THE PYRROLIZIDINE ALKALOIDS

Studies on the Pyrrolizidine base Retronecanol

by

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CONTENTS

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Summary

Degradative and biosynthetic studies were performed on retronecanol, obtained from monocrotaline, the alkaloid present in Crotalaria spectabilis. Previously two different theories regarding the biosynthesis of the pyrrolizidine bases had been proposed. Conflicting results in this school emphasised the need for a critical examination of the degradative procedure.

Heliotridane, derived from retronecanol, was submitted to the Hofmann degradation. The product of the reaction was shown by gas-liquid chromatography to be a mixture with at least five components. Attempts to separate and purify the components were unsuccessful, although some information regarding their identity was deduced.

The mass spectra of retronecanol, heliotridane and heliotridene were obtained and plausible rationalisations of these spectra are presented.

In order to circumvent the difficulties previously encountered in the degradation, attempts were made to obtain retronecanone from retronecanol by oxidation on a small scale. Three different oxidants, however, failed to give the required product.

Plants of Crotalaria spectabilis were fed with [5- ^{14}C]-ornithine, followed by degradation of the active monocrotaline obtained to give the activities of carbon atoms 1 and 1' of the

pyrrolizidine base. The results are consistent with a theory which invokes a symmetrical intermediate in the biogenesis of ring B of the pyrrolizidine unit.

INTRODUCTION

INTRODUCTION

The presently described work is a continuation of research done into the biosynthesis of the Senecio alkaloids and is concerned, in particular, with the build-up of the basic pyrrolizidine structure in these alkaloids.

1. THE PYRROLIZIDINE ALKALOIDS

The recent appearance of an excellent and comprehensive review on the pyrrolizidine alkaloids by F.L. Warren [1], following on earlier reviews [2, 3], precludes repetition here. It will suffice to point out briefly some of the features of these alkaloids as they relate to the present work.

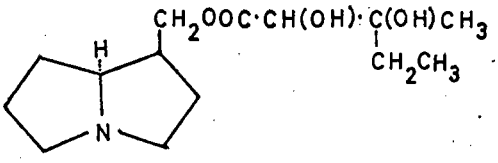
The pyrrolizidine alkaloids are, for the most part, a group of basic substances composed of hydroxylated methyl-pyrrolizidines, esterified by one or two monobasic carboxylic acids or by a dicarboxylic acid. Some free, unesterified pyrrolizidine bases have been isolated and the pyrrolizidine base may occur as the N-oxide in the plant. These alkaloids have now been isolated from the plant families Compositae, Leguminosae, Boraginaceae, Santalaceae and Gramineae. Recently other alkaloids, such as the sulphur-containing Rhizophoraceae alkaloid cassipourine [4], which contain the unhydroxylated pyrrolizidine skeleton have been isolated and are being studied.

Even more recently new types of pyrrolizidine - containing alkaloids have been isolated from the family Orchidaceae [4a]. An example is the glycoside nervosine. Some examples of the pyrrolizidine alkaloids are given in Chart I. Physiologically, some of these alkaloids have been shown to be responsible for hepatic cirrhosis.

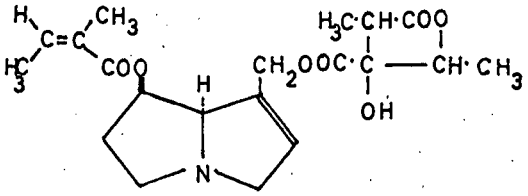
Considerable information regarding the basic hydrolysis products of the alkaloids, the so-called "necine" bases, has accumulated. Most of them have 1-methylpyrrolizidine (1) as carbon skeleton. They may be hydroxylated at positions 1, 2 and/or 7 and there may be unsaturation between positions 1 and 2. Differences in stereochemistry give rise to different necines. Some of the more frequently encountered bases, with their stereochemistry, are presented in Chart II.

Biosynthetically the pyrrolizidine skeleton can be derived from two molecules of ornithine. Previous workers [5,6,7, 8, 9] have shown [1, 4- ^{14}C] putrescine, [2- ^{14}C] ornithine, [5- ^{14}C] ornithine and [^{3}T] proline to be precursors of the base retronecine in Crotalaria spectabilis, Senecio douglasii, S. isatideus and S. sceleratus. In addition [2- ^{14}C] acetate has been shown to impart activity to the bases in S. isatideus and C. spectabilis. In view of the parallel nature of the theories regarding the metabolic pathways leading to the pyrrolizidine and pyrroline moeities in alkaloids, it is useful and pertinent to discuss briefly the present state of knowledge regarding the biosynthesis

