0.01 mc.) contained in a sealed "poly-top" tube. A current of warm air was blown over the leaf spores to assist take-up. Over 99% of the radioactivity was absorbed by the plant. After three days, the plant was crushed under alcohol and the residues were extracted by a method kindly commended to us by Dr. G. F. Smith and described[56] in his work on folicanthine. The basic fraction (0.1% incorporation) was chromatographed on alumina, and weight and activity histograms were plotted for the various fractions (Fig.6) The presence of an alkaloidal impurity (fractions 4 - 7) of lower specific activity than the chimonanthine (fractions 8 - 10) was clearly indicated. This may be significant in view of the isolation by Scott and his co-workers[65] of meso-chimonanthine from a crude sample of natural

Fig. 6. Chromatography of tryptophan feeding

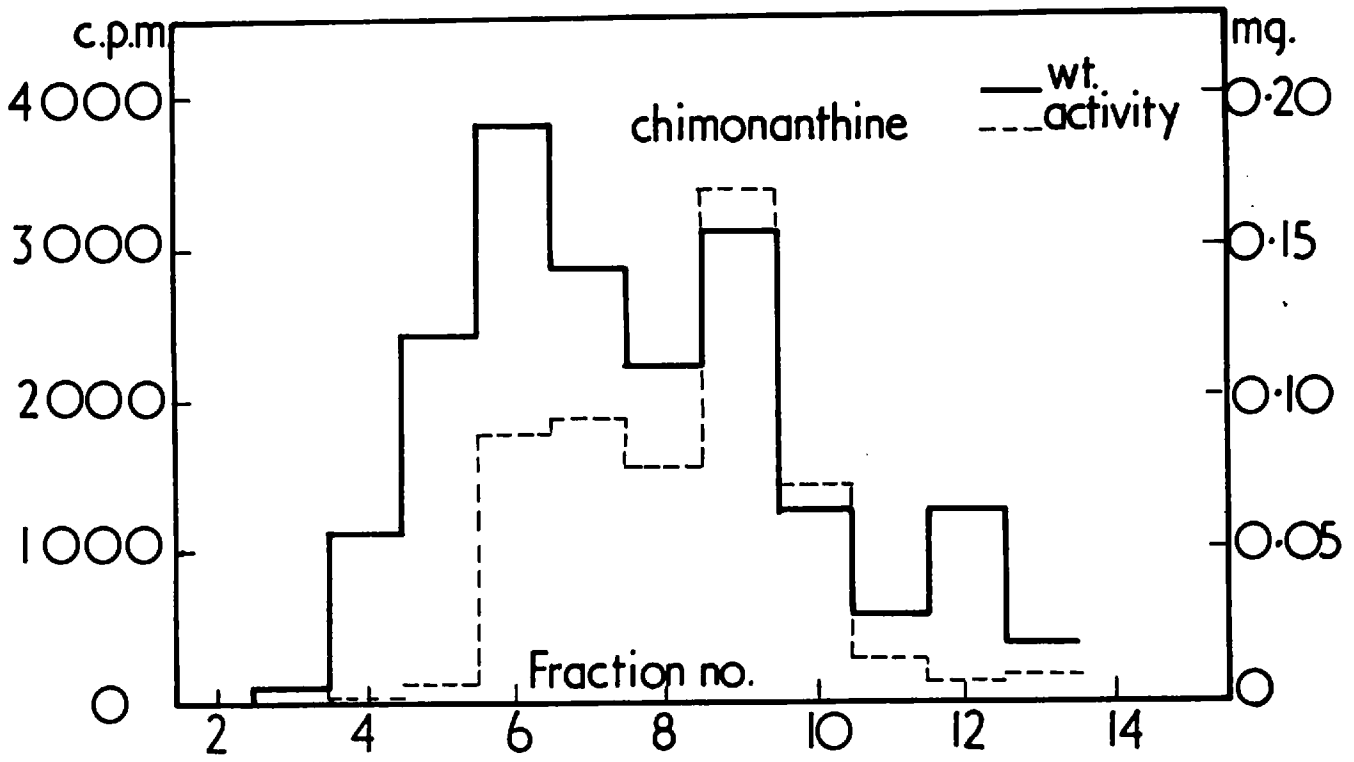
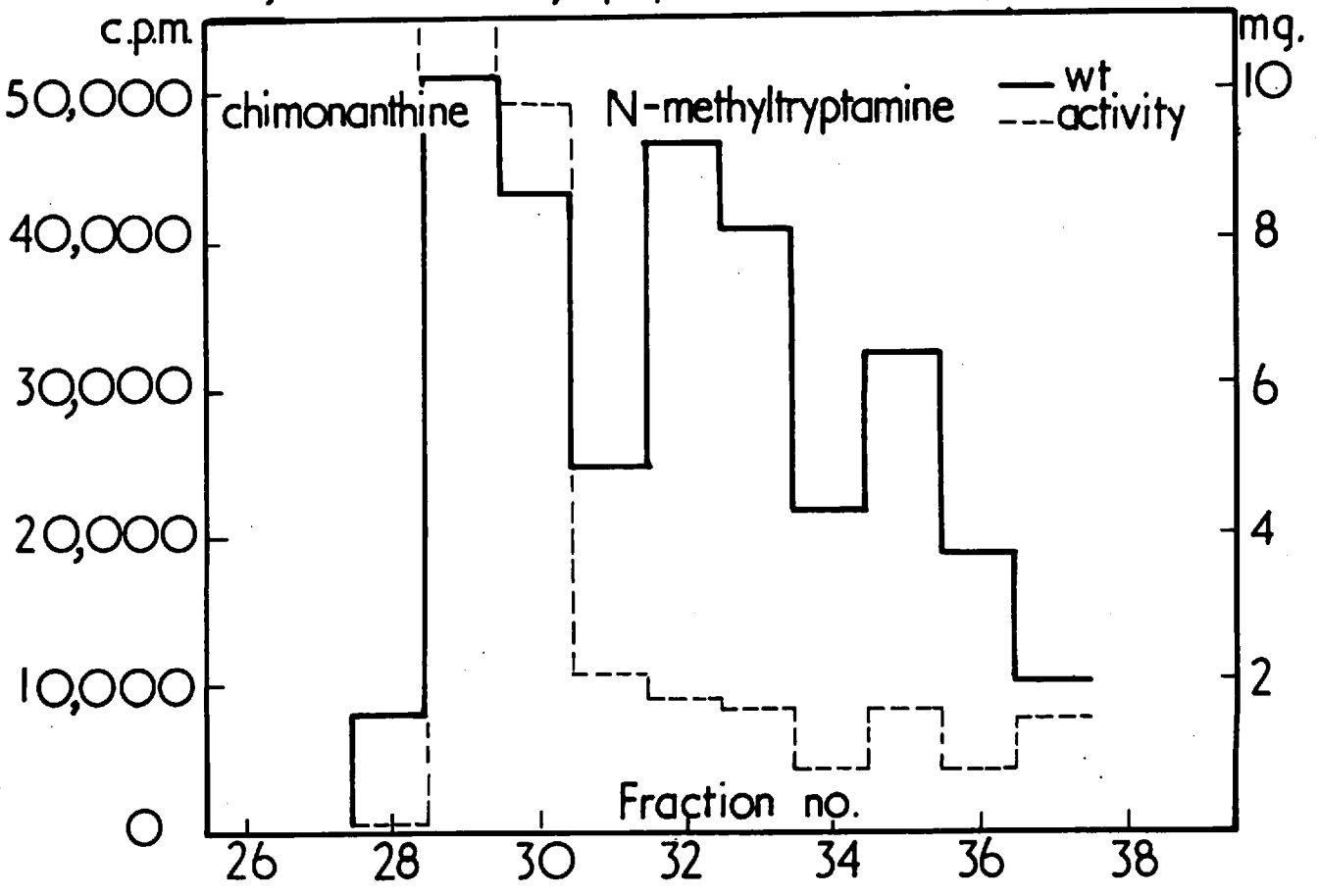


Fig. 7. Chromatography of N-methyltryptamine feeding



(-)-chimonanthine. The chimonanthine fractions (8 - 11) were diluted with inactive (-)-alkaloid obtained partly by extraction of Chimonanthus leaves and partly by Dr. G. F. Smith's gift of a sample. There was a modest drop in activity on crystallisation of the diluted material from benzene but on recrystallisation the specific activity of the alkaloid remained constant (0.06% incorporation).

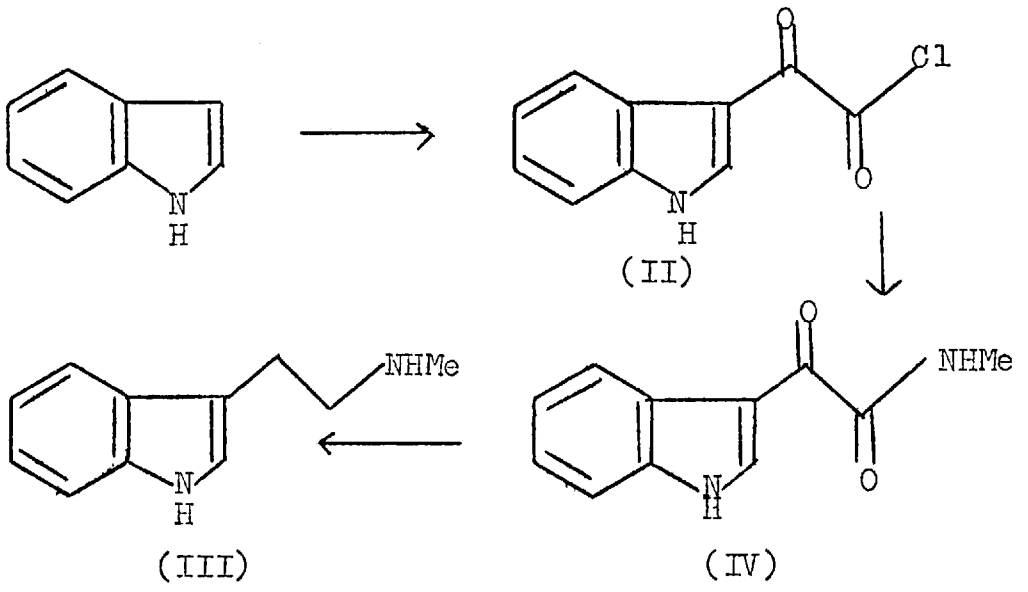
This result showed that detached leaves were capable of elaborating the alkaloid with reasonable efficiency - incorporations of 0.01% or lower are not uncommon in work with certain species (see, for example, ref.66). As supplies of chimonanthine were very low, no degradative work was performed. In cases where indole alkaloids derived from tryptophan labelled in the side-chain have been degraded (Chapter Four), the labelling position has accorded with expectation and no "scrambling" has been observed.

N-Methyltryptamine feeding.

The chemical conversion of N-methyltryptamine into (±)-chimonanthine by a one-step process[65] provides some support for the amine or its equivalent being the

immediate biological precursor of the dimeric structure. To test this point, the synthesis of N-[methyl-^{14}C] methyltryptamine was undertaken, in collaboration with Dr. R. H. Prager. It is interesting to note that the alkaloid dipterine, isolated from the Chenopodiaceae Girgensohnia diptera, is N-methyltryptamine.[67]

The route chosen for the synthesis was a modification of that described in the literature. 3-Indoleglyoxylyl-chloride (II) was obtained in high yield from the condensation of oxalyl chloride with indole.[68,69] The preparation of N-methyl-3-indoleglyoxamide (IV) from the acid chloride is briefly described in the literature[68] but attempts to repeat this procedure gave amorphous mixtures. However, a modification using methylamine in dimethylformamide gave a 38% yield of pale yellow crystalline product. The m.p. of the amide was 97° higher than that quoted[68] but its infrared and n.m.r. spectra were in accordance with the assigned structure. Reduction with lithium aluminium hydride and aluminium chloride in dioxan[70] gave N-methyltryptamine (III), which was converted into the hydrochloride. Another method,[71] involving reduction of N-methyl-N-tosyltryptamine with sodium and ammonia, was less satisfactory.



The sequence was repeated with [^{14}C]methylamine hydrochloride to give the required active amine in 30% radiochemical yield. Assay by thin-layer chromatography showed that 90% of the activity was present in the N-methyltryptamine spot and the precursor (0.03 mc.) was fed without further purification to the plant at pH 3-4. For dilution of the extracted basic fraction, synthetic (±)-chimonanthine, generously provided by Professor A. I. Scott, was used. Since the amount of racemic material used for dilution was large relative to the amount of natural chimonanthine present in the plant, this procedure should not have been a reason for appreciable decrease in activity in later recrystallisation. Inactive dipterine was also added to the crude basic fraction as unmetabolised N-methyltryptamine was very likely to be a contaminant.

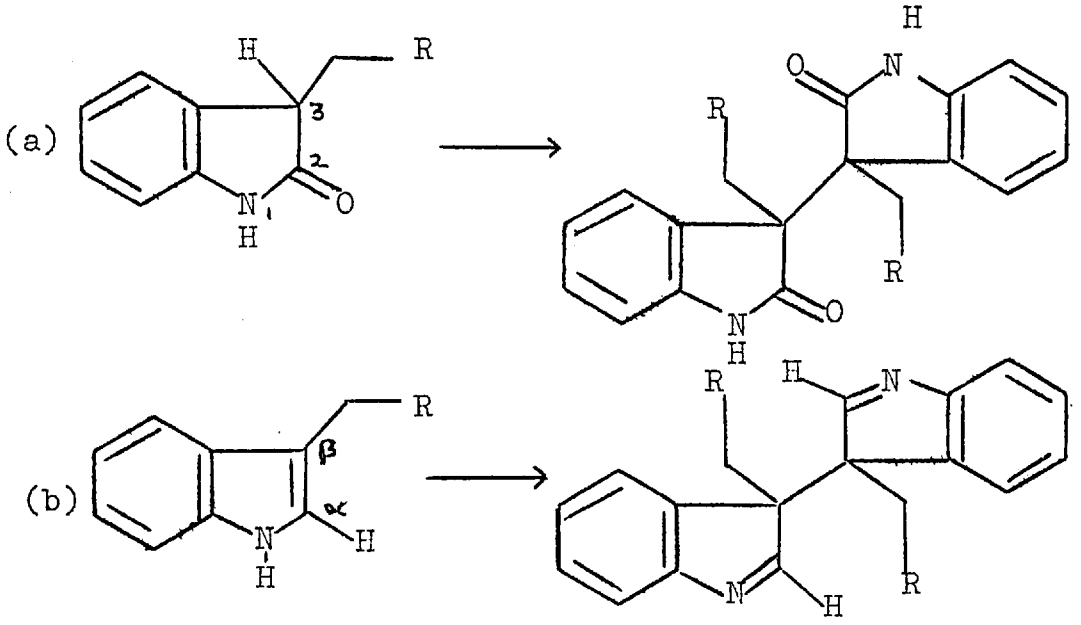
Clear separation of the two bases proved difficult but was effected after three chromatographic runs on alumina (1000-fold excess). The specific activity of the chimonanthine fractions (29 and 30) from the second chromatography (Fig.7) indicated high activity in the alkaloid (3% incorporation) and little in the dipterine. However, rechromatography of the chimonanthine fractions caused a drastic drop in activity and the presence of a highly active component with similar chromatographic properties to chimonanthine was clearly indicated. Four crystallisations from benzene caused a further drop in activity; a steady value was not obtained but the incorporation was less than 0.011%. A small amount of activity (0.1%) was found in the recovered N-methyltryptamine, which was recrystallised to constant activity from petroleum ether and converted to the corresponding hydrochloride of the same specific activity.

It thus appears that N-methyltryptamine is not as an efficient a precursor for chimonanthine as is tryptophan. The tentative conclusion is that methylation occurs after dimerisation. Prager[72] observed similarly that N-methyltryptophan and N-methyltryptamine were not incorporated into ajmaline whereas tryptophan was.

Another possibility is that the oxidative dimerisation does not involve simple tryptamine units. This point will be discussed in the next section.

Synthesis of [2-^{3}H]-labelled indole derivatives.

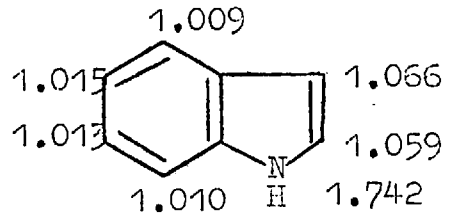
Two attractive mechanisms for the biological dimerisation of indole derivatives are suggested by the chemical syntheses referred to earlier: namely routes (a) and (b). The nature of R is as yet not determined but the decision between an oxindole or an indole intermediate can be made by labelling the α-proton [* in (b)]. This proton must clearly be lost in the first route and retained in the second.



The experimental approach involves the synthesis of tryptophan and tryptamine derivatives labelled with tritium at C-2 and with ^{14}C at a convenient carbon atom in the molecule. The ^{14}C label would then serve as an internal reference for determining loss or retention of tritium during conversion into chimonanthine. DL-[alanine-3-^{14}C]Tryptophan is commercially available and labelled tryptamine can be obtained from it by decarboxylation. The problem therefore is to label the molecule specifically at C-2 with tritium.

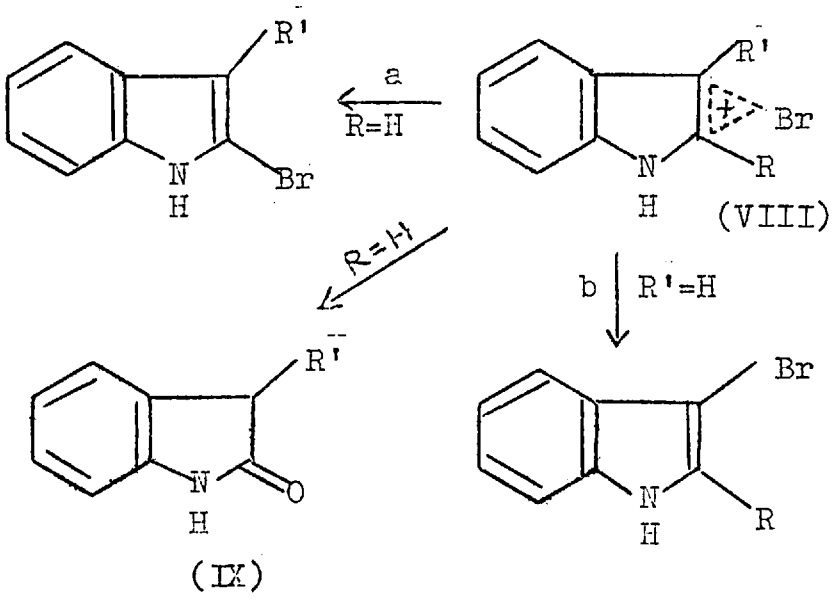
In general, electrophilic substitution of the indole nucleus occurs at position 3; if this is blocked, the 2-position is attacked. The benzene ring of indole is only attacked if positions 2 and 3 are already occupied and vigorous conditions are required. Selective attack of the 2-position was reported in a few cases in the earlier literature. For instance, Borgo and Mazzaro[73] described the formation of 2-chloroindole and then 2,3-dichloroindole on treatment of indole with sulphuryl chloride. The identity of the monochloro-derivative was not, however, proved and it is more likely to be the 3-substituted compound. Speeter and Anthony[74] found that the compound formulated as 2-indoleglyoxamide was in

fact 3-indoleglyoxamide. Substitution reactions of indoles are summarised by Julian, Meyer, and Printy[75]; the reactivity of the various positions of the indole nucleus to electrophilic attack can be simply represented[76] by the system (VII), showing π-electron densities calculated by the molecular orbital method.



(VII)

Recorded instances of the bromination of indoles in non-aqueous media are summarised by Witkop and his co-workers.[77] They postulate an intermediate bromonium ion (VIII) to account for α- and β-substitution and for the formation of oxindoles (IX) by oxidation of indoles in aqueous media with N-bromosuccinimide.



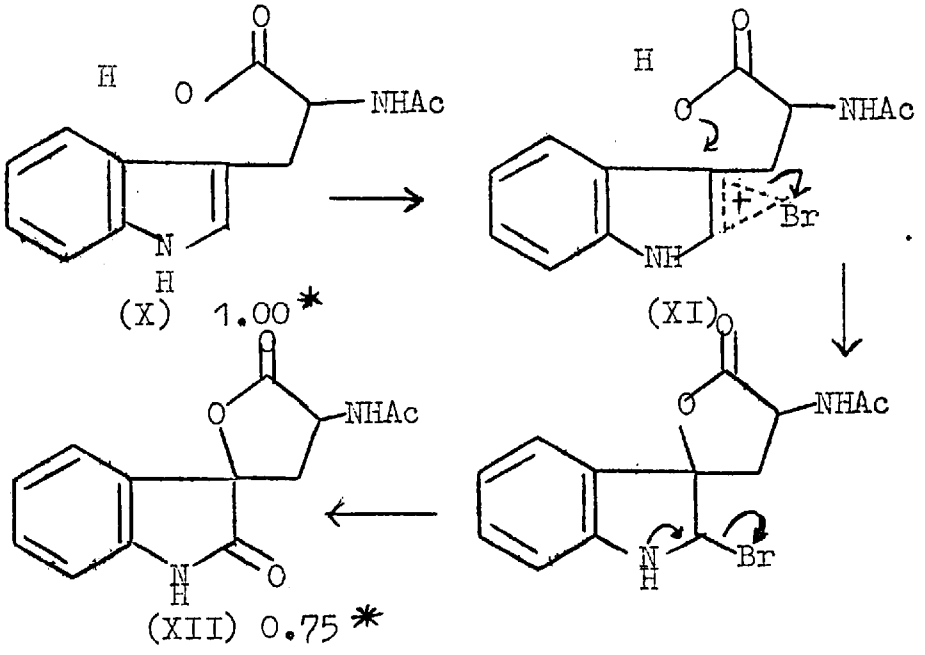
Several deuteration studies have been carried out with indole itself and with substituted indoles. Koizumi and his co-workers[78] found that between pH 7 and 2.5 the NH proton of indole exchanges rapidly with deuterium. Between pH 2.5 and 0.5 the β-proton also exchanges, and below pH 0.5 (> 0.8N HCl) it is stated that further exchange of the α-proton occurs. Hinman and Whipple[79] point out, however, that α-exchange was not proved in this work since the results were obscured by extensive dimerisation and exchange in the benzenoid ring may have been taking place. The Japanese workers[80] found exchange was much slower under alkaline conditions.

Pfleninger and his co-workers[36] obtained hepta-deuteroindole by heating the base with aqueous alkali at 300°. They also prepared indole specifically deuterated in the 5- and in the 6-position by reduction of the 2-carboxylic acid of the corresponding bromo-compound. Witkop and his co-workers[81] and Jardine and Brown[82] found that N-deuterated indoles could be prepared in neutral solution by evaporation from solvent containing D_2O. Jardine and Brown[82] further state that considerable deuteration (50% isotopic purity) occurs at the 2-position by this procedure. However, Hinman and Baunan[83] state that it is difficult to reconcile the latter result with their own work or that of Koizumi.[78] These workers prepared [1,3-^{2}H]indole by refluxing a solution of indole in D_2O containing 5 x 10^{-3}M H_2SO_4 and this in turn was converted to [3-^{2}H]indole (87% isotopic purity) by refluxing the dideutero-compound with water. Hinman and Whipple[79] found that for substituted indoles such as 1,3-dimethylindole and skatole, slow exchange of the α-proton occurred in acid solution. This result is of course consistent with the observation that electrophilic substitution of the α-position occurs when the 3-position is blocked.

It thus seemed possible that, for the present work, selective exchange of the α-hydrogen of tryptophan could be induced by using appropriate conditions. Preliminary deuteration experiments were carried out with indole, and the extent of substitution at the 2- or 3-position was followed by n.m.r.[81] Exchange of the 2- and 3-protons and of the equivalent of two benzene protons was observed on heating indole in D_2O in the presence of pre-reduced Adams catalyst. Only 3-deuteration was observed under alkaline conditions. The exchange of tryptophan was then investigated. However, in this compound the chemical shift of the α-proton is decreased to a lower τ value and the hydrogen is not distinguishable from the benzenoid ones. Under alkaline conditions, little change was observed in the n.m.r. spectrum. However, heating with 4N DCl for 2 hours, or with 6N D_2SO_4 for ½ hour, caused a 4/5ths decrease in the integration area of the aromatic protons. Heating with 4N DCl for 6 hours caused, allowing for the 4% dilution of tryptophan protons added, complete exchange of the five ring protons.

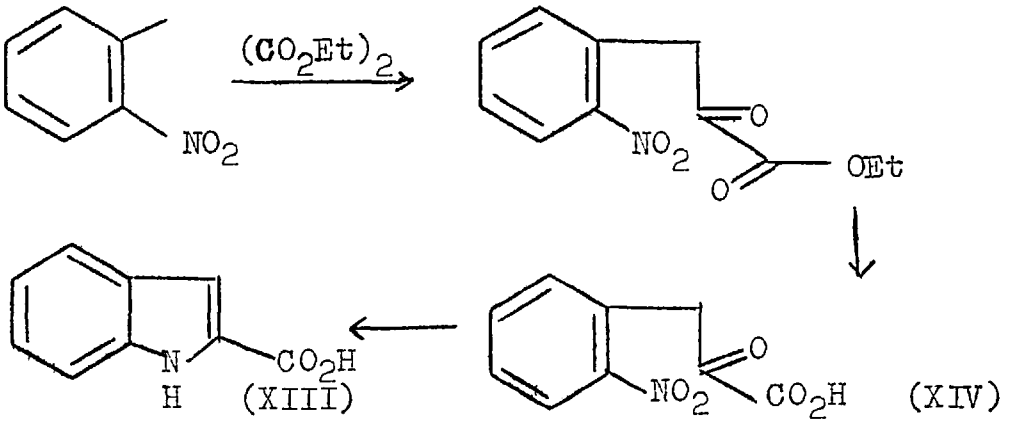
The same procedure was therefore used to prepare generally tritiated tryptophan. Isolation of the amino-acid as its hydrochloride and precipitation several

times at the iso-electric point to remove -ND, $-ND_2$, and $-CO_2D$, gave DL[2,4,5,6,7-^{3}H]tryptophan. The tryptophan was converted to its N-acetyl derivative[84] for counting and the activity at the 2-position was assayed by conversion to the lactone (XII) by treatment with one mole of N-bromosuccinimide.[85] This compound was chosen as it contains neither 2 nor 3-protons; the formation of a simple oxindole might conceivably involve migration of a proton from the 2- to the 3-position. The formation of the lactone can be explained by a concerted displacement reaction on the transient bromonium ion (XI). The α-position was found to contain 25% of the total activity; equal distribution of the label would require 20% at each position.



This generally labelled precursor was suitable for use and it was in fact fed as a doubly labelled precursor with DL-[alanine-3-^{14}C]tryptophan. However, in view of the low supply of inactive chimonanthine for dilution it was deemed necessary to reserve this for dilution of alkaloid derived from tryptophan labelled specifically at the 2-position, as this would enable any loss of tritium to be determined more accurately.

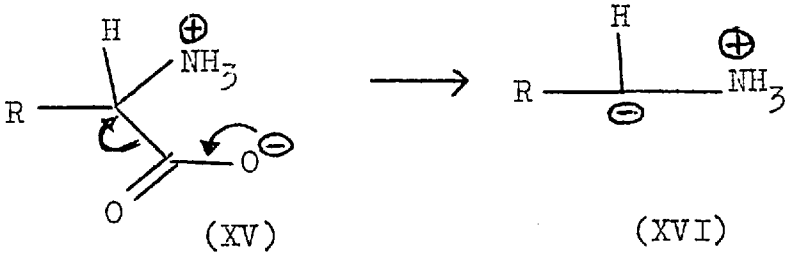
Decarboxylation of indole-2-carboxylic acid (XIII) is known to proceed smoothly[86] and it was thought that this would be a useful process for introducing tritium specifically into the 2-position. Accordingly, the acid was synthesised from o-nitrotoluene and diethyl oxalate by a modification[87] of the Reissert synthesis, followed by reductive cyclisation of the resulting o-nitrophenylpyruvic acid (XIV) with ferrous sulphate and ammonia.[88]



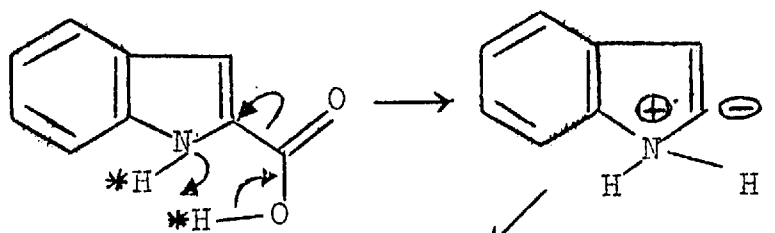
The acid was recrystallised from D_2O to introduce two deuterium atoms [ND and CO_2D] and then decarboxylated at 240°. Deuterated indole in 9% chemical yield was obtained on chromatography of the pyrolysis product. This yield was sufficient for the present work but could probably be improved by heating with copper chromite in quinoline.[89] The n.m.r. spectrum of the deuterated product showed the absence of any peak in the α-proton region but a new band, probably due to an impurity, appeared in the benzenoid region. The pyrolysis was repeated with tritiated material, and the indole was diluted with inactive material and recrystallised from water, without any loss of activity. The specific gravity of the labelled indole was higher than that calculated on the assumption that only one labelled atom had been introduced into the molecule, and it was therefore advisable to assay the material.

It could not be assumed that the label was confined exclusively to the 2-position. Not only could the mildly acidic conditions of the pyrolysis have caused some exchange in the 3-position but also the mechanism of decarboxylation could conceivably have involved migration of a proton from the 2- to the 3-position.

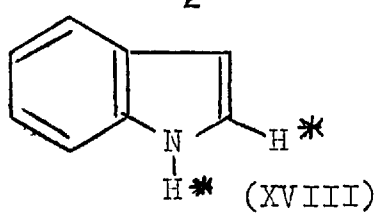
It is known that α-amino-acids (XV) such as tyrosine and tryptophan, but not their sodium salts or hydrochlorides, decarboxylate fairly readily to give good yields of the corresponding amine. This would appear to involve generation of a zwitterion (XVI). If a similar mechanism occurs in the decarboxylation of indole-2-carboxylic acid (XVII) then the label in the recrystallised indole should be present exclusively in the 2-position (XVIII). However, an alternative mechanism can be written whereby the acid (XIX) forms an indolenine intermediate (XX) and in this case, heavy labelling of the 3-position would occur.



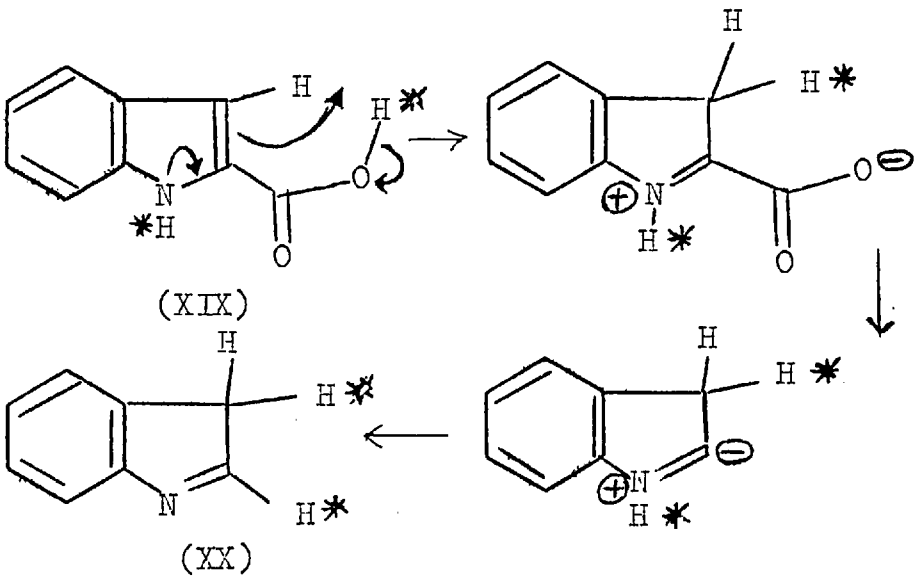
(XV) (XVI)



(XVII)



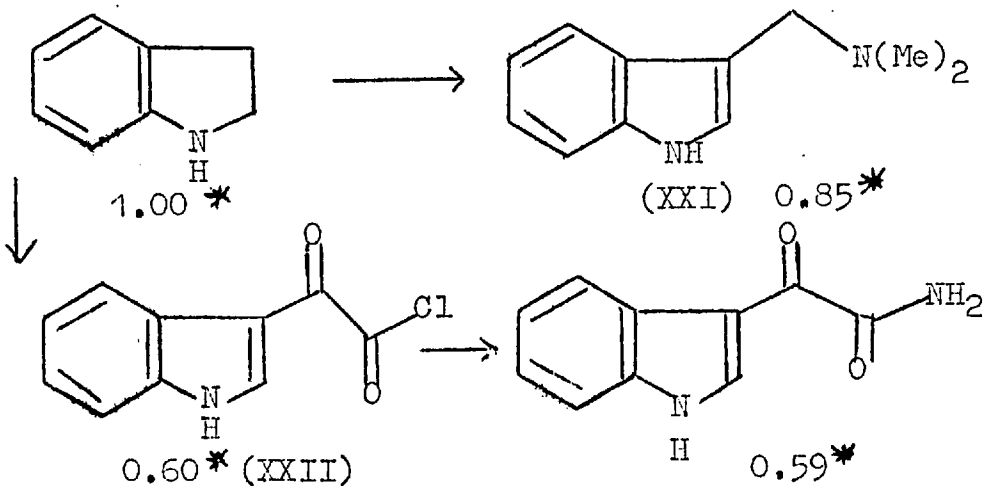
(XVIII)



(XIX)

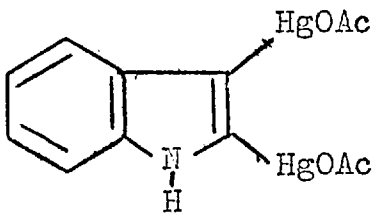
(XX)

It was thus necessary to assay the activity at the 2- and 3-positions by reactions not involving strongly acidic conditions. For the 3-position, gramine (XXI) seemed a suitable derivative. This was prepared[90] and only 16% of the activity was found at this position. Condensation with oxalyl chloride to give the acid chloride (XXII) caused a larger decrease in activity (40%) but obviously this could have occurred by acid-catalysed exchange. The conversion of gramine to tryptophan and hence tryptamine under non-acidic conditions can be accomplished by modification of known procedures[90] and the present synthesis constitutes a route to the required [2-^{3}H] precursors.

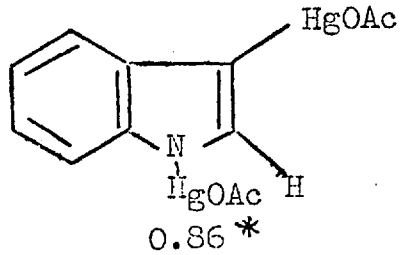


Although there can be little doubt that the α-position is heavily labelled it seemed worthwhile to attempt to determine the activity precisely. The exact value in the labelled tryptophan can, of course, be determined by formation of the lactone (XII) described earlier. Surprisingly little is known about the substitution of indoles at the 2-position. Chlorination with sulphuryl chloride[73] and alkylation both involve acidic conditions. It seemed that the preparation of indole dimercuriacetate, which can be accomplished quantitatively under neutral conditions,[91] would be a suitable reaction. However, when the labelled indole was converted into this derivative, only a small drop in activity was observed. In fact, the derived dimercuriacetate had the same activity as the gramine described

earlier. Clearly the structure (XXIII) assigned to the derivative,[91] and apparently still accepted,[92] is incorrect and the alternative form (XXIV) or even the indolenine (XXV) are more likely. It is possible that in the original work, isomerisation of the first formed compound, possibly (XXIV), occurred during recrystallisation from hot acetic acid.

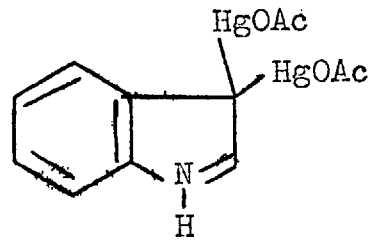


(XXIII)



0.86 *

(XXIV)



(XXV)

EXPERIMENTAL

M.p.'s were taken on a Kofler block. Merck aluminium oxide G was used for thin-layer chromatography. [^{14}C]Activities were measured by plating out thin films (*ca.* 0.5 mg./cm.^{2}) and were not corrected for self-absorption or back-scattering. A gas-flow (methane) proportional counter, constructed by Dr. D. W. Turner, was used, giving a background of *ca.* 20 counts per min. (c.p.m.) and an efficiency of 50%). [^{3}H]Activities were measured, unless otherwise stated, in dimethylformamide-xylene scintillator solution on an I.D.L. measuring head. The efficiency (14-20%) for each system was determined by addition of [1,2-^{3}H]hexadecane, corrected for ageing, as an internal standard. Measurements were made in duplicate and samples were counted for a period of time sufficient to give a standard deviation from the mean of less than 5%. Incorporations were calculated from the total activity of the administered precursor, the specific activity of the purified alkaloid, and the yield of crude alkaloid after chromatography. Proton magnetic resonance spectra were determined by Mrs. A. I. Boston on a Varian A60 spectrometer at 60 Mc.p.s.,

using tetramethylsilance as an internal standard. Reference to more detailed prooedures in laboratory notebooks is made by giving the appropriate page numbers after each side-heading. The above remarks apply to both Parts I and II of the Experimental section.

PART I

Isolation of alkaloids. 105. Non-viable seeds (100 g.) of Delphinium elatum L. (Carters New Giant Flowered Mixed) were extracted with 2½% acetic acid by the procedure of Cookson and Trevett.[93a] Thin-layer chromatography (chloroform, Dragendorff spray reagent[94]) showed the presence of six alkaloids in the crude basic fraction. The alkaloid mixture (1.6 g.) in chloroform (1 ml.) was placed on a column of grade III alumina (50 g.) in benzene. Elution was carried out with a linear gradient of ethyl acetate in benzene, the ethyl acetate concentration increasing by 1% per 20 ml. fraction collected. The course of elution was followed by ultraviolet absorption at 280 mμ (Fig.1, p.53). Ethyl acetate (15%) in benzene eluted delpheline (281 mg., 0.45%) m.p. 216-217° (from acetone) (lit.,[93a] 216-219°), $[\alpha]_D$ -25° in chloroform (lit.,[93a] -26° in ethanol). (Found: C, 66.4; H, 8.9; N, 2.9. Calc. for $C_{25}H_{39}O_6N$: C, 66.8; H, 8.7; N, 3.2%). Ethyl acetate (25%) in benzene eluted alkaloid 2 (104 mg., 0.17%) m.p. 140-144° (sinters at 135°) after drying at 60°, $[\alpha]_D$ + 24° (C = 0.7 in ethanol), $\lambda_{max.}$ 278 mμ ($E^{1\%}_{1\ cm}$ 683),

$\nu_{max.}$ 3550 and 1730 cm.$^{-1}$ Ethyl acetate (32%) in benzene eluted impure alkaloid 3 (55 mg., 0.09%). Ethanol (5%) in ethyl acetate eluted methyl-lycaconitine (177 mg., 0.28%) m.p. 125-128° (lit.,[95] 128°), $[\alpha]_D$ + 50° (C = 0.65 in ethanol) (lit.,[95] + 49°, c = 2 in dry alcohol), $\nu_{max.}$ 3500 and 1715 cm.$^{-1}$ Ethanol eluted alkaloids 5 and 6 (147 mg., 0.23%), $\nu_{max.}$ 3450, 1695, and 1370 cm.$^{-1}$ Plant material (70 g. dry wt.) gave a lower yield of delpheline (0.16%). D.Wellbyii Hemsl. plants (1.17 Kg.) collected just before flowering gave a basic fraction (0.3%) containing seven alkaloids, none of which had the same R_F values as the alkaloids from D.elatum or lycoctonine, obtained by hydrolysis of methyl-lycaconitine.[95]

Desethyl-N-nitrosodelpheline. 39. Prepared from delpheline (100 mg.) by the method of Cookson and Trevett as needles m.p. 250-251° (ether-acetone) in 15% yield. Delpheline (67%) was recovered.

Attempted photoxidation of N-ethylpiperidine. 69. The infrared spectrum of N-ethylpiperidine (13 g.) in benzene containing eosin (0.3 g.) remained unchanged after 18 hrs.

Dehydrodelpheline. 111. Prepared from delpheline (200 mg.) by the method of Cookson and Trevett.[93a] The basic product was chromatographed on grade III alumina (10 g.). Ethyl acetate (25%) in benzene eluted dehydrodelpheline (90 mg.), m.p. 134-135°, star clusters. Ethanol (5%) in ethyl acetate eluted a yellow oil (64 mg.) containing no bromine.

Mercuric acetate treatment of delpheline. 113, 117. The product obtained from delpheline (250 mg.) using the conditions of Cookson and Trevett[93a] was chromatographed on Woelm alumina (20 g.). Ethyl acetate (10%) in benzene eluted dehydrodelpheline (35 mg.). Ethanol (4%) in ethyl acetate eluted a yellow oil (65 mg.), $\lambda_{max.}$ 205 mμ ($E^{1\%}_{1\ cm}$ 98), $\nu_{max.}$ 3580 and 1720 cm.$^{-1}$ Small scale oxidations using a 2-4 molar excess of mercuric acetate gave dehydrodelpheline and unchanged delpheline. One reaction product (7-fold excess) was separated by chromatography on Woelm alumina into four components, including dehydrodelpheline (15%) and oxodelpheline (30%). The lactam was identified by comparison of its infrared spectrum with that of oxodelpheline obtained from delpheline (28 mg.) by potassium permanganate oxidation.[93a]

Estimation of delpheline content of young plants. 49. The whole plant was washed with distilled water, blotted dry, and crushed under ethanol in a beaker. The residues were dried to constant wt. and then soaked in 2½% acetic acid (150 ml.). After three days, the acidic solution was filtered, neutralised with saturated sodium bicarbonate solution, refiltered, and extracted with ether (3 x 75 ml.) and ethyl acetate (2 x 75 ml.). The combined organic extracts were treated with celite 545, dried over sodium sulphate, and evaporated *in vacuo*. The crude alkaloid fraction was run on several chromatoplates (8 x 8") in chloroform. A thin strip on each side of the developed chromatogram was sprayed with Dragendorff reagent and the delpheline bands from the untreated alumina were scraped off, eluted with chloroform, and the extracts made up to a standard volume (5, 10, or 25 ml.). A suitable aliquot was evaporated in a conical flask and the weight of delpheline determined by the colorimetric method of Cookson and Trevett.[93b]

Zeisel determinations. 55. The usual microanalytical procedure[98] for *O*-methyl and *N*-ethyl groups was followed on samples (*ca.* 5 mg.) of active alkaloid. The evolved methyl or ethyl iodide was collected in ethanolic,

freshly distilled triethylamine,[99] which was then evaporated; The residual quaternary salt was plated in the usual way. Delpheline obtained from a [2-^{14}C] acetate feeding had an activity of 91±5 c.p.m./mg. (0.004% incorporation), unchanged on rechromatography and crystallisation from acetone and ethanol. A Zeisel determination showed no significant labelling in the methoxyl (3 x 2%) or N-ethyl groups (0%).

Mevalonate II feeding. 81. A young plant (dry wt. 2330 mg.) was fed through a cut stem with DL-[2-^{14}C] mevalonic acid (0.067 mc.). Over 98% of the radioactivity was taken up. After four days, the plant was extracted in the usual manner. The basic fraction was run on three chromatoplates and the eluted delpheline fractions (5470 c.p.m.) were diluted with inactive alkaloid (60 mg.) and crystallised from acetone. Samples for counting were plated from chloroform solution; the final count was 2,3 c.p.m./mg. (0.000% incorporation). The petroleum ether extract (70 mg., 3.15% incorporation) from the same plant was heated on the steam-bath with aqueous alcoholic 10% potassium hydroxide (4 ml.). The non-saponifiable fraction counted at 70, 770 c.p.m./mg., (13 mg., 1.25% incorporation). Chromatography on

grade III alumina (10 g.), eluting with benzene-chloroform (1:1), gave a crude phytosterol fraction (3.6 mg., 236950 c.p.m./mg., 1.15% incorporation). Inactive β-sitosterol (30 mg.) was added and the diluted material crystallised from ethanol. Samples for counting were plated from benzene solution. The recrystallised sterol had m.p. 138-139° lit., e.g.[96] 140-141°, 1.49 x 10^5 c.p.m./mMole.

	c.p.m./mg.	
cryst.	850,	942
recryst.	362,	364
recryst.	347,	373
recryst.	339,	371

The β-sitosterol was converted into its acetate, which was recrystallised from aqueous ethanol and from acetone-methanol to give needles m.p. 118-119°, 1.66 x 10^5 c.p.m./mMole (0.017% incorporation). Samples for counting were plated from chloroform solution.

	c.p.m./mg.
cryst.	362
recryst.	367
recryst.	363

Mevalonate IV feeding. 155. Four young plants (1760, 1950, 2040, and 2760 mg. dry wt.) were fed with [2-^{14}C]mevalonate (0.04 mc.). The isolated delpheline was totally inactive. A portion (3 mg.) of the non-saponifiable fraction (20 mg., 6258 c.p.m./mg.) from the lipid extract was chromatographed on grade III alumina (Table 1, p.59). Further aliquots were diluted with β-sitosterol (2.6 + 51.0 mg.) and stigmasterol (2.9 + 62.7 mg.). The β-sitosterol was crystallised from ether-methanol and recrystallised from methanol. The acetate (sodium acetate and acetic anhydride) had the same specific activity (5.5 x 10^4 c.p.m./mMole). Incorporation was 0.017%.

	c.p.m./mg.
cryst.	238, 246
recryst.	137, 139
acetate	120, 125

The stigmasterol fraction was recrystallised from acetone-methanol and ether-methanol m.p.154-158° (lit., e.g.[97] 168°) acetate m.p. 142-143° from ethanol (lit.,[97] 143-144°), 8.7 x 10^3 c.p.m./mMole (0.004% incorporation).

	c.p.m./mg.
cryst.	144, 148
recryst.	60, 68
acetate	19, 21
recryst.	18, 20

Formate feeding. 121. Seven young plants (350, 500, 810, 835, 1015, 1190, and 2650 mg. dry wt.) were fed with sodium [^{14}C]formate (0.1 mc.). Over 99% of the radioactivity was taken up. After 2 days, the plants were extracted in the usual manner to give a crude basic fraction (72 mg., 0.25% incorporation) which was diluted with inactive alkaloid mixture (78 mg.) and chromatographed on grade III alumina (50 g.). Total activity, weight, and ultraviolet absorption curves were plotted for 60 fractions eluted using a linear gradient of ethyl acetate in benzene, followed by ethanol (5%) in ethyl acetate. The purified delpheline from fractions 16-21 was inactive.

Serine feeding. 95. A young plant (1690 mg. dry wt.) was fed with L-[3-^{14}C]serine (0.01 mc.). The basic fraction (17 mg.) was separated on two chromatoplates (8 x 8"). The purified delpheline was inactive.

Methionine V feeding. 129. Six young plants (387, 470, 547, 833, 770, and 1160 mg.) were fed with L-[methyl-^{14}C]methionine (0.05 mc.). Over 99% of the radioactivity was taken up. After 2 days the plants were extracted in the usual manner. Incorporation into the non-saponifiable lipid fraction was 0.013%. Inactive alkaloid extract (163 mg.) was added to the crude basic material (46 mg., 0.76% incorporation), and the mixture was chromatographed on grade III alumina (Fig.4, p.61). Total incorporation into the eluted alkaloids was 0.34%, of which the delpheline fractions represented 0.045% and the methyl-lycaconitine fractions 0.21%. Fractions 16-19 (28 mg., 715 c.p.m./mg.) were diluted with inactive delpheline (28 mg.) and rechromatographed (Fig.5, p.63). Fractions 15-20 from the rechromatography were crystallised from acetone to constant activity, 1.1 x 10^5 c.p.m./mMole (0.025% incorporation).

	c.p.m./mg.
chromat.	220
cryst.	232
recryst.	226 256
recryst.	252 270
recryst.	245 ± 8

Degradative results are summarised in Table 2, p.64.

Hydrolysis of delpheline with sulphuric acid. 143. The active delpheline (245 ± 8 c.p.m./mg., 1.1 x 10^5 c.p.m./mMole) was hydrolysed by a procedure based on the work of Sarkar.[100] The alkaloid (6.0 mg.) in 20% sulphuric acid (10 ml.) was slowly distilled at constant volume. The distillate was collected in dimedone (85 mg.) in water (10 ml.). After 50 ml. had distilled the precipitated formaldehyde dimedone derivative (1.3 mg., 33%) m.p. 180-184° was collected. The activity (18 ± 3, 21 ± 6 c.p.m./mg.) remained essentially the same after chromatography on grade III alumina (1 g.) (14 ± 3, 20 ± 2 c.p.m./mg.) m.p. 184-188°. This result was confirmed by a second hydrolysis. A blank experiment under the same conditions gave no dimedone derivative.

Differential centrifugation procedure. 203. Leaves. (19.5 g.) of young delphinium plants (sown December 1962) were washed with distilled water, blotted dry, and cut into small segments, which were macerated under a buffer solution (40 ml. of 0.35M NaCl, 0.01M K_2PO_4, pH 7.4). The homogenate was strained through eight sides of cheese-cloth and the filtrate was spun at 200 x g. to remove whole cells, nuclei, and cell debris. Five pellets were collected by preparative differential

centrifugation on the Serval RC-2 automatic superspeed refrigerated centrifuge and the Spinco Model L preparative ultra-centrifuge (Table 4, p.71). The procedure was repeated with roots (20 g.). A sample of the chloroplast suspension was purified by density gradient.

Chlorophyll estimation. 205. The pellet was suspended in ether (25 ml.). A 1 ml. aliquot was evaporated and the residue redissolved in 80% acetone (4 ml.). The optical density values at 645 mμ and 663 mμ were determined on a Unicam SP.500 spectrophotometer using 10 mm. cells. The chlorophyll content (mg.) is calculated from the equation: $C = (20.2\ D_{645} + 8.02\ D_{663}) \times \frac{25 \times 4}{1000}$. This is derived from simultaneous equations using specific absorption coefficients for chlorophyll a and b as given by MacKinney.[101]

PART II

Isolation of chimonanthine. 169. Dry, powdered leaves (142 g.) of Chimonanthus fragrans, Lindle, were extracted by the method that Hodson and Smith used for the isolation of folicanthine from Calycanthus occidentalis Hook and Arn.[56] Petroleum ether extraction (for 36 hrs) gave a green residue, which was dissolved in ether (500 ml.) and extracted with 5% tartaric acid (50, 25, 25 ml.). The combined acid extracts were washed with ether (25 ml.), basified with 4N NaOH, and extracted with ether (5 x 100 ml.). The ethereal extracts were dried (Na_2SO_4) and evaporated at room temperature to give crude basic material (49 mg.) which was chromatographed on grade V alumina (10 g., 50 g.). Benzene-chloroform (1:1) eluted impure (-)-chimonanthine (20 mg.) which was recrystallised from benzene/petroleum ether. Maceration of a second batch of leaves under ethanol in a Waring blender gave residues that were difficult to work up cleanly. Thin-layer chromatography showed the presence of another alkaloid besides chimonanthine in all the basic extracts. The higher running spot had an R_F value of 0.8 with acetone or chloroform/ethanol

(9:1) and was not a chimonanthine solvate. The two spots coalesced in methanol (R_F 0.8). Chimonanthine ran at R_F 0.4 to 0.6 in acetone and at R_F 0.8 in chloroform-acetic acid-ethanol (96:4:1).[102]

Tryptophan feeding. 157. The stem of an excised leaf (2530 mg. dry wt.) was placed in a solution of DL-[alanine-3-^{14}C]tryptophan (2 ml.; 0.01 mc.). The tube was sealed so as to be airtight, and warm air was blown gently across the leaf spores to assist uptake. After 3 days, the plant material was extracted as described above. The basic fraction was chromatographed on grade III alumina (10 g.). Chloroform (25%) in benzene eluted chimonanthine (0.35 mg.; 21900 c.p.m./mg.) preceded by an alkaloidal impurity (0.34 mg.; 9200 c.p.m./mg.) (Fig.6). Fractions 8-11 were combined, diluted with inactive (-)-chimonanthine (27 mg.), and recrystallised from benzene. Incorporation into the purified alkaloid was 0.060%.

	c.p.m./mmole
chromat.	6.47×10^6
cryst.	$1.07, 1.10 \times 10^4$
recryst.	$8.10, 8.69 \times 10^3$
recryst.	$8.20, 8.37 \times 10^3$

Indole-3-glyoxylyl chloride. 171. Prepared by the method of Shaw and his co-workers[69] as pale yellow prisms in 90% yield, m.p. 128-129°, rapidly oxidising on exposure to air.

N-[^{14}C]methyl-3-indoleglyoxamide. 177. Prepared by the method of R. H. Prager.[72] [^{14}C]Methylamine hydrochloride (2.6 mg., 0.1 mc.) was heated in dry dimethylformamide (0.5 ml.) to effect solution and added to the acid chloride (17.2 mg.) in a dry ampoule. Washings of the methylamine tube (2 x 0.1 ml.) were added, the solution cooled to room temperature, and dry redistilled triethylamine (6 drops) added. The ampoule was sealed and left overnight. The reaction mixture was pipetted into saturated sodium bicarbonate solution (2 ml.) and stirred. Chloroform extracts (4 x 1 ml.) of the alkaline mixture were washed with N HCl (2 x 1 ml.) and water (1 ml.). The washings were extracted with chloroform (0.5 ml.). Evaporation of the dried chloroform extracts afforded the amide (6.5 mg., radiochemical yield 83%) m.p. 200-205°. An inactive sample of the amide had m.p.225-225.5° (isopropanol) (lit.,[68] 128-129°), R_F 0.7 with methanol and chloroform-acetic acid-ethanol

(96:4:1) detected by Prochazka reagent[102] (Found: C, 65.3; H, 5.0; N, 13.8. Calc. for $C_{11}H_{10}N_2O_2$: C, 65.3; H, 5.0; N, 13.9).

N-[^{14}C]Methyltryptamine. 177. A solution of the amide (6.5 mg.) in dioxan (1 ml.) was added over 30 mins. to a refluxing suspension of lithium aluminium hydride (200 mg.) and aluminium chloride (40 mg.) in dioxan (5 ml.). The pink complex immediately formed was slowly decolorised. After refluxing for 5 hrs., the reaction mixture was cooled, treated with ether (10 ml.) and cautiously poured into saturated sodium sulphate solution. The organic phase was removed and the aqueous layer was washed with ether (3 x 5 ml.). The combined extracts were extracted with 2N HCl (3 x 5 ml.), which was basified with ammonia and extracted with chloroform (4 x 5 ml.) to give an oil (2.2 mg.). An aliquot was run on a chromatoplate in methanol and the eluted N-methyltryptamine spot (R_F 0.4) was found to contain 90% of the activity (radiochemical yield 40%). An impurity ran at R_F 0.8. The neutral fraction from the work up (3.1 mg.) was further reduced for 5 hrs., but gave only the basic product R_F 0.8. An inactive

sample of the amine had m.p. 87-88° (petroleum ether 80-100°) (lit., e.g.[81] 86-87°), R_F 0.2 with methanol. A satisfactory nitrogen analysis was not obtained but carbon and hydrogen determinations on freshly recrystallised material were in agreement with the calculated values (Found: C, 75.9; H, 8.1; N, 15.5. Calc. for $C_{11}H_{14}N_2$: C, 75.8; H, 8.1; N, 16.1).

N-Methyltryptamine hydrochloride. 179. The amine (33 mg.) was dissolved in ethanol and ethereal 4N HCl (0.5 ml.) added. On dilution with ether and standing in the cold, flakes were obtained m.p. 179-179.5° (Found: C, 62.5; H, 6.9; N, 14.3. Calc. for $C_{11}H_{15}N_2Cl$: C, 62.7; H, 7.2; N, 13.3).

N-Methyltryptamine feeding. 181. The precursor (0.033 mc., 90% amine) in distilled water (1 ml.) was fed at pH 3-4 to several shoots (4.9 g. dry wt.). An aliquot of the feeding solution was reserved for later comparison. Over 99% of the radioactivity was taken up. After 2 days, the shoots were worked up to give basic material (3 mg.) which was diluted with inactive N-methyltryptamine (40.8 mg.) and synthetic (±)-chimonanthine (17.8 mg.). The mixture was separated by 3 chromatographic runs on grade V alumina (10 g.,

50 g., 50 g.) (Fig.7). The elution of the bases was followed by thin-layer chromatography. With methanol as the developing solvent, chimonanthine ran at R_F 0.8 whereas N-methyltryptamine ran at R_F 0.2 to 0.4. With acetone, chimonanthine gave a brown spot R_F 0.5 which turned yellow on standing (staining with iodine); N-methyltryptamine appeared as a darker spot on the origin. To the chimonanthine fractions (13.6 mg.), eluted by benzene-chloroform (1:1), was added further inactive alkaloid (11.1 mg.). To the N-methyltryptamine fractions (20.6 mg.), eluted by chloroform-ethyl acetate (7:3), was added inactive base (25.1 mg.). The recrystallised chimonanthine (benzene) showed an incorporation of less than 0.011%.

	c.p.m./mg.		c.p.m./mMole
chromat.	3244,	3140	1.10×10^6
cryst.	818,	862	2.91×10^5
recryst.	317,	328	1.11×10^5
recryst.	131,	134	4.57×10^4
recryst.	102,	110	3.67×10^4

The recrystallised N-methyltryptamine (petroleum ether b.p. 40-60°) had a higher activity (≡ 0.097% incorporation), retained on conversion into the hydrochloride

(ethanol-ether).

	c.p.m./mg.	c.p.m./mmole
chromat.	446, 458, 562	8.51×10^4
cryst.	364, 344	6.16×10^4
recryst.	367, 357	6.30×10^4
recryst.	354, 350	6.12×10^4
hydrochloride	308, 328	6.67×10^4

Deuteration of tryptophan. 183. Tryptophan (51 mg.) was dissolved in D_2O containing potassium t-butoxide (55 mg., 2 moles), aromatic region (τ 2.6), methine quintuplet centered at τ 6.55, methylene triplet centered at τ 7.05. Thionyl chloride (0.4 ml.) was reacted with D_2O (2 ml.) and the resulting sulphur dioxide was blown out in a stream of dry nitrogen. Tryptophan (200 mg.) was added; the hydrochloride precipitated but redissolved on heating on the steam-bath. After 2 hrs., the solution was cooled and the deposited hydrochloride filtered off, dried, and dissolved in alkaline D_2O. The equivalent of 4 aromatic protons had exchanged. A longer period of heating (6 hrs.) gave complete exchange of the five aromatic protons.

Tritiation of tryptophan. 187. Tryptophan (100 mg.) was added to thionyl chloride (0.2 ml.) in tritiated water (0.11 g.; 55 mc.) and distilled water (0.4 ml). After 6 hrs. heating on the steam-bath, the amino-acid (10 mg.) was precipitated at its iso-electric point and washed with water (2 x 1 ml.). An aliquot (0.936 mg.) was diluted with inactive tryptophan (100.8 mg.). The diluted amino-acid was converted to the N-acetyl derivative[84] (10.2×10^5 c.p.m./mMole) and radio-assayed by preparation of α-acetamido (dioxindole-3)-propionic acid lactone (7.59×10^5 c.p.m./mMole), using the procedure of Hinman and Bauman.[85]

O-Nitrophenylpyruvic Acid. 191. The acid was prepared from o-nitrotoluene and diethyl oxalate, by a modification of the Reissert synthesis devised by Mayer and Balle,[87] as pale yellow needles m.p. 119-120°, 22% yield. The method of Wright and Collins[103] gave a product that was more easily purified and a better yield (40%).

Indole-2-carboxylic acid. 193. The reductive cyclisation of o-nitrophenylpyruvic acid was carried out as described by Kermack, Perkin, and Robinson to give needles m.p. 208° (sinters at 190°) in 20% yield.

The acid (51 mg.) was recrystallised from D_2O (10 ml.) or precipitated (150 mg.) from D_2O (5 ml.) containing potassium t-butoxide (124 mg.) by acidification.

[2-^{2}H]Indole. 195. The deuterated acid (200 mg.) was heated[86] at 240° in an evacuated micro-Carius tube (with a plug of glass wool) for 2 hrs. The cooled tarry product was extracted with benzene and chromatographed on grade III alumina (3 g.). Benzene-chloroform (5:1) eluted indole (10%), with no peak corresponding to α-hydrogen in its n.m.r. spectrum. Indole-3-glyoxylyl chloride was prepared from the deuterated indole, but decomposed on standing.

[2-^{3}H]Indole. 197. Indole-2-carboxylic acid (400 mg.) was dissolved in distilled water (4.8 ml.) containing tritiated water (0.19 g.), 38 mc.) and potassium t-butoxide (310 mg., 1.1 mole). The tritiated acid (350 mg.; 4.28×10^7 c.p.m./mMole) was precipitated with conc. H_2SO_4 and dried. Pyrolysis as described above yielded tritiated indole (33 mg., 9%), which was diluted with inactive material (3.825 g.) and recrystallised from water, off-white plates, m.p.51-52°.

	c.p.m./mMole	d.p.m./mMole
chromat.	2.60, 2.72 x 10^5	1.50, 1.56 x 10^6
cryst.	2.42, 2.61 x 10^5	1.40, 1.51 x 10^6

Derivatives of labelled indole. 199, 201. Gramine[90] was obtained in 90% yield, m.p. 127-128° (acetone). Indole-3-glyoxylyl chloride (96%) and the corresponding amide (88%) were prepared by the methods of Shaw and his co-workers[69]

	c.p.m./mMole	d.p.m./mMole
gramine	2.42, 2.59 x 10^5	1.23, 1.31 x 10^6
acid chloride	1.57, 2.23 x 10^5	0.91, 1.29 x 10^6
amide	1.54, 1.58 x 10^5	0.89, 0.91 x 10^6

Indole dimercuriacetate. 207. Prepared by the method of Mingoia.[91] The compound was counted without recrystallisation in dimethylsulphoxide-xylene scintillator solution, 1.83 x 10^5 c.p.m./mMole, 1.30 x 10^6 d.p.m./mMole.

N-Tosyltryptamine. 165. Prepared by the method of Hoshino and Kabayashi[71] m.p.115-116° (ethanol), 80% yield.

N-Methyl-N-tosyltryptamine. 167. Prepared by the method of Hoshino and Kabayashi[71] m.p. 113-114°. The N-tosyl compound was shown up with the Prochazka reagent[102] as a light brown spot (R_F 0.5, ethyl acetate); the N-tosyl-N-methyl compound appeared as a darker spot at R_F 0.8 to 0.9.

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