BIOSYNTHESIS OF THE LYCOPODIUM ALKALOIDS

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By

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SCOPE AND CONTENTS:

The biosynthesis of two Lycopodium alkaloids of remarkably different structures, lycopodine and cernuine, was studied by tracer methods. The modes of incorporation of lysine, cadaverine, Δ^1-piperideine and pelletierine into the two alkaloids were compared.

The results obtained clearly show that the modes of biosynthesis of lycopodine and of cernuine are analogous and that these alkaloids are true dimers of a C_8N unit. However, since only one pelletierine unit is incorporated into the alkaloids, pelletierine is not the monomeric C_8N unit which serves as the precursor of the alkaloids.

ACKNOWLEDGEMENTS

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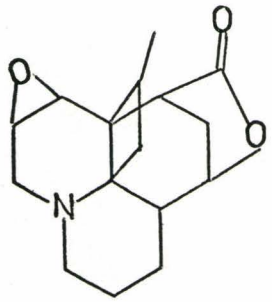
(i) Structure and Biogenesis of the Lycopodium Alkaloids

The Lycopodium alkaloids are a group of nitrogenous bases which are found in the clubmosses, the members of the family Lycopodiaceae. The first alkaloid to be isolated from a Lycopodium species was reported in 1881 when Boedeker[1] described a base from Lycopodium complanatum L. which he called lycopodine. Structural studies were begun in the nineteen-forties. It was not, however, until 1957, that the first structure of a Lycopodium alkaloid, annotinine (1), was elucidated by Wiesner and co-workers.[2] In 1960 the structure of the most commonly found of these alkaloids, lycopodine (2), was solved.[3] Since then, the number of alkaloids isolated and characterized has increased rapidly and future studies will undoubtedly reveal new structural types.[4]

The majority of the Lycopodium alkaloids possess the carbon skeleton of lycopodine. Other structural types known in this series are exemplified by annotinine (1), lyconnotine (3), annotine (4), serratinine (5), lycodine (6), selagine (7), luciduline (8), cernuine (9), and alopecurine (10).

Among the various structural types of the Lycopodium alkaloids, the $C_{16}N_2$ skeletons of the lycodine-obscurine and of the cernuine groups are the most revealing from a biogenetic point of view. These structures can be dissected into two halves whose skeleton, in turn, corresponds to that of the C_8N piperidine alkaloids, of which coniine (11) and pelletierine (12) are typical examples.



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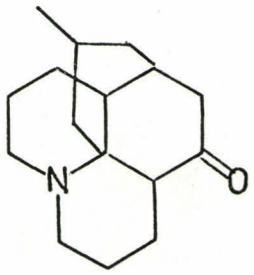

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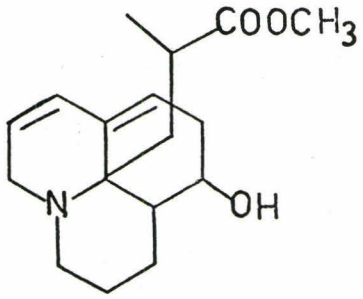

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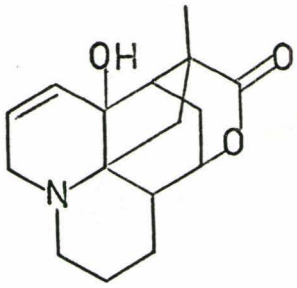

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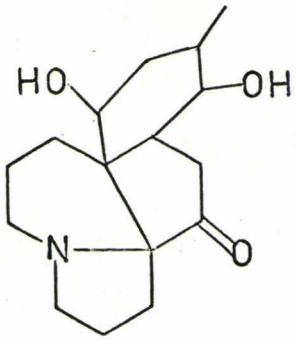

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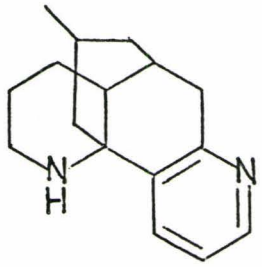

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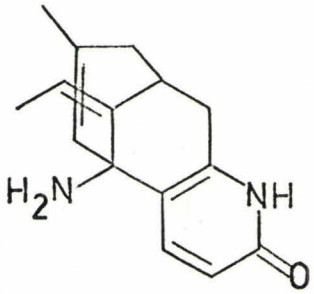

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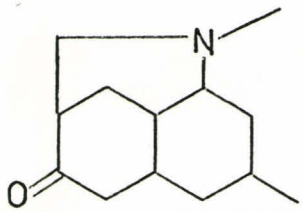

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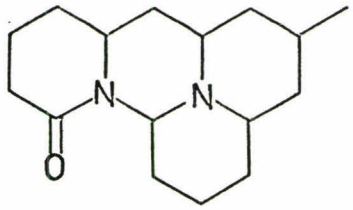

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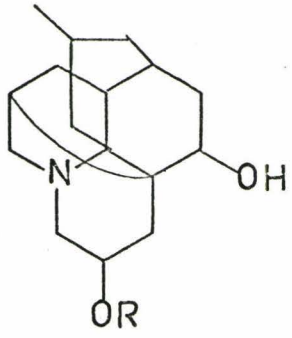

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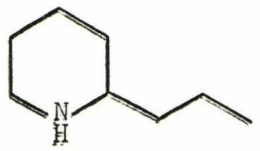
(R = COC_6H_5)

Fig. 1. Representative Lycopodium alkaloids.



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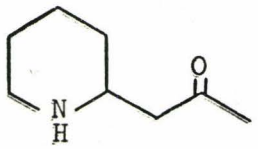

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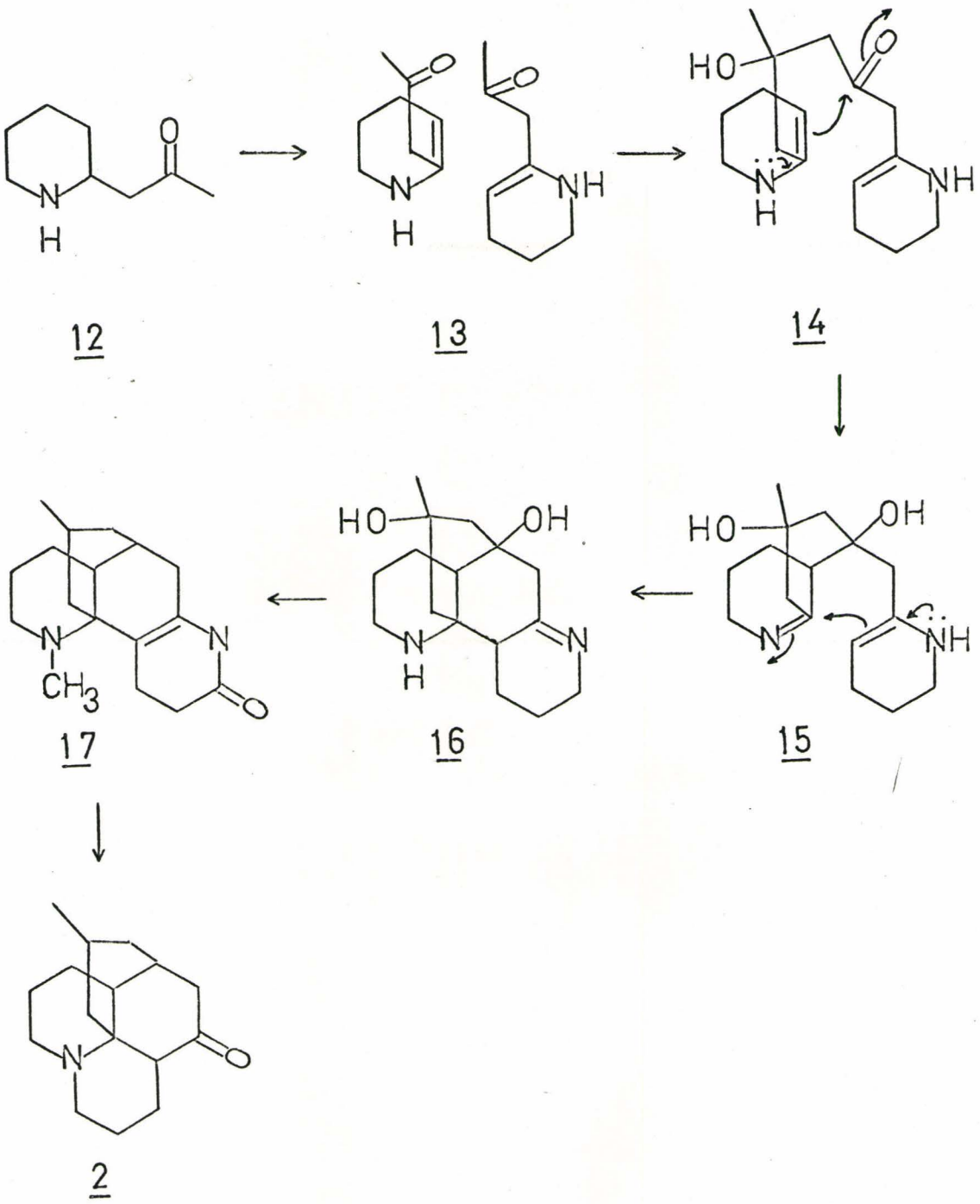
This visual dissection of the $C_{16}N_2$ skeletons of the lycodine-obscurine and of the cernuine groups of alkaloids led to the proposal[5] that there might be a biogenetic relationship between pelletierine and the Lycopodium alkaloids.

This biogenetic scheme, the pelletierine hypothesis, envisaged that the Lycopodium alkaloids originate from two pelletierine units, which, in turn, were derived from lysine and acetate or from related fragments. A hypothetical sequence, whose order of step is arbitrary, will serve as an illustration (Fig. 2). Aldol condensation between the methyl carbon of one pelletierine derived unit 13 and the carbonyl carbon of the other yields 14. Ring closure gives the carbon-nitrogen skeleton 16 of obscurine (17). Rearrangement of this skeleton with the elimination of a nitrogen atom results in lycopodine (2). An important feature of this hypothesis is that the $C_{16}N$ group of alkaloids (e.g., lycopodine) is derived from the $C_{16}N_2$ series (e.g. α-obscurine).

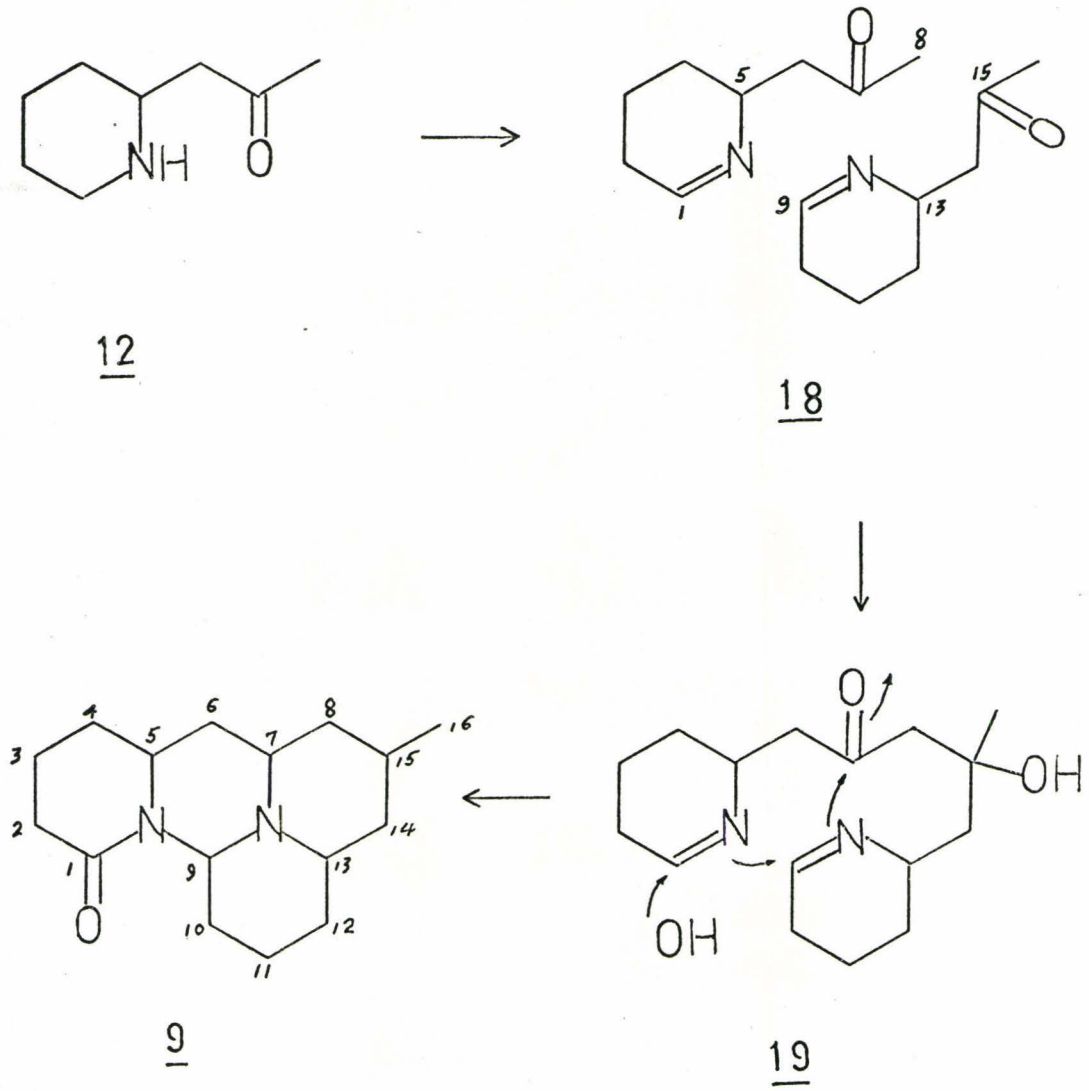
Aldol condensation of the methyl group (C-8) of one pelletierine (12) derived unit 18 with the carbonyl group (C-15) of the other furnishes 19 (Fig. 3). Ring closure followed by adjustment of oxidation states affords cernuine (9).

An earlier biogenetic hypothesis put forward to account for the origin of the Lycopodium alkaloids on the basis of structural relations



12 13 14 15 16 17 2

Fig.2. The pelletierine hypothesis of the biogenesis of the Lycopodium alkaloids - lycopodine type.



12 18 19 9

Fig.3. The pelletierine hypothesis of the biogenesis of the Lycopodium alkaloids - cernuine.

is the polyacetate hypothesis.[6] Condensation of two eight-carbon chains, derived by head to tail union of four acetate units, i.e., 3,5,7-triketooctanoic acid (20) accounts for the basic framework (I) as well as many structural details of the Lycopodium alkaloids. A hypothetical scheme, whose order of steps is arbitrary is outlined in Fig. 4.

The polyacetate hypothesis seemed to be very plausible in relating the structures of various Lycopodium alkaloids and was generally accepted when first proposed by Conroy. The numbering system of the alkaloids which is now in use was proposed by Wiesner[7] on the basis of the polyacetate hypothesis and illustrates the manner of combination of the two C_8 chains to yield C-1 to -8 and C-9 to -16 of the Lycopodium alkaloids. Recent experimental evidence on the biosynthesis of lycopodine does not support the polyacetate hypothesis, but is consistent with the pelletierine hypothesis.

The polyacetate hypothesis is also applicable to cernuine. In fact, Ayer and co-workers[8] predicted the correct structure of cernuine based upon the degradation product of the alkaloid and the polyketide biogenetic scheme. Dehydrogenation of cernuine (see Section III (ii)) gave 2-n-butyl-4-methyl-6-n-pentylpyridine (27). If the C-4 methyl of the pyridine (27) represented the C-methyl group of cernuine and if this group was derived from the terminal methyl group of one of the two polyacetate chains, a possible biogenetic scheme could be derived as shown in Fig. 5. Aldol condensation of the C-15 carbonyl of one chain with C-8 of the other gave 26. Condensation with two molecules of ammonia followed by adjustment of the oxidation states led to the correct structure 9 for cernuine.

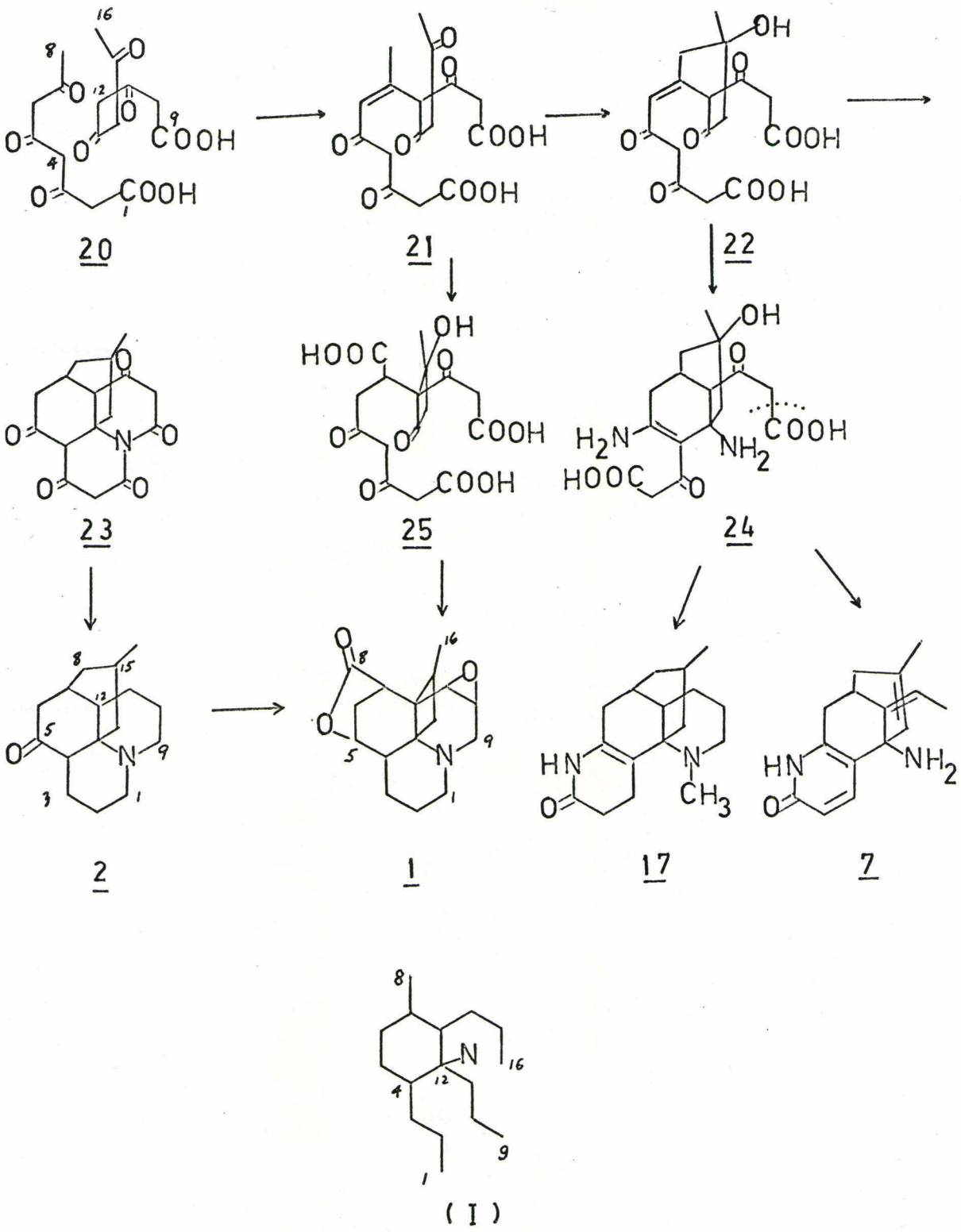


Fig.4. The polyacetate hypothesis.