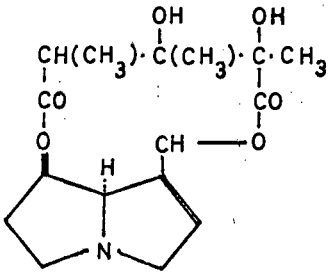
CHART I



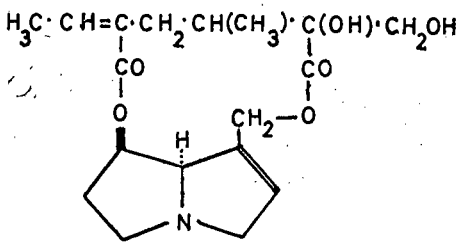
STRIGOSINE



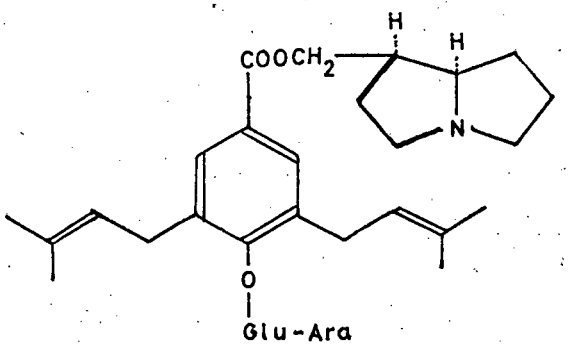
LATIFOLINE



MONOCROTALINE

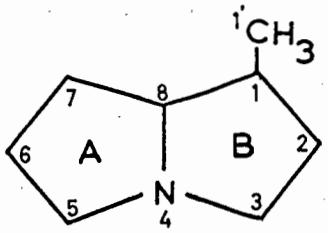


RETRORSINE

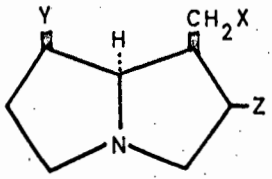


NERVOSINE

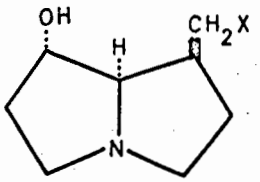
CHART II



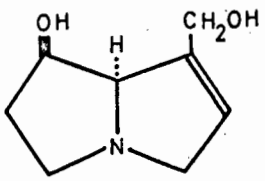
(1)



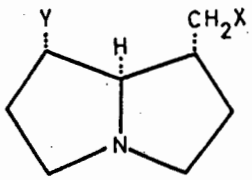
	X	Y	Z
Heliotridane	H	H	H
Retronecanol	H	OH	H
(-)-Isoretronecanol	OH	H	H
Platynecine	OH	OH	H
Rosmarinecine	OH	OH	OH



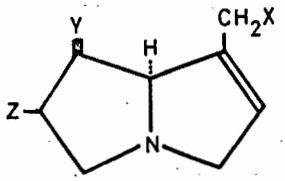
Oxyheliotridane	X=H
Dihydroxyheliotridane	X=OH



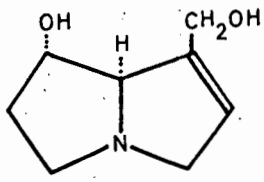
Hastanecine and/or
Turniforcidine



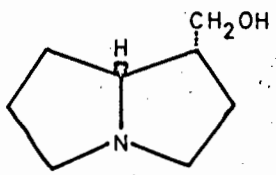
Macronecine	X=Y=OH
(-)-Trachelanthamidine	X=OH, Y=H
Pseudoheliotridane	X=Y=H



Retronecine	X=Y=OH, Z=H
Supinidine	X=OH, Y=H, Z=H
Desoxyretronecine	X=H, Y=OH, Z=H
Crotanecine	X=Y=Z=OH



Heliotridine



(+)-Isoretronecanol,
Lindelofidine

of the pyrroline ring, in particular in that system which has been best-studied, namely nicotine.

2. BIOSYNTHESIS OF ALKALOIDAL PYRROLINE RINGS

An intensive study has been made in recent years of the biosynthesis of the pyrrolidine ring in nicotine (2). Feeding experiments showed [2- ^{14}C] ornithine, [1- ^{14}C] putrescine, [2- ^{14}C] glutamic acid and several other 2-, 3- and 4- carbon moeities to be incorporated into the pyrrolidine ring. On the basis of degradative studies on the active alkaloid obtained from such feeding experiments, Leete et al (11, 12) proposed the glutamate-symmetrical intermediate hypothesis (Chart III) for the biosynthesis of the pyrrolidine ring in nicotine.

Cyclisation of glutamic-δ-semialdehyde (3), obtained from glutamic acid and ornithine, gives $\Delta^{1,2}$-pyrroline-5-carboxylic acid (4) which, on decarboxylation, forms the symmetrical, mesomeric Δ^{1}-pyrrolinium anion (5). Incorporation of this anion, or the resulting Δ^{1}-pyrroline (6), into nicotine (2) leads to equal labelling of carbon atoms 2′ and 5′ (radioactive ^{14}C atoms are starred). An alternative pathway results from decarboxylation of ornithine to the symmetrical putrescine and conversion of the latter to Δ^{1}-pyrroline (6). Under similar conditions, however, incorporation of putrescine into nicotine was only 25% that of ornithine.

Proline may be incorporated by one of the pathways shown in Chart IV. Ornithine and proline have been shown to be

CHART III

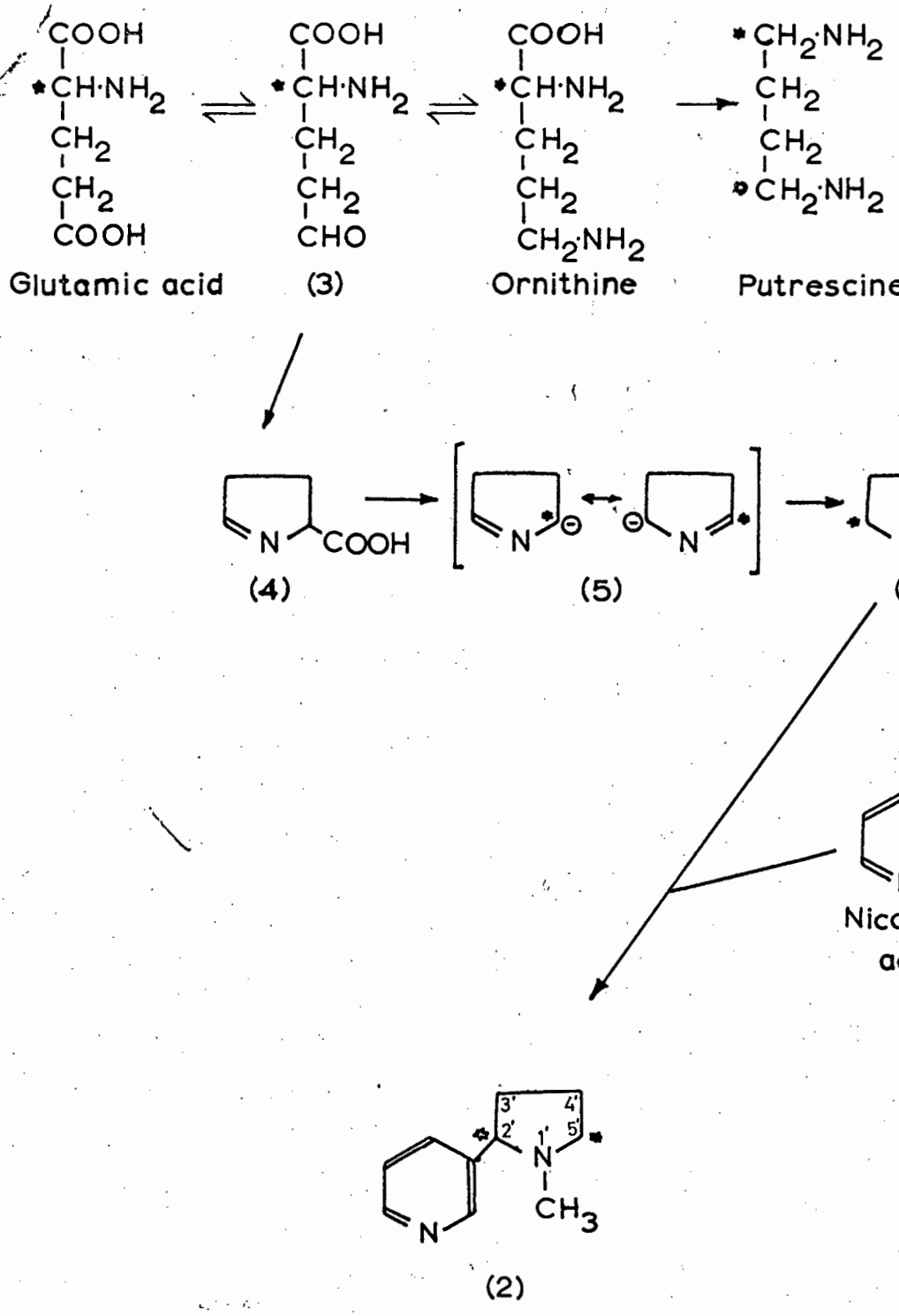
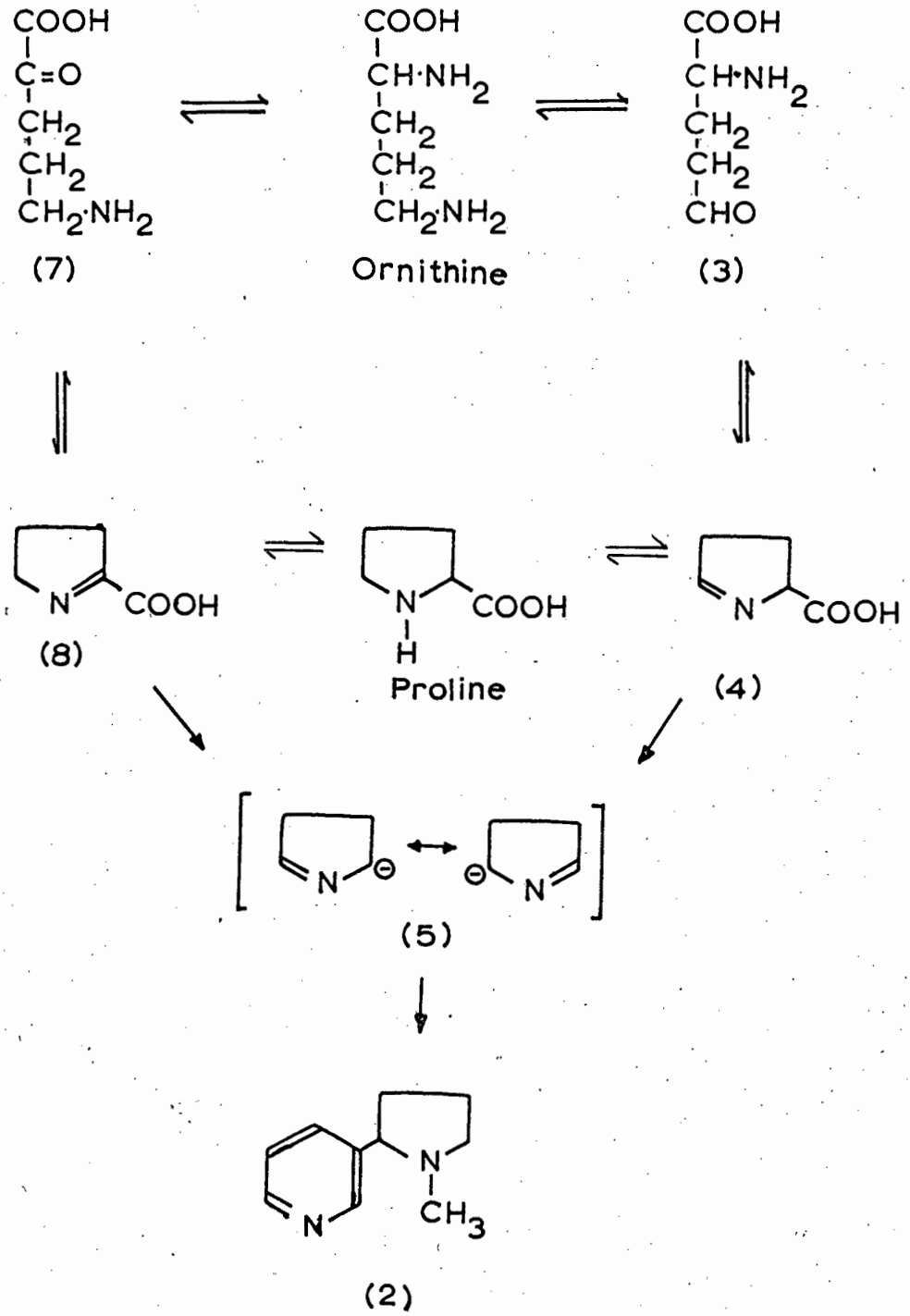


CHART IV

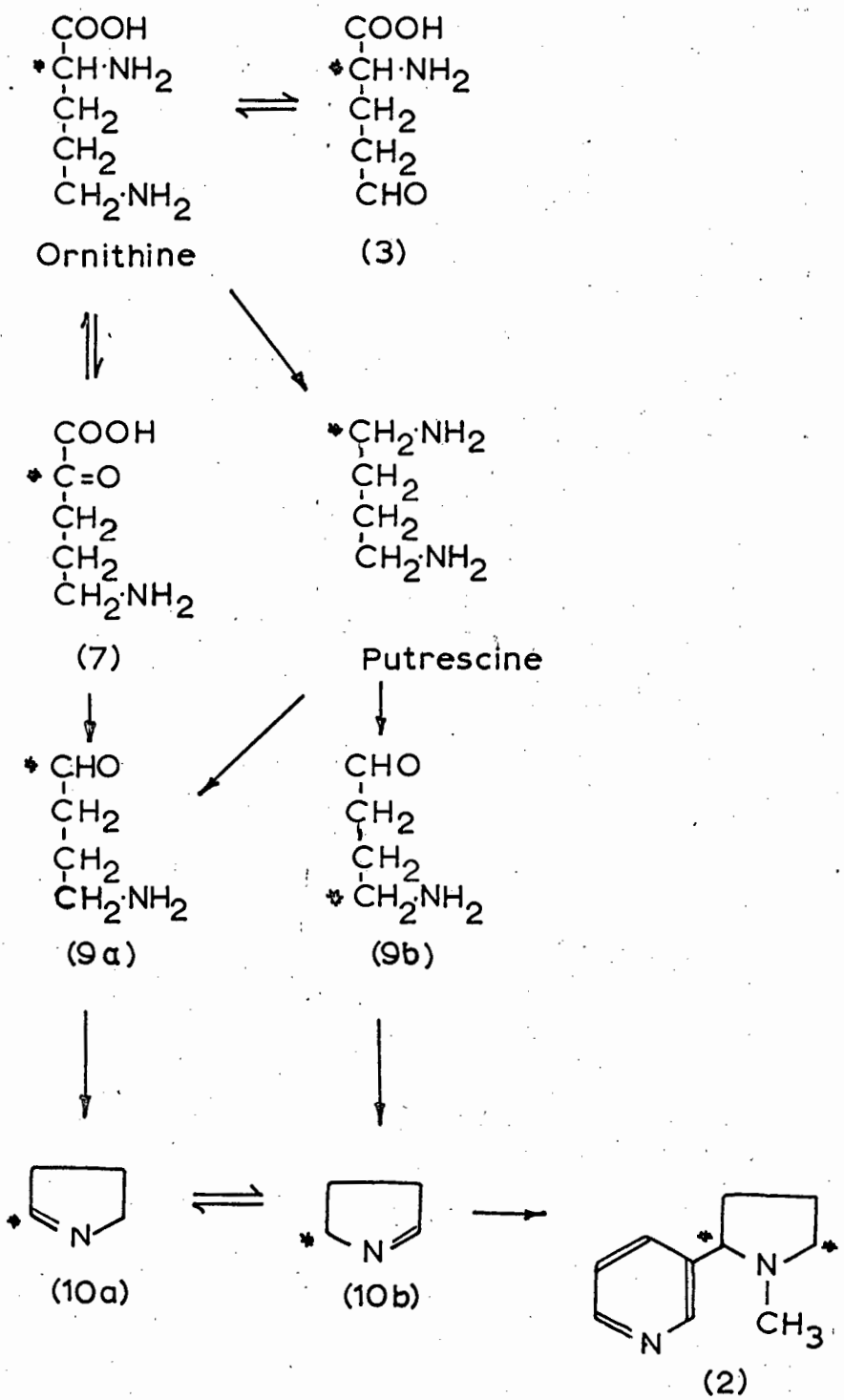

COOH
C=O
CH2
CH2
CH2·NH2
(7)
COOH
CH·NH2
CH2
CH2
CH2·NH2
Ornithine
COOH
CH·NH2
CH2
CH2
CHO
(3)
N
COOH
(8)
N
H
COOH
Proline
N
COOH
(4)
N
N
(5)
N
CH3
(2)

interconvertible by several pathways, amongst which is the sequence : ornithine to α - keto- δ -aminovaleric acid (7) to $\Delta^{1,2}$-pyrroline-2-carboxylic acid (8) to proline. This is of special interest in view of the theory [9] that α-keto- δ -amino-valeric acid could be a precursor in pyrrolizidine biosynthesis.

Subsequent to his earlier publications, Leete has modified his hypothesis (Chart III) on the basis of the results of feeding experiments with ^{15}N-labelled precursors[13]. The modified scheme is presented in Chart V. Transamination of ornithine is followed by decarboxylation of the resulting α-keto- δ -aminovaleric acid (7), giving 4-aminobutanal (9a). Cyclisation of this would afford Δ^{1}-pyrroline (10a). The tautomeric equilibrium between 10a and 10b would give rise to equal labelling at carbon atoms 2′ and 5′ in the final product, nicotine. The results from the ^{15}N feeding experiments could also readily be explained by the alternate pathway *via* the symmetrical putrescine, but this pathway seems the less likely in view of the low incorporation of [1- ^{14}C] putrescine into nicotine.

Pyrroline rings in alkaloids appear to be almost exclusively derived from glutamate *via* ornithine or proline, although details of ring-closure and attachment to other parts of the molecule may vary from one alkaloid to another. Until fairly recently the glutamate was visualised as being derived

CHART V

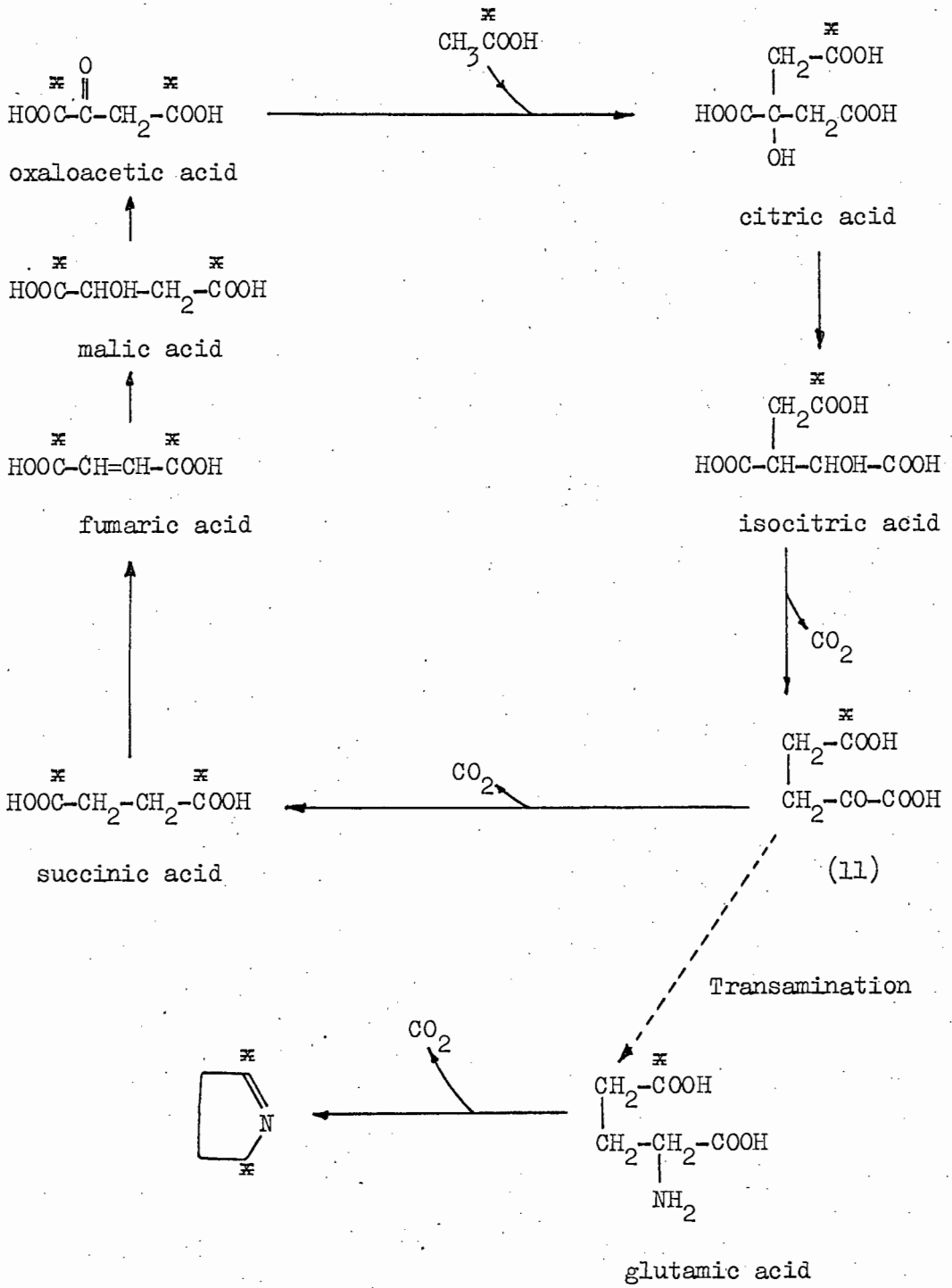

COOH
CH·NH2
CH2
CH2
CH2·NH2
Ornithine
COOH
CH·NH2
CH2
CH2
CHO
(3)
COOH
C=O
CH2
CH2
CH2·NH2
(7)
CH2·NH2
CH2
CH2
CH2·NH2
Putrescine
CHO
CH2
CH2
CH2·NH2
(9a)
CHO
CH2
CH2
CH2·NH2
(9b)
N
(10a)
N
(10b)
N
N
CH3
(2)

exclusively from α-keto-glutaric acid (11), an intermediate in the tricarboxylic acid (Krebs) cycle (Chart VI). It has now been shown, however, that glutamate is derived in ways other than through the Krebs cycle in various biological systems.

As support for the Krebs cycle pathway to glutamate in the biosynthesis of nicotine was cited the results of feeding experiments with simple 2-, 3- and 4-carbon pre - cursors such as acetate, glycerol, propionate and aspartate. The labelling patterns obtained in nicotine from such tracer experiments were generally regarded as being consistent with the glutamate-symmetrical intermediate hypothesis, the glutamate having been derived via the Krebs cycle. However, large experimental errors were found with some precursors.

From Chart VI it can be seen that, theoretically, the feeding of [1- ^{14}C] acetate or any active intermediate that can be built up from [1- ^{14}C] acetate, for example [1- ^{14}C] citrate or [1- ^{14}C] succinate, would give pyrrolidine ring activity only at carbons 2′ and 5′ in nicotine. Furthermore, those atoms would be equally labelled. With [2- ^{14}C] acetate the labelling pattern is more complex and depends on the number of times the Krebs cycle is negotiated (14). After a large number of cycles the activity at each of the carbons 2′ and 5′ would be 16.5% of the total pyrrolidine ring activity, the residual activity being equally divided between positions 3′ and 4′. In practice, although some precursors gave labelling

CHART VI

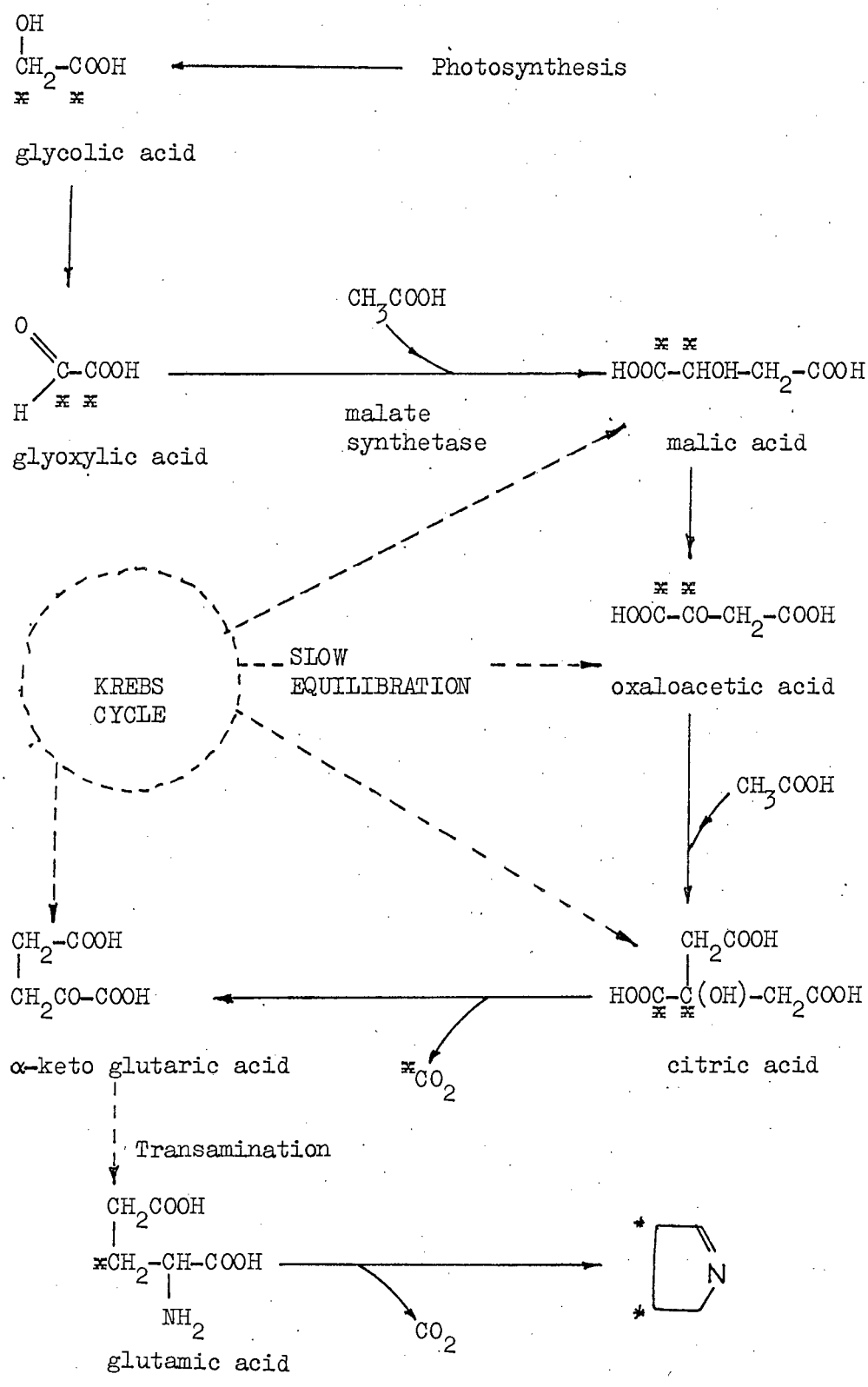

CH3COOH
HOOC-C-CH2-COOH
O
oxaloacetic acid
HOOC-CHOH-CH2-COOH
malic acid
HOOC-CH=CH-COOH
fumaric acid
HOOC-CH2-CH2-COOH
succinic acid
CH2-COOH
HOOC-C-CH2COOH
OH
citric acid
CH2COOH
HOOC-CH-CHOH-COOH
isocitric acid
CO2
CH2-COOH
CH2-CO-COOH
(11)
CO2
Transamination
CO2
N
CH2-COOH
CH2-CH2-COOH
NH2
glutamic acid

patterns close to the theoretical, with others an inordinately large experimental error had to be invoked to make the results consistent.

In an effort to explain these anomalies, Rapoport et al [14, 15] conducted some elegant feeding experiments with $^{14}CO_2$ on plants of the species Nicotiana glutinosa. The plants of N. glutinosa were grown under carefully controlled conditions in an atmosphere containing $^{14}CO_2$ and the nicotine extracted and degraded. Initially only carbon atoms 2′ and 5′ were isolated and counted. Their activity was consistently found to be equal, in accordance with the symmetrical intermediate hypothesis. However, the incorporation of activity at these two carbons was very much less than 50% of the total pyrrolidine ring activity, implying higher activity at carbons 3′ and 4′, which, on the basis of the symmetrical intermediate hypothesis, would be equally labelled. This labelling pattern could not be reconciled with the formation of glutamate through the Krebs cycle after fixation of CO_2 in the plant.

Other known modes of glutamate biosynthesis were considered. The glyoxalate condensation pathway,[16] the citramalic pathway [16] and the two routes followed in the micro-organisms Rhodospirillum rubrum and Clostridium kluyveri [14] could not, however, be invoked to explain the labelling pattern in nicotine from Nicotiana glutinosa. Consequently a plausible new glutamate biosynthetic pathway was put forward (Chart VII) in which glutamate is built up

CHART VII


OH
CH2-COOH
Photosynthesis
glycolic acid
CH3COOH
O
C-COOH
H
HOOC-CHOH-CH2-COOH
glyoxylic acid
malate synthetase
malic acid
HOOC-CO-CH2-COOH
KREBS CYCLE
SLOW EQUILIBRATION
oxaloacetic acid
CH3COOH
CH2-COOH
CH2CO-COOH
CH2COOH
HOOC-C(OH)-CH2COOH
α-keto glutaric acid
CO2
citric acid
Transamination
CH2COOH
CH2-CH-COOH
NH2
CO2
N
glutamic acid

outside the Krebs cycle, the intermediates only slowly equilibrating with those of the Krebs cycle. It can be seen that fixation of $^{14}CO_2$ would result in activity at positions 3′ and 4′ in nicotine. Active CO_2 would also be incorporated into the acetate entering the pathway but at a much lower rate, thus explaining the higher activity postulated at positions 3′ and 4′ than at 2′ and 5′ in nicotine.

Unfortunately for this theory, a further set of experiments by Rapoport et al [17] enabled all the carbon atoms of the pyrrolidine ring in nicotine to be isolated and assayed separately for activity after feeding Nicotiana glutinosa with $^{14}CO_2$. Surprisingly, activities at positions 2′, 3′ and 5′ were found to be equal, whilst that at carbon 4′ was about four times as much. This now casts suspicion upon the whole symmetrical intermediate theory and, to date, no alternative explanation has been forthcoming consistent with all the available experimental data. This impasse has its parallel in pyrrolizidine biosynthesis, where conflicting sets of results have been reported regarding a similar symmetrical intermediate.

3. BIOGENESIS OF THE PYRROLIZIDINE BASES

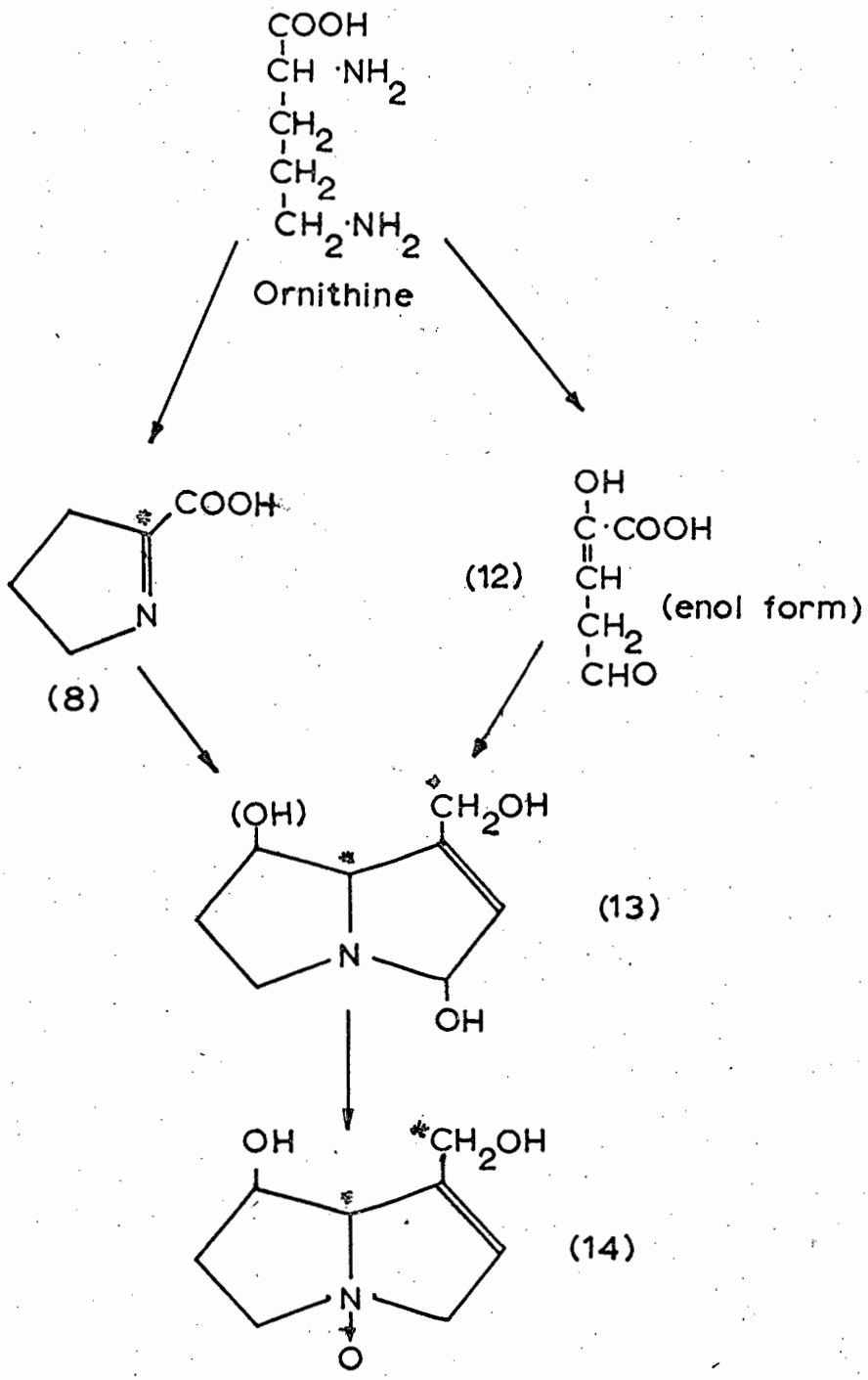
The biogenesis of the pyrrolizidine bases has been studied by several schools. Nowacki and Byerrum [5], following up the earlier suggestion of Robinson [18], showed that [2- ^{14}C]

ornithine was incorporated into monocrotaline (Chart I). The pyrrolizidine ring may well be built up from two molecules of ornithine.

Hughes [8, 9] fed [2- ^{14}C] ornithine to Senecio isatideus plants and performed degradations and counting experiments on the resulting active retrorsine. The activity at carbon 1′ of the base retronecine (15) was found to be 26% of the total retronecine activity. The residual activity (71% of the total) was found at the combined carbons 7 and 8, presumably mostly at position 8 since no conceivable pathway could account for activity at position 7. It is very significant that, in these experiments, little or no activity was found at positions 3 or 5.

These results prompted Hughes to put forward a bio-synthetic pathway (Chart VIII) involving two unsymmetrical intermediates, each derived from an ornithine molecule. Ornithine is converted to $\Delta^{1,2}$-pyrroline-2-carboxylic acid (8) and also to α-keto-glutamic-δ-semialdehyde (12). Condensation of (8) and (12), with decarboxylation, affords the pyrrolizidine unit (13). Rearrangement would give isatinecine (14). This pathway accounts for hydroxylation at positions 1′ and 2, $\Delta^{1,2}$ unsaturation and N-oxide formation, but cannot account for hydroxylation frequently encountered at position 7 in these bases. This may, however, arise from a hydroxylated precursor or from hydroxylation subsequent to ring closure.

CHART VIII


COOH
CH·NH2
CH2
CH2
CH2·NH2
Ornithine
COOH
N
(8)
(12)
OH
C·COOH
CH
CH2
CHO
(enol form)
(OH)
CH2OH
N
OH
(13)
OH
CH2OH
N
O
(14)

Results conflicting with those of Hughes were obtained by Bottomley and Geissmann [6], working with Senecio douglassii plants. These workers extracted retronecine (15) from plants which had been fed [1,4- ^{14}C] putrescine, [2- ^{14}C] ornithine and [5- ^{14}C] ornithine. Treatment of the active base with osmium tetroxide, followed by reaction with periodate, gave carbon 1′ as formaldehyde, counted as the dimedone derivative. In all three feeding experiments, carbon 1′ was found to have 25% of the retronecine activity. This is only possible if a 4-carbon symmetrical intermediate such as succinic dialdehyde (16) is involved in the build-up of ring B of retronecine. The α-keto-glutamic-δ-semialdehyde (12) can give rise to succinic dialdehyde (16) by decarboxylation.

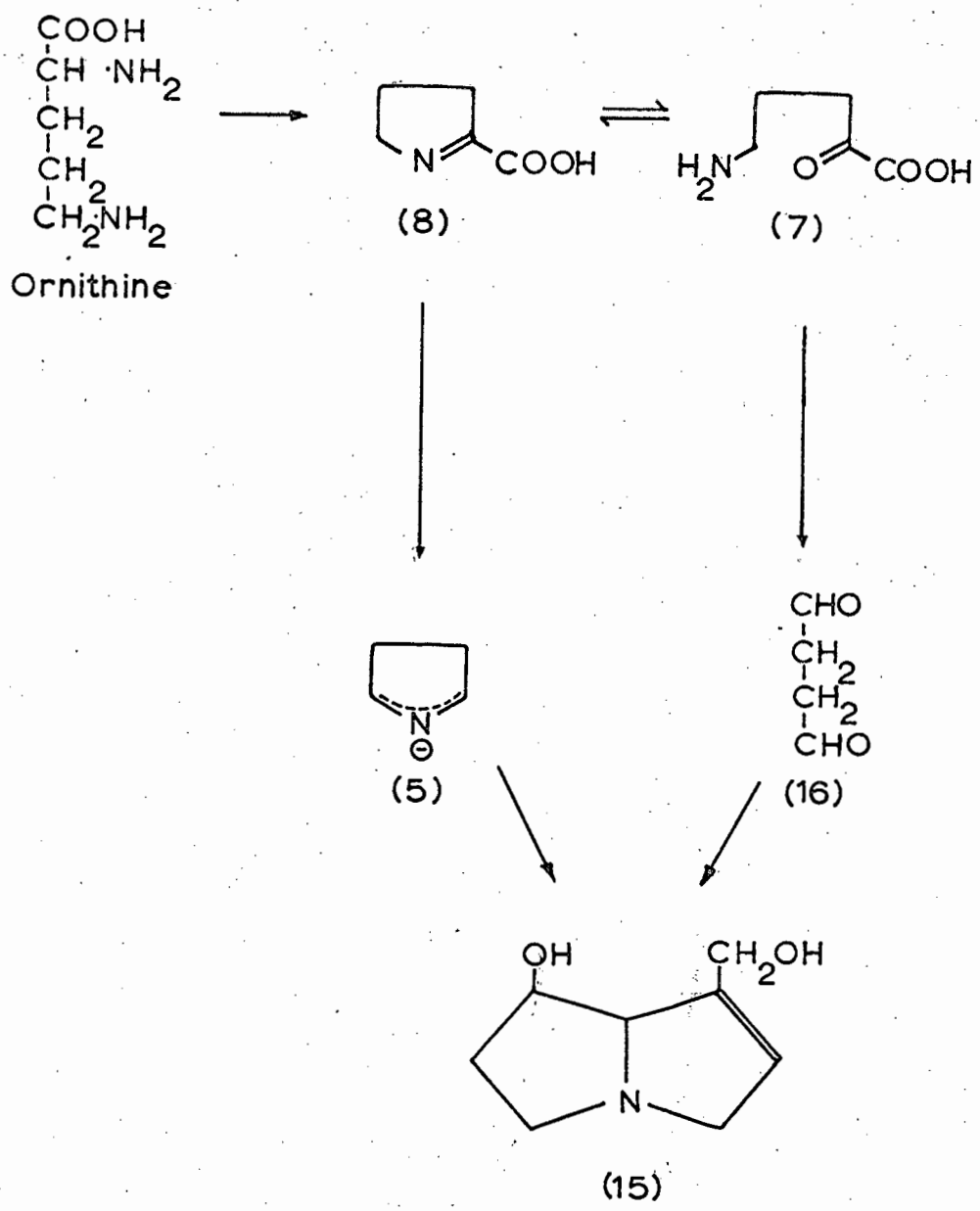
Morgan [7] has subsequently confirmed these latter results in the case of retronecine from Senecio sceleratus fed with [5- ^{14}C] ornithine, finding 25% of the activity at carbon 1′ by way of a Kuhn-Roth oxidation. Using essentially the same degradative procedure as Hughes, Morgan also isolated and counted the activity of carbon 5 in retronecine which had [5- ^{14}C] ornithine as precursor. If an unsymmetrical intermediate, such as the $\Delta^{1,2}$-pyrroline-2-carboxylic acid (8) invoked by Hughes, were involved in the build-up of ring A, an activity approaching 50% of the total should have been expected at carbon 5 of his active base. However, if a symmetrical intermediate such as the mesomeric pyrrolinium

anion (5) were involved, an activity of 25% of the total would be expected at carbon 5. Morgan found 17.4% of the total activity at carbon 5, which favours a symmetrical intermediate and conflicts with Hughes' results. The low figure obtained could be rationalised by the hypