STUDIES IN ALKALOID BIOSYNTHESIS

a thesis

submitted by

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ABSTRACT

The biosynthesis of alkaloids derived from tyrosine is reviewed, on the basis of recent tracer studies in the field. Evidence for the most widely accepted concept for the biosynthesis of the Amaryllidaceae alkaloids has been accumulated by a series of tracer experiments. Hypothetical phenolic precursors were synthesised containing a labelled carbon atom, and their incorporation into alkaloids of the family investigated. Various degradations to establish the labelling pattern have been carried out, and in the light of available evidence a plausible biosynthetic route to two of the alkaloids has been proposed. Unambiguous proof is presented for the derivation of the methylenedioxy group of haemanthamine from the methoxyl group of one of the precursors synthesized. A series of experiments to establish the obligatory nature of various precursors has been carried out, in particular, one phenolic precursor has been shown to be a necessary intermediate in alkaloid biosynthesis.

ACKNOWLEDGEMENTS

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C O N T E N T S

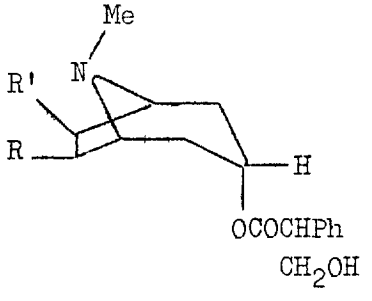
BIOSYNTHESIS OF ALKALOIDS DERIVED FROM TYROSINE

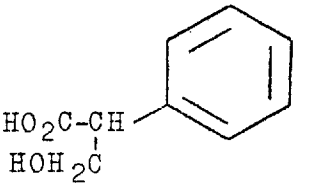
Due to the recent publication of an excellent comprehensive review on biosynthesis,[1] this review is brief, and contains in addition only the more recent publications.

"Biosynthesis from tyrosine" must be understood to include all its close metabolic relations such as phenylalanine, tyramine and p-hydroxyphenylpyruvic acid, the interconversions of which are well established.[2]

1. Hyoscyamine and Hyoscine

The ester alkaloids hyoscyamine (I) and hyoscine (II) which occur in several solenaceous plants, for example, Atropa-belladonna, Datura stramonium and Hyoscyamus niger, contain an acid moiety tropic acid (III).

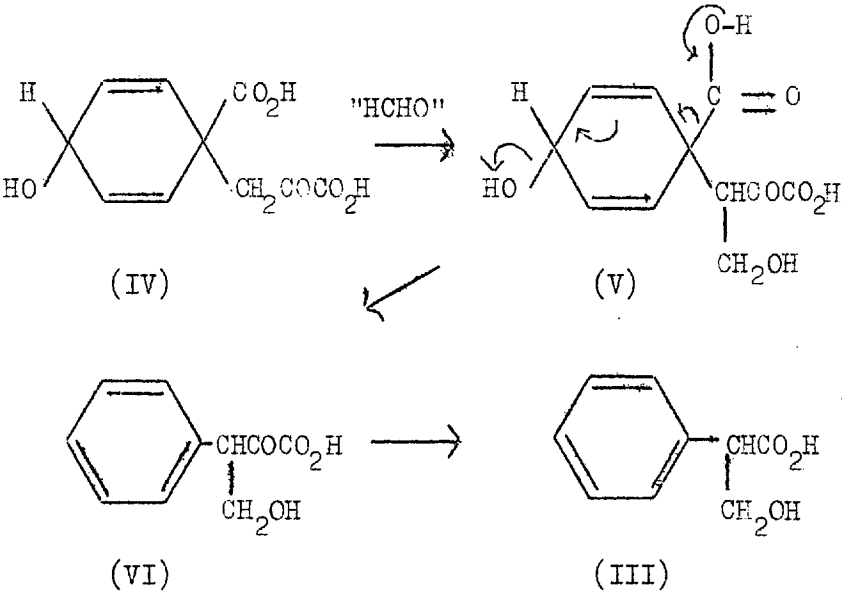




(I, R = R' = H) (III)
(II, R-R' = -O-)

Trautner[3] suggested that tropic acid is related biogenetically to the terpenes because of its branched three-carbon side chain. More recently Wenkert[4] proposed that

prephenic acid (IV), a well established intermediate in the biosynthesis of aromatic compounds, was a suitable precursor of the acid moiety. One plausible route from prehenic acid is illustrated.



An aldol condensation between prephenic acid (IV) and formaldehyde (or its biological equivalent) would give compound (V), which, on decarboxylation and dehydration, could give rise to the aromatic acid (VI). Oxidative decarboxylation would then give tropic acid (III).

When [3-^{14}C] phenylalanine[5] was fed to young Datura stramonium plants, the isolated hyoscyamine and hyoscine were radioactive. Hydrolysis of the alkaloids gave tropic acid containing essentially all the radioactivity, the tropine and oscine residues being virtually inactive. The tropic acid, in

both cases, was oxidised to benzoic acid, decarboxylation yielding carbon dioxide containing all the radioactivity. This indicates that phenylalanine is a direct precursor of tropic acid, carbon atom-3 of the amino acid providing carbon atom-2 of tropic acid.

In parallel experiments [^{14}C] sodium formate and [^{14}C]-formaldehyde were fed, and rather surprisingly found not to be a source of the hydroxymethylene group of the acid.

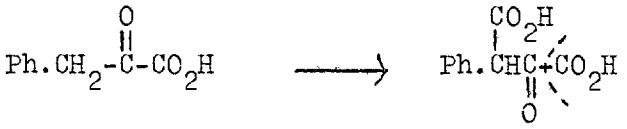
A further series of experiments was carried out using [2-^{14}C] phenylalanine[6], when the isolated alkaloids were again radioactive. Hyoscyamine on hydrolysis gave tropic acid, which was dehydrated to give atropic acid (VII). Oxidation with osmium tetroxide gave α-phenylglyceric acid (VIII) which yielded formaldehyde containing all the radioactivity, on cleavage with sodium periodate.

(VII) (VIII)

Carbon atom-2 of phenylalanine provides carbon atom-3 (the hydroxymethylene group) of tropic acid, and explains the lack of incorporation of formate.

Rather surprisingly, carboxyl labelled tropic acid was obtained from feeding experiments with [^{14}C] tryptophan[7]. A

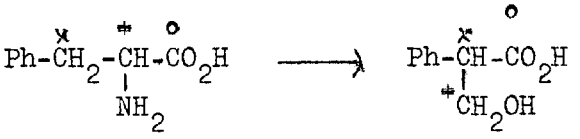
plausible explanation of this was proposed by Leete[6], who suggested tryptophan was metabolised to give radioactive carbon dioxide which could then be incorporated by the following scheme.



(IX) (X)

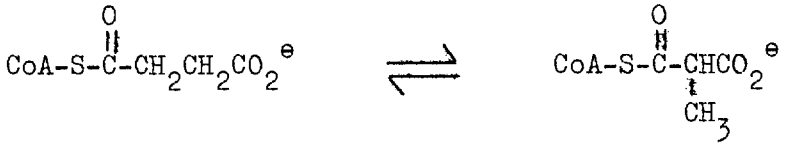
Carboxylation of phenylpyruvic acid (IX) followed by a reductive decarboxylation of (X) could lead to tropic acid, and account for the incorporation of carbon dioxide.

Further experiments[8] showed that the carboxyl group of tropic acid was in fact derived from the carboxyl group of phenylalanine. [1-^{14}C] Phenylalanine was fed to D.stramonium plants, and the radioactive hyoscyamine degraded as described previously to give inactive formaldehyde and phenylglyoxylic acid isolated as its oxime. The oximino acid on decarboxylation gave benzonitrile, the liberated carbon dioxide containing all the radioactivity. Hydrolysis of the nitrile gave inactive benzoic acid, as did direct oxidation of tropic acid. The results of the tracer experiments are summarised below.



×, +, o, denote labelled atoms in separate experiments.

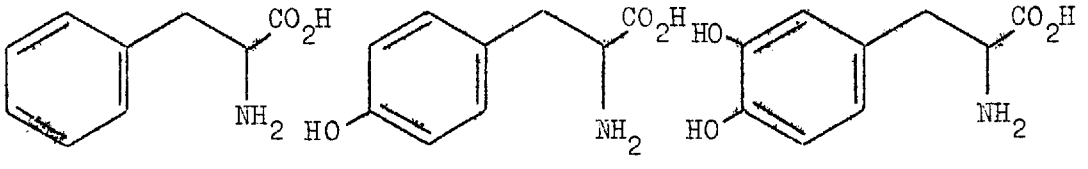
The relatively high incorporation (0.25%) of the carboxyl label without scrambling suggests that the side chain of tropic acid is in fact derived from the phenylalanine side chain by a novel intramolecular rearrangement. A close analogy is the reversible rearrangement of succinnyl coenzyme-A (XI) to methyl-malonyl coenzyme-A (XII)[9].



(XI) (XII)

2. Phenylethylamine Alkaloids

It was suggested as long ago as 1910 [10,11] that the main precursors of the phenylethylamine alkaloids are the aromatic amino acids phenylalanine, (XIII), tyrosine (XIV), and dihydroxyphenylalanine (XV).

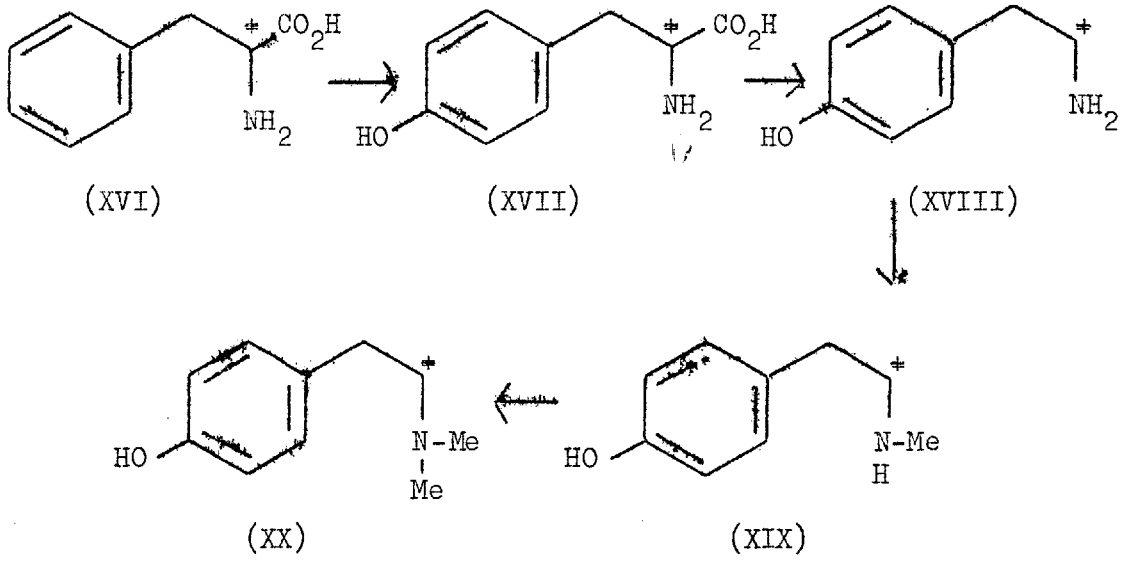


(XIII) (XIV) (XV)

Hordenine

Early work[12] showed that formate but not choline could act as a source of the N-methyl groups in hordenine (XX) and N-methyltyramine (XIX). Later work[13,14] showed methionine was the most efficient precursor (d and l forms equally efficient), although betaine[14] was also incorporated.

Decarboxylation, oxidation and methylation are well known reactions in living systems, and the following pathway to the simple alkaloids is now well established.



(XVI) (XVII) (XVIII) (XX) (XIX)

[1-^{14}C] Tyramine (XVIII) was fed to sprouting barley[15] when the isolated hordenine (XX) and N-methyltyramine (XIX) were found to be radioactive, the latter having the much higher specific activity. Oxidation of O-acetylhordenine with permanganate gave the corresponding inactive benzoic acid, and Hofmann degradation gave inactive trimethylamine, establishing that the activity was

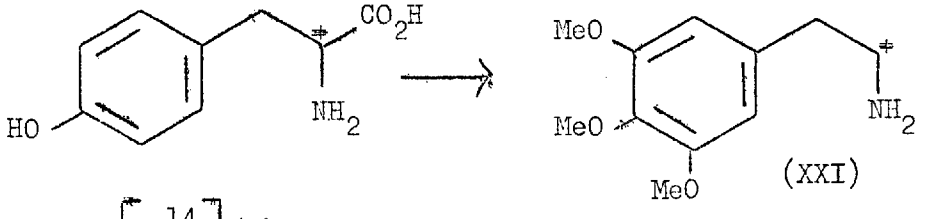
at the position indicated. Degradation of N-methyltyramine also established incorporation of [^{14}C] tyramine without scrambling. These results establish that tyramine is a direct precursor of these alkaloids.

Similar results were obtained by feeding [2-^{14}C]-phenylalanine and [2-^{14}C] tyrosine[16], which were also shown to be direct precursors of hordenine and N-methyltyramine. The higher incorporations into N-methyltyramine in all cases furnish good evidence for the fact that N-methyltyramine is closer to the precursors on the biosynthetic pathway than is hordenine.

Phenylalanine and tyrosine are known to arise by separate routes in Escheri coli[17] and probably do so in wheat and buckwheat[18], so the major pathway in barley may not involve phenylalanine, simply going from shikimic acid to prephenic acid and hence tyrosine.

Mescaline

Mescaline (XXI), a well known hallucinogen provided another simple case for investigation[18].



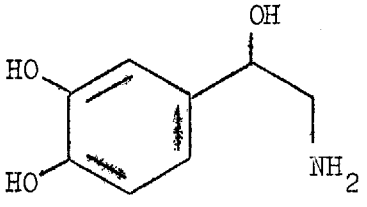
(XXI)

[2-^{14}C] (±)-Tyrosine was fed to the cactus Anhalonium lewinii Hennings (peyote) by direct injection, and root feeding. The isolated mescaline was radioactive, and permanganate oxidation gave inactive trimethoxybenzoic acid, establishing that the

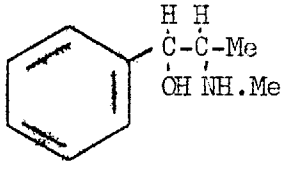
tyrosine is incorporated without scrambling, and is a direct precursor of the alkaloid.

(-)-Ephedrine and (+)-Norpseudoephedrine

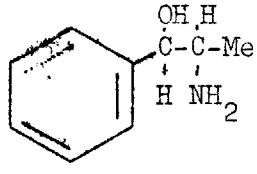
(-)Ephedrine (XXII) and (+)norpseudoephedrine (XXIII) have also been the subjects of tracer studies. Both these simple phenylethylamine alkaloids contain a benzylic hydroxyl group, and a C-methyl group, and are closely related to noradrenaline (XXIV).



(XXIV)



(XXII)



(XXIII)

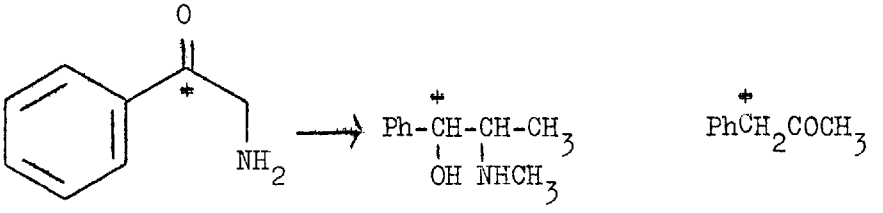
The biosynthesis of noradrenaline has been investigated in animal systems[20] and is thought to involve hydroxylation of dopamine (3,4-dihydroxytyramine) derivable from tyrosine.

Administration of [3-^{14}C] (±)-phenylalanine to an excised shoot from the bush Catha edulis[21] (Arabian tea) gave radioactive (+)-norpseudoephedrine. The purified alkaloid was oxidised to benzoic acid and decarboxylated with copper chromite in quinoline to give labelled carbon dioxide, containing all the activity. Phenylalanine is consequently a direct precursor of this alkaloid.

Elegant work using ^{15}N-labelled phenylalanine and

^{15}N-alanine[22] showed that no transamination to or from the general nitrogen pool in the plants occurs in the case of ephedrine. ^{15}N-Phenylalanine, but not ^{15}N-alanine, gave rise to radioactive ephedrine in Ephedra distachya, suggesting, in this case at least, that the amino acid rather than a biological equivalent, for example, phenylpyruvic acid, is used in the biosynthesis. Incorporation of $[^{14}C]$ formate into the C-methyl group of the alkaloid, suggested that this did not come from the carboxyl group of the amino acid, but by addition of a C_1 unit after decarboxylation.

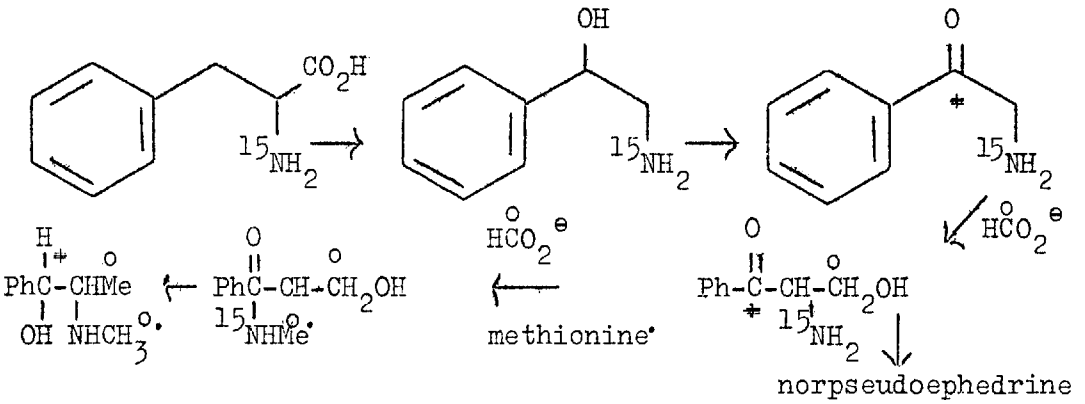
Experiments with $[^{14}C]\omega$-aminoacetophenone (carbonyl-labelled) (XXV), using the same plants[23], gave radioactive ephedrine which was degraded in the following manner.



(XXV) (XXVI) (XXVII)

The labelled ephedrine (XXVI) was treated with sulphuric acid to give the ketone (XXVII) and inactive methylamine. Oxidation of the ketone with permanganate gave benzoic acid and subsequently carbon dioxide containing all the activity. The specific incorporation of ω-aminoacetophenone suggests it is a direct precursor. The following scheme is compatible with the

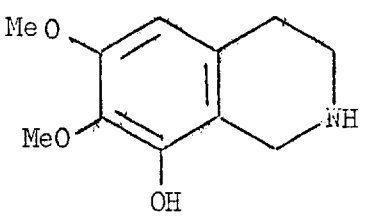
experimental results. (The labelled atoms resulting from the separate experiments are indicated).



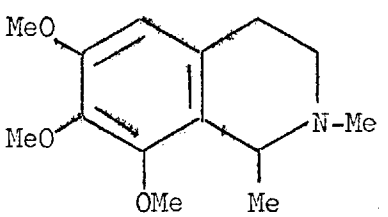
The stages of methylation, for instance, are hypothetical and without experimental verification, and other pathways are equally acceptable.

3. Isoquinoline Alkaloids

The simple isoquinoline alkaloids, can be derived in principle from tyrosine or related amino acid. Thus anhalamine (XXVIII) and pellotine (XXIX) can be derived from tyrosine and formaldehyde, and tyrosine and acetaldehyde, or their equivalents, respectively. Laboratory analogies under physiological conditions are well authenticated.[24, 25]



(XXVIII)

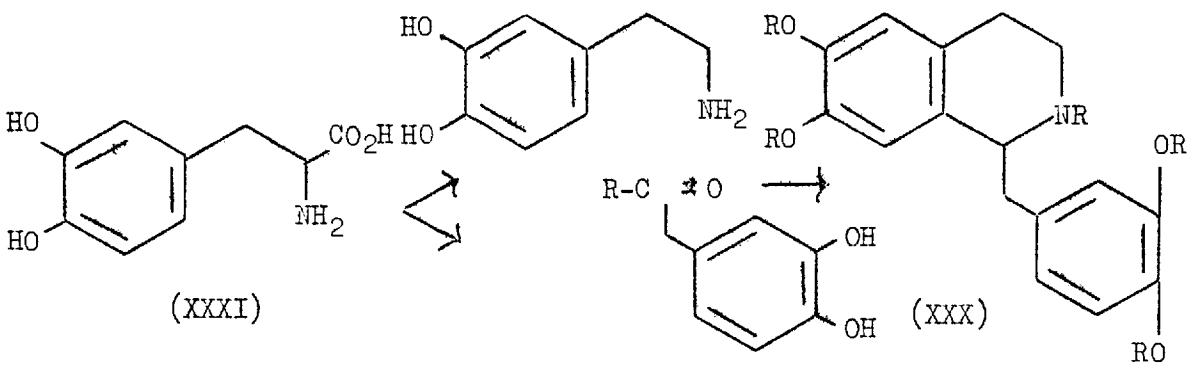


(XXIX)

Pellotine

When [2-^{14}C] tyrosine was fed to the cactus Lophora williamsii radioactive pellotine (XXIX) was isolated.[26] Degradation of the alkaloid should show the labelled atom to be in the predicted position.

The benzylisoquinoline system norlaudanosoline (XXX,R=H) could arise from two molecules of dihydroxyphenylalanine (XXXI) according to the following scheme.[10]

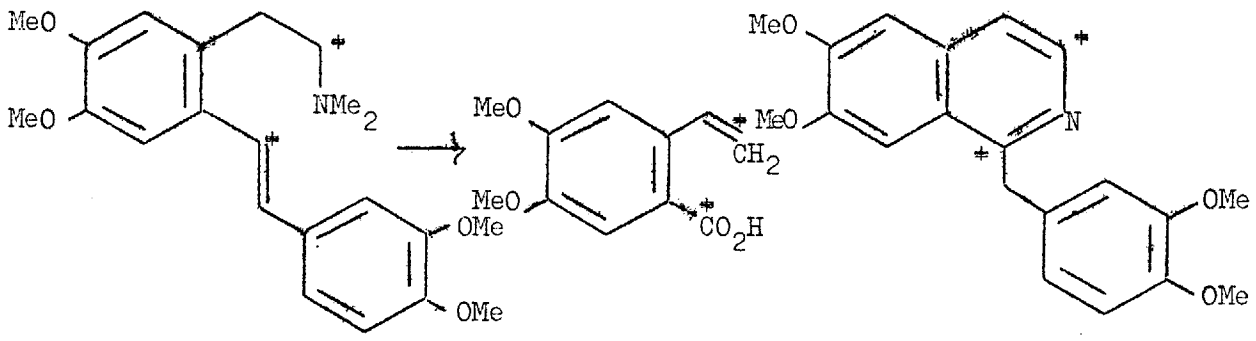


Methylation at some stage could give laudanosine (XXX., R=Me), whilst dehydrogenation would give papaverine (XXXII). On the basis of this scheme, tyrosine should be a precursor of papaverine.

Papaverine

When [2-^{14}C] tyrosine was fed to plants of Papaver somniferum[27], radioactive papaverine was isolated. The position of the labels was determined according to the following scheme.

The labelled papaverine was converted to its methiodide, and reduced to laudanosine with potassium borohydride. Hofmann degradation gave the base (XXXIII) which was oxidised and subjected to further Hofmann degradation to give (XXXIV), which contained



(XXXIII) (XXXIV) (XXXII)

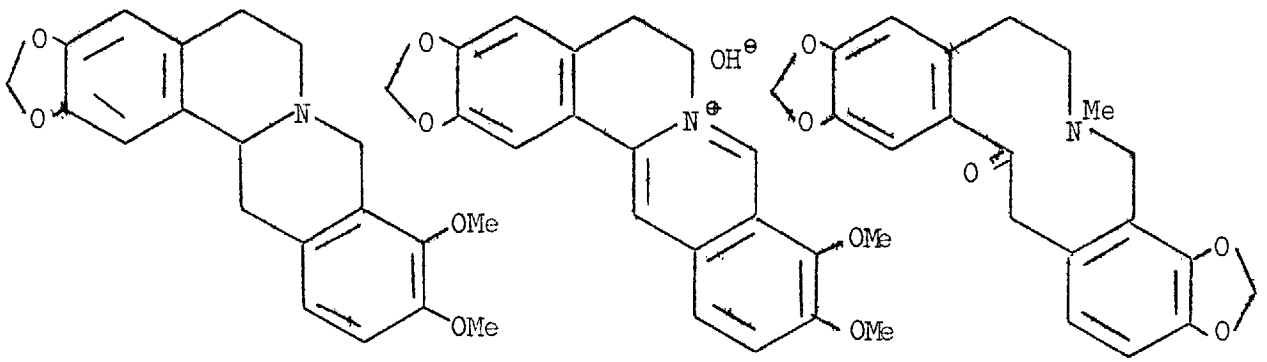
all the activity. Ozonolysis and decarboxylation isolated the labelled carbon atoms, each of which carried half the activity of the original alkaloid. This important result, with specific labelling in the predicted positions, establishes the use of tyrosine as a direct precursor of the alkaloid. The lack of radioactivity in any other positions, indicates the use of two molecules of tyrosine, without degradation when scrambling of the label would occur, in the biosynthesis.

In further work, using $[1\text{-}^{14}C]$ norlaudanosoline, the same group[28] obtained radioactive papaverine from Papaver somniferum fed with the labelled precursor. The alkaloid was shown to be specifically labelled in the predicted position by degradation, and furnishes further proof for the postulated pathway.

Many other alkaloids of this family can in fact be built up from the basic norlaudanosoline skeleton.[11,29]

Condensation with a one-carbon unit could give canadine (XXXV), whilst dehydrogenation would lead to berberine (XXXVI).

Oxidative cleavage of the canadine skeleton could give protopine (XXXVII).



