BIOSYNTHESIS OF LYCOPODINE

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By

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The biosynthesis of lycopodine was studied by feeding radioactive acetate, acetoacetate, lysine and pelletierine to Lycopodium tristachyum. Partial degradation of the radioactive lycopodine recovered from these experiments revealed specific incorporation of these precursors. The labelling pattern obtained is discussed in the light of the two major biogenetic hypothesis advanced for the Lycopodium alkaloids.

The results obtained disproved Conroy's polyacetate hypothesis. They are consistent with the hypothesis that regards these alkaloids derived from lysine and acetate.

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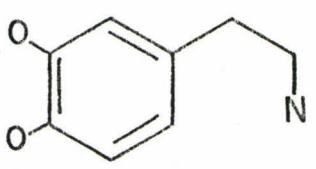
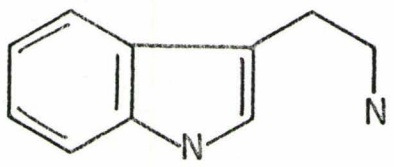
1. Introduction

The study of the biosynthesis of alkaloids has become an increasingly important facet of alkaloid chemistry. A proper understanding of the biological processes involved in the synthesis of these nitrogenous substances began when organic compounds labelled with carbon-14 and with other isotopes became readily available. Yet long before tracers became available organic chemists were wondering about the biosynthesis of these natural products and speculated about their mode of formation.

Hypothetical biogenetic schemes were proposed, which were based on the recognition that families of alkaloids contained common structural features. Thus, the structural unit A was observed in many alkaloids, particularly those of the isoquinoline group. Another example of a structural feature common to a large number of alkaloids is represented by structure B, as found in alkaloids of the indole group.

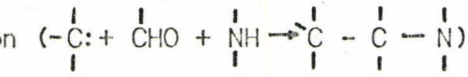


A



B

These two structural units are also present in the amino acids phenyl alanine and tryptophan, respectively, and this relationship suggested that amino acids might be implicated in the biosynthesis of alkaloids. Thus, Pictet suggested, as long ago as 1906 (1) that the indole nucleus is derived from the amino acid tryptophan and Winterstein and Trier postulated, in 1910, that the isoquinoline alkaloids were derived from phenylalanine (2).

This biogenetic approach, i.e., of structural relations, was complemented by a correlation of alkaloidal structures on the basis of a unifying reaction mechanism. Thus, the biosynthesis of a wide variety of alkaloidal structures was rationalized, in chemical terms, by the operation of a few simple organic reactions, e.g., aldol condensations, oxidative coupling of phenols and Mannich-type reactions. This last reaction, of particular relevance to the biosynthetic work discussed in the following sections, consists of the condensation of an amine, a carbonyl compound and a carbanion  (3).

These hypotheses and more recent contributions by Barton (4), Woodward (5), Wenkert (6) and others, established the main outlines of alkaloid biogenesis. Following development of isotope labelling techniques, in the late 1940's, and under the stimulus of these biogenetic postulates, experimental work was begun. Since then, the knowledge accumulated has not only revealed the striking simplicity by which these natural products are formed, but has also shown that many of these biogenetic speculations were basically correct.

The isotopic tracer method consists in feeding to the intact

organism a postulated precursor which is labelled with an isotopic atom at a single known position. Carbon-14 has found the widest application in investigations of alkaloid biosynthesis, but other radioactive isotopes, e.g., tritium, as well as stable isotopes such as deuterium, carbon-13, nitrogen-15 and oxygen-18 have also been used. Radioisotopes are preferred because of the greater sensitivity of the methods of measurement of radioactivity, as compared with measurements of heavier isotopes. This permits the quantity of labelled precursor which is administered, to be kept at a minimum, a condition required to minimize alterations in the normal steady-state conditions of the living organism. After an arbitrary period of growth of the plant in contact with the tracer, the desired compound is isolated, purified and degraded to localize the label within it. If activity within it is confined to a single site, or to several specific sites, incorporation of radioactivity is shown to have taken place in a non-random manner. The substrate is then said to have been incorporated specifically and is regarded as a precursor.

Further proof that the intact carbon skeleton of the precursor has been incorporated into the product can be obtained by feeding other radiomers of the precursor, followed by recovery of the activity at the predicted positions of the product. Multiply labelled precursors of defined isotopic distribution, are particularly useful in this respect. Thus, intact incorporation of the precursor is proven if the product retains the same isotopic distribution as that of the precursor.

Complementary to a study of specificity of conversion of a precursor into a product is the study of the efficiency of precursor incorporation, i.e., the extent to which several related or alternative

substrates are incorporated into the same product. Unfortunately, several ambiguities involved in work with plants, make any conclusions derived from such comparative experiments of doubtful validity. Some of the factors involved are the following:

a) permeability, rate of transport and rate of utilization before reaching the actual site of synthesis can be quite different for different substrates which are administered, so that expressing yields as a fraction of substrate supplied is misleading.

b) natural variations in a small number of individuals make comparisons under "standard conditions" difficult.

c) the amount of final product isolated is a function of past rates of synthesis and of further metabolism and never measures the amount of final product which has been formed during the biosynthetic experiment.

The most powerful single approach to the study of precursor-product relationships in biosynthetic studies is the specificity of precursor incorporation and it is from studies of this type that most of the present knowledge of alkaloid biosynthesis has been obtained. The tracer work has been successful in relating many of the alkaloidal structural groups to intermediates of primary metabolism and in establishing the biosynthetic anatomy of their origin.

For many families of alkaloids this first stage of the biosynthetic investigation is now complete. The next step will involve the testing of the biosynthetic sequences which have been proposed on the basis of the preliminary incorporation studies. This will require

the isolation of proposed intermediates and the study of the mechanism of each step in the metabolic pathway. This, in turn, will require the isolation of the enzymes involved in each of these transformations and also a knowledge of the kinetics of the processes involved. These tasks are expected to be far more difficult to achieve than the rather simple initial 'precursor-product' stage.

Thus, in vitro studies of alkaloid biosynthesis using enzymes extracted from plants have, so far, given few results (7,8). These negative findings may be an indication that alkaloid synthesis requires contributions from other parts of the plant. It is difficult to determine the real site of alkaloid synthesis because of translocations of intermediates and because different steps in the synthesis may not take place in the same tissues.

A serious limitation of the tracer technique not mentioned so far is the fact that tracer results alone do not establish the normal and obligatory occurrence of a metabolic pathway. It is likely that many non-specific enzymes and reaction pathways exist in plants. Thus, foreign substances, which have been forced into the organism, may be utilized in the synthesis of an alkaloid even though they are not normally present in the system. Examples of contradictory results obtained in biosynthetic experiments which can be attributed to the existence of these alternative pathways will be mentioned in the following section.

Ideally, it should be shown that the compound fed is a normal constituent of the system and that the rate of the proposed steps is

consistent with the normal overall rate of formation of the product. The enzymes responsible for the synthesis of the alkaloid should also be isolated and characterized. Only when this has been done can obligatory routes of biosynthesis be rigorously defined.

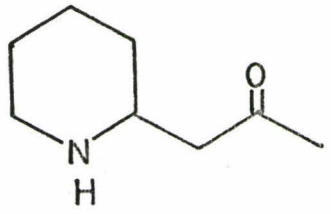
2. Biosynthesis of Piperidine and related alkaloids

A substituted piperidine ring is found in nature in a variety of alkaloidal structures, e.g., pelletierine (1), coniine (2), sedamine (3) anabasine (4), lupinine (5), lobinaline (6), etc. (Figure 1). According to classical biogenetic theory, the piperidine ring present in these alkaloids is derived from lysine. Robinson, who made this proposal, envisaged the biogenesis of these bases to proceed by a condensation of lysine, or of its derivatives, 5-aminopentanal or Δ^1-piperideine (7), with an "acetone derivative", such as acetoacetic acid or other appropriate metabolites (3). The fact that some of these alkaloids were synthesized in the laboratory using these hypothetical intermediates gave further support to this view and stimulated work along these lines (9,10).

The tracer work of the last ten years has confirmed the general validity of this hypothesis, but has also shown that the piperidine ring may be derived from precursors other than lysine, namely, acetic acid and mevalonic acid. In these instances, structural analogy proved to be misleading.

Lysine as the precursor of the piperidine ring

In higher plants, the carbon skeleton of lysine is derived from pyruvate and aspartate with the intermediate formation of diaminopimelic acid (11). This reaction pathway, known as the diaminopimelic pathway is outlined in Figure 2. In certain algae and fungi the biosynthesis of lysine is known to take place by a different and distinct pathway involving acetate and α-ketoglutarate. The catabolism of lysine in plants is not well known, but work on animals and micro-organisms has



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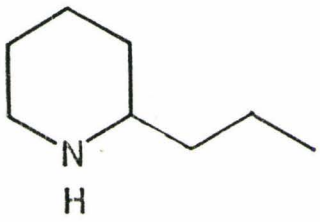

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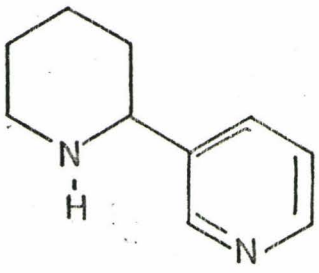

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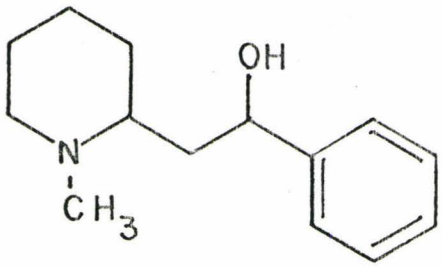

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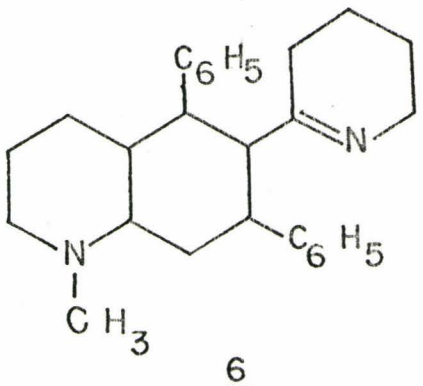

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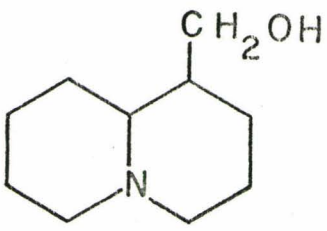

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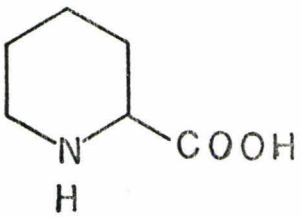

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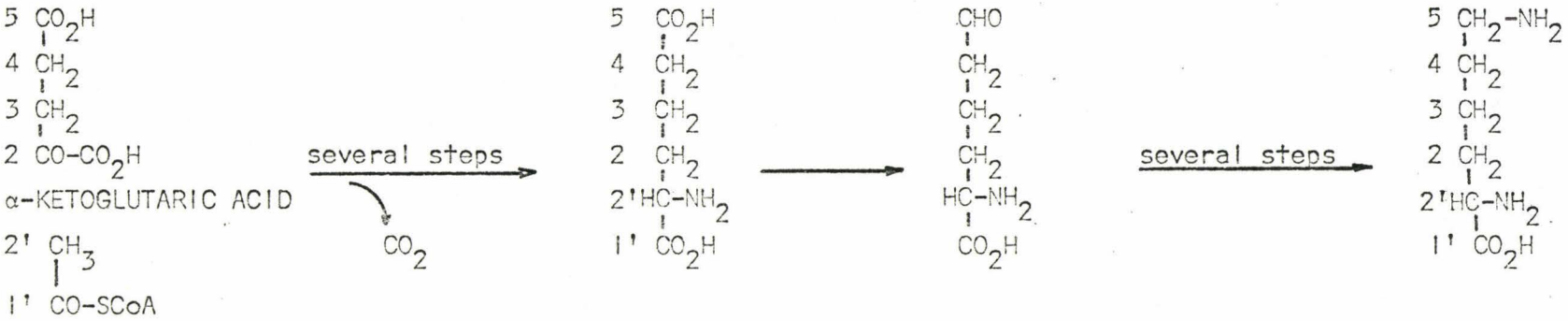
FIGURE 1 : Representative Piperidine Alkaloids

FIGURE 2

THE BIOSYNTHESIS OF LYSINE

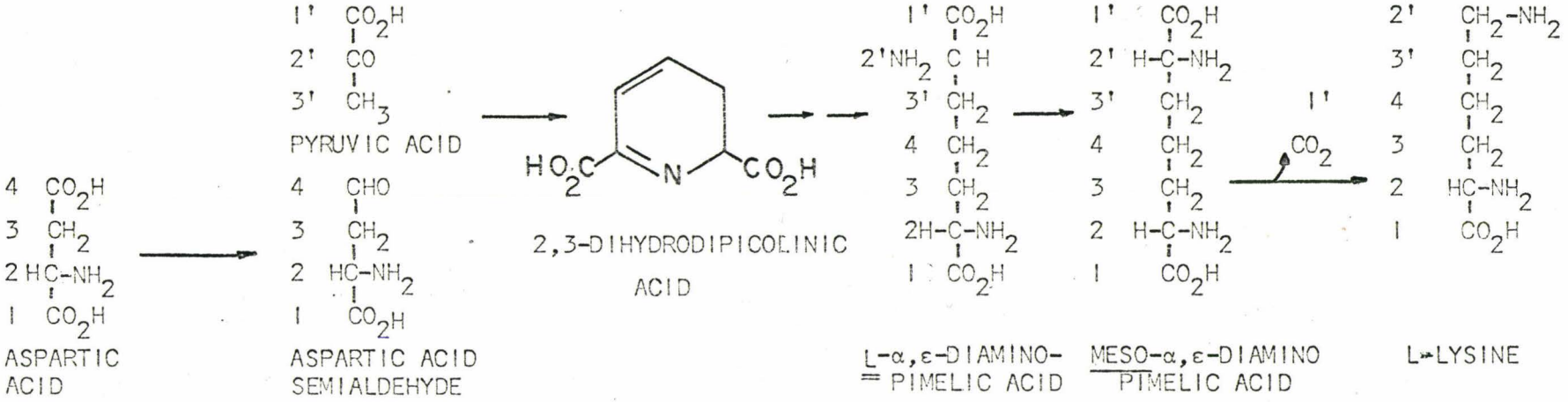
α-AMINOADIPIC PATHWAY



The numbering refers to the carbon atoms of a α-ketoglutaric acid (1-5) and of acetate (1'-2')

The numbering refers to the carbons of aspartic acid (1-4) and pyruvic acid (1'-3').

DIAMINOPIMELIC PATHWAY



shown that pipecolic acid (8) is an intermediate in the degradation, which leads to acetic acid and carbon dioxide as ultimate products. Pipecolic acid has also been found in plants and tracer work has confirmed its metabolic relationship to lysine. According to a recent paper, pipecolic acid is formed from lysine in bean plants and in Sedum acre by way of 6-amino-2-ketocaproic acid (9) (12). This conclusion was inferred because the $^{3}H:^{14}C$ ratio found in pipecolic acid was identical with that of the 6-^{3}H,6-^{14}C-lysine administered to bean plants and to Sedum acre.

Lysine has indeed been shown to supply the C_5N unit present in several piperidine alkaloids. In Nicotiana glauca, activity from 2-^{14}C-lysine was shown to be incorporated exclusively at C-2 of the piperidine ring of anabasine (4) (13). In Sedum acre, all the activity present in sedamine (3), after the plant had been fed 6-^{14}C-lysine, was found at C-6 of the piperidine ring (14). Likewise, 2-^{14}C-lysine and 6-^{14}C-lysine administered in separate experiments to Sedum sarmentosum yielded radioactive N-methylpelletierine (10) labelled solely at C-2 and C-6, respectively (15). These results clearly indicate that lysine is the specific precursor of the piperidine ring of these alkaloids. They also show that incorporation of lysine takes place by way of a non-symmetrical intermediate, probably 6-amino-2-ketocaproic acid (9) or its cyclized derivatives Δ^1-piperideine-2-carboxylic acid (11) and Δ^1-piperideine (7), as shown in Figure 3. The intermediacy of the derivative 7 has been verified in the biosynthesis of anabasine (16).

Further evidence that the 2-amino nitrogen of the precursor is lost during the biosynthetic sequence and that the ε-amino nitrogen

is incorporated into the alkaloid was obtained by feeding doubly labelled precursors. Thus, when 6-^{15}N,2-^{14}C-lysine was administered to Nicotiana glauca, the anabasine nucleus showed a ^{15}N : ^{14}C ratio identical with that of the precursor. When 2-^{15}N,2-^{14}C-lysine was tested as precursor, the radioactive anabasine was not enriched in ^{15}N (17). Somewhat similar experiments were carried out with Sedum acre and Sedum sarmentosum. The alkaloids isolated from specimens of these plants to which 6-^{3}H,6-^{14}C-lysine had been fed, showed a ^{3}H : ^{14}C ratio identical with that of the precursor. Since loss of the 6-amino nitrogen would have been accompanied by loss of tritium from C-6, it is the 2-amino rather than the 6-amino nitrogen which is lost from lysine in the biosynthetic sequence (12).

The origin of the substituents at the ring carbon adjacent to nitrogen in these alkaloids has also been investigated. Classically, sedamine and N-methylpelletierine were considered to arise by condensation of a lysine derivative, 5-aminopentanal or its cyclized derivative, Δ^{1}-piperideine, with the ß-keto acids benzoylacetic acid or aceto acetic acid, derived from phenylalanine and acetate, respectively. The experimental results are in complete agreement with these expectations. Thus, when 2,3-$^{14}C_2$-phenylalanine, of known isotopic distribution, was administered to Sedum acre, the radioactive sedamine isolated showed an isotopic distribution identical with that of the doubly labelled precursor (14). Likewise, 1-^{14}C-acetate was incorporated almost exclusively at the carbonyl carbon of the N-methylpelletierine side chain, as expected for incorporation via acetoacetic acid (15). The N-methyl group present in these two alkaloids was shown to be derived from (methyl-^{14}C)-methionine (14,15).

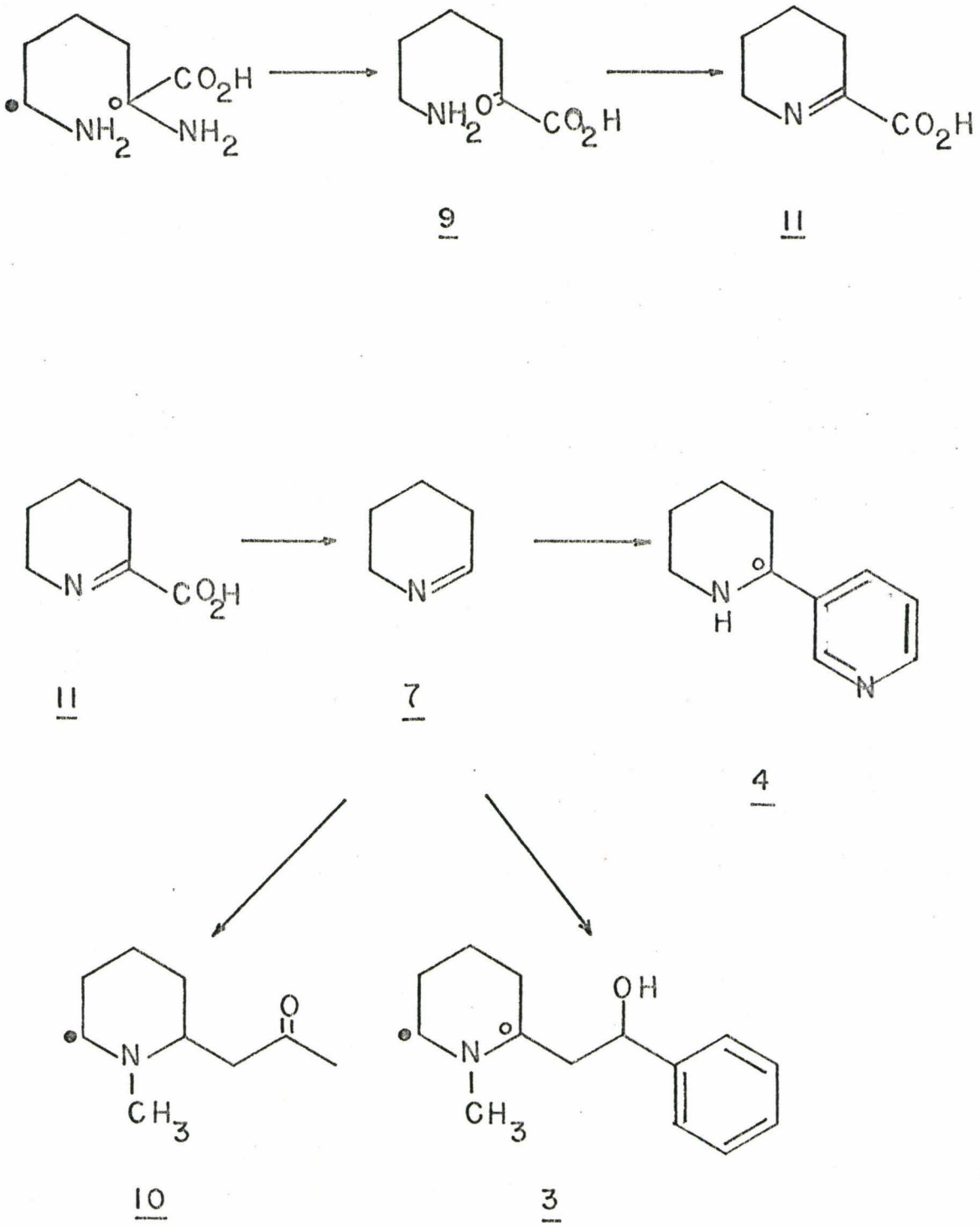


FIGURE 3 : Incorporation of Lysine into some Piperidine Alkaloids

It should be stressed that the exact nature and the steps by which these fragments are formed and combined is not yet known. What is known with certainty from the results of these experiments is that the C_5N nucleus is derived from lysine and that the side-chain is derived, in one case, from a C_6-C_2 residue afforded by phenylalanine and, in the other, from acetate units. The pyridine ring present in anabasine is derived from nicotinic acid (18).

Lysine has also been shown to be the precursor of another quite different group of alkaloids, the lupin alkaloids. The accumulated evidence indicates that lysine enters the skeleton of the lupin alkaloids by way of a symmetrical intermediate. This is in sharp contrast with the mode of incorporation of lysine discussed above. Figure 4 shows the incorporation of 2-^{14}C-lysine and of 1,5-^{14}C-cadaverine into several quinolizidine alkaloids. Approximately one quarter of the total specific activity of lupinine (5) (19) and one sixth of that of sparteine (12) (20), lupanine (13) (21), hydroxylupanine (14)(21) and matrine (15)(22) was found to be at the starred carbon atoms. These results are interpreted as shown in the same figure. Again, we must observe that the manner in which the lysine residues are modified and combined is not known in detail. Some interconversions between these bases have also been studied. Thus, activity from radioactive lupinine enters sparteine in Lupinus luteus, but this conversion is not reversible (23). The same irreversibility has been shown in the series sparteine→lupanine→13-hydroxy-lupanine (23).

The observed incorporation of lysine and cadaverine into these alkaloids points to a symmetrical intermediate in their biosynthesis.

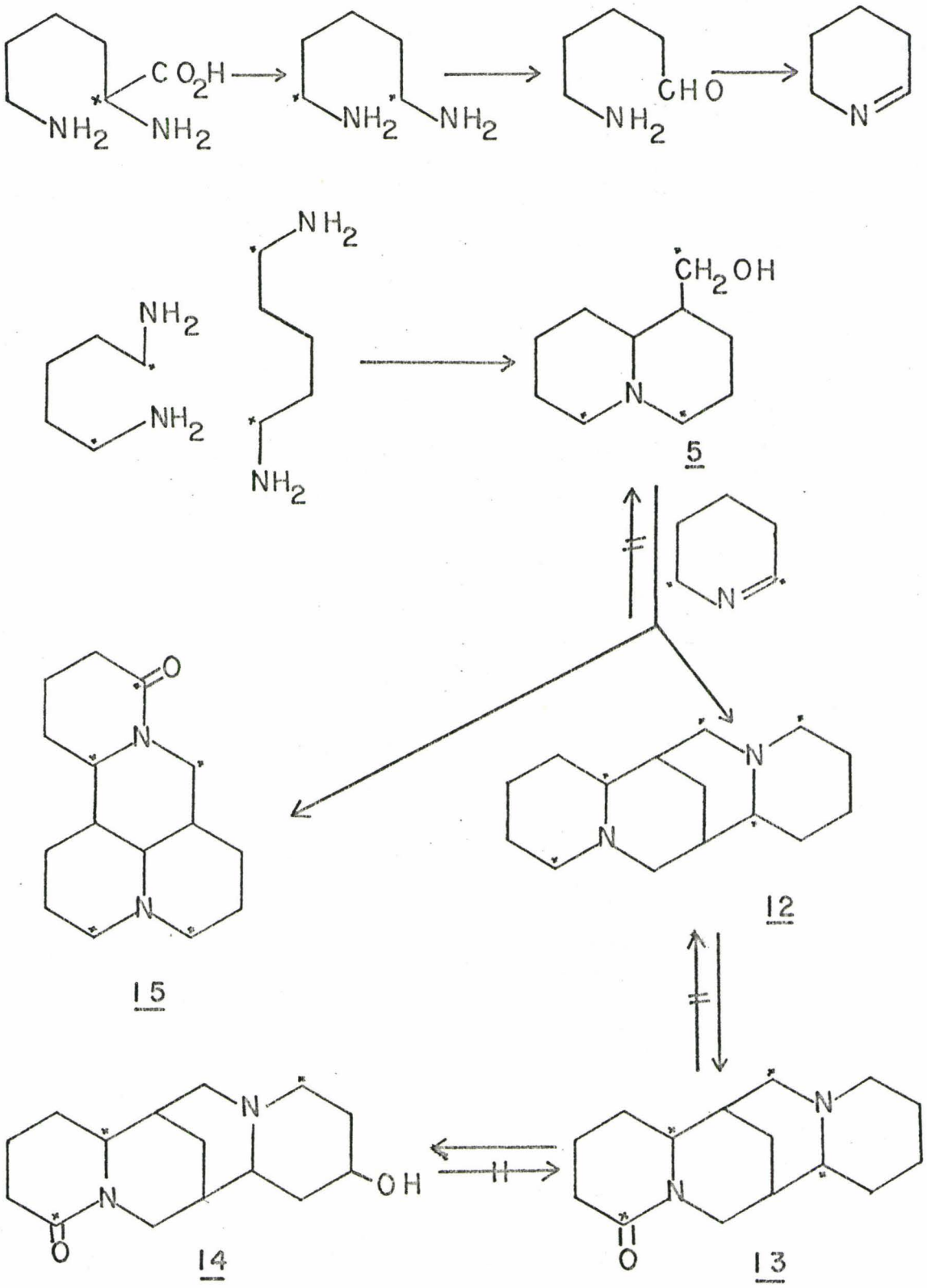


FIGURE 4 : Incorporation of Lysine into Lupine Alkaloids

Cadaverine itself, or its derivatives, 5-aminopentanal or Δ^1-piperideine (7) are regarded as the normal precursors of these bases. Results of experiments with doubly labelled precursors were compatible with the intermediacy of cadaverine in the pathway (20). Other positive evidence in this respect is the fact that diamine oxidase activity, capable of converting cadaverine to 5-aminopentanal, has been demonstrated in a preparation from lupin seedlings (24). Despite these findings, the role of cadaverine in alkaloid biosynthesis is, in general, uncertain. Thus, decarboxylation of lysine has not yet been demonstrated nor has cadaverine been found in plants. Although diamine oxidase activity has been shown in a number of plants it has not been detected in several others (Nicotiana and Datura species, among others) (25). The strongest argument against participation of cadaverine (and of putrescine as well) comes from incorporation studies. Since these diamines are symmetrical, their incorporation into, say, anabasine (or nicotine) would require equal incorporation of both nitrogen atoms into the products. But it has been shown that the 2-amino nitrogen of lysine or ornithine is lost in both cases, whereas the 6 (or 5)-amino group is retained. It follows that a symmetrical molecule cannot be a normal intermediate in the pathway from lysine (or ornithine) to these alkaloids. Cadaverine and putrescine are, nevertheless, incorporated into anabasine and nicotine, respectively (in fact, they are incorporated much more efficiently than the parent aminoacids themselves). These facts have been interpreted in a number of ways but the most likely explanation of these contradictory results lies in the recognition of the operation that alternative or parallel routes to a given product are available

and in the inability of the tracer method to distinguish true from foreign precursors and normal from aberrant metabolic pathways. The history of the biosynthesis of nicotine is highly instructive in this respect (26,27).

Acetate as the precursor of fatty acids and steroids

In recognition of the key role played by acetic acid in primary and secondary metabolism and because of its relevance to the biogenesis and biosynthesis of the Lycopodium alkaloids, a brief survey of its general metabolism and incorporation into natural products is included here.

Acetyl-Coenzyme A arises mainly by catabolism of sugars, with pyruvic acid as an intermediate (28). Other routes leading to acetyl-CoA are the oxidative degradation of fatty acids and the catabolism of several amino acids. The formation of acetate by degradation of lysine was mentioned earlier. On the other hand, acetyl-CoA is the starting point and the building unit of two important primary metabolites, the fatty acids and the steroids. Variations in the pathway leading to these fundamental substances give rise to numerous secondary metabolites, e.g., the polyketides, plant terpenes and, also alkaloids.

The unbranched hydrocarbon chain of the fatty acids is built up in a sequence of two-carbon units of acetic acid joined head-to-tail (29). In this sequence, acetic acid is activated by conversion firstly to a thiol ester by attachment to the sulfydryl group of Coenzyme A and secondly, by carboxylation to malonyl-CoA. Equations 1 and 2 represent these processes. Malonyl-CoA is the chain extending agent. It is not only

more reactive than acetyl-CoA but the accompanying decarboxylation (Eq.3) drives the reaction in the forward sense. This fact is important since the direct self-condensation of acetyl-CoA to yield acetoacetyl-CoA is thermodynamically unfavourable.

1. $CH_3COOH \xrightleftharpoons{Mg^{++}\ CoASH,\ ATP} CH_3CO\text{-}SCoA$ (CoASH = Coenzyme A)

2. $CH_3CO\text{-}SCoA \xrightarrow[Biotin,\ ATP,\ Mg^{++}]{CO_2} CH_2(COOH)CO\text{-}SCoA$

3. $CH_3CO\text{-}SCoA + CH_2(COOH)CO\text{-}SCoA \rightleftharpoons CH_3CO\text{-}CH_2CO\text{-}SCoA + CO_2 + CoASH$

$$K_{eq} = 2 \times 10^{-2} \qquad (29);$$

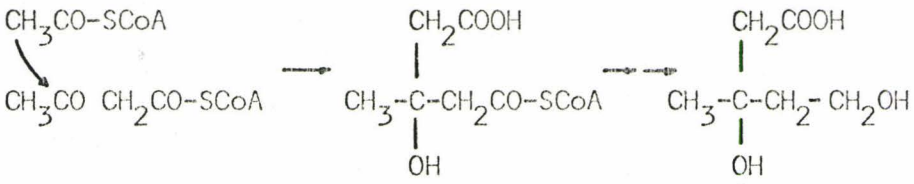
4. $CH_3CO\text{-}SCoA + CH_3CO\text{-}SCoA \rightleftharpoons CH_3CO\ CH_2CO\text{-}SCoA + CoASH$

$$k_{eq} = 1.6 \times 10^{-5} \qquad (30)$$

The chain extending process in fatty acid biosynthesis involves reductions of the β-keto groups, dehydrations and hydrogenations of the growing acid. The polyketides or acetogenins arise by a modification of this primary metabolic pathway. Here the reduction steps are missing or are incomplete, and a linear polyketo acid is generated, which is further transformed by internal cyclizations, aromatizations, alkylations, etc. Such events rationalize the biosynthesis of a large number of aromatic and homocyclic compounds found in nature in plants and micro-

organisms (31). Many tracer experiments have been undertaken with acetate as precursor and the results provide a solid experimental basis for this polyacetate theory. The expected alternation of activity from the methyl and carboxyl positions of acetate is almost always observed (31b).

The second group of fundamental substances which are derived entirely from acetate are the steroids; the biosynthesis of the plant terpenes is intimately related to this metabolic pathway. Here, the intermediate is mevalonic acid which is derived from acetyl-CoA and acetoacetyl-CoA. Subsequent transformations of mevalonic acid yield isopentenyl pyrophosphate, the true biological unit involved in the biosynthesis of this group of substances (31).



mevalonic acid

These two metabolic pathways, the polyketide and the mevalonic routes, are the biochemical basis of the empirical rules known as the "polyacetate rule" and the "isoprene rule" which are so useful in structural studies of natural products.

Acetate as the precursor of alkaloids

The hemlock plant Conium maculatum contains a group of piperidine alkaloids, e.g. coniine (2), γ-coniceine (16), conhydrine (17), etc. It has been conclusively shown that the carbon chain of these alkaloids is derived by linear combination of acetate units, in the

manner shown in Figure 5 (32). Unambiguous degradations showed that 1-^{14}C-acetate was incorporated into coniine without randomization. Each of the starred carbon atoms in coniine carried approximately one quarter of the activity in the intact alkaloid. The other carbons were essentially unlabelled. Incorporation of acetate into lysine and then into coniine is ruled out by the labelling pattern obtained. The role of γ-coniceine as the immediate precursor of the other alkaloids in Conium maculatum has been shown by tracer work and by incorporation studies with carbon dioxide-14 (33,34). The exact nature of the nitrogen source and the stage at which it is introduced into the products remains to be elucidated.

The biosynthesis of carpaine skeleton (18) in Carica papaya was investigated by feeding acetate, lysine and mevalonate. In agreement with Leete's proposal for the polyketide origin of coniine, the experiments showed acetate to be by far the best incorporated of the three precursors (35).

Other examples of acetate as a specific precursor of the piperidine ring in nitrogenous compounds are found in a number of steroidal and monosesqui- and diterpene alkaloids. Skytanthine (20) from Skytanthus acutus and actinidine (19) from Actinidia polygama are examples of such compounds. The ultimate precursor of their carbon skeleton is acetate which, as expected, has been found to be incorporated by way of mevalonic acid (36,37).

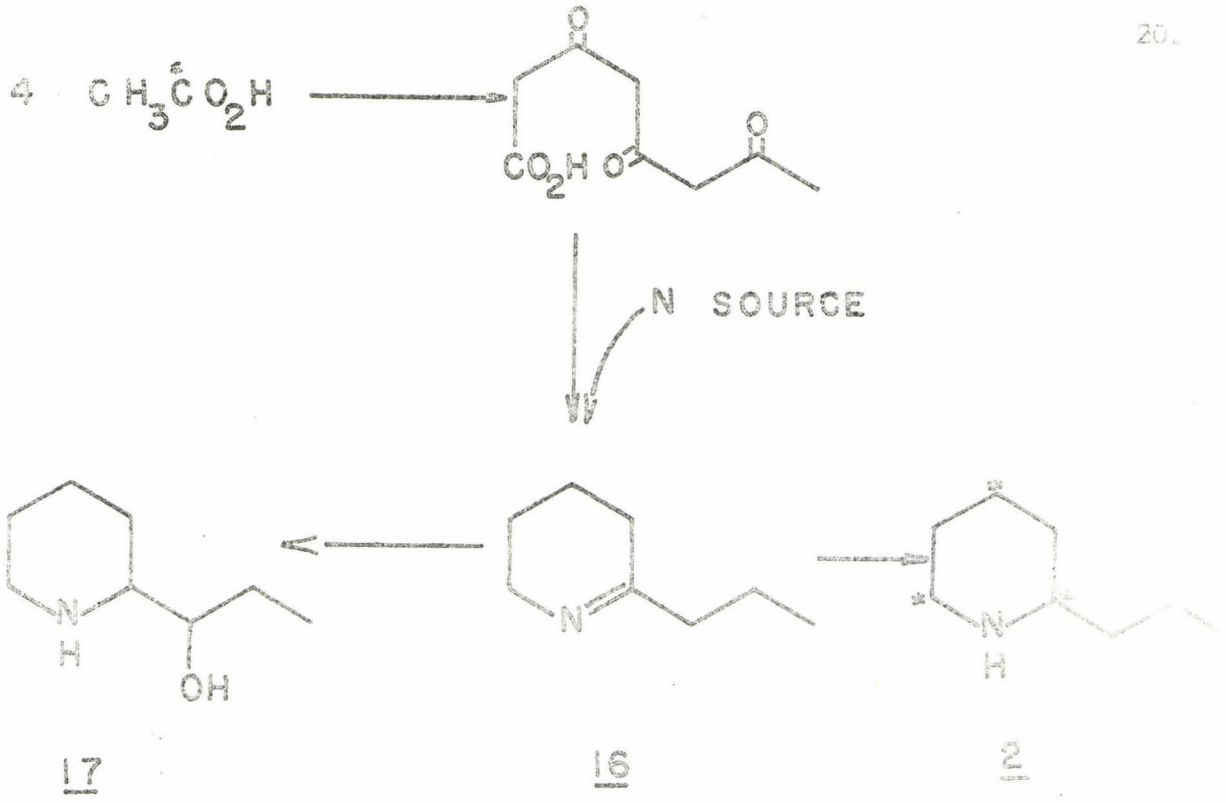
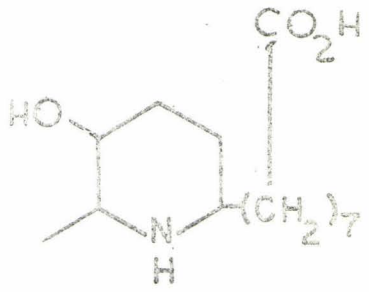


FIGURE 5 : Biosynthesis of Hemlock Alkaloids



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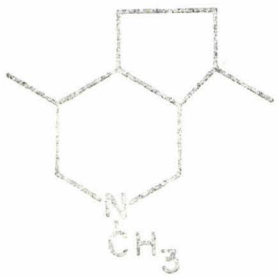

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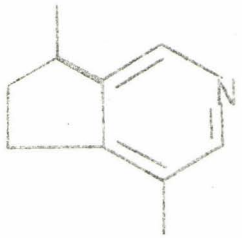

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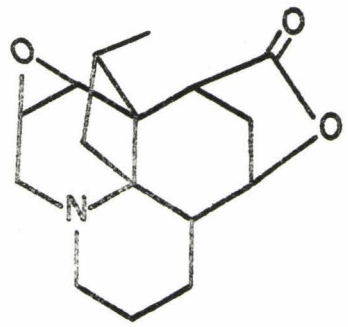
3. Biogenesis of the Lycopodium alkaloids

The Lycopodium alkaloids are a unique group of nitrogenous substances elaborated by the club-mosses of the Lycopodiaceae family. Since 1957 when the structure of annotinine (21) was first elucidated by Wiesner and co-workers (38), the number of alkaloids isolated and characterized has increased greatly and new structural types are still being discovered (39). Lycopodine (22) is the most commonly found of these bases and the majority of the Lycopodium alkaloids possess its carbon skeleton. Other structural types are exemplified by annotinine (21) lyconnotine (23), annotine (24), lycodine (25), serratinine (26), luciduline (27), cernuine (28) and alopecurine (29) (R = CO C_6H_5).

The polyketide hypothesis

Shortly after the structure of annotinine became known, Conroy proposed a biogenetic scheme to account for the origin of the alkaloids (40). He postulated that they arose by the condensation of two unbranched eight-carbon chains, which were in turn, derived from acetate. According to this hypothesis, the biogenesis of lycopodine proceeds by the sequence shown below (Scheme I). Even though the order of the steps is arbitrary, each one is plausible in terms of the mechanisms of known reactions. Thus, the ring junctions are formed by a condensation between an activated methylene group (acetate methyl) and a carbonyl carbon atom (acetate carboxyl) in the manner of an aldol condensation. A Mannich type reaction between the carbonyl carbon C-13, the methylene group C-4 and ammonia would lead to the intermediate 32, which upon lactam formation with the free carboxyl group gives the



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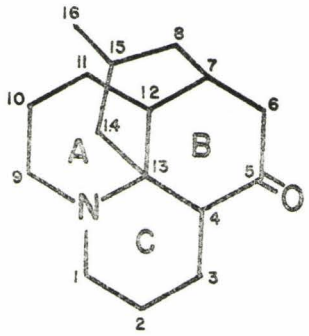

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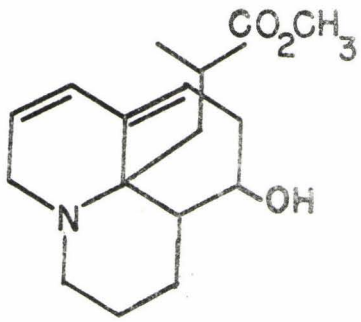

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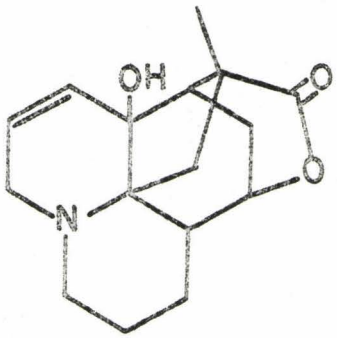

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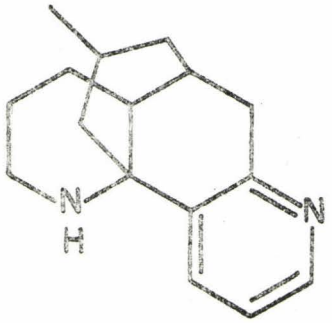

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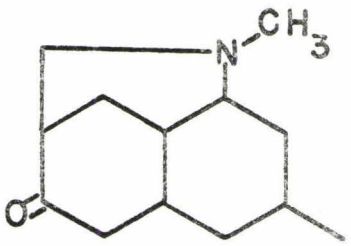

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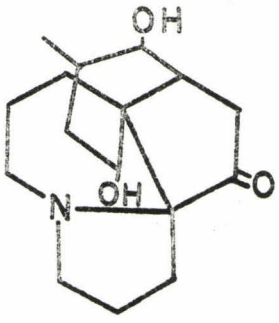

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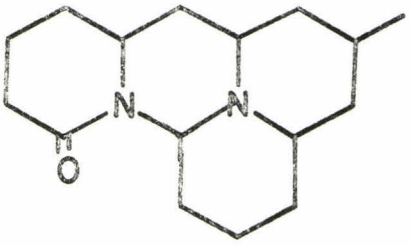

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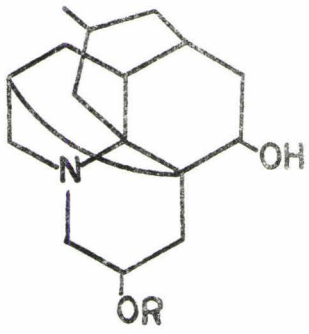

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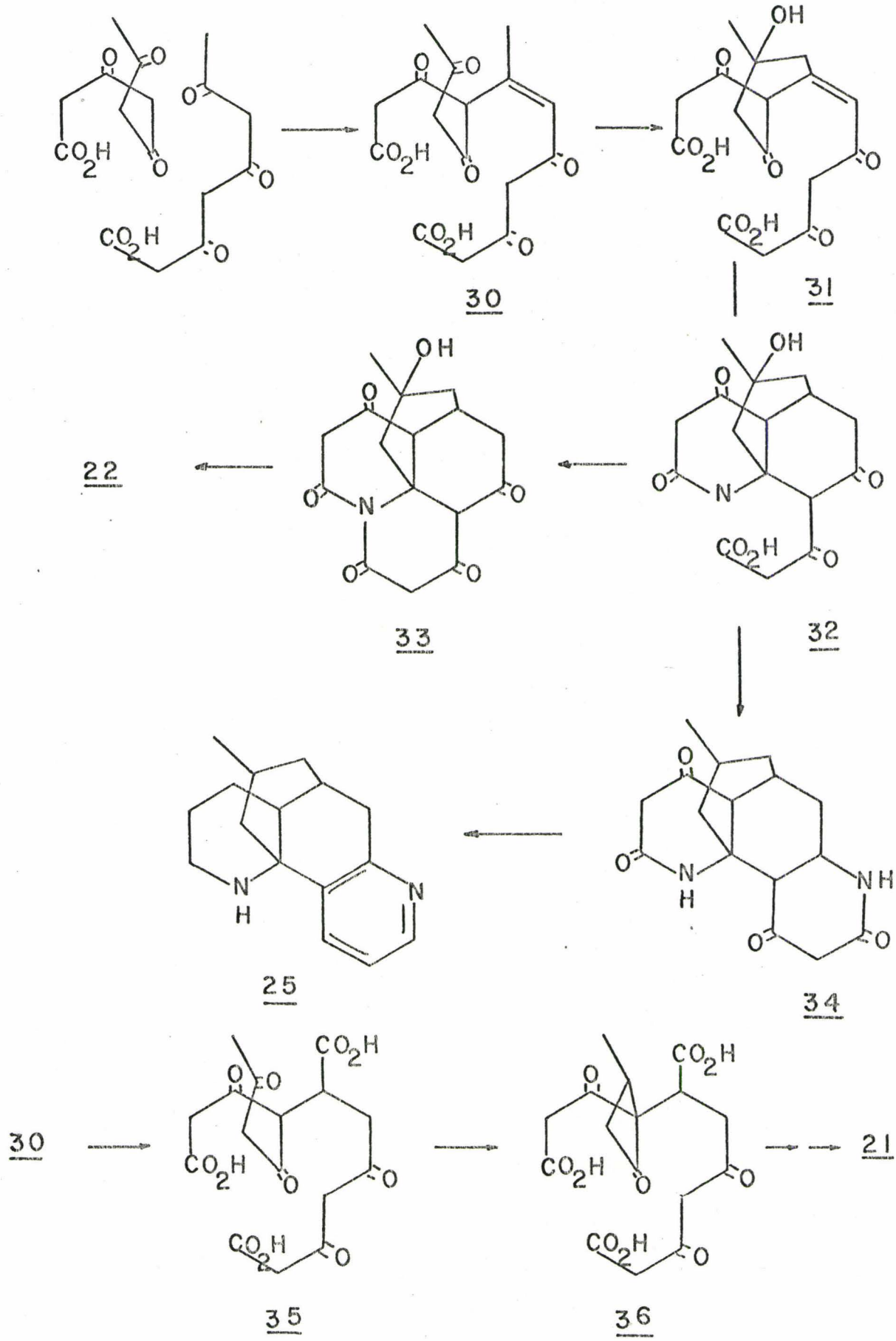
FIGURE 6 : Representative Lycopodium Alkaloids



SCHEME I : Conroy's Polyacetate Hypothesis

immediate precursor 33 of the lycopodine-type alkaloids. The numbering system which is in general use for the Lycopodium alkaloids was originally proposed by Wiesner on the basis of this polyacetate hypothesis (41).

The same hypothetical intermediate 32 can serve as the progenitor of the dinitrogenous alkaloids exemplified by lycodine (25). Insertion of a second nitrogen at C-5, followed by appropriate lactam closure leads to the immediate precursor 34. Conroy's scheme provides for the formation of annotinine by oxidation of the methyl carbon atom C-8, to the level of carboxyl. The cyclobutane ring would then be formed by an alternative aldolization between C-15 and C-12, to give the intermediate 36. Mannich condensation and lactamization (as in 31 → 33) then results in the annotinine ring system. Wiesner suggested as an alternative that annotinine might be derived from an alkaloid with the lycopodine skeleton by cleavage of the C-8–C-15 bond, followed by recyclization of C-15 at C-12 (41). This suggestion implies that lycopodine might be the central intermediate in the biosynthesis. This view is supported by the fact that lyconnotine (23) and annotine (24), which are found in L. annotinum together with annotinine, could also be formed in the same way, whereas their formation by direct condensation of two polyacetyl chains is not feasible.

Conroy's hypothesis was proposed at a time when the structure of only a few Lycopodium alkaloids was known. New structural types which were discovered could also be accommodated by the hypothesis. Conversely, biogenetic considerations based on the polyacetate scheme played an important role in structural studies, notably in the

elucidation of the structures of cernuine and lycocernuine, lycodine, serratinine, fawcettidine and fawcettimine and, recently, luciduline. The usefulness of the hypothesis as a tool in structural studies led to its general acceptance. Ayer and co-workers, for example, arrived at the correct structure of cernuine on the basis of biogenetic considerations, backed by degradations experiments (42). The pyridine derivative 37 was isolated from a dehydrogenation of cernuine with palladium-charcoal. If the C-methyl group of the pyridine nucleus represents the C-methyl of cernuine, and if this, in turn, corresponded to the terminal methyl group of one of the hypothetical eight-carbon chains of Conroy's hypothesis, then a plausible biogenetic scheme for cernuine would be as shown in Figure 7 (42).

A similar and equally fruitful combination of chemical experiments and biogenetic reasoning led to the assignment of structure 27 to luciduline, a novel type of alkaloid isolated from L. lucidulum (43). Selenium dehydrogenation of luciduline gave 2,6-dimethylnaphthalene (38) in good yield. This degradation product accounted for twelve of the thirteen carbon atoms of the intact alkaloid; this finding suggested that luciduline might contain the carbon framework 39, which was readily accounted for in terms of the scheme shown in Figure 8 (43).

The new structural type represented by the serratinine ring system has also been accommodated by the polyacetate scheme. Inubushi et al (44) suggested that serratinine (26) might be formed by a rearrangement of an intermediate with the lycopodine skeleton such as lycodoline (40). The presence of lycodoline in L. serratum and the

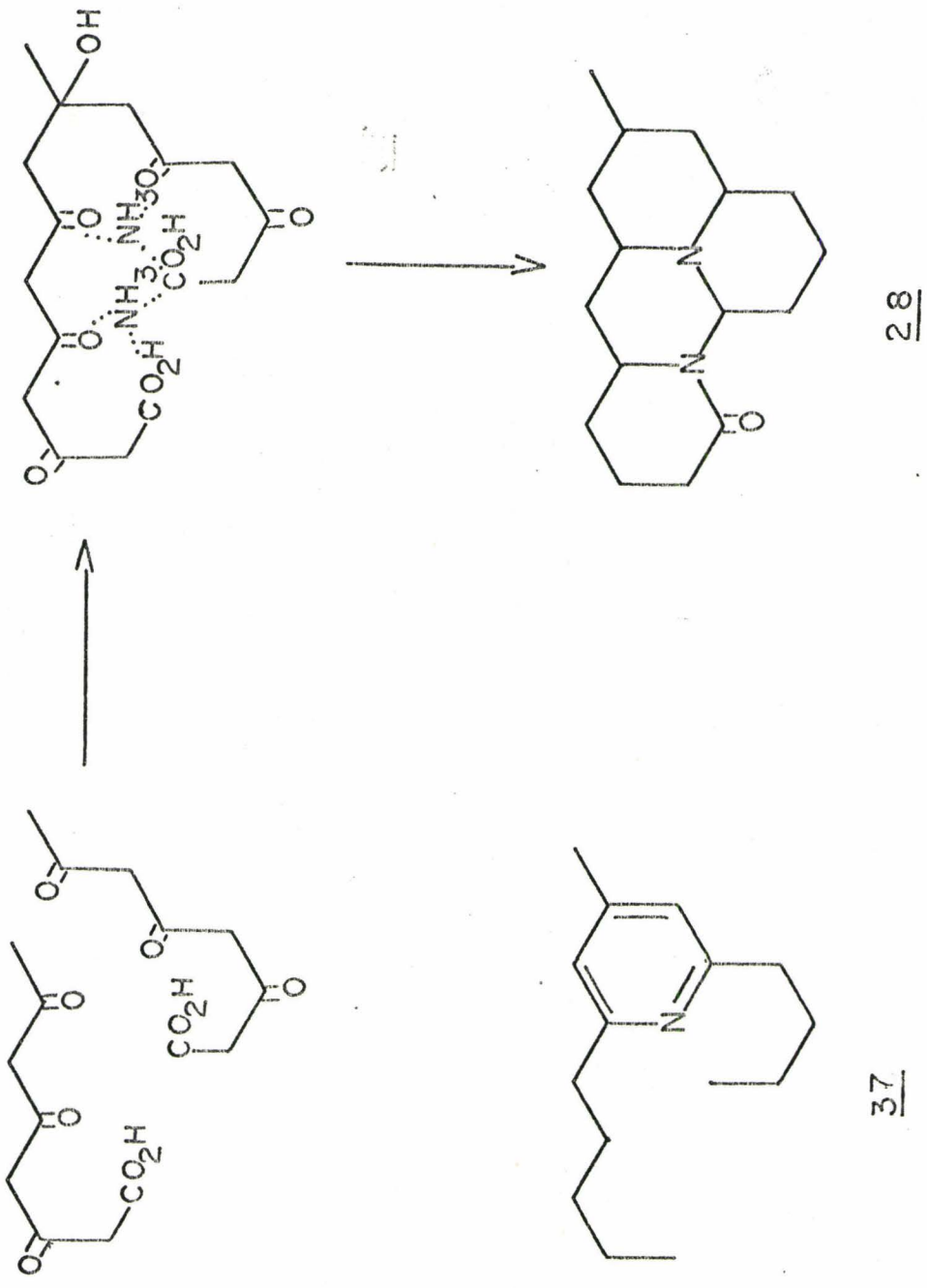


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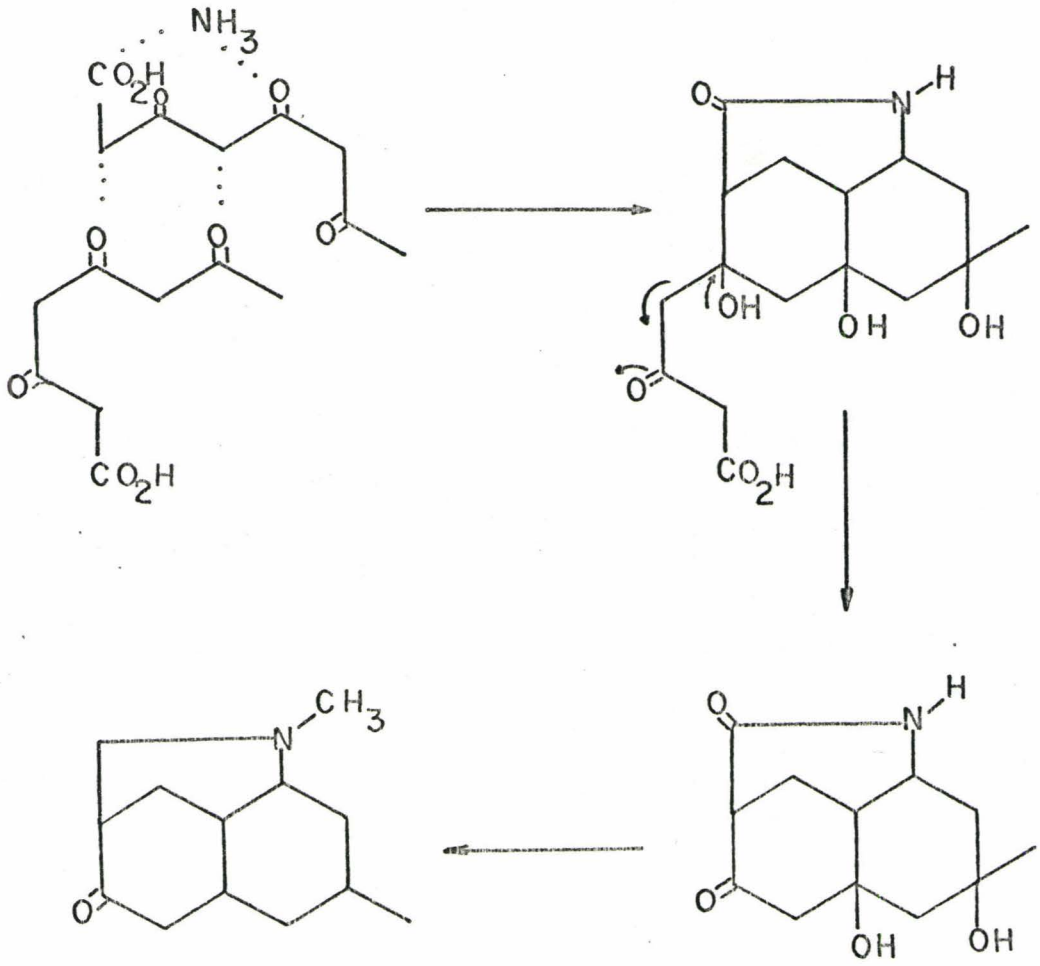
FIGURE 7 : Biogenesis of Cernuine



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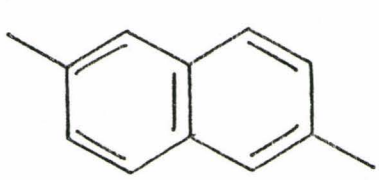

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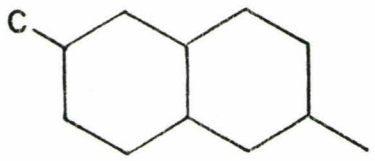

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FIGURE 8 : Biogenesis of Luciduline

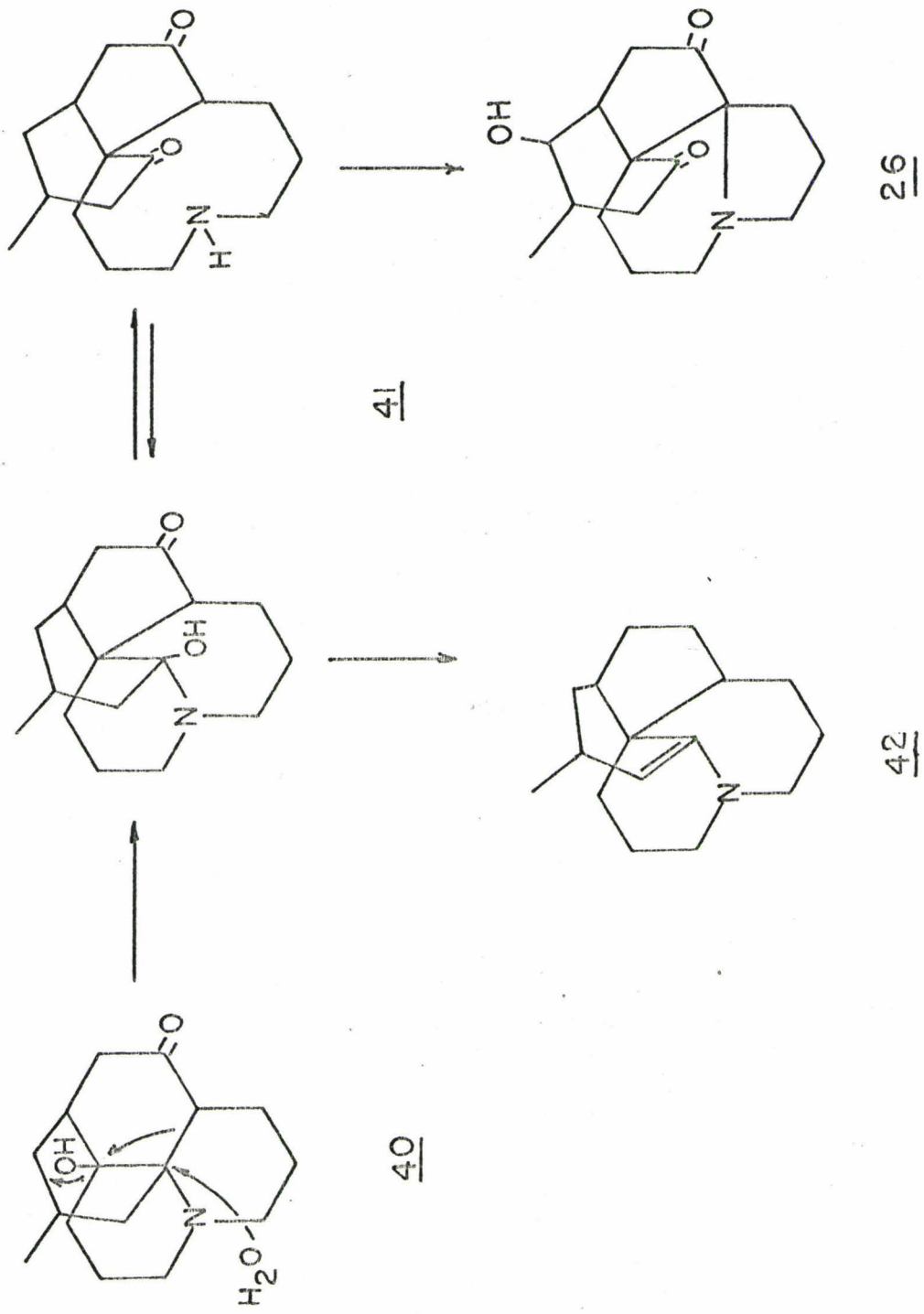


FIGURE 9 : Biogenesis of Serratinine

natural occurrence of the hypothetical intermediates postulated for such a rearrangement (fawcettimine, 41 and fawcettidine 42) (45), lend strong support to the proposed biogenetic scheme (Figure 9).

Experimental evidence in support of Conroy's hypothesis was sought by Leete and Louden (46). Activity from 1-^{14}C-acetate was incorporated into annotinine in L. annotinum. Distribution of activity in the acetic acid isolated by Kuhn-Roth oxidation of annotinine was not compatible with the Conroy scheme.

Pelletierine hypothesis

A new hypothesis concerning the biogenesis of these alkaloids was put forward at a time when no experimental data had yet been advanced in connection with this problem (47).

According to this hypothesis the $C_{16}N_2$ series of Lycopodium alkaloids, e.g., lycodine, would arise by condensation of two pelletierine units. The novelty of this proposal resided in the biogenetic derivation of the $C_{16}N$ ring system of lycopodine and the lycopodine-type alkaloids by rearrangement of an intermediate with a $C_{16}N_2$ carbon skeleton. Thus, it was postulated that the biogenetic origin of lycopodine took place by C-N cleavage of an $C_{16}N_2$ intermediate, e.g., 43, (Figure 10) followed by loss of nitrogen and recyclization. This hypothesis relates the Lycopodium alkaloids biogenetically to the dimeric Lobelia alkaloids, e.g, lobinaline (6), whose derivation from phenacylpiperidine, the phenyl analog of pelletierine has been shown in Lobelia cardinalis (48).

Pelletierine has been reported to occur in Sedum acre (49), in Punica granatum (49) and in Withania somnifera (50), but has not been

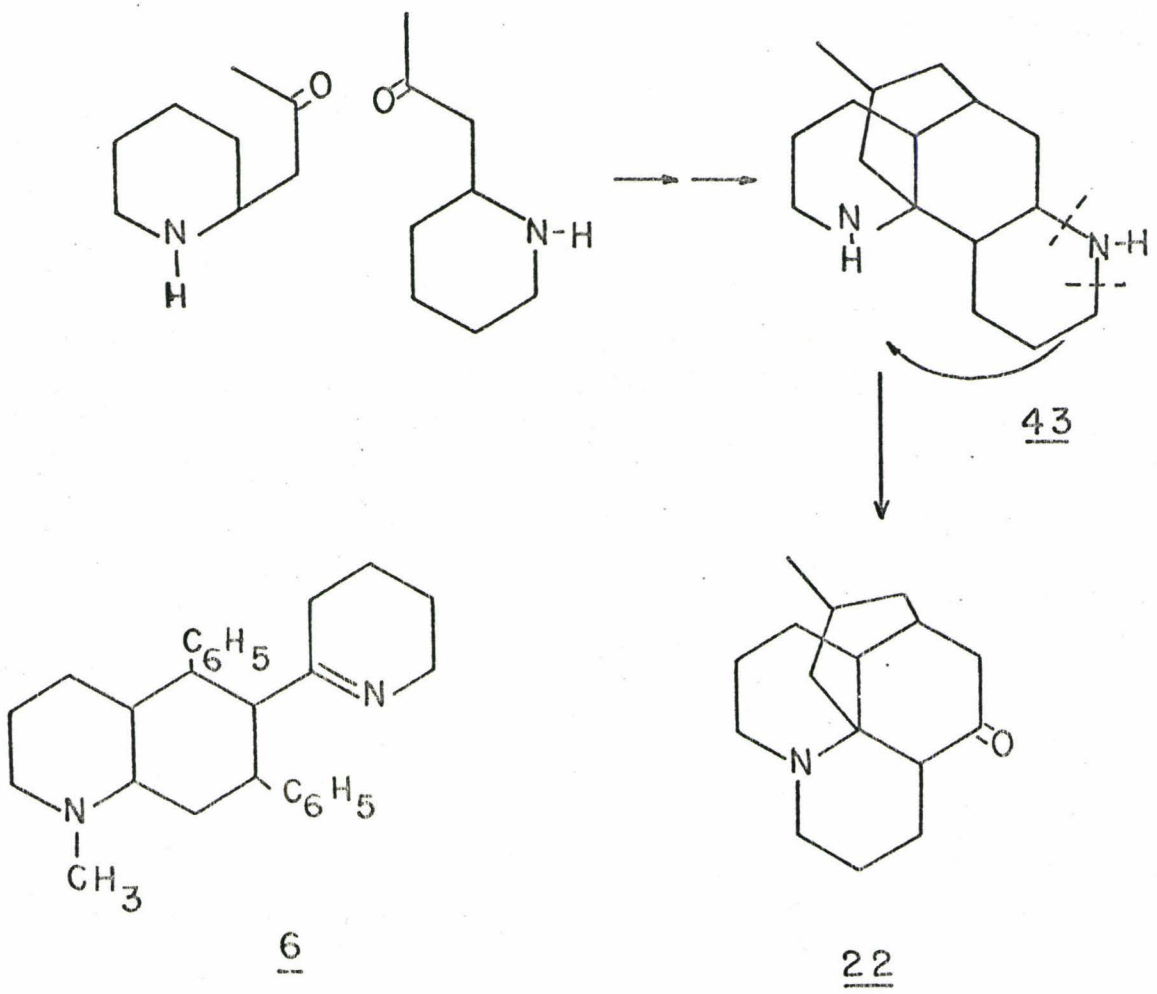


FIGURE 10 : Biogenesis of Lycopodine. Pelletierine Hypothesis

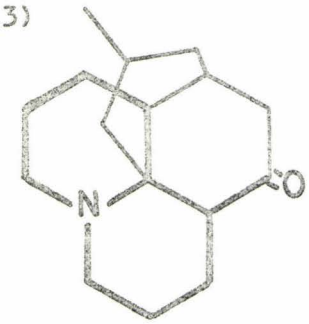
observed in any of the Lycopodium species examined so far. Although, as noted before, it is desirable to demonstrate the occurrence of every postulated intermediate of a biosynthetic sequence in the system under study; failure to find evidence of the presence of such intermediates may be due to a rapid turnover of these metabolic entities and/or their transformation to apparently unrelated products. An analogous case of an alkaloid which occurs in one plant species and serves as the precursor of a more complex alkaloid in another plant species in which its presence has not been demonstrated, is that of hygrine, found in

Nicandra physaloides, which serves as the precursor of hyoscyamine in Datura stramonium (51). Hygrine has not yet been detected in Datura species (52).

The pelletierine hypothesis rationalizes equally well the biogenesis of other Lycopodium alkaloids. Thus, the origin of annotinine, serratinine and, notably cernuine and luciduline, can be easily accommodated by this biogenetic scheme. In fact, considering the Lycopodium alkaloids of known structure, the polyketide hypothesis and the pelletierine hypothesis cannot be distinguished on the basis of structure alone. Application of the tracer method, as we shall presently show, differentiates between these alternatives.

Other biogenetic hypotheses

Some other hypothetical precursors have been postulated in connection with the biogenesis of the Lycopodium alkaloids (41,53). Of these we shall only mention the suggestion made by Wiesner that lysine might be involved in the biogenetic route since lycopodine contains the lupinine skeleton (41), whose derivation from lysine has already been discussed (cf. p. 13)

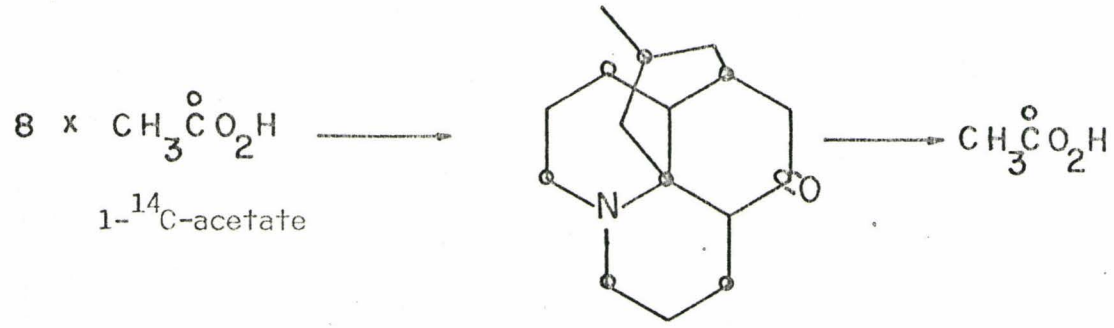


It was the objective of this work to study the biosynthesis of the Lycopodium alkaloids and in particular, to test the validity of the two major biogenetic hypotheses.

II. BIOSYNTHESIS OF LYCOPODINE

Clearly, the study of the biosynthesis of the Lycopodium alkaloids in general and of lycopodine in particular, required as an initial stage, a study of the incorporation of acetate and lysine into the alkaloids. Incorporation of ^{14}C-labelled acetate and determination of the labelling pattern in the resulting alkaloid was crucial in order to differentiate between the polyketide and the pelletierine hypothesis.

According to the polyketide hypothesis, acetic acid, obtained by Kuhn-Roth degradation of lycopodine, representing C-15,16 of the alkaloid, should carry 1/8 (12.5%) of the activity present in lycopodine, regardless of whether 1-^{14}C-acetate of 2-^{14}C-acetate had been utilized as precursors.

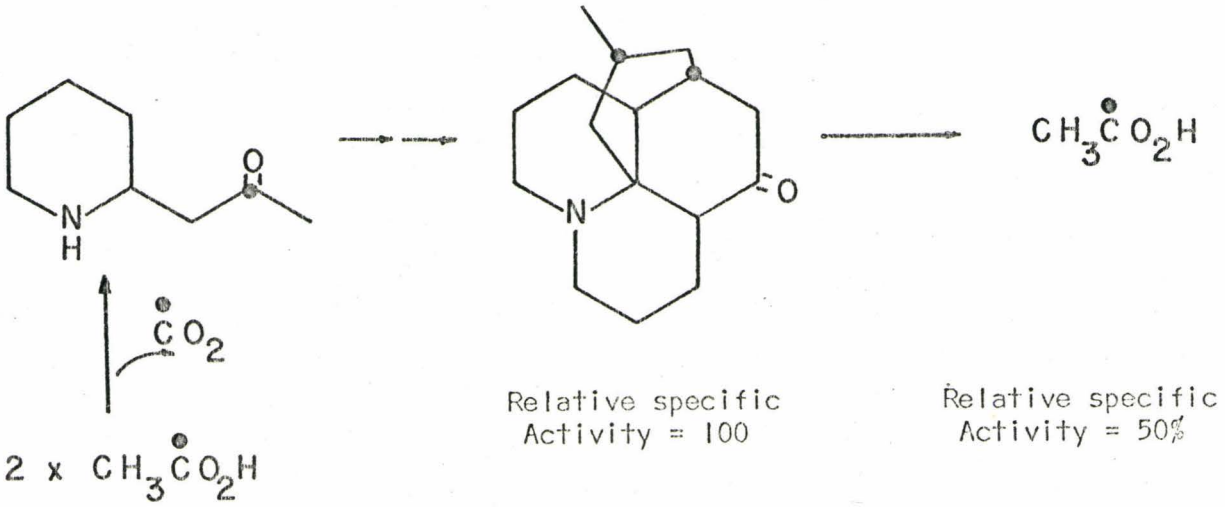


Relative Specific Activity = 100

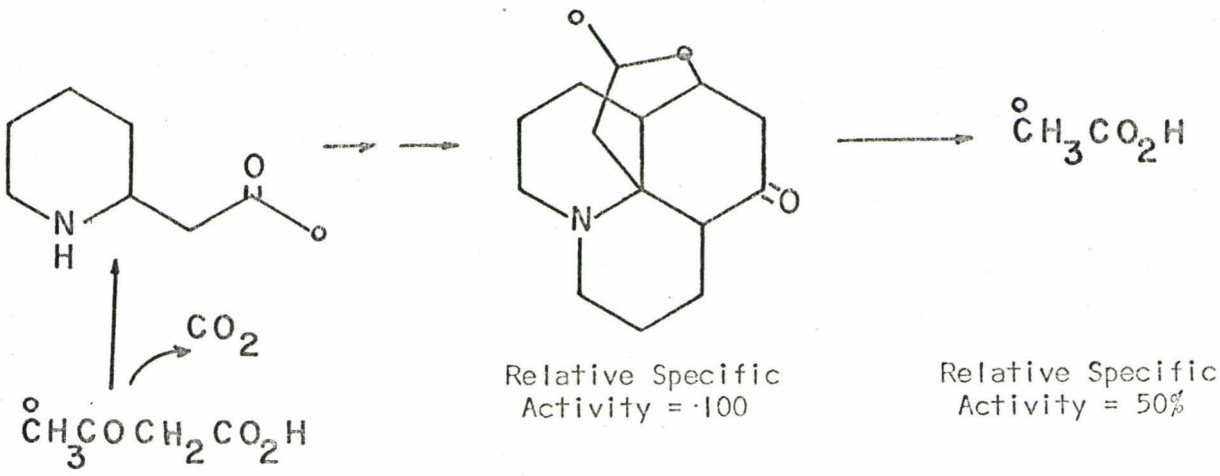
Relative specific Activity = 12.5%

On the other hand, the pelletierine hypothesis predicts an entirely different labelling pattern. If incorporation of acetate into the side-chain of pelletierine had taken place according to classical biogenetic postulates (which were proven to be correct, as discussed

earlier (page 10), and then into lycopodine by condensation of two such pelletierine units, this would result in activity from 1-^{14}C-acetate equally distributed between C-15 and C-7 of lycopodine, whereas 2-^{14}C-acetate would share its label between C-6,8,14 and 16.



A set of additional experiments with acetoacetic acid derivatives would complement the results obtained with acetic acid. The pelletierine hypothesis demands the intact incorporation of two C_3-units derived by decarboxylation of acetoacetate into lycopodine in such a manner that the label from 4-^{14}C-acetoacetate would be equally distributed between C-16 and C-8 whereas, 3-^{14}C-acetoacetate would be expected to share its label between C-15 and C-7 of the alkaloid.



Specific incorporation of lysine, on the other hand, was demanded only by the pelletierine hypothesis. Non incorporation of this precursor, however, would not necessarily mean that the polyketide instead of the pelletierine hypothesis was valid. No definite conclusions can be drawn from such negative results since failure to incorporate radioactivity into a product might be due to permeability factors, breakdown of the administered precursor before reaching the site of synthesis or lack of biosynthesis of the alkaloid in the plant at the time chosen for the experiment. The expected distribution of activity in lycopodine, after administration of radioactive lysine, would depend on the mode of incorporation of this precursor into pelletierine (vide infra). Nevertheless, it was anticipated that the carbon atoms, C-5 and C-9 of lycopodine would harbour at least a fraction of the activity, if 2-^{14}C-lysine and 6-^{14}C-lysine, respectively, were to be incorporated.

Lycopodium tristachyum was chosen for the biosynthetic experiments carried out in the present work. This species, found in Ontario, contains lycopodine as the major alkaloid, which can be easily separated from other minor bases. The other alkaloids present in L. tristachyum are lycodine, anhydrodihydrolycopodine, L 15 ($C_{20}H_{31}NO$), L 13 ($C_{16}H_{25}NO$) and nicotine (54).

Incorporation of a number of labelled precursors into lycopodine was tested. The feeding experiments were carried out by the wick-method and also with fresh cuttings. Eleven feeding experiments were performed and radioactive lycopodine was isolated in each case. Table 1 summarizes all the pertinent information. Radiochemical yields, as percent of

total activity recovered in lycopodine, are listed in Table 1a. The results obtained showed that of all compounds tested, pelletierine was by far the most efficient precursor of lycopodine. Thus, from Experiment II, approximately 0.25% of the total radioactivity fed to L. tristachyum was recovered in the isolated lycopodine, whereas this recovery dropped to 0.03 and 0.003% when 6-^{14}C-lysine and 1-^{14}C-acetate (Experiments 5 and 1 respectively) were tested as precursors. Acetate was incorporated with a lower efficiency than lysine. It should also be noted that better incorporation of radioactivity was obtained by using cuttings than with wick feedings.

The degradations of the radioactive samples of lycopodine which were carried out in order to isolate individual carbon atoms or smaller fragments of the alkaloids are outlined below. A detailed discussion of the chemistry of these degradative reactions is presented in Chapter III.

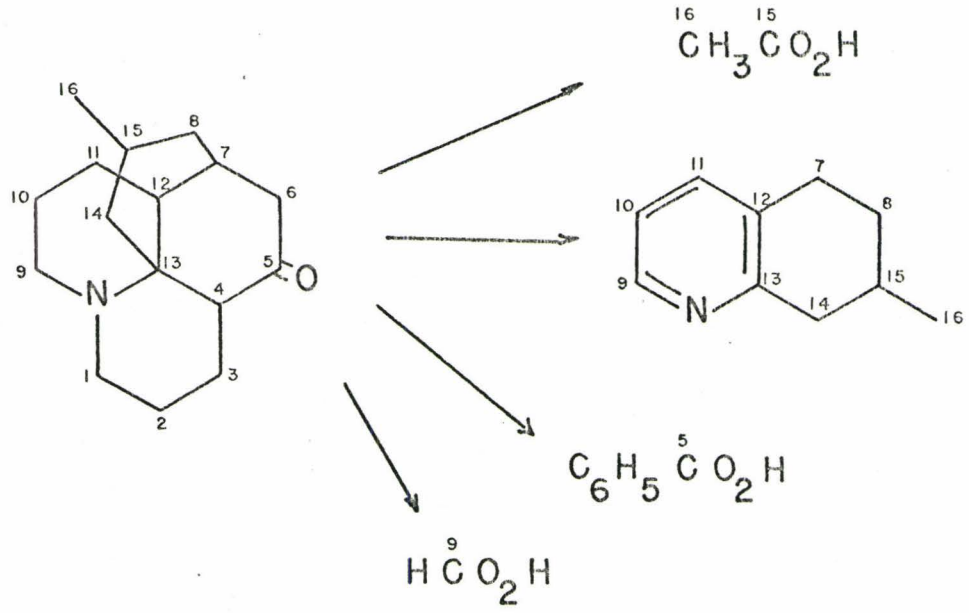


TABLE I

Expt.No.	Compound Administered	Nominal Specific Activity mCi/mmole	mode of Administration	Date	Weight of Dry Plant (g)	Yield	Lycopodine Specific Activity [f]
1	Sodium 1-^{14}C-Acetate[a]	2.0	wick	July 1966	160	81[e]	6.12 ± 0.11
2	Sodium 2-^{14}C-Acetate[a]	2.0	wick	July 1966	450	350[e]	1.84 ± 0.07
9	Sodium 3-^{14}C-Acetoacetate[ad]	4.2	wick	June 1968	35	95	16.9 ± 0.65
10	Sodium 4-^{14}C-DL-β-Hydroxy-butyrate[b]	8.6	wick	August 1968	95	205	8.61 ± 0.22
3	2-^{14}C-DL-Lysine[a]	3.3	wick	Sept. 1967	300	540[e]	2.88 ± 0.05
7	2-^{14}C-DL-Lysine[a]	3.3	cuttings	Sept. 1967	70	230	13.0 ± 0.29
4	6-^{14}C-DL-Lysine[c]	9.2	wick	Sept. 1967	220	380	0.91 ± 0.03
5.	6-^{14}C-DL-Lysine[c]	9.2	cuttings	Sept. 1967	430 (fresh wt)	350	12.2 ± 0.29 (^{14}C
	4,5-$^{3}H_2$-DL-Lysine[a]	2.9×10^3					
8	2-^{14}C-Pelletierine[d]	1.0	wick	June 1968	55	180	41.4 ± 0.75 (^{14}C
	4,5-$^{3}H_2$-Pelletierine[d]	10	wick				
11	2,3'-$^{14}C_2$-Pelletierine[d]	0.65	wick	August 1968	55	165	180 ± 2.5

a. New England Nuclear Corporation

b. Radiochemical Centre

c. Centre Energie Atomique, France

d. See Experimental

e. isolated by column chromatography (see experimental)

f. counts $min.^{-1}$ $mmole^{-1}$) $\times 10^{-3}$

TABLE Ia

Expt. No.	Compound Administered	Nominal Total Activity mCi	Lycopodine Specific Activity (counts/min/mmole) $\times 10^{-3}$	Lycopodine Total Activity* mCi $\times 10^{6}$	Recovery* (%)
1	Sodium 1-^{14}C-Acetate	0.1	6.12	3.0	0.003
2	Sodium 2-^{14}C-Acetate	0.1	1.84	4.0	0.004
9	Sodium 3-^{14}C-Aceto-Acetate	0.1	16.9	10	0.01
10	Sodium 4-^{14}C-DL-β-Hydroxybutyrate[b]	0.1	8.61	12.5	0.01
3	2-^{14}C-DL-Lysine	0.1	2.88	9.4	0.01
7	2-^{14}C-DL-Lysine	0.1	13.0	1.8	0.002
4	6-^{14}C-DL-Lysine	0.1	0.91	2.0	0.002
5	6-^{14}C-DL-Lysine	0.1	12.2	26	0.03
8	2-^{14}C-Pelletierine	0.065	41.4	45	0.08
11	2,3'-$^{14}C_2$-Pelletierine	0.072	180	200	0.25

* See Appendix A

Distribution of label in lycopodine obtained from polyketide precursors

Table 2 shows the results obtained in the degradation of radioactive lycopodine isolated after administration of acetate and acetate-related precursors. The recovery of 47% of the activity of lycopodine derived from 1-^{14}C acetate, and 21% of that derived from 2-^{14}C-acetate (experiments 1 and 2 respectively), in the fragment C-15,16, were in complete disagreement with the values predicted by the polyketide hypothesis; the experimental values not only differed from the expected figures but they differed from each other. These results demonstrated that the polyketide hypothesis is not valid. Acetic acid is indeed a specific precursor of lycopodine, but it is not incorporated by way of two tetraacetyl chains, as predicted by Conroy's hypothesis.

The labelling pattern obtained in these experiments is consistent with the pelletierine hypothesis. According to its predictions, C-15 should carry 50% of the activity present in lycopodine after administration of 1-^{14}C-acetate, and C-16 should carry 25% of the activity in the alkaloid isolated from the 2-^{14}C-acetate experiment.

The results obtained from the experiments with acetoacetic acid derivatives are also shown in Table 2. It can be seen that label from 3-^{14}C-acetoacetate was incorporated as expected (50% in acetic acid) but no definitive conclusions regarding the intact incorporation of acetoacetate can be drawn from this experiment since this labelling pattern cannot be distinguished from that of 1-^{14}C-acetate. On the other hand, the results shown for incorporation of 4-^{14}C-β-hydroxybutyrate indicate that the C_3-unit is not incorporated intact but by way of discrete

TABLE 2

INCORPORATION OF POLYKETIDE PRECURSORS INTO LYCOPODINE

PRECURSOR:	1-^{14}C-Acetate		2-^{14}C-Acetate		3-^{14}C-Aceto-Acetate		4-^{14}C-β-Hydroxy-butyrate	
EXPT. No.	1		2		9		10	
PRODUCT :	SA[a]	RSA[b]	SA	RSA	SA	RSA	SA	RSA
Lycopodine	6.12±0.11	100±2	1.84±0.07	100±4	8.89±0.34[c]	100±4	8.61±0.22	100±3
Acetic acid (as α-naphthyl amide (C-15, 16)	2.89±0.10	47±2	0.39±0.02	21±1	4.56±0.14	51±3	1.75±0.06	20±1
PREDICTED RSA[b] of Acetic Acid on the basis of:								
Polyketide hypothesis		12.5		12.5				
Pelletierine hypothesis		50		25				
Intact incorporation of C_3 unit						50		50

a. Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-3}

b. Relative specific activity:percent (lycopodine = 100)

c. Obtained by recrystallizing a mixture of 66 mg lycopodine, specific activity (16.89 ± 0.65) x 10^{3} counts min^{-1} $mmole^{-1}$, and 50 mg carrier lycopodine

acetate units. (A feeding experiment with 4-^{14}C-acetoacetate itself failed to yield radioactive lycopodine.) The breakdown of acetoacetate, when administered to plants, yielding two equivalents of acetic acid correspondingly labelled, might be due to biological degradation of this metabolite before reaching the site of synthesis of the alkaloid. The existence of a rapid equilibrium between acetoacetyl-CoA and acetyl-CoA, in favour of acetyl-CoA, was mentioned before (page 17). Similar results have been reported elsewhere (15).

Distribution of label in lycopodine obtained from lysine

The radioactive samples of lycopodine obtained from Experiments 3, 4, 5, and 7 (see Table 3) were degraded by the series of reactions described earlier.

C-5 and C-9 were isolated individually. It was found that each of these carbon atoms carried one quarter (25%) of the activity present in the intact alkaloid, regardless of whether 2-^{14}C-lysine (Experiments 3 and 7) or 6-^{14}C-lysine (Experiments 4 and 5) had been tested as precursors. Such specific incorporation of activity from lysine is consistent with the pelletierine hypothesis, provided it is assumed that lysine is incorporated into the piperidine nucleus of pelletierine in a symmetrical manner.

If this assumption is made, the remaining activity would be expected to be distributed between C-1 and C-13, but these carbon atoms were not isolated individually. However, C-13 together with C-9 is contained in the fraction of the molecule represented by 7-methyl-5,6,7,8-tetrahydroquinoline. It was found that this degradation product carried one half of the activity in the alkaloid isolated from

TABLE 3

INCORPORATION OF LYSINE INTO LYCOPODINE

PRECURSOR :	2-^{14}C-DL-Lysine				6-^{14}C-DL-Lysine					
EXPT. No. :	3		7				4		5	
PRODUCT	SA[a]	RSA[b]	SA	RSA	SA	RSA	SA	RSA	SA	RSA
Lycopodine	2.88±0.05	100±2	13.01±0.29	100±2	2.87±0.08[c]	100±3	0.91±0.03	100±3	12.20±0.29	100±2
Benzoic acid (C-5)	0.69±0.02	24±1	3.34±0.07	26±1	-	-	-	-	3.08±0.09	25±1
Formic Acid (as α-naphthyl-amid) (C-9)	-	-	-	-	0.63±0.02	22±1	0.23±0.01	25±1	-	-
7-Methyltetra-hydroquinoline perchlorate (C-7 to 16)	1.35±0.08	47±3	-	-	-	-	-	-	-	-

a Specific activity (counts min^{-1} $mmole^{-1}$) $\times 10^{-3}$

b Relative specific activity : percent (lycopodine = 100)

c Obtained by recrystallizing a mixture of 56 mg lycopodine, specific activity (13.01 ± 0.29) $\times 10^{3}$ counts min^{-1} $mmole^{-1}$, and 197 mg carrier lycopodine

Experiment 3 (2-^{14}C-lysine). Since the individual isolation of C-5 and C-9 showed that activity from lysine had entered lycopodine in a non-random manner, and since half of the activity in this quinoline derivative must be at C-9, it is very likely that the other half of its activity is indeed located at C-13.

Visual inspection of the lycopodine carbon skeleton, coupled with the incorporation data obtained so far, reveals only one possible arrangement of the two five-carbon-chains derived from lysine: one of these lysine fragments is accounted for in the carbon atoms C-9,10,11,12 and 13, while the other lysine unit is present in the chain from C-1 through to C-5. Since C-5 has been shown to carry 25% of the total activity present in lycopodine, it can be inferred that C-1 is the most likely site to contain the remaining, unaccounted activity. If this assignment is correct, it can be concluded that each of the carbon atoms C-5, C-9, C-13 and C-1 carried one quarter of the total activity in the alkaloid, derived from 2-^{14}C- or from 6-^{14}C-lysine.

Interpretation of the incorporation data in terms of the pelletierine hypothesis

Three general conclusions can be drawn from the labelling pattern obtained in these lysine experiments:

i) a symmetrical intermediate lies in the pathway of incorporation of lysine into lycopodine.

ii) two units derived from lysine are utilized in the biosynthesis of lycopodine.

iii) these two lysine fragments are incorporated with equal efficiency into the two segments of the alkaloid.

These conclusions are clearly derived from the labelling pattern obtained, i.e., the recovery of one quarter of the total activity present in lycopodine in each of the carbon atoms mentioned above. Incorporation of lysine by way of a non-symmetrical intermediate would have been accompanied by an entirely different labelling pattern. Activity from 2-^{14}C-lysine would have been confined to C-5 and C-13, whereas activity from 6-^{14}C-lysine would have been distributed between C-9 and C-1 only.

Thus, incorporation of lysine into lycopodine proceeds by a pathway different from the one observed for most of the piperidine alkaloids discussed in Chapter I, but is related, in this respect, to the biosynthesis of the lupin alkaloids. This similarity is also observed in the manner in which these two lysine-derived C_5N units are joined together to yield a $C_{10}N$ structural unit, as found in lycopodine and lupinine.

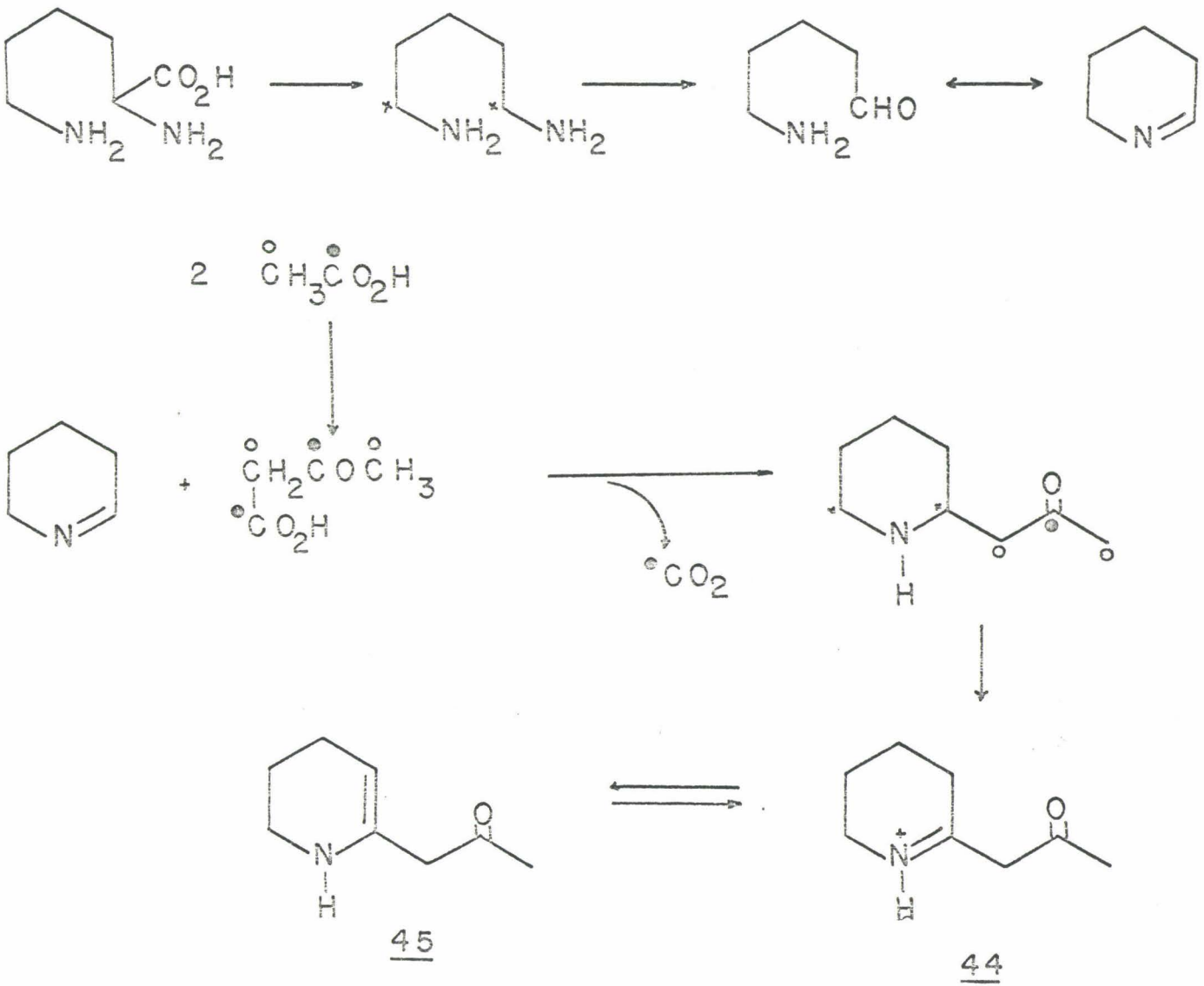
As already mentioned, one of these lysine units makes up the piperidine ring present in lycopodine (ring A). The second lysine-derived fragment generates ring C + C-5. These two segments combined constitute the above mentioned $C_{10}N$ structural unit. One half of the activity in the intact alkaloid was found to be equally distributed between C-5 and C-9. Since these carbon atoms must originate from separate lysine-units, this finding was an indication that these two segments are incorporated with equal efficiency into the final product. This equal labelling of the two "halves" of lycopodine might be interpreted to indicate that the "doubling step" in the biosynthetic sequence involves two identical lysine-derived metabolites. It will be shown later that

such a conclusion might be wrong.

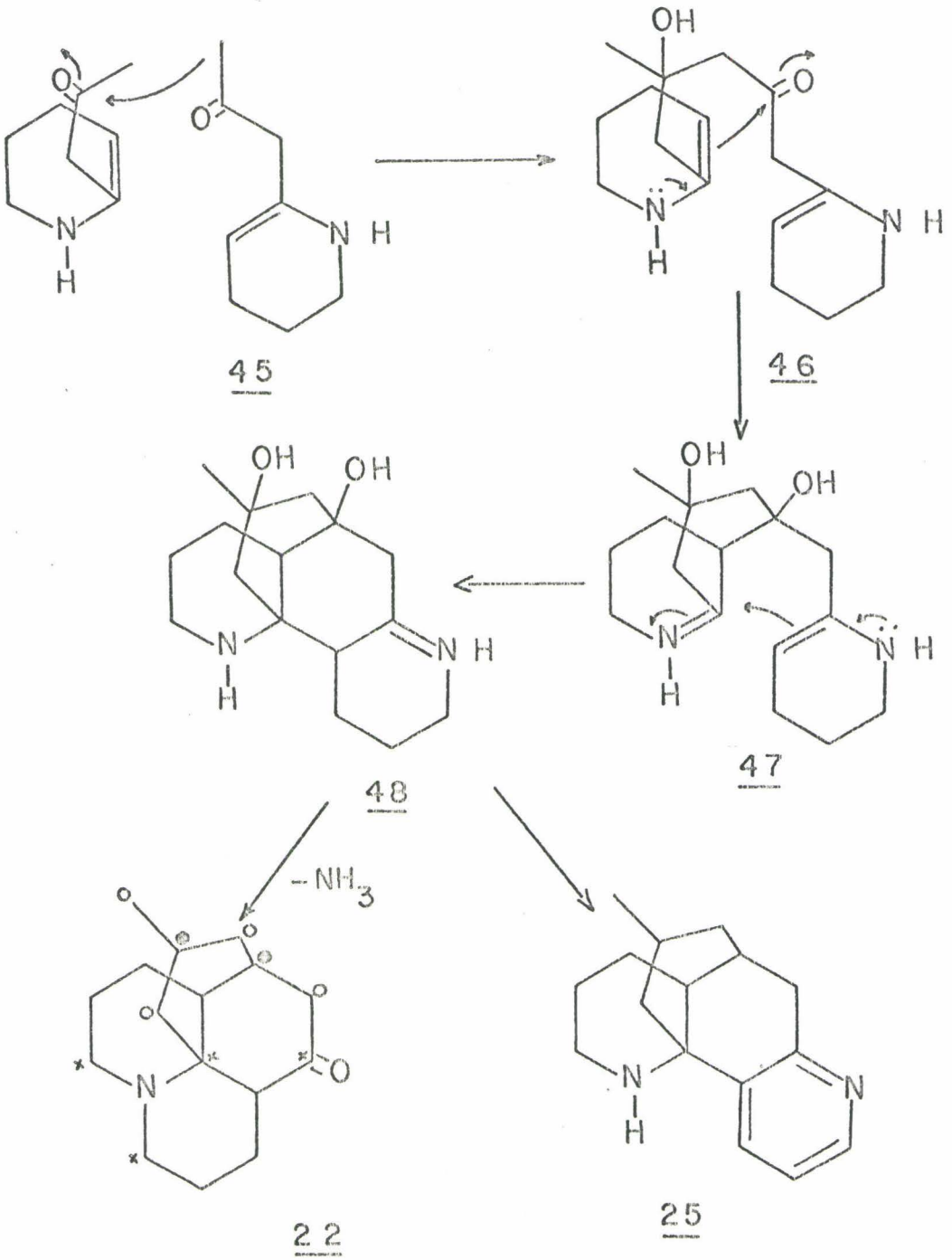
The experimental results discussed so far were entirely consistent with the predictions advanced in the simple model of the pelletierine hypothesis. This experimental evidence allows us to fill some of the gaps in the biosynthetic events and to propose a plausible mechanism for the metabolic sequence. Thus, the self condensation of pelletierine to yield the $C_{16}N_2$ and $C_{16}N$ series of Lycopodium alkaloids would proceed as shown in Scheme 2a and 2b.

The evidence in support of the origin of pelletierine (or rather of N-methylpelletierine), by way of condensation of a non-symmetrical lysine derivative and acetoacetate, has already been discussed. If pelletierine were indeed a precursor of lycopodine it cannot originate in the same manner. A simple modification of the route, interposition of cadaverine between lysine and Δ^1-piperideine (Scheme 2a). in the origin of pelletierine in Lycopodium species, would reconcile the observed results with the pelletierine hypothesis. Accordingly, activity from either 2-^{14}C-lysine or from 6-^{14}C-lysine would be expected to be equally distributed between C-2 and C-6 of the hetero-ring in pelletierine, since cadaverine, derived by decarboxylation of lysine, is a symmetrical molecule. Entry of label from radioactive acetate would be confined to the side-chain of pelletierine, in the manner to be expected from the mechanism of the condensation of lysine and acetoacetate.

These findings pose the question why pelletierine should be produced in two different ways. A plausible reason for this divergency might be found in the different enzymic properties of the systems involved,



SCHEME 2a : Biosynthesis of Lycopodine: Pelletierine Hypothesis



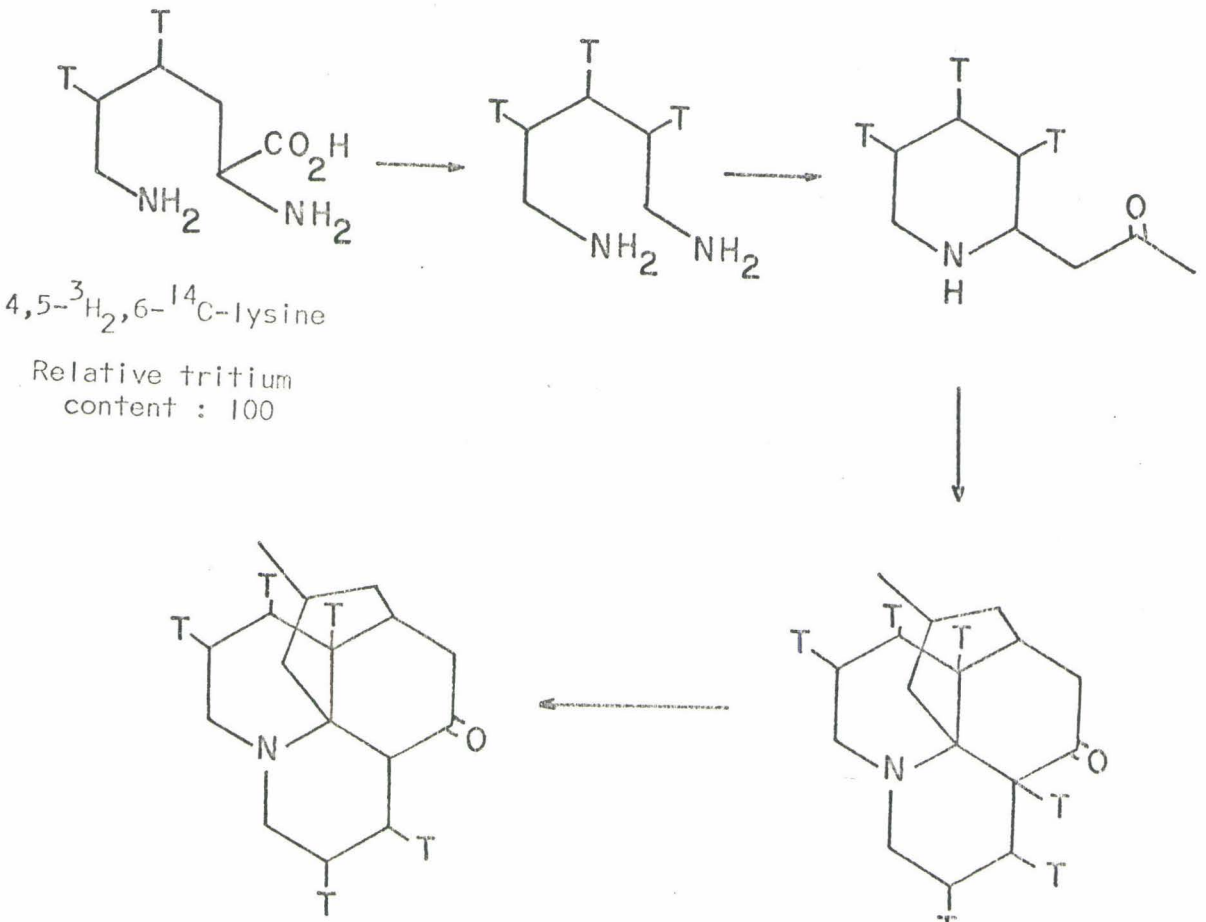
SCHEME 2b : Biosynthesis of Lycopodine: Pelletierine Hypothesis

but this is, of course, tantamount to admitting that an answer to this question is beyond our present knowledge.

Although the order of the steps in the combination of the two pelletierine units is unknown, we prefer the formation of the C_{15}-C_8 bond, as shown in 46, as the initial condensation step. The next step is considered to be preceded by suitable activation of the condensing unit, as shown in the tautomerization between 44 and 45. According to this scheme, lycopodine arises from an intermediate with a ring system as found in lycodine (48), by hydrolytic cleavage of one of the hetero-rings, loss of ammonia and recyclization to the lycopodine ring system. The possible derivation of annotinine (21) from lycopodine has already been mentioned.

The pattern of incorporation into lycopodine of label from acetate and from lysine, demanded by the pelletierine hypothesis, is as shown in 22. Thus, recovery of approximately one half and one quarter of the activity of the intact alkaloid in acetic acid (C-15, 16) is consistent with the postulated incorporation of 1-^{14}C-acetate and 2-^{14}C acetate respectively, by way of the condensation of acetoacetate and a C_5N unit derived from lysine. The other half of the original activity in lycopodine from the 1-^{14}C-acetate experiment is expected to be located, according to this scheme, at C-7. The isolation of this carbon atom, and the ones expected to carry the rest of the activity in the 2-^{14}C-acetate experiment, was not attempted.

One further piece of evidence consistent with this scheme was provided by the observation that the incorporation of tritiated lysine (Experiment 5) into lycopodine was accompanied by a loss of 21.5% of the tritium content relative to ^{14}C. This lowering of the ^{3}H : ^{14}C ratio can be rationalized, in terms of the proposed reaction sequence as follows:

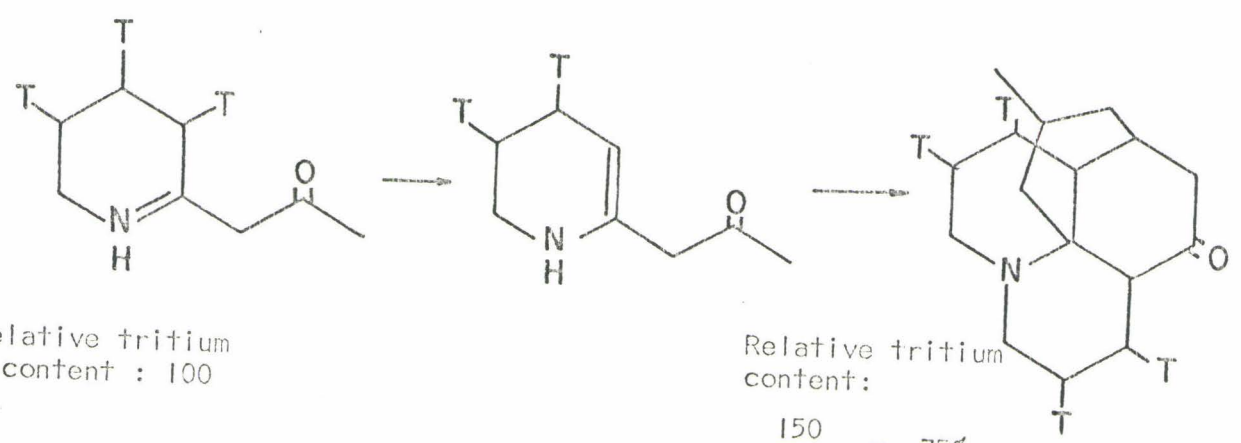


4,5-$^{3}H_2$,6-^{14}C-lysine

Relative tritium content : 100

Relative tritium content :

$\frac{162.5}{200}$ = 81.25%



Relative tritium content : 100

Relative tritium content:

$\frac{150}{200}$ = 75%

FIGURE II : Incorporation of 4,5-$^{3}H_2$,6-^{14}C-Lysine into Lycopodine

The proposed scheme predicts a minimal loss of 18.75% of the tritium present in the precursor. It also predicts a maximal loss of 25% if we assume that all the tritium present at C-3 of pelletierine is lost in the fast equilibrium postulated in the reaction sequence shown in Scheme 2a.

The observed value of 21.5% (Table 5) is in agreement with these predictions. These expected values are approximate since we are assuming non-stereospecific removal of hydrogen in the condensation steps and the absence of isotope effects.

Several requirements had to be met in order to fully support this pelletierine hypothesis. Firstly, the scheme is not valid if pelletierine is not specifically incorporated. Further, two pelletierine-derived units must be incorporated. Also, the hypothesis demands a common biogenetic pathway for both the $C_{16}N_2$ and the $C_{16}N$ series of Lycopodium alkaloids.

Pelletierine as a precursor of Lycopodine

Radioactive lycopodine of high specific activity was obtained from two experiments (Experiments 8 and 11) with labelled pelletierine*. The radioactive lycopodine obtained from the feeding experiment with 4,5-$^{3}H_2$, 2-^{14}C-pelletierine (Experiment 8) was degraded to yield benzoic acid representing the carbonyl carbon of lycopodine. According to the postulated scheme benzoic acid should carry one half of the ^{14}C-activity in the intact alkaloid. Instead, only 1% of the original activity was found at this position (Table 4). In the same experiment, the ^{3}H : ^{14}C ratio in lycopodine was found to be identical with the ^{3}H : ^{14}C ratio in the precursor (see Table 5).

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* The synthesis of radioactive samples of this precursor is described in the Experimental Section (page 70).

The preservation of the ^{3}H : ^{14}C ratio in this experiment appears to indicate incorporation of one or two intact pelletierine units into lycopodine. The absence of activity at C-5, however, was an indication that pelletierine was not incorporated into the half of the alkaloid containing this carbon atom.

It remained to obtain conclusive evidence for the incorporation of an intact pelletierine unit into the other half of the molecule.

Evidence that label from pelletierine had indeed been incorporated only into that half of the alkaloid was obtained by degrading the lycopodine to 7-methyl-5,6,7,8-tetrahydroquinoline (49), representing C-7 to C-16 of the intact alkaloid. It was found that this degradation product carried all the activity in the starting material (Table 4). Although these results indicated the incorporation of only one pelletierine unit into lycopodine, further proof of the intact incorporation of the precursor was required since label from pelletierine might have gone into lycopodine indirectly.

In order to clarify this point, pelletierine doubly labelled with ^{14}C (Table 4) was tested as precursor (Experiment II). Preservation of the isotopic distribution of this doubly labelled precursor in the product would not only prove the specific and intact incorporation of a single pelletierine unit into lycopodine but it would also show, unambiguously, the position of this structural unit in the lycopodine ring system (cf. page 66).

Accordingly, the radioactive alkaloid from this feeding experiment was degraded to 7-methyl-5,6,7,8-tetrahydroquinoline and, in a separate degradation, to acetic acid. It was found that all the

activity of lycopodine was retained in the quinoline derivative (C-7 to C-16). Acetic acid, representing C-15, 16 of lycopodine carried 18% of the activity present in the intact alkaloid (Table 4). Since the distribution of label in 2,3-$^{14}C_2$-pelletierine had been found to be as shown in Table 6 (e.g., 80% in C-2 of the hetero-ring and 18% in C'-3 of the side chain), these results showed conclusively the specific incorporation of one intact pelletierine unit in the manner shown (Figure 12).

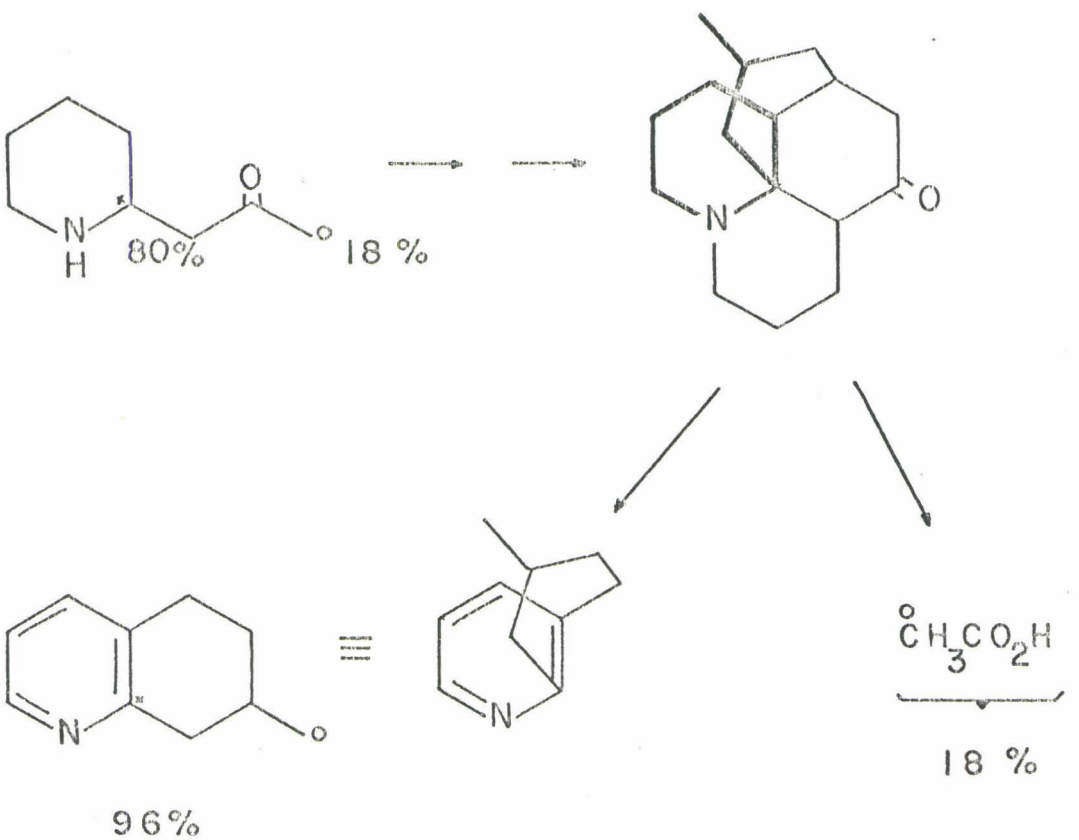


FIGURE 12 : Specific Incorporation of Pelletierine into Lycopodine

TABLE 4

INCORPORATION OF PELLETIERINE INTO LYCOPODINE

PRECURSOR :	2-^{14}C-Pelletierine				2,3'-$^{14}C_2$-Pelletierine			
EXPT. No. :	8				11			
PRODUCT :	SA[a]	RSA[b]	SA	RSA	SA	RSA	SA	RSA
Lycopodine	41.39±0.75	100±2	6.32±0.15[c]	100±2	25.95±0.47[d]	100±2	7.15±0.10[d]	100±1
Benzoic acid (C-5)	0.51±0.05	1±0.1	-	-	-	-	-	-
Acetic acid (as α-naphthyl amid (C-15, 16)	-	-	-	-	4.74±0.06	18±0.4	-	-
7-Methyltetra-hydroquinoline Hydrochloride (C-7 to 16)	-	-	5.92±0.23	94±4	-	-	6.88±0.19	96±3

a Specific acitivity (counts min^{-1} $mmole^{-1}$) $\times 10^{-3}$

b Relative specific activity : percent (lycopodine = 100)

c obtained by recrystallizing a mixture of 35 mg lycopodine, specific activity (41.39±0.75) $\times 10^3$ counts min^{-1} $mmole^{-1}$, and 190 mg carrier lycopodine

d obtained from the original lycopodine (specific activity (1.80 ± 0.025) $\times 10^5$ counts min^{-1} $mmole^{-1}$) of Experiment No.10 by dilution with carrier lycopodine

TABLE 5

INCORPORATION OF $^{3}H, ^{14}C$-LABELLED PRECURSORS

EXPT. No.	PRECURSOR	$^{3}H, ^{14}C$ RATIO IN PRECURSOR	$^{3}H, ^{14}C$ RATIO IN LYCOPODINE
5	4,5-$^{3}H_2$,6-^{14}C-DL-Lysine	10.7 ± 0.2	8.4 ± 0.1
8	4,5-$^{3}H_2$,2-^{14}C-Pelletierine	9.5 ± 0.1	9.6 ± 0.1

TABLE 6

INCORPORATION OF 2,3'-$^{14}C_2$-PELLETIERINE INTO LYCOPODINE

DISTRIBUTION OF LABEL IN

2,3'-$^{14}C_2$-PELLETIERINE			LYCOPODINE		
	SA[a]	RSA[b]		SA[a]	RSA[b]
Pelletierine	59.75±0.98[c]	100±2	Lycopodine	25.95±0.47	100±2
Acetic Acid (as α-naphthylamide) (C-2',3')	10.88±0.10	18±0.3	Acetic acid (as α-naphthylamide) (C-15, 16)	4.74±0.06	18±0.4
Pipecolic Acid (nucleus, C-1')	47.24±0.84	79±2			

a Specific activity (counts min^{-1} $mmole^{-1}$) $\times 10^{-3}$

b Relative specific activity : percent

c obtained by mixing a small sample (total activity 0.2 μC) of the original 2,3'-$^{14}C_2$-pelletierine hydrochloride used in Experiment II with inactive pelletierine hydrochloride (350 mg), and recrystallizing the mixture to constant activity

Clearly, even though the results discussed so far indicate intact incorporation of pelletierine, they do not support the view that lycopodine is a modified dimer of pelletierine. It is necessary, then, to modify the pelletierine hypothesis in order to accommodate these experimental results.

A modified pelletierine hypothesis

The nature of the experimental evidence gathered has narrowed down considerably the number of alternatives. Thus, any plausible biosynthetic scheme has to account for the following experimental findings:

a) the carbon skeleton of lycopodine is not derived from polyacetyl chains,

b) two units derived from lysine supply the two C_5 chains (C-9 to C-13, and C-1 to C-5) which make up the $C_{10}N$ structural unit of lycopodine,

c) the remaining six carbon atoms which complete the $C_{16}N$ carbon skeleton of lycopodine are composed of two C_3 fragments which are, in turn, derived from acetate units,

d) each of these C_3 units is derived from two acetate equivalents, joined head-to-tail, with one terminal carboxyl group being lost from each fragment at some stage of the assembly,

e) a C_5N-C_3 unit or a closely related fragment, is an intermediate in the biosynthetic pathway.

With these considerations in mind, a few alternative possibilities can be discussed.

One of the attractive features of the pelletierine hypothesis was the fact that it accounted not only for the biosynthesis of lycopodine but also for the biosynthesis of a number of Lycopodium alkaloids of

completely different structures (e.g., lycodine, cernuine, etc.). It is tempting to consider alternative schemes which retain as one of their essential points the notion that these alkaloids arise by a condensation of two pelletierine or some very closely related structures. One such equivalent "monomeric unit" might be a compound such as 50, whose origin in terms of a lysine equivalent and acetate fragments, as demanded by the tracer data obtained, might be depicted as shown below. An alternative derivation of 50 might involve a stepwise condensation of Δ^1-piperideine with two malonyl-CoA units.

50

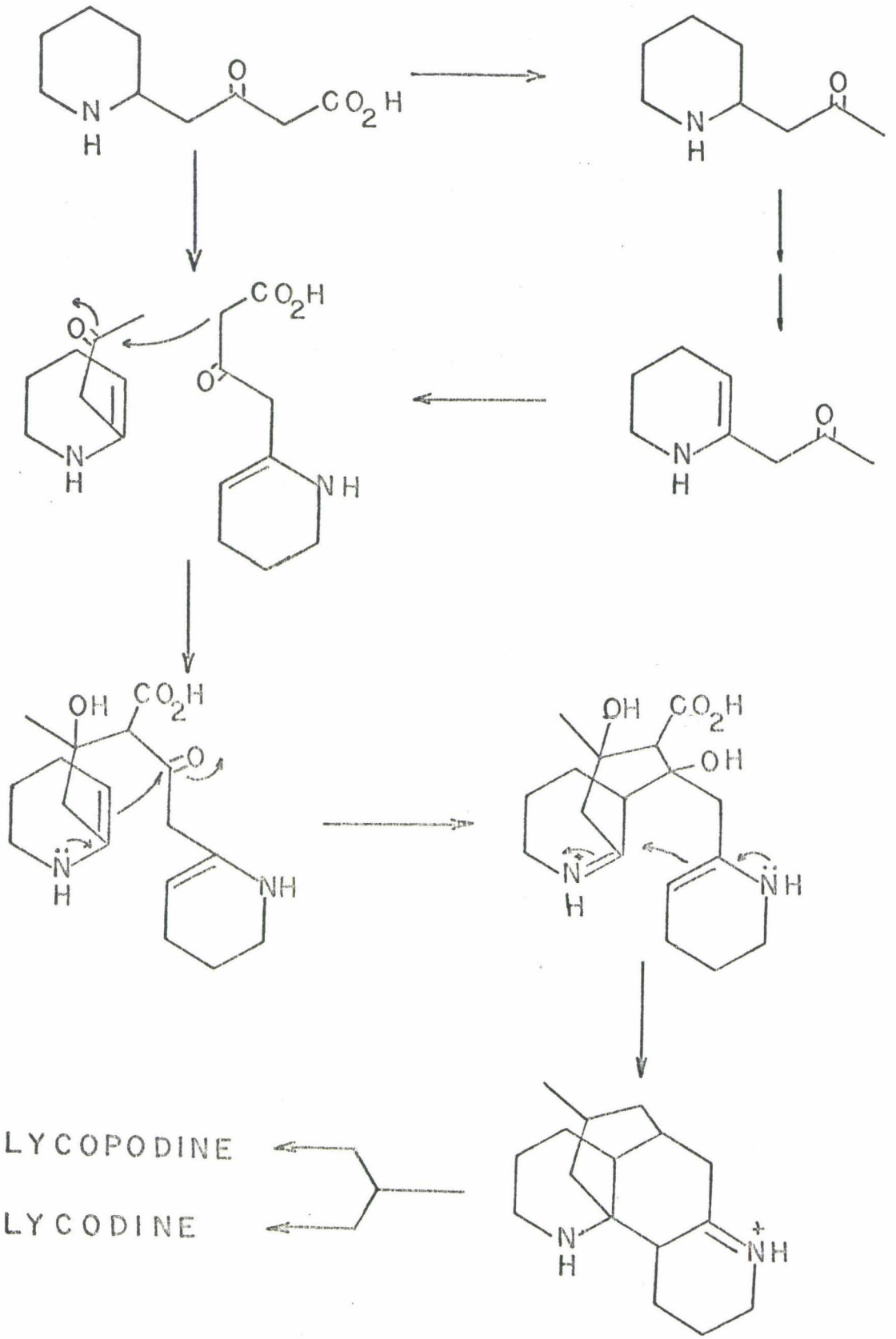
It should be noted that decarboxylation of 50 leads to a structure indistinguishable from the one obtained by a "normal" condensation of acetoacetate and Δ^1-piperideine. Evidence from tracer experiments with ^{14}C-acetate is equally compatible with both alternatives. If structure 50 is postulated as the monomeric precursor, its condensation to yield a dimeric intermediate in the biosynthesis of lycopodine could take place in a manner analogous with the reaction sequence proposed in the pelletierine hypothesis. The extra carboxyl group would serve a twofold purpose:

a) it would provide a necessary point of attachment to the enzyme site and

b) it would further activate the nucleophilicity of the methylene carbon which initiates the condensation by forming the C_{15}-C_8 bond in lycopodine.

In the sequence drawn below (Scheme 3) one of these monomeric precursors, the one which will eventually yield the C-methyl bearing group, is represented in its decarboxylated form, in keeping with the role played by pelletierine. Also, both condensing units are drawn as their respective oxidized enamine derivatives. Such a scheme is entirely consistent with the labelling pattern obtained in the pelletierine, as well as in the lysine and acetate experiments.

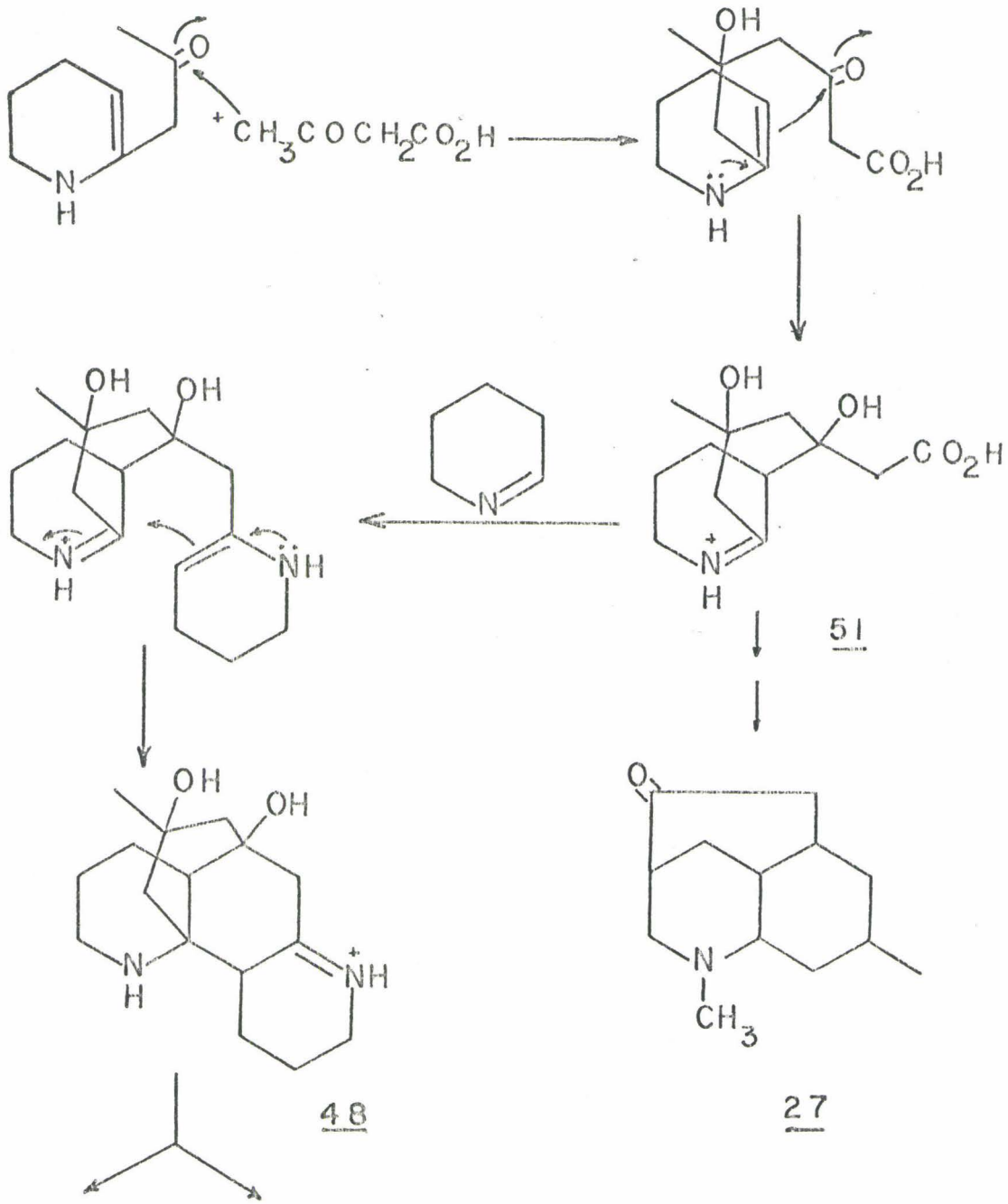
Thus, it is conceivable that the biosynthesis of lycopodine does indeed take place by a pathway which involves a common monomeric unit and that the dimerization step takes place after an irreversible modification of one of these metabolic units. Such modification, decarboxylation, for example, would allow pelletierine to find its way into one branch only of the metabolic route. The intermediacy of the precursor 50 accounts, apparently, for the finding that lysine is incorporated with equal efficiency into both segments of lycopodine, a fact that, as mentioned before, could be taken as evidence in support of the condensation of two identical units of monomeric precursor to yield a true dimer as a biosynthetic intermediate. This point should not, however, be over emphasized. In fact, the symmetrical incorporation of lysine into both segments of lycopodine might also be due to a redistribution of activity between intermediates, which can occur during long-term experiments and it could also be partly, coincidental.



SCHEME 3 : Biosynthesis of lycopodine, alternative hypothesis

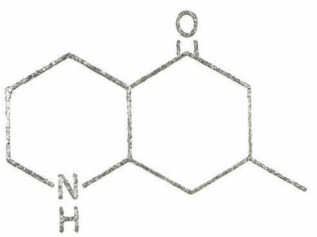
Further information regarding this matter might be obtained by additional feeding experiments with other potential precursors, such as cadaverine or Δ^1-piperideine, compounds which would be expected to be incorporated into lycopodine more efficiently than lysine, which is channelled into many other plant constituents. The incorporation of cadaverine or Δ^1-piperideine into both segments of the alkaloid in short-term experiments would be expected to be more sensitive to quantitative variations in the metabolic pathway.

Another plausible reaction sequence in which the intermediacy of pelletierine is retained is shown below (Scheme 4). Here the central idea is the participation of pelletierine as a close or immediate precursor of one segment of the alkaloid while the remainder of the molecule is assembled in a stepwise manner from metabolic units whose incorporation into lycopodine has already been shown, i.e., acetoacetate or related fragments and a lysine derivative. Thus, condensation of pelletierine, written as its oxidized enamine derivative, with aceto-acetic acid (or alternatively, by a stepwise condensation with two acetate equivalents), followed by ring closure leads to the immonium ion 51. Condensation of this intermediate with a lysine-derived fragment and subsequent decarboxylation and ring closure leads to the intermediate 48, the immediate precursor of the $C_{16}N_2$ and the lycopodine alkaloids envisaged by the pelletierine hypothesis. The labelling pattern and the experimental data reported in this work are consistent with this scheme. One additional attractive



SCHEME 4 : Biosynthesis of Lycopodine, alternative hypothesis

feature of this reaction sequence is the intermediacy of a structure such as 51, which is thought to be involved in the biosynthesis of luciduline (27) (55). In this connection, the quinoline derivatives 57 and 58 might be expected to serve as alternative precursors of lycopodine.



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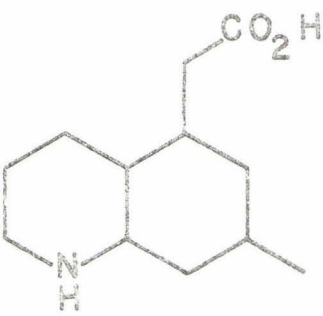


58

Finally, one essential feature of these alternative schemes (and of the original pelletierine hypothesis as well) deserves further comment. This refers to the biogenetic relationship between the $C_{16}N_2$ and $C_{16}N$ series of Lycopodium alkaloids.

In all the schemes considered it has been assumed that the alkaloids have a common biogenetic origin. Any postulated precursor, whether it be pelletierine or the intermediates 50 and 51, should be incorporated in the same manner into alkaloids of the $C_{16}N_2$ and the $C_{16}N$ series in the same plant. Unfortunately, it was not possible, in the present work to secure experimental evidence to support this view. Although radioactive lycodine was isolated from L. trystachium after administration of 2,3'-$^{14}C_2$-pelletierine (Experiment II), its degradation to acetic acid, by Kuhn-Roth oxidation, gave inconclusive results (presumably due to insufficient purification of lycodine). If two intact pelletierine units had been incorporated into lycodine, the acetic acid isolated should have

carried 9% of the activity present in lycodine, but if incorporation of pelletierine into this dinitrogenous alkaloid had taken place in the same manner as into lycopodine then, clearly, the activity in this degradation product should have corresponded to 18% of the activity in the intact alkaloid.

It would be desirable to settle this matter. Clearly, further experimental work should be oriented in this direction. If it were demonstrated that the $C_{16}N_2$ alkaloids, such as lycodine and cernuine, are indeed dimers of pelletierine, this would imply that alkaloids of one and the same taxon could have completely different biogenetic origins, a situation without precedent in biosynthetic studies in alkaloids. On the other hand, proof of partial incorporation of pelletierine into these alkaloids would give indirect support to alternative schemes, such as the ones discussed here.

III. DEGRADATION METHODS

The degradation of radioactive alkaloids to isolate individual carbon atoms is a fundamental step in any biosynthetic study. If non-random incorporation of tracers into products is to be proven, the total activity of the intact product must be accounted for in terms of the activity at individual sites. This aim is achieved by devising degradative schemes which lead to the unambiguous isolation of individual atoms or groups of atoms, whose radioactivity can then be determined.

Lycopodine does not lend itself very readily to the reactions commonly employed to degrade the carbon skeleton of a molecule and the isolation of individual carbon atoms. Many of the initial structural studies were hampered because of its lack of reactivity. Thus, the carbonyl oxygen of lycopodine was originally attributed (56) to an ether function. Hoffman and Emde degradations, two very common reactions employed in the degradation of alkaloids, were unsuccessful. The reaction that proved to be the key to the unravelling of the structure of lycopodine (57), the opening of rings A and B with cyanogen bromide, could not be applied to our purposes because of the low yields obtained. Since the amount of radioactive products are small, the chemical yield of every reaction in a given degradative scheme must be reasonably high. In the same communication in which the Von Braun reaction was discussed (57), the authors described the reaction of lycopodine with phenyllithium. The product phenyldihydrolycopodine 52, was fully characterized. We made use of this reaction since it provides an easy and reliable way of isolating

carbon 5, the carbonyl carbon. Oxidation of phenyldihydrolycopodine with hot aqueous potassium permanganate yields benzoic acid whose carboxyl group represents the carbonyl carbon of lycopodine. C-5 of lycopodine was one of the carbon atoms at which activity from 2-^{14}C-lysine and from 6-^{14}C-lysine was expected (cf. Chapter I). The other carbon atoms at which activity was predicted to reside were one or more of the C-9, C-1 and C-13. Of these carbon atoms, C-9 was thought to be the one most easily accessible. Oxidation of the methylene at C-9 to form the lactam 53 would open the way to the isolation of C-9 by the scheme described below. This lactam had been obtained by an entirely different route (58) and there was no ambiguity about its structure.

Permanganate oxidation of lycopodine in acetone gave a 50% yield of crude lactam. This method was originally developed by Ayer and co-workers (59) for the oxidation of lycopodine in a large scale (5 g). It was necessary to modify the procedure when the reaction is scaled down to 100-200 mg of starting material. Sodium borohydride reduction of the carbonyl group at C-5 leaves the molecule ready for phenylation of the lactam carbonyl which by subsequent hot aqueous permanganate oxidation would yield C-9 as the carboxyl group of benzoic acid. The phenylation reaction, however, was unsuccessful. Several attempts, under different reaction conditions, failed to yield the desired product. C-9 was eventually isolated as the formyl derivative 54, obtained by the series of reactions shown in Figure 13. Permanganate oxidation of lycopodine under acidic conditions gives a mixture of products; amongst these is the N-formylamino acid 55, which is obtained in 25-30% yield (60).

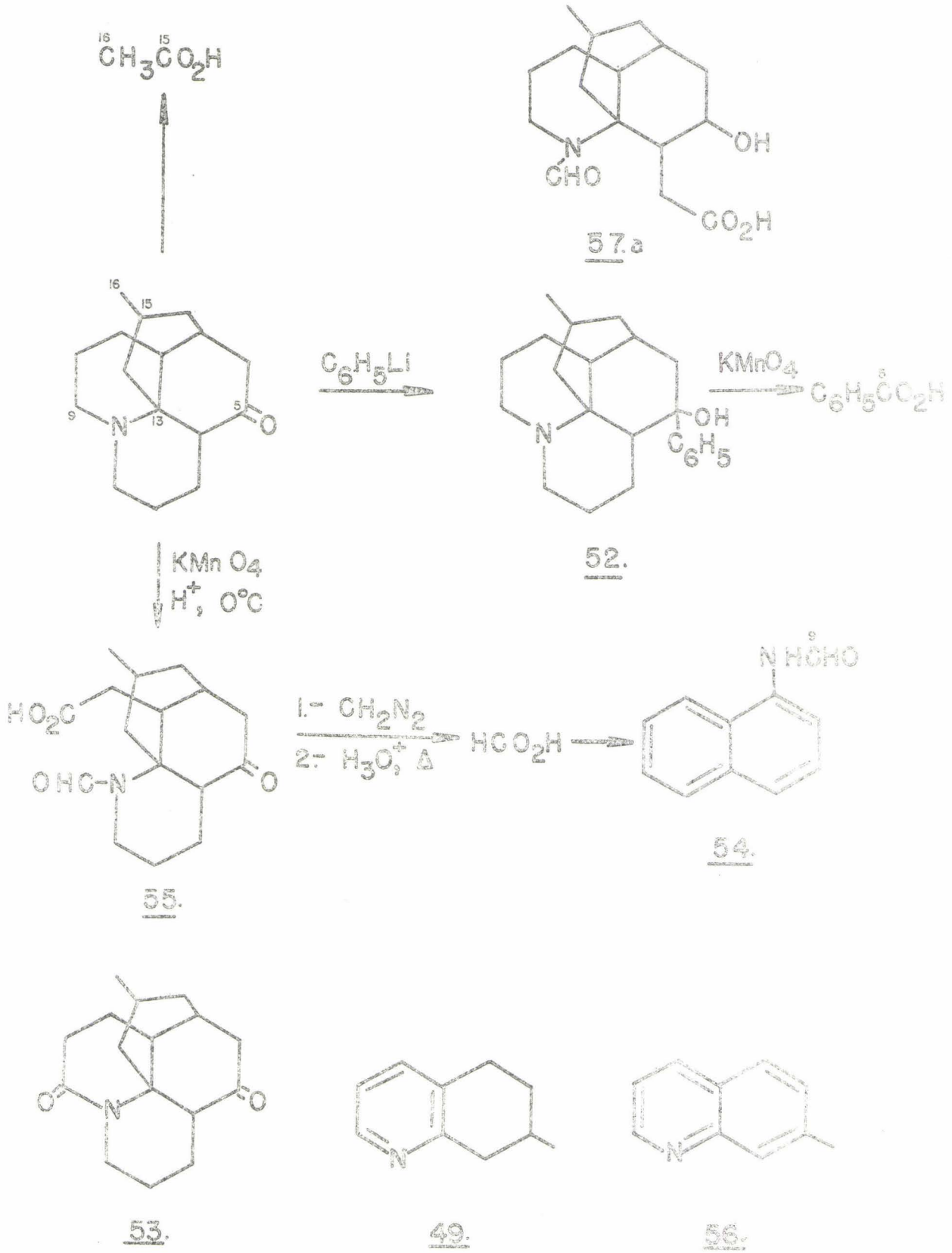


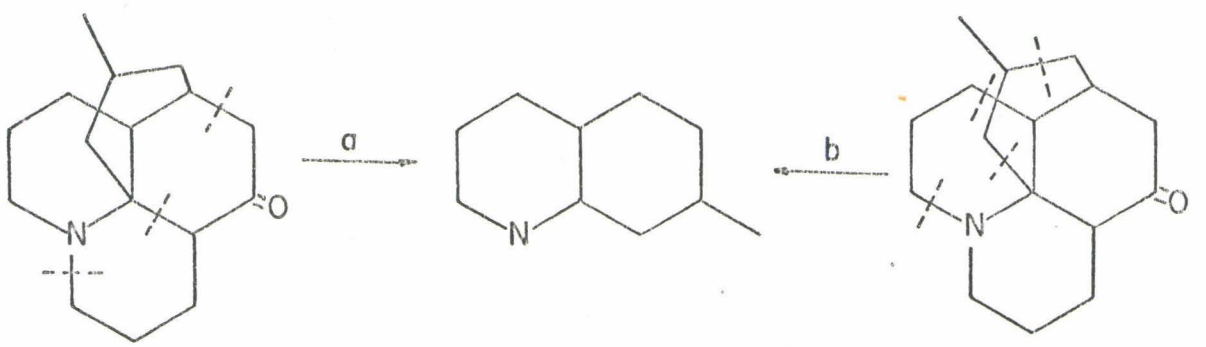
FIGURE 13 : Degradation Methods

Again, the original method had to be modified in order to adapt it to our purposes. Esterification, followed by acid hydrolysis, liberates formic acid which is separated from the reaction mixture by steam distillation. For radioactivity counting, formic was transformed into N-formyl- α-naphthyl-amide (54).

There is, at present, no conclusive proof that the formic acid isolated corresponds entirely to C-9. It is conceivable that oxidation at C-1 might also take place forming the corresponding derivative. Indirect evidence that this is not so comes from the finding that permanganate oxidation of lycopodine in acetone gives exclusively lactam 53; in other words, oxidation proceeds only by attack at the C-9 methylene group. Further indirect evidence for the position was obtained by Law (60) : the hydroxy acid derived by borohydride reduction of the N-formylamino acid 55 failed to lactonize in refluxing benzene containing p-toluenesulfonic acid. The alternative structure would lead to 57, which might be expected to lactonize. A more conclusive proof regarding the origin of the formic acid might be obtained by converting the N-formylamino acid to the known lactam 53.

The individual isolation of C-1 and C-13 was not attempted but a fragment containing C-13, together with C-9, but not C-1 or C-5, was obtained. This fragment was 7-methyl, 5,6,7,8-tetrahydroquinoline (49). The dehydrogenation of lycopodine, one of the first degradation reactions carried out on this alkaloid (56) yields a mixture of products among which several methylquinoline have been identified. Marion and Manske concluded from these experiments that a reduced quinoline skeleton was present in lycopodine. Since the same product, e.g., 7-methylquinoline 56, was obtained when the dehydrogenation was carried out under very mild conditions

($Pd-BaSO_4$ under nitrogen and by heating lycopodine with phthalic anhydride) they concluded that no rearrangement had taken place in the formation of this product. This observation is supported by the finding that obscurine also yields 7-methylquinoline under the same experimental conditions (61). Visual inspection of lycopodine shows that a 7-methylquinoline skeleton may arise in at least two different ways (which do not involve molecular rearrangements), as shown below, where the dashed lines indicate the bonds that are broken in the course of the reaction (obscurine and lycodine, on the other hand, yield a 7-methylquinoline skeleton in only one possible way). Path a, is expected to be preferred since it involves the cleavage of three bonds, whereas path b requires the cleavage of four bonds and a greater number of chemical changes.



In our hands, dehydrogenation of lycopodine by the methods described by Marion and Manske did not lead to the isolation of 7-methylquinoline in amounts sufficient for further purification and radioactivity assay. Treatment of lycopodine over Zn-dust, however, yielded a complex mixture of products from which we isolated 7-methyl-quinoline, 7-methyldecahydroquinoline and 7-methyl-5,6,7,8-tetradhydro-

quinoline. Only this last derivative was isolated in amount sufficient for radioactivity assay. This compound was transformed into several crystalline derivatives : perchlorate, hydrochloride, methiodide, etc. Its identity was confirmed by comparison with a synthetic sample (62). The fact that this degradation product was optically active (62) supports path a. Apparently, the asymmetry at C-15 of lycopodine has been retained in the degradation product. It would be unlikely that this 7-methyl-quinoline derivative would show optical activity had it been derived through path b.

Lycopodine possesses a secondary $CH-CH_3$ group (C-15 - C-16). This entity can be easily isolated as acetic acid after vigorous oxidation with chromic acid (Kuhn-Roth oxidation). The Kuhn-Roth oxidation of lycopodine and some derivatives was studied in detail by Harrison (63). He showed that, under the reaction conditions, acetic acid was the only volatile acid isolated from lycopodine itself. The acetic acid can be further degraded by a Schmidt rearrangement to methylamine and carbon dioxide, the methyl group of the amine corresponding to C-16 of lycopodine. The insufficient amount of acetic acid obtained in these degradations did not allow us to perform this last degradation but, in our view, this is not a serious omission considering the data obtained (a Schmidt degradation would have been imperative if, for example, the values obtained from both the 1-^{14}C-acetate and 2-^{14}C-acetate experiments had been identical).

IV EXPERIMENTAL

1. Administration of labelled compounds and isolation of alkaloids

Lycopodium tristachyum Pursh, ground cedar, a clubmoss whose creeping stem is buried 3-5 inches below ground, was found in the vicinity of Huntsville, Ontario, near Algonquin Provincial Park. Attempts to propagate the plant in the greenhouse were unsuccessful.

Labelled compounds were administered to intact plants (Experiments 1-4, 8-11) and to excised shoots (Experiments 5 and 7). The first experiments were carried out in the bush. More recent wick feeding experiments were carried out in the greenhouse. Pieces of turf (approximately 3 ft. x 2 ft. x 8 in. deep) containing a clump of the club-moss were collected in the bush and transported to the greenhouse. The wick-feeding experiment was carried out on the following day. Cotton thread was inserted into approximately 20 shoots. The end of each thread was placed into a small glass receptacle The labelled compound, dissolved in glass-distilled water (10 ml), was placed into the receptacles and was absorbed into the plant through the cotton wicks. After the original tracer solution had been absorbed the receptacles were repeatedly refilled with glass-distilled water. The plants were kept in contact with the tracer for 48 hours, and were then harvested. Shoots and subterranean stems were dried separately and the material was then taken to the laboratory for extraction and work-up.

In another feeding method (Experiments 5 and 7) shoots, 2 to 3 inches in length, from a 3 x 2 ft. clump of club-moss, were cut and packed, cut surfaces downward, into three 100 ml. beakers. The aqueous solution containing the radioactive tracer was divided among the beakers.

Glass distilled water (3 ml) was added to each beaker after 24 hours, when most of the original solution had been absorbed and the experiment was continued for a further 24 hours.

A summary of the feeding experiments which were carried out is presented in Table I. The nominal total activity administered is presented together with the radiochemical yields, in Table Ia.

Sodium 3-^{14}C-acetoacetate (Experiment 9) was prepared by hydrolysis of ethyl 3-^{14}C-acetoacetate (Radiochemical Centre) (66). 2-^{14}C-pelletierine (Experiment 8 and 11) and 4,5-$^{3}H_2$-pelletierine were synthesized from 2-^{14}C-DL-lysine and 4,5-$^{3}H_2$-DL-lysine, respectively, and inactive acetoacetic acid (67). 3'-^{14}C-pelletierine (Experiment 11) was prepared from 4-^{14}C-acetoacetic acid generated by hydrolysis of ethyl 4-^{14}C-acetoacetate and Δ^1-piperideine obtained from inactive lysine*.

Isolation of Lycopodine

Preliminary experiments indicated that the subterranean stems of L. tristachyum contained little alkaloid. Labelled lycopodine was obtained from the aerial parts of the plant.

Green shoots of L. tristachyum were dried and ground to a fine powder in an Osterizer blender. The powder was moistened with 1M ammonia (5-100 ml) and was continuously extracted with ether for 48 hours. The ether solution was extracted with hydrochloric acid (0.5M, 4 x 25 ml), the aqueous extract was washed with ether and was then basified with 1M ammonia. The alkaloids were extracted into ether (3 x 30 ml), the ether extract was

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* The synthesis of the radioactive precursors tested in Experiments 8 and 11 was carried out by R.N. Gupta.

dried (Na_2SO_4) and the solvent evaporated to yield a mixture of bases. Lycopodine and lycodine were separated from this residue by extraction with warm hexane. The hexane extract was concentrated to dryness and the residue was sublimed at 110° and 1×10^{-3} mm, yielding a crystalline sublimate which consisted of lycopodine containing not more than 1-3% lycodine, as shown by gas liquid chromatography (glc) (5% SE30, on Chromosorb 1/8" x 4 ft. 200°, 15 ml/min of He). The sublimate was dissolved in a little boiling hexane and the solution was kept overnight. Lycopodine, which crystallized (m.p. 115°), was sublimed at 90° and 1×10^{-3} mm. The purity of the product was checked by glc and by radiochromatography of a thin layer chromatogram (tlc) (Silica Gel coated with 0.1N NaOH; developed with Chloroform-Methanol, 1:1, Rf. 0.48), in a Radiochromatogram scanner, Model 7201, Packard Instrument Company.

Even though glc analysis indicated a single component, in several instances the product required further purification to remove a radiochemical contaminant whose presence was detected by tlc and radioscanning. Lycopodine was dissolved in ether and the solution was neutralized with an ethereal solution of perchloric acid. The solvent was evaporated and the lycopodine perchlorate so obtained was recrystallized from hot methanol. Melting point 276-278°. (Lit. 278°), (56).

Several modifications of the above procedure were employed in early experiments. In Experiment 5, the shoots of the plant were homogenized in methanol, the methanol solution was filtered and concentrated, the residue was then extracted with hydrochloric acid and the acid solution was basified with ammonia. The alkaloidal fraction was then extracted into ether and worked up as already described. In Experiments 1-3, the

basic fraction was separated into its components by column chromatography on alumina. Lycopodine and lycodine were separated from the other basic components by elution with benzene.

Isolation and purification of lycodine

Lycodine is present in L. trystachyum to a very small extent, usually not more than 1% with respect to lycopodine. It is very soluble in hexane and is difficult to separate from lycopodine by fractional crystallization or column chromatography. Accordingly, isolation was attempted only after addition of inactive lycodine, which, in turn, required a high degree of incorporation of the radioactive tracer into lycodine. For this reason, lycodine was isolated only from Experiment II. Inactive lycodine (10 mg) was added to the mother liquors from the crystallization of lycopodine, the solvent evaporated to dryness and the residue dissolved in 25 ml of ethanol. An excess of $NaBH_4$ was added and the solution left at room temperature during 16 hours. The residue after work-up of the reaction mixture, was placed on top of a column filled with alumina and lycodine was eluted with benzene. Under these conditions, dihydrolycopodine, the sodium borohydride reduction product of lycopodine, was not eluted. Lycodine was further purified by preparative thin layer chromatography and sublimation.

2. Degradation of lycopodine

Carbon-5 as benzoic acid

Lycopodine, 100 mg (0.4 mmoles) was dissolved in 50 ml of dry ether and added dropwise to a solution of phenyllithium (Alpha Inorganics, 1.9M, 5-8ml) in ether, and the mixture was refluxed 4 hours in an atmosphere of nitrogen. The solution was cooled and poured into concentrated hydrochloric acid (5 ml) to which crushed ice (10 g) had been added. The ether layer was discarded, the aqueous layer was washed with ether (2 x 30 ml) and was basified with ammonia. The product was extracted into ether (5 x 30 ml), the ether solution was dried (Na_2SO_4), the solvent evaporated and the residue (105 mg) recrystallized from hexane, yielding phenyldihydro-lycopodine, melting at 150°-153° (lit. 154°-155°), (57). Glc (5% SE 30 on Chromosorb W, 200°, 1/8-in. x 4 ft.) revealed the presence of traces of lycopodine. The product was oxidized without further purification.

Finely ground potassium permanganate (0.5 g) was added in small portions to a suspension of phenyldihydrolycopodine (80 mg) in hot water (25 ml). The mixture was refluxed with vigorous stirring during 8 hours. The mixture was cooled, acidified with 0.5M hydrochloric acid and decolorized by addition of sodium bisulphite. The solution was extracted with ether (3 x 40 ml), the extract dried (Na_2SO_4), the solvent evaporated and the residue sublimed to yield benzoic acid (14-20 mg, 40-50% yield), melting at 119-120°.

Carbon-9 as formic acid

A solution containing lycopodine (250 mg, 1 mmole) and oxalic acid (125 mg) in water (25 ml) was cooled in an ice bath. Potassium permanganate (525 mg) was added in small portions over 2 hours with vigorous stirring.

After addition was complete, stirring of the solution was continued for 4 hours at 0° and for 2 hours at room temperature, cooled to 0°, and gaseous sulphur dioxide passed through the suspension, resulting in a clear pale yellow solution.

The acidic solution was extracted six times with chloroform to remove acidic and neutral products. These were separated by shaking the combined organic extract twice with aqueous ammonia, acidifying the latter with dilute hydrochloric acid and extracting again with chloroform to remove acidic products. Neutral material remained in the original chloroform extract. The neutral and basic material was not investigated in detail.

The crude acidic products were sublimed to yield pure N-formyl-aminoacid 55, 70-78 mg (24-30% yield).

The crystalline N-formylamino acid 55, 70 mg, was converted into the methyl ester by reaction with an excess of freshly distilled diazomethane. After sublimation at 140° and 1×10^{-3} mm the methyl ester melted at 114-116 (Lit. 117°) (60). Yield : 56 mg.

The methyl ester (55 mg) was dissolved in sulphuric acid (1M, 10 ml) and the mixture refluxed two hours. Steam was then passed through the reaction mixture until 50 ml of distillate, containing volatile acids, had been collected. The distillate was neutralized with 0.1M sodium hydroxide (1.10 - 1.25 ml), and the solution was evaporated to dryness. The residue was dissolved in water (2 ml) containing α-naphthylamine hydrochloride (20 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (50 mg). N-formyl-α-naphthylamide 54 precipitated after a few minutes. After sublimation at 120° and 1×10^{-3} mm, recrystallization

from benzene, and resublimation, the product melted at 137° - 138° (Lit. 139°) (64), and was identical (mass spectrum, melting point, mixture melting point) with an authentic specimen.

Carbons-15 and -16 as acetic acid

A solution of chromic acid (2 g) in water (3 ml) was added to a solution of lycopodine (100 mg) in sulfuric acid (2M, 5 ml). The mixture was refluxed two hours. Steam was then passed through the mixture until 50 ml of distillate had been collected. The distillate was neutralized with sodium hydroxide (0.1M, 2.0 - 2.5 ml) the solution evaporated and the sodium acetate (50-60%) so obtained converted into acetyl-α-naphthylamide (65).

Carbons-7 to -16 as 7-methyl-5,6,7,8-tetrahydroquinoline

An intimate mixture of lycopodine (250 mg) and zinc powder (8 g) was placed in a Carius tube (15 x 1 cm) and more zinc powder (8 g) was added. The tube was evacuated and sealed, and was heated at 290° - 310° for 24 hours. A yellow liquid condensed in the cold end of the tube protruding from the furnace. Glc analysis of the reaction mixture furnished variable amounts of 7-methyldecahydroquinoline (A), 7-methyl-5,6,7,8-tetrahydroquinoline (B), 7-methylquinoline, a dimethylquinoline and several other uncharacterized compounds. Preparative glc (20% Carbowax 20M on Chromosorb W, coated with 5% KOH, 3/8 in. x 10 ft.), 175° and a helium flow rate of 15 ml/min gave A and B with retention times 30 and 51 min. respectively.

7-methyl-5,6,7,8-tetrahydroquinoline 49, was obtained as a liquid. Its mass spectrum showed peaks at m/e 147 (100 molecular ion), 146 (30), 132 (51), 105 (71), identical with the mass spectrum of a synthetic sample (62).

This product was further purified by sublimation of its hydrochloride, melting point 153-156°.

Oxidation of 2,3'-$^{14}C_2$-pelletierine

2,3'-^{14}C-pelletierine hydrochloride, (150 mg) (obtained by mixing a small sample, total activity ca. 0.2 μC) of the original 2,3'-^{14}C-pelletierine hydrochloride used in Experiment II with 350 mg of inactive pelletierine hydrochloride, and recyrstallizing the mixture to constant activity), was dissolved in 3 ml. of sulfuric acid 1M. To this solution, chromic acid (120 mg) in water (3 ml) was added and the mixture was kept at 90° for 4 hours. Steam was passed through the reaction mixture until 50 ml of distillate had been collected. The distillate was neutralized with sodium hydroxide (0.1M, 5 ml) and the solution was evaporated to dryness. The acetic acid so obtained was converted into acetyl-α-naphthylamide.

Pipecolic acid, isolated from the remaining aqueous reaction mixture by a method already described in the literature (14), was purified by sublimation, m.p. 279°.

3. Radioactivity assay

A weighed amount of the compound was transferred to an aluminum planchette and dissolved in two drops of a 1% solution of collodion in dimethylformamide. For benzoic acid, one or two drops of an aqueous sodium hydroxide solution were added (2% w/v) in order to avoid any loss of sample by evaporation. The solution was covered with a circle of lens tissue, which caused it to spread evenly over the surface of the planchette, and was evaporated under an infra red drying lamp. Radioactivity was assayed on a Nuclear Chicago Corporation gas flow system (Model 4342). ^{3}H : ^{14}C ratios were measured by liquid scintillation counting (Mark,I liquid scintillation computer, Model 6860, Nuclear Chicago). Activity due to ^{3}H and ^{14}C was determined simultaneously, by external standardization counting with ^{133}Ba. Samples were dissolved in methanol or methanol-water and the solution was dispersed in a solution of Liquifluor (Nuclear Chicago) diluted 25 times with toluene. Duplicate samples of each compound were counted under comparable conditions of quenching.

Each sample was prepared in triplicate and counted for 1000 counts for ten readings. The planchette holders for each sample were counted for background for 200 counts (ten readings). The confidence limits shown in the results (Tables I - 6) are standard deviations of the mean. The complete calculation of the specific activities of a typical compound, from the data obtained from measurements in triplicate samples, will be presented in Appendix B.

SUMMARY

The biosynthesis of lycopodine has been studied by feeding radioactive acetate, acetoacetate, lysine and pelletierine to L. tristachyum. Degradations of the radioactive lycopodine obtained in these experiments showed specific incorporation of label in each case. The labelling pattern found in lycopodine obtained from the acetate experiments disproved Conroy's polyacetate hypothesis of the biogenesis of the Lycopodium alkaloids. The observed distribution of activity from acetate into lycopodine can be interpreted as incorporation via two units derived from acetoacetate or related fragments.

The distribution in lycopodine of label from lysine showed that two lysine-derived units were incorporated into the alkaloid and that incorporation of lysine takes place by way of a symmetrical intermediate. The results of the acetate and lysine experiment are consistent with the hypothesis that the lycopodium alkaloids are modified dimers of pelletierine. This hypothesis was tested by feeding radioactive pelletierine to L. tristachyum. It was found that pelletierine was specifically incorporated into lycopodine, but that only one intact pelletierine unit entered the alkaloid.

These experimental results demand modification of the pelletierine hypothesis. Alternative biosynthetic schemes, consistent with the experimental data, are considered.

APPENDIX A

1 Curie = 3.6×10^{10} dps = 2.2×10^{11} dpm

1 mCurie (mCi) = 3.6×10^{7} dps = 2.2×10^{9} dpm

$$\text{Total activity (counts min}^{-1}\text{)} = \text{Specific Activity} \times \frac{\text{weight (mg)}}{\text{mmole}}$$

$$\text{Total activity (dpm)} = \text{Total activity (counts min}^{-1}\text{)} \times \frac{100}{30}$$

(100/30 = efficiency of counting system)

$$\text{Total activity (mCi)} = \frac{\text{Total activity (dpm)}}{2.2 \times 10^{9} \text{ (dpm)}}$$

$$\text{Recovery (\%)} = \frac{\text{total activity recovered (mCi)}}{\text{total activity fed (mCi)}}$$

APPENDIX B

Experiment 8 : Lycopodine perchlorate-^{14}C

Planchette	1665	1666	1667
weight taken (mg)	.955	.665	.460
mmole	347.7	347.7	347.7
Self-absorption factor (68)	.874	.877	.886
Number of counts	1000	1000	1000

Definitions and Symbols (69):

time (min) for 1000 counts = x

number of time readings = $n = 10$

average value for 10 readings = $\bar{x}$

$$\text{mean} = \bar{x} = \frac{\Sigma x_i}{n}$$

$$\text{variance} = \frac{\Sigma[(x_i-\bar{x})^2]}{n}$$

$$\text{standard deviation} = \sigma = \sqrt{\Sigma[(x_i-\bar{x})^2]/n}$$

$$\text{best estimate of} \quad \sigma = \sqrt{\Sigma[(x_i-\bar{x})^2]/n-1}$$

	Time readings for 1000 counts	$x - \bar{x}$	$(x-\bar{x})^2$
1	9.69	0.20	0.040
2	9.27	0.62	0.384
3	10.11	0.22	0.048
4	10.06	0.17	0.029
5	9.55	0.34	0.115
6	10.08	0.19	0.036
7	9.81	0.08	0.006
8	10.18	0.29	0.084
9	9.91	0.02	0.0004
10	10.23	0.34	0.115

$\Sigma x = 98.89$ $\qquad$ $\Sigma(x-\bar{x})^2 = 0.861$

$\frac{\Sigma x}{n} = 9.89$ $\qquad$ $\Sigma(x-\bar{x})^2/n-1 = 0.861/9$

$= 0.0956$

$$\text{Count rate} = \frac{1000}{9.89} \pm \frac{1000}{9.89} \cdot \sqrt{\frac{0.0956}{(9.89)^2}}$$

$$= 101.11 \pm 3.16 \text{ counts min}^{-1}$$

The background was determined in the same way and was found to be 3.62 ± 0.277 counts min^{-1}. The reading for the radioactive sample was corrected for background in the following manner:

$$101.11 - 3.62 \pm \sqrt{(3.16)^2 + (0.277)^2}$$

$$= 97.49 \pm 3.17 \text{ counts min}^{-1}$$

Correction is now applied for weight and self-absorption

$$(97.49 \pm 3.17) \times \frac{347.7 \text{ mg mmole}^{-1}}{.955 \times 0.874}$$

$$= (4.0611 \pm 0.132) \times 10^4 \text{ counts min}^{-1} \text{ mmole}^{-1}$$

The corresponding values derived from planchettes 1666 and 1667 were, respectively:

$(4.1971 \pm 0.1236) \times 10^4$ and $(4.2122 \pm 0.141) \times 10^4$ counts $\text{min}^{-1}\text{mmole}^{-1}$

The mean specific activity for these triplicate readings was found:

$$\frac{4.0611 + 4.1971 + 4.2122}{3} \pm \frac{\sqrt{(0.132)^2 + (0.1236)^2 + (0.141)^2}}{3} \times 10^4$$

$$= (4.156 \pm 0.070) \times 10^4 \text{ counts min}^{-1} \text{ mmole}^{-1}$$

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