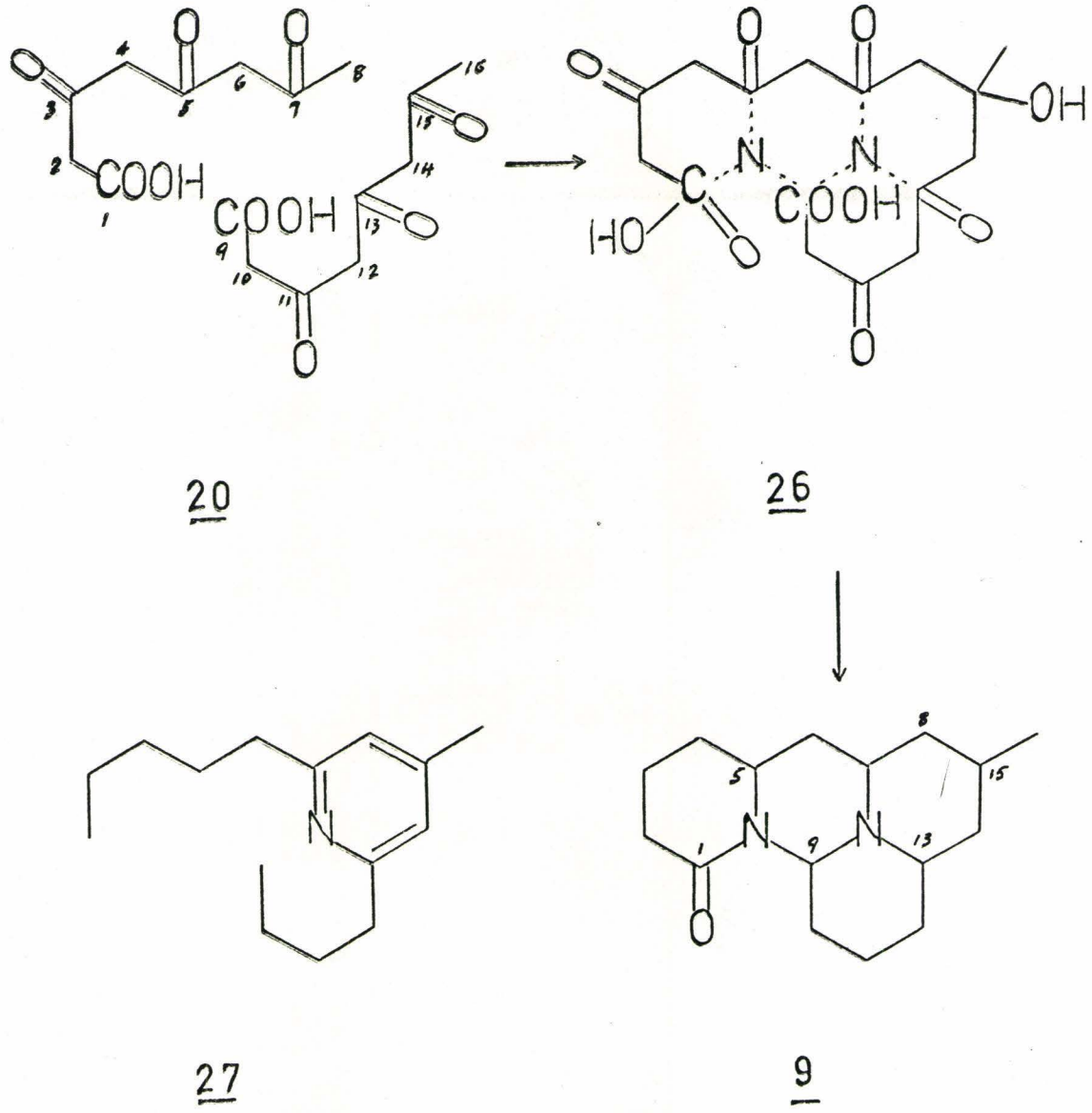


Fig.5. The biogenesis of cernuine - the polyacetate hypothesis.

(ii) Biosynthesis of Pelletierine and Other Lysine Derived Piperidine Alkaloids

It has been shown by tracer studies that the amino acid lysine provided the five-carbon unit of the piperidine ring of anabasine(35); the major alkaloid of Nicotiana glauca,[9] of sedamine(36), an alkaloid of Sedum acre,[10] of N-methylpelletierine(34), a base found in Sedum sarmentosum[11] and in Punica granatum,[12] of lobinaline(37), the major alkaloid of Lobelia cardinalis,[13] and of lobeline(38), an alkaloid of Lobelia syphilitica.[14]

Radioactive N-methylpelletierine[15] was obtained from S. sarmentosum which had been kept in contact with 6-^{14}C-lysine. Degradation of the alkaloid showed that the activity was localized exclusively at C-6 of the piperidine nucleus, demonstrating nonrandom and nonsymmetrical incorporation of lysine. Doubly labelled 4,5-$^{3}H_2$, 6-^{14}C-lysine, and 6-^{3}H, 6-^{14}C-lysine were both incorporated into N-methylpelletierine in S. sarmentosum without change of the $^{3}H : ^{14}C$ ratio. These results further strengthen the evidence that an intact C_5N chain, derived from lysine, supplies the skeleton of the piperidine nucleus of the alkaloids, and strongly suggest that the α- rather than the ε-amino group of lysine is lost in the pathway of N-methylpelletierine biosynthesis.

The sidechain of N-methylpelletierine has been shown to be derived from acetate,[16,17] a potential precursor of acetoacetate. However, direct incorporation into the sidechain of an intact three-carbon unit derived from acetoacetate could not be demonstrated.[15]

It is evident from these observations that lysine is incorporated into the piperidine nucleus of N-methylpelletierine in a

nonsymmetrical manner. ϵ-Amino-α-ketocaproic acid(29), arising by α-deamination of lysine, is the likely intermediate. Decarboxylation of its cyclized form, Δ^1-piperideine-2-carboxylic acid(30) gives Δ^1-piperideine(31) whose double bond is now known to be fixed.[18,19] Attachment of the propanone sidechain at C-2, the electrophilic center of the piperideine nucleus furnished N-methylpelletierine (Fig. 6).

Other naturally occurring 2-substituted piperidine alkaloids, such as anabasine and sedamine have been shown to be derived in a similar manner. Thus radioactivity from 2-^{14}C-lysine was incorporated exclusively into C-2 of the piperidine ring of anabasine.[20,21] In isolated roots of *N. glauca*,[22] anabasine contained the same $^{15}N : {}^{14}C$ ratio as that of the administered substrate, ϵ-^{15}N, α-^{14}C-lysine. On the other hand, it contained no ^{15}N when α-^{15}N, α-^{14}C-lysine was used even though ^{14}C was located at the predicted site. Degradation of sedamine, isolated from *S. acre* roots, showed that label from 2-^{14}C- and 6-^{14}C-lysine was incorporated solely into C-2 and C-6 of the piperidine nucleus, respectively.[23] When 6-^{3}H, 6-^{14}C-lysine was administered to *S. acre*, the sedamine isolated showed a $^{3}H : {}^{14}C$ ratio identical with that of the precursor.[24] It is evident from these findings that it is the ϵ-amino group of lysine which supplies the nitrogen atom of the piperidine nucleus, and it is the α-carbon atom of lysine which is transformed into C-2 of the piperidine ring. The reaction sequence of incorporation of lysine into these alkaloids is shown in Fig. 6. Δ^1-Piperideine has now been shown to be a precursor of the piperidine nucleus of anabasine.[19] Since incorporation of lysine and of Δ^1-piperideine takes place in a nonsymmetrical manner, a symmetrical

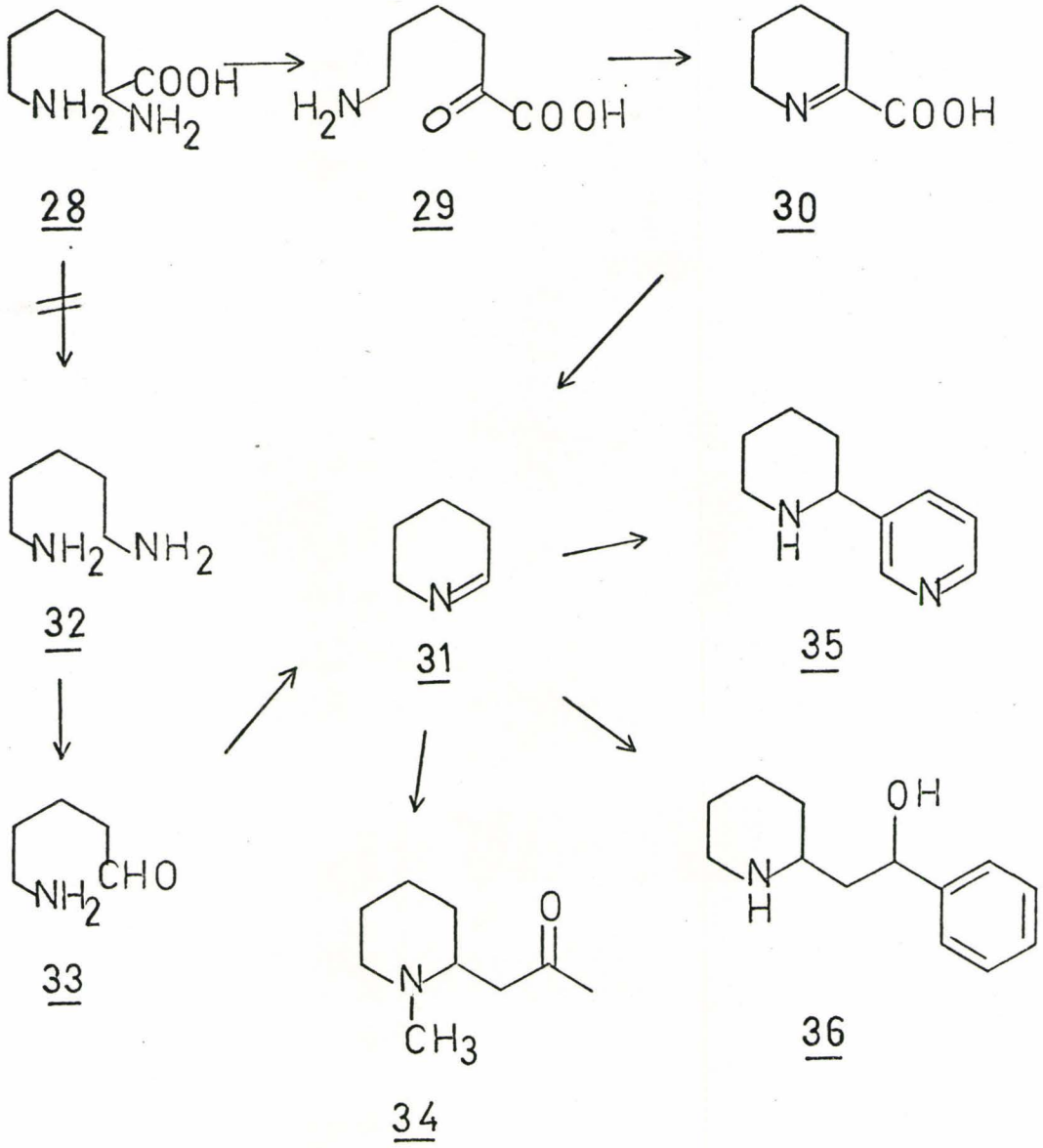
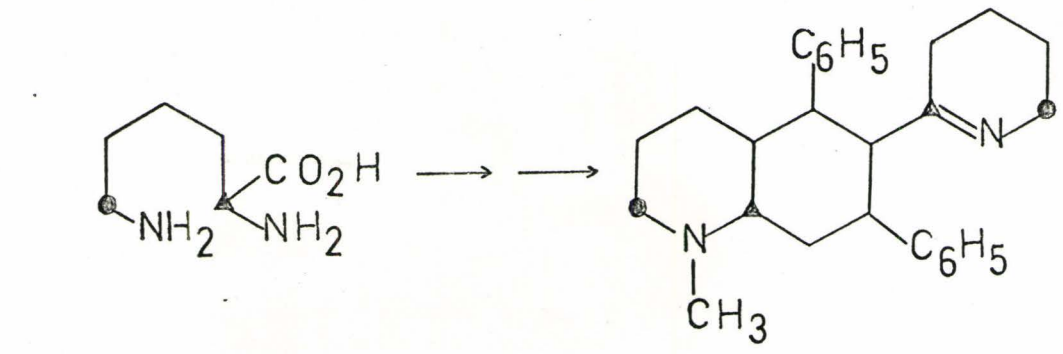


Fig. 6a. Incorporation of lysine into the piperidine alkaloids.



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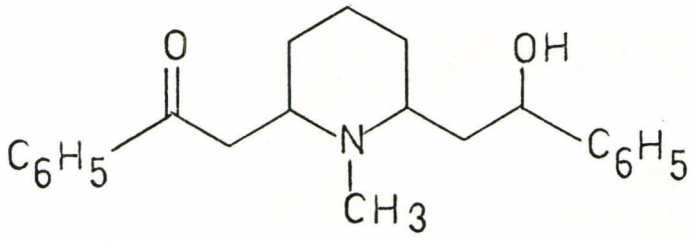

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Fig.6b. Incorporation of lysine into the piperidine alkaloids.

compound such as cadaverine (32) cannot be a normal intermediate. Nevertheless, cadaverine has been shown to be incorporated into anabasine,[21] presumably because it is convertible into Δ^1-piperideine when supplied to the system. The utilization of cadaverine in the N. glauca plant is now considered as an "aberrant reaction".[19]

Only preliminary results are available for the biosynthesis of the Lobelia alkaloids. The piperidine nuclei of lobinaline (37) and of lobeline (38) have been shown to arise from lysine.[25,16] Partial degradation showed that lysine entered the piperidine nucleus of lobinaline non-symmetrically, a mode of incorporation similar to that found to occur in other lysine-derived piperidine alkaloids.

(iii) Biosynthesis of Lycopodine

The biosynthesis of lycopodine[26,27] was studied in intact plants and in excised shoots of the clubmoss Lycopodium tristachyum Pursh. Radioactivity from labelled acetate was incorporated into lycopodine. Approximately half of the activity present in the intact lycopodine isolated from an experiment in which 1-^{14}C-acetate had been administered to the plant was localized at C-15 and C-16, whereas these two carbon atoms contained only 21% of the activity of lycopodine when 2-^{14}C-acetate served as the precursor. When ^{14}C-lysine was administered to the plants, the lycopodine isolated was also active. Both C-5 and C-9 contained a quarter of the activity present in the intact lycopodine regardless of whether 2-^{14}C or 6-^{14}C-lysine had been the precursor. One of the degradation products, 7-methyltetrahydroquinoline (44), (representing C-7 to -16 of lycopodine) contained about 50% of the activity of the alkaloid isolated from 2-^{14}C-lysine feeding experiment. Since C-9 contained 25%

of the activity, it was likely that the remaining 25% resided at C-13. The remaining 25% of the activity of lycopodine was inferred to be localized at C-1. The observed distribution of activity leads to the inference that two C_5-units derived from lysine are incorporated into the two halves of lycopodine with equal efficiency. These results indicate that lycopodine does not originate from two polyacetate chains as suggested by Conroy, but they are consistent with the pelletierine hypothesis (with modification).

The observed distribution of label may be interpreted as follows. Condensation of lysine and acetoacetate (derived from two acetate units) gives pelletierine which dimerises to yield an intermediate from which lycopodine is derived. It must be noted that lysine is incorporated into lycopodine via a symmetrical intermediate, in contrast to its mode of incorporation into N-methylpelletierine in Sedum sarmentosum.

Pelletierine has been tested as precursor of lycopodine in L. tristachyum. When 4,5-$^{3}H_2$, 2-^{14}C-pelletierine was administered to the plants, the lycopodine isolated showed a $^{3}H : {}^{14}C$ ratio identical to that of the precursor. But whereas 7-methyltetrahydroquinoline contained 94% of the activity present in the intact alkaloid, C-5 of the alkaloid contained no radioactivity. These results indicate that lycopodine is not a modified dimer of pelletierine. A pelletierine unit serves as a precursor of only one C-8 unit, C-9 to -16, of the lycopodine molecule. Evidence for the intact incorporation of a pelletierine moiety into the C_8-unit, C-9 to -16, is strengthened by the results of the experiment with 2,3'-$^{14}C_2$-pelletierine. All the activity of the lycopodine obtained from this feeding experiment was confined to the quinoline derivative

(C-7 to -16), and the fraction of activity in C-15,16 was identical with that at the C-methyl group of the precursor.

These results establish conclusively, that radioactivity from lysine, acetate and pelletierine is incorporated nonrandomly into lycopodine, and that these compounds are thus specific precursors of the alkaloid.

The mode of incorporation of acetate is incompatible with the polyacetate hypothesis of lycopodine biosynthesis, but conforms entirely to the prediction of the pelletierine hypothesis. The mode of incorporation of lysine and of pelletierine was unexpected, however, in terms of this hypothesis. Whereas the hypothesis demanded incorporation of two pelletierine units, only one was incorporated. Whereas nonsymmetrical incorporation of lysine was expected, entry of lysine in fact took place by way of a symmetrical intermediate.

It was the objective of this thesis to gather further evidence on the biosynthesis of the Lycopodium alkaloids.