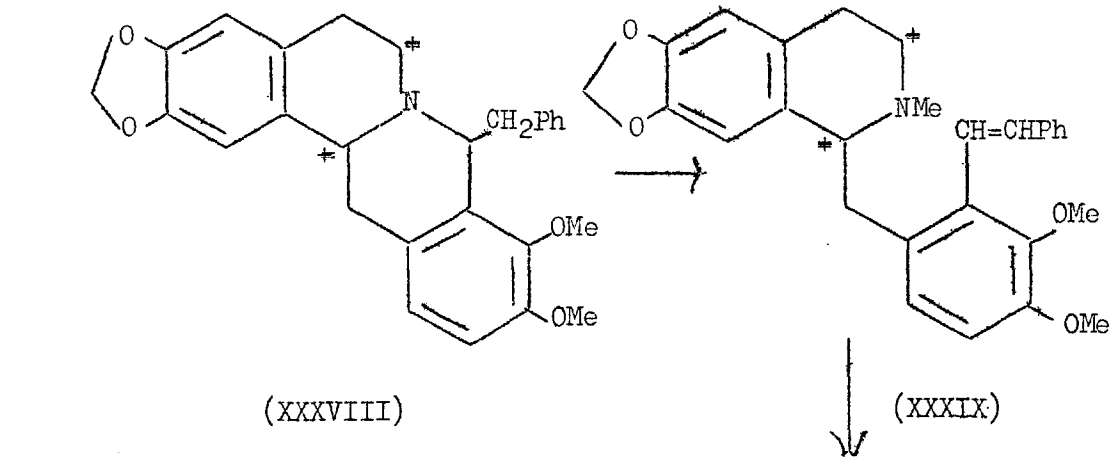
(XXXV) (XXXVI) (XXXVII)

Berberine

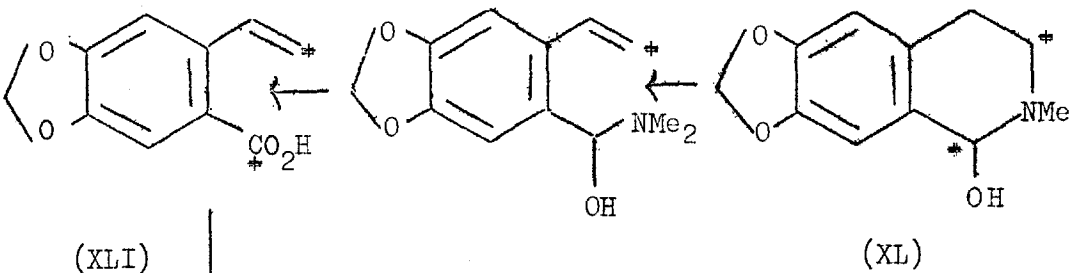
Radioactivity was detected qualitatively in berberine (XXXVI) isolated from plants of Berberis vulgaris fed with [2-^{14}C]-phenylalanine.[30] [2-^{14}C] Tyrosine gave radioactive berberine from plants of Coptis vulgaris but no degradation to establish the labelling pattern has been reported.[31] Another group of workers isolated radioactive berberine from plants of Hydrastis canadensis fed with [2-^{14}C] tyrosine[32,33] and [2-^{14}C] dopamine[33]. The degradation of berberine from the tyrosine fed plants was carried out according to the annexed scheme.[34]

Radioactive berberine was subjected to a Grignard reaction with benzyl chloride, and reduced with sodium borohydride to give (XXXVIII). Hofmann degradation gave de-N-methyltetrahydrostyryl-berberine (XXXIX) which on oxidation gave hydrastinine (XL) containing all the activity. The hydrastinine, subjected to Hofmann degradation, followed by oxidation, gave the vinylpiperonylic

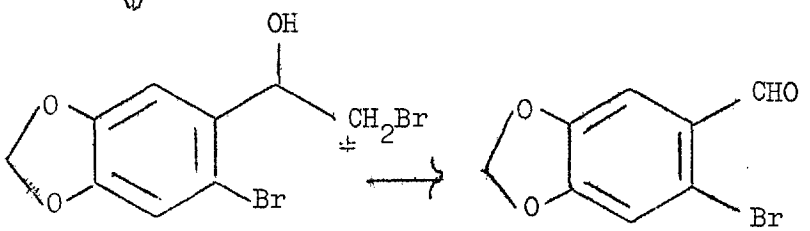
Berberine Degradation



(XXXVIII) (XXXIX)



(XLI) (XL)



(XLII) (XLIII)

acid (XLI), which on bromodecarboxylation gave the bromide (XLII). The bromide and carbon dioxide each contained half the activity of the original alkaloid. Oxidation of the bromide gave inactive bromopiperonyldehyde (XLIII) isolated as its acetone derivative.

The labelling pattern, in agreement with theory, shows that tyrosine acts as a direct precursor of berberine, two tyrosine molecules being involved in the biosynthesis.

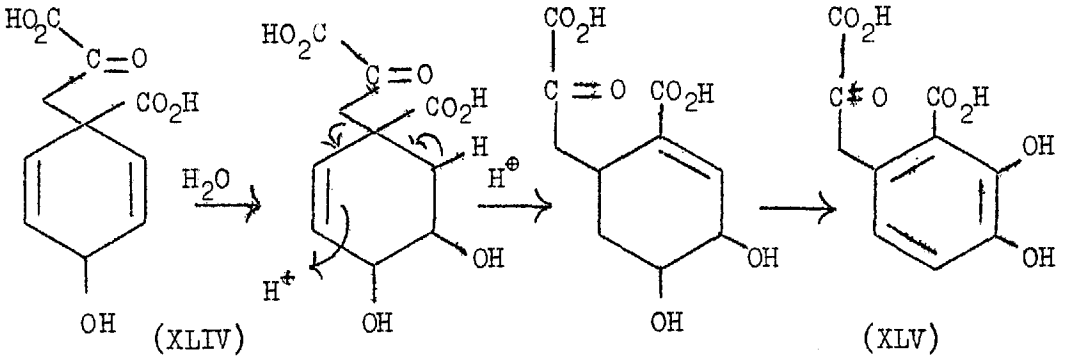
Protopine

Although no tracer studies with tyrosine or related compounds have been carried out, work with methionine and formate has been undertaken.[35] Dicenta hybrids were fed with [^{14}C]-methionine, [^{14}C] choline and [^{14}C] formate. The methionine methyl served as a precursor of the methylendioxy and N-methyl groups, as did formate, although to a considerably less extent, whilst choline was ineffective. The results suggested that the methylendioxy group arose from oxidation of an O-methyl group followed by cyclisation on an adjacent hydroxyl, rather than by formylation followed by reduction and ring closure.

Phthalide Isoquinoline Alkaloids

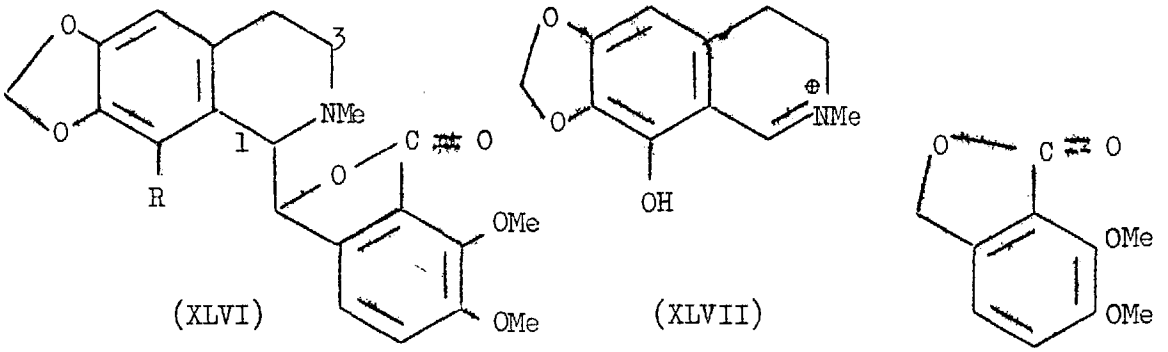
Two possible routes to the phthalide isoquinoline alkaloids have been proposed.[11,36] The classical route[11] depends on an oxidative modification of the canadine skeleton derivable, in turn, from two molecules of tyrosine. The other pathway[36]

involves the hydration of prephenic acid (XLIV) with subsequent rearrangement and transformations.



(XLIV) (XLV)

Reaction of the o-carboxyphenylpyruvic acid with a phenylethylamine, also derived from prephenate, would give rise to the phthalide isoquinoline alkaloids for example, hydrastine (XLVI, R=H)

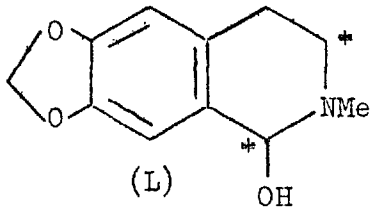


(XLVI) (XLVII) (XLVIII)

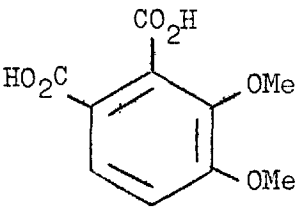
Hydrastine

Papaver somniferum plants were fed with [^{14}C] generally labelled tyrosine,[37] and radioactive narcotoline (XLVI, R=OH) was isolated. The alkaloid was cleaved to give cotarnoline (XLVII) and meconine (XLVIII), the activities of which suggested that narcotoline was derived from two molecules of tyrosine, but the evidence was indicative rather than conclusive.

Preliminary work with the plants Hydrastis canadensis[32] showed that [2-^{14}C] (±)-tyrosine was incorporated into hydrastine (XLVI, R=H) with specific labelling. Oxidation gave inactive opianic acid (XLIX) and hydrastinine (L) containing all the activity.



(L)



(XLIX)

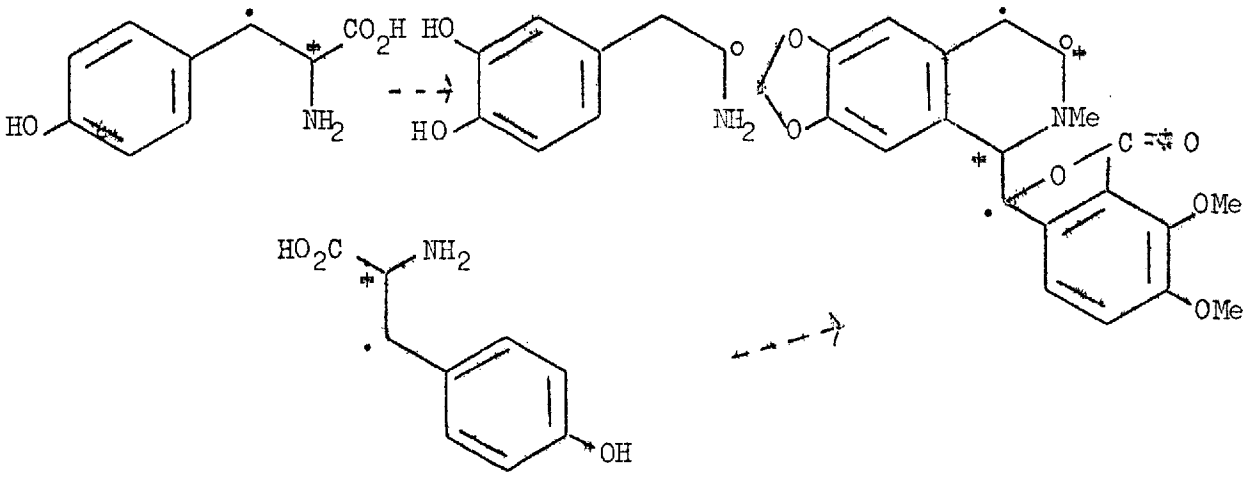
The hydrastinine was degraded as described earlier from berberine (page 14).

In parallel experiments[32] [2-^{14}C] phenylalanine and [^{14}C]-generally-labelled glucose were incorporated with much lower efficiency. These results suggest that the conversion of phenylalanine to tyrosine is minimal in Hydrastis[18,38] , and are in accordance with classical[11] and contrary to Wenkert's theory.[36]

Although the degradation shows that tyrosine serves as a specific precursor of hydrastine, two molecules participating in the biosynthesis, an interesting feature was observed. The labelling was unequal in the two positions, being higher in the 3 position, in contradiction to the results on papaverine[27] and berberine.[34] This indicates different utilization of tyrosine in deriving the two fragments responsible for formation of the alkaloid. If two similarly derived fragments "dimerise" to give the alkaloid equal labelling should be observed. If, on the

other hand, dissimilar fragments are involved, as seems likely in this case, equal labelling of the predicted positions would be fortuitous. The relative incorporations in this case depend on a number of factors, including the number of metabolic steps prior to the "dimersation", and the pool sizes of each branch intermediate.

Further experiments were carried out with $[\alpha\text{-}^{14}C]$-dopamine and $[\beta\text{-}^{14}C]$ tyrosine fed to plants of Hydrastis canadensis[33] in parallel experiments. Isolation of radioactive hydrastine in both cases, was followed by degradation as described previously, the results showing that dopamine acts as a specific precursor of the alkaloid, but only one unit is involved in the biosynthesis. These results confirm that tyrosine is a common precursor of both branches, (not necessarily at the point of branching) and indicate that dopamine is only associated with one branch. Dopamine may not be an obligatory precursor, but only the source of a true intermediate. The results of the tracer experiments are summarised below.



Morphine Alkaloids

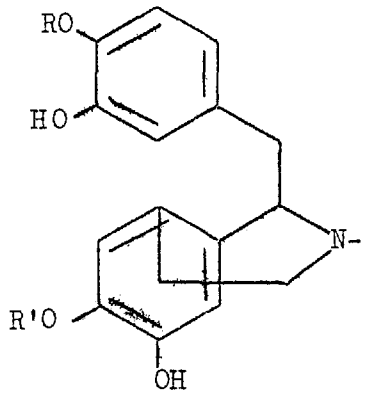
Morphine and related isoquinoline alkaloids have long been thought to arise by oxidative coupling of the aromatic rings of a benzylisoquinoline molecule such as norlaudanosoline,[11] derivable in turn from two molecules of dihydroxyphenylalanine.[10] Of the many mechanisms for the cyclisation,[11,39,40,41] the most satisfying is that of Barton and Cohen. This scheme, involving the concept of radical pairing, was based on knowledge gained during structural work on Pummerer's ketone.

Norlaudanosoline (LI) (where R,R' are suitable blocking groups, e.g. part of an enzyme surface, readily added or removed from phenolic hydroxyl groups to ensure specific coupling processes, the state of the nitrogen being unspecified) on oxidation by a one-electron transfer process would give the diradical (LII). Coupling of this diradical would give the dienone (LIII) which could tautomerise as shown to give the oxide bridge in (LIV). Simple changes are then required to give rise to thebaine (LV), morphine (LVI, R=H) and codeine (LVI, R=Me).

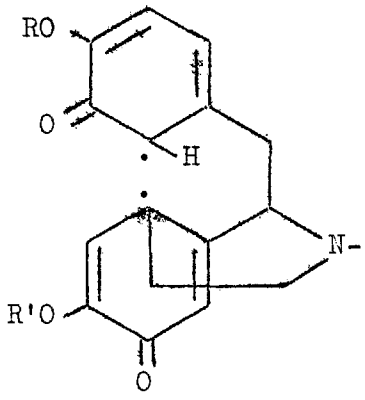
The majority of aporphine alkaloids can be derived from the same type of precursor by interchange of the coupling positions. Thus an ortho-ortho coupling of the norlaudanosoline skeleton (written as in LVII) would give rise to alkaloids of the corytuberine family (LVIII), whilst ortho-para coupling would lead to the glaucine type skeleton (LIX).

Alkaloids such as crebanine (LXI) whose biogenesis is

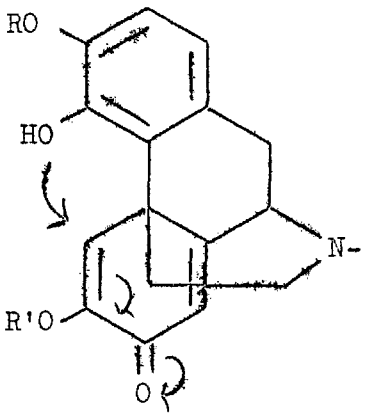
Biogenesis of the Morphine Alkaloids (Barton and Cohen)



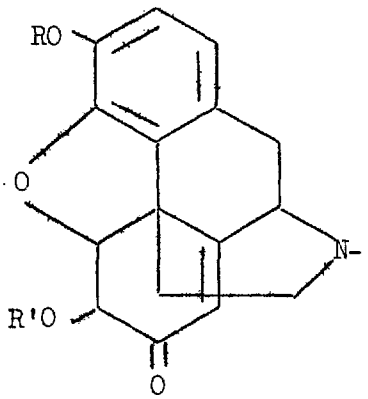
(LI)



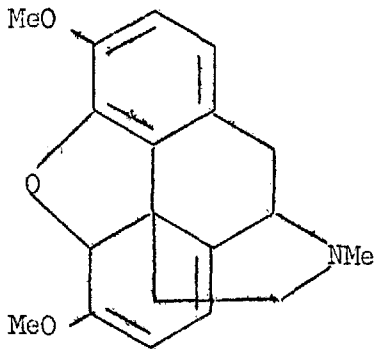
(LII)



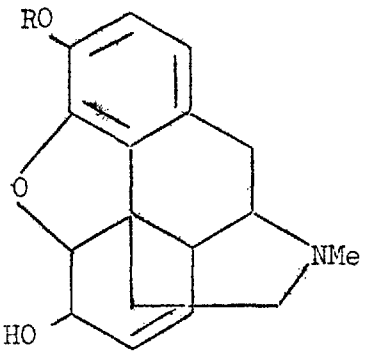
(LIII)



(LIV)



(LV)



(LVI)

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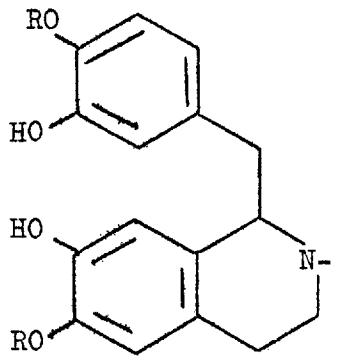
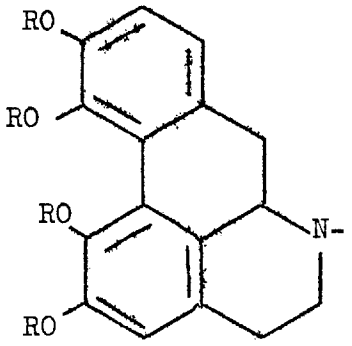
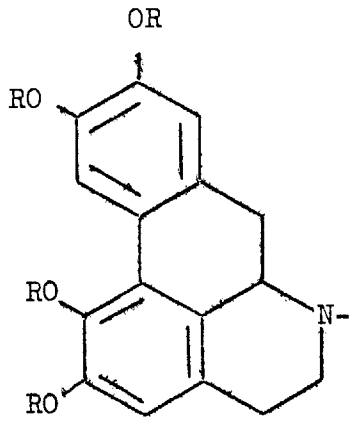


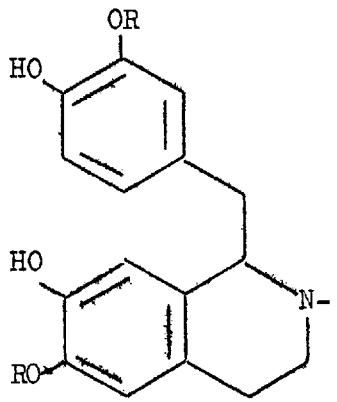
(LVII)

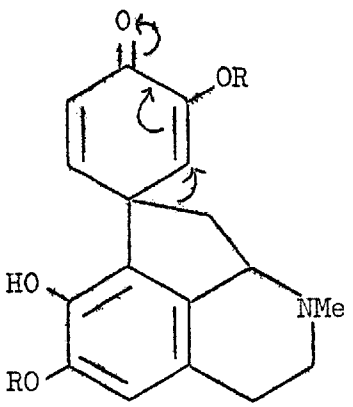


(LVIII)

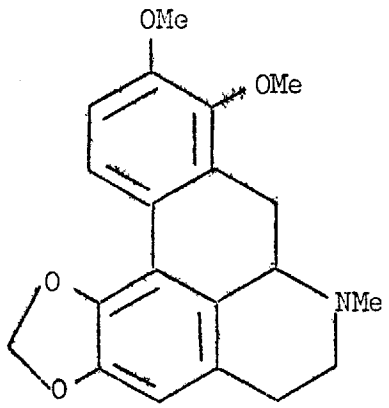


(LIX)

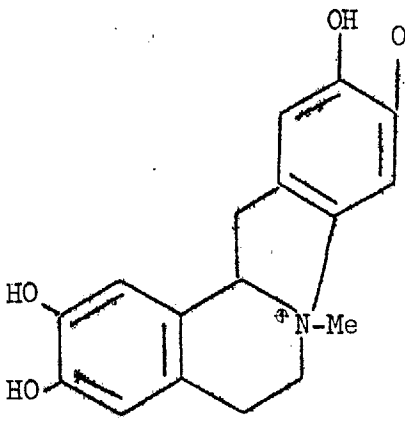




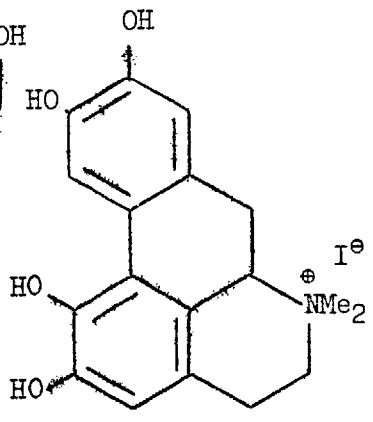
(LX)



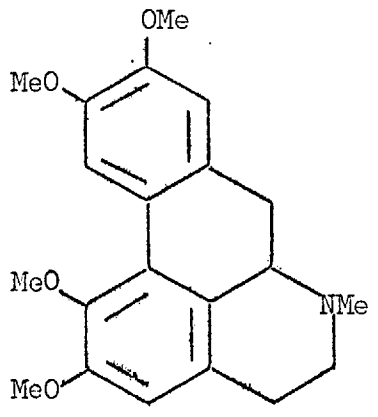
(LXI)



(LXII)



(LXIII)



(LXIV)

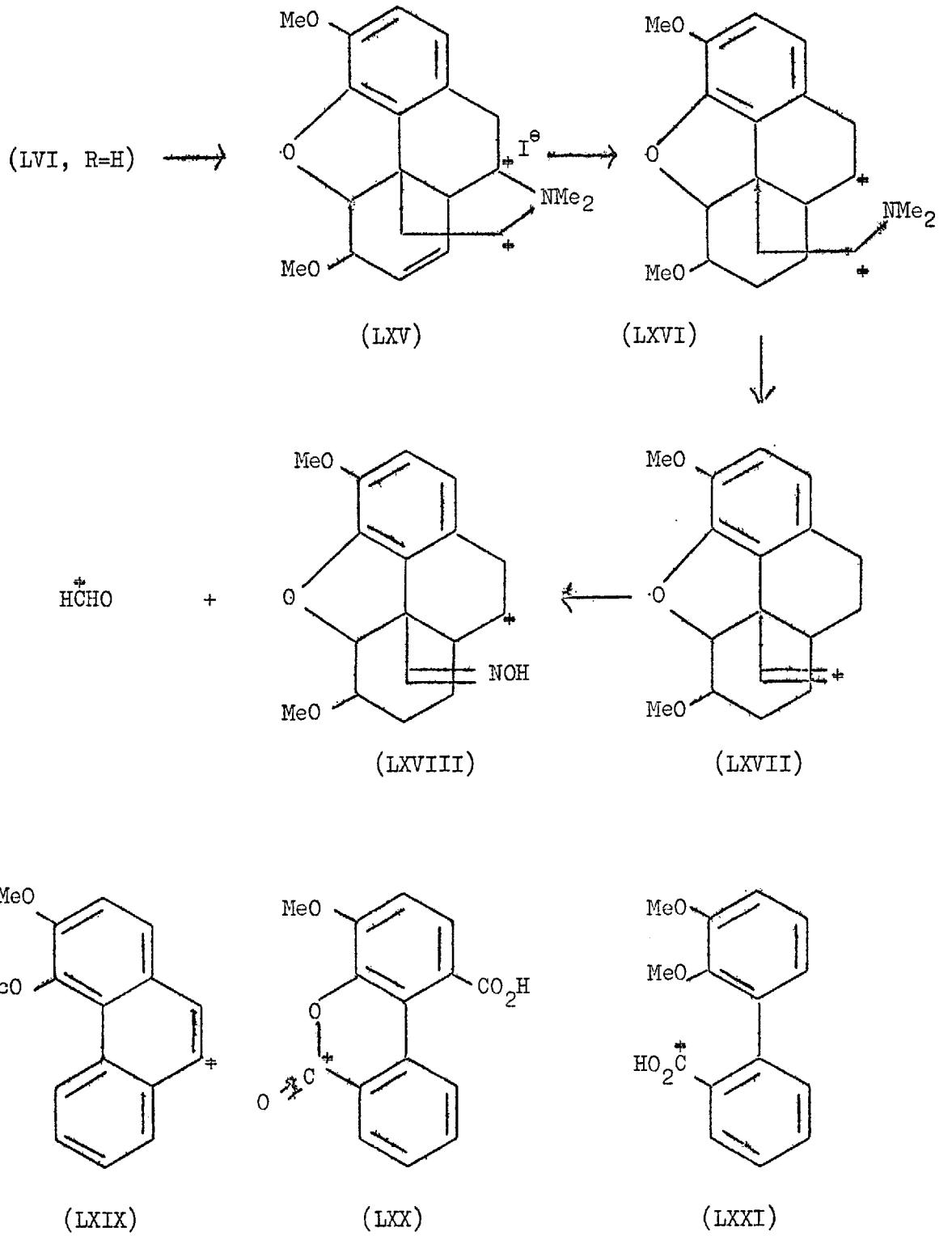
not immediately obvious can also be incorporated in the general scheme by a series of reactions involving dienone-phenol rearrangements of an intermediate such as (LX).

Early attempts to simulate the oxidative coupling process in the laboratory were unsuccessful, [42, 43] mild oxidation of norlaudanosoline giving dehydronorlaudanosoline (LXII). Recently,[44] oxidation of norlaudanosoline methiodide with ferric-chloride, however, has resulted in a high yield of (LXIII), identical with the product obtained by demethylation and quaternisation of glaucine (LXIV).

Tracer studies on the morphine group of alkaloids have led to a more complete biosynthetic picture of this field than perhaps any other. Early work[45,46] established that two molecules of tyrosine or its equivalent are built into morphine. Two groups independently fed $\left[2\text{-}^{14}C\right]$ tyrosine and $\left[2\text{-}^{14}C\right]$ phenyalanine to Papaver somniferum plants and isolated the radioactive morphine, thebaine and codeine[45]. Tyrosine was a much more efficient precursor than phenylalanine. Degradation of labelled morphine by one group[45] was accomplished as follows.

Methylation and quaternisation gave compound (LXV) which was subjected to Hofmann degradation, and reduced to give the tetrahydrocodemethine (LXVI). Further Hofmann degradation gave the morphenol derivative (LXVII), which on oxidation gave formaldehyde and a major component isolated as its oxime (LXVIII), each containing half the activity, and establishing the position of

Degradation of Morphine



one labelled atom. Later work[47] established the position of the second labelled carbon atom. The methine was treated with sodium ethoxide to give the phenanthrene purified as its acetate (LXIX). Chromic acid oxidation gave a quinone which was cleaved by periodate to give the diphenic acid. Hydrolysis of the acetyl group gave the coumarin (LXX) which was decarboxylated with strong acid. Vigorous alkaline hydrolysis in the presence of dimethyl sulphate gave the dimethoxy acid (LXXI), which was decarboxylated with copper chromite in quinoline, to give radioactive carbon dioxide containing half the activity of the original alkaloid.

An alternative degradation[46] supported the latter work. The phenanthrene (LXIX), containing half the activity of the alkaloid, was oxidised to give phthalic acid. A Schmidt reaction on the acid gave anthranilic acid containing one quarter of the original morphine activity, this the author reasoned was due to the equivalence of the carboxyl groups of the acid, and means half the activity is situated in one of these, or shared between both. The former being the more plausible alternative, the results agree with the full degradative work. The specific incorporation of tyrosine into morphine with essentially the same activity at the two predicted positions is good evidence that the morphine skeleton is built up from tyrosine (or its equivalent) via norlaudanosoline in accordance with theory.[11]

Further confirmation came from the incorporation of

[^{14}C] norlaudanosoline into morphine in Papaver somniferum plants.[48] Degradation of the alkaloid[49] showed that specific incorporation had taken place, and the incorporation was higher than that of any other precursor used. The high incorporation of such an aromatic system suggests that morphine is in fact derived by a coupling reaction from such a precursor, and that norlaudanosoline is close to it on the biosynthetic pathway.

Evidence for the later stages of the biosynthesis has rapidly accumulated. Studies with [^{14}C] carbon dioxide[49] and [2-^{14}C] tyrosine[50] have established a biosynthetic route from thebaine through codeine to morphine. Tyrosine was found to be a precursor of all three alkaloids in Papaver somniferum, and the rate of incorporation investigated. The results[50] showed a rapid uptake of activity into thebaine, followed by a rise in codeine, then a steady fall in both relative to morphine. Similar results were obtained using [^{14}C] carbon dioxide.

Confirmation of these results came from a series of elegant experiments using [^{14}C] generally-labelled alkaloids.[51] When labelled thebaine was fed to the plants, radioactive codeine and morphine could be isolated. Feeding of labelled codeine resulted in the isolation of essentially inactive thebaine, the morphine being radioactive, whilst on administration of labelled morphine, both thebaine and codeine isolated were inactive. These results not only establish the pathway from thebaine to morphine, but establish O-demethylation of alkaloids as a metabolic

pathway, since no significant reversibility of any of the steps was encountered. The high incorporations (ca. 5-7%) obtained indicate the transformations must be a major biosynthetic pathway, and dispose of the theory of methylation as a terminal step used by plants as a deactivating process.[52]

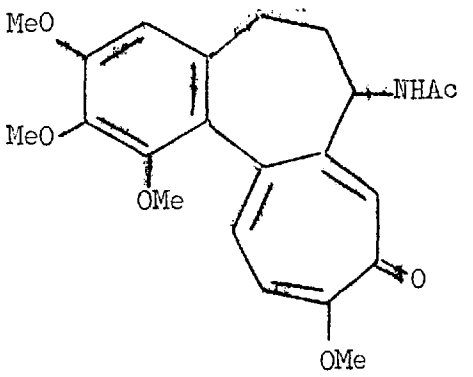
In one case[53] a higher incorporation of glucose than of tyrosine was achieved in Papaver somniferum, although it is generally accepted[54] that tyrosine is formed from glucose. The low incorporation of tyrosine coupled with this fact led one author[51] to suggest that tyrosine incorporation was by a minor or aberrant pathway. The same author degraded labelled morphine[55] obtained from $[^{14}C]$ carbon dioxide and came to the conclusion that the hydrophenanthrene skeleton was not derived from two identical molecules, in contradiction with all other work.[37,45,46].

The weight of evidence at present, however, indicates the use of two tyrosine molecules to provide norlaudanosoline, which has also been established as a precursor of the morphine alkaloids, followed by oxidative coupling to give the morphine skeleton. Experiments with methylated norlaudanosoline type precursors should establish the methylation sequence.

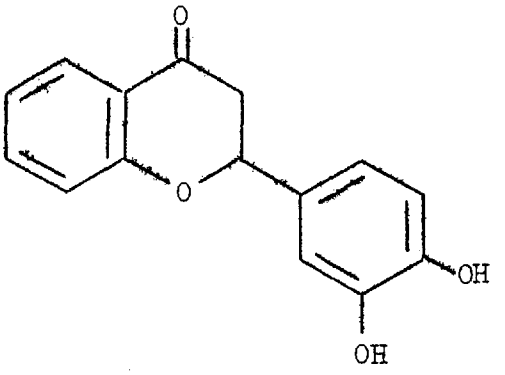
Colchicine

Several schemes have been postulated for the biogenesis of colchicine. One suggestion[56] is that colchicine (LXXII) may be biogenetically related to the flavones (LXXIII), the tropolone ring being derived from the catechol ring.[57] Another scheme

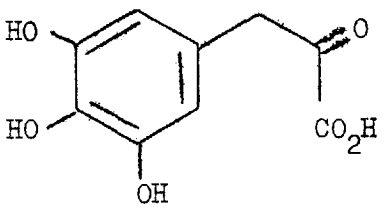
Biogenetic Schemes for Colchicine



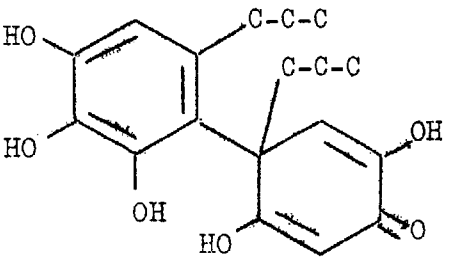
(LXXII)



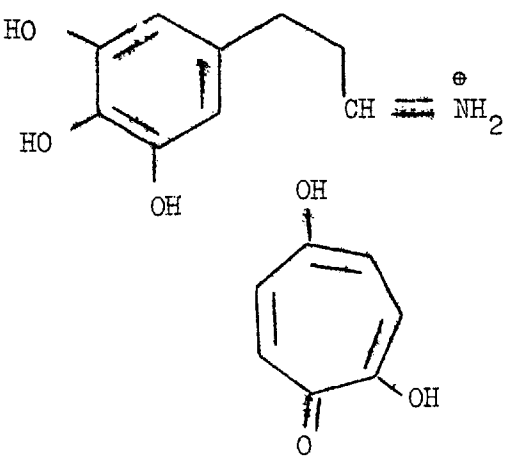
(LXXIII)



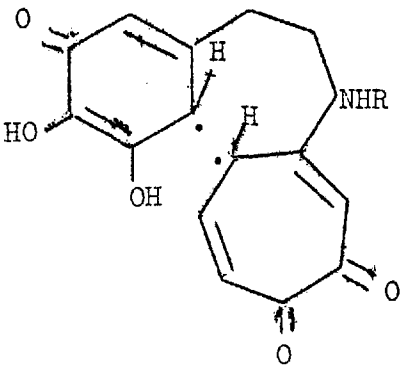
(LXXIV)



(LXXV)



(LXXVI)



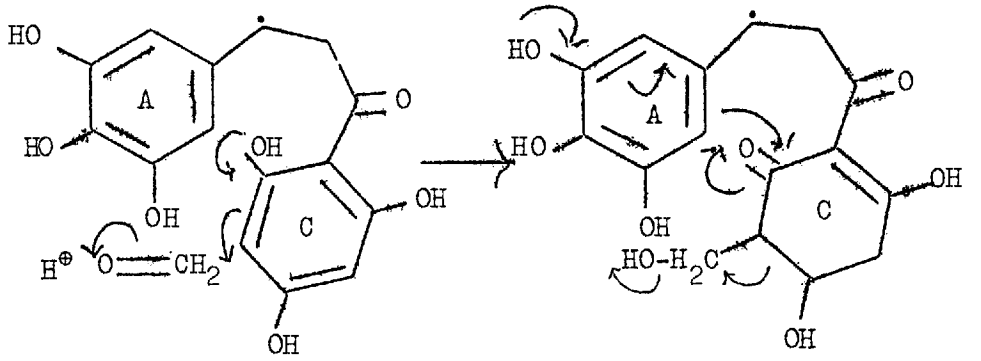
(LXXVII)

postulates the oxidative coupling of two molecules of 3,4,5-trihydroxyphenylpyruvic acid[58] (LXXIV) to yield (LXXV), which on fission of the quinonoid ring followed by cyclisation of the fragments, could give the colchicine skeleton. Wenkert on the other hand[59] conceived the formation of colchicine from condensation of a protonated Schiff's base and preformed 5-hydroxytropolone (LXXVI), whilst Scott[60] suggested that colchicine arose by oxidative coupling of a preformed tropolone ring (LXXVII).

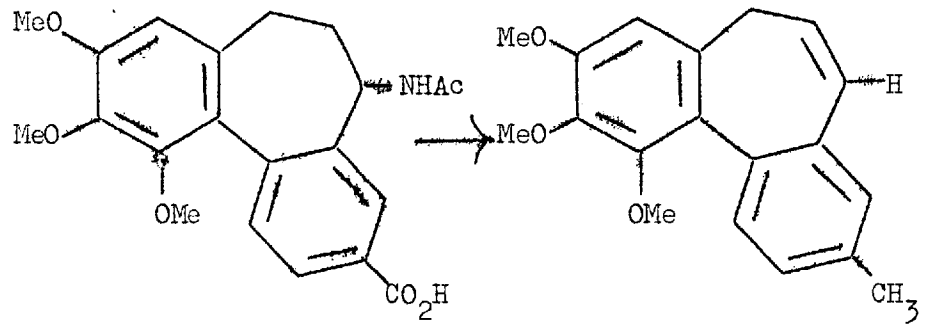
Tracer studies were carried out with [3-^{14}C] phenylalanine, when radioactive colchicine was isolated from Colchicum byzantinum[62]. Oxidation of the colchicine with ferricyanide gave 3,4,5-trimethoxyphthalic anhydride, which was treated with concentrated acid to give trimethoxybenzoic acid. Decarboxylation with copper chromite in quinoline gave carbon dioxide, containing essentially all the activity. This result was compatible with Wenkert's hypothesis, but had the wrong labelling pattern for the other schemes.

The authors favoured a relationship to flavanoid biogenesis and modified one scheme[56] accordingly. In this scheme[61] ring A of colchicine corresponds to the 3,4-dihydroxyphenyl substituent, and the acetate derived ring C reacts with formaldehyde to give (LXXVIII). Ring enlargement, followed by condensation with ring A gives the colchicine skeleton (LXXIX), the labelling pattern of which is compatible with their results.

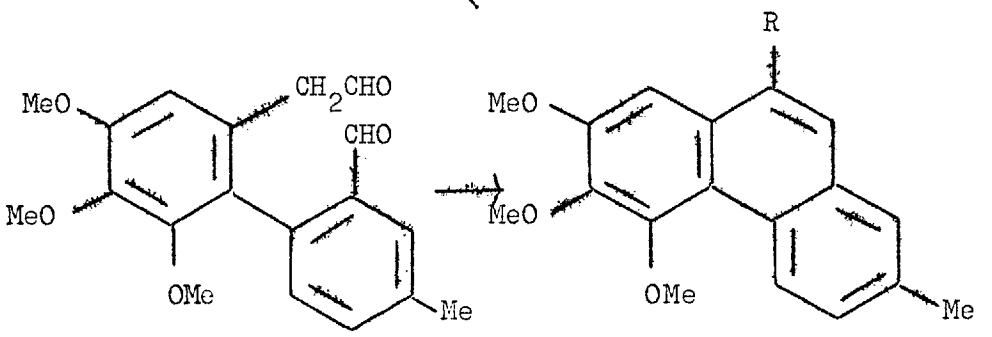
Independent work[62] was carried out with [2-^{14}C] tyrosine



(LXXVIII) (LXXIX)



(LXXX) (LXXXI)



(LXXXII) (LXXXIII)

fed to Colchicum autumnale, the radioactive colchicine being degraded as shown in the annexed acheme. Rearrangement of colchicine gave allocolchiceine (LXXX), which was reduced with lithium aluminium hydride, hydrogenolysed and subject to Hofmann degradation to give the neutral product (LXXXI). Hydroxylation and cleavage with osmium tetroxide and periodate gave the dialdehyde (LXXXII) which was cyclised to the phenanthrene (LXXXIII, R=CHO). Oxidation to the acid (LXXXIII, R = CO_2H), and decarboxylation gave virtually inactive carbon dioxide. None of the activity resided at the predicted position, and the most reasonable interpretation of the above results is that the amino acid is degraded to a C_6 - C_1 unit before incorporation.

Both groups of workers [62,63] fed [1-^{14}C] acetate, which led to the surprising conclusion that the tropolone ring is not derived from acetate, all activity residing in the N-acetyl group.

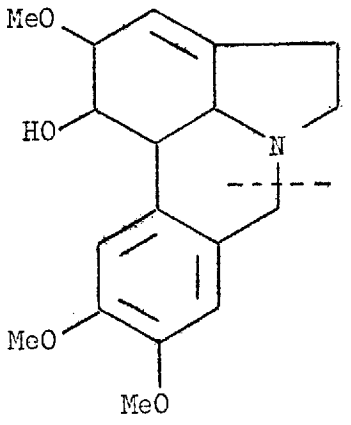
All that has in fact, been established is that phenylalanine is a direct precursor of the aromatic ring of colechicine, the biosynthesis of the tropolone residue remaining unknown.

BIOSYNTHESIS OF AMARYLLIDACEAE ALKALOIDS

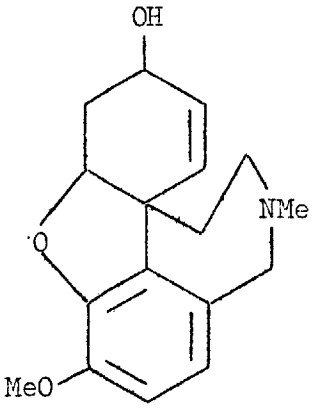
Introduction

The Amaryllidaceae alkaloids comprise an extensive group of alkaloids which have been the subject of intensive investigation during the past few years, and an excellent review of their chemistry has recently been published by Wildman.[64]

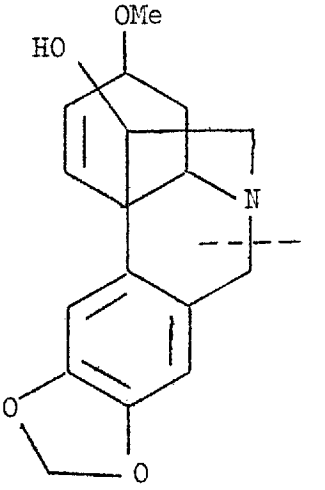
The alkaloids can be divided into three main classes based on (a) a pyrrolophenanthridine skeleton, for example,(LXXXIV) galanthine, (b) dibenzofuran skeleton, for example, galanthamine, (LXXXV), and (c) an ethanophenanthridine ring system, for example, haemanthamine (LXXXVI).



(LXXXIV)



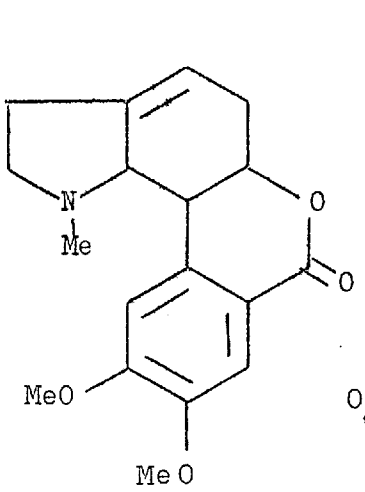
(LXXXV)



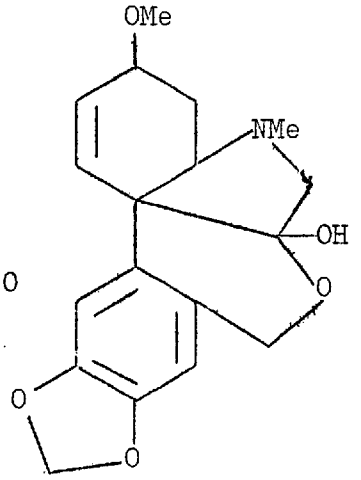
(LXXXVI)

The other classes of alkaloids can, in principle at least, be derived from the main classes by processes of cleavage, rearrangement and recyclisation. Oxidative cleavage of (LXXXIV) at the indicated position could lead to the alkaloids based on a

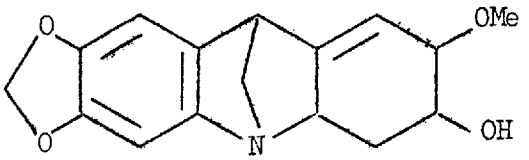
benzpyranoindole, for example homolycorine (LXXXVII). Oxidative cleavage of the haemanthamine type skeleton followed by an internal oxidation-reduction, and recyclisation would give rise to tazettine (LXXXVIII), one of the most structurally complex of the Amaryllidaceae alkaloids. Yet another rearrangement with migration of the methylenedioxyphenyl ring, can give rise to the recently isolated alkaloids based on a methanomorphanthridine skeleton, for example montanine (LXXXIX).



(LXXXVII)



(LXXXVIII)

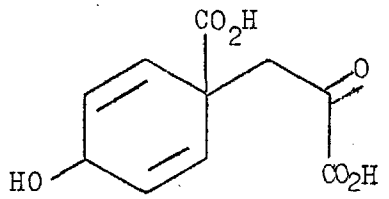


(LXXXIX)

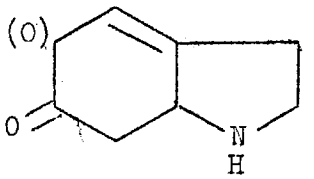
In spite of the apparent diversity of structures shown by the Amaryllidaceae alkaloids they are, in fact, very closely related. On investigation, it can be seen that all the alkaloids contain a common hydroaromatic C_6-C_2 unit and an aromatic C_6-C_1 unit. Theories for their biogenesis have been proposed by Wenkert[4], and Barton and Cohen.[41]

Wenkert suggested that the alkaloids arise from the pyrroles (XCI) and (XCII), derived from prephenic acid (XC) by ammination and hydration.

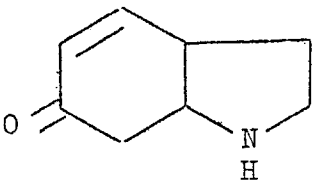
Biogenesis of Amaryllidaceae Alkaloids(Wenkert)



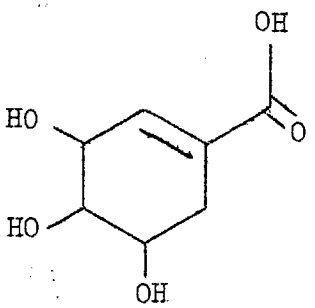
(XC)



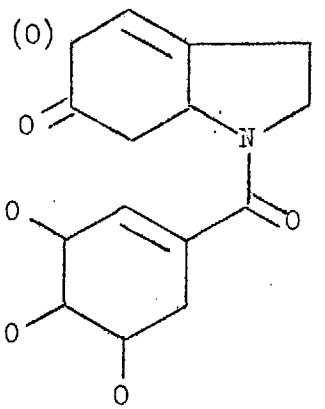
(XCI)



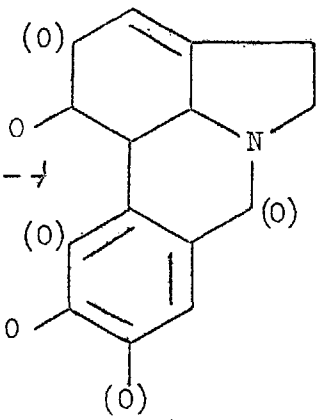
(XCII)



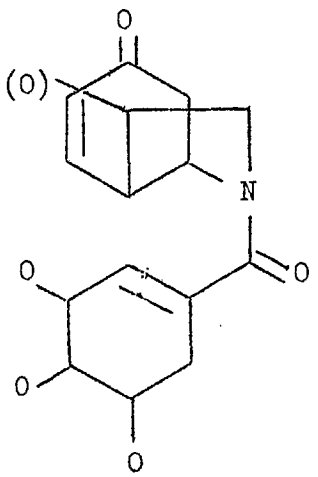
(XCIII)



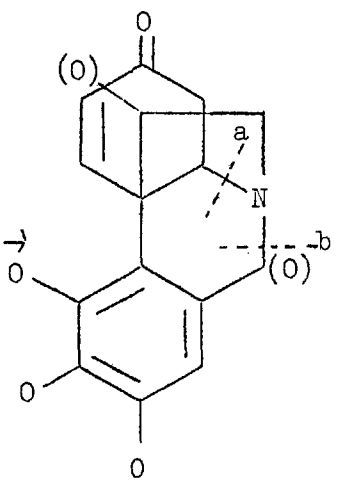
(XCIV)



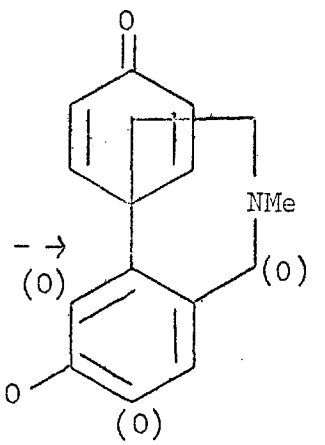
(XCV)



(XCVI)



(XCVII)



(XCVIII)

Condensation of the pyrrole (XCI) with shikimic acid (XCIII) would give the intermediate (XCIV). A Michael reaction followed by subsequent hydration-dehydration and oxidation-reduction changes could give rise to the lycorine skeleton (XCV).

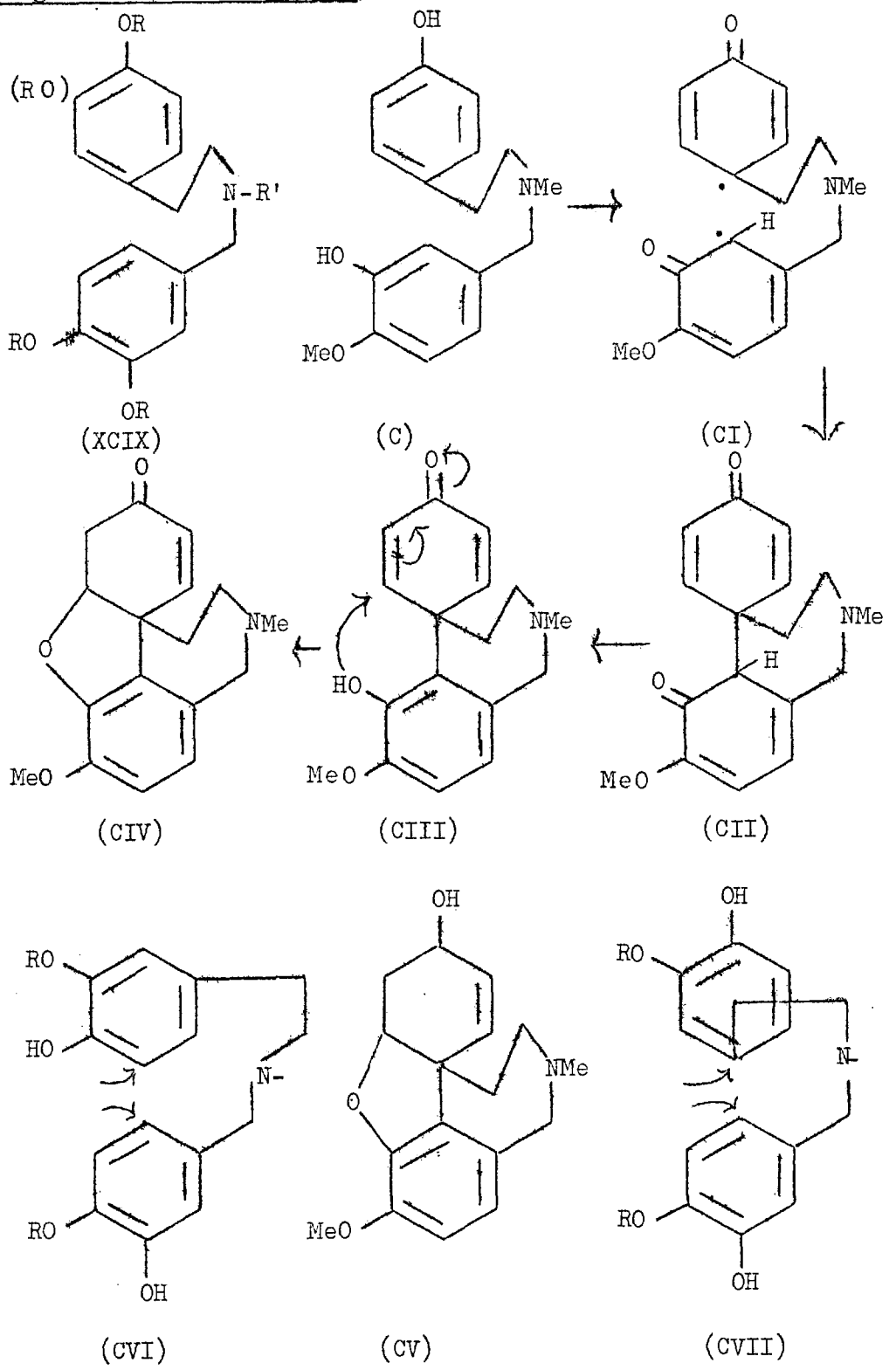
The second pyrrole (XCII) by similar condensation with shikimic acid would give the intermediate (XCVI), which by similar sequences of reactions could give rise to the haemanthamine skeleton (XCVII). Quaternisation of the nitrogen, followed by cleavage at bond "a" would lead to the dienone (XCVIII), which by simple changes would give the galanthamine skeleton. Cleavage at bond "b" would give the tazettine skeleton.

Wenkert's theory accurately locates all the oxygen atoms in the alkaloid family.

Barton and Cohen, on the other hand, suggested that the alkaloids arise from the oxidative coupling of the two phenolic rings of a precursor of general type (XCIX, where R = H or suitable blocking groups, which could be alkyl or part of an enzyme surface). Ortho and para coupling reactions, specifically controlled by the blocking groups can then account for the variety of structures in the family.

Oxidation of the N-benzylphenylethylamine (C) would give the diradical (CI), which on radical pairing would give the dienone (CII). Aromatisation of the bottom ring, followed by addition of the hydroxyl group across the unsaturated system in (CIII), would give the oxide bridge in (CIV). Reduction of the

Biogenesis (Barton & Cohen)



unsaturated ketone would then give galanthamine (CV).

A similar sequence of reactions involving an ortho-para coupling of the precursor as indicated, (CVI), would give rise to the lycorine skeleton. The same precursor (written as CVII) could give the haemanthamine skeleton by a para-para coupling between the indicated positions.

The same precursor (XCIX) can, in fact, embrace all the alkaloids in the family.

Support for the latter theory has come from the isolation of the alkaloids belladine[65] (XCIX, R' = Me), the fully methylated precursor in Amaryllis belladonna, and narwedine[66] (CIV), a postulated intermediate in the pathway, with galanthamine in Texas daffodils.

The greatest weight of evidence for the concept of Barton and Cohen has come from the verification of the predicted structure (CV) for galanthamine with its in vitro synthesis by Barton and Kirby.[67] Although, of course, this synthesis does not prove that the biosynthesis of galanthamine necessarily follows the same route, it does increase the plausibility of the concept.

Oxidation of the phenol (C), $[^{14}C]$ labelled in its N-methyl group, with a variety of oxidising agents in the presence of racemic inactive narwedine showed that narwedine was formed, and in fact yields up to 1.4% of the alkaloid were isolated from the large amount of polymer formed in the reaction. The isolated (±)-narwedine was then reduced to give (±)-galanthamine as the

major product. Resolution of the racemic galanthamine was carried out by a novel technique. It was found that recrystallisation of (±)-narwedine in the presence of (-)-galanthamine gave partly resolved (+)-narwedine. Reduction of (+)-narwedine so obtained gave (+)-galanthamine which, in turn, resolved (±)-narwedine to give (-)-narwedine. Reduction gave (-)-galanthamine identical with the naturally occurring alkaloid.

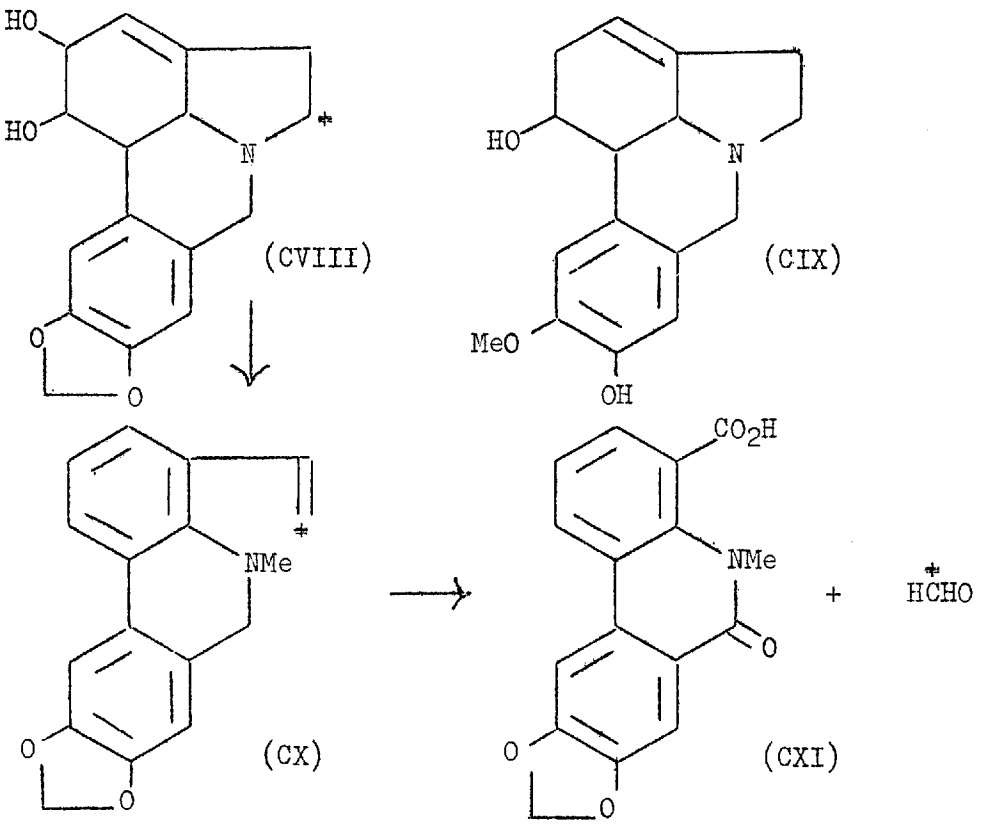
On the basis of a biogenetic relationship with naturally occurring (-)-galanthamine, narwedine isolated from the plant would be expected to have a negative rotation, contrary to that of $[\alpha]_D$+100° observed by Boit.[66] Repetition of this isolation, in collaboration with the present author (exptl. P.83), gave (+)-narwedine with an even lower rotation $[\alpha]_D$+52°. Crystallisation of (±)-narwedine from benzene in the presence of (-)-galanthamine also gave pure narwedine with a similar rotation. It may well be that the compound exists in the plant in the racemic form, or is racemised on work up, or it mayyet belong to the (-) series, the positive rotations observed being simply due to the above-mentioned phenomenon.

Early Tracer Studies

There is an essential difference between the two biogenetic concepts outlined earlier. Wenkert postulated the derivation of the hydroaromatic C_6-C_2 unit from a hydroaromatic precursor which never becomes aromatic, whilst Barton and Cohen suggested that the C_6-C_2 unit is, in fact, derived from an

aromatic precursor. In preliminary work, Barton and Kirby[68] isolated radioactive galanthamine from King Alfred daffodils fed with [2-^{14}C] tyrosine and [^{14}C] phenol (C), but the necessary degradations to establish the labelling pattern were not carried out. Radioactive lycorine was also isolated from the tyrosine-fed plants.

In an independent study,[69] Battersby and Binks isolated radioactive lycorine (0.23% incorporation) (CVIII), and norpluviine (CIX) from Twink daffodils fed with [2-^{14}C] tyrosine. The lycorine labelling pattern was established as follows.

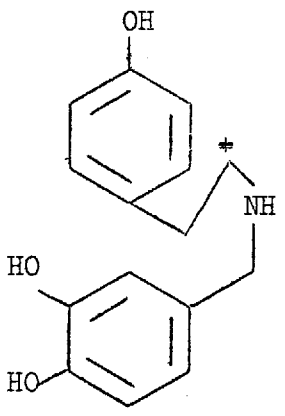


Methylation of the radioactive lycorine gave a mixture of α and β-lycorine methiodide, which were subjected to Hofmann degradation. The resulting methine (CX) was hydroxylated and

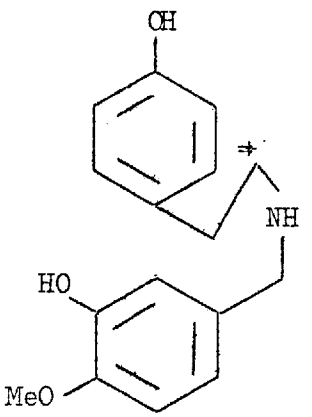
cleaved to give formaldehyde containing essentially all the activity. The residue, without isolation was oxidised to the lactam acid (CXI) which, as expected, was virtually inactive.

It is generally accepted that the conversion of prephenic acid to tyrosine is irreversible,[70] and the above results thus established that tyrosine can provide the hydroaromatic C_6-C_2 unit in accordance with the concept of Barton and Cohen and contrary to that of Wenkert.

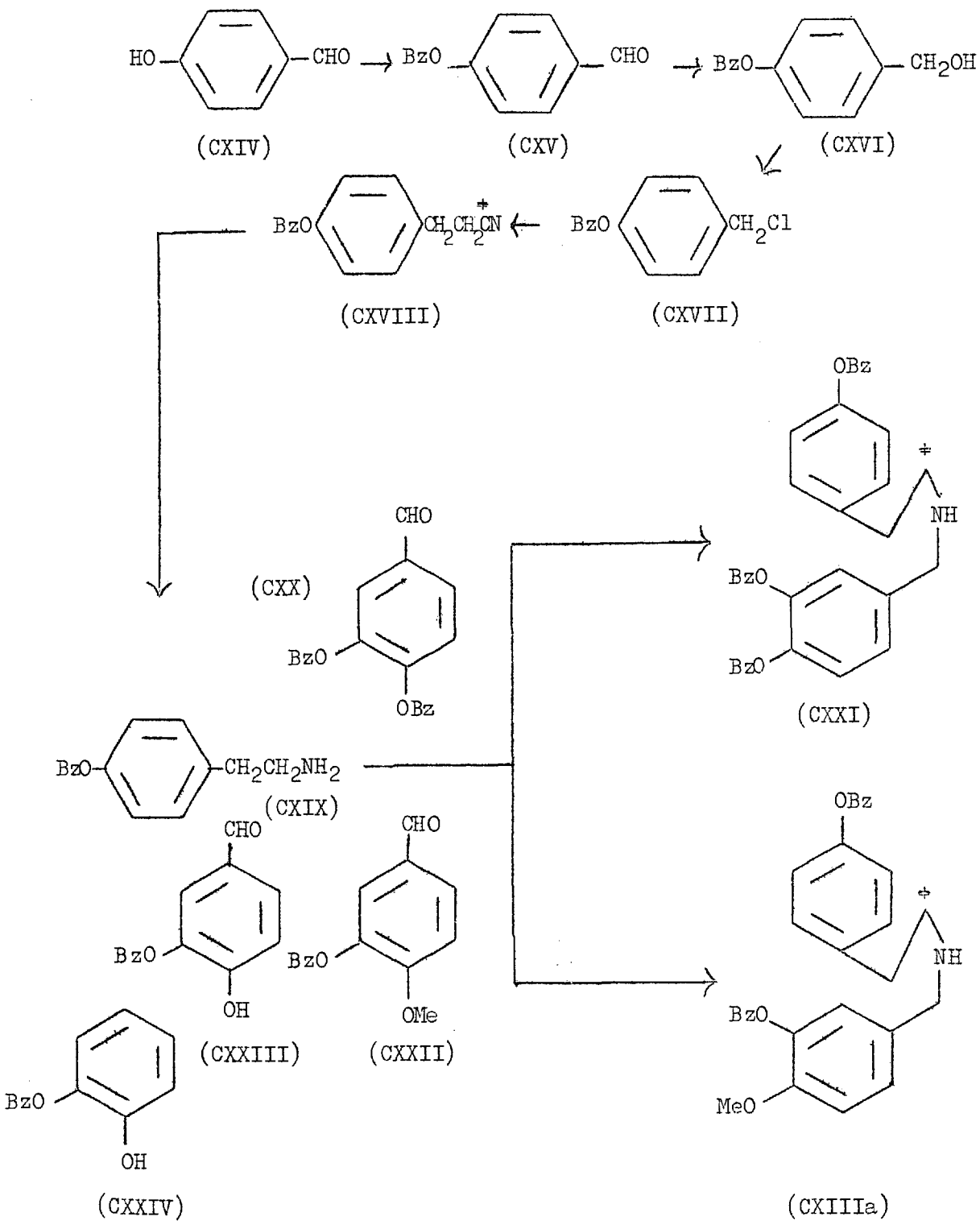
Confirmation of Barton and Cohen's theory can, of course, only come from tracer studies using postulated precursors in the biosynthesis. A plausible precursor of all three main types of alkaloid in the family is norbelladine (CXII), and the bulk of the author's work revolved around the use of [α-^{14}C] norbelladine, and [α-^{14}C]-4-hydroxy-N-(3-hydroxy-4-methoxybenzyl) phenylethylamine (CXIII), (hereafter referred to as O-methylnorbelladine).



(CXII)



(CXIII)


HO
CHO
BzO
CHO
BzO
CH2OH
(CXIV)
(CXV)
(CXVI)
BzO
CH2CH2CN
BzO
CH2Cl
(CXVIII)
(CXVII)
OBz
NH
BzO
BzO
(CXXI)
CHO
(CXX)
BzO
OBz
BzO
CH2CH2NH2
(CXIX)
CHO
CHO
BzO
OH
BzO
OMe
(CXXIII)
(CXXII)
OBz
NH
BzO
MeO
BzO
OH
(CXXIV)
(CXIIIa)

Synthesis of precursors

The precursors were synthesised, labelled in the indicated positions, according to the annexed scheme.

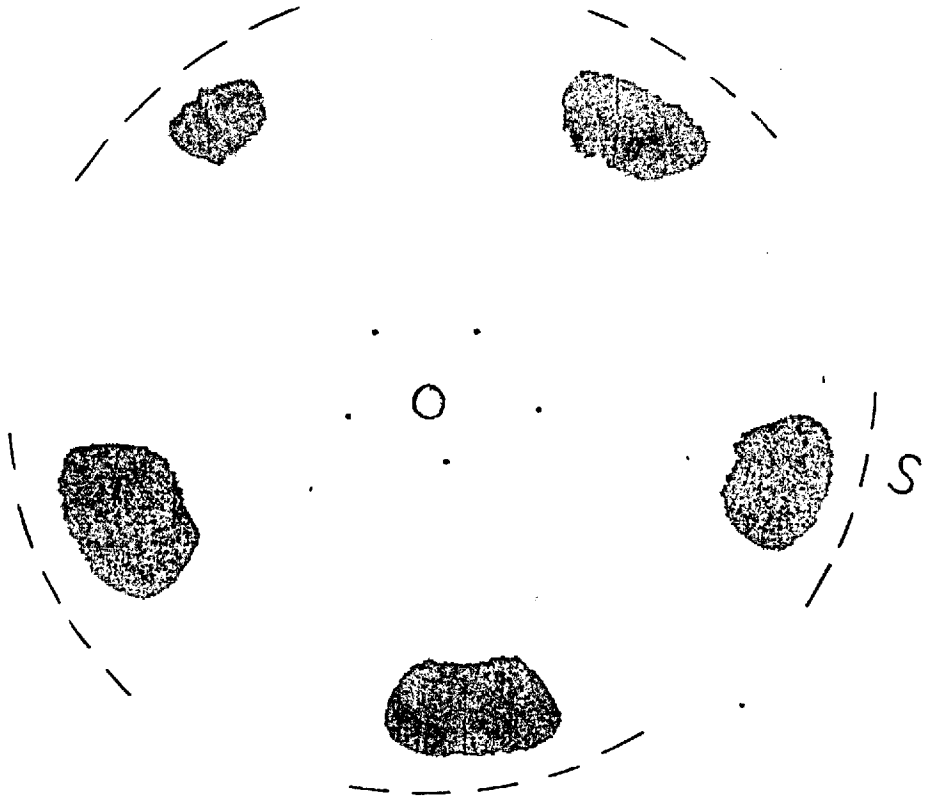
p-Hydroxybenzaldehyde (CXIV) on treatment with excess benzyl chloride, in ethanolic alkali gave p-benzyloxybenzaldehyde (CXV), which was reduced by potassium borohydride, in suspension, to give the alcohol (CXVI). Treatment of the alcohol with thionyl chloride under the usual conditions gave the benzyl chloride (CXVII).

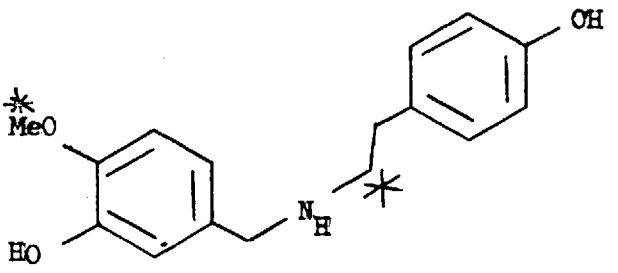
The attempted synthesis of the cyanide (CXVIII) under the usual conditions was unsuccessful, extensive solvolysis occurring. The synthesis was accomplished, in good yield, by the novel use of dimethylsulphoxide as solvent, later reported by other workers.[71] Reduction of the cyanide (CXVIII) with lithium aluminium hydride in ether, gave the key intermediate O-benzyl-tyramine (CXIX).

Condensation of O-benzyltyramine with O,O-dibenzyl-protocatechuic aldehyde (CXX) prepared by treatment of protocatechuic aldehyde with excess benzyl chloride and potassium carbonate in acetone, and reduction of the resulting imine with potassium borohydride gave the tribenzyl ether (CXXI), purified as its hydrochloride. Hydrogenolysis of (CXXI) using 10% palladium carbon catalyst gave norbelladine (CXII), purified as its hydrochloride.

A similar condensation of O-benzyltyramine with O-benzylisovanillin (CXXII) followed by similar steps to the above,

Autoradiograph of circular paper chromatogram of doubly-labelled precursor (CXIII)





O = Origin

S = Solvent front

gave O-methylnorbelladine (CXIII).

The singly-labelled precursors were prepared by essentially the above procedure, using $[^{14}C]$ sodium cyanide for introduction of the labelled atom into the precursors.

The doubly-labelled O-methylnorbelladine used in some experiments was prepared by an analogous procedure, using $[^{14}C]$-O-benzylisovanillin. This was synthesised as further illustrated in the scheme (method of Mr. G.M. Thomas). Partial benzylation of catechol under usual conditions gave the monobenzyl ether (CXXIV). This was subjected to Reimer Tiemann conditions to give the aldehyde (CXXIII). Methylation with $[^{14}C]$ methyl iodide then gave the labelled aldehyde (CXXII) used in some experiments.

The doubly-labelled precursor was assayed for purity by dilution analysis, and autoradiography of circular paper chromatograms. The compound was shown to be pure both chemically and radiochemically, and in particular, was free from $[^{14}C]$-tyramine. A typical autoradiograph is shown on P.42.

Tracer studies using Galanthus Elwesii Snowdrop

Preliminary experiments were carried out with the early flowering Galanthus elwesii snowdrop. It was hoped to investigate the biozynthesis of tazettine as well as the major alkaloids lycorine and galanthamine, but no tazettine could be isolated from the amounts of plant material used.

Flowering snowdrops, in similar stages of growth, were

fed with [2-^{14}C] tyrosine and [^{14}C] norbelladine, respectively. The bulbs were partly uncovered, and feeding accomplished by direct injection into the bulbs. The plants so suffered no physical harm, being indistinguishable in appearance over the course of the experiments from control plants. The isolated alkaloids after rigorous purification were radioactive, as shown in the following table.

Feeding Expt.	Incorporation (%)	
	galanthamine	lycorine
tyrosine	0.135	0.04
norbelladine	0.053	ca. 0.03

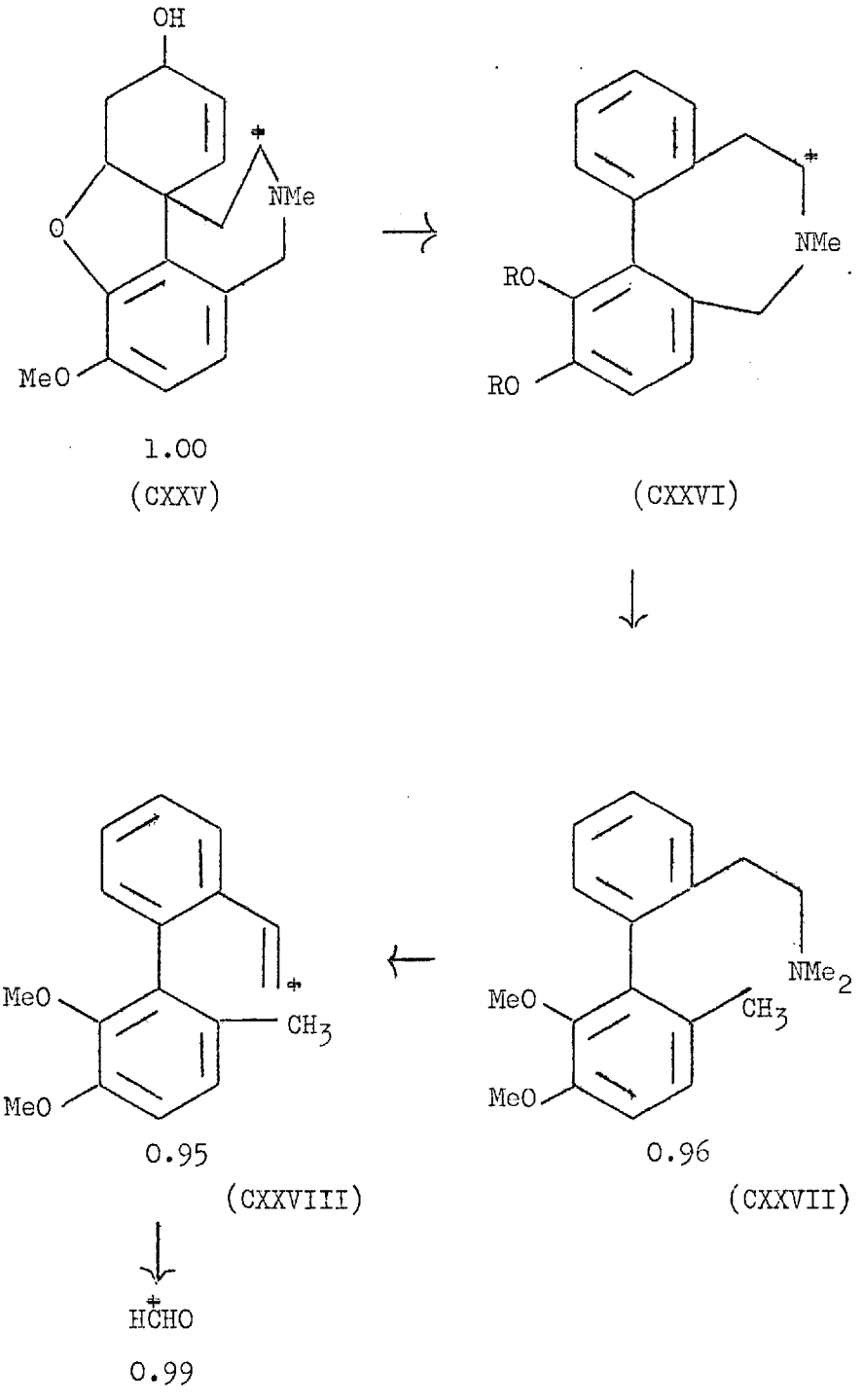
The value for incorporation of norbelladine into lycorine is approximate, since anomolous results were obtained on dilution with inactive alkaloid.

It can be seen that norbelladine is incorporated into the alkaloids with comparable efficiency to tyrosine, in agreement with theory.

The degradation of the radioactive galanthamine obtained from the lycorine experiment was carried out according to the procedure of Kobayashi and Uyeo,[72] with slight modification.

Treatment of galanthamine (CXXV) with aqueous hydrobromic acid, under reflux, gave apogalanthamine (CXXVI. R = H) which was methylated to give the dimethyl ether (CXXVI. R = Me). Emde degradation of the corresponding methochloride gave the base (CXXVII), which was further degraded by Hofmann elimination to

Degradation of Galanthamine



1.00
(CXXV)

(CXXVI)

0.95
(CXXVIII)

0.96
(CXXVII)

0.99

give the vinyldiphenyl (CXXVIII), containing all the activity of the original alkaloid. Ozonolysis under controlled conditions gave formaldehyde, isolated as its dimedone derivative, containing all the activity. The residue was essentially inactive. (The activities of the degradation products are shown with that of galanthamine arbitrarily taken as 1.00).

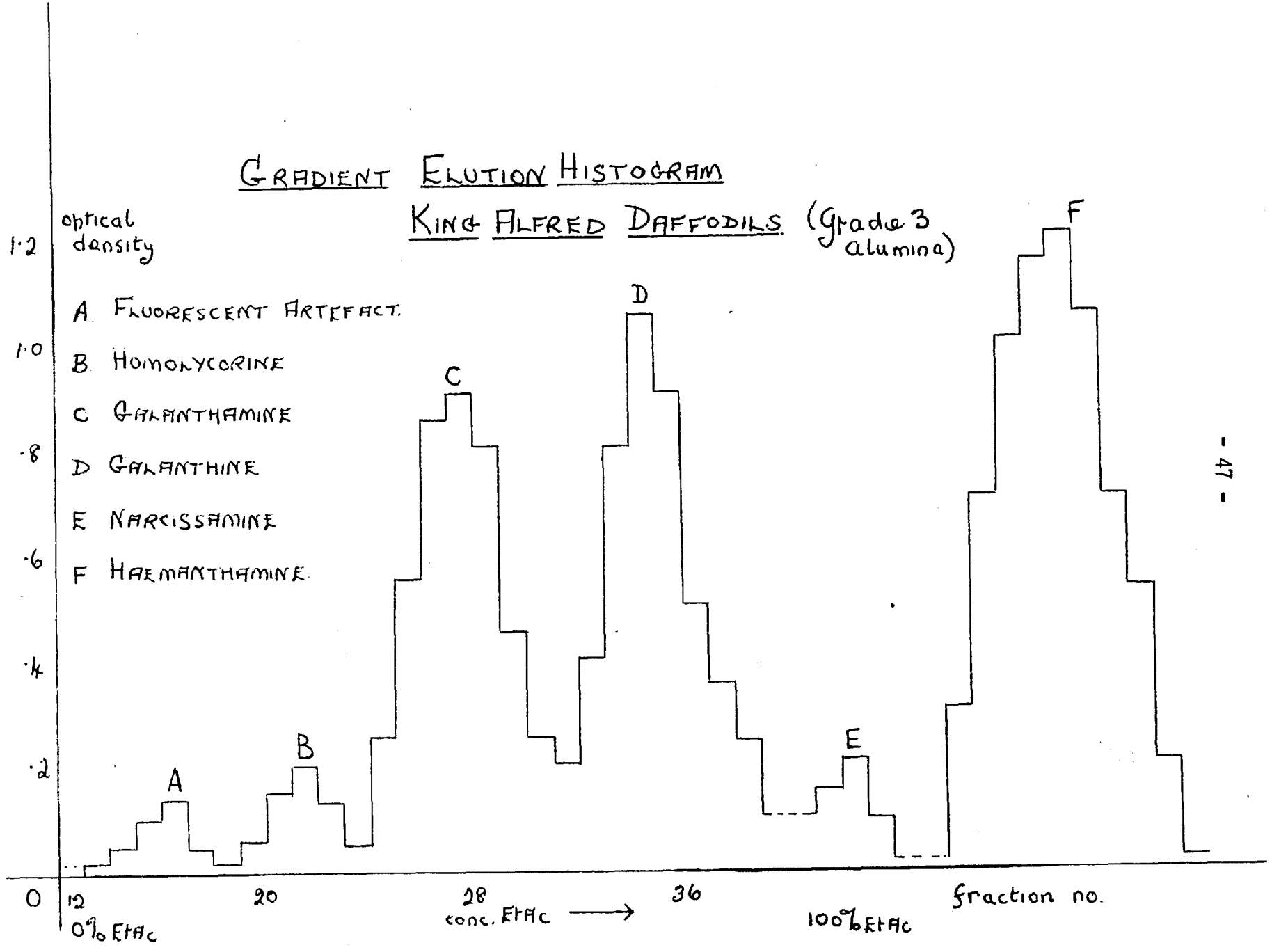
Tyrosine thus acts as a direct precursor of galanthamine in Galanthus elwesii, capable of providing C_6-C_2 unit of the hydroaromatic ring, in accordance with theory. The comparable incorporation of norbelladine suggests that it, too, is a true precursor of the alkaloid, since if degradation occurred to smaller fragments prior to incorporation, a lower value would be anticipated. The labelling pattern has not been established, however, in this case.

Tracer Studies using King Alfred Daffodils

The bulk of the biosynthetic studies were carried out with King Alfred daffodils. The plants were worked up according to the general procedure of Fales, Guiffrida and Wildman,[73] and separated by a gradient elution technique on alumina, elution of fractions being followed in the U.V. A typical gradient elution histogram is shown on P. 47. It can be seen that roughly equal amounts of galanthamine, galanthine and haemanthamine, alkaloids from each of the three major classes, are obtained, and render the plant as an excellent choice for comparative tracer studies. Only minute quantities of the other alkaloids present in the plant

GRADIENT ELUTION HISTOGRAM

KING ALFRED DAFFODILS (Grade 3 alumina)



were obtained from the amount of plant material used.

In comparative experiments, flowering King Alfred daffodils in a similar stage of growth were fed with [2-^{14}C]-tyrosine, [^{14}C] norbelladine and [^{14}C] O-methylnorbelladine. Feeding was accomplished by direct injection of aqueous solutions of the precursors into the hollow stalks of the mature plants. Injections were made at about pH_7, and followed by a further injection of distilled water. The plants suffered no apparent harm, compared with control plants, except in one case (P.68.), when injections of narwedine were found to cause a rapid poisoning of the plant.

The plants were worked up after eight days, and the alkaloids isolated. The results are tabulated below.

	Incorporation (%)		
Feeding expt.	Galanthamine	Galanthine	Haemanthamine
tyrosine	0.012	0.007	0.20
norbelladine	0.014	0.0036	0.25
O-methylnorbelladine	0.0	0.0	0.036

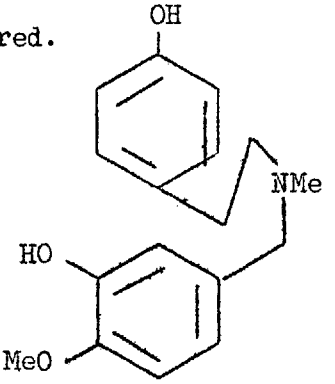
The radioactive galanthamine from the plants fed with norbelladine was degraded as described in earlier work (P.45.). The isolated formaldehyde again contained essentially all the radioactivity, the residue being inactive, the results are summarised below.

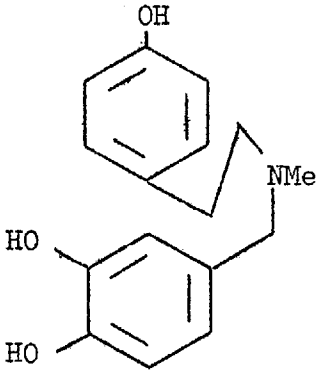
	Fraction of original activity
Galanthamine	1.00
apogalanthamine (CXXVI)	0.96
vinyldiphenyl (CXXVIII)	1.01
formaldehyde dimedone	0.94

This result establishes that norbelladine can provide at least the C_6-C_2 hydroaromatic unit, of galanthamine, and the incorporation, comparable with that of tyrosine, suggests but does not prove, that no degradation takes place.

Work by Mr. G.M. Thomas[74] showed that the compounds (CXXIX) and (CXXX), hereafter called N,O-dimethylnorbelladine and N-methylnorbelladine respectively, are precursors of galanthamine (0.014% and 0.018% incorporation respectively), comparable in efficiency to tyrosine and, in particular, no transmethylation had occurred.

(CXXIX)





(CXXX)

From the results it can be seen that not one of the compounds fed is a very efficient precursor of galanthine in King Alfred, whilst norbelladine is a particularly efficient precursor of haemanthamine.

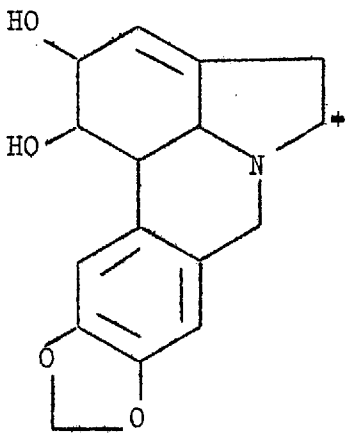
Perhaps the most surprising result is the incorporation of O-methylnorbelladine into haemanthamine (0.036%). At first sight, this compound would not be expected to be a precursor of this alkaloid, since it contains a methoxyl group on the aromatic ring, whilst haemanthamine contains a methylenedioxy group attached to the corresponding position. This may be reasonably

explained by degradation of the precursor to a C_6-C_2 unit prior to incorporation, or alternatively cleavage of the methoxyl group with incorporation of the resultant norbelladine. The lack of observed incorporation into galanthamine and galanthine would then be presumably due to the comparative inefficiency of the cleavage.

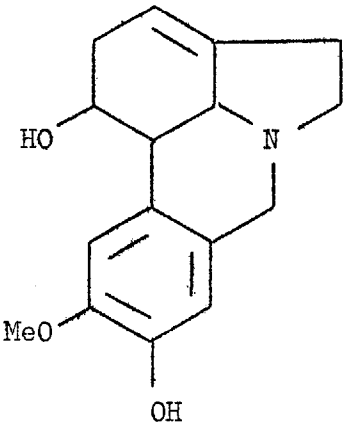
The most interesting possibility, however, is that the methoxyl group of the precursor may be the source of the methylenedioxy group in haemanthamine. Although Kirkwood[35](P.15.) suggested this possibility as a result of tracer studies on the alkaloid protopine, no direct evidence has in fact, been put forward to date. In view of later described work using multiple labelled precursors (P.53.), this possibility seemed the most reasonable, and O-methylnorbelladine has now been shown to be a direct precursor of haemanthamine (P.56.).

From the results (on P.48) it can also be seen that although O-methylnorbelladine may well be a precursor of haemanthamine, it is not a precursor of galanthamine.

Complementary independent work was carried out by Battersby, Binks, Brewer, Fales and Wildman[75] using $[^{14}C]$-norbelladine synthesised by an alternative route. Norbelladine was fed to Twink daffodils when radioactive lycorine (CXXXI) and norpluviine (CXXXII), with incorporations of 0.24% and 0.73% respectively were isolated.



(CXXXI)



(CXXXII)

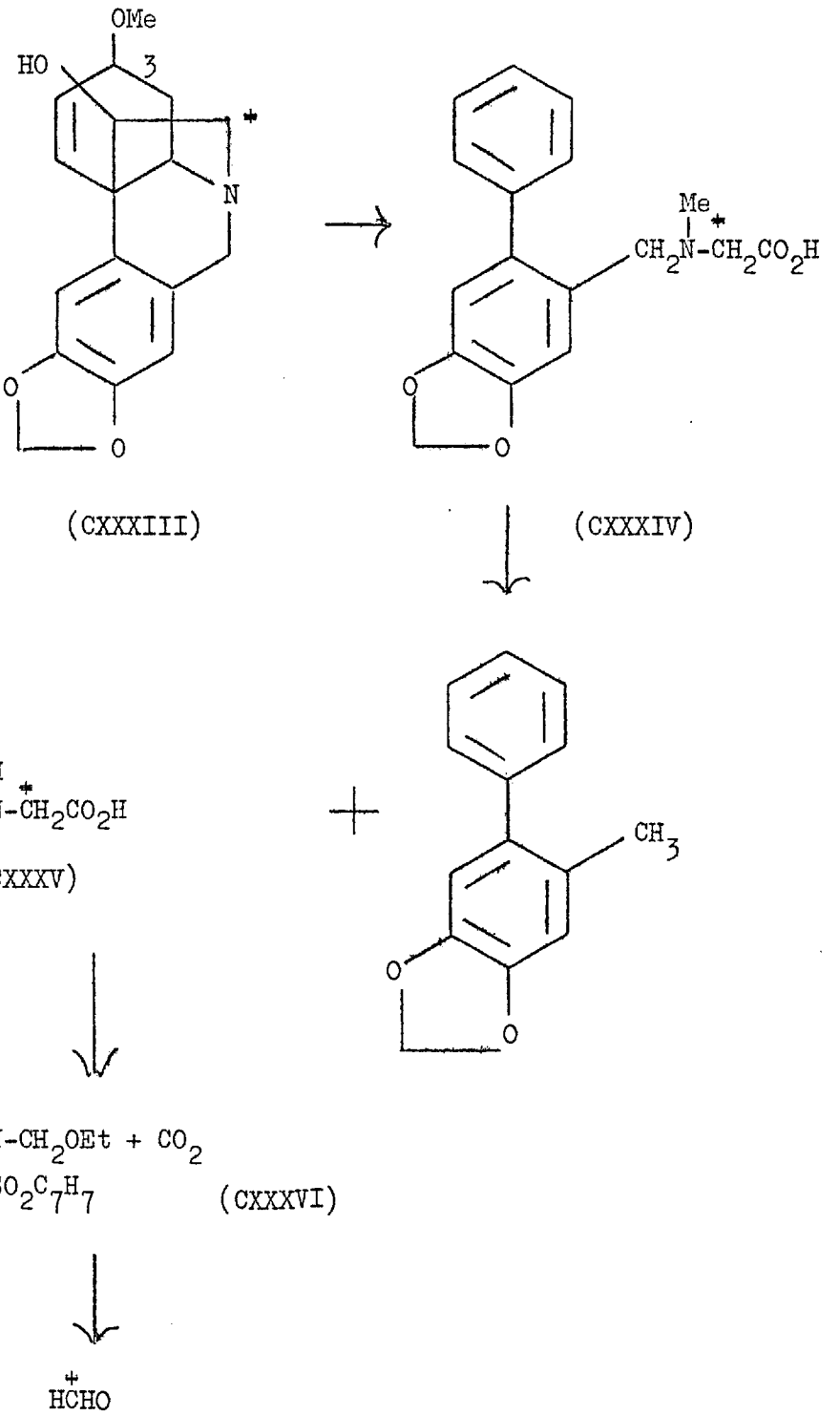
The lycorine was degraded as described in their earlier work (P.38.) when the results showed that norbelladine could supply at least the C_6-C_2 hydroaromatic unit of lycorine. Moreover, the higher incorporation of norbelladine into norpluviine than lycorine was in keeping with closer proximity to norpluviine on the biosynthetic pathway.

Further work[76] confirmed that tyrosine and norbelladine were both precursors of haemanthamine. Radioactive haemanthamine (CXXXIII) and crinamine (C_3 epimer) were isolated from Twink daffodils fed with both precursors, and the haemanthamine in both cases degraded according to the annexed scheme.

Haemanthamine was converted to the amino acid (CXXXIV) which was hydrogenolysed to give sarcosine (CXXXV) isolated as its tosylate. Kolbe electrolysis gave the sulphonamide (CXXXVI) which gave on hydrolysis formaldehyde, containing all the activity.

Tyrosine and norbelladine are thus direct precursors of haemanthamine in Twink daffodils, in accord with the results from

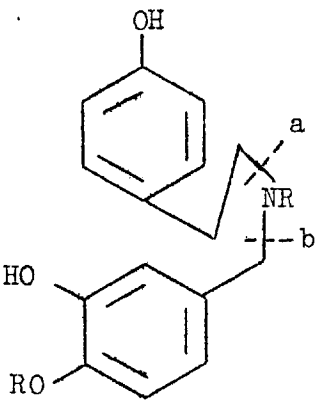
Degradation of Haemanthamine (Battersby)



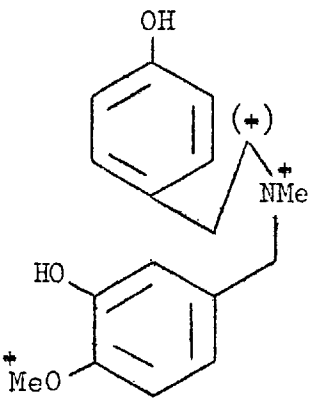
King Alfred daffodils, (P. 48), although the necessary degradations were not carried out in the latter case.

All results are thus in keeping with theory, further supporting the view that oxidative coupling of phenolic intermediates is an important step in the alkaloid biosynthesis.

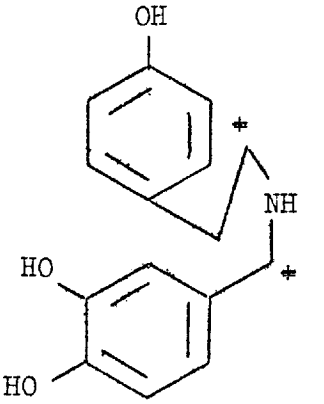
All the experiments described do not exclude the possibility of rupture of the precursors (CXXXVII) at bonds "a" and (or) "b" prior to incorporation, since only the incorporation of the C_6-C_2 fragment without scrambling is established by the degradations.



(CXXXVII)



(CXXXVIII)



(CXXXIX)

Evidence is now available for the intact incorporation of these precursors.

The doubly-labelled precursor (CXXXVIII) was incorporated into galanthamine[77] (Mr. G.M. Thomas) the ratio of the labels remaining the same in the alkaloid as in the precursor, excluding fission at bond "b" and establishing the lack of selective

demethoxylation. Further experiments with the triply labelled precursor (CXXXVIII) (Mr. G.M. Thomas), established lack of fission at bond "a", the ratio of the three labels remaining constant within the limits of experimental error, going from precursor to alkaloid.

An extensive study by Battersby, Brewer, Fales, Highet and Wildman[78] showed that the doubly labelled norbelladine (CXXXIX) was incorporated intact into lycorine, crinamine (CXL) and belladine, (CXLI), with incorporations of 0.07%, 0.0009% and 2.64% respectively, in Nerine bowdenii.

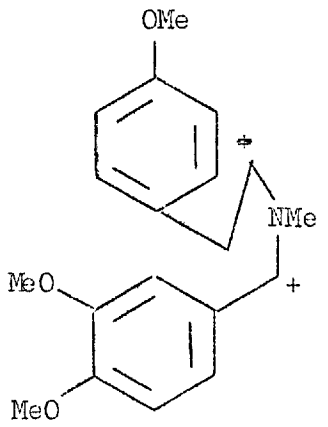
The precursor (CXXXIX) was methylated to give belladine (CXLI) which was cleaved by cyanogen bromide to give the bromide (CXLIII, R= CH_2Br), and cyanamide (CXLII, R = CN). These were converted to the aldehyde (CXLIII, R = CHO), and urea (CXLII, R = $CONH_2$) to establish the ratio of the two labels.

The isolated belladine by similar procedure gave the same fragments. The aldehyde (CXLIII, R = CHO), on oxidation and decarboxylation gave carbon dioxide, whilst the other labelled atom was isolated by Hofmann degradation of belladine, with cleavage of the resulting styrene (CXLIV).

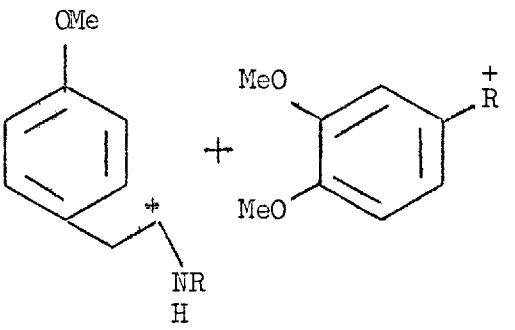
The radioactive crinamine was degraded by the procedure used for haemanthamine (P. 52.).

The radioactive lycorine was degraded as described earlier to the lactam acid (CXLV) which was decarboxylated and converted to the phenanthridinium derivative (CXLVI) which was

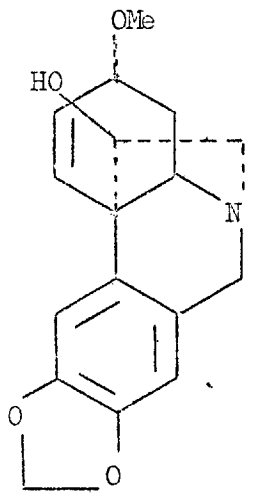
Degradation of Belladine (Battersby et al)



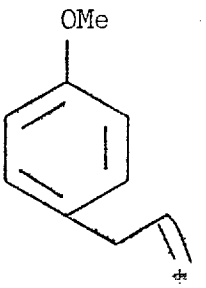
(CXLI)



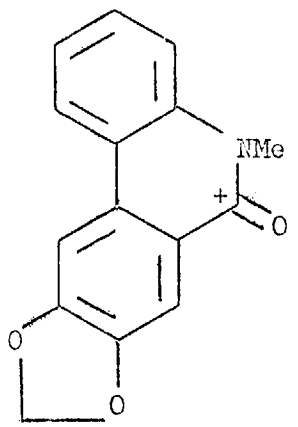
(CXLII) (CXLIII)



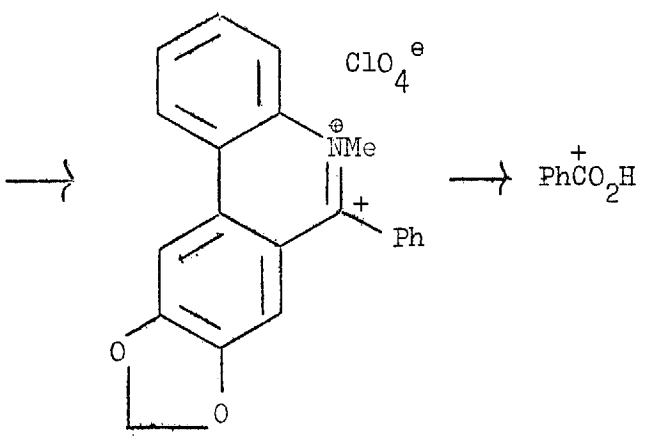
(CXL)



(CXLIV)



(CXLV)



(CXLVI)

oxidised to benzoic acid.

The ratio of the labels in the alkaloids and precursor were essentially the same in all cases.

Intact incorporation of the phenolic precursors (CXXXVII) into Amaryllidaceae alkaloids is thus rigorously established, and gives added support to the concept of Barton and Cohen.

Doubly labelled Experiments on Haemanthamine

The intact incorporation of the above precursors, together with the lack of selective demethoxylation observed in the case of the N,O-dimethyl precursor (CXXXVIII) suggested that O-methylnorbelladine (P.50.) might well be a true precursor of haemanthamine. The present work using the doubly labelled precursor (CXLVII) rigorously establishes this.

The doubly labelled precursor was synthesised, and assayed for purity as described earlier (P.43.). The ratio of the labelled atoms was determined by cleavage of the methoxyl group in a modified Zeisel apparatus using hydriodic acid, the liberated methyl iodide being trapped as its quaternary salt with triethylamine.[79]

Doubly labelled precursor (4.55% of total activity in the methoxyl group) was fed to a flowering King Alfred daffodil in the usual way, the plant was worked up after eight days, and radioactive haemanthamine isolated (incorporation 1.0%). The isolated galanthamine and galanthine were both inactive in agreement with earlier work (P.48.).

Degradation of the alkaloid carried out in the following manner, established the ratio of the labelled atoms in the alkaloid.

Hydrolysis of the methylenedioxy group was carried out using the procedure of Kirkwood[35] with 20% sulphuric acid. The formaldehyde, isolated as its dimedone derivative, was found to be radioactive (4.73% of total activity of alkaloid), containing essentially all the activity associated with the methoxyl group of the precursor.

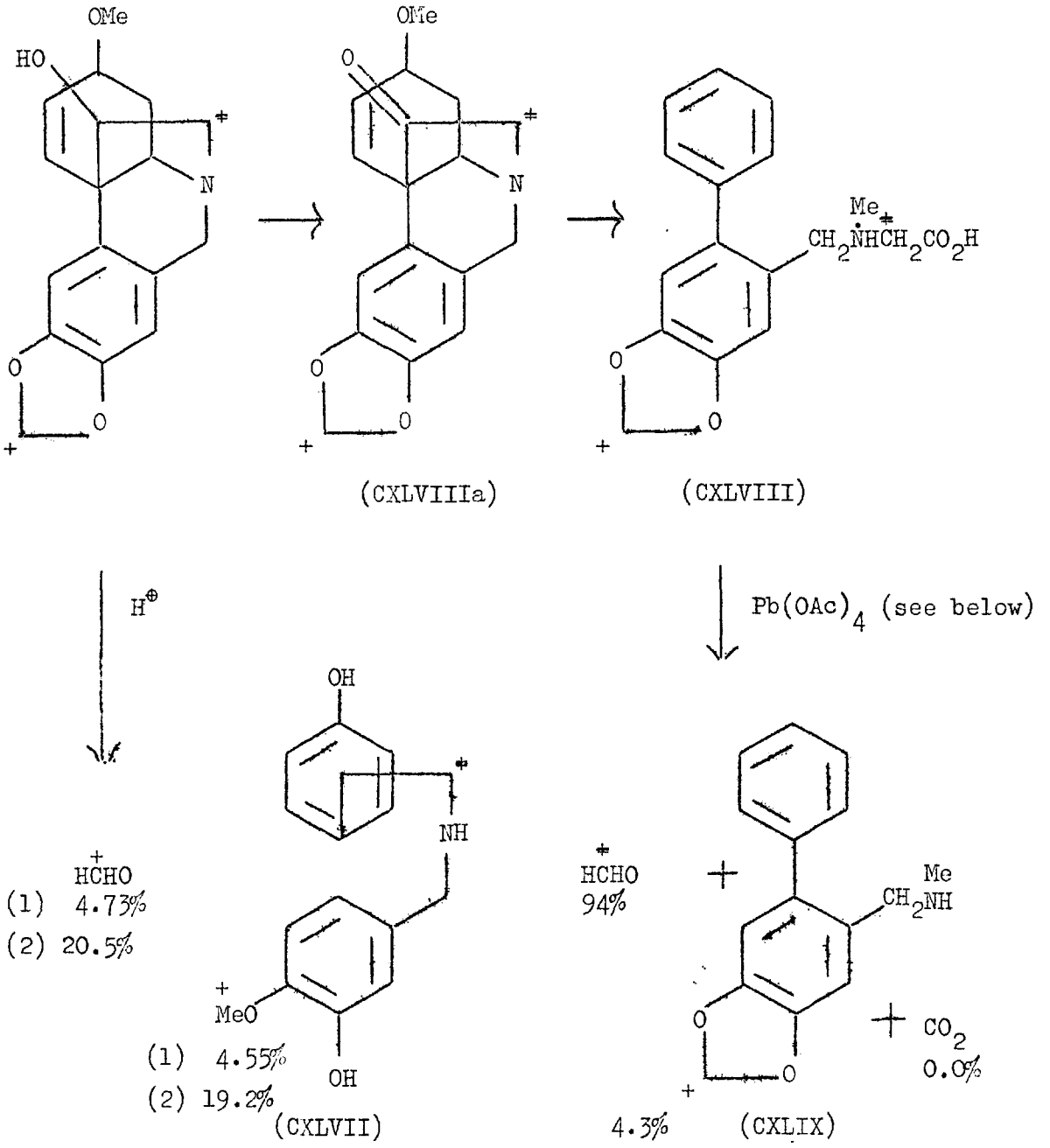
To establish conclusively that no cleavage had occurred, with resultant scrambling of the labels, the haemanthamine was degraded as shown in the annexed scheme.

The radioactive haemanthamine was converted to the N-(6 phenylpiperonyl)-N-methylglycine (CXLVIII) by standard techniques as described previously.[80]

Oxidation of this compound with lead tetraacetate in glacial acetic acid gave formaldehyde, carbon dioxide and N-methyl -6-phenylpiperonylamine (CXLIX), isolated as its hydrochloride. The alkaloids can be seen to be specifically labelled in the predicted positions, the ratios of the labelled atoms remaining essentially constant in both the precursor and alkaloid.

In a repeat experiment doubly labelled O-methylnorbelladine (19.2% of total activity in methoxyl group), was fed to two flowering plants, and the radioactive haemanthamine (incorporation 0.036%) and inactive galanthamine and galanthine isolated.

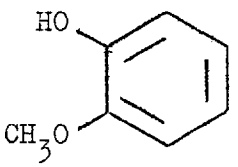
Degradation of Haemanthamine



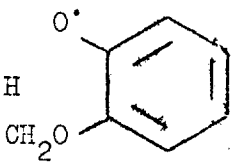
A series of model experiments with lead tetraacetate, and a series of unsuccessful experiments with a variety of other oxidising agents are described in the experimental section (P.98.).

Hydrolysis of the radioactive alkaloid as described previously gave formaldehyde from the methylenedioxy group, containing essentially the same activity (20.5% of total).

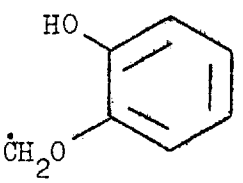
These experiments establish conclusively that O-methyl-norbelladine is a direct precursor of haemanthamine, and in particular the methylenedioxy group, in this case at least, arises from the methoxyl group of the precursor. Although it has been suggested that the methoxyl group can act as a source of the methylenedioxy group in alkaloids,[35] no direct evidence had been put forward prior to this work.



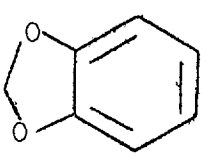
(CL)



(CLI)



(CLII)



(CLIII)

One possible mechanism is via the generation of a phenoxide radical adjacent to a methoxyl group as in (CLI). Hydrogen abstraction from the methyl group would give the methylene radical in (CLII), which by a further one electron oxidation could give rise to the methylenedioxy group in (CLIII).

To summarise all the preceeding work, it has been shown that tyrosine and various norbelladine type precursors act as

direct precursors of the Amaryllidaceae alkaloids, and in particular O-methylnorbelladine is not a precursor of galanthamine but is a precursor of haemanthamine. In the light of these results one plausible route to the alkaloids is illustrated, in Chart I.

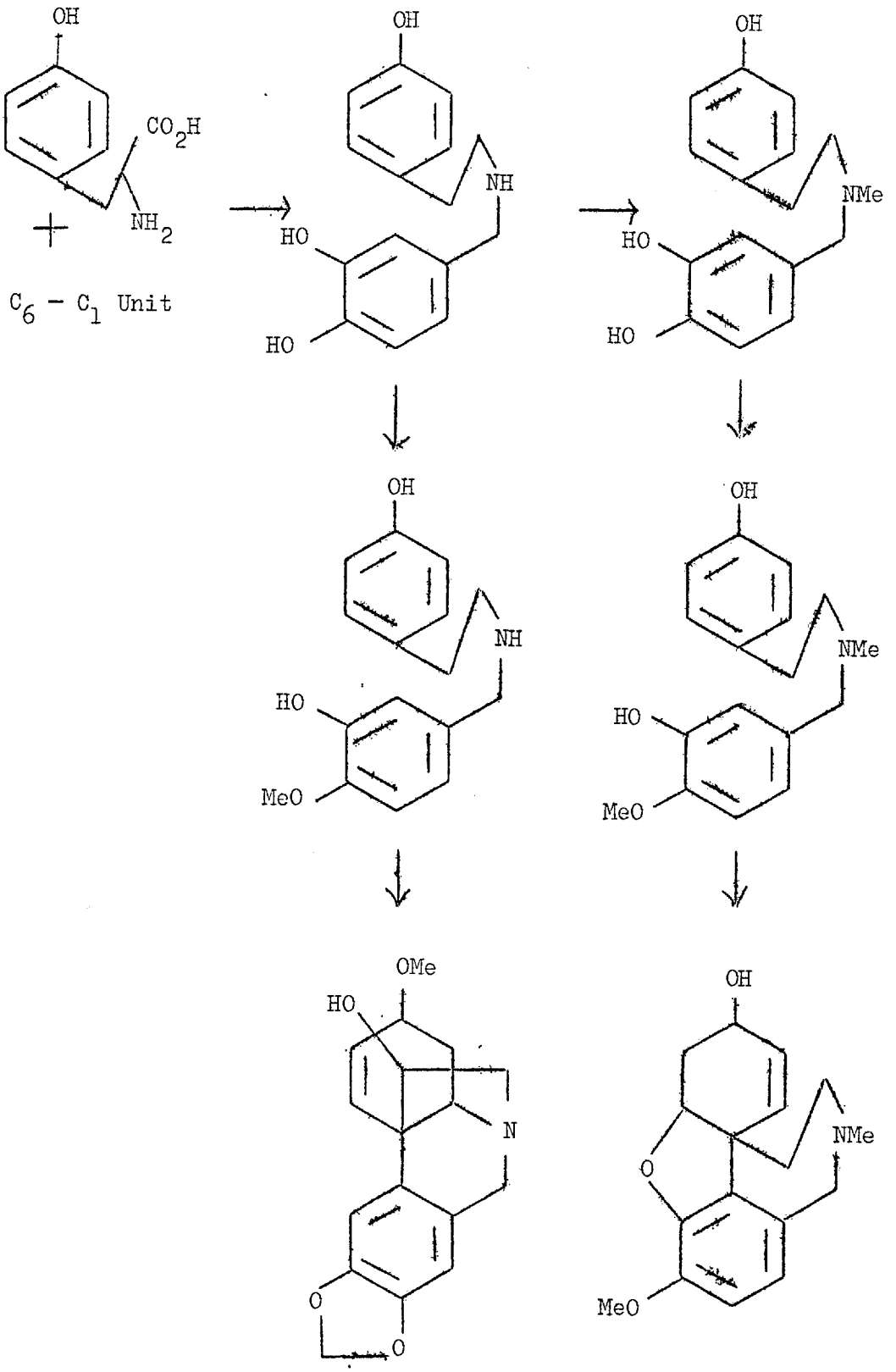
On the basis of the annexed scheme prior N-methylation of norbelladine would direct the biosynthesis to galanthamine, whilst prior O-methylation of the precursor would lead to haemanthamine. Although the experimental results establish that O-methylnorbelladine is a direct precursor of haemanthamine, it is not necessarily an obligatory precursor and other pathways to the alkaloids are, of course, plausible.

Nature of the C_6-C_1 Unit

Although the origin of the hydroaromatic C_6-C_2 unit has now been firmly established, it arising from tyrosine or a close metabolic relation, little is known about the origin of the aromatic ring and benzylic carbon atom. It has been suggested[81,82] that these arise from a C_6-C_1 unit.

Jeffs[81] fed [3-^{14}C] tyrosine to Haemanthus natalensis plants and obtained radioactive haemanthamine, haemanthidine (CLIV) and 6-hydroxycrinamine (CLV) with incorporations of 0.97%, 0.19% and 1.08% respectively. The haemanthamine was degraded to the key intermediate (CLVI) which was oxidised with ninhydrin to give carbon dioxide containing all the activity. A further oxidation using N-chlorotoluene p-sulphonamide gave carbon dioxide

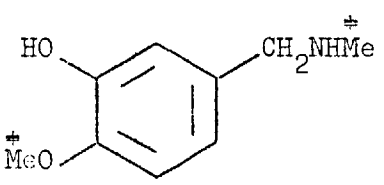
CHART I



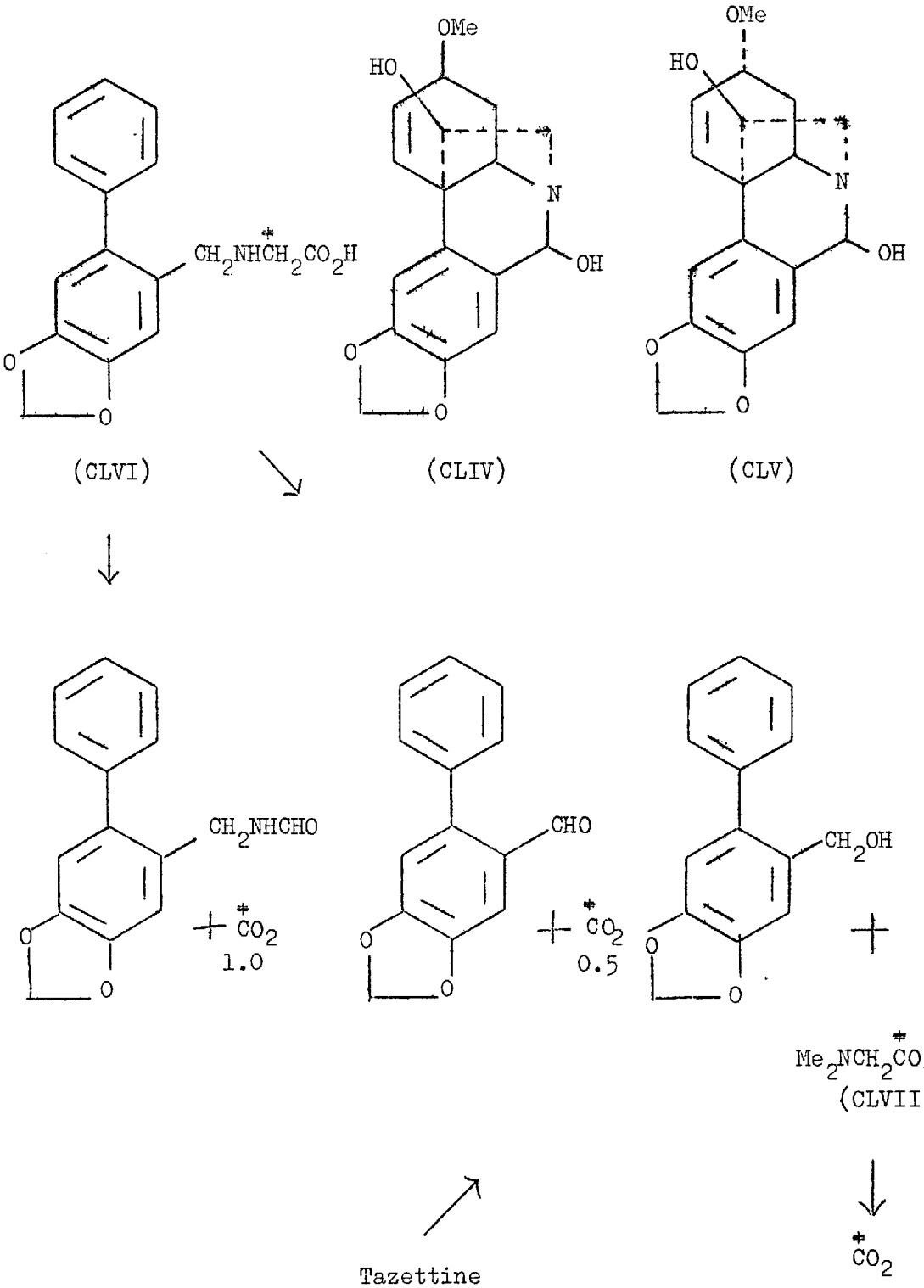
containing half the activity of the original alkaloid. These results established that the benzylic carbon atom was inactive, and that tyrosine is not a precursor of the aromatic ring.

In independent work[82] [3-^{14}C] tyrosine was fed to Sprekelia formosissima and radioactive haemanthamine, haemanthidine and tazettine isolated. The haemanthamine was degraded as previously. The haemanthidine was converted to tazettine which was subjected to Hofmann degradation to give the amino acid (CLVII) which was decarboxylated. In all cases the benzylic position was inactive, in accordance with the preceeding results that tyrosine is not a precursor of the lower ring, suggesting that either a C_6-C_1 unit is not the precursor of the aromatic ring and benzylic carbon atom, or, if so, it is not derived from tyrosine.

[^{14}C] Generally labelled tyrosine was fed to King Alfred daffodils when radioactive galanthamine was obtained. (Work of Mr. G.M. Thomas). Conversion of the alkaloid to apogalanthamine (CXXVI), followed by oxidation gave phthalic anhydride, the activity of which suggested that tyrosine was a precursor of both hydroaromatic and aromatic rings in galanthamine although less efficient a precursor of the aromatic ring. [3-^{14}C] phenylalanine has been shown to be a precursor of the C_6-C_1 unit in lycorine.[83]



(CLVIII)


OMe
HO
N
OH
O
O
O
CH2NHCH2CO2H
(CLVI)
(CLIV)
(CLV)
CH2NHCHO
CHO
CH2OH
+ CO2
1.0
+ CO2
0.5
+
Me2NCH2CO2H
(CLVII)
CO2
Tazettine
(LXXXVIII)

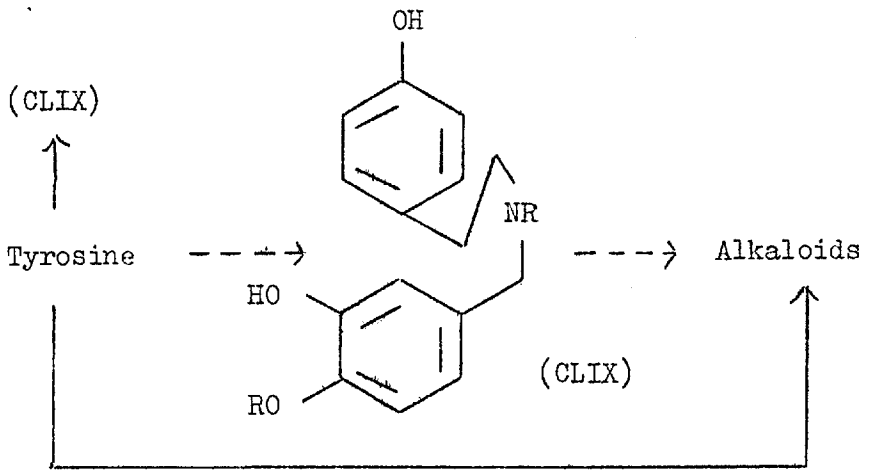
The doubly labelled benzylamine (CLVIII) (work of Mr.G.M. Thomas), was incorporated into galanthamine[17] but with complete loss of the N-methyl label. This suggests that in the case of galanthamine, at least, the C_6-C_1 unit may well be of the isovanillin or protocatechuic aldehyde type.

Intermediate Trapping Experiments

This section of the work deals with a series of experiments planned to establish the obligatory nature of various precursors used in the preceeding section.

It would not be wise to place too much significance on the values obtained for the incorporation of the precursors in tracer experiments, and the low values often obtained should not detract from their significance. There may, indeed, be many reasons for the low values obtained. Failure of the precursor to reach the site of alkaloid synthesis, use of the precursor for other purposes such as peptide synthesis, and degradation of the precursor itself may all contribute to the low values obtained. Alternatively the plant may not be actively engaged in alkaloid synthesis during the course of the experiment, and some attempts were made to determine the optimum feeding time in some cases (P. 93).

Conclusive evidence for the obligatory nature of a precursor in the biosynthesis can only come from intermediate trapping experiments.



alternate pathway

I n the scheme outlined above, if the compound (CLIX), is an obligatory intermediate on the pathway from tyrosine to the alkaloids it must be present, at least in transient amounts in the plant, at some stage. The intermediate may be derived from tyrosine in the plant but not neccessarily in the pathway from tyrosine to the alkaloids, the pathway may not involve the proposed intermediate at all, or the intermediate may enter the biosynthesis by conversion to a true precursor, or an aberrant pathway.

If, consequently, $\left[{}^{14}C\right]$ tyrosine were administered to the plant together with large chemical amounts of an obligatory precursor two effects should be observed. The presence of large amounts of the intermediate should reduce the incorporation of labelled tyrosine into the alkaloid by dilution at the intermediate stage, and secondly, the isolated intermediate should be radioactive. Either factor on its own, either inhibition or recovery of radioactive intermediate, is not

sufficient to establish the obligatory nature of the intermediate. A series of experiments with various proposed intermediates was carried out.

1. Norbelladine and O-methylnorbelladine

Recovery of both these phenols after administration to the plant proved difficult, as they could not be purified by chromatography on alumina or silica gel. Chromatography on Kieselgel and celite gave a suitable separation of the phenols from the alkaloids. Control experiments showed that alkaloid synthesis in the plant was unaffected by the amounts of phenols administered.

In comparative experiments, sprouting King Alfred plants in a similar stage of growth were fed by direct injection into the bulbs with $[2\text{-}^{14}C]$ tyrosine and norbelladine, and $[2\text{-}^{14}C]$ and O-methylnorbelladine respectively. In a control experiment, O-methylnorbelladine was added on work up to a plant injected with $[2\text{-}^{14}C]$ tyrosine. This would not affect incorporation of tyrosine, but simply trap any stationary amount of compound present in the plant. The plants were worked up in the usual way and the results are summarised below.

	Incorporation (%)		
Feeding expt.	Galm.	Galn.	Haem.
(a) tyrosine+norbelladine	0.0	0.0	0.02
(b) tyrosine+O-methylnorbelladine	0.0	0.0	0.021
(c) tyrosine (control)	0.013	0.03	0.13

It can be seen that in both cases the added precursor produces a significant decrease in tyrosine incorporation into the alkaloids, compared with the control experiment.

The phenolic fractions in each case were chromatographed on paper in a butanol:acetic acid:water system. In experiments (b) and (c), two spots appeared on development, the faster running one (Rf.0.82) running alongside authentic O-methyl-norbelladine. In experiment (a) no trace of norbelladine (a catechol) appeared. The radioactivity was shown in all cases to be confined to the phenols, but on dilution with authentic precursor dropped to zero.

No conclusions can be drawn from these results, since both precursors caused inhibition, but the recovered precursors were inactive. O-Methylnorbelladine is not incorporated into galanthamine and galanthine, and the inhibition observed may simply be due to the close similarity of the precursor to the true intermediate.

2. Narwedine

A fully grown plant was fed with $[2\text{-}^{14}C]$ tyrosine and inactive narwedine, a plausible precursor of galanthamine, and the alkaloids isolated. The results are summarised below.

Incorporation (%)

Galanthamine	galanthine	haemanthamine	narwedine
0.0	0.0	0.065	0.0

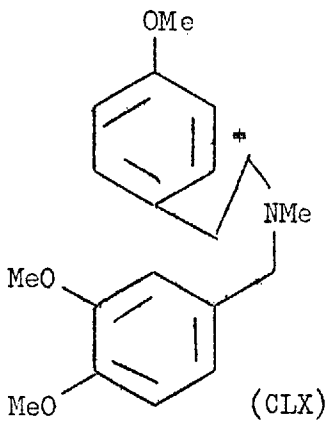
Again the presence of the precursor produces inhibition, but the recovered narwedine was surprisingly inactive. This may be due to the poisoning of the plant which was observed during administration of the alkaloid, possibly inhibiting alkaloid synthesis.

3. N,O-dimethylnorbelladine[67] (C)

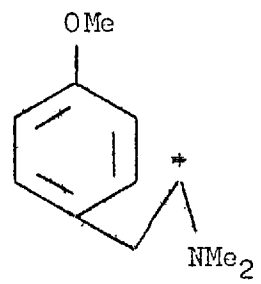
[2-^{14}C] Tyrosine and N,O-dimethylnorbelladine were fed to flowering King Alfred daffodils and the alkaloids and phenol, in this case, separated by chromatography on alumina. The recovered phenol was purified by dilution with authentic phenol, and the results summarised below.

	Incorporation (%)		
Galanthamine	galanthine	haemanthamine	N,O-dimethylnorbelladine
0.0	0.0	0.049	0.0016

Significant inhibition of tyrosine incorporation into all alkaloids was obtained and the recovered phenol was radioactive. The purity of the recovered radioactive phenol was confirmed by a partial degradation.



(CLX)



(CLXI)

The phenol was methylated with diazomethane to give belladine (CLX) which was subjected to Emde degradation to give O-methylhordenine (CLXI). Further degradation was not possible due to the small amount of material available.

The incorporation of tyrosine into the precursor, together with the inhibition observed establish that this precursor is an obligatory intermediate in the alkaloid biosynthesis. No definite conclusions can be drawn from the results of the remainder of the experiments.

In all the intermediate trapping experiments described previously, the phenolic fractions contained a slow running constituent of unknown identity. In attempts to identify this phenol, the phenolic fraction from plants fed with $\left[^{14}C\right]$ generally-labelled glucose was chromatographed and the total activity shown to reside in the phenolic components. The phenolic fractions were eluted, and in separate experiments diluted with likely phenols, and purified to constant radioactive count. In this manner, it was established that the slow running phenol was not hordenine, N-methyltyramine, tyramine or tyrosine, the most likely alternative being a phenolic alkaloid. The fast running spot was shown not to be N,O-dimethylnorbelladine.

EXPERIMENTAL

Unless otherwise stated, melting points were determined on the Kofler block; specific rotations were measured in chloroform solution at room temperature, ultraviolet absorption spectra were determined in ethanol on a Unicam SP 500 or SP 700 spectraphotometer, and infrared spectra were taken on a Perkin Elmer 137 or Unicam SP 200 instrument. Radioactive materials used were obtained from the U.K.A.E.C. radiochemical centre at Amersham, and samples were counted in a gas-flow (methane) proportional counter and are not corrected for self absorption. Unless specified to the contrary, activities were measured as thin films (0.5 mg.-1.0 m.g. cm^2.) of material plated on nickel planchettes. Petroleum ether refers to the fraction of b.p. 60-80^{o}.

SYNTHESIS OF PRECURSORS

p-Benzyloxybenzaldehyde (CXV)

p-Hydroxybenzaldehyde (6.1g.), recrystallised from water, benzyl chloride (6.3g.) and potassium hydroxide (2.8g.) in ethanol (20 ml.) were heated under reflux for six hours on a steam bath, in an atmosphere of nitrogen.[84] The solvent was then evaporated, water added, the product ether extracted, and washed with water. The ethereal extract was dried (Na_2SO_4), solvent evaporated, and the product recrystallised from aqueous alcohol (7.5g., 73%), m.p. 72° (lit. m.p. 72°).[85]

p-Benzyloxybenzyl alcohol (CXVI)

p-Benzyloxybenzaldehyde (7.5g.) in methanol (25 ml.) was shaken with excess potassium borohydride (5g.) at room temperature overnight. The excess borohydride was decomposed by addition of water, and the product extracted into ether. The ether extract was washed with water, dried (Na_2SO_4) and the solvent evaporated. The product recrystallised from benzene/petroleum as plates (7g., 93%), m.p. 87.5-88° (lit. m.p. 86-87°).[86]

p-Benzyloxybenzyl chloride (CXVII)

The alcohol (7g.) in benzene (100 ml.) was added slowly to a solution of thionyl chloride (7 ml.) in boiling benzene (20 ml.) containing pyridine (0.1 ml.). The solution was heated

under reflux for one hour, cooled and treated with ice-water. The benzene layer was washed successively with sodium bicarbonate and water, dried (Na_2SO_4) and solvent removed under vacuum. The crude product was recrystallised from petroleum ether (60-80°) (5.4g. 70%) m.p. 77.5-78.5° (Found: C, 72.2; H, 5.9. $C_{14}H_{13}OCl$ requires C, 72.3; H, 5.6%).

p-Benzyloxybenzyl cyanide (CXVIII)

Attempted synthesis using potassium cyanide in various solvents

The chloride (0.5g.) and potassium cyanide (0.15 g.) in ethanol (10 ml.) were treated under reflux for periods from 4-16 hours. On filtration, and evaporation of solvent an oil was obtained showing no trace of nitrile in its infra-red spectrum.

Repeated attempts using water, aqueous acetone, dioxan as solvent gave similar results. The attempted synthesis using silver cyanide in acetonitrile as solvent were also unsuccessful. Silver cyanide (0.15g.) suspended in acetonitrile, (dried by distillation from phosphorus pentoxide) and the halides (0.25g.) were heated under reflux for eighteen hours. The oil obtained after filtration and removal of solvent showed no trace of nitrile in its infra-red spectrum.

Synthesis using dimethyl sulphoxide as solvent

The nitrile was prepared using sodium cyanide (dried

at 100°) in dimethyl sulphoxide, freshly distilled under reduced pressure before use. p-Benzyloxybenzyl chloride (2g.) in dimethyl sulphoxide (10 ml.) was added to excess sodium cyanide (0.5g.) in the same solvent (20 ml.). The solution was heated at 100° for three hours, with occasional stirring. The reaction mixture was treated with water (100 ml.), and ether extracted. The ethereal extract was washed with water, dried, and the solvent removed under vacuum. The crude product was recrystallised from petroleum ether (60-80°) to give plates (1.4g. 73%), m.p.68-69° (lit m.p. 69°)[87]. (Found: C, 80.6; H, 5.9; N, 6.3. Calculated C, 80.7; H, 5.8; N, 6.3% for $C_{15}H_{13}ON$).

Experiments using p-benzyloxybenzyl bromide, m.p.86-87° (Found: C, 60.7; H, 4.9. $C_{14}H_{13}OBr$ requires C,60.6; H,4.7%). prepared from the alcohol using phosphorus tribromide, gave lower yields of the nitrile from the much darker reaction mixture.

O-Benzyltyramine (CXIX)

p-Benzyloxybenzyl cyanide in anhydrous ether (20 ml.) was added over a period of one hour to a suspension of lithium aluminium hydride (5g.) in ether under reflux. After a further thirty minutes, the excess lithium aluminium hydride was decomposed with water, the ether layer separated, and the aqueous layer extracted with further quantities of ether (2 x 20 ml.) The total ethereal extract was washed with water, and dried (Na_2SO_4).

After removal of the solvent, the amine, m.p. 50° was converted to its hydrochloride (0.8g. 68%) using ethanolic hydrogen chloride. The hydrochloride crystallised from ethanol-ether as plates, m.p. 202-204°. (Found: C, 68.2; H, 6.9; N, 5.5. $C_{15}H_{18}ONCl$ requires C, 68.3; H, 6.8; N, 5.3%).

O,O-Dibenzylprotocatechuic aldehyde (CXX)

Protocatechuic aldehyde (1.5g.) was treated with excess benzyl chloride (4 ml.) and anhydrous potassium carbonate (3g.) in acetone (20 ml.) under reflux for 10 hours in an atmosphere of nitrogen, with stirring. The solution was filtered and subjected to steam distillation to remove excess benzyl chloride. The aldehyde solidified, and was recrystallised from methanol, (3.2g.) m.p. 89-91° (lit. m.p. 91°).[88]

O, O, O-Tribenzylnorbelladine

O-Benzyltyramine (0.45g.) in methanol (10 ml.) was added to excess O,O-dibenzylprotocatechuic aldehyde (0.65g.) in methanol (15 ml.) and the solution allowed to stand at room temperature for one hour. In one experiment the resulting imine crystallised out (m.p. 101-102°, I.R. 1640 $cm.^{-1}$ imine), but in general was reduced directly in methanol with excess potassium borohydride (0.5g.) for three hours. Excess solvent was removed under vacuum, the product diluted with water, and extracted into ether. Concentrated hydrochloric acid (2 ml.) was added, the

hydrochloride filtered off, and recrystallised to give (0.88g., 78%) as plates, m.p. 148-149° from ethanol. (Found: C, 76.3; H, 6.7; N, 2.4, $C_{36}H_{36}O_3NCl$ requires C, 76.4; H, 6.4; N, 2.5%).

Norbelladine (CXII)

The tri-benzyl ether (0.5g.) in methanol (25 ml.) containing concentrated hydrochloric acid (0.1 ml.) and 10% palladium/Carbon catalyst (50 mg.) was hydrogenolysed. Uptake of hydrogen was complete after ca. thirty minutes. The product was filtered, the solvent removed under vacuum to give norbelladine hydrochloride (0.24g. 91%) as plates, m.p. 175-176° from ethanol/ether. (Found: C, 61.3; H, 6.3; N, 4.6, $C_{15}H_{18}O_3NCl$ requires C, 60.9; H, 6.1; N, 4.7%).

4-Benzyloxy-N-(3-benzyloxy-4-methoxybenzyl) phenylethylamine (CXIIIa)

Condensation of O-benzyltyramine (0.45g.) with excess O-benzylisovanillin and reduction with potassium borohydride, under the same conditions as for norbelladine gave the dibenzyl ether characterised as its hydrochloride (0.8g. 85%), m.p.123-124° (decomposition), from ethanol-ether. (Found: C, 73.3; H, 6.7; N, 2.5; $C_{30}H_{32}O_3NCl$ requires C, 73.6; H, 6.5; N, 2.9%).

4-Hydroxy-N-(3-hydroxy-4-methoxybenzyl) phenylethylamine (O-Methylnorbelladine).(CXIII)

Hydrogenolysis of the corresponding amine hydrochloride dibenzyl ether (0.3g.) under the same conditions as for norbelladine

gave O-methylnorbelladine hydrochloride (.16g., 81%), m.p.205-207° ethanol-ether. (Found: C, 61.8; H, 6.5; N, 4.9, $C_{16}H_{20}O_3NCl$ requires C, 62.0; H, 6.5; N, 4.5%).

RADIOCHEMICAL SYNTHESIS OF NORBELLADINE

$[^{14}C]$ sodium cyanide (0.1 mC, 0.98 mg.) was diluted with sodium cyanide (4.2 mg.) dissolved in dimethyl sulphoxide (0.2 ml.) and excess p-benzyloxybenzyl chloride (30 mg.) in dimethyl sulphoxide (0.5 ml.) added. The solution was heated at 100° for three hours, cooled, and diluted with saturated sodium chloride solution. The product was extracted into ether (4 x 2ml.), carrier nitrile (20 mg.) being added in the first ether portion, and the ether extract washed with water (2 x 2ml.).

After evaporation of the ether, the residue was dried, dissolved in anhydrous ether (5 ml.) and added to excess of lithium aluminium hydride (50 mg.) in ether (5 ml.) under reflux, during one hour. After a further half-hour, water (0.4 ml.) was added, the ether layer decanted off, and the residue extracted with further quantities of ether (4 x 1ml.). The total ether extract was washed with water (2 x 2ml.) and 4N hydrochloric acid (0.2 ml.) added. The precipitated hydrochloride was washed thoroughly with ether (4 x 2ml.), and 4NNaOH (0.4 ml.) added. The tyramine was extracted with ether (4 x 1ml.) washed with water (2 x 1ml.) and dried.

To the tyramime (36 mg.) in methanol (1 ml.) was added excess 0,0-dibenzylprotocatechuic aldehyde (150 mg.) in methanol (8 ml.), and the solution allowed to stand for three hours. Excess sodium borohydride (100 mg.) was then added, and the solution left for a further four hours. The solvent was then evaporated, water (3 ml.) added, and the product extracted into ether (4 x 2ml.), and washed with water (2 x 2 ml.)4N hydrochloric acid (0.2 ml.) was added, the hydrochloride centrifuged off, and washed with ether (6 x 2 ml.). The product was recrystallised from ethanol. Hydrogenolysis was carried out in methanol (5 ml.) using 10% palladium/carbon catalyst (50 mg.). The product was centrifuged off, residue washings (6 x 2 ml.) added, and the solvent evaporated. The product was recrystallised from ethanol/ethyl acetate, (29 mg.) m.p. and mixed m.p. 205-207°. The precursor was diluted with a known amount of inactive material, recrystallised and counted. (0.039 mC., 39% overall yield).

RADIOCHEMICAL SYNTHESIS OF DOUBLY-LABELLED PRECURSOR

[^{14}C] O-Benzylisovanillin (method of Mr. G.M. Thomas) (CXXII)

Catechol was converted into its monobenzyl ether[90] using benzyl chloride and potassium hydroxide under the usual conditions. The monobenzyl ether was then treated with chloroform and alkali, under Reimer-Tiemann conditions, to give O-benzylprotocatechuic aldehyde, m.p. 113-114° (lit. m.p. 113-114°).[91]

[^{14}C] methyl iodide (0.1 mC.) was distilled into a solution of the O-benzylprotocatechuic aldehyde (9 mg.) in dry acetone (5 ml.) containing anhydrous potassium carbonate (50 mg.). The reaction vessel was sealed in vacuo, and heated at 60° for 12 hours with stirring. Inactive methyl iodide (1 ml.) was then added and the mixture refluxed for a further 6 hours to complete the methylation. Inactive O-benzylisovanillin (18 mg) was added, the product centrifuged, and the filtrate evaporated, and washings (4 x 1 ml.) added. The product was chromatographed on alumina (Grade III, 3g.) elution with benzene giving O-benzylisovanillin (25 mg.).

[^{14}C] O-methylnorbelladine

[2-^{14}C] tyramine (37 mg.) prepared in the usual manner was condensed with the [^{14}C] O-benzylisovanillin (40 mg.). The resulting imine was reduced with borohydride, ether extracted (4 x 2 ml.) and evaporated. The product was dissolved in methanol (1 ml.) and excess inactive O-benzylisovanillin (15 mg.) in methanol (5 ml.) added. Reduction with borohydride and work up in the usual way gave the benzyl ether, tyramine free, recrystallised from ethanol (29 mg.). Hydrogenolysis in the usual way gave, after recrystallisation, the doubly-labelled precursor (13.5 mg.), m.p. 205-207°.

The precursor was assayed for purity as described below

by autoradiography of a paper chromatogram, and by dilution analysis using inactive precursor. (0.0084 mC.).

Autoradiography

The precursor (ca 1 mg.) was dissolved in ethanol (0.5 ml.) and spotted on to a circular chromatography paper, in different concentrations. The paper was developed in the butanol:acetic acid:water (4:1:5) system, and carefully dried. The paper was exposed to Kodak X-Ray film 'Kodirex' for two days. Development of the film showed that the radioactivity was coincident with the spots (Rf. 0.9) corresponding to the precursor obtained by spraying the paper with Pauli's [92] spray. The precursor was not contaminated with radioactive or chemical impurities, and in particular was free from [^{14}C] tyramine.

Dilution analysis

The precursor (0.50 mg.) was diluted with inactive material to 50 mg., counted, recrystallised and counted again, the count remaining essentially constant.

Precursor (diluted) 2.54×10^6 c.p.m./m.Mole.

2.53×10^6 c.p.m./m.Mole.

mean count = $\underline{2.53 \times 10^6}$ c.p.m./m.Mole.

Precursor (recryst) 2.51×10^6 c.p.m./m.Mole.

2.61×10^6 c.p.m./m.Mole.

mean count = $\underline{2.56 \times 10^6}$ c.p.m./m.Mole.

The corresponding singly-labelled precursor was prepared by the same procedure using inactive O-benzylisovanillin and [^{14}C] tyramine.

ISOLATION OF ALKALOIDS

The general procedure used was that of Fales, Giuffrida and Wildman.[73]

1. Galanthus Elwesii Snowdrop[64] - galanthamine 0.055%
lycorine 0.029%
haemanthamine 0.0028%
tazettine 0.027%

The bulbs (205 g.) were macerated in a Waring blender, and extracted with 1% tartaric acid in ethanol (250 ml.). After 24 hours the extract was filtered and the residue extracted with a further portion of ethanolic tartaric acid (250 ml.) The total crude extract was filtered (celite) evaporated to small bulk (ca. 100 ml.), and diluted with water (350 ml.) and 2N hydrochloric acid (20 ml.). The solution was extracted with chloroform (3 x 50 ml.), the chloroform washings extracted with 2N acid (5 x 30 ml.) and the combined aqueous solutions made alkaline with sodium carbonate. The solution was extracted with chloroform (10 x 40 ml.), the extracts dried (Na_2SO_4) and evaporated to give crude alkaloid (530 mg.).

Crude alkaloid (250 mg.) was chromatographed on Grade III alumina (30g.) to give the following fractions.

Fraction 1.

ethyl acetate:benzene (2:5) 55 mg. gave Galanthamine (46 mg.) m.p. 124-127°.

Fraction 2.

ethanol:chloroform (1:20) 25 mg. - gum possibly haemanthamine not investigated further.

Fraction 3.

ethanol:chloroform (1:10) 17 mg. - lycorine, m.p. 270-290°

Alternative procedure (Boit) [93]

Crude alkaloid (250 mg.) was extracted with 2N sodium hydroxide (10 x 10 ml.). The "phenolic" fraction was buffered to pH_7 by addition of glacial acetic acid followed by dilute aqueous ammonia. The neutral solution was extracted with chloroform (5 x 20ml.) washed with water, and dried. Evaporation gave a gum showing no trace of tazettine in its ultra-violet spectrum, but a peak at 1730 cm^{-1} (acetate) in its infra-red. Hydrolysis of the "acetate" with ethanolic potassium hydroxide, concentration and extraction into chloroform gave a gum containing no tazettine on chromatography.

2. King Alfred Daffodils[64] - lycorenine 0.006%
galanthamine 0.03%
galanthine 0.012%
haemanthamine 0.039%
methylpseudolycorine 0.002%
narcissamine 0.006%

The bulbs (140g.) were macerated and extracted with 1% tartaric acid in ethanol (2 x 150 ml.). The extract was filtered, evaporated to small bulk (ca. 50 ml.), and diluted with water

(150 ml.) and 2N hydrochloric acid (15 ml.). The acid solution was washed with chloroform (3 x 30 ml.) and the washings extracted with 2N hydrochloric acid (5 x 20ml.). The combined acid extracts were made alkaline with aqueous sodium carbonate, and extracted with chloroform (10 x 30 ml.). The chloroform extract was dried (Na_2SO_4) and the solvent removed under vacuum to give crude basic extract (320 mg.). The crude alkaloid was chromatographed on Grade III alumina (25g.) by a continuous elution technique using benzene (500 ml.) and ethyl acetate (500 ml.), 10 ml fractions corresponding to ca. 2% increases of concentration of ethyl acetate in benzene were taken to give the following fractions.

Fraction 1.

ca. 16% ethyl acetate gave a fluorescent artefact (3mg.).

Fraction 2.

ca. 23% ethyl acetate gave gum (22 mg.) probably homolycorine not investigated further.

Fraction 3.

40 mg. gum gave galanthamine (30 mg.) m.p. 128-130°.

Fraction 4.

49 mg. gum gave galanthine (22 mg.) m.p. 85-100°, remelt 164-165°.

Fraction 5.

46 mg. gum gave haemanthamine (31 mg.) m.p. 199-201°.

3. Texas Daffodils[64] - galanthamine 0.0044%
narwedine 0.0055
haemanthamine 0.0209
lycorine 0.067
pluviine 0.0022

The bulbs (2 kg.) were ground and extracted with ethanolic tartaric acid in the usual manner. The final chloroform extract was evaporated to low bulk, filtered to remove lycorine, and evaporated to dryness. The residue (740 mg.) was then chromatographed in benzene on alumina (grade III) (50 g.), narwedine and galanthamine being eluted with benzene-ethyl acetate (9:1). After rechromatography on alumina (grade III) the narwedine, still mixed with a little galanthamine, crystallised from benzene to give prisms (25 mg.), m.p. and mixed m.p. 188-190°, $[\alpha]_D$ + 52° (c 1.0).

Crystallisation of an artificial mixture of (±)-narwedine (10 mg.) and (-) -galanthamine (5 mg.) from benzene gave narwedine, $[\alpha]_D$ + 40° (c 0.35) and $[\alpha]_D$ + 57° (c 0.42) in two separate experiments.

4. Chlidanthus Fragrans[64] - chlidanthine 0.0156%
lycorine 0.0228
tazettine 0.05

Resting bulbs (300 g.) worked up in the usual way gave no tazettine.

TRACER STUDIES ON GALANTHUS ELWESII SNOWDROP

(a) Tyrosine

[2-^{14}C](±)-tyrosine hydrochloride (0.01 mC.) was diluted with l-tyrosine hydrochloride (20 mg.) in water (6 ml.), and injected into the bulbs of 30 flowering snowdrops (2x0.1 ml.) each bulb), over the course of one week. The whole plants, (wet weight 85 g.) were worked up after a further three days. Lycorine (40 mg.) precipitated on evaporation of the chloroform extract to low bulk. Removal of the solvent gave crude alkaloid (243 mg.) which was chromatographed on alumina to give galanthamine (29 mg.).

Alkaloid	Activity (c.p.m./m.Mole)
lycorine hydrochloride (cryst)	3.49×10^4
- recryst.	3.56×10^4
- perchlorate (cryst)	3.60×10^4

Incorporation of tyrosine into lycorine = 0.04%

galanthamine (cryst)	1.49×10^4
- hydrochloride (cryst)	1.54×10^4
- recryst.	1.55×10^4

Incorporation of tyrosine into galanthamine = 0.13(5)%

Degradation of [^{14}C] galanthamine

The degradation was carried out according to the method of Uyeo and Kobayashi[72] with some modifications.

[^{14}C] galanthamine hydrochloride (ca. 6 mg.) was diluted with inactive alkaloid (190 mg.), dissolved in water, basified with sodium hydroxide, extracted into chloroform (4 x 2 ml.) and dried (Na_2SO_4). Removal of solvent gave the free base (176 mg.).

Apogalanthamine (CXXVI)

The free base (174 mg.) was heated under reflux with 45% aqueous hydrobromic acid (1.7 ml.) under nitrogen for four hours. Apogalanthamine hydrobromide, m.p. 228-230°, crystallised out on cooling, and was filtered off, dissolved in water, and the free base precipitated by aqueous ammonia. The free base was collected, and dried over phosphorus pentoxide at room temperature (110 mg.).

O,O-Dimethylapogalanthamine (CXXVI, R = Me)

Apogalanthamine (110 mg.) in ethanol (5 ml.) was treated with an excess of diazomethane in ether (40 ml.) for two days. The solvent was removed, and the product chromatographed on alumina (grade III). Elution with benzene-ethyl acetate (9:1) gave O,O-dimethylapogalanthamine as an oil (73 mg.). Elution with ethyl acetate-benzene (1:1) gave a further quantity of material (12 mg.), m.p. 212-214° (dec.), presumably partially methylated product, which was not investigated further. Treatment of the dimethyl ether with methyliodide (1 ml.) in methanol (3 ml.) under reflux for thirty minutes gave the

methiodide (114 mg.), m.p. 143-150°.

Emde base (CXXVII)

The methiodide (114 mg.) was converted into its methochloride by shaking with freshly prepared silver chloride in water (5 ml.). The insoluble silver salts were filtered off and the filtrate (15 ml.) heated with 4% sodium amalgam (10 g.) on a steam bath, until evolution of gas ceased (4-6 hours). The oil which separated was taken up in ether (10 ml.), washed with water, and dried (Na_2SO_4). Concentration gave the Emde base (53 mg.) which was converted into its methiodide (93 mg.), m.p. 220-223°.

2-Methyl-5,6-dimethoxy-1^1-vinyldiphenyl (CXXVIII)

Uyeo's Hofmann elimination gave low yields, and was modified according to the following procedure. The methiodide (93 mg.) was converted into its methohydroxide by shaking with freshly prepared silver hydroxide, and sodium hydroxide ($\frac{N}{10}$, 1.75 ml) was added. Evaporation of solvent at 50° in a rotary evaporator, and distillation of the residue at 100-140° in vacuo (1.5×10^{-6} mm) gave a colourless oil. This was taken up in ether, washed with acid and water, dried (Na_2SO_4) and evaporated to give the vinyldiphenyl (22 mg.).

Cleavage of the vinyldiphenyl

Owing to the low yield of formaldehyde obtained in

Uyeo's procedure, a more desirable method was sought. Attempted hydroxylation and cleavage using potassium permanganate and osmium tetroxide under various conditions resulted in low yields of formaldehyde. The cleavage was efficiently accomplished by ozonolysis under controlled conditions.

The olefine (22 mg.) was dissolved in ethyl chloride (10 ml.) and ozonised at -25° (cardice/carbon tetrachloride bath). Uptake of ozone was followed by the change in U.V. spectrum (240-260 mu) and was complete after forty minutes. The solvent was evaporated, zinc dust (1 g.) added, and the product steam distilled, the distillate being collected in aqueous dimedone solution. The formaldehyde was isolated as its dimedone derivative (14 mg.), m.p. 179-184°, which was recrystallised from aqueous ethanol. The residue was extracted into ether (3 x 10 ml), washed with water and dried (Na_2SO_4). Removal of solvent gave the formyldiphenyl residue (12 mg.) as an oil which was not purified further.

	c.p.m./m.Mole (x 10^{-3})	
galanthamine	4.75	1.00
O,O-dimethylapogalanthamine	4.56	0.96
vinyldiphenyl	4.45	0.95
formaldehyde dimedone	4.72	0.99
formyldiphenyl residue.	inactive	0.0

(b) Norbelladine

[^{14}C] Norbelladine hydrochloride (21 mg.), (0.026 mC) in water (6 ml.) was injected into the bulbs of thirty flowering plants (2 x 0.1 ml. each bulb), the whole plants being worked up three days after the last injections. No lycorine was precipitated on evaporation to low bulk, and the total crude alkaloid (174 mg.) was chromatographed on alumina (grade III) to give the following fractions.

Fraction 1.

benzene-ethyl acetate (7:3) galanthamine (30 mg.)
21 mg., m.p. 125-127°.

Fraction 2.

ethyl acetate-ethanol (9:1) lycorine (5 mg.)., m.p. 235-250°.

Alkaloid	Activity (c.p.m./m.Mole)
lycorine hydrochloride (cryst)	4.88×10^5
- (recryst)	3.37×10^5
- (diluted)	3.67×10^5

Anomolous results were obtained on further crystallisation, so the value is not reliable.

Incorporation of norbelladine into lycorine ca. 0.03%

Alkaloid	Activity (c.p.m./m.Mole)
galanthamine (cryst)	1.55×10^5
- hydrochloride (cryst)	1.44×10^5
- (recryst)	1.46×10^5

Galanthamine hydrochloride (ca. 2 mg.) was diluted with

inactive material (16 mg.), the free base regenerated, and oxidised using active manganese dioxide (200 mg.) in chloroform (5 ml.). The product was filtered and chromatographed on alumina (grade III) (5 g.), elution with benzene ethyl acetate (10:1) giving narwedine (12 mg.), m.p. 188-190°.

Alkaloid	Activity (c.p.m./m.Mole)
galanthamine (cryst)	1.55×10^4
narwedine (cryst)	1.60×10^4
- (recryst)	1.65×10^4

Incorporation of norbelladine into galanthamine = 0.05%

TRACER STUDIES ON KING ALFRED DAFFODILS

Flowering King Alfred daffodils were injected with $[2\text{-}^{14}C]$ (±)-tyrosine hydrochloride (0.007 mC), $[^{14}C]$ norbelladine hydrochloride (0.039 mC), and $[^{14}C]$ O-methylnorbelladine hydrochloride (0.022 mC), singly labelled as described on page 76, all approximately 25 mg. in water (10 ml.) at pH6. Each compound was injected into the hollow flower stalks of three plants, distilled water being injected after the final injection. The three separate experiments were performed concurrently, and the bulbs were worked up after eight days.

(a) Norbelladine

The whole plants (wet weight 326 g.) were worked up in the usual way and gave crude alkaloid (701mg.), separated by gradient elution to give the following fractions.

Fraction 1.

88 mg. gave galanthamine (63 mg.), m.p. 115-125°.

Fraction 2.

86 mg. gave galanthine (52 mg.), double m.p. 80-100°, 161-162°.

Fraction 3.

96 mg. gave haemanthamine (65 mg.), m.p. 195-197°.

Alkaloid	Activity (c.p.m./m.Mole)
haemanthamine (cryst)	4.57×10^5
- (recryst)	3.20×10^5
- (recryst)	3.64×10^5
- picrate (cryst)	4.06×10^5
- (recryst)	3.90×10^5

Incorporation of norbelladine into haemanthamine = 0.25%

galanthamine (cryst)	2.61×10^4
- hydrochloride (cryst)	2.05×10^4
- (recryst)	2.00×10^4
converted to narwedine (cryst)	1.98×10^4

Incorporation of norbelladine into galanthamine = 0.014%

galanthine (cryst)	6.98×10^3
- (recryst)	5.14×10^3
- hydrochloride (cryst)	5.06×10^3
- (recryst)	4.89×10^3

Incorporation of norbelladine into galanthine = 0.0036%

Degradation of galanthamine

[^{14}C] galanthamine hydrochloride (49 mg.) was diluted with inactive material (151 mg.), and converted into the free base (175 mg.). The degradation was carried out in the same way as for tyrosine injected snowdrops. (page84). Galanthamine (173 mg.) on treatment with aqueous hydrobromic acid gave apogalanthamine (112 mg.).

Methylation in the usual way gave O,O-dimethyl-apogalanthamine (70 mg.) converted into its methiodide (112 mg.), m.p. 141-148°.

The methiodide was subject to Emde degradation via the methochloride to give the Emde base (52 mg.) and converted to its methiodide (90 mg.), m.p. 221-223°.

The modified Hofmann elimination on the corresponding methohydroxide gave the vinyldiphenyl (23 mg.).

Ozonolysis under controlled conditions gave formaldehyde dimedone (12 mg.), m.p. 183-186°, purified by recrystallisation.

	c.p.m./m.Mole (x 10^{-3})	
galanthamine	3.06	1.00
apogalanthamine	2.93	0.96
vinyldiphenyl	3.08	1.01
formaldehyde dimedone	2.87	0.94
formyldiphenyl residue	inactive	0.0

(b) Tyrosine

The whole plants (wet weight 315 g.) on work up in the usual manner gave crude alkaloid (601 mg.). Gradient elution from alumina (grade III, 50 g.) gave the following fractions.

Fraction 1.

78 mg. gave galanthamine (59 mg.), m.p. 125-128°.

Fraction 2.

86 mg. gave galanthine (65 mg.), m.p. 82-100°.

Fraction 3.

102 mg. gave haemanthamine(76 mg.), m.p. 194-196°.

Alkaloid	Activity (c.p.m./m.Mole)
galanthamine (cryst)	5.66×10^3
- hydrochloride (cryst)	3.59×10^3
- (recryst)	3.48×10^3
- (recryst)	3.37×10^3
converted to narwedine (cryst)	3.34×10^3

Incorporation of tyrosine into galanthamine = 0.012%

galanthine (cryst)	3.51×10^3
- hydrochloride (cryst)	2.00×10^3
- (recryst)	1.98×10^3
- picrate (cryst)	1.87×10^3

Incorporation of tyrosine into galanthine = 0.007%

haemanthamine (cryst)	18.71×10^4
- (recryst)	4.46×10^4

Alkaloid	Activity (c.p.m./m.Mole)
haemanthamine (recryst)	4.61×10^4
- picrate (cryst)	4.64×10^4

Incorporation of tyrosine into haemanthamine = 0.20%

In another series of experiments tyrosine was fed to King Alfred daffodils at different stages of growth in an attempt to determine the optimum conditions for incorporation.

(i) Tyrosine into resting bulbs

Resting bulbs (200 g.) were injected with [2-^{14}C]-tyrosine (0.007 mC), and the bulbs worked up after one week to give crude alkaloid (578 mg.). Gradient elution in the usual way gave the following fractions.

Fraction 1.

76 mg. gave galanthamine (36 mg.), m.p. 127-130°.

Fraction 2.

110 mg. gave galanthine (46 mg.), m.p. 78-95°.

Fraction 3.

88 mg. gave haemanthamine (53 mg.), m.p. 194-197°.

All alkaloids were inactive after purification.

(ii) Tyrosine into sprouting bulbs

Sprouting bulbs were injected with tyrosine (0.005 mC), the plants (95 g.) being worked up in the usual way to give crude alkaloid (190 mg.). Gradient elution in the usual way gave the following fractions.

Fraction 1.

41 mg. gave galanthamine (29 mg.), m.p. 125-129°.

Fraction 2.

44 mg. gave galanthine (35 mg.), m.p. 83-98°.

Fraction 3.

45 mg. gave haemanthamine (32 mg.), m.p. 197-200°.

Alkaloid	Activity (c.p.m./m.Mole)
galanthamine (cryst)	27.24×10^3
- hydrochloride (cryst)	9.02×10^3
- (recryst)	4.47×10^3
- (recryst)	4.74×10^3

Incorporation of tyrosine into galanthamine = 0.013%

(c) O-Methylnorbelladine

The whole plants (wet weight 350 g.) gave crude alkaloid (572 mg.) separated by gradient elution to give the following fractions.

Fraction 1.

63 mg. gave galanthamine (47 mg.), m.p. 123-126°.

Fraction 2.

125 mg. gave galanthine (93 mg.), double m.p. 84-103°, 161-165°.

Fraction 3.

120 mg. gave haemanthamine (83 mg.), m.p. 196-200°.

Alkaloid	Activity	Incorporation
galanthamine	Inactive	0.0%
galanthine	Inactive	0.0%

Alkaloid	Activity (c.p.m./m.Mole)
Haemanthamine (cryst)	2.095×10^4
- (recryst)	2.28×10^4
- (recryst)	2.26×10^4
- picrate (cryst)	2.12×10^4
- (recryst)	2.16×10^4

Incorporation of O-methylnorbelladine into haemanthamine = 0.036%

EXPERIMENTS WITH DOUBLY LABELLED PRECURSORS

1. One fully grown King Alfred daffodil was injected in its flower stalk with doubly labelled O-methylnorbelladine (page78) (11.2 mg., 0.0084 mC.) in water (3 ml.).

The precursor was assayed for purity as described earlier. The ratio of the labels was determined by cleavage of the methyl ether in a modified Zeisel apparatus, the methyl iodide being trapped as triethylamine methiodide, using the micro-analytical technique.

O-Methylnorbelladine (ca. 1.5 mg.) was diluted to 50 mg. with inactive material and recrystallised. The precursor (ca. 8 mg.) together with phenol (ca. 20 mg.), ammonium iodide (ca. 20 mg.), 5% gold chloride solution (1 drop), acetic anhydride (0.5 ml.) and hydriodic acid (Analar microanalytical reagent 1.5 ml.) were heated under reflux, in an atmosphere of nitrogen. The liberated methyl iodide was swept through cadmium sulphate

solution and trapped in triethylamine in ethanol (1:1) (10 ml.).[79] Heating was continued for forty minutes, the apparatus being swept with nitrogen for a further fifteen minutes. Evaporation of the solvent gave triethylamine methiodide, m.p. 230°(dec.). Work up of the plant (180 g.) in the usual way gave crude alkaloid (313 mg.) separated by gradient elution to give the following fractions.

Fraction 1.

47 mg. gave galanthamine (35 mg.), m.p. 127-130°.

Fraction 2.

51 mg. gave galanthine (39 mg.), m.p. 77-95°.

Fraction 3.

85 mg. gave haemanthamine (58 mg.), m.p. 198-200°

Alkaloid	Activity(c.p.m./m.Mole)
galanthamine	inactive
galanthine	inactive
haemanthamine (cryst)	3.93×10^5
- (recryst)	3.77×10^5
- (recryst)	3.21×10^5
- (recryst)	3.23×10^5
- picrate (cryst)	3.51×10^5

Incorporation into haemanthamine = 1.00%

Hydrolysis of haemanthamine

Hydrolysis of the alkaloid was accomplished using 20% sulphuric acid according to the method of Kirkwood.[35,94]

Haemanthamine (21 mg.) was dissolved in 20% sulphuric acid (40 ml.) and the solution heated until water began to distil over. Water was then added at the rate of distillation to keep the concentration of acid constant. The water was distilled into a solution of dimedone (200 mg.) in water (25 ml.) and heating continued until 150 ml. had distilled over, the formaldehyde dimedone crystallising out on cooling (11 m.g., 56%), m.p. 186-188°.

Precursor (dil) mean count = 8.46×10^5 c.p.m./m.Mole.

triethylamine methiodide mean count = 3.64×10^4 c.p.m./m.Mole (4.30% of total)

- repeat = 4.10×10^4 c.p.m./m.Mole (4.85% of total)

Haemanthamine (6 mg.) was diluted with inactive alkaloid (18 mg.) and crystallised.

haemanthamine mean count = 7.50×10^4 c.p.m./m.Mole

formaldehyde dimedone (cryst) mean count = 3.59×10^3 c.p.m./m.Mole

- (recryst) = 3.49×10^3 c.p.m./m.Mole (4.73% of total)

Degradation of haemanthamine [80]

Oxohaemanthamine (CXLVIIIa)

Haemanthamine (60 mg.) in dry pyridine (0.25 ml.) was added to chromic oxide (65 mg.) in pyridine (0.75 ml.), and kept overnight at room temperature, with shaking. The product was filtered through a sintered glass funnel, after pouring into ice-water (5 ml.). The precipitate was washed with chloroform

(5 x 5 ml.) and the washings used to extract the aqueous pyridine filtrate. The total chloroform extract was dried (Na_2SO_4) and evaporated to give a gum which was extracted with hot benzene. The benzene extract was chromatographed on alumina (grade III, 5g.), elution with benzene-ethyl acetate (4:1) giving a colourless gum 30 mg. (I.R.ν_{max}1748 cm.$^{-1}$) which did not crystallise. The gum was dissolved in methanol (2 ml.) and heated with methyl iodide (1 ml.) to give oxohaemanthamine methiodide (44 mg.), m.p.243-245° (aqueous ethanol).

N-(6-phenylpiperonyl)-N-methylglycine (CXLVIII)[80]

A solution of oxohaemanthamine methiodide (40 mg.) in water (0.5 ml.) was heated with 10% sodium hydroxide (1 ml.), under nitrogen, for thirty minutes on a steam bath. A precipitate of the hydrated sodium salt (40 mg.) appeared on cooling.

Model Degradation of N-methyl-N-benzylglycine

Benzaldehyde (1 g.) in methanol (5 ml.) was treated with excess aqueous methylamine (30%, 3 ml.), and the resulting imine reduced with excess potassium borohydride (5 g.) at room temperature. The resulting amine was extracted into ether (5 x 10 ml.) and purified as its hydrochloride (0.82 g.), m.p. 175-176° (lit.m.p.176°)[99] (ethanol/ether). The amine (0.50 g.) was reacted with ethyl chloroacetate (0.75 g.) in ethanol (2 ml.), the precipitated amine hydrochloride was filtered off, and the mother liquors evaporated to dryness to give the ester as an oil. The ester was hydrolysed by heating with concentrated hydrochloric acid for

ten minutes, and the amino acid isolated as its hydrochloride, m.p. 160-170°. The free acid, m.p. 187-189° (lit. m.p.189-191°)[95] was obtained by treatment with one equivalent of ethanolic sodium hydroxide.

Attempted decarboxylation and demethylation with hydrogen iodide

N-Benzyl-N-methylglycine (10 mg.) was refluxed with hydriodic acid (2 ml. analytical reagent) phenol (5 mg.), ammonium iodide (20 mg.), and acetic anhydride (0.5 ml.), in a modified Zeisel apparatus at 350°, the conditions used for cleavage of N-methyl groups. The methyl iodide liberated was isolated as triethylamine methiodide (13 mg.), (0.96 mole). No further quantity of methyl iodide distilled on prolonged heating.

Attempted oxidation with mercuric acetate[96]

The amino acid (170 mg., 1 m.mole) was treated with mercuric acetate (448 mg., 4 m.moles) in acetic acid (10 ml.), the system being flushed with carbon dioxide-free nitrogen. The carbon dioxide evolved was precipitated as barium carbonate from a standard solution of barium hydroxide. Approximately one m.mole of carbon dioxide was obtained from the free acid, the acid hydrochloride or the sodium salt. No formaldehyde was obtained under the conditions of the experiments.

Attempted oxidation with N-bromo-succinimide[97]

The amino acid (179 mg., 1 m.mole) treated with N-bromo-succinimide (1 m.mole or excess) gave carbon dioxide, but no

trace of formaldehyde even on prolonged heating at 100°. The reaction mixture was basified and extracted with ether (5 x 10 ml.). The ether extract was extracted with dilute acid (5 x 10 ml.), which was neutrallised and re-extracted with ether, no basic material being obtained. The first ethereal extract on evaporation yielded benzaldehyde (15 mg.), characterised as its dimedone derivative, m.p. 193-194°.

Glycine (150 mg., 2 m.mole) was added to N-bromosuccinimide (356 mg., 2 m.moles), and succinimide (356 mg.) in 1 M sodium acetate in acetic acid solution (4 ml.) at 30°. The system was flushed with nitrogen and the carbon dioxide (1.8 m.moles) liberated trapped in barium hydroxide. No formaldehyde was obtained, addition of excess mercuric oxide in dilute sulphuric acid resulted in the evolution of a further quantity of carbon dioxide (0.6 ml.), indicating the oxidation of formaldehyde to formic acid.

Oxidation with lead tetra-acetate

(i) N-benzylglycine [95]

N-benzylglycine (201 mg.), in water (2 ml.), was added to lead tetra-acetate (443 mg.) in acetic acid (10 ml.) and heated on a steam bath for three hours. The system was flushed with nitrogen (carbon dioxide-free), and evolved carbon dioxide (0.81 m.mole) estimated by absorption in standard barium hydroxide solution (200 ml.) which was back titrated with 0.1 N hydrochloric

acid. The formaldehyde liberated was characterised as its dimedone derivative (100 mg.), (32%). The solution was basified, and extracted with ether (5 x 5 ml.). The ether extract was extracted with acid (5 x 5 ml.), the acid extract was neutrallised and re-extracted into ether. The basic fraction was converted to its hydrochloride, to give benzylamine hydrochloride (75 mg., 52%), m.p. and mixed m.p. 247-248° (lit. m.p. 248°).[98]

(ii) N-Benzyl-N-methylglycine

N-Benzyl-N-methylglycine (215 mg.) treated with lead tetra-acetate (443 mg.) under the same conditions gave carbon dioxide (0.9 m.mole), formaldehyde dimedone (83 mg., 28%) and N-methylbenzylamine hydrochloride (90 mg., 57%), m.p. and mixed m.p. 175-176°. (lit. m.p. 176°).[99]

(iii) N-Methylpiperonylamine

Piperonal (5 g.) was treated with 25% aqueous methylamine (5 g.) in methanol (50 ml.), and allowed to stand for three hours. The resulting imine was reduced with excess potassium borohydride, the excess borohydride destroyed with water, methanol removed, and the product ether extracted (3 x 20 ml.). The amine was purified as its hydrochloride, (3.5 g.), m.p. 194-195°. (lit m.p. 194°).[100]

N-Methylpiperonylamine hydrochloride (201 mg.) in water (2 ml.), was treated with lead tetra-acetate (443 mg.) in glacial

acetic acid (10 ml.) on a steam bath for three hours. No carbon dioxide or formaldehyde was obtained, and work up of the basic solution gave N-methylpiperonylamine-hydrochloride (110 mg.), (55% recovery).

(iv) N-(6-Phenylpiperonyl)-N-methylglycine

The sodium salt-hydrate (20 mg.) in water (1 ml.) was added to lead tetra-acetate (27.3 mg.) in glacial acetic acid (5 ml.). The reaction mixture was heated at 100° for three hours, and swept with nitrogen to give carbon dioxide (0.88 equivalent), and formaldehyde dimedone (8mg., 44%). The reaction mixture was worked up in the usual way to give 6-phenyl-N-methylpiperonylamine hydrochloride (8.2 mg., 59%), m.p. 159-160°. (lit. m.p. 160°).[101]

Degradation of $\left[{}^{14}C\right]$ haemanthamine

Doubly labelled haemanthamine (see page 96) (15 mg.) was diluted with inactive alkaloid (60 m.g.).

Oxohaemanthamine

Haemanthamine (70 mg.) on oxidation with chromic oxide (80 mg.) in pyridine (2 ml.) for two days gave oxohaemanthamine (37 mg.) converted to its methiodide (54 mg.).

N-(6-Phenylpiperonyl)-N-methylglycine

The methiodide (54 mg.) on treatment with 10% sodium hydroxide (1.5 ml.) gave the sodium salt (45 mg.).

Lead tetra-acetate oxidation

Oxidation of the sodium salt hydrate (25 mg.) with lead tetra-acetate (34 mg.) gave barium carbonate (12 mg.) The formaldehyde dimedone separating as a partly crystalline oil was filtered off, washed thoroughly with water, taken up in benzene, and dried (Na_2SO_4). The product was chromatographed on alumina (grade III) elution with benzene-ethyl acetate (1:1) giving formaldehyde dimedone, m.p. and mixed m.p. 190-191° (7mg.). Work up of the reaction mixture in the usual way gave N-methyl-6-phenylpiperonylamine hydrochloride (9 mg.), m.p. 159-160°.

	Activity (c.p.m./m.Mole)	
haemanthamine	6.29×10^4	
formaldehyde dimedone (cryst)	5.56×10^4	
- (recryst)	5.92×10^4	(94% of total)
barium carbonate	inactive	
6-phenyl-N-methylpiperonylamine hydrochloride	2.70×10^3	(4.30% of total)

2. Doubly labelled O-methylnorbelladine (25 m.g., 0.011 mC) in water (5 ml.) was injected into the stalks of two flowering plants. The plants (235 g.) were worked up after eight days to give crude alkaloid (467 mg.). The ratio of labels was determined by the Zeisel technique described previously, the precursor being assayed for purity by inverse dilution analysis. The crude alkaloid was separated in the usual way to give the

following fractions.

Fraction 1.

22 mg. gave galanthamine (15 mg.), m.p. 126-129°.

Fraction 2.

19 mg. gave galanthine(16 mg.), m.p. 76-95°.

Fraction 3.

48 mg. gave haemanthamine (43 mg.), m.p. 198-200°.

Alkaloid	Activity (c.p.m./m.Mole)	Incorporation
galanthamine	inactive	0.0%
galanthine	inactive	0.0%
haemanthamine (cryst)	2.97×10^4	
- (recryst)	2.79×10^4	
- picrate (cryst)	2.75×10^4	
- (recryst)	2.77×10^4	

Incorporation of O-methylnorbelladine = 0.036(5)%

Hydrolysis

$[^{14}C]$ haemanthamine (30 mg.) in 20% sulphuric acid (40ml) was hydrolysed as described previously to give formaldehyde dimedone (16 mg.), m.p. 186-189° (56%).

	Activity (c.p.m./m.Mole)	
O-methylnorbelladine (0.23 mg. diluted to 60 mg.)	5.52×10^5	
triethylamine methiodide	1.06×10^5	
repeat	1.06×10^5	(19.1(5)% of total)

	Activity (c.p.m./m.Mole)	
haemanthamine	2.77×10^4	
formaldehyde dimedone (cryst)	5.62×10^3	
- (recryst)	5.73×10^3	(20.5% of total)

INTERMEDIATE TRAPPING EXPERIMENTS

1. Isolation of O-methylnorbelladine

The phenolic base was insoluble in chloroform, benzene, ethyl acetate and ether. The free base was obtained from its hydrochloride in aqueous solution by addition of sodium bicarbonate, the precipitated base being washed with water, and dried, m.p. 179-180°.

(a) Chromatography of free base

O-Methylnorbelladine could not be eluted (methanol) from alumina (grade V) or silica gel (acid washed). Attempted chromatography on neutral silica (obtained by careful neutralli-sation of the acid washed material with dilute ammonia and washing, and reactivated at 140°) gave no separation from haemanthamine.

(b) Acetate of O-methylnorbelladine

The hydrochloride (100 mg.) was treated with excess acetic anhydride (2 ml.) in pyridine (2 ml.), overnight. The solution was then diluted with water and evaporated to give an oil (140 mg.) which did not crystallise. Chromatography on alumina (grade III) gave, on elution with ethyl acetate-benzene (1:4) the triacetate (60 mg.) further purified by distillation

at 180° (10^{-4} m.m.), (Found C, 65.9; H, 6.3%; $C_{22}H_{25}O_6N$ requires C, 66.2; H, 6.3%.).

(c) Thin layer chromatography

Chromatography using kieselgel plates in ethanol chloroform (1:1), and development with modified Dragendorf[92] reagent showed separation of haemanthamine (Rf.0.9) and O-methylnorbelladine streaking to Rf.0.5.

Recovery of O-methylnorbelladine from King Alfred daffodil

A sprouting bulb (80 g.) was injected with O-methylnorbelladine (50 mg.) in water (0.6 ml.) and left for seven days. Work up in the usual way, except that the acid extract was buffered at pH_7 before chloroform extracting, gave crude mixture (220 mg.). The crude extract was chromatographed on kieselgel-belite (1:1), (50 g.), elution with chloroform giving alkaloid mixture (161 mg.), and further elution with methanol, giving O-methylnorbelladine (22 mg.) converted to its hydrochloride, m.p. 202-203°. The alkaloid extract was shown to be free from phenol by thin layer chromatography, and developing with Pauli's spray (for phenols). Gradient elution chromatography in the usual fashion gave galanthamine (22 mg.); galanthine (24 mg.) and haemanthamine (27 mg.), i.e. the biosynthesis of alkaloids was unaffected by the presence of O-methylnorbelladine.

2. Norbelladine

The phenolic base, insoluble in organic solvents was

obtained by precipitation from its hydrochloride.

(a) Chromatography of free base

Norbelladine could not be recovered from adsorption on alumina (grade V), acid washed, or neutral silica gel.

(b) Acetate

Acetylation of norbelladine (100 mg.) as for O-methyl-norbelladine gave an oil (160 mg.) which could not be eluted from alumina (grade V).

(c) Thin layer chromatography

Chromatography using the same system as for O-methyl-norbelladine gave a separation of haemanthamine (Rf 0.9) and norbelladine (streaking to Rf. 0.4).

(d) Chromatography on kieselgel

Norbelladine (15 mg.) was chromatographed on kieselgel: celite (1:1, 10g.), elution with methanol gave the phenol (12 mg.), converted to its hydrochloride, m.p. 113-115°.

TRACER STUDIES

Three sprouting bulbs (in identical state of growth) were injected separately with (a) [2-^{14}C] tyrosine (0.01 mC.) and inactive O-methylnorbelladine (80 mg.); (b) [2-^{14}C] tyrosine (0.01 mC.) and inactive norbelladine (80 mg.); and (c) [2-^{14}C] tyrosine (0.005 mC.), in water (5 ml.) at pH_6. The plants were worked up after ten days.

(a) O-Methylnorbelladine (Typical work up)

The bulb (79 g.) was macerated, and extracted with 1% tartaric acid in ethanol (2 x 250 ml.). The combined extract was filtered (celite) and evaporated to low volume (ca.50 ml.), and diluted with water (300 ml.), and 2NHCl (30 ml.). The acid extract was extracted with chloroform (3 x 40 ml.) and the chloroform solution extracted with 2N acid (5 x 40 ml.) and added to the bulk. The extract was neutrallised with sodium bicarbonate and extracted with chloroform (10 x 40 ml.). Concentration of the extract gave crude alkaloid (261 mg.). The mixture was chromatographed on kieselgel celite (1:1, 50 g.); elution with ethanol:chloroform (1:1) gave crude alkaloid (198 mg) (phenol free), and elution with methanol gave crude phenolic mixture (20 mg.). The alkaloid fraction was chromatographed in the usual way to give the following fractions.

Fraction 1.

40 mg. gave galanthamine 27 mg., m.p. 121-125°.

Fraction 2.

48 mg. gave galanthine 35 mg., m.p. 174-194°.

Fraction 3.

49 mg. gave haemanthamine 25 mg., m.p. 195-198°.

Alkaloid	Activity (c.p.m./m.Mole)	Incorporation
galanthamine	inactive	0.0%
galanthine	inactive	0.0%

Alkaloid	Activity (c.p.m./m.Mole)
haemanthamine (cryst)	1.30×10^5
- (recryst)	1.43×10^5
- picrate (cryst)	1.41×10^5
- (recryst)	1.44×10^5

Incorporation into haemanthamine = 0.021%

The crude phenol (1100 c.p.m./mg.) was dissolved in 1N-hydrochloric acid (2 ml.). The mixture was chromatographed on Whatman paper No.1. using descending solvent technique in a n-butanol:acetic acid:water system (4:1:5). The phenol was run against authentic samples of O-methylnorbelladine, norbelladine, tyramine and tyrosine. Development with diazotised sulphanilic acid showed two spots in the unknown mixture, one of Rf. 0.82 (cf. O-methylnorbelladine, Rf. 0.83) and the other with Rf.0.51 (cf. tyramine 0.56). In separate experiments portions (0.1 ml.) were streaked on paper; thin strips developed to show the positions of the phenols. The paper was then cut into strips and the phenols eluted separately with methanol. The areas remaining were also examined for activity.

Distribution of radio-activity (2 x 0.1 ml. fractions chromatographed)

Origin	0 c.p.m.
Area Rf.0.51	320 c.p.m.
Area Rf.0.82	270 c.p.m.
Solvent front	37 c.p.m.

(The total extract from each fraction was plated and counted). The remaining areas were essentially inactive.

The fast running phenol (Rf. 0.82) from four runs (total count 520 c.p.m.) was diluted with authentic inactive O-methylnorbelladine hydrochloride (25 mg.), and recrystallised, when the activity dropped to zero.

In separate experiments the slow running phenol (Rf.0.51) was eluted and diluted with tyramine (20 mg.) and N-methyl-tyramine (20 mg.) respectively. In both cases the activity was lost on recrystallisation.

(b) Norbelladine

The bulb (82 g.) on work up in the usual way gave basic fraction (170 mg.). Separation on kieselgel gave crude alkaloid (150 mg.) and phenol mixture (23 mg.). Gradient elution chromatography in the usual way gave the following fractions.

Fraction 1.

30 mg. gave galanthamine, (20 mg.), m.p. 125-126°.

Fraction 2.

27 mg. gave galanthine, (19 mg.), m.p. 74-97°.

Fraction 3.

37 mg. gave haemanthamine, (25 mg.), m.p. 198-200°.

Alkaloid	Activity (c.p.m./m.Mole)	Incorporation
galanthamine	inactive	0.0%
galanthine	inactive	0.0%

Alkaloid	Activity (c.p.m./m.Mole)
haemanthamine (cryst)	3.24×10^4
- (recryst)	1.83×10^4
- (recryst)	1.79×10^4
- picrate (cryst)	1.80×10^4

Incorporation into haemanthamine = 0.02%

The phenolic mixture (23 mg.) was dissolved in 1N-acid (2 ml.) and fractions run against authentic norbelladine, tyrosine, tyramine and N-methyltyramine. No fast running spot (cf. norbelladine Rf.0.77) showed up on spraying, but the slow running phenol (Rf. 0.50) was still present. Elution, and dilution with the tyramine and N-methyl tyramine in separate experiments showed the phenol not to be either of these.

(c) Tyrosine (control experiment)

The plant (75 g.) was worked up in the usual way, with the addition of O-methylnorbelladine (80 mg.) during work up, to give total basic fraction (254 mg.). Separation on kieselgel gave crude alkaloid (220 mg.) and phenol (21 mg.).

Gradient elution from alumina gave the following fractions.

Fraction 1.

38 mg. gave galanthamine,(20mg.), m.p. 128-130°.

Fraction 2.

41 mg. galanthine, (29 mg.), m.p. 80-100°.

Fraction 3.

55 mg. gave haemanthamine, (30 mg.), m.p. 195-201°.

Alkaloid	Activity (c.p.m./m.Mole)
galanthamine (cryst)	6.44×10^3
- hydrochloride (cryst)	5.15×10^3
- (recryst)	5.20×10^3
converted to narwedine (cryst)	5.15×10^3
Incorporation into galanthamine = 0.013%	
galanthine (cryst)	1.43×10^4
- hydrochloride (cryst)	1.15×10^4
- (recryst)	1.18×10^4
- picrate (cryst)	1.17×10^4
Incorporation into galanthine = 0.031%	
haemanthamine (cryst)	3.86×10^4
- (recryst)	3.93×10^4
- picrate (cryst)	3.99×10^4
Incorporation into haemanthamine = 0.13%	

The phenol (600 c.p.m./mg.) was dissolved in 1N acid (2 ml.) and fractions chromatographed on paper and run against O-methylnorbelladine, N-methyltyramine and tyramine. Two spots appeared on spraying a fast running spot (Rf. 0.82), similar to O-methylnorbelladine, and a slow running one (Rf. 0.51) similar

to tyramine. The fast running phenol was eluted, diluted with inactive O-methylnorbelladine (18 mg.), and recrystallised to lose the activity. In similar experiments it was established that the slow running phenol was neither tyramine nor N-methyl-tyramine.

Distribution of radio-activity (2 x 0.1 ml.fractions streaked and chromatographed)

Origin	0 c.p.m.
area Rf. 0.51	250 c.p.m.
area Rf. 0.82	206 c.p.m.
solvent front	30 c.p.m.

The remainder of the paper was essentially inactive.

(d) Narwedine (CIV)

A fully grown plant was injected with [2-^{14}C] tyrosine (0.01 mC.), and inactive (±) narwedine hydrochloride (50 mg.) in water (3 ml.). The plant was worked up in the usual way after eight days to give crude alkaloid (392 mg.). Chromatography on alumina (grade 3) in the usual fashion gave the following fractions.

Fraction 1.

narwedine (26 mg.), m.p. 185-187°.

Fraction 2.

58 mg. gave galanthamine (44 mg.), m.p. 122-126°.

Fraction 3.

53 mg. gave galanthine, (40 mg.), m.p. 81-101°.

Fraction 4.

57 mg. gave haemanthamine, (40 mg.), m.p. 194-201°.

Alkaloid	Activity (c.p.m./m.Mole)	Incorporation
narwedine	inactive	0.0%
galanthamine	inactive	0.0%
galanthine	inactive	0.0%
haemanthamine (cryst)	3.78×10^4	
- (recryst)	3.82×10^4	
- picrate (cryst)	3.80×10^4	
- picrate (recryst)	3.79×10^4	

Incorporation into haemanthamine = 0.065%

(e) N,O-dimethylnorbelladine (CXXIX)[67]

Two fully grown plants were injected with [2-^{14}C]-tyrosine (0.02 mC) and the phenol hydrochloride (100 mg.) in water (30 ml.) over seven days. The bulbs (394 g.) were worked up after a further three days, to give crude alkaloid (537 mg.).

Preliminary experiments showed that the phenol could be recovered from adsorption on alumina (grade V). A mixture of the phenol (40 mg.) and haemanthamine (40 mg.) was chromatographed on alumina (grade V), (10 g.). Elution with ethyl acetate: benzene (3:2) gave haemanthamine (35 mg.), m.p. 199-201°, and elution with ethanol:chloroform (1:20) gave the phenol (35 mg.), m.p. 131-134°.

The crude alkaloid (537 mg.) was chromatographed on alumina (grade V) to give the following fractions.

Fraction 1. - ethyl acetate-benzene (1:5)

99 mg. gave galanthamine (62 mg.), m.p. 126-130°.

Fraction 2. - ethyl acetate-benzene (2:5)

105 mg. gave galanthine (87 mg.), m.p. 81-94°.

Fraction 3. - ethyl acetate-benzene (2:5)

112 mg. gave haemanthamine (63 mg.), m.p. 199-201°.

Fraction 4. - ethanol-chloroform (1:20)

28 mg. gave phenol hydrochloride (29 mg.), m.p. 212-220°.

Alkaloid	Activity(c.p.m./m.Mole)	Incorporation
galanthamine	inactive	0.0%
galanthine	inactive	0.0%
haemanthamine (cryst)	3.36×10^4	
- (recryst)	3.04×10^4	
- (recryst)	2.85×10^4	
- picrate (cryst)	2.77×10^4	

Incorporation into haemanthamine = 0.049%

Circular paper chromatography of the phenolic fraction in butanol:acetic acid:water (4:1:5) and development with Pauli's spray showed a major component running alongside authentic phenol. The phenol hydrochloride (28 mg.) was diluted with authentic inactive hydrochloride (30 mg.),and recrystallised to constant count.

	Activity (c.p.m./m.Mole)
N,O-dimethylnorbelladine	4.27×10^4
- diluted	2.91×10^3
- recryst.	1.99×10^3
- recryst.	2.06×10^3
- recryst.	1.92×10^3

Incorporation into N,O-dimethylnorbelladine = 0.0016%

Model degradation of N,O-dimethylnorbelladine

N,O-dimethylnorbelladine (40 mg.) in methanol (5 ml.), was methylated in the usual way with diazomethane. Evaporation of the solvent gave an oil which was chromatographed on alumina (grade III). Elution with benzene gave belladine (36 mg.) which was converted to its methiodide (36 mg.), m.p. 205°. (lit.m.p. 206°)[102] The methiodide was converted to its methochloride by shaking with freshly prepared silver chloride in water (5 ml.). The filtrate was treated with 4% sodium amalgam at 100° for six hours. The oil which separated was taken up in ether (10 ml.) washed with water and extracted with 1N acid (4 x 5 ml.). The acid extract was basified and the solution extracted with ether (4 x 5 ml.). The ethereal solution was dried (Na_2SO_4) and evaporated to give O-methylhordenine which was converted to its hydrochloride (8 mg.), m.p. 172-174°. (lit. m.p. 176°).[103]

Degradation of $\left[{}^{14}C\right]$ N,O-dimethylnorbelladine

Methylation of the phenol (30 mg.) according to the above procedure gave belladine (22 mg.) converted to its hydrochloride (18 mg.), m.p. 182-186°. (lit. m.p. 182-185°).[67]

Regeneration of the free base, conversion to the methiodide and Emde degradation of the product gave O-methylhordenine (5 mg.).

Alkaloid	Activity (c.p.m./m.Mole)
O-methyl-N-methylbelladine hydrochloride	2.02×10^3
belladine-hydrochloride	2.12×10^3
O-methylhordenine hydrochloride	1.99×10^3

INVESTIGATION OF UNKNOWN PHENOL

Synthesis of hordenine (XX)

N-Methyl-p-benzyloxyphenylacetamide

p-O-Benzylphenylacetic acid (1 g.) was treated with excess oxalyl chloride (0.5g.) in benzene (10 ml.). The resulting acid chloride without purification was treated with excess aqueous methylamine, and the resulting amide extracted into ether (2 x 20 ml.), washed with water and dried (sodium sulphate). The crude amide was recrystallised from benzene/pet ether (1.7g.), m.p. 145°. (lit. m.p. 145°).

O-Benzyl-N,N-dimethyltyramine

The amide (500 mg.) was heated under reflux in dry tetrahydrofuran (5 ml.) with sodium hydride (500 mg.) for three hours, methyliodide (2 ml.) was then added, and the reaction mixture heated overnight. The solvent was then removed, excess sodium hydride decomposed by water, and the amide extracted into ether and dried (sodium sulphate). Solvent was evaporated to give an oil (I.R. showed no N-H band) which was reduced directly with

lithium aluminium hydride (400 mg.) in dry ether (5 ml.). Excess lithium aluminium hydride was decomposed with water, and the product extracted into ether. The ether extract was extracted with dilute acid (4 x 10 ml.), the acid extract neutrallised and re-extracted into ether (4 x 10 ml.). Theether extract was dried (sodium sulphate) and evaporated to give O-benzyl-hordenine purified as its hydrochloride (345 mg.), m.p.208-210° dec.

Hordenine

O-Benzyl hordenine hydrochloride (250 mg.) was hydrogenolysed under the usual conditions to give hordenine hydrochloride (180 mg.), m.p. 170-173°. The product was recrystallised from ethanol/ether, m.p. 175-176°.(Lit.m.p. 176°)[98]

Identification of Phenol

Three flowering King Alfred bulbs were injected with [^{14}C]-generally-labelled glucose (0.02 mC) and worked up after eight days. Chromatography on kieselgel:celite (1:1, 50 g.), gave crude alkaloid (650 m.g.), and phenolic fraction (39 mg.).

The phenolic fraction was acidified with concentrated hydrochloric acid (0.1 ml.), and dissolved in ethanol (2 ml.). The phenolic mixture was run on paper (see page109) together with norbelladine, O-methylnorbelladine, O,N-dimethylnorbelladine, tyramine, N-methyltyramine, hordenine and tyrosine. Development with Pauli's spray showed two spots, one (Rf. 0.82) similar to O,N-dimethylnorbelladine, and the other (Rf. 0.51) lying between tyramine and tyrosine. Fractions were streaked on paper, run in

the usual way, and the phenols eluted.

Distribution of radio-activity (2 x 0.5 ml. fractions streaked and chromatographed)

origin	0 c.p.m. (total)
area Rf. 0.5	10,750 c.p.m.
area Rf. 0.82	1,770 c.p.m.
solvent front	74 c.p.m.

The remainder of the paper was essentially inactive. The fast running phenol was diluted with inactive O,N-dimethyl-norbelladine (25 mg.), and recrystallised when the activity dropped to zero.

Rf. values of Phenols

Trapping Experiments (P.107.)	Rf. Phenols isolated	
(a) Tyrosine & O-methylnorbelladine	0.51	0.82
(b) Tyrosine & norbelladine	0.50	-
(c) Tyrosine & O-methylnorbelladine	0.51	0.83
Glucose fed plants	0.51	0.82

Standards used for comparison	Rf.
Tyrosine	0.40
Tyramine	0.56
N-methyltyramine	0.60
Hordenine	0.65
Norbelladine	0.77
O-methylnorbelladine	0.83

The above values are for a butanol:acetic acid:water (4:1:5) system, using Whatman paper No.1.

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