THE ROLE OF 2-PIPERIDINEACETIC ACID IN THE BIOSYNTHESIS OF LYCOPODINE

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By

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SCOPE AND CONTENTS:

The role of 2-piperidineacetic acid in the biosynthesis of lycopodine was tested by feeding experiments with β-^{14}C-2-piperidineacetic acid hydrochloride. The compound was synthesized starting from 2-picolylchloride which was treated with K^{14}CN yielding the labeled 2-pyridineacetonitrile. Conversion of the nitrile to the ester was followed by reduction of the pyridine ring and hydrolysis of the ester function. The labeled β-^{14}C-2-piperidineacetic acid was obtained in 60% radiochemical yield. When administered to L. tristachyum, alone or along with radioactive lysine, β-^{14}C-2-piperidineacetic acid showed very low incorporation into lycopodine. The incorporation of lysine into lycopodine showed however that the alkaloid was being synthesized in the plant in the course of the feeding experiments. These results cast doubt on the hypothesis that 2-piperidineacetic acid is an intermediate on the pathway to lycopodine from lysine.

ACKNOWLEDGEMENTS

It is a pleasure to express my gratitude to my research supervisor, Dr. D. B. MacLean, for his advice and encouragement during the course of this investigation.

Thanks are also due to Dr. I. D. Spenser for his guidance on several steps of the experimental work.

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HISTORICAL INTRODUCTION

The alkaloids are a large group of naturally-occurring compounds containing nitrogen. They are, in most cases, basic in character and physiologically active. Extensive studies centered on their isolation, their structure determination, and their synthesis have been carried out over the years.

Speculation on the biogenetic origin of alkaloids (1) was based initially on the visual dissection of their molecular skeletons into simpler structural units, and precursor-product relationship were advanced on the basis of known chemical reactions. The Mannich reaction (2), the condensation of an amine, an aldehyde and a carbanion $-\overset{|}{\underset{|}{C}}^{-} + C\langle^{O}_{H} + HN\langle \longrightarrow -\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-N\langle$, and the oxidative coupling reactions of phenols have figured prominently in schemes for the biosynthesis of a large number of alkaloids.

Real progress in the study of alkaloid biosynthesis began in the late 1940's following the development of isotope labeling techniques (3,4). The general experimental procedure in biosynthetic work using isotopic labels consists in administration to the intact organism of a postulated precursor labeled with an isotope (^{3}H, ^{14}C, ^{15}N are widely used), and isolation and purification of the desired compound after a period of growth of the organism in contact with the tracer. The alkaloid is then assayed for isotope content and degraded to determine the distribution of label within it. Radioisotopes are used because of the great sensitivity

of the methods of measurement of radioactivity, and at the same time, they permit a minimum change in the normal conditions of the living organism since only a small quantity of labeled compound is administered. Varied techniques are available for the administration of labeled substrate into intact plants, excised shoots, and tissue culture.

The substrate is regarded as a precursor if activity is incorporated in a non-random manner into the product. Specific incorporation of a labeled substrate is demonstrated by feeding with multiply labeled precursors. Even though an intact precursor is specifically incorporated, an obligatory precursor-product relationship is not necessarily established. Sometimes it is not easy to discover whether the synthesis of a compound from a given precursor is a normal biosynthetic pathway, that is, the way in which the substance is synthesized in the intact organism. Precursor sequences which have been postulated on the basis of incorporation efficiencies or of dilutions of different substrates are of doubtful validity for the following reasons. Cell permeability may be different for different substrates. In higher plants, the pathway leading to a given product may differ in detail from one organ to another. The time, and sometimes the modes of feeding may be important.

Techniques that have been developed to strengthen a demonstrated precursor-product relationship are: detection of postulated intermediates by carrier dilution, and isotope competition, i.e., depression of incorporation of label into a product by an intermediate on the pathway.

Over the last twenty years, the first stages of the investigation of alkaloid biosynthesis have been achieved. Some of the postulated

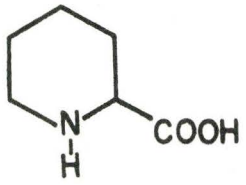
intermediates along the biosynthetic pathways have been isolated (5,6) and their incorporation tested in experiments with living plants.

It is clear that enzymes must be involved in alkaloid formation since most of the alkaloids found in nature are optically active. The majority of amino acids which are constituents of higher plants have the L- configuration, and it has been established in several cases that L-amino acids and not D- amino acids serve as precursors of the alkaloids (7). The isolation of enzymes which catalyze the individual steps of a postulated biosynthetic sequence and a knowledge of the kinetics of the processes involved will finally establish the normal obligatory metabolic pathways. To date, little is known of the enzymes of the alkaloid biosynthesis (8) and only a few cases have been investigated *in vitro*.

Since the piperidine skeleton is present in the Lycopodium alkaloids and since lysine is involved in the biosynthesis of both Lycopodium alkaloids and piperidine alkaloids, it is worthwhile to discuss briefly the biosynthesis of piperidine alkaloids from lysine as precursor.

I. LYSINE AND THE BIOSYNTHESIS OF PIPERIDINE ALKALOIDS

The piperidine nucleus is found in the structure of many naturally-occurring compounds. Amino acids such as pipecolic acid (1) and its derivatives, bases containing α or α,α' substituted piperidines such as anabasine (2), pelletierine (3), sedamine (4) and lobeline (5), condensed systems such as pseudopelletierine (6) and lobinaline (7), and quinolizidine alkaloids such as lupinine (8) and sparteine (9) are examples of compounds whose structures contain the piperidine system.



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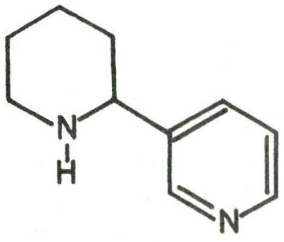

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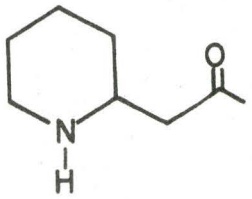

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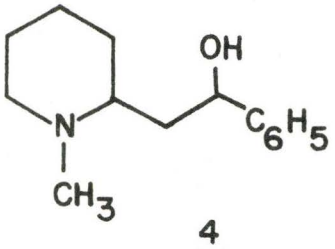

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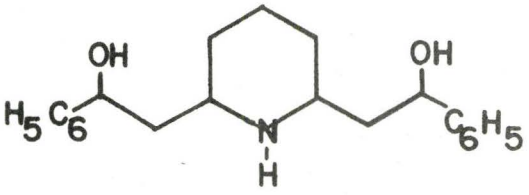

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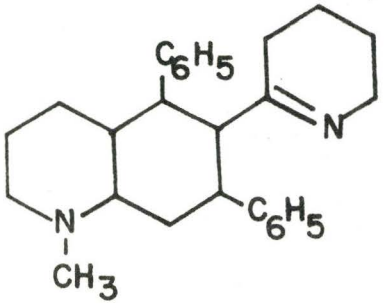

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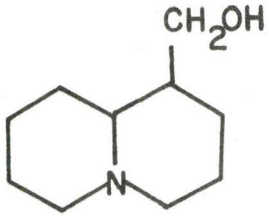

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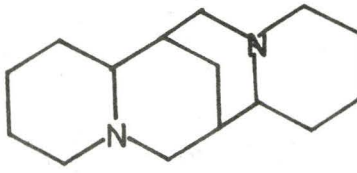

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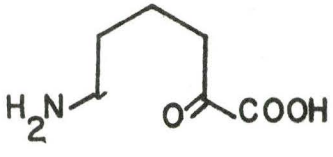

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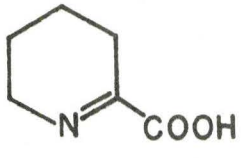

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Figure 1

Label from ^{14}C-lysine and ε-^{15}N-lysine was incorporated into pipecolic acid and its derivatives in a number of plants (9,10,11). It has been suggested that α-keto-ε-aminocaproic acid (10) and Δ'-piperideine-2-carboxylic acid (11) are the intermediates of the metabolic pathway from lysine to pipecolic acid.

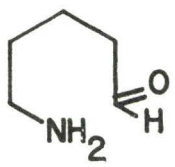
In Nicotiana glauca, 2-^{14}C lysine is incorporated into C-2 of the piperidine ring of anabasine (12); the ε-amino group of lysine supplied its nitrogen atom (13). In Sedum acre L., a specific incorporation of 2-^{14}C lysine and 6-^{14}C lysine into C-2 and C-6 respectively of the piperidine ring of sedamine has been demonstrated (14). All activity in the radioactive N- ethylpelletierine isolated from excised shoots of Sedum sarmentosum to which 6-^{14}C lysine has been administered was found at C-6 of its nucleus (15). Activity from 2-^{14}C and from 6-^{14}C lysine was incorporated selectively into lobinaline (16) which is a major alkaloid of Lobelia cardinalis and whose structure is a modified sedamine dimer. Experiments with doubly labeled lysine (4,5-^{3}H, 6-^{14}C lysine is incorporated into N-methylpelletierine of Sedum sarmentosum) suggested that the α-amino nitrogen is lost during the biosynthetic sequence (15). These findings are consistent with the proposed pathway, in which, 5-aminopentanal (12) and its cyclized form, Δ'-piperideine (13) exist as intermediates. It has been known that the double bond of Δ'-piperideine is fixed (17,18), and indeed, activity from 6-^{14}C-Δ'-piperideine is specifically incorporated into C-6 of the piperidine nucleus of anabasine. The proposed existence of Δ'-piperideine as an intermediate along a metabolic pathway is supported by the fact that,

the compound has been obtained by enzymic oxidation of 1,5-diaminopentane (cadaverine) (19). A chemical synthesis of pelletierine has been carried out from lysine and acetoacetic acid following the biogenetic scheme suggested by Robinson (18).

For the quinolizidine alkaloids found in plants of the Lupin family, lysine serves as precursor by way of the symmetrical intermediate cadaverine (14). 2-^{14}C-lysine and 1,5-^{14}C-cadaverine were administered to various plant species elaborating lupin alkaloids. The observed incorporation of lysine and cadaverine into lupinine (20) (15), sparteine (21,22) (16), lupanine (23) (17) and matrine (24,25) (18) is shown in Scheme 1. The same distribution of label within these alkaloids was found regardless of whether 2-^{14}C-lysine or 1,5-^{14}C-cadaverine served as precursor, which indicates that lysine enters the Lupin alkaloids by way of a symmetrical intermediate. Results from feeding experiments with doubly labeled lysine (2-^{14}C, α-^{15}N-lysine) into L. luteus showed that isotopic nitrogen from α-^{15}N-lysine was incorporated into sparteine.

Despite the findings of incorporation of cadaverine into alkaloids, the role of cadaverine in alkaloid biosynthesis is uncertain. It has been shown that a symmetrical intermediate cannot exist on the pathway from lysine to anabasine since only the ε-amino nitrogen of lysine is retained.

Yet, cadaverine is incorporated into anabasine much more efficiently than lysine itself. One half of the label was found at C-2 of the piperidine ring when 1,5-^{14}C-cadaverine was administered. This suggests that the piperidine ring can be formed without the obligatory inter-



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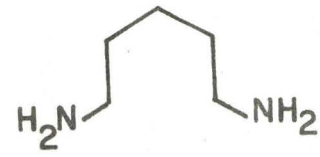

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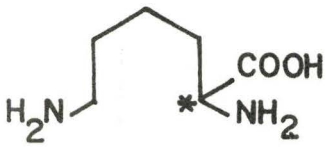
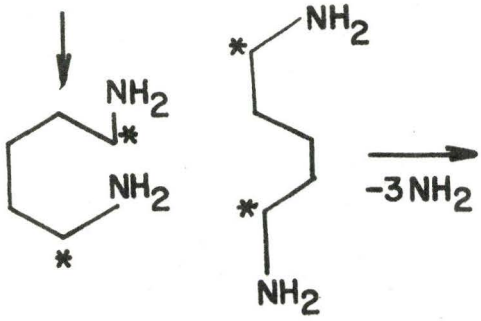
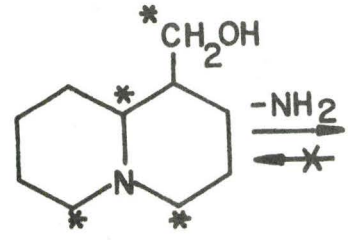


Lysine



Cadaverine



Lupinine
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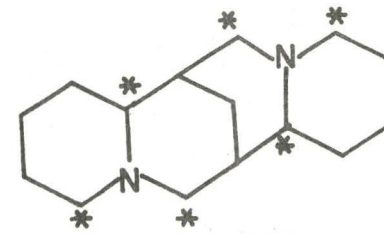
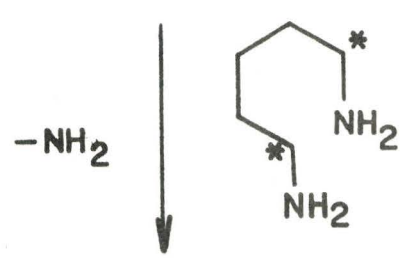
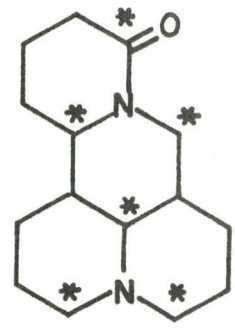
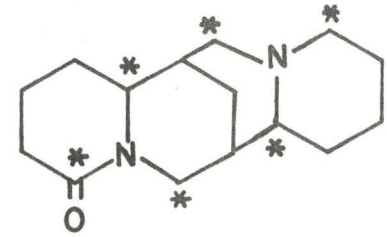


Sparteine
16





Matrine 18



Lupanine 17

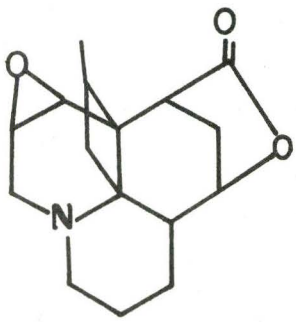
mediacy of α-keto-ε-aminocaproic acid. A possible explanation for these contradictory results is that there are alternative routes to a given product and the plants cannot distinguish foreign precursors from normal ones in its biosynthetic pathway. Another rationalization lies in the recognition of the enzymes involved in the metabolic sequence, which will be discussed later in the section, "biosynthesis of lycopodine".