(iv) Some Outstanding Problems in the Biosynthesis of the Lycopodium Alkaloids

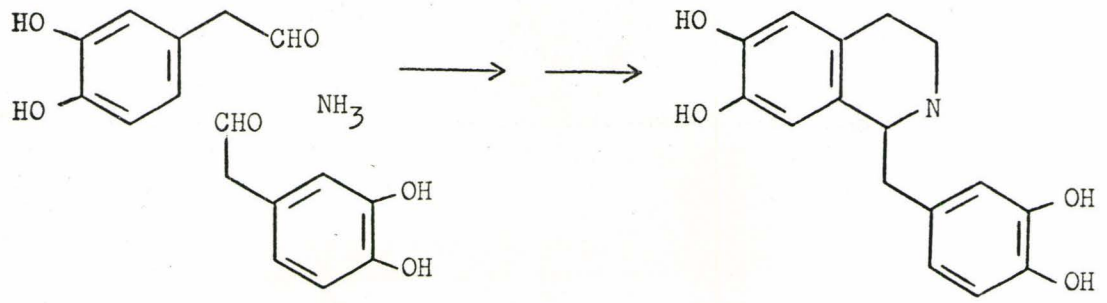
The fact that label from 2-^{14}C-lysine and from 6-^{14}C-lysine is distributed equally among carbon atoms 1,5,9 and 13 of the intact lycopodine suggests that a symmetrical intermediate lies on the pathway from lysine to lycopodine. Cadaverine (32), a symmetrical molecule, is most likely to be the intermediate in question. Decarboxylation of 2-^{14}C-lysine and of 6-^{14}C-lysine would yield 1-^{14}C-cadaverine, which, being a symmetrical molecule, would be incorporated symmetrically into lycopodine. It is well established that in microorganisms cadaverine

is derived from lysine by decarboxylation. The enzyme is L-lysine decarboxylase and there is evidence that pyridoxal-5'-phosphate is the coenzyme.[28] Enzymatic oxidation of cadaverine, catalyzed by the diamine oxidase, to 5-aminopentanal (33) which spontaneously cyclizes to Δ^1-piperideine (31) is well documented.[29, 30,31] Synthesis of pelletierine under chemical conditions, by condensation of Δ^1-piperideine and acetoacetic acid, was first carried out successfully in 1949.[32] This chemical model strengthens the view that Δ^1-piperideine is the immediate precursor of the heterocyclic nucleus of the piperidine alkaloids.

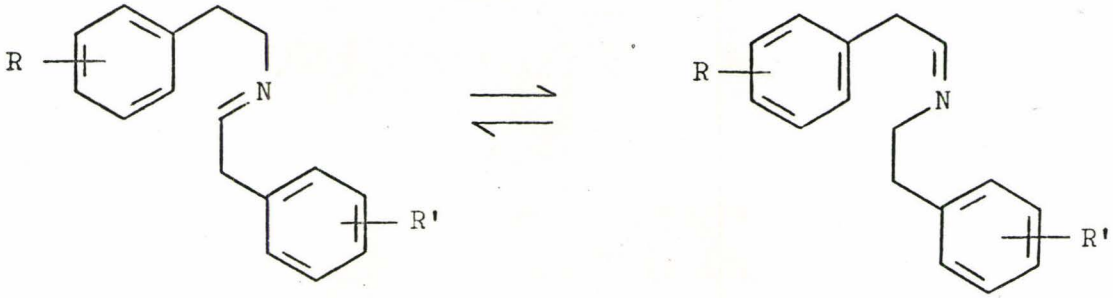
Thus it is desirable to test whether or not the predicted intermediates, cadaverine and Δ^1-piperideine are indeed incorporated into lycopodine. Radioactive cadaverine and Δ^1-piperideine should be administered into the plants. If 1,5-^{14}C-cadaverine is incorporated into lycopodine, it is anticipated that C-1, -5, -9 and -13 should each carry 25% of the activity of the intact alkaloid. If 2-^{14}C-Δ^1-piperideine is incorporated into the alkaloid, the pattern of labelling will depend upon the mode of its entry to lycopodine. However, it is also anticipated that C-1, -5, -9 and -13 should each carry part or all of the activity present in the intact alkaloid.

The fact that lysine and acetate are both incorporated equally into two halves of lycopodine leads to the inference that lycopodine is a true dimer of a C_8N unit. It had been assumed that biosynthetic utilization of two units of a single radioactive precursor to form an end product would inevitably be associated with equal labelling in the two segments of the latter. However the assumption is not valid. Equal

labelling of the two halves of a dimeric product is obligatory only if two identical units (II) are directly involved in the "doubling step"[33] (direct doubling), or if the intermediate formed from the combinations of two non-identical molecules becomes symmetrical[34] (III). If, however,



(II)



(III)

the product is formed by combination of two non-identical molecules and a symmetrical intermediate does not pertain, then the ratio of radioactivities in the two halves of the product will depend on a number of factors. The most important of these are:

(a) the number and rate of metabolic steps in each branch of the sequence prior to 'doubling'

(b) the size of the metabolic pool of each branch intermediate

(c) possible utilization and feed-in of branch and 'dimeric' intermediates

(d) the turn-over rate of the product

Equal incorporation into both halves of the product will be approached in a single-dose feeding experiment at infinite time, provided there is no leakage or feed-in of the intermediates, and provided the product continuously accumulates.

The equal labelling in both halves of lycopodine obtained from lysine and acetate feeding experiments appears to indicate that lycopodine is a true dimer of a C_8N unit, consistent with the hypothesis that lycopodine is generated from two pelletierine units whose sidechain is derived from acetate and whose piperidine nucleus arises from lysine. But pelletierine supplies only one C_8 unit, C-9 to -16, of lycopodine. It follows either, that if pelletierine is an obligatory precursor of lycopodine, then the dimerization hypothesis is wrong, or that if the dimerization hypothesis is correct, then pelletierine cannot be an obligatory intermediate in the biosynthesis of lycopodine. Hence it is important to gain additional evidence on the mode of incorporation of pelletierine into lycopodine, and to confirm that it is incorporated into one C_8 unit only.

Further questions must be posed: Why is the mode of incorporation of lysine into pelletierine, the putative intermediate, different in Lycopodium from the manner in which it enters N-methylpelletierine in S. sarmentosum?

If pelletierine is not a true intermediate, what is the true monomeric precursor of lycopodine, and what is the explanation for the incorporation of pelletierine?

Does the same sequence and the same type of incorporation of lysine and pelletierine apply to other Lycopodium alkaloids, such as

cernuine, whose structure is entirely different from that of lycopodine?

It was the objective of this work to attempt to gain evidence which might contribute to a solution of these questions.

II. BIOSYNTHESIS OF LYCOPODINE

(i) Materials and Methods

Lycopodium tristachyum, which contains lycopodine as the major alkaloid, was chosen for the biosynthetic experiments carried out in the present work. Feeding experiments were carried out by keeping the fresh cuttings of the plant in contact with the radioactive substrate for two days.

Four feeding experiments were performed and radioactive lycopodine was isolated in each case. Table 1 summarizes all the pertinent information.

The degradation scheme[27] outlined in Fig. 7 was used in determining the pattern of labelling of the intact lycopodine. Carbon 5 was isolated as the carboxyl group of benzoic acid and carbon 9 was isolated as formic acid. The 7-methyltetrahydroquinoline isolated represented C-7 to -16 of lycopodine.

(ii) Further Precursors of the Lysine-Derived Moieties of Lycopodine

The radioactive lycopodine obtained from the feeding experiment with 1,5-^{14}C-cadaverine was degraded by the series of reactions described earlier. Each of C-5 and C-9, which were isolated individually, contained 25% of the activity present in the intact alkaloid. The quinoline derivative contained half of the activity of the alkaloid (Table 2). It is likely that the activity was distributed equally between C-9 and C-13. The remaining 25% of the activity of the alkaloid is reasonably assumed to reside at C-1 as discussed before in the lysine feeding experiments.[27]

TABLE 1

INCORPORATION OF PELLETIERINE, CADAVERINE AND Δ^1-PIPERIDEINE INTO LYCOPODINE. CHEMICAL AND RADIOCHEMICAL YIELDS

Expt. No.	Compound Administered	Nominal Total Activity mCi	Nominal Specific Activity mCi / mmole	Weight of dry plant (g)	Yield (mg)	Lycopodine Specific Activity (Counts min^{-1} $mmole^{-1}$) x 10^{-4}
1	6-^{14}C-Pelletierine	0.04	1.0	46	133	13.1 ± 0.27
2	1,5-^{14}C-Cadaverine[a]	0.06	3.2	36	100	72.8 ± 1.5
3	2-^{14}C-Δ^1-Piperideine	0.1	0.4	35	88	104.4 ± 1.6
4	6-^{14}C-Δ^1-Piperideine	0.1	0.8	38	104	190.4 ± 4.1

[a] New England Nuclear.

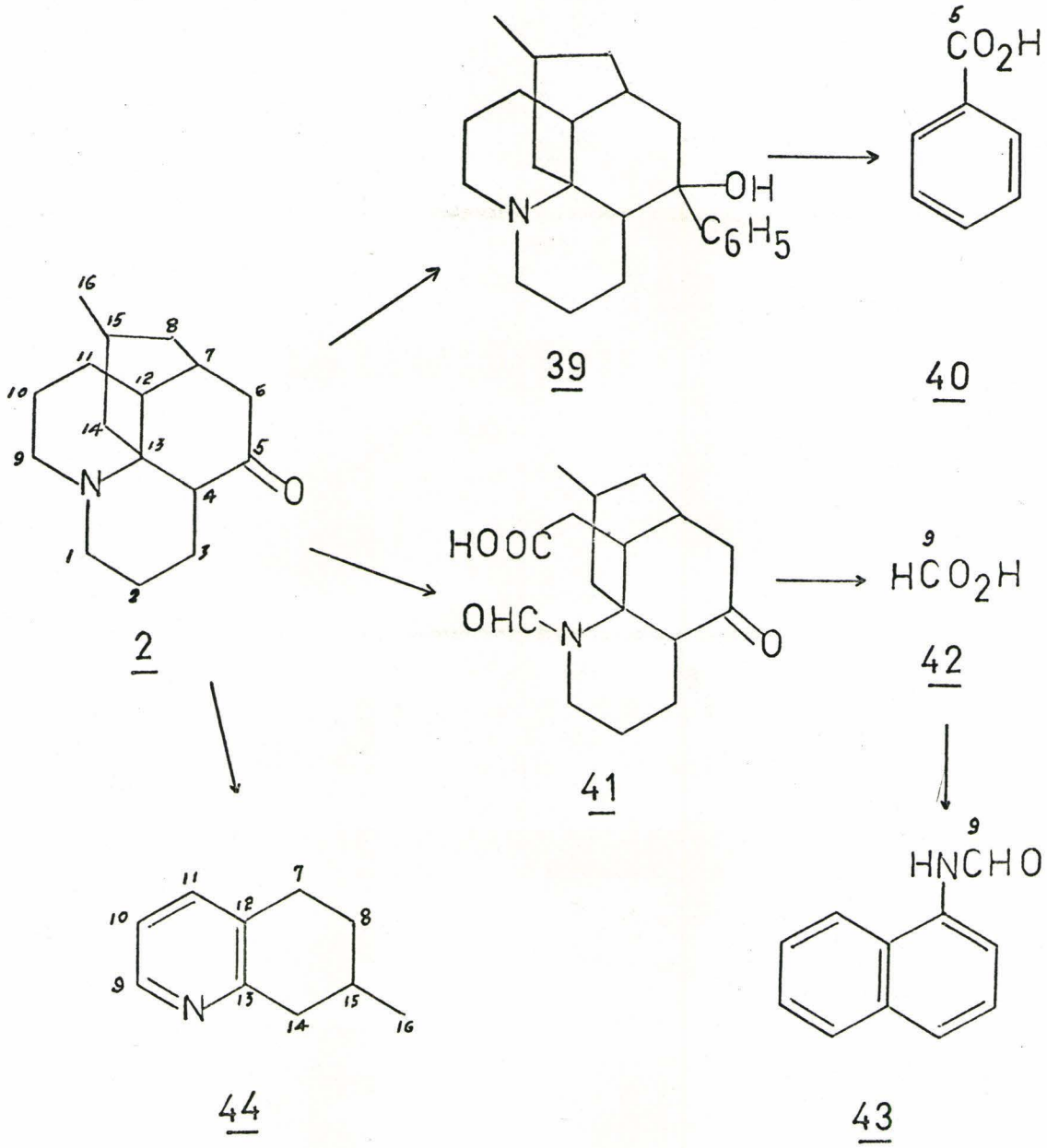


Fig.7. Degradation of lycopodine.

TABLE 2

INCORPORATION OF CADAVERINE AND PELLETIERINE INTO LYCOPODINE

Precursor	1,5-^{14}C-Cadaverine		6-^{14}C-Pelletierine	
Expt. No.	2		1	
Product	SA[a]	RSA[b]	SA	RSA
Lycopodine	30.76 + 0.62[c]	100 ± 2	8.7 ± 0.2[d]	100 ± 2
Benzoic Acid (C-5)	7.73 ± 0.38	25.1 ± 1.3	-	-
Formic Acid (as α-Naphthylamide) (C-9)	7.55 ± 0.21	24.5 ± 0.8	9.0 ± 0.2	103.8 ± 2.7

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

[b] Relative specific activity, percent (lycopodine = 100)

[c] Obtained by approximately 25 fold dilution of the lycopodine, specific activity (7.28 ± 0.15) x 10^5 counts min^{-1} $mmole^{-1}$, with inactive lycopodine.

[d] Obtained from the original lycopodine (specific activity (13.1 ± 0.27) x 10^4 counts min^{-1} $mmole^{-1}$) of Expt. 1 by dilution with inactive carrier.

These results show that cadaverine is incorporated into lycopodine specifically and strongly suggest that it is an intermediate on the pathway of the incorporation of lysine into lycopodine, as predicted in Section I (iv).

Degradation of the radioactive samples of lycopodine obtained from the feeding experiments with Δ^1-piperideine showed a different incorporation pattern. Lycopodine derived from 2-^{14}C-Δ^1-piperideine contained 50% of the activity at C-5, and none at C-9. When 6-^{14}C-Δ^1-piperideine was fed, there was no activity at C-5 but 50% of the activity of the alkaloid was localized at C-9. On the other hand, the 7-methyltetrahydroquinoline (44) harboured half of the activity present in lycopodine regardless of whether 2-^{14}C- or 6-^{14}C-Δ^1-piperideine was administered (Table 3). It is very likely that C-5 and C-13 each contained 50% of the activity of lycopodine when 2-^{14}C-Δ^1-piperideine was the precursor, and C-1 and C-9 each carried half of the activity present in lycopodine when 6-^{14}C-Δ^1-piperideine was used. The degradation results indicate that Δ^1-piperideine is incorporated into lycopodine nonrandomly, and that it is thus a specific precursor of the alkaloid.

The mode of incorporation of cadaverine and Δ^1-piperideine into lycopodine suggests that these compounds are obligatory precursors of lycopodine, as predicted. The results are entirely consistent with the following sequence of steps from lysine into lycopodine (Fig. 8). Lysine (28) is converted into cadaverine (32) by enzymatic decarboxylatation. Enzymatic oxidation of cadaverine gives 5-aminopentanal (33) which is in equilibrium with the ring closure derivative, Δ^1-piperideine (31) whose

TABLE 3

INCORPORATION OF Δ^1-PIPERIDEINE INTO LYCOPODINE

Precursor	2-^{14}C-Δ^1-Piperideine				6-^{14}C-Δ^1-Piperideine			
Expt. No.	3				4			
Product	SA[a]	RSA[b]	SA	RSA	SA	RSA	SA	RSA
Lycopodine	3.14 ± 0.05[c]	100 ± 2	0.384 ± 0.11[c]	100 ± 3	13.82 ± 0.29[d]	100 ± 2	2.42 ± 0.06[d]	100 ± 2
Benzoic Acid (C-5)	1.62 ± 0.05	51.7 ± 1.6	-	-	0.14 ± 0.03	0.98 ± 0.18	-	-
Formic Acid (as α-naphthylamide) (C-9)	0.03 ± 0.01	1.1 ± 0.2	-	-	-	-	1.26 ± 0.02	51.8 ± 1.7
7-Methyltetrahydroquinoline Hydrochloride (C-7 to -16)	-	-	0.190 ± 0.007	49.4 ± 2.3	-	-	-	-

[a] Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}

[b] Relative specific activity percent (lycopodine = 100)

[c] Obtained from the original lycopodine (specific activity (104.4 ± 1.6) x 10^4 counts min^{-1} $mmole^{-1}$) of Expt. 3 by dilution with inactive lycopodine.

[d] Obtained from the original lycopodine (specific activity (190.4 ± 4.1) x 10^4 counts min^{-1} $mmole^{-1}$) of Expt. 4 by dilution with carrier.

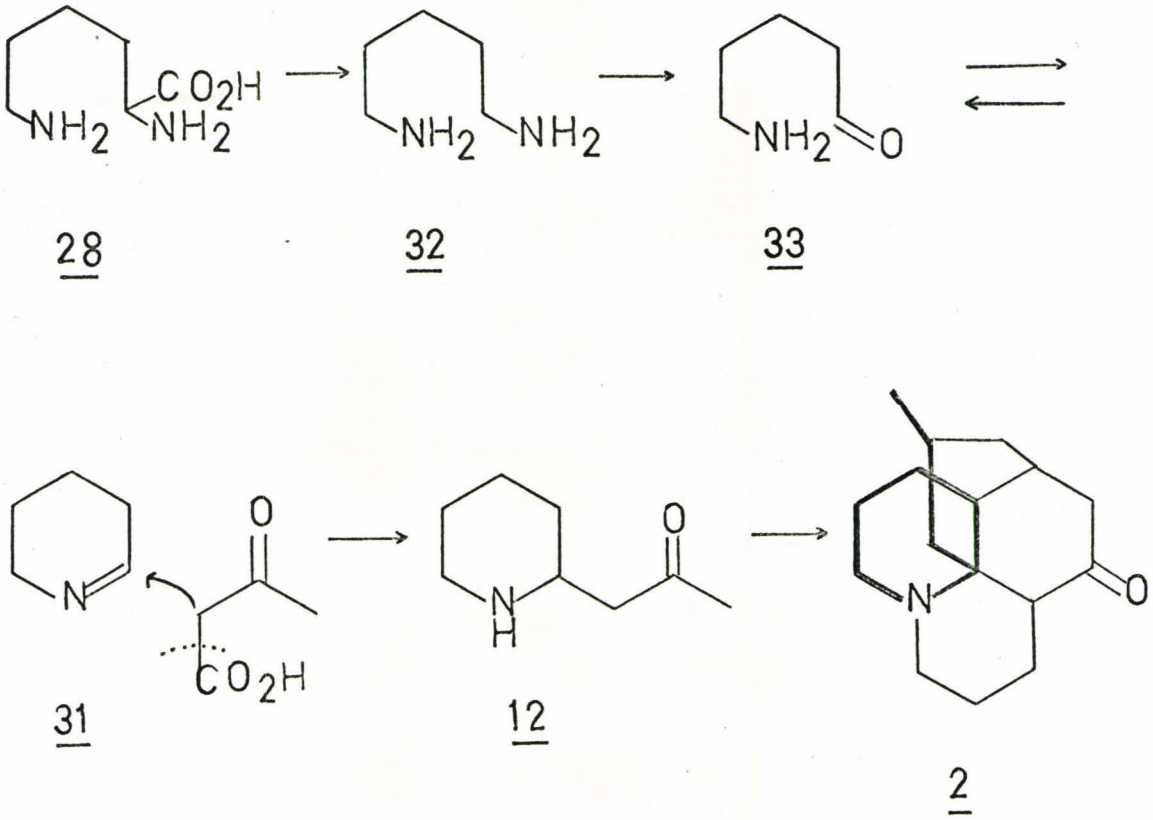


Fig.8. The mode of incorporation of lysine into lycopodine.

double bond is found to be fixed in this system, in agreement with results reported[18,19] in other plant species. Attachment of a C_3 unit, derived by decarboxylation of acetoacetate, at C-2, the electrophilic center of the piperideine nucleus affords pelletierine(12) which supplies the C_8 unit, C-9 to -16, of lycopodine. The exact nature of the precursor of the other C_8 unit of lycopodine is not known.

Although pelletierine has been shown to be incorporated into one C_8 unit of lycopodine, its intermediacy on the pathway of the biosynthesis of lycopodine has been questioned. Again, the equal labelling into two halves of lycopodine of activity from cadaverine and from Δ^1-piperideine strengthens the point that lycopodine is derived from direct coupling of two identical C_8N units. Possible monomeric precursors other than those suggested[27] will be discussed in a later section. The suggestions must accommodate the following observations.

1. Lysine supplies two C_5 units, C-1 to -5 and C-9 to -13 of lycopodine with equal efficiency.
2. The mode of incorporation of lysine is symmetrical. Cadaverine and Δ^1-piperideine are intermediates on the pathway from lysine to lycopodine.
3. The C_5 unit, C-6 to -2 of Δ^1-piperideine, is incorporated equally into the two segments, C-1 to -5, and C-9 to -13, of lycopodine.
4. The three-carbon fragment of acetoacetate furnishes the two sections, C-6 to -8, and C-14 to -16, of lycopodine with equal efficiency.

(iii) Further Evidence on the Specific Incorporation of Pelletierine into Lycopodine

Even though incorporation of an intact pelletierine moiety into the C_8 unit, C-9 to -16, of lycopodine is demonstrated by experiments with 4,5-$^{3}H_2$, 2-^{14}C-pelletierine and 2,3'-$^{14}C_2$-pelletierine, the entire activity of the product derived from these precursors was not accounted for, due to limitations of the degradation procedures.[27]

To complete the evidence on the specific incorporation of pelletierine, an experiment with 6-^{14}C-pelletierine was performed. The radioactive lycopodine obtained from this feeding experiment was degraded. Formic acid, obtained from C-9 of lycopodine, contained all the radioactivity of the intact alkaloid (Table 2). This result clearly and conclusively demonstrates that only one C_8 unit, C-9 to -16, of lycopodine is derived from pelletierine.

III. BIOSYNTHESIS OF CERNUINE

(i) Materials and Methods

Lycopodium cernuum,* the Lycopodium species from which lycocernuine and cernuine were isolated, was chosen for the biosynthetic experiments carried out in the present work. This species, found in Jamaica, is the first species of Lycopodium examined which does not contain alkaloids closely related to lycopodine.[8]

A number of radioactive substrates were tested as precursors of the alkaloids of L. cernuum. Two methods were used for the administration of tracers. In each case feeding experiments with intact plants were carried out by the wick method. In addition fresh cuttings were packed with cut stem downward into a 100 ml beaker containing tracer solution. Six feeding experiments were performed and radioactive cernuine was isolated from each. However, the lycocernuine isolated in each case contained only residual activity. Pertinent information on the six feeding experiments is summarized in Table 4.

Degradation of cernuine was carried out by the scheme outlined in Fig. 9. The acetic acid representing C-15 and -16 of cernuine was obtained by Kuhn-Roth oxidation. β-Alanine (48) and γ-aminobutyric acid (49), originally thought to represent exclusively C-1 to -3 and

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*A voucher specimen of the species used in these experiments is deposited in the herbarium of the University of the West Indies, Mona, Jamaica. We are greatly indebted to Dr. C. D. Adams for authenticating the samples.

TABLE 4

INCORPORATION OF PRECURSORS INTO CERNUINE

Expt. No.	Compound Administered	Nominal Total Activity mCi	Nominal Specific Activity mCi / mmole	Weight of dry plant (g)	Cernuine Yield (mg)	Cernuine Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}
5	2-^{14}C-DL-Lysine[a]	0.1	3.3	77	12.5	32.1 ± 1.2
6	4,5-$^{3}H_2$, 2-^{14}C-DL-Lysine			77	23	16.5 ± 0.8 (^{14}C)
	from 4,5-$^{3}H_2$, DL-lysine[a]	1.0	5.4×10^3			
	from 2-^{14}C-DL-lysine[a]	0.1	3.3			
7	6-^{14}C-DL-Lysine[b]	0.1	48	63	20	33.2 ± 1.3
8	1,5-^{14}C-Cadaverine[a]	0.1	3.2	92	30	27.8 ± 1.2
9	6-^{14}C-Δ^7-Piperideine	0.1	0.8	92	31	15.8 ± 0.6
	from 6-^{14}C-DL-lysine[b]	0.1	48			
10	4-^{3}H, 6,2'-$^{14}C_2$-Pelletierine			164	45	17.8 ± 0.6 (^{14}C)
	from[c] 4-^{3}H-Pelletierine	0.4	10			
	from[c] 6-^{14}C-Pelletierine	0.03	1			
	from[c] 2'-^{14}C-Pelletierine	0.02	0.25			

[a] New England Nuclear

[b] Commissariat à l'Energie Atomique, France

[c] See experimental

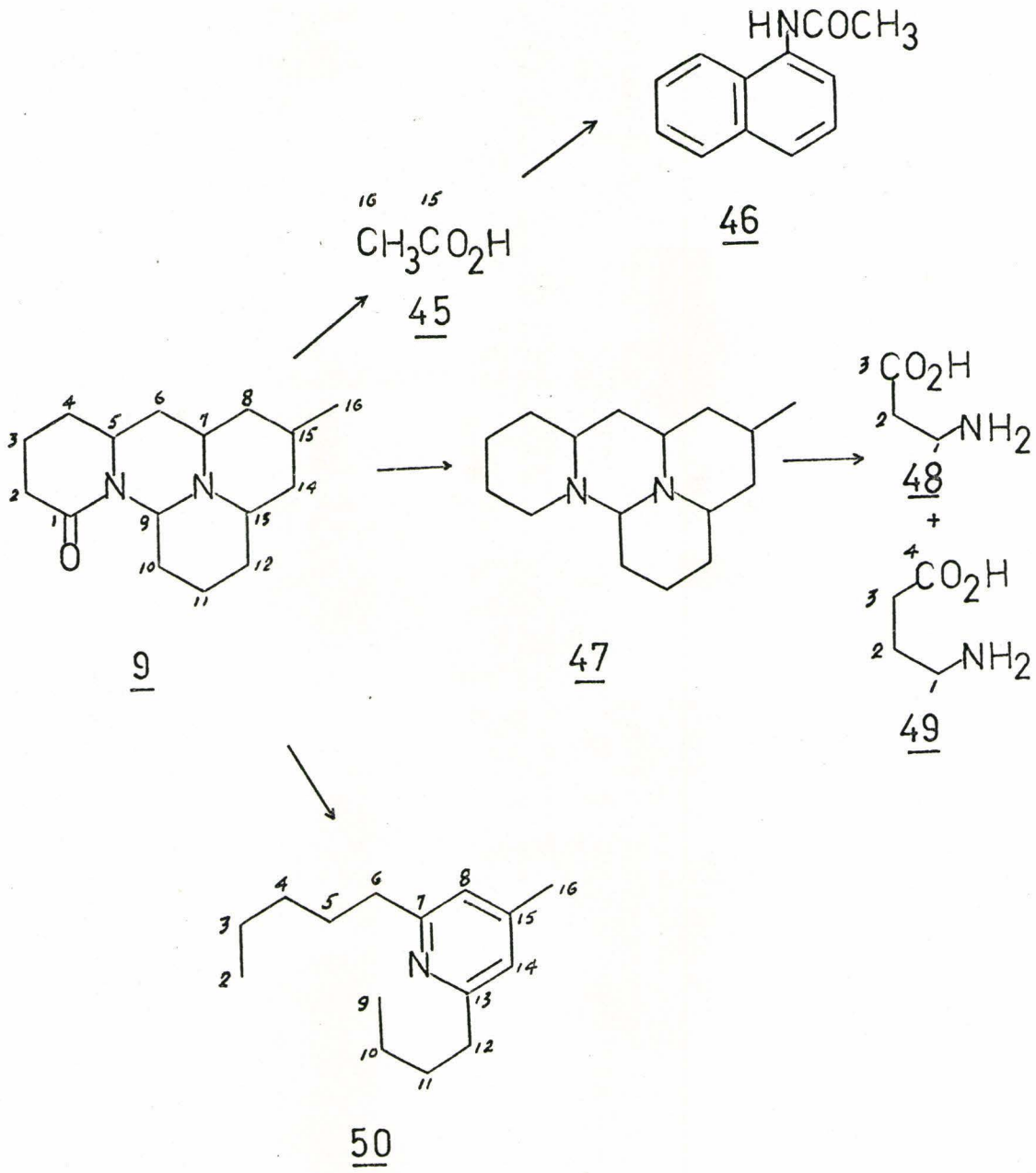

Fig.9. Degradation of cernuine.

C-1 to -4 respectively* were isolated from the chromic acid oxidation of the lithium aluminium hydride reduction product(s) of cernuine. Dehydrogenation of cernuine gave 2-n-butyl-4-methyl-6-n-pentylpyridine (50), representing all carbon atoms but C-1 of cernuine.

(ii) The Chemistry of the Degradation of Cernuine

Cernuine does not lend itself readily to the reactions commonly employed to degrade the carbon skeleton of a molecule and the isolation of the individual carbon atoms. The carbonyl group was thought to be reactive toward phenylation, and it was hoped that oxidation of the phenylation product would give benzoic acid (54) whose carboxyl group represented C-1 of the alkaloid. However, the attempted phenylating of the carbonyl group with phenyllithium under usual conditions was unsuccessful.

It is reported[8] that lithium aluminium hydride in ether reduced the amide carbonyl group of cernuine (9), to give the diacidic base dihydrodeoxycernuine (47). Lithium aluminium hydride in ether, however, reduced both allocernuine (55) and epiallocernuine (51) to dihydrodeoxy-epiallocernuine (51) which could arise only with epimerization at C-9 of the former. Due to the strained nature of its ring system, allocernuine (55) is isomerized to the thermodynamically favoured epiallocernuine (57) when refluxed in methanol even in the absence of reducing agents.[35] This can be rationalized[36] in terms of the hexahydropyrimidine system present in allocernuine. In a polar solvent, the zwitterion (56) must be formed to a certain extent. Ring closure of 56 results in the

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*See page 36.

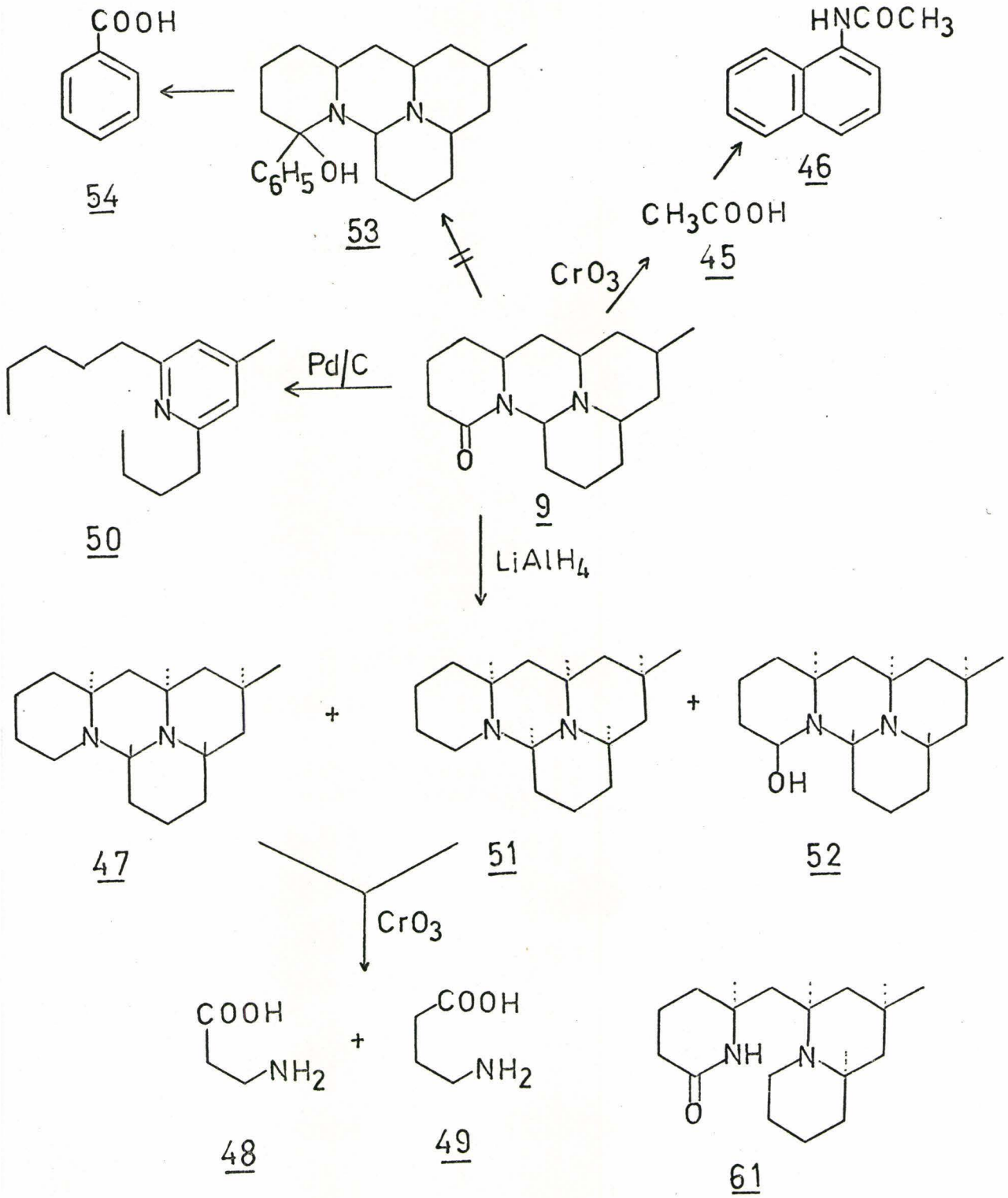

Fig.10. Degradation of cernuine.

more stable isomer epiallocernuine (57) (Fig. 11). The isomerization of allocernuine to the epiallo series during the hydride reduction can also be explained on the same grounds.[35] The isomerization, catalyzed by aluminium hydride, a Lewis acid, could take place before reduction, or could conceivably occur at the carbinolamine stage of the reduction, as depicted in Fig. 11.

The reduction of cernuine with lithium aluminium hydride was carried out under several different conditions. When commercially available anhydrous ether was used as solvent, a mixture of products was obtained, of which dihydrodeoxycernuine (47) was the major component. Minor products were not positively identified but were presumably dihydrodeoxyepiallocernuine (51) and the partially reduced product, dihydrocernuine (52). This assignment was based on the observations that the mass spectrum of the mixture of products showed two molecular weight peaks at m/e 264 and 248. The mixture showed three components on VPC however. The major peak represented dihydrodeoxycernuine (47) (I.R. no NH absorption, no carbonyl absorption, m/e 248 after purification by VPC). The minor peaks did not correspond to that of dihydroallocernuine (61).* One of the minor peaks, however, did correspond to that of dihydrodeoxyepiallocernuine.* When the ether used as solvent was dried with sodium hydride, the major reduction product was again dihydrodeoxycernuine. It was accompanied by a small amount ($<7\%$) of its isomer dihydrodeoxyepiallocernuine. (Characteristics of reduction

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*We are greatly indebted to Professor W. A. Ayer for supplying authentic samples of these compounds.

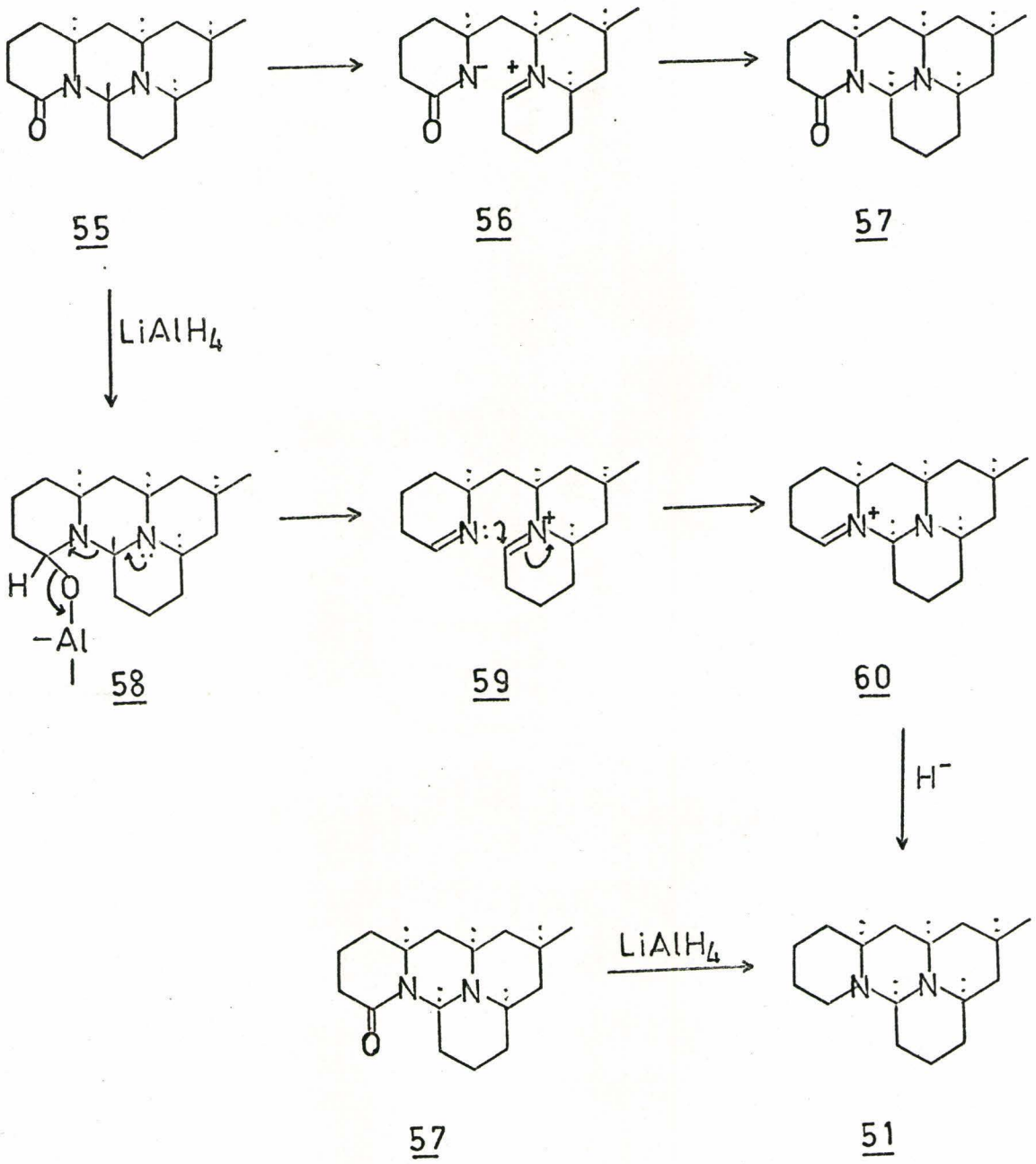

Fig.11. Reduction and rearrangement of allocernuine.

mixture: Mass spectrum showed one molecular peak only, m/e 248. Vapor phase chromatography showed two components, of which the major one was dihydrodeoxycernuine and the minor one corresponded to dihydrodeoxyepiallocernuine. I.R. showed absence of NH and carbonyl absorption.) These results can be rationalized as follows: Commercially available "anhydrous" ether contains sufficient water to destroy some of the lithium aluminium hydride, so that the reduction does not go to completion. The isomerization of cernuine to the epiallo series is explicable as follows: In the course of reduction, the aluminium hydride catalyzes the isomerization as depicted in Fig. 12. (c.f. isomerization of allocernuine). However, this rearrangement is so unfavourable that it takes place to a small extent only.

Chromic acid oxidation of dihydrodeoxycernuine liberated β-alanine (48) and γ-aminobutyric acid (49) representing C-1 to -3 and C-1 to -4, respectively, of cernuine.* The dinitrophenyl derivatives of the amino acids were separated by thin layer chromatography and purified by sublimation.

Vigorous catalytic dehydrogenation of cernuine with palladium on charcoal gives 2-n-butyl - 4-methyl - 6-n-pentylpyridine (50).[8] This pyridine derivative represents all carbon atoms, except C-1, of cernuine. For the purpose of radioactivity analysis, the pyridine derivative, an oily liquid, was converted to the crystalline picrolonate derivative. The only other solid derivative which has been described, the chloroplatinate,[8] is not suitable for this purpose.

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*This inference will be modified in the light of later experimental evidence.

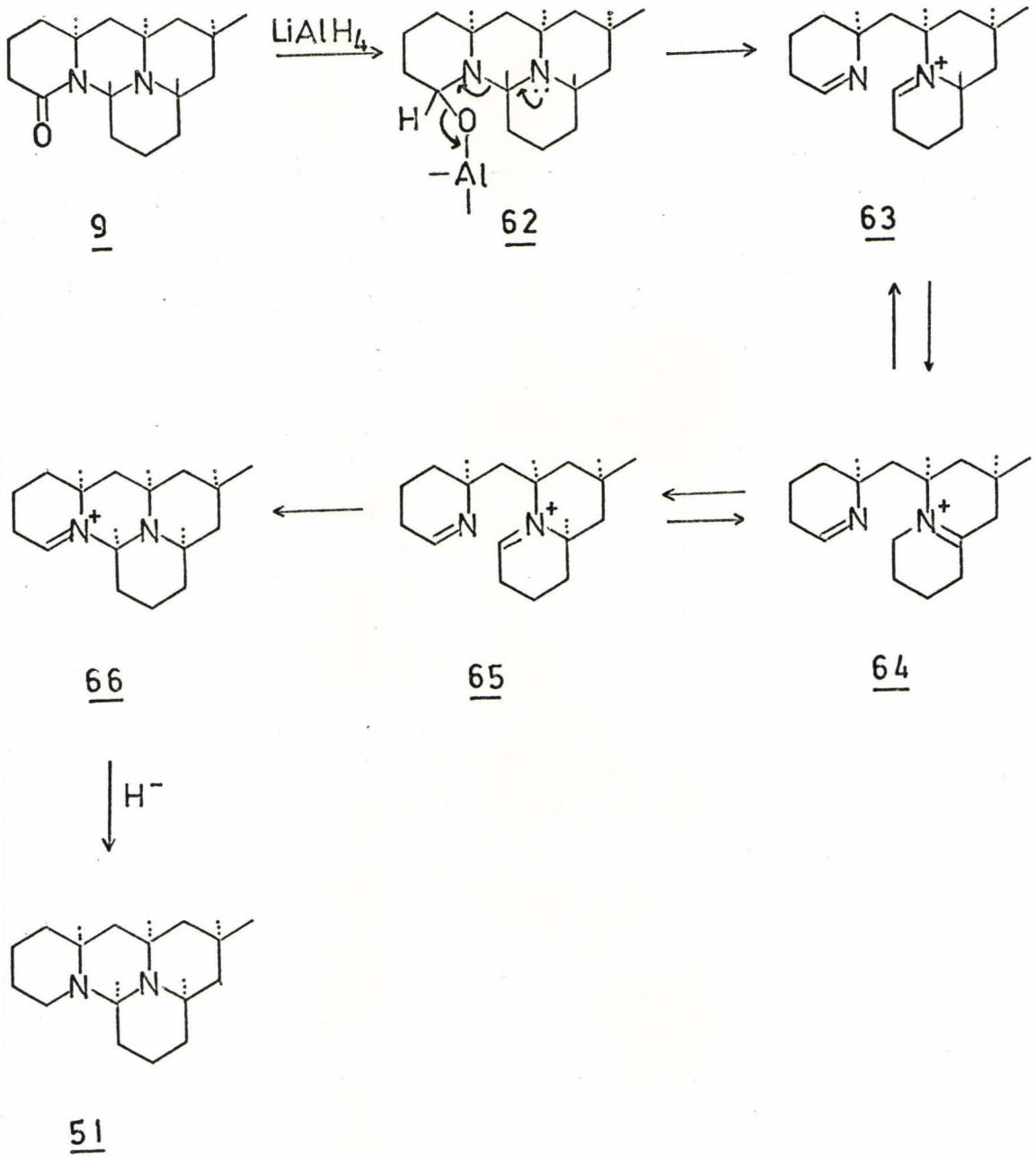


Fig.12. Reduction and rearrangement of cernuine.

The C-methyl group (C-16) of cernuine and the adjacent carbon atom (C-15) are easily isolated as acetic acid by Kuhn-Roth oxidation. The acetic acid was separated from the oxidation mixture by steam distillation and converted into the α-naphthylamide derivative (46) for radioactivity analysis.

(iii) Precursors of Cernuine

The radioactive samples of cernuine isolated from the feeding experiments using labelled lysine, cadaverine, Δ^1-piperideine and pelletierine as precursors, were partially degraded by the reactions already discussed. The results are summarized in Tables 5 - 7.

Even though degradation of the active samples of cernuine was only partial, and the activity of the intact alkaloid was not accounted in its entirety in terms of the activity at individual centers, the results present a clear cut answer to be given to the question, "Is the mode of incorporation of the substrates under investigation, lysine, cadaverine, Δ^1-piperideine and pelletierine, into cernuine similar to or different from the manner in which they enter lycopodine?"

The results shown in Tables 5 - 7 clearly indicate that the incorporation patterns of the precursors into both alkaloids are analogous.

Degradation of cernuine obtained from the plants to which 2-^{14}C- and 6-^{14}C-lysine had been administered, yielded β-alanine, representing C-1 to -3 of cernuine, which contained 25% of the activity present in the intact alkaloid, regardless of which radiomer of lysine had served as the precursor. This result parallels the findings on the incorporation of lysine into lycopodine. Two C_5-units, comprising C-2 to -6 of lysine, are incorporated into the two "halves" of lycopodine (C-1 to -5 and C-9

TABLE 5

INCORPORATION OF LYSINE, CADAVERINE, AND Δ^1-PIPERIDEINE INTO CERNUINE

recursor	2-^{14}C-Lysine		4,5-^{3}H, 2-^{14}C-Lysine		6-^{14}C-Lysine		1,5-^{14}C-Cadaverine		6-^{14}C-Δ^1-Piperideine	
xpt. No.	5		6		7		8		9	
roduct	SA[a]	RSA[b]	SA	RSA	SA	RSA	SA	RSA	SA	RSA
ernuine	2.02 ± 0.06[c]	100 ± 2.8	3.18 ± 0.04[c]	100 ± 1	3.04±0.03[c]	100 ± 1	3.61 ± 0.04[c]	100 ± 1	4.02 ± 0.08[c]	100 ±
NP-β-Alanine	0.479±0.012	23.8 ± 0.9	-	-	-	-	-	-	-	-
-n-Butyl-4-ethyl-6-n-entylpyridine	-	-	2.22 ± 0.02	69.7 ± 1.1	2.22 ± 0.02	73.1 ± 1.1	2.66 ± 0.03	73.8 ± 1.1	2.15 ± 0.02	53.6±
NP-γ- Amino-utyric Acid	0.510 ± 0.034	25.3 ± 1.8								

Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}

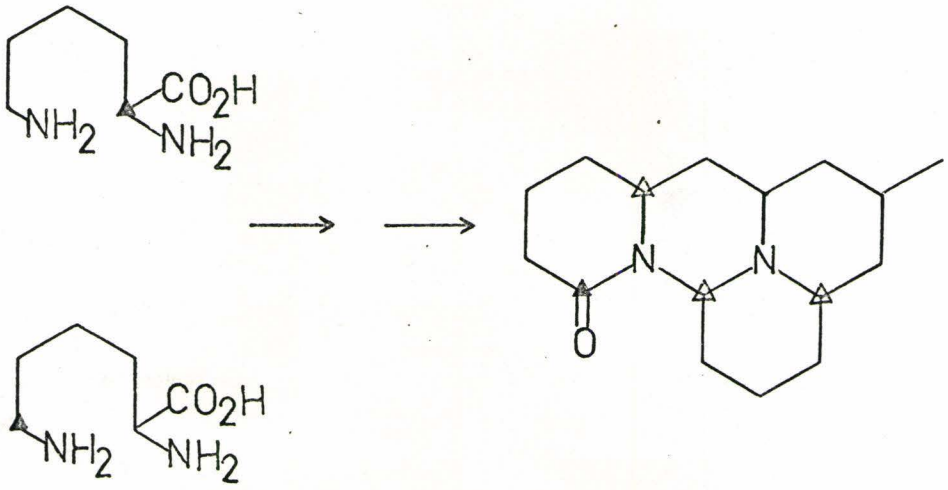
Relative specific activity percent (cernuine = 100)

Obtained from the original samples of cernuine, specific activity [(32.1 ± 1.2), (16.5 ± 0.8), (33.2 ± 1.3), (27.8 ± 1.2), and (15.8 ± 0.6)] x 10^4 counts min^{-1} $mmole^{-1}$, of Expt. 5, 6, 7, 8, and 9 respectively.

to -13) with equal efficiency.[27] One quarter of the activity of the intact alkaloid was recovered at each of two individual carbon atoms of lycopodine. It was inferred that each of these two C_5 units is incorporated into lycopodine in a symmetrical manner and must be derived from lysine by way of a symmetrical intermediate. The recovery of one quarter of the activity of cernuine from 2-^{14}C- and 6-^{14}C-lysine in β-alanine is consistent with the interpretation that two C_5 units, derived from lysine are incorporated into cernuine. Such a mode of incorporation would place 25% of the total activity at each of C-1, C-5, C-9 and C-13 of the alkaloid. Of these carbon atoms only one, C-1, is included in β-alanine, which as predicted contains one quarter of the activity. Equal distribution of activity from 2-^{14}C- and from 6-^{14}C-lysine is explicable only in terms of symmetrical C_5 units, comprising C-2 to -6 of lysine, supplying C-1 to -5 and C-9 to -13 of the alkaloid with equal efficiency, a case parallel to that of lycopodine.

If this interpretation is correct, 75% of the activity of cernuine is inferred to be distributed equally among carbon atoms 5, 9 and 13 (Fig. 13). This prediction is consistent with the finding that the pyridine derivative (50), representing all carbon atoms but C-1 of cernuine, harboured 75% of the activity present in the intact alkaloid regardless of whether 2-^{14}C- or 6-^{14}C-lysine was used as precursor. This result confirms that C-1 of cernuine carries a quarter of the activity, as inferred above.

Cadaverine is incorporated into lycopodine in the same manner as lysine. The evidence now presented shows that its mode of incorporation into cernuine is analogous.

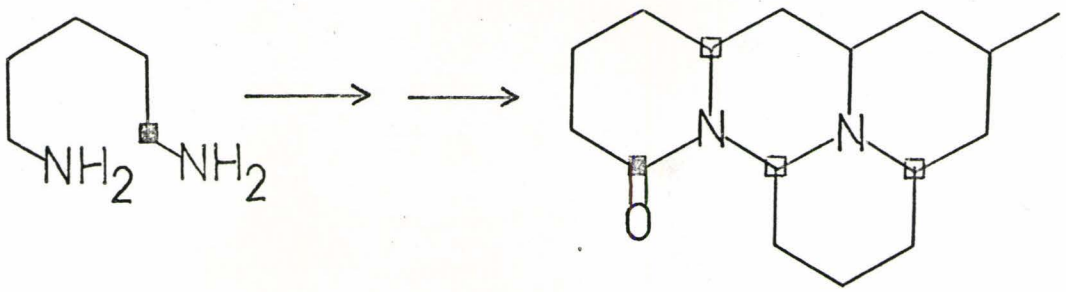


28 9

Fig.13. Incorporation of lysine into cernuine. Sites of activity derived from 2-^{14}C-lysine and 6-^{14}C-lysine, established by degradation (▲) (~25% of total activity, from either radiomer) or inferred(△).

The pyridine derivative contained 75% of the activity of the intact cernuine which was isolated from the feeding experiment with 1-^{14}C-cadaverine. This result shows that a quarter of the activity of cernuine is localized at C-1. This nonrandom incorporation of activity into cernuine from 1-^{14}C-cadaverine shows conclusively that it is a specific precursor of the alkaloid. Cadaverine is also a specific precursor of lycopodine, and activity from 1-^{14}C-cadaverine is distributed equally among carbon atoms 1, 5, 9 and 13. C-1 of cernuine carries a quarter of the activity present in the intact alkaloid when 1-^{14}C-cadaverine is the precursor, similar to the percentage of activity resides at C-1 of lycopodine obtained from the same manner. It is reasonable to infer that the pattern of labelling of cernuine and of lycopodine when 1-^{14}C-cadaverine is used as precursor is identical, i.e., each of carbon atoms 1, 5, 9 and 13 carries a quarter of the activity of the intact alkaloid (Fig. 14). It is important to note that cadaverine is incorporated equally into the two halves of cernuine.

As anticipated, Δ^1-piperideine serves as a specific precursor of cernuine. Half of the activity of the intact cernuine isolated from the feeding experiment with 6-^{14}C-Δ^1-piperideine was localized at C-1 (Table 5). Again, this result corresponds to the findings in the parallel experiments on the incorporation of Δ^1-piperideine into lycopodine, which were described earlier in this thesis. Each of C-1 and C-9 carried half of the activity of lycopodine. Thus C-9 of cernuine is inferred to accommodate the remaining half of the activity. The degradation result indicates that Δ^1-piperideine is a specific precursor of cernuine. The mode of incorporation is consistent with the evidence that the position of the double



32 9

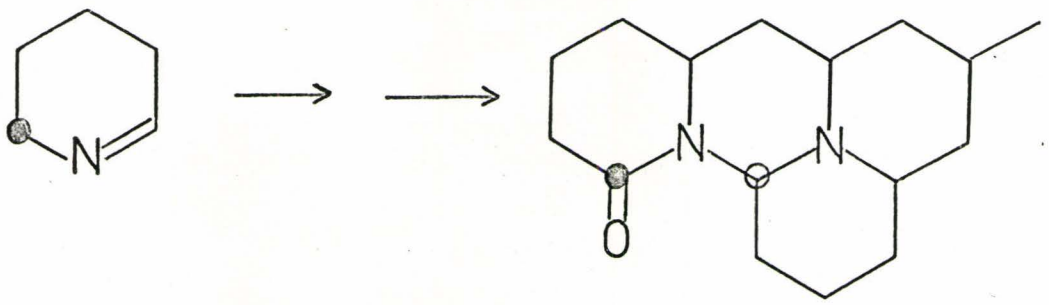
Fig.14. Incorporation of cadaverine into cernuine. Sites of activity derived from 1-^{14}C-cadaverine, established by degradation (■) (~25%) or inferred (□).

bond in Δ^1-piperideine is fixed.[18,19] It follows that two intact moieties of Δ^1-piperideine enter cernuine in a non-symmetrical manner and that the identity of their carbon atoms is maintained. These two units supply the two piperidine nuclei of the alkaloid (C-1 to -5, and C-9 to -13) with equal efficiency (Fig. 15).

The fact that the individuality of C-2 and C-6 of labelled Δ^1-piperideine is maintained within the product, whereas the corresponding carbon atoms, C-2 and C-6, of labelled lysine become identical on route to cernuine strongly suggests the randomization of C-2 and C-6 of lysine must take place at a stage of the metabolic route prior to the formation of Δ^1-piperideine. Once Δ^1-piperideine is formed, the equilibration of these two centers does not occur. Cadaverine, a symmetrical molecule as well as a specific precursor of cernuine is most likely to be the intermediate between lysine and Δ^1-piperideine. It is formed from lysine by decarboxylation[28] and is convertible to Δ^1-piperideine by enzymatic oxidation.[29,30,31] The evidence clearly shows that the sequence lysine $\longrightarrow$ cadaverine $\longrightarrow$ Δ^1-piperideine constitutes part of the biosynthetic pathway to cernuine (Fig. 16).

The quantitative distribution within cernuine, of label from lysine, cadaverine and Δ^1-piperideine (Table 5) shows that in every case each of the two C_8N units contains half of the activity of the intact alkaloid, a case similar to that in lycopodine. As discussed before, equal incorporation of activity into two halves of a "dimeric" product is obligatory only if two identical units of "monomeric" precursor combine to yield a true dimer as a biosynthetic intermediate.

Indications are strong that cernuine is a true dimer of a C_8N unit, consistent with the pelletierine hypothesis or its modification



31 9

Fig.15. Incorporation of Δ^1-piperideine into cernuine. Sites of activity from 2-^{14}C-Δ^1-piperideine, established by degradation (●) (~50%) or inferred (○).

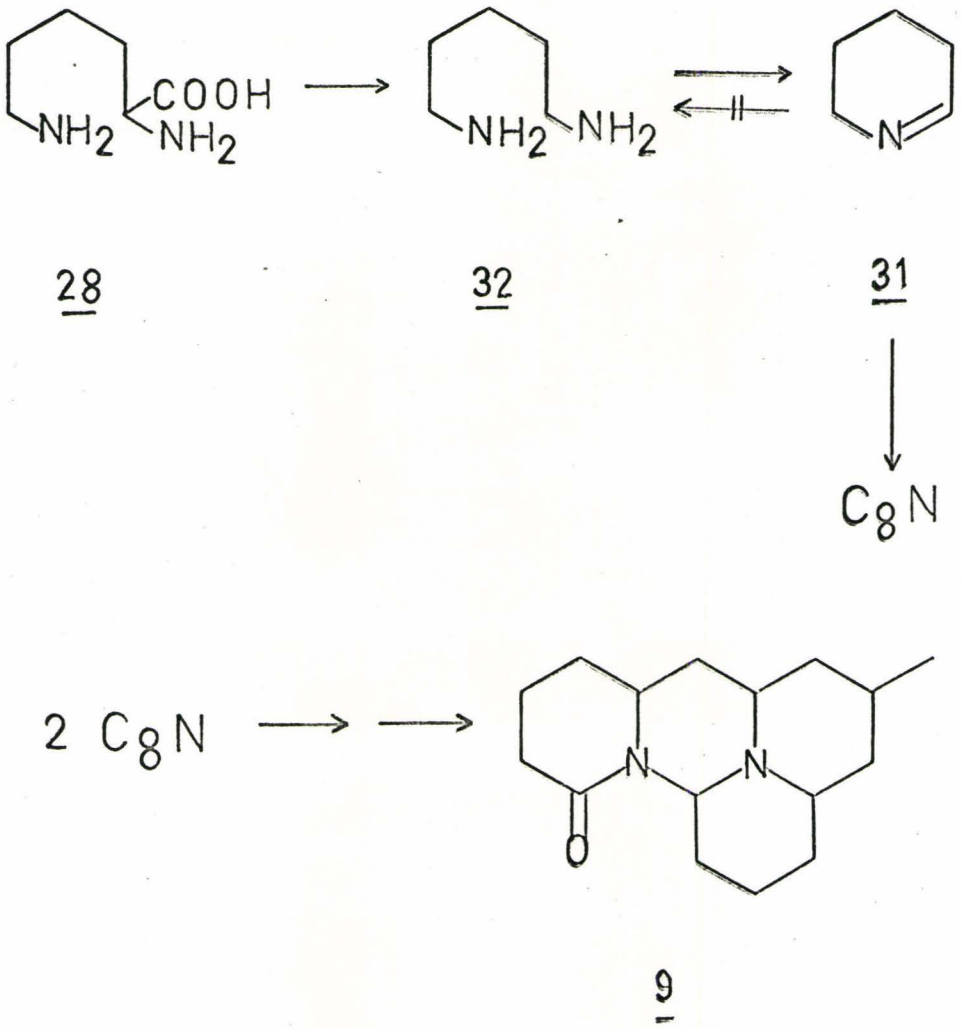


Fig.16. Biosynthesis of cernuine. The established sequence: lysine (28) ⟶ cadaverine (32) ⟶ Δ^1-piperideine (31) and the dimerization hypothesis.

but incompatible with the polyacetate hypothesis (Fig. 16).

Another piece of evidence supporting the sequence which leads from lysine to the C_8N unit, and is followed by the dimerization of the C_8N units, to give cernuine, is provided by the result of the feeding experiment with doubly labelled 4,5-$^3H_2$, 2-^{14}C-lysine. As shown in Fig. 17, the loss of tritium demanded by the scheme is 37.5%. In other words, the ^3H:^{14}C ratio of the intact cernuine should be 62.5% of that of the precursor lysine. The observed result (Table 6) agrees with this expected value within experimental error.

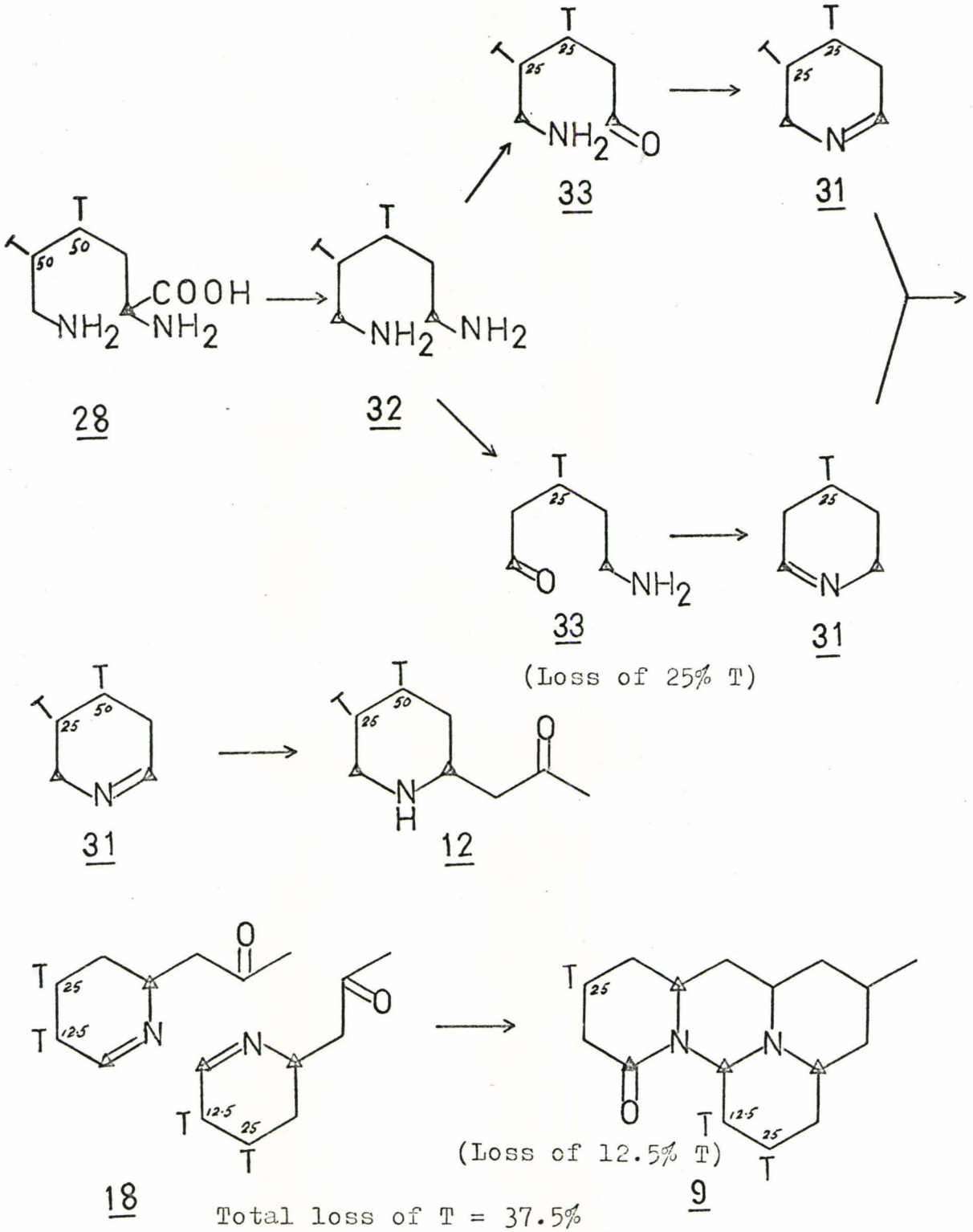
The results on the incorporation of lysine, cadaverine and Δ^1-piperideine are parallel in the case of cernuine and of lycopodine. It was of importance to test whether pelletierine is incorporated into cernuine and whether the mode of its incorporation into cernuine corresponds to that observed in the case of lycopodine.

An intermolecularly triply labelled sample, 3-^3H, 6, 2'-$^{14}C_2$-pelletierine, was administered to the plants. The cernuine isolated had a ^3H:^{14}C ratio identical with that of the precursor (Table 6). This result clearly indicates that pelletierine is incorporated intact into cernuine, consistent with the pelletierine hypothesis. The distribution of label within the alkaloid was established by partial degradation. The acetic acid, obtained from C-15 and -16 of cernuine contained 41% of the activity of the intact alkaloid, identical with the fraction of activity at C-2' of the precursor (Table 7). These results clearly demonstrate that pelletierine supplies only one C_8N unit, C-9 to -16, of cernuine. Entry of two intact pelletierine moieties into the alkaloid would have led to a Kuhn-Roth acetate containing only 20% of the activity

TABLE 6

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

Precursor	$^{3}H : ^{14}C$ Ratio In Precursor	$^{3}H : ^{14}C$ Ratio In Cernuine
4,5-$^{3}H_2$, 2-^{14}C-DL-Lysine	10.6 ± 0.4	6.5 ± 0.04
3-^{3}H, 6, 2'-$^{14}C_2$-Pelletierine	12.4 ± 0.1	12.2 ± 0.1



Total loss of T = 37.5%

Fig.17. Incorporation of lysine into cernuine. Loss of tritium relative to ^{14}C from 4,5-$^{3}H_2$,2-^{14}C-lysine.

TABLE 7

INCORPORATION OF PELLETIERINE INTO CERNUINE

$4\text{-}^{3}H, 6,2'\text{-}^{14}C_2$-Pelletierine		Cernuine	
Total Activity Administered (^{14}C) (dmp) x 10^{-7} in		Specific Activity (^{14}C) (counts $min^{-1} mmole^{-1}$) x 10^{-4}	
$6\text{-}^{14}C$-Pelletierine	6.77 ± 0.01		
$2'\text{-}^{14}C$-Pelletierine	4.64 ± 0.01	Kuhn/Roth Acetate	0.81 ± 0.06
Total	11.41 ± 0.01	Intact Cernuine‡	1.99 ± 0.03
$\frac{2'\text{-}^{14}C}{\text{Total}}$ =	$(41 \pm 1)\%$	$\frac{\text{Kuhn/Roth Acetate}}{\text{Intact Cernuine}}$ =	$(41 \pm 1)\%$

‡Obtained from the cernuine, specific activity $(17.8 \pm 0.6) \times 10^4$ counts min^{-1} $mmole^{-1}$ (Expt. 10) by dilution with inactive carrier.

of the intact cernuine. These observations suggest that pelletierine is incorporated intact into only the C_8N unit with the methyl group in the skeleton of cernuine, i.e., C-9 to -16, of the alkaloid (Fig. 18). These findings are completely compatible with the results in the case of lycopodine. It follows that cernuine is not a modified dimer of pelletierine, as might have been anticipated on the basis of structural relations.

Since pelletierine is incorporated into only one C_8N unit, C-9 to -16, of cernuine, C-1 to -3 cannot carry any activity, and β-alanine which, as discussed earlier was inferred to represent C-1 to -3 of the alkaloid, should be inactive. The β-alanine isolated from the degradation of the intact cernuine nevertheless contained radioactivity, a result which was in complete contradiction with the finding that the Kuhn-Roth acetate contained 41% of the activity.

An explanation of these apparently irrational findings is mandatory:

Under the condition in which the oxidation is carried out, i.e., in strongly acidic medium, dihydrodeoxycernuine (47) exists as (67) which is in equilibrium with its ring opened form 68a due to the repulsion of the two adjacent positive charges on the nitrogen atoms. 1,3 migration of a proton gives 68b and 68c which on oxidation liberates β-alanine from C-9 to -11 as well as from C-1 to -3 (Fig. 19). C-9 should carry 60% of the radioactivity of the intact cernuine and the β-alanine obtained from the chromic acid oxidation thus contains radioactivity (β-alanine - 20%, γ-aminobutyric acid - 38%).

In the light of the conclusion that chromic acid oxidation of dihydrodeoxycernuine liberates β-alanine not only from C-1 to -3 but

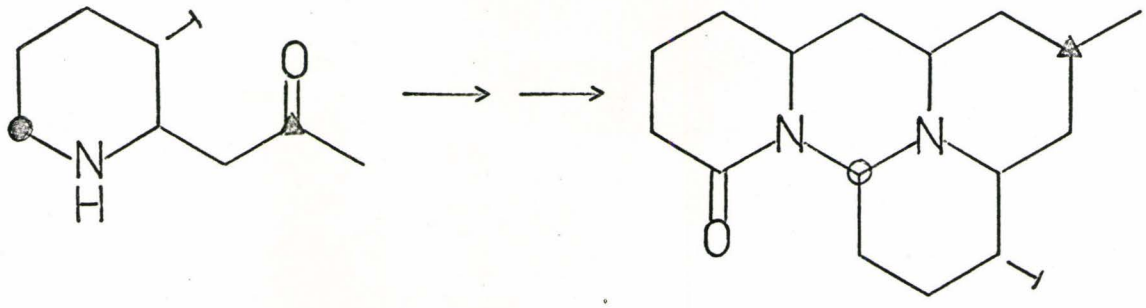


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Fig.18. Incorporation of pelletierine into cernuine. Sites of activity established by degradation (▲) (40% of total activity) or inferred (O, T).

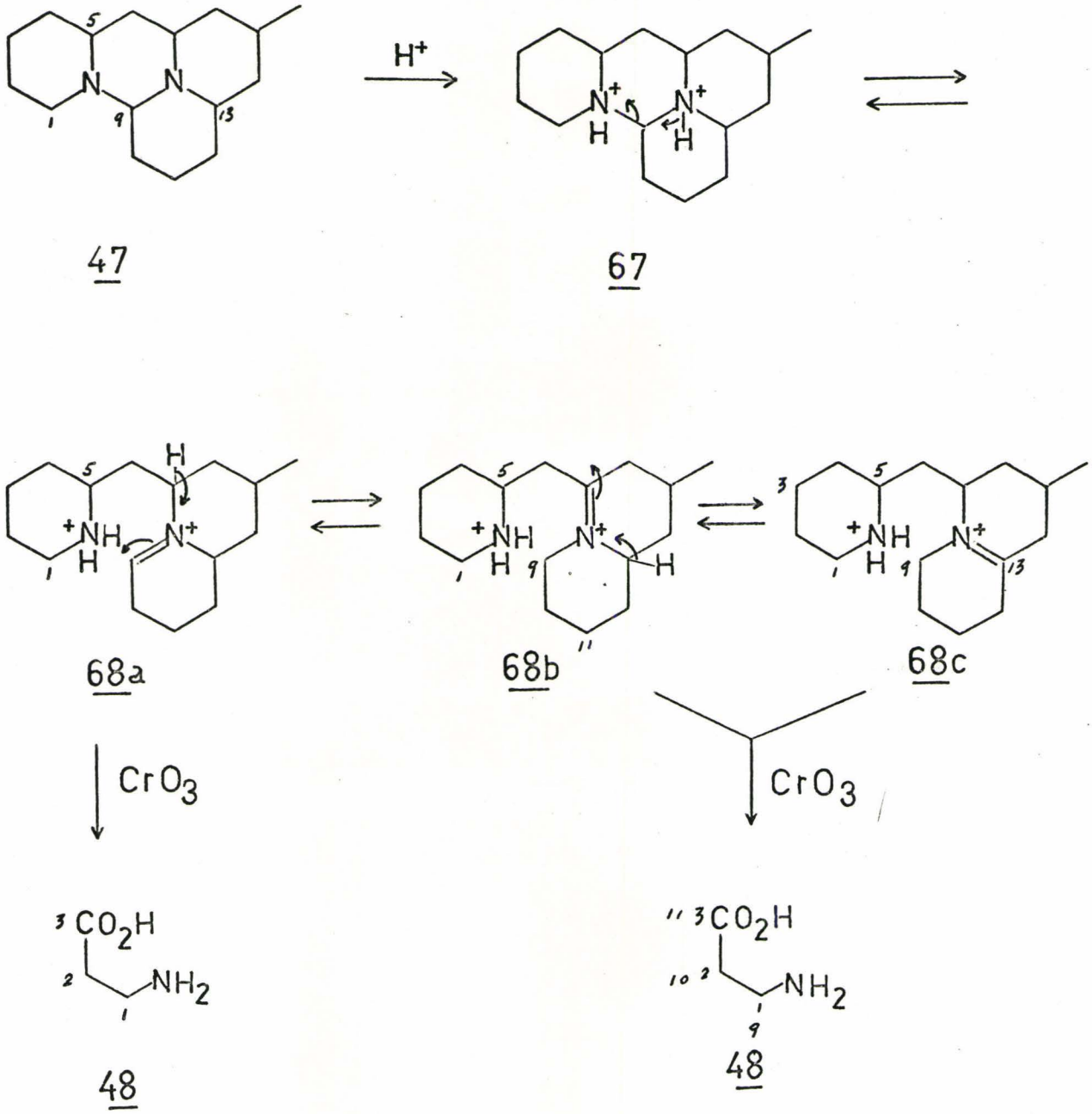

Fig.19. Rearrangement and oxidation of dihydrodeoxy-cernuine under strong acidic medium.

also from C-9 to -11, the interpretation of the lysine results must be re-examined. Fortunately, their interpretation is not affected by this new evidence. The results from the dehydrogenation of cernuine shows that C-1 contains 25% of the activity of the intact alkaloid regardless of whether 2-^{14}C- or 6-^{14}C-lysine had been the precursor. The fact that the β-alanine so obtained carries a quarter of the activity of the intact cernuine strengthens the indication that C-9 also carries 25% of the activity. Otherwise, the β-alanine, derived from C-1 to -3 and C-9 to -11 would have carried activity other than 25% of that present in the intact alkaloid.

IV. THE BIOSYNTHESIS OF THE LYCOPODIUM ALKALOIDS. THE PRESENT STATUS.

It has been shown conclusively that lysine, cadaverine and Δ^1-piperideine are specific precursors of both lycopodine and cernuine, two Lycopodium alkaloids with remarkably different skeletons, yet common structural features. This evidence supports the view that alkaloids of one and the same taxon have a common biogenetic origin, and makes it very likely that the same precursors generate other Lycopodium alkaloids.

Lysine is incorporated into the Lycopodium alkaloids symetrically, and its decarboxylation product, cadaverine, is a normal intermediate on the pathway. Oxidation of cadaverine is known to give 5-aminopentanal which spontaneously cyclizes to Δ^1-piperideine[29,30,31]. It has now been shown that two intact units of Δ^1-piperideine are incorporated into both lycopodine and cernuine. Thus it is very likely that this compound serves as the immediate precursor of the piperidine nuclei of the alkaloids.

The quantitative distribution within lycopodine and cernuine, of label from all the tracers which have been tested as precursors presents a consistent pattern. In every case it can be inferred that each of the two C_8 units, C-1 to -8 and C-9 to -16, contains one half of the activity present in the intact alkaloids. This equal incorporation of radioactivity into the two segments of lycopodine and cernuine indicates that the alkaloids are modified dimers of a "monomeric" precursor.

Pelletierine was thought to be the monomeric precursor in question. Conclusive evidence for the intact incorporation of pelletierine into the alkaloids is presented in this thesis. However, indications are strong that, if the alkaloids are modified dimers, pelletierine is not a normal precursor. This conclusion follows from the observations that pelletierine is incorporated into only one of the two C_8 units, C-9 to -16, of the alkaloids. Lysine is incorporated into pelletierine, if it is a normal intermediate, symmetrically in contrast to its mode of entry to N-methylpelletierine.[15] Pelletierine has not been observed in any of the Lycopodium species examined.

This conclusion gives rise to the apparent paradox that pelletierine can replace one but not the other of two identical C_8N moieties, serving as precursors of the dimeric alkaloids.

This paradox can be resolved: It is suggested that 2-allylpiperidine (69) rather than pelletierine serves as the monomeric C_8N unit, from which the Lycopodium alkaloids are ultimately derived. A hypothetical sequence for the biosynthesis of lycopodine is shown in Fig. 20. Oxidation of 69 gives 70 which is in equilibrium with 71. Dimerization of this oxidized derivative (71) of 2-allylpiperidine is initiated by protonation at the terminal side chain carbon. In the dimerization step, the protonated species 72 may be replaced by pelletierine, if the latter is supplied to the system, whereas the non-protonated species 71 cannot be so replaced. An analogous sequence applies to the biosynthesis of cernuine (Fig. 21). The oxidized derivative of 2-allylpiperidine (70) is in equilibrium with 74 whose dimerization is also initiated by protonation at the terminal side chain

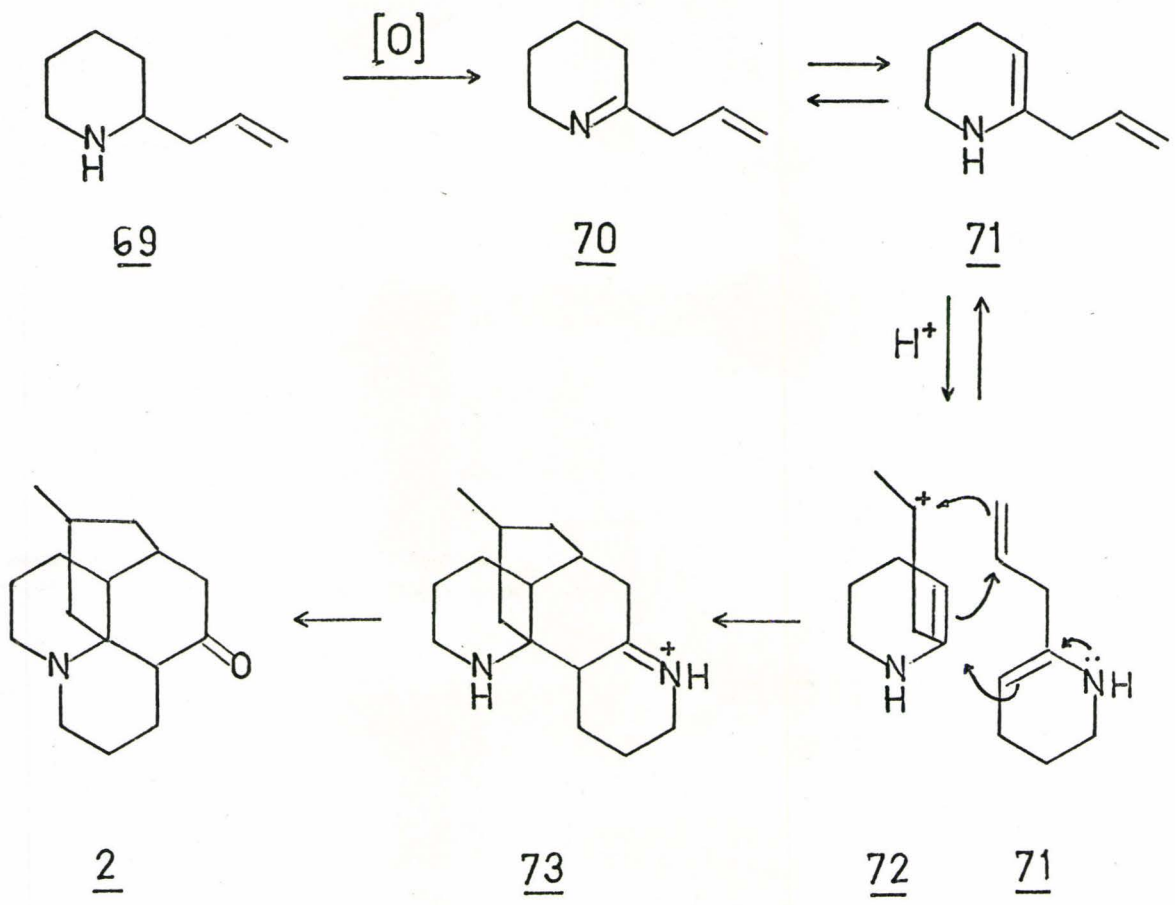

Fig.20. Alternative dimerization hypothesis for the biosynthesis of the Lycopodium alkaloids - lycopodine.

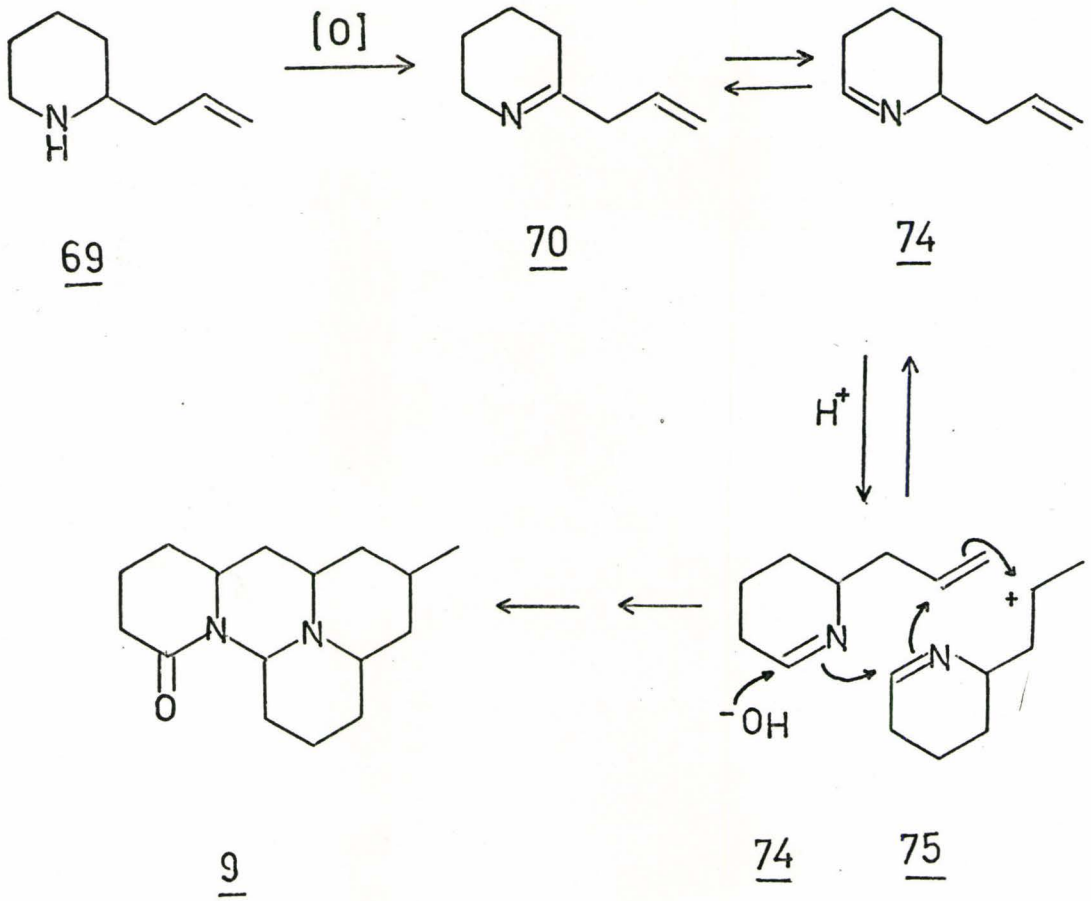

Fig.21. Alternative dimerization hypothesis for the biosynthesis of the Lycopodium alkaloids - cernuine.

carbon (or initiated by the attack of a hydroxyl ion at the electrophilic center of the piperideine nucleus). Again, in the dimerization step the protonated species 75 but not the non-protonated species 74 may be replaced by pelletierine, if this latter compound is available.

This alternative hypothesis can be tested as follows: Lysine has been shown to be a specific precursor of lycopodine and cernuine. If 2-allylpiperidine was the obligatory monomeric precursor, it should be incorporated into the alkaloids more efficiently than lysine. Thus, by administration of labelled lysine alone and together with inactive 2-allylpiperidine to the plants, the following indications may be gained. If the presence of inactive 2-allylpiperidine does not affect the percentage of incorporation of lysine into the alkaloids, it indicates that 2-allylpiperidine is not likely to be a normal intermediate on the pathway. If the presence of 2-allylpiperidine depresses the incorporation of lysine into the alkaloids, it indicates that the former is a normal intermediate of the biosynthesis of the alkaloids. In any case, 2-allylpiperidine should be isolated either directly or by carrier dilution, i.e., addition of an inactive sample of 2-allylpiperidine and reisolation of the specimen from plants grown in contact with labelled lysine. If the reisolated specimen is radioactive, it strongly indicates that 2-allylpiperidine is an intermediate in the formation of the alkaloids.

If the synthesis of the labelled 2-allylpiperidine or its oxidized derivative is successful, this putative substrate must then be administered to the plant. Equal and specific incorporation of label into two halves of the Lycopodium alkaloids will support this new dimerization hypothesis. Further experimental work should be oriented in this direction.

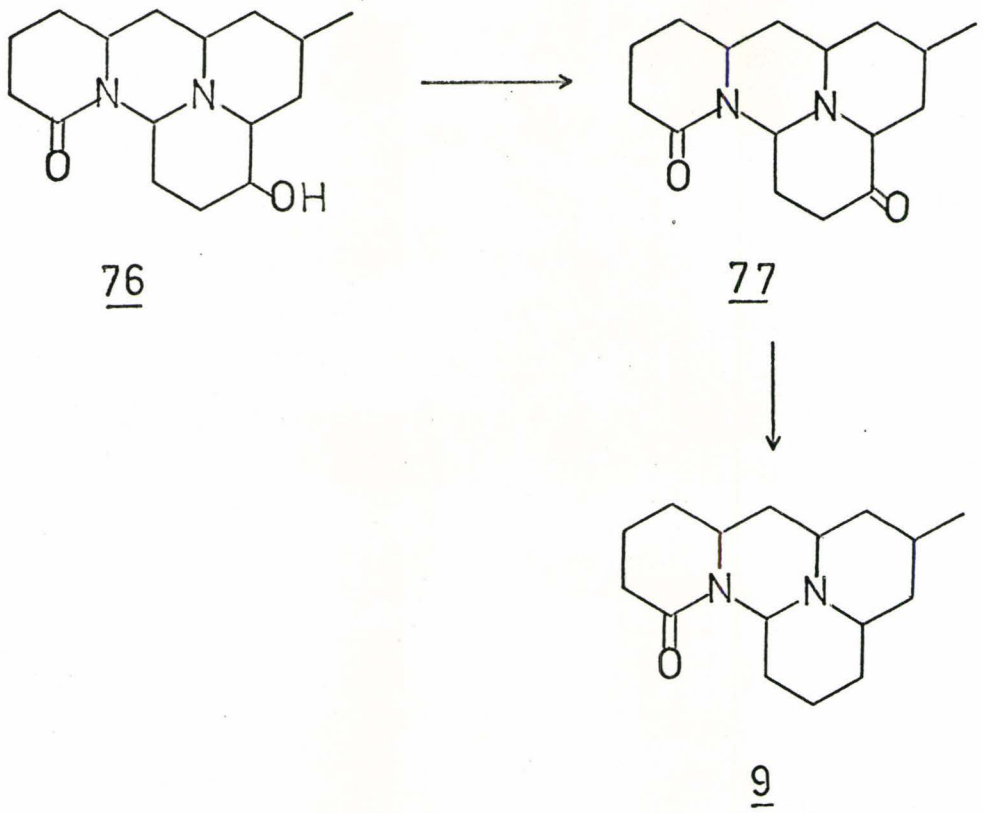

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Fig.22. Conversion of lycocernuine (76) into cernuine.

V. EXPERIMENTAL

(i) Administration of Labelled Compounds

A. To Lycopodium Tristachyum

Lycopodium tristachyum plants*, found in the vicinity of Huntsville, Ontario, near Algonquin Provincial Park, were transported to the greenhouse. Shoots of 3 to 4 inches high were cut and packed with cut surfaces downward into two 100 ml beakers. The radioactive compound was dissolved in water (10 ml) and the solution was divided between the beakers. Glass distilled water was added to each beaker when most of the original solution had been absorbed. The shoots were kept in contact with the tracer for 48 hours.

A summary of the feeding experiments carried out is presented in Table 1.

B. To Lycopodium Cernuum

Lycopodium cernuum, a subtropical lycopodium species growing in Jamaica, was chosen for the investigations carried out in the present work. Two methods were used for the administration of tracers. Feeding experiments with intact plants were carried out in the bush in Jamaica by the wick method. Cotton thread was inserted into the shoots and the

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*We are indebted to Dr. J. S. Pringle, Royal Botanical Gardens, Hamilton for the taxonomic identification of the species used in our experiments. A voucher specimen of the experimental plant is deposited in the herbarium of Algonquin Provincial Park, Ontario.

end of each thread was placed into a small glass beaker. The tracer solution (10 ml) was divided among the beakers and was absorbed by the plants through the cotton wicks. After the original tracer solution had been absorbed, the beakers were repeatedly refilled with glass-distilled water. The plants were kept in contact with the tracer for 48-72 hours and were then harvested.

In addition fresh cuttings (12 to 18 inches high) were packed with cut stems downward into a 100 ml beaker. The labelled compound, dissolved in glass-distilled water (10 ml), was added to the beaker. Refill and harvest were carried out in the same manner as above.

A summary of the feeding experiments which were carried out is outlined in Table 4 .

The 6-^{14}C-pelletierine (experiment 1 and 10) and 4-^{3}H-pelletierine were synthesized from 6-^{14}C-DL-lysine (Commissariat à l'Energie Atomique, France) and 4-^{3}H-DL-lysine (Commissariat à l'Energie Atomique, France), respectively, and inactive acetoacetic acid.[18] 2'-^{14}C-pelletierine was prepared from 3-^{14}C-acetoacetate (New England Nuclear) and Δ^{1}-piperideine obtained from inactive lysine.* An intermolecularly triply labelled sample, 4-^{3}H,6,2'-$^{14}C_2$-pelletierine, was obtained by uniting these samples. 2-^{14}C- and 6-^{14}C-Δ^{1}-piperideine were obtained from 2-^{14}C- and 6-^{14}C-lysine respectively.[37]

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*The synthesis of these labelled precursors was carried out by Dr. R. N. Gupta.

(ii) Isolation of the Alkaloids

Isolation of Lycopodine[27]

Green shoots of L. tristachyum were dried and ground to a fine powder in an Osterizer blender. The plant powder was moistened with ammonia (0.6 M, 1 ml/gm) and was continuously extracted with ether for 48 hours. The ether solution was extracted with hydrochloric acid (0.6 M, 5 x 20 ml). The aqueous extract was washed twice with ether, basified with ammonia (1.2 M), and was then extracted with ether (5 x 20 ml). The ether extract was washed with distilled water and was dried over anhydrous sodium sulphate. The residue obtained after evaporation of ether was extracted with hexane (2 x 5 ml). The hexane extract, which contained lycopodine and lycodine, was evaporated to dryness and the residue was sublimed at 110° and 1 x 10^{-3} mm, yielding a crystalline sublimate which consisted of lycopodine and about 2% of lycodine as shown by vapour phase chromatography (VPC) (5% SE 30, on chromosorb, 1/8 in. x 4 ft., 200°, 20 ml/min of nitrogen). The sublimate was crystallized from hot hexane yielding pure lycopodine which was further purified by sublimation at 90° and 1 x 10^{-3} mm, m.p. 115° (Lit. 116°).[4]

Isolation of Cernuine and Lycocernuine

Finely ground L. cernuum (500 gm) was moistened with ammonia (0.6 M, 1 ml/gm) and was continuously extracted with chloroform for 48 hours. The chloroform extract was concentrated to 1/10 of its volume and ether was added. The solution was then extracted with hydrochloric acid (0.6 M, 5 x 30 ml). The aqueous extract was washed twice with ether, basified with ammonia (1.2 M), and was extracted with chloroform (5 x 25 ml). The dried chloroform extract (anhydrous sodium sulphate)

was concentrated almost to dryness and a small amount of acetone was added. The crystalline solid, lycocernuine (110 mg), was recrystallized from acetone and melted at 228-229° (Lit. 230-231°).[8]

The mother liquors from the crystallization were dissolved in benzene and subjected to chromatography over basic alumina (cf. Ref. 8) (40 g activity III). Elution with benzene gave a mixture of minor bases. Elution with benzene-ether (1:1) gave cernuine (55 mg) and further elution gave lycocernuine (10 mg). Elution with ether gave more lycocernuine (8 mg). Elution with chloroform gave a mixture of minor bases.

When neutral alumina (40 g activity I) was used for chromatography, elution with benzene and benzene-ether (1:1) gave a mixture of minor bases. Elution with ether gave cernuine, and elution with chloroform gave first a mixture of cernuine and lycocernuine and then lycocernuine. Elution with methanol gave a mixture of minor bases.

The cernuine was sublimed at 110° and 3×10^{-3} mm, and was recrystallized from hexane, m.p. 101-102° (Lit. 103-104°).[8] In several instances, especially in the case of radioactive samples, the cernuine was dissolved in ether and the solution was neutralized with an ethereal solution of perchloric acid. The solvent was evaporated and the cernuine perchlorate so obtained was recrystallized from ethanol-ether, melted at 110° (Lit. 110°).[4]

Several modifications of the above procedure were also employed. When ether instead of chloroform was used as extracting solvent, the extraction of alkaloids was incomplete. The alkaloid extract on the basis of VPC analysis contained twice as much cernuine as lycocernuine whereas the reverse was true when a better solvent such as chloroform

(see above) or methanol (see below) was used. Thus when ether was used, not all of the lycocernuine was extracted.

Methanol was employed as solvent in large scale extraction.[8] Finely ground L. cernuum was extracted at room temperature with methanol for 36 hours and the methanolic extract was concentrated at reduced pressure. This process was repeated twice and the combined extracts were warmed with hydrochloric acid (0.6 M). The hydrochloric acid insoluble portion was removed by filtration and then extracted again with hydrochloric acid. The combined filtrates were extracted with ether and made strongly basic with ammonia. The alkaloidal fraction was then extracted into chloroform and worked up as already described.

Conversion of Lycocernuine into Cernuine[8]

Dehydrolycocernuine (77)

A solution of lycocernuine (200 mg) in acetone (40 ml) was cooled in an ice bath, and Jones' reagent[32] (0.2 ml) was added. After 20 min most of the solvent was removed, water (10 ml) added, and the resulting solution extracted with chloroform (6 x 10 ml). Evaporation of the chloroform yielded dehydrolycocernuine (115 mg). Basification of the aqueous solution and extraction with chloroform yielded a mixture (65 mg) of lycocernuine and dehydrolycocernuine (VPC). This mixture was again subjected to oxidation with Jones' reagent. Worked up as above, yielded dehydrolycocernuine (45 mg).

Wolff-Kishner Reduction of Dehydrolycocernuine

Dehydrolycocernuine (160 mg) was dissolved in diethylene glycol (12 ml) and hydrazine hydrate (1.6 ml) was added to the solution under a nitrogen atmosphere. The solution was heated at 100-130° for 1 hour,

two pellets of sodium hydroxide were then added, and the temperature was raised to 180-190° for 3 hours. The cooled solution was diluted with water and extracted with chloroform (5 x 15 ml). The chloroform extract was washed with water and dried over sodium sulphate. The concentrated chloroform solution was filtered through alumina to remove traces of diethylene glycol. Evaporation of the chloroform yielded cernuine (97 mg) which was further purified by sublimation and crystallization from hexane, m.p. 103-104°.

(iii) Degradation of the Alkaloids

Degradation of Lycopodine[27]

Carbon-5 as Benzoic Acid

A solution of lycopodine (100 mg) in anhydrous ether (50 ml) was added dropwise to a solution of phenyllithium in ether (6 ml) and the mixture was refluxed 4 hours under nitrogen atmosphere. The cooled solution was poured into a stirred solution of concentrated hydrochloric acid (5 ml) and crushed ice (10 gm). The ether layer was discarded. The aqueous layer was washed with ether (2 x 30 ml), basified with ammonia, and extracted with ether (5 x 30 ml). Evaporation of the dried ether solution (sodium sulphate) yielded phenyldihydrolycopodine (39) which was recrystallized from hexane.

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 for 8 hours. The cooled mixture was acidifed with hydrochloric acid (0.6 M), decolorized by passing through sulphurdioxide and extracted with ether (3 x 40 ml). Evaporation of the dried extract (sodium sulphate) gave a

residue which yielded benzoic acid on sublimation (14 - 20 mg).

Carbon - 9 as Formic Acid

A solution containing lycopodine (250 mg) 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. The solution was then stirred for 4 hours at 0° and 2 hours at room temperature. The excess potassium permanganate and manganese dioxide were destroyed by passing sulphur dioxide through the solution at 0°.

The acidic solution was extracted six times with chloroform which was in turn extracted twice with aqueous ammonia (0.6 M). The aqueous extract was acidified with hydrochloric acid (0.6 M) and extracted with chloroform. The dried chloroform extract (sodium sulphate) was evaporated and the crude acidic residue sublimed to yield pure N-formylaminoacid (41) (70-78 mg).

The crystalline N-formylaminoacid (41) (70 mg) was converted into the methyl ester by treating with an excess of freshly distilled diazomethane. The methyl ester, purified by sublimation at 140° and 1×10^{-3} mm, melted at 114-116° (Lit. 117°).[39] Yield: 56 mg.

The solution of the methyl ester (55 mg) in sulphuric acid (1 M, 10 ml) was refluxed for 2 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 sodium hydroxide (0.1 M), and 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 (43) which precipitated after a few minutes, was sublimed at 120° and 1 x 10^{-3} mm, recrystallized from benzene and resublimed, m.p. 137-138° (Lit. 139°).[40]

Carbon Atoms -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 and more zinc powder (8 g) was added. The tube was evacuated, sealed, and heated at 290° - 310° for 24 hours. A yellow liquid condensed in the cold end of the tube protruding from the furnace. The reaction mixture, as revealed by VPC, consisted of 7-methyldecahydroquinoline (A), 7-methyl-5,6,7,8-tetrahydroquinoline (B), 7-methylquinoline, a dimethylquinoline and several other uncharacterized compounds. Preparative VPC (20% Carbowax 20 M 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. The tetrahydroquinoline derivative (44) was further purified by sublimation of its hydrochloride, m.p. 153-156°.

Degradation of Cernuine

Reduction of Cernuine[8]

Cernuine (250 mg) was added to a slurry of lithium aluminium hydride (0.50 g) in anhydrous ether (20 ml, dried over sodium hydride), and the resulting mixture was refluxed for 20 hours and then worked up as follows.[41] The cooled (ice bath) mixture of reaction products and excess hydride were decomposed by dropwise addition of water (0.5 ml) followed by sodium hydroxide (4 M, 0.5 ml). More water (21 ml) was then added and the mixture was stirred vigorously for another 20 min, filtered with suction, and the precipitate was washed thoroughly with

hot water and then with ether. The filtrate was extracted with ether (5 x 10 ml) and the combined ether extract and washings were washed with water, dried over sodium sulphate and concentrated at reduced pressure. The crystalline product was sublimed at 100° and 4×10^{-3} mm. The mass spectrum of the sublimed product showed a m/e 248 peak which was regarded as the molecular ion peak. Two components were detected however, by V.P.C. (1% Carbowax 20 M + 5% KOH on Chromosorb W, 4 ft. x 0.125 in. O.D., 130-210° at 10°/min. and a helium flow rate of 40 ml/min.). The retention time of the minor component ($< 7\%$) was 2 min., identical with that of an authentic sample of dihydrodeoxyepiallocernuine. The retention time of the major component was 3.2 min.* The major component was regarded as dihydrodeoxycernuine[8] (see section III, (ii)). These two components were not further separated and were used for the oxidation described below.

β-Alanine and γ-Aminobutyric Acid from Cernuine

The above reduction product (192 mg) of cernuine was dissolved in hot sulphuric acid (6 M, 4 ml) and chromium trioxide (500 mg) in water (2 ml) was added in several portions. The resulting mixture was heated under reflux with stirring for 48 hours. The cooled mixture was extracted with ether several times. The ether layer was rejected. The aqueous layer was diluted with water (10 ml) and warmed, and hot aqueous barium hydroxide solution was added until the solution was neutral. The precipitate was filtered off, washed well with hot water and the filtrate was evaporated

*A sample of the reduction product mixed with the authentic dihydrodeoxyepiallocernuine was injected and only two components with retention times 2 and 3.2 min. respectively were observed. The intensity of the peak of the minor component increased with the amount of the authentic dihydrodeoxyepiallocernuine added.

to dryness. The residue was dissolved in water (1 ml), and aqueous sodium bicarbonate (1.2 M) was added until the pH of the solution was 9. 1-Fluoro-2,4-dinitrobenzene (FDNB) solution in methanol (10%, 1.5 ml) was then added. The resultant mixture was shaken frequently for a period of one hour and the pH was maintained at 8 - 9 with additional bicarbonate solution. After one hour, water (4 ml) and bicarbonate solution (0.5 ml) were added and the excess FDNB was removed by extracting the solution with ether (2 x 6 ml). The solution was then acidified with hydrochloric acid (6 M) and extracted with ether (3 x 6 ml). The dried (sodium sulphate) ether extract was concentrated and the yellow dinitrophenyl (DNP) amino acids were separated by preparative thin layer chromatography (Silica Gel plate, developed with benzene-pyridine-acetic acid (80:20:2)).

Three yellow bands were observed. The major band showed an R_f value (R_f 0.69) identical with that of an authentic sample of N-2,4-dinitrophenyl-β-alanine. A second band (R_f 0.84) corresponded to N-2,4-dinitrophenyl-γ-aminobutyric acid. The minor band (R_f 0.28) was not further examined, but may have been N-2,4-dinitrophenylglycine.[42] The amino acid derivatives were eluted separately from the absorbant with methanol. The methanolic extract of the β-alanine band (R_f 0.69) which was the most abundant was evaporated to dryness. The residue was dissolved in water (1 ml) and hydrochloric acid (6 M, 1 ml) was added, and the mixture was extracted with ether (3 x 5 ml). The ether layer was washed with water (2 ml), dried (sodium sulphate) and evaporated to dryness. The residue was sublimed at 160-170° and 1 x 10^{-3} mm when the DNP derivative of β-alanine was obtained as a yellow crystalline solid. Yield: 8 mg. The band of DNP derivative of γ-aminobutyric acid (R_f 0.84) was worked up similarly.

Carbons-15 and -16 as Acetic Acid

A solution of chromic acid (2 g) in water (3 ml) was added to a solution of cernuine (100 mg) in sulphuric acid (2 M, 5 ml). The resultant mixture was heated under reflux for two hours. Steam was then passed through the mixture until 50 ml of distillate had been collected. The distillate was neutralized with sodium hydroxide (0.1 M) and evaporated to dryness. The sodium acetate so obtained was converted into the acetyl-α-naphthylamide (43) which was purified by sublimation at 135° and 1 x 10^{-3} mm. The white sublimate was dissolved in a little benzene. On addition of petroleum ether 1-acetamidonaphthalene, melting point 158°-159° (Lit. 159-160°),[43] separated as colorless needles.

Dehydrogenation of Cernuine[8]

Cernuine (100 mg), 5% palladium on charcoal (300 mg), and freshly distilled tetralin (3 ml) were heated in a sealed tube at 300° for 7 days. The cooled reaction mixture was diluted with ether, the catalyst filtered off and washed well with ether and then with a little methanol, and the filtrate was extracted with hydrochloric acid (0.6 M, 4 x 15 ml). The acid extract was made basic with aqueous ammonia and extracted with chloroform. The residue remaining after evaporation of the dried (sodium sulphate) chloroform extract showed three main spots on t.l.c. and was separated by preparative t.l.c. on silica gel plates (0.5 mm layers, developed with ether-ammonia (75 ml:2 drops)). The more polar component (R_f 0.65), which was the most abundant one (20 mg), was eluted from the absorbent with chloroform and subjected to pot-to-pot distillation at 50° and 4 x 10^{-3} mm to yield 2-n-butyl-4-methyl-6-n-pentylpyridine (14 mg), identical with an authentic sample.* The pyridine derivative

*We are greatly indebted to Professor W. A. Ayer for supplying this sample.

was converted into the picrolonate in the usual manner and recrystallized from ether-ethanol, (m.p. 137-139°). It was also converted into the chloroplatinate and recrystallized from ethanol containing a drop of hydrochloric acid. The orange crystals so obtained melted at 186-188° (Lit. 189-190°).[8] The minor components of the dehydrogenation were not examined.

Radioactivity Measurements

Radioactivity was assayed on samples of finite thickness on aluminium planchettes (Nuclear Chicago Corporation) with a low background gas flow Geiger counter (Nuclear Chicago Corporation, Model 4342). Corrections for background and self-absorption[44] were made. Samples for counting were prepared as follows: Solid materials (0.1 - 1.2 mg) were weighed on aluminium planchettes, and dissolved in suitable solvent system. Two drops of a 1% solution of collodion in dimethylformamide was suitable for most substances. For volatile substances like benzoic acid, one or two drops of an aqueous sodium hydroxide solution (0.5 M) were added in order to prevent loss of sample by evaporation. The solution was covered with a circle of lens tissue to ensure even distribution of the sample. The planchettes were thoroughly dried under an infrared lamp before counting.

^{3}H:^{14}C ratios were measured by liquid scintillation counting (Mark I, Liquid Scintillation Computer, Model 6860, Nuclear Chicago). Radioactivity due to ^{3}H and ^{14}C was determined simultaneously by external standardization counting with ^{133}Ba. Samples were dissolved in benzene or methanol 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.

The confidence limits shown in the results (Table 1-7) are standard deviations of the mean. The calculation of the specific activity of a typical compound, from the data obtained from measurements in duplicate sample, will be outlined in the Appendix.

SUMMARY

The biosynthesis of two Lycopodium alkaloids of different skeletal structures, lycopodine and cernuine, was studied by tracer methods. The mode of incorporation of different substances into the two compounds was compared.

Cadaverine, Δ^1-piperideine and pelletierine are incorporated into lycopodine in Lycopodium tristachyum. Degradations of the radioactive lycopodine obtained from these experiments show specific incorporation of label in each case. The distribution in lycopodine of label from cadaverine leads to the conclusion that two C_5 units derived from this precursor are incorporated into the two halves of the alkaloid with equal efficiency. The pattern of label in lycopodine obtained from the experiments with Δ^1-piperideine shows that two Δ^1-piperideine moieties are incorporated equally into the two halves of the alkaloid in such a way that the identity of their carbon atoms is maintained.

Specific incorporation of one pelletierine unit into the fragment C-9 to -16 of lycopodine is confirmed.

The biosynthesis of cernuine was studied by administering radioactive lysine, cadaverine, Δ^1-piperideine and pelletierine to L. cernuum. Partial degradation of the radioactive cernuine shows specific incorporation of label in each case. The labelling pattern found in cernuine obtained from every experiment clearly shows that the mode of incorporation of lysine, cadaverine, Δ^1-piperideine and pelletierine into cernuine is similar to the manner in which they enter lycopodine. Thus, lysine is incorporated into the two halves of cernuine with equal efficiency.

Two C_5 units derived from lysine by way of a symmetrical intermediate supply C-1 to C-5 and C-9 to C-13 of the alkaloid. Cadaverine yields the expected pattern of label and is presumably the symmetrical intermediate in question. Two Δ^1-piperideine moieties are incorporated equally into two halves of cernuine in such a way that the identity of C-2 and C-6 of the precursor units is maintained. Pelletierine is incorporated intact into cernuine, but only one of the two C_8N units, C-9 to C-16, of the alkaloid is supplied by pelletierine.

Thus, it is clear that the modes of biosynthesis of lycopodine and of cernuine are analogous even though the two compounds are remarkably different in structure. Their skeletons are derived from lysine and acetate and the sequence lysine $\longrightarrow$ cadaverine $\longrightarrow$ Δ^1-piperideine constitutes part of the pathway of the biosynthesis of the alkaloids. The fact that only one pelletierine unit is incorporated into the alkaloids shows that the compounds are not modified dimers of pelletierine as predicted by the pelletierine hypothesis.[5] A modification of this hypothesis is discussed. It is suggested that 2-allylpiperidine rather than pelletierine serves as the monomeric precursor of the alkaloids.

APPENDIX

Experiment 3: Lycopodine obtained from 2-^{14}C-Δ^1-Piperideine

Planchette No.	4004	4005
Weight Taken (mg)	0.433	0.412
Molecular Weight	247.2	247.2
Self-Absorption Factor[44]	0.874	0.873

Definitions and Symbols:[45]

time (min) for 1000 counts = X

number of time readings = n

mean = $\bar{X} = \frac{\Sigma X_i}{n}$

Variance = $\Sigma[(X_i - \bar{X})^2]/n$

Standard deviation = $\sigma = \sqrt{\Sigma[(X_i - \bar{X})^2]/n}$

Best estimate of = $\sqrt{\Sigma[(X_i - \bar{X})^2]/n-1}$

Planchette 4004	X	$\lvert X - \bar{X} \rvert$	$(X - \bar{X})^2$
1	19.87	0.43	0.185
2	19.83	0.47	0.221
3	20.72	0.42	0.176
4	19.97	0.33	0.109
5	20.15	0.15	0.022
6	20.68	0.38	0.144
7	20.56	0.26	0.068
8	20.57	0.27	0.073
9	20.53	0.23	0.053
10	20.12	0.18	0.032

APPENDIX (cont'd)

$\Sigma X = 203.0$

$\bar{X} = \frac{\Sigma X}{10} = 20.30$

$\Sigma(X - \bar{X})^2 = 1.083$

$\Sigma(X - \bar{X})^2/n-1 = \frac{1.083}{9} = 0.120$

$$\text{Count rate} = \frac{1000}{20.30} \pm \frac{1000}{20.30} \times \sqrt{\frac{0.120}{(20.30)^2}}$$

$$= 49.35 \pm 0.83 \text{ counts min}^{-1}$$

The background, determined in the same manner, was found to be 2.19 ± 0.13
The reading for the radioactive sample was corrected for background:

$$49.35 - 2.19 \pm \sqrt{(0.83)^2 + (0.13)^2}$$
$$= 47.16 \pm 0.84 \text{ counts min}^{-1}$$

Corrections for self-absorption and normalization to specific activity

$$= (47.16 \pm 0.84) \times \frac{247.2 \text{ mg mmole}^{-1}}{0.433 \text{ mg} \times 0.874}$$

$$= (3.075 \pm 0.056) \times 10^4 \text{ counts min}^{-1} \text{ mmole}^{-1}$$

The corresponding value derived from planchette 4005 was $(3.202 \pm 0.079) \times 10^4$

$$\text{The mean specific activity} = \left(\frac{3.075 + 3.202}{2} \pm \frac{\sqrt{(0.056)^2 + (0.079)^2}}{2} \right) \times 10^4$$

$$= (3.138 \pm 0.048) \times 10^4 \text{ counts min}^{-1} \text{ mmole}^{-1}$$

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