II. BIOGENESIS OF LYCOPODIUM ALKALOIDS

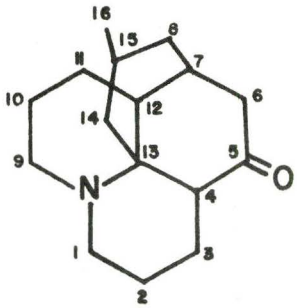
A. Introduction

The alkaloids of Lycopodium species were first studied in 1881 by Bödeker who isolated an alkaloid, now known as lycopodine, from Lycopodium complanatum but serious investigations on Lycopodium alkaloids were not conducted until the late 1930's. The first alkaloid whose structure was elucidated was annotinine (19), followed shortly thereafter by lycopodine (20), α- and β-obscurine (21, 22), lycodine (23), and selagine (24). New structural types that have been found recently are luciduline (25), serratinine (26), alopecurine (27) and annopodine (28). Approaches to the chemical synthesis of annotinine were first reported in 1965, and the total synthesis was completed by the group of Wiesner in 1967 (25,26). Lycopodine has been synthesized recently by the groups of Ayer (27) and of Stork (28).

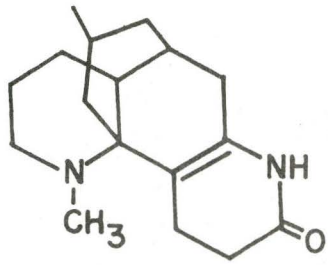
Conroy (29) proposed a biogenetic scheme for the origin of the Lycopodium alkaloids at a time when the structures of only a few alkaloids were known. The condensation of two 3,5,7-triketo-octanoic acid chains, derived from acetate, in an aldol condensation followed by a Mannich reaction between a carbonyl carbon, a methylene and ammonia



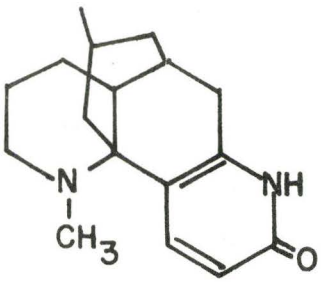
Annotinine 19



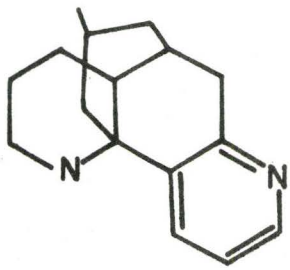
Lycopodine 20



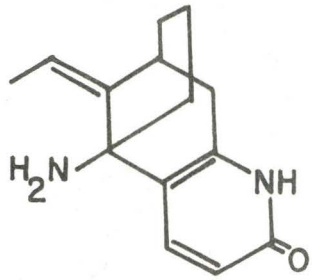
α-Obscurine 21



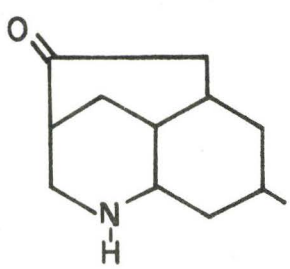
β-Obscurine 22



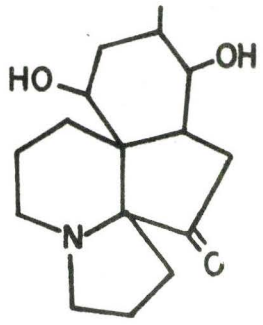
Lycodine 23



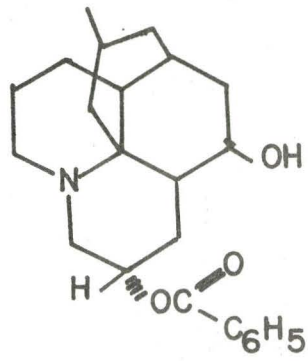
Selagine 24



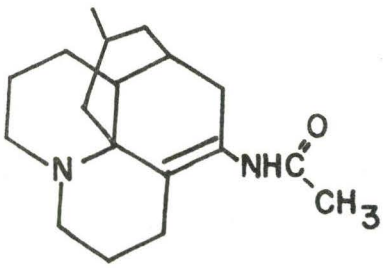
Luciduline 25



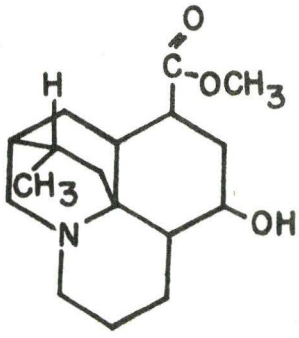
Serratinine 26



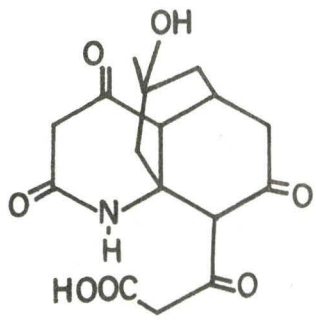
Alopecurine 27



Flabelline 30



Annopodine 28



29

Figure 3

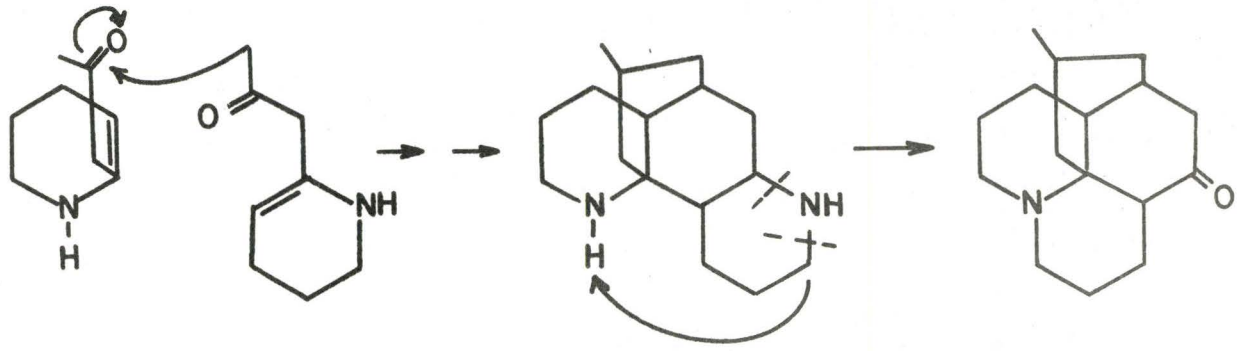
would give the immediate precursor (29) of the lycopodine type alkaloids. Conroy's hypothesis will also accommodate the structure of annotinine, the dinitrogeneous alkaloid, cernuine, and the new structural types found in serratinine, alopecurine, and luciduline. Experiments with 1-^{14}C and 1-^{14}C acetate (30) have ruled out this polyacetate hypothesis. Nevertheless, Conroy's scheme played an important role in the structural studies of the Lycopodium alkaloids, e.g. cernuine, lycodine, serratinine and luciduline; and the numbering system for these alkaloids was proposed by Wiesner on the basis of this polyketide hypothesis.

B. The Pelletierine Hypothesis

A close look at the structures of the Lycopodium alkaloids reveals that they all belong to two groups: the $C_{16}N_2$ series, e.g. lycodine, obscurine, cernuine and their analogues, and the $C_{16}N$ series, e.g., lycopodine, annotinine, annopodine ... except luciduline which has a $C_{12}N$ skeleton. As the $C_{16}N_2$ and the $C_{16}N$ alkaloids were both found together in several species of Lycopodium plants (lycopodine, lycodine and obscurine in L. obscurum L. var. dendroideum and in L. flabelliforme var. ambiguum), one might anticipate that they have the same biogenetic origin.

The pelletierine hypothesis states that the $C_{16}N$ alkaloids are derived from a binitrogeneous precursor. The $C_{16}N_2$ skeleton of the lycodine, obscurine and cernuine group may be dissected into two halves, C-1 to C-8 and Nb, and C-9 to C-16 and Na, which correspond to the C_8N piperidine alkaloids. A dimerization of a suitable C_8N monomer will form the $C_{16}N_2$ structure. It was suggested that this C_8N unit was

pelletierine.



The $C_{16}N$ lycopodine-type alkaloids may be derived from the $C_{16}N_2$ lycodine-obscurine skeleton by a C_5-Nb cleavage, followed by extrusion of nitrogen and recyclization. This proposal is supported by the fact that most of the $C_{16}N$ alkaloids have an oxygen function at C-5 where the nitrogen Nb is attached in the $C_{16}N_2$ series. Flabelline (30) has the lycopdine skeleton with an acetamido group at C-5, and so does serratinidine which has a modified lycopodine type structure. Luciduline may be considered to arise from a condensation of one pelletierine unit and one C_4 unit derived from acetate; alternatively, it may arise from two pelletierine units with loss of four carbons.

III. BIOSYNTHESIS OF LYCOPODINE

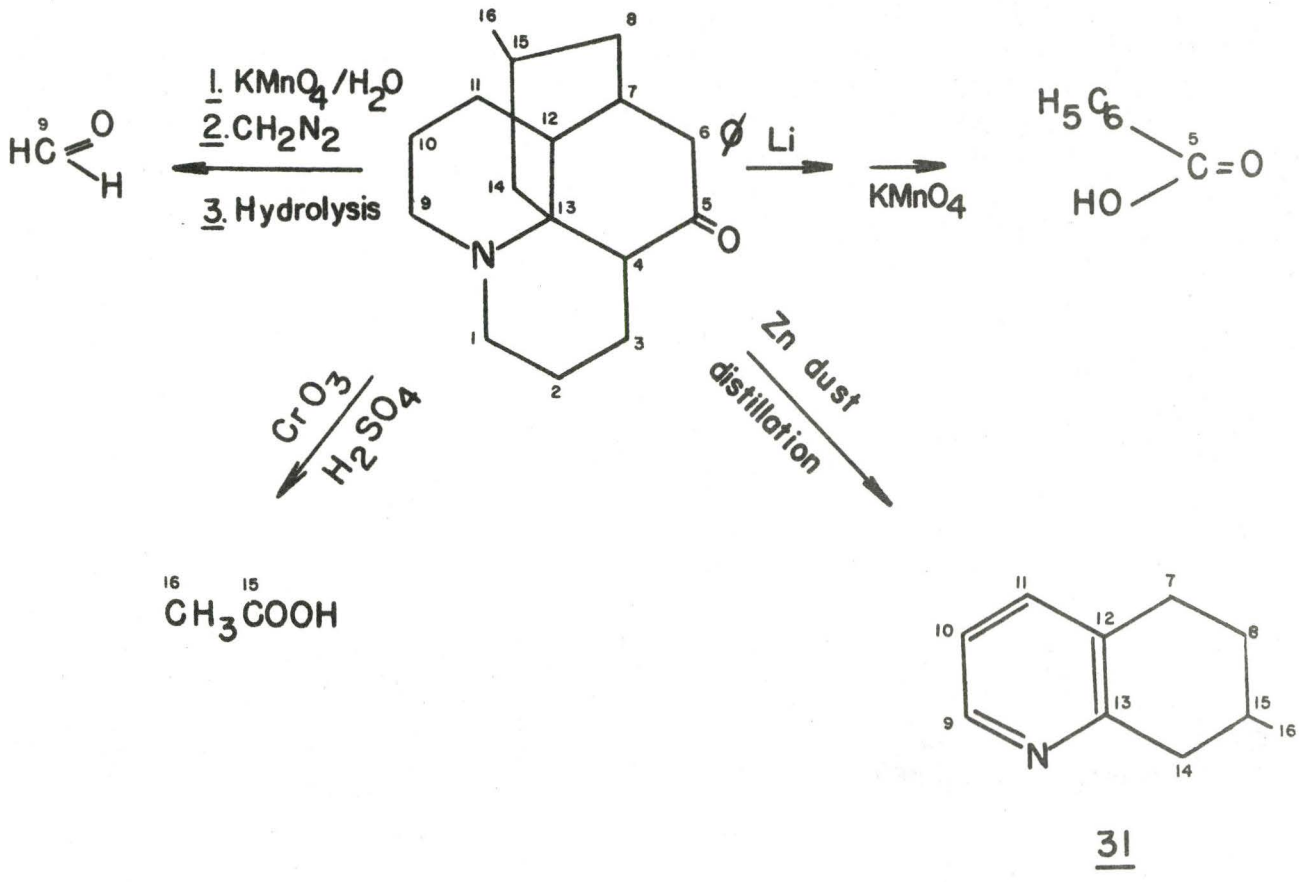
As it was pointed out before in the section on the biosynthesis of piperidine alkaloids, the piperidine nucleus of N-methylpelletierine was derived from lysine in a non-symmetrical manner. Acetate is the precursor of the side chain of N-methylpelletierine. A dimerization of

two pelletierine units leading to Lycopodium alkaloids would predict a specific incorporation of pelletierine as well as its postulated precursors, e.g. lysine, acetate ... into these alkaloids. Experiments designed to test this hypothesis were undertaken.

The species chosen for experiment was Lycopodium tristachyum Pursh, and the alkaloid studied was lycopodine. The radioactive lycopodine obtained from feeding experiments was degraded according to Scheme 2. The C-5 and C-9 atoms were isolated individually, the former as formic acid and the latter as benzoic acid. C-15 and C-16 were obtained together as acetic acid, and C-7 to C-16 were isolated as (+)7 methyl-5,6,7,8-tetrahydroquinoline (31).

The pelletierine hypothesis predicts that one-half of the activity of the labeled lycopodine should be found in the Kuhn-Roth acetate (C_{15} and C_{16}) when 1-^{14}C acetate is fed, and one-quarter of the activity when 2-^{14}C acetate serves as precursor. The experimental results were in accord with the prediction (30). Experiments with 3-^{14}C acetoacetate showed the same pattern of incorporation of 1-^{14}C acetate, and the result from labeled 4-^{14}C-β-hydroxybutyrate as precursor indicated that, similar to the case of N-methylpelletierine (15) the C_4 unit of acetoacetate had been degraded to acetate prior to incorporation.

If lysine is incorporated into both halves of lycopodine in the same non-symmetrical manner as it is incorporated into N-methylpelletierine, one half of the activity of lycopodine derived from 2-^{14}C-lysine should be located at C-5 and the other half should reside at C-13. One half of the activity of lycopodine derived from 6-^{14}C-lysine should be



Scheme 2

found at C-9 and the rest should be at C-1. The results of feeding experiments with 2-^{14}C and 6-^{14}C-lysine indicate that lysine is indeed incorporated into the two halves of lycopodine, but the incorporation follows a symmetrical pattern. With either 2-^{14}C or 6-^{14}C-lysine as precursor, one-quarter of the activity of the labeled lycopodine was located at C-9 and one-quarter at C-5. The 7-methyl,5,6,7,8-tetrahydroquinoline, which contains both C-9 and C-13, had approximately one-half

of the activity of the intact alkaloid. Since it was demonstrated that one-quarter of the activity resided at C-13, it is likely that the remaining 25% is located at C-1. The labeling pattern suggested that a symmetrical molecule such as cadaverine exists along the metabolic pathway of lycopodine from lysine.

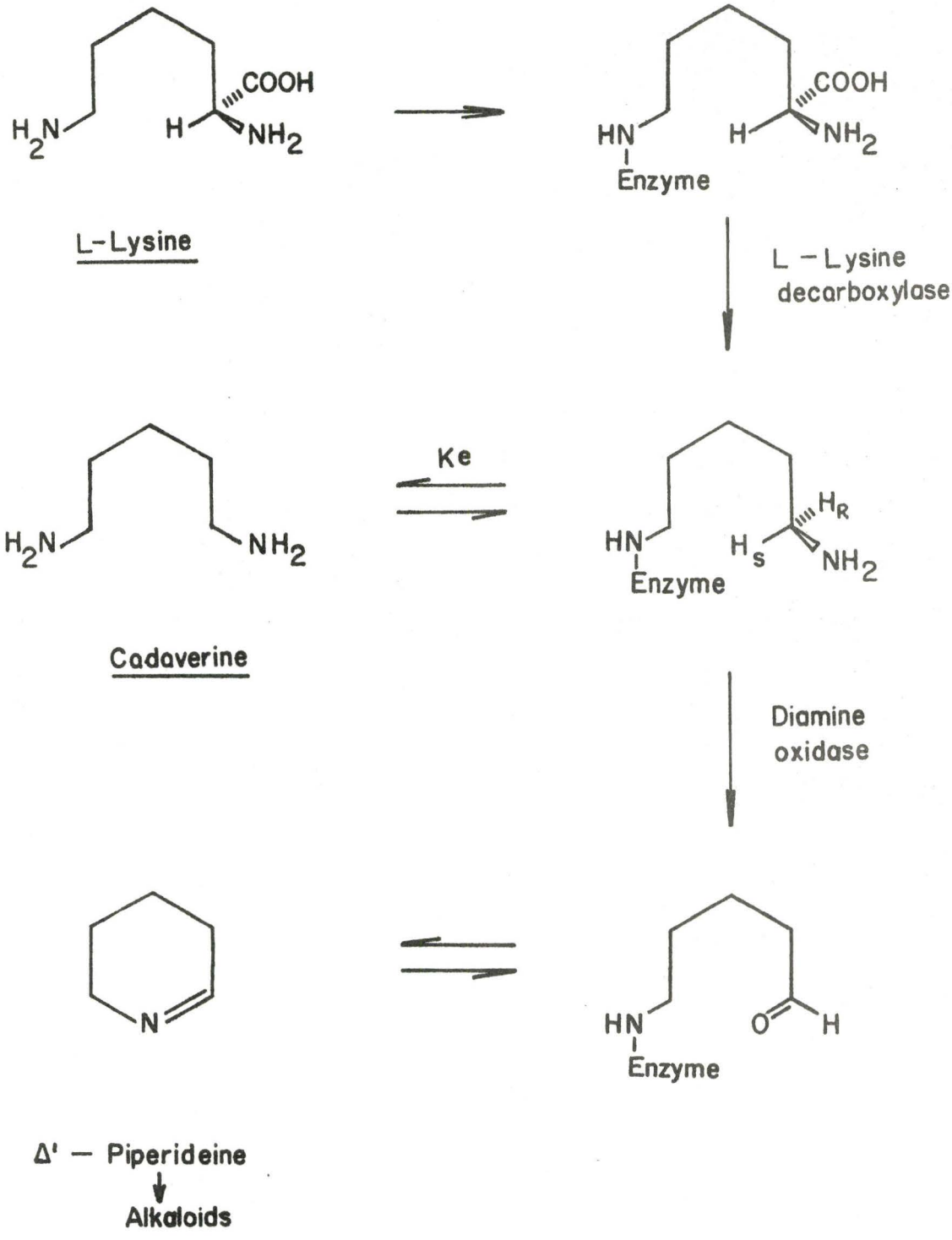
The lycopodine obtained from an experiment with 4,5- $^{3}H_2$, 6-^{14}C-lysine showed a loss of 22% of tritium relative to ^{14}C. This is consistent with the postulated existence of cadaverine as an intermediate (30). In fact, 1,5-^{14}C-cadaverine has been shown to be incorporated into lycopodine with the same distribution of activity as lysine, and the incorporation of cadaverine is much more efficient (100 times) than that of lysine. As it was suggested that pelletierine is a precursor of lycopodine, the above findings pose the question why the formation of pelletierine in Lycopodium plants follows a different route from that of N-methylpelletierine in S. sarmentosum.

Further studies were carried out with 2-^{14}C and 6-^{14}C-Δ'-piperideine as labeled substrates (31). One-half of the activity of lycopodine derived from 2-^{14}C-Δ'-piperideine was located at C-5 and the rest was at C-13. With 6-^{14}C-Δ'-piperideine as precursor, one-half of the activity of labeled lycopodine was found at C-9 and the other half was inferred to be at C-1. These results show that 2 molecules of Δ'-piperideine were incorporated equally into lycopodine, and the identity of C-2 and C-6 of Δ'-piperideine was preserved during the biosynthetic sequence. If both cadaverine and Δ'-piperideine are obligatory intermediates on the pathway to lycopodine from lysine, the incorporation pattern suggests

that the symmetrical intermediate, cadaverine, must lie between lysine and Δ^1-piperideine since once the latter is formed, randomization of C-2 and C-6 does not occur. It is known that cadaverine is formed by decarboxylation of lysine and is convertible to Δ^1-piperideine in irreversible enzymic reactions (19). A rationalization of the different biosynthetic routes of alkaloids from lysine (via symmetrical and non-symmetrical intermediates) could be met if one considers that enzyme-bound cadaverine and not cadaverine itself was formed from a stereospecific decarboxylation of lysine. The magnitude of the dissociation constant of this cadaverine-enzyme complex will determine whether lysine is incorporated into alkaloids in a symmetrical or non-symmetrical manner as shown in Scheme 3.

The distribution of label from all the tested tracers (acetate, lysine, cadaverine, Δ^1-piperideine) is consistent with the following conclusion. Two identical units, C-1 to C-8 and C-9 to C-16, combined to form the lycopodine skeleton. These C_8 units are derived from lysine and acetate. The fragments C-6 to C-8 in the first half and C-14 to C-16 in the second half of lycopodine are derived from acetate, while the two lysine fragments give C-1 to C-5 and C-9 to C-13.

Pelleterine was postulated to be the monomeric precursor of lycopodine and labeled pelletierine is predicted to enter equally into each "half" of the lycopodine skeleton. The radioactivity of lycopodine derived from 2-^{14}C pelletierine was confined to one "half" of the molecule (C-9 to C-16) isolated as 7-methyl,5,6,7,8-tetrahydroquinoline. The other "half" contained no radioactivity. Similarly, all the activity of lycopodine was recovered at C-9 when 6-^{14}C pelletierine served as


COOH
H
NH2
H2N
L-Lysine
HN
Enzyme
L – Lysine
decarboxylase
Ke
HR
Hs
Cadaverine
Diamine
oxidase
N
O
Δ' — Piperideine
Alkaloids

Scheme 3

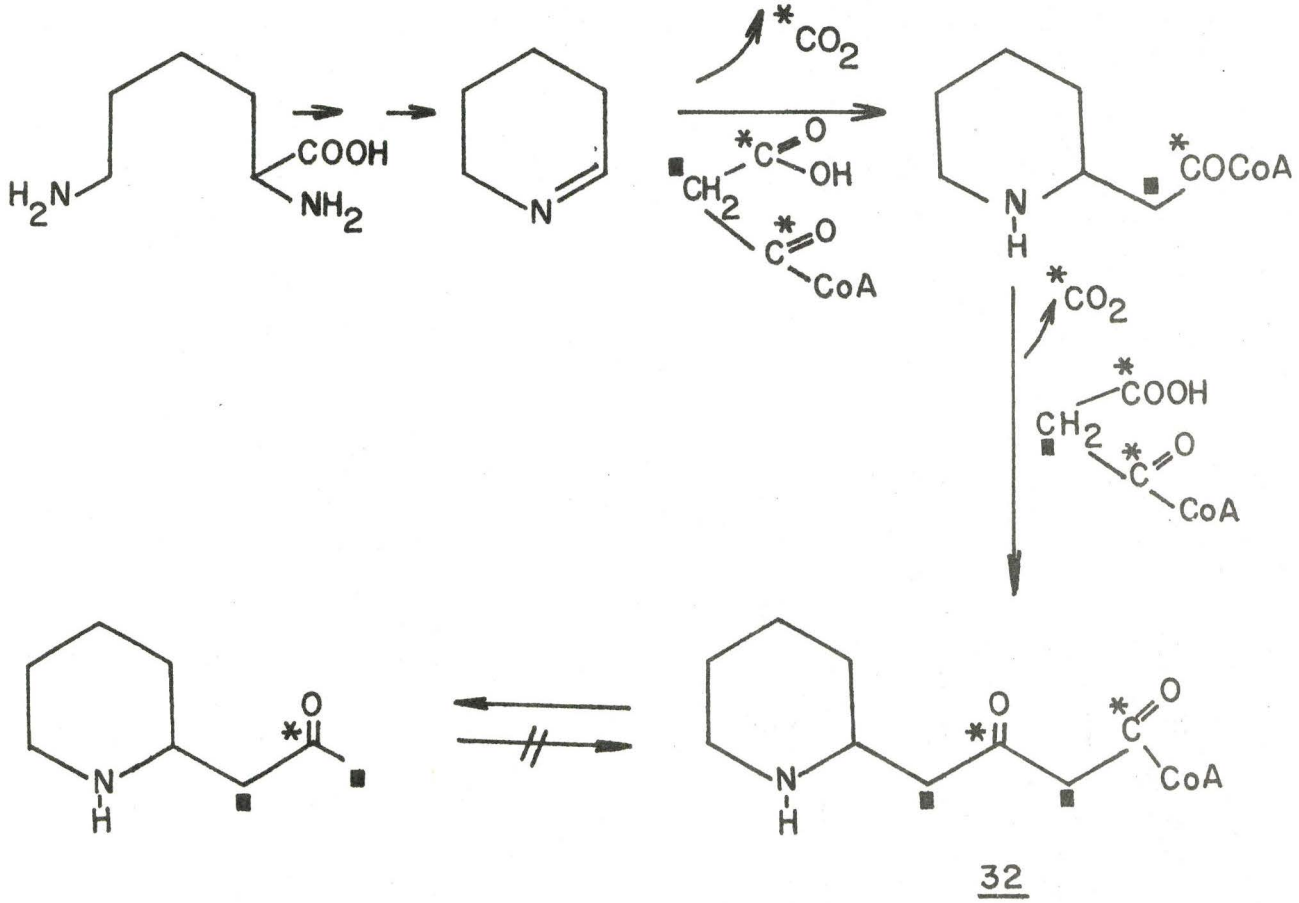
precursor. A feeding experiment with doubly labeled 4,5-$^{3}H_2$, 2-^{14}C-pelletierine showed the same $^{3}H/^{14}C$ ratio in lycopodine and in the precursor. Similarly, a feeding experiment with 2,3'$^{14}C_2$-pelletierine showed that an intact pelletierine unit was incorporated into lycopodine but only into the C-9 to C-16 fragment, the other "half" is not derived from this precursor.

Two alternatives were suggested following those experiments (32).

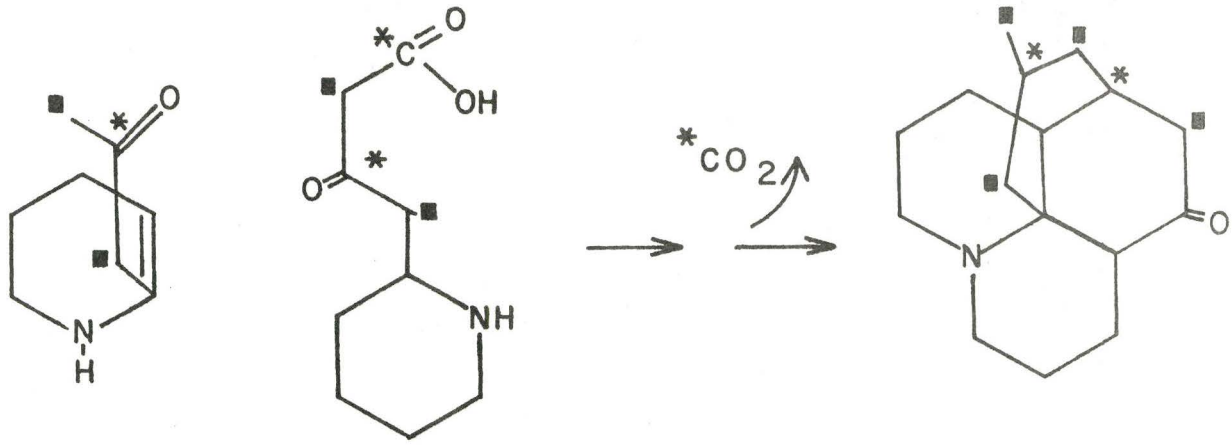
(i) Lycopodine is indeed a dimer of some structural unit which is derived from lysine and acetate. Pelletierine, although it is not the normal precursor, can replace one but not the other of two identical monomers. 2-allylpiperidine (32) was postulated as the C_8N monomeric unit. A feeding experiment with 2-^{14}C allylpiperidine showed that this hypothesis is untenable (32).

(ii) Lycopodine is not a dimer of two identical monomer units but pelletierine is an obligatory intermediate of the C-9 to C-16 fragment. The other half is derived from lysine in the same pathway as pelletierine (via cadaverine and Δ'-piperideine) and acetate, but pelletierine is not its precursor.

This hypothesis has been supported by the demonstration that pelletierine is an intermediate in the pathway to lycopodine. Activity from labeled lysine, cadaverine and Δ'-piperideine enters both C_8 units of lycopodine, whereas pelletierine serves as precursor of the C-9 to C-16 unit only. If pelletierine is indeed an intermediate on the biosynthetic pathway of lycopodine, addition of inactive pelletierine to the plants would suppress the incorporation of labeled cadaverine or


H2N
COOH
NH2
N
*CO2
*C
O
OH
CH2
*C
O
CoA
N
H
*COCoA
*CO2
*COOH
CH2
*C
O
CoA
N
H
O
*
N
H
O
*
*C
O
CoA
N
H

32

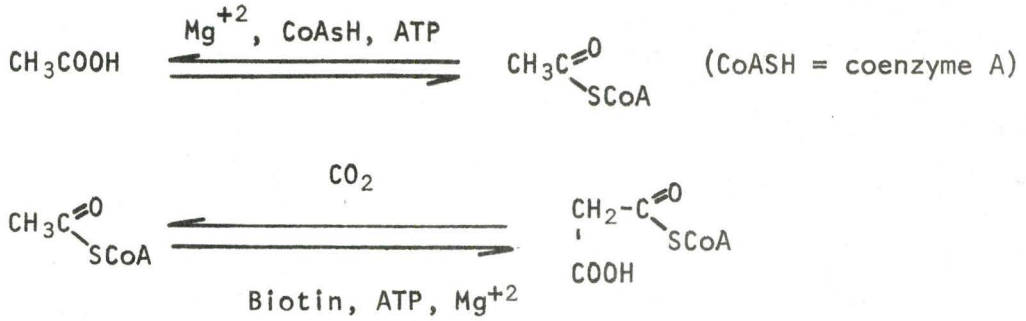

O
*
N
H
*C
O
OH
O
*
NH
*CO2
*
*
*
O
N

Scheme 4

Δ'-piperideine into the C-9 to C-16 unit, and in the limit, all the activity should be found in the C-1 to C-8 unit of lycopodine.

Experiments showed that pelletierine is indeed an intermediate on the pathway from lysine to lycopodine. Most of the activity was recovered at C-5 when 2-^{14}C Δ'-piperideine was administered along with added inactive pelletierine. Similarly, most of the activity was located in the first half of lycopodine when 1,5-^{14}C cadaverine and inactive pelletierine were administered to the plants. It was also shown that pelletierine was present in the plants at a time when lycopodine is being formed. Radioactive pelletierine (isolated as pelletierine hydrochloride) was recovered from Lycopodium plants, to which 2-^{14}C Δ'-piperideine and 1,5-^{14}C cadaverine had been administered along with unlabeled pelletierine.

These results are consistent with the second alternative in which lycopodine is not a true dimer, and pelletierine is the precursor of the C-9 to C-16 unit only. To reconcile this finding with the observed equal incorporation into the two halves of lycopodine from acetate, lysine and its derivatives, model 32 was proposed as a precursor of pelletierine provided that the transformation from the former to the latter is irreversible. The metabolic sequence was postulated as shown in Scheme 4. In the proposed pathway, malonate units are involved. It is known that acetyl-coenzyme A is transformed to malonyl-CoA by carboxylation according to the route below:



If malonate units are involved in the biosynthesis of lycopodine, and are derived from acetate molecules, the results of feeding experiments with acetate as precursor are still compatible with the biosynthetic sequence of Scheme 5. The involvement of malonate units may be tested by feeding experiments with labeled malonate. This postulated precursor should incorporate specifically into the two halves of lycopodine with the same distribution of label as in the case of acetate (Scheme 4).

This novel route also suggested that 2-piperidineacetic acid, or rather 2-piperidineacetyl-coenzyme A, is a precursor of lycopodine. It is the purpose of this work to test this hypothesis, and particularly the role of 2-piperidineacetic acid in the biosynthesis of lycopodine.

DISCUSSION OF RESULTS

It has been suggested that 2-piperidineacetyl-CoA is a precursor in the biosynthesis of lycopodine and that it is formed by the condensation of one Δ'-piperideine unit and one malonyl-CoA unit. Two conditions must be met to establish a precursor-product relationship. 1) a specific incorporation of 2-piperidineacetic acid into lycopodine and 2) the formation of this substrate within the system at the time the alkaloid is being formed. To determine if piperidineacetic acid is a precursor, feeding experiments should be done with labeled 2-piperidineacetic acid and the lycopodine from these experiments isolated and assayed for radioactivity. If the alkaloid is radioactive, degradation should be carried out, and the distribution of label determined. It was the purpose of this work to synthesize labeled 2-piperidineacetic acid and test its incorporation into lycopodine.

Pelletierine has been synthesized by a condensation of Δ'-piperideine and sodium acetoacetate in aqueous solution at room temperature. Δ'-Piperideine may be derived from its trimer, α-tripiperideine, which is prepared from N-chloripiperidine (33), or from lysine (18). The original intent of this work was to synthesize 2-piperidineacetic acid, in a manner similar to the synthesis of pelletierine, by condensation of Δ'-piperideine and a malonate derivative. In this way, one may obtain, in principle, 2-piperidineacetic acid with labeled atoms on the piperidine ring and/or at the side chain by using radioactive lysine (^{14}C or ^{3}H)

and radioactive malonate derivatives (1-^{14}C or 2-^{14}C malonate).

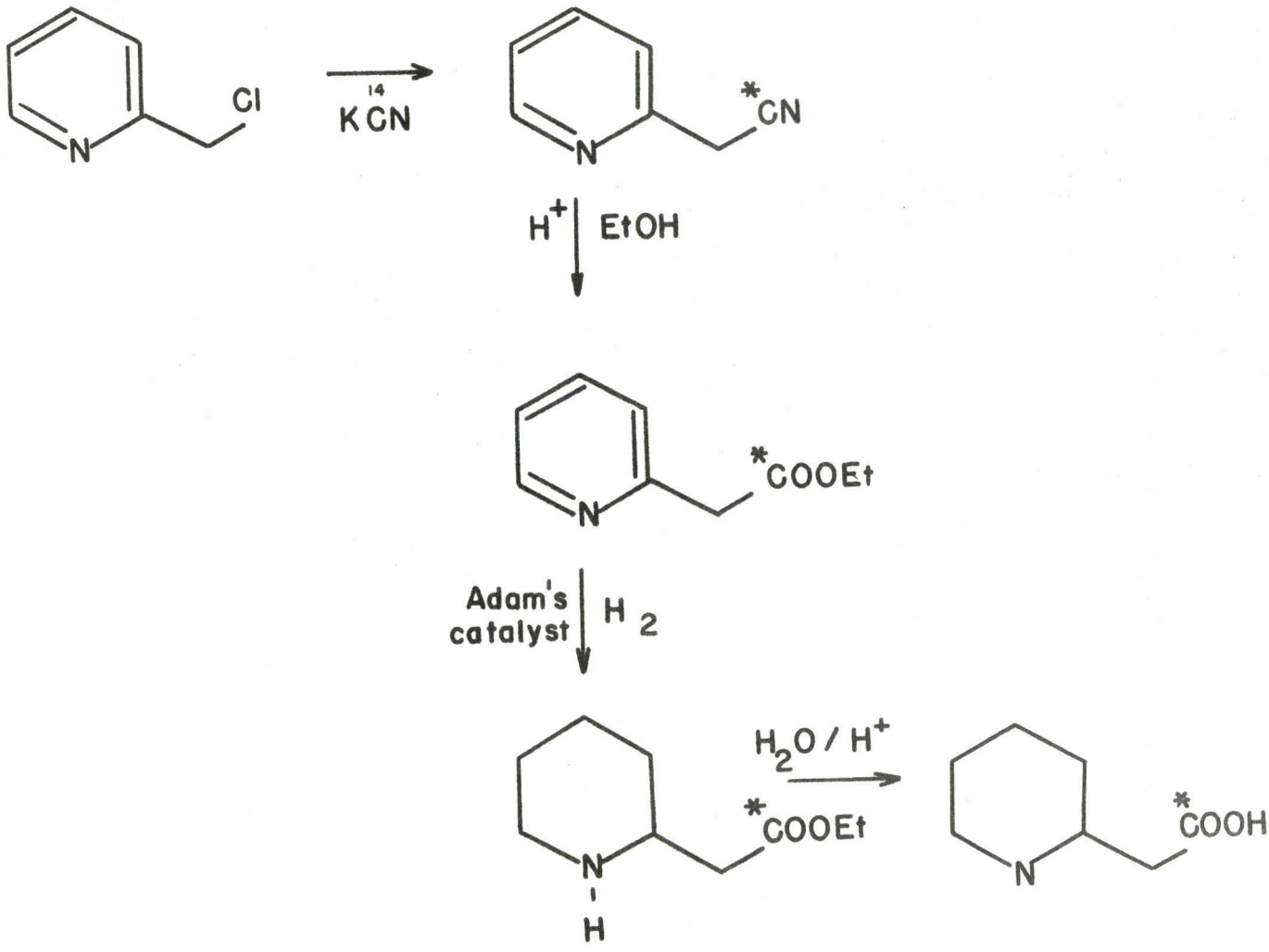
In the same way that lysine is incorporated symmetrically into the two "halves" of lycopodine, the hypothesis (Scheme 4) predicts that 2-piperidineacetic acid will be incorporated into both halves of lycopodine. Thus, in a feeding experiment with 2-piperidineacetic acid labeled at C-2, the distribution of label is expected to be between C-5 and C-13. Similarly, lycopodine should be equally labeled at C-9 and C-1 when 6-^{14}C-2-piperidineacetic acid serves as precursor. Lycopodine isolated from feeding experiment with β-^{14}C-2-piperidineacetic acid should have equal distribution of label between C-7 and C-15.

A sample of 2-piperidineacetic acid was prepared by oxidation of 2-piperidineethanol. It was identified by its mass spectrum and its melting point, and by comparison on t.l.c. with an authentic sample. This synthetic sample was used as a reference in the subsequent work.

Several attempts to elaborate 2-piperidineacetic acid from a condensation of Δ'-piperideine and malonate derivatives (malonic acid, diethylmalonate, malonitrile) under various conditions (aqueous solvent at different pH's, pH = 3, 7, and 9, ethanol/H_2O) were unsuccessful. Accordingly, another synthetic scheme was sought.

A search of the literature (34) reveals that 2-picolylchloride may be converted to ethyl 2-pyridineacetate by the procedure outlined in Scheme 5. Extension of this synthesis to 2-piperidineacetic acid was expected and was proved to proceed readily by hydrogenation of the pyridine ring followed by hydrolysis of the ester. Although this

scheme does not permit the synthesis of a doubly labeled precursor, it seemed an attractive and simple route for the synthesis of the acid singly labeled at the β position in the side chain.



Scheme 5. Synthesis of β-^{14}C-2-piperidineacetic acid

Experiments with radiotracers are valid and interpretations of results meaningful if careful consideration is given to certain basic factors. The amount of activity employed should be restricted to the minimum necessary to permit reasonable counting rates in the sample to be assayed. The specific activity of the tracer must be sufficiently high so that the amount of labeled compound administered does not disturb the normal conditions of the system. For safe handling of radioisotopes the highest permissible dose of activity of ^{14}C used should not exceed 1 mC (35). The ratio of $K^{14}CN/KCN$ used should be as high as possible so that an optimum specific activity of the compound synthesized is obtained. This ratio, of course, is related to the amount required to permit satisfactory chemical manipulation and to give the desired yield. The whole sequence of the synthesis of 2-piperidine-acetic acid using non-radioactive material was carried out in the same way as it should be done in the synthesis using radiotracer. The conditions of the reactions, the amount of the starting materials used, and the overall yield of the product were established before the synthesis of the radioactive compound was undertaken.

The original reaction sequence consists of 4 steps: the introduction of CN, conversion of the cyano group to the ester, hydrogenation of the ring and hydrolysis of the ester group to the acid. The first two steps were carried out as in the procedure described by Winterfeld and Flick (34). In this procedure, 2.20 equivalents of KCN were used for each equivalent of 2-picolylchloride hydrochloride, and the solvent was a mixture of water-ethanol. Attempts to reduce the amount of

potassium cyanide used in the reaction using DMSO as solvent, in order to obtain a maximum radio chemical yield, failed to give a complete reaction, and therefore, a good chemical yield of the product. The reaction was then carried out with the free base, 2-picolylchloride, which was prepared from the hydrochloride salt and used immediately. It was found that equal equivalents of 2-picolylchloride, and potassium cyanide in ethanol-water gave a yield of 85%. The conversion of the nitrile compound to the ester was realized in 90% yield using the established procedure. It has been known that the pyridine ring can be hydrogenated at low pressure (3-4 atm) using Rh on C and platinum oxide catalyst. A hydrogenation at atmospheric pressure is more suitable for a synthesis of a radioactive compound since the hazard of contamination is minimized. Various solvent systems were investigated (ethanol/HCl, acetic acid) to carry out hydrogenation at atmospheric pressure. The best solvent system found for the synthesis was aqueous HCl (10%) with Adam's catalyst at room temperature. The ethyl 2-piperidineacetate was obtained in 90% yield. In the procedure using non-radioactive material, the product from the hydrogenation was isolated and identified by its mass spectrum, by n.m.r. and i.r. before proceeding to the last step. In the synthesis of the radioactive compound, however, ethyl 2-piperidine-acetate was not isolated. The ethyl β-^{14}C-2-piperidineacetate hydrochloride formed from the hydrogenation reaction in aqueous HCl solution was checked on t.l.c. with an authentic sample in order to ensure that the reaction was complete. Then the acidic aqueous solution of the ester was refluxed, and the hydrolysis completed in a 95% yield. The

product was obtained as β-^{14}C-2-piperidineacetic acid hydrochloride and this product was used in the tracer experiments.

Two feeding experiments (Table I) were undertaken using the procedure employed successfully in previous studies (30,31). In the first experiment, β-^{14}C-2-piperidineacetic acid alone was administered. The crude alkaloid isolated from this feeding was radioactive. The sample was subjected to careful chromatography and a number of fractions collected and examined. The lycopodine (fraction I, 70 mg), which was isolated from the basic alumina column and purified further by crystallization and sublimation, had a mean specific activity = 5.27 ±.04 x 10^2 counts min^{-1} $mmole^{-1}$. The observed activity is about 10-20 times less than the activity of lycopodine isolated from feeding experiments with radioactive acetate and lysine. The low activity of lycopodine precluded degradation of the sample.

A second fraction (fraction II, 3 mg) eluted from the column showed no radioactivity measurable by liquid scintillation counting. This minor alkaloid, after being purified by sublimation, had an Rf value corresponding with that of an authentic cample of dihydrolycopodine ($C_{16}H_{27}NO$). Its mass spectrum showed a molecular ion peak at m/e = 249, and an intense ion at m/e = 192 like dihydrolycopodine, but further investigation to confirm the identity of this alkaloid has not been carried out.

A third fraction (fraction III, 10 mg) contained a compound which is strongly fluorescent under a U.V. lamp. This fraction was subjected to t.l.c. (silica gel on aluminum sheet, eluant = $CHCl_3$/MeOH:

TABLE I

	Compounds administered	
	β-^{14}C-2-piperidineacetic acid	β-^{14}C-2-piperidineacetic acid L-4-^{3}H-Lysine
Nominal total activity (^{14}C, Millicurie)	0.1	.02
Mode of administration	cuttings	cuttings
Date	July 72	August 72
Weight of dry plants (g)	60	80
Yield (Lycopodine, mg)	70	80
Lycopodine - Specific activity (counts $min^{-1}mmole^{-1}$)	$(5.27 \pm .04)10^2$	$(1.63 \pm 0.9)10^2$
$^{3}H/^{14}C$ ratio in precursor		4.89 ±.60
$^{3}H/^{14}C$ ratio in lycopodine		81.80 ±3.36
Recovery of activity (^{14}C)	0.68×10^{-4}%	1.2×10^{-4}%

1:3). The fluorescent compound has an Rf = 0.71, but assay of radioactivity by the radioscanner showed no radioactivity in this fluorescent compound, instead, a single radioactive peak was shown at Rf = 0.54, total activity = 5.5×10^{-6} mC. The total activity of this component is about 80 times greater than the total activity of the lycopodine isolated (0.067×10^{-6} mC). However, this compound is not detectable on t.l.c. with various reagents (Dragendorff's, iodine vapour) nor with a U.V. lamp and must exist in minute amount in L. tristachyum. It is apparent that β-^{14}C-2-piperidineacetic acid is incorporated into it much more readily than into lycopodine. The Rf value of this material is very close to the Rf value of lycodine (Rf = .57) which exists in minute amount in L. tristachyum and of β-obscurine (Rf = .54). However, in using the carrier dilution method, no activity was detectable in the crystals of β obscurine or of lycodine recovered from crystallization of these alkaloids in the presence of the radioactive compound.

A second experiment in which a mixture of β-^{14}C-2-piperidineacetic acid and L-4-^{3}H-lysine ($^{3}H/^{14}C = 4.9$) was administered to L. tristachyum yielded radioactive lycopodine. The lycopodine, isolated after purification by crystallization and sublimation, showed a specific activity in $^{14}C = (1.63 \pm .09)10^{2}$ counts min^{-1} $mmole^{-1}$ and a ratio $^{3}H/^{14}C = 82$. The ratio of $^{3}H/^{14}C$ indicates that at a time when lycopodine is being produced in the plants and lysine is incorporated into it, 2-piperidineacetic acid is not incorporated into the product according to the proposed metabolic sequence.

The results of this work cast doubt upon the validity of the hypothesis put forward in Scheme 4. However, one may not discard completely the suggested biosynthethic sequence since several factors must still be considered. 2-piperidineacetyl-Coenzyme A and not 2-piperidineacetic acid was proposed as an intermediate on the pathway. It is possible that the plants cannot transform the fed substrate to the CoA derivative. The permeability of the substrate through the cell membranes and the rate of transport of the compound to the site of synthesis in the plants may also affect the incorporation of the proposed precursor. A checking experiment, in which the ester of β-^{14}C-2-piperidineacetic acid is administered should be done in the future to clarify this point.

If 2-piperidineacetic acid is indeed an intermediate on the pathway from lysine to lycopodine, similarly to the case of pelletierine, one should be able to detect the presence of 2-piperidineacetic acid in the plants by carrier-dilution in feeding experiments in which other confirmed precursors are administered (lysine, cadaverine, or Δ'-piperideine). Experiments along this line are now underway in this laboratory but the results at this stage are inconclusive.

EXPERIMENTAL

Apparatus, Methods and Materials

The n.m.r. spectra were run on a Varian T-60 instrument with samples dissolved in $CDCl_3$ or DMSO-d6. The infrared spectra of samples were recorded using a Perkin Elmer 337 spectrometer. A C.E.C. 21-110B double focusing mass spectrometer at an ionization potential of 70 eV and at an ionization current of 100 μA was used to record the mass spectra.

Melting points were determined on a Kofler Micro Hot Stage and are uncorrected.

Potassium cyanide-^{14}C (1 mCi, specific activity = 54.2 mCi/mmole) and L-4-^{3}H-lysine (200 μCi) were obtained from the Radiochemical Centre, Amersham, England.

Solid samples were assayed for radioactivity on a gas flow Geiger counter (Nuclear Chicago Corporation Model 4342). A weighed amount of the compound (from .3 mg to 1.0 mg) was deposited on an aluminum planchette, and dissolved in 2 drops of a solution of collodion (2%) in dimethylformamide. An even spreading of the sample over the surface of the planchette was obtained by covering the solution with a disc of lens tissue. The planchette was dried under an infrared lamp and cooled before counting.

Liquid scintillation counting was carried out on a Mark I Liquid Scintillation Counter (Model 6860, Nuclear Chicago Corporation). Samples

were dissolved in methanol or methanol-benzene, enough solvent was added to make up a certain volume (e.g. 1 ml), a predetermined amount of the solution (e.g. 100) was pipetted into a counting vial, mixed well with 10 ml of Aquasol solution (a ready-to-use xylene-based liquid scintillation counting solution from New England Nuclear) and counted.

Preparation of 2-piperidineacetic acid

Ten grams of 2-piperidineethanol was dissolved in 10 ml of distilled water. To the ice-cooled solution was added dropwise a mixture of 20 g of chromic acid, 40 g of conc. sulfuric acid and 300 ml of water. The addition of reagents was completed after one hour and the stirring of the mixture was continued for 3 hours at room temperature. The solution was made basic with a saturated aqueous solution of $Ba(OH)_2$, excess of $Ba(OH)_2$ was neutralized with CO_2. The mixture was filtered over celite, and the filtrate was evaporated to dryness. The residue was dissolved in methanol and the product was crystallized from methanol-ether. White needles of 2-piperidineacetic acid were obtained (8 g, yield = 72%) which melted at 218°C (lit. value = 214°C) (36). It was compared on t.l.c. with an authentic sample of 2-piperidineacetic acid obtained from Dr. A.I. Meyers.* The mass spectrum of the compound showed the molecular ion peak at m/e = 143. Its i.r. spectrum showed broad bands in the region of 2800-3300 cm^{-1} and a sharp band at 1730 cm^{-1}.

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*Department of Chemistry, Louisiana State University of New Orleans.

Preparation of 2-piperidineacetic acid hydrochloride salt

2-piperidineacetic acid was dissolved in aqueous HCl solution (10%). The solvent was evaporated to dryness, and the compound was sublimed at 120°C, 10^{-2} mm. It melts at 160-162°C. Its i.r. spectrum was recorded and was in accord with the structure (Fig. 3). It has the same Rf value as that of 2-pyridineacetic acid using acidic eluant (Rf = 0.39, eluant = n-butanol-acetic acid-water = 4:2:1 on silica gel).

Preparation of 2-picolylchloride hydrochloride

To freshly distilled 2-pyridylmethanol (300 mg) cooled in an ice-bath, distilled thionyl chloride (1.0 g) was added dropwise with stirring. The mixture was refluxed mildly for 2 hours, cooled, 20 ml of dried benzene was added and the mixture was stirred for one hour to dissolve the excess of thionyl chloride. The benzene phase was decanted, and the procedure was repeated with 10 ml of fresh benzene. The residue was dissolved in 3 ml of ethanol and the precipitate was collected after cooling the solution. Recrystallization was effected with ethanol-ether. Brown needles of the hygroscopic 2-picolylchloride hydrochloride (300 mg) were obtained, yield = 85%. Melting point = 128-130°C (lit. value = 128-129°C). Its n.m.r. and i.r. spectra were taken (Fig. 4a, 4b) and are in accord with the structure.

Preparation of 2-pyridineacetonitrile

The inactive material was prepared following the procedure described by Winterfeld and Flick (34). 2-pyridineacetonitrile was obtained as a yellow liquid. Boiling point = 80° at 0.4 mm. Its n.m.r

and i.r. spectra were taken (Fig. 5a, 5b). The spectra were compatible with the assigned structure.

Preparation of ethyl 2-pyridineacetate

The compound was prepared from 2-pyridineacetonitrile (34). The product is a yellow liquid, boiling point = 126°-127°C at 17 mm Hg. Its n.m.r. spectrum was taken (Fig. 6). The spectrum is in accord with the structure.

Preparation of ethyl 2-piperidineacetate

Ethyl 2-pyridylacetate (165 mg) was dissolved in 10 ml of aqueous HCl, 40 mg of Adam's catalyst was added, and the hydrogenation was carried out at atmospheric pressure and room temperature for 10 hours. The solution was filtered, made basic with aqueous $NaHCO_3$ solution and then extracted with $CHCl_3$. The chloroform extract was dried over anhydrous Na_2SO_4, evaporated, and the residue was distilled under vacuum at 80°C, 10^{-2} mm Hg. Ethyl 2-piperidineacetate was obtained as a yellow liquid (150 mg, 90% yield). Its mass spectrum showed a molecular ion peak at m/e = 171. Its n.m.r. and i.r. spectra were recorded (Fig. 7a, 7b) and they are in accord with the structure. The compound has an Rf value = .35 on t.l.c. (silica gel, eluant = $CHCl_3$).

Preparation of β-^{14}C-2-pyridineacetonitrile

2-picolylchloride hydrochloride (270 mg) was added to 10% $NaHCO_3$ in aqueous solution (10 ml) and the mixture was extracted with 3 x 5 ml of $CHCl_3$. The chloroform solution was dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated at 30°C under vacuum (water pump). 2-picolyl-

chloride (200 mg) was obtained as a volatile pink liquid.

To the 2-picolylchloride in the reaction flask, dried potassium cyanide (113 mg) and $K^{14}CN$ (1.2 mg, 1 mCi, specific activity = 54.2 mCi/mmole) were added, together with 12 mg of KI. Five milliliters of an ethanolic aqueous solution ($EtOH/H_2O$: 3:2) was used to carry out the transfer of the radioactive material into the reaction flask, and also as the solvent for the reaction. The solution was stirred and refluxed for 2 hours. A drying tube fitted on the condenser contained solid KOH to absorb any $H^{14}CN$ gas formed. The reaction was allowed to stand overnight. The ethanol was distilled off with continuous addition of water until 4 ml of distillate was collected. Saturated aqueous sodium carbonate solution (5 ml) was added, and the mixture, without transfer from the original flask was continuously extracted with ether for 24 hours, and again with fresh ether for another 24 hours. The ethereal phases were combined, and the extract evaporated by passing through it a stream of dry nitrogen. The residue was distilled under vacuum at $60^{o}C$, 10^{-2} mm. The product was identical on t.l.c. with an authentic sample (silica gel, thickness 2 mm, developed with ether-chloroform = 1:1, Rf = .56).

Preparation of ethyl β-^{14}C-2-pyridineacetate

The distillation tube containing the product obtained previously was fitted on a three necked flask containing 20 ml of absolute ethanol. The alcohol was refluxed until the β-^{14}C-2-pyridylacetonitrile was completely dissolved and transferred into the flask. The distillation tube was quickly replaced by a condenser and the mixture cooled in an ice salt bath. A stream of dry HCl gas (dried by passing through a trap

of conc. H_2SO_4) was passed through for 45 minutes, with cooling and stirring of the mixture. The mixture was refluxed overnight and the ethanol was distilled off under vacuum. The white residue was transferred into the continuous extraction flask with 10 ml of H_2O and 10 ml of saturated aqueous Na_2CO_3 solution was added. The mixture was continuously extracted with ether for 24 hours, and again with fresh ether for another 24 hours. The ether phases were combined, evaporated in a stream of nitrogen, and the mixture was distilled under vacuum at 90°C, 10^{-2} mm. The product was identical on t.l.c. with an authentic sample (silica gel, developed with ether-chloroform = 1/1, Rf = 0.60).

Preparation of β-^{14}C-2-piperidineacetic acid hydrochloride

The part of the distillation tube containing the radioactive material prepared above was cut off and dropped into a 50 ml hydrogenation flask. Aqueous hydrochloric solution (10%, 10 ml) was added, and then 40 mg of Adam's catalyst. Hydrogenation was carried out at room temperature and at atmospheric pressure for 15 hours. The catalyst was then filtered off, and rinsed with 20 ml of 10% hydrochloric acid. The combined filtrates were refluxed for 10 hours. The solvent was then distilled off under vacuum and the residue was sublimed at 10^{-2} mm, 120°C. The sublimate was recrystallized from methanol-ether. β-^{14}C-2-piperidineacetic acid hydrochloride was obtained (1st crop of crystals: 108 mg, 2nd crop: 50 mg) as white needles, specific activity = .62 mCi (mmole). Its purity was checked on t.l.c. against the authentic sample (silica gel, using n-butanol/acetic acid/water: 4:2:1 as solvent). The thin layer chromatogram was checked for radioactivity in a radioscanner.

One single radioactive peak showed at Rf = 0.39. Radioactivity of the product was also assayed on aluminum planchettes in a Geiger counting system. Total chemical yield from 2-picolylchloride hydrochloride = 67%. Total radio chemical yield = 60%.

Administration of labeled β-^{14}C-2-piperidineacetic acid hydrochloride

Lycopodium tristachyum Pursh plants were found near Algonquin Provincial Park. The green shoots (165 g wet weight) were cut from the plants, and packed with cut surfaces downward into two 50 ml beakers. An aqueous solution (20 ml) of β-^{14}C-2-piperidineacetic acid hydrochloride (29 mg, 100 μCi) was divided between the two beakers. After the tracer solution had been absorbed, distilled water was repeatedly added to the beakers. The plants were kept in contact with the tracer for 2 days, and then dried in an oven at 45-50°C for 30 hours. The beakers were rinsed with distilled water, and the rinsing liquid was assayed for radioactivity, 99% of the tracer was absorbed by the plants. The dried plants (60 g) were ground to a fine powder in a blender (Osterizer Galaxie 10) and extracted in the manner described below.

Isolation of Lycopodine

The powder was extracted with methanol for 3 days in a Soxhlet extractor, and again with fresh methanol for 18 hours more. The methanolic extract was evaporated under vacuum. The residue was stirred overnight in 200 ml of 5% hydrochloric acid. The aqueous acidic solution obtained after filtration was extracted with ether (3 x 50 ml), basified with 2M ammonia, and then extracted with chloroform (6 x 50 ml). The

chloroform extract was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was extracted with warm hexane, the hexane extract was evaporated and the residue was sublimed at 110°C, 10^{-2} mm. The yellowish sublimate was recrystallized twice from hexane.

The purity of the lycopodine obtained was checked by radio chromatography of a thin layer chromatogram (silica gel, developed with $CHCl_3$/Methanol: 1:3). The analysis showed that lycopodine contained a highly active impurity (Rf = 0.06).

Purification of Lycopodine

The mother liquors from the various crystallization and the crystals of lycopodine were combined and passed through a column (40 x 2 cm) of basic alumina (28 g). The solvents used for eluting the alkaloids were in the following sequence: benzene, benzene-ether, ether, ether-chloroform. Two hundred fractions of 20 ml were collected, and one in every five fractions was checked for radioactivity in a liquid scintillation counter, and also chromatographed on t.l.c. (silica gel, developed with chloroform/methanol: 1:3).

Fractions 17-77 (eluted with benzene) which contained lycopodine (Rf = .30) were combined, the residue was crystallized from hexane, and the crystals of lycopodine obtained were sublimed at 90°C, 10^{-2} mm. The sublimate (70 mg) was checked for radioactivity on an aluminum planchette. Mean specific activity for a duplicate reading = $(5.27 \pm .04)10^2$ counts min^{-1} $mmole^{-1}$.

Fractions 112-116 were combined (eluted with benzene/ether, Rf = .05) which corresponds with the Rf value of an authentic sample of

dihydrolycopodine. No radioactivity was detectable in this sample.

Fractions 117-132 (eluted with $CHCl_3$) were combined. This fraction contains a compound which is strongly fluorescent under a U.V. lamp (Rf = 0.71) and a radioactive compound, shown by the radioscanner, which has Rf = .51, total activity = 5.5×10^{-6} mC. Neither compound has been identified.

Isolation of lycopodine from feeding experiment with β-^{14}C-2-piperidine acetic acid and L-4-^{3}H-lysine

L-4-^{3}H-lysine (1 mCi) was dissolved in 10 ml water. One ml of this solution was withdrawn and added to an aqueous solution of 5.7 mg (20 μCi) of β-^{14}C-2-piperidineacetic acid hydrochloride. A sample of this mixture was assayed for radioactivity in a liquid scintillation counter, and $^{3}H/^{14}C$ ratio was measured (4.89 ±0.6). The solution was fed to 165 g of fresh cuttings of L. tristachyum and the plants were dried, ground and extracted with methanol in the same way as in the previous feeding experiment.

The crude alkaloidal mixture extracted by chloroform was passed through a column of basic alumina (30 g, 30 x 1.5 cm) and lycopodine was obtained after elution with benzene. The lycopodine was crystallized from distilled hexane, and sublimed under reduced pressure. The sublimate was assayed for radioactivity in a liquid scintillation counter.

The isolation and identification of other minor constituents of the crude extract was not carried out.

DEFINITIONS

1 Curie = 3.6×10^{10} dps = 2.2×10^{12} dpm

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

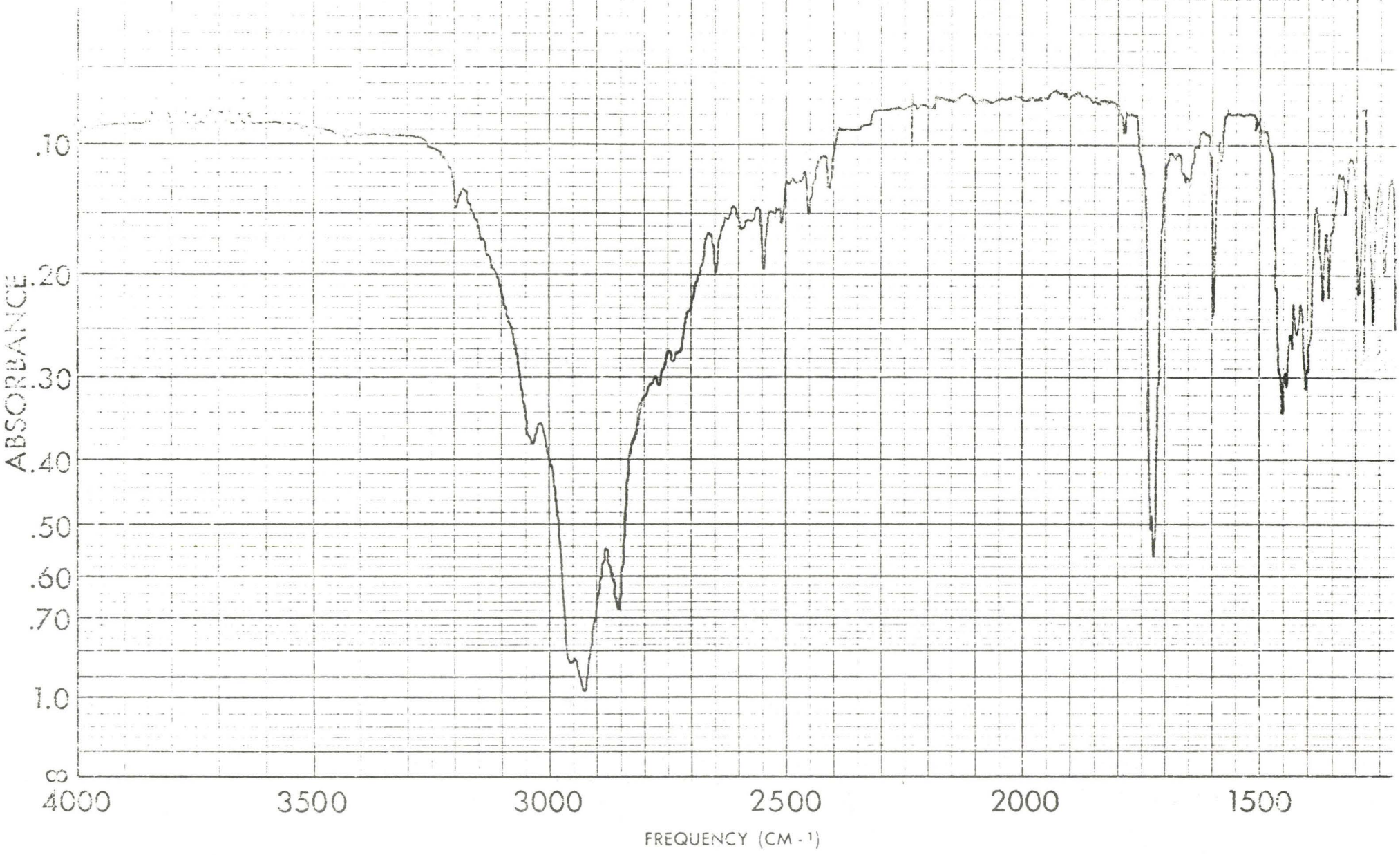
$$\text{Specific activity (counts min}^{-1}\text{ mmole}^{-1}) = \frac{\text{Total activity (counts min}^{-1})}{\text{weight (mg)}} \times \text{mmole (mg)}$$

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

(30/100 = efficiency of the Geiger counting system)

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

$$\text{Radio chemical yield (\%)} = \frac{\text{Total activity recovered (mCi)}}{\text{Total activity fed (mCi)}} \times 100$$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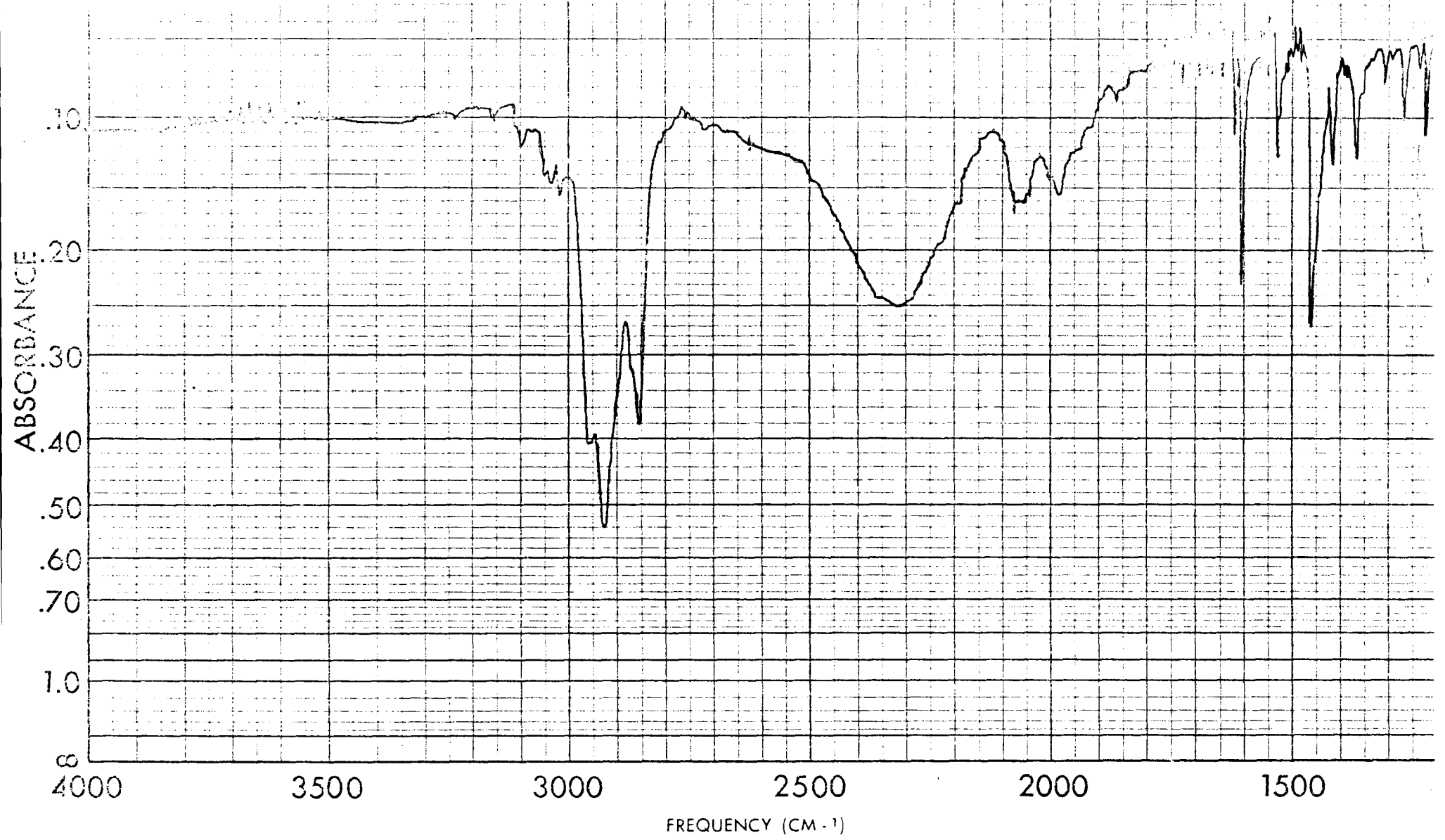
FREQUENCY (CM-1)

Figure 3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FREQUENCY (CM-1)

Figure 4a

Figure 4b

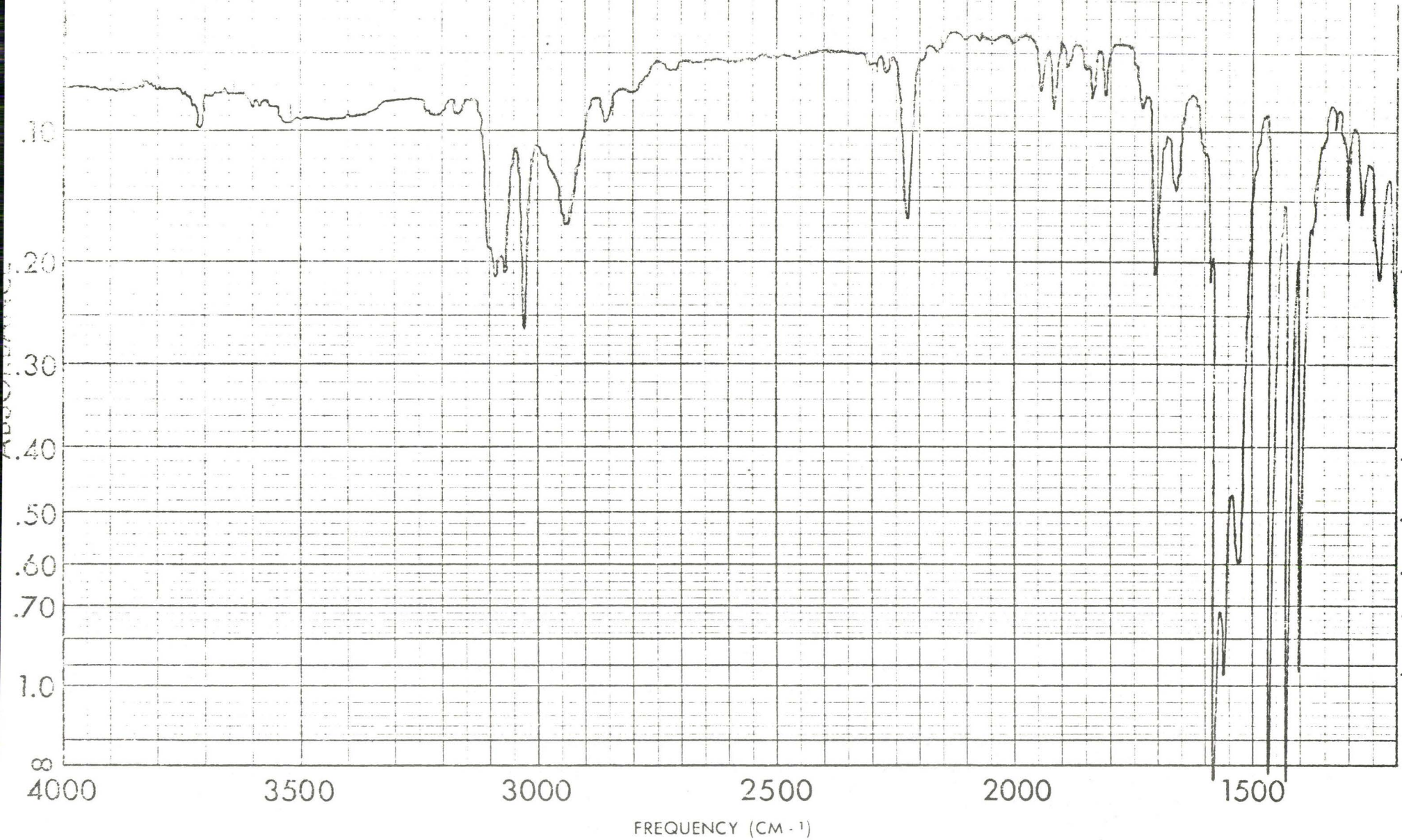
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Figure 5a

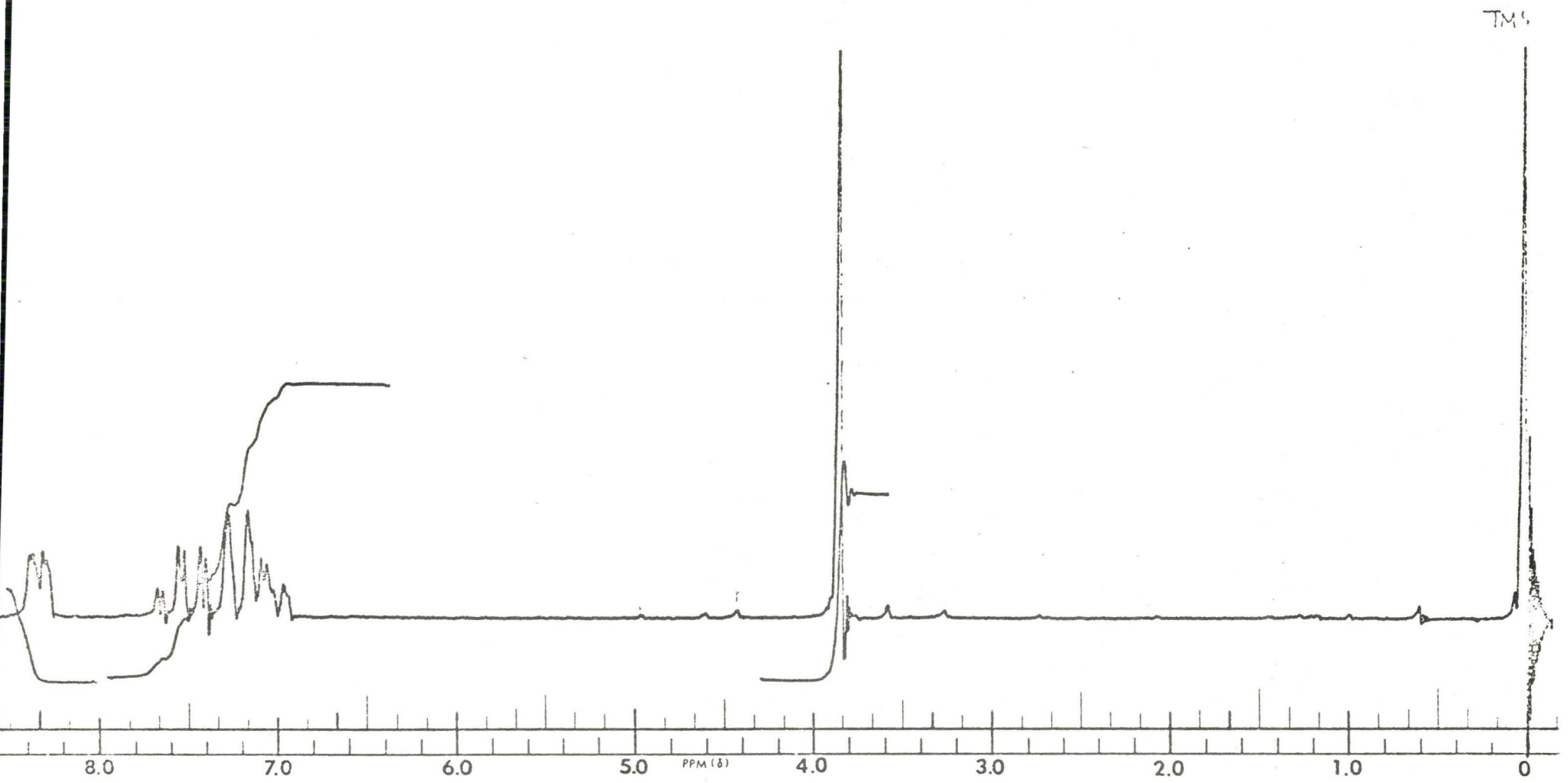


Figure 5b

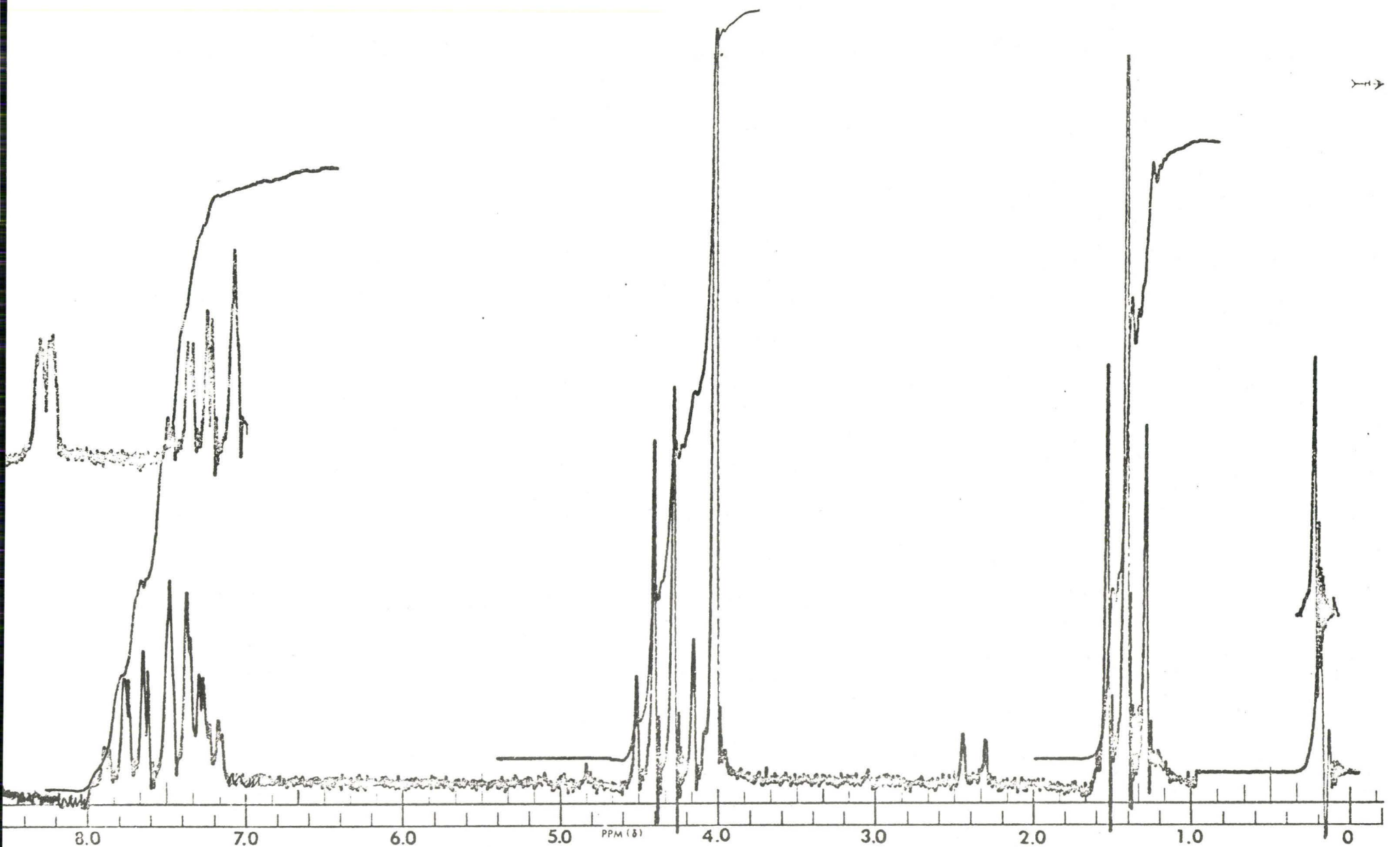
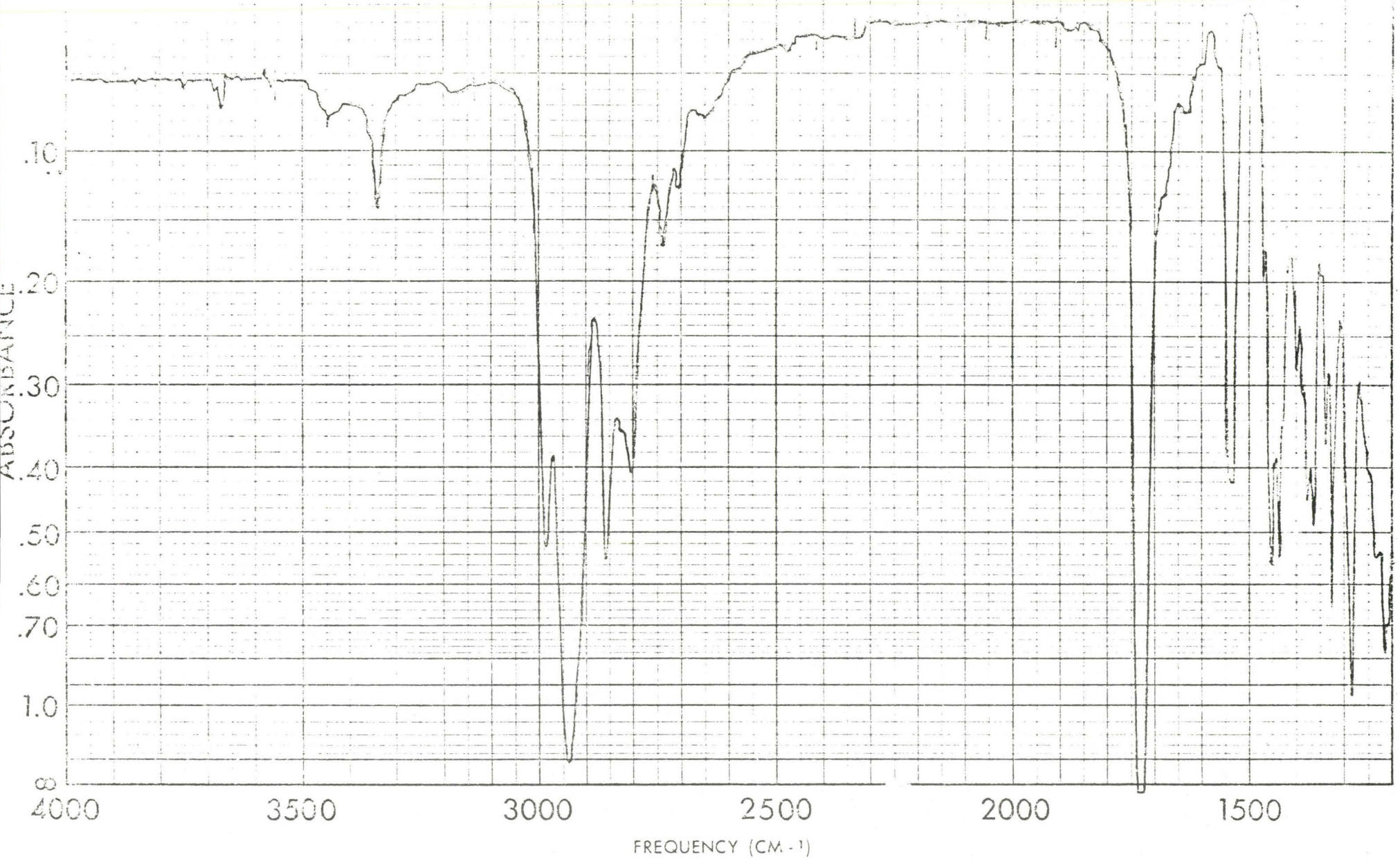


Figure 6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FREQUENCY (CM.-1)

Figure 7a

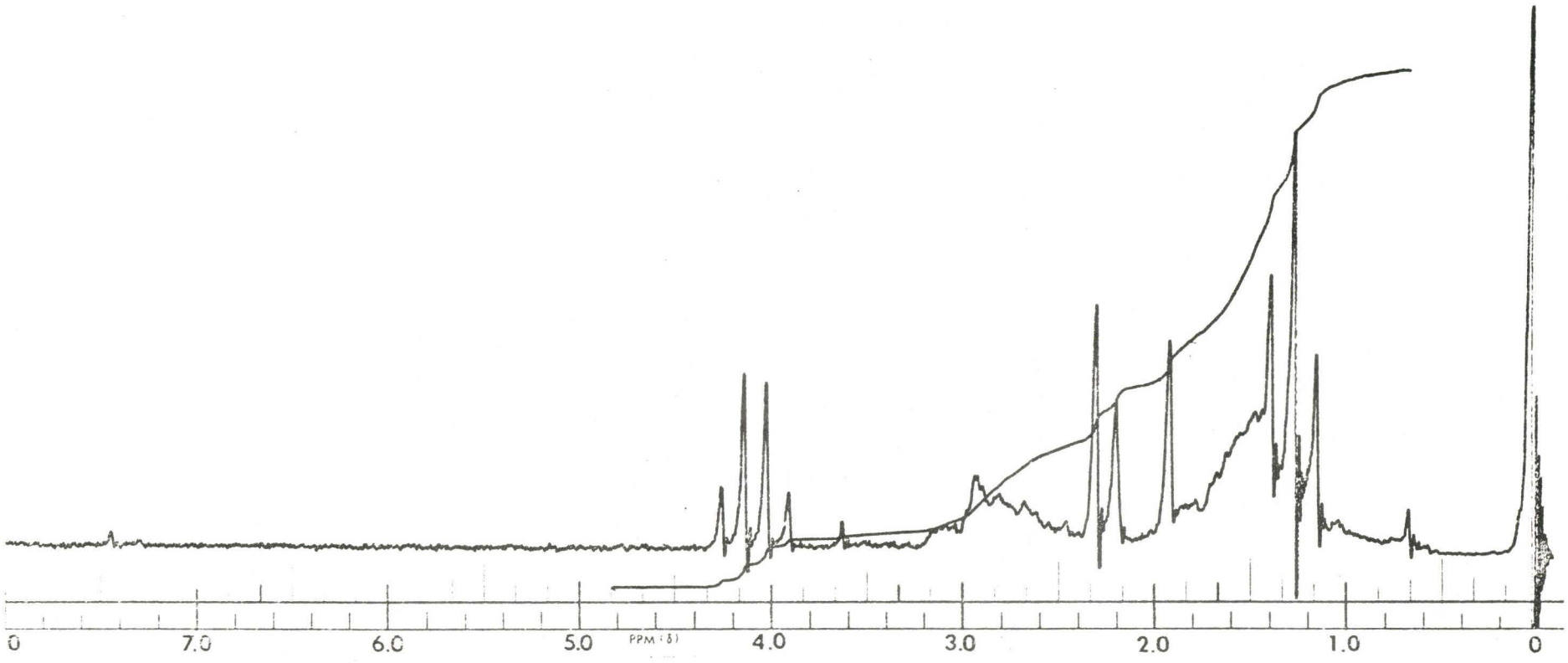

Figure 7b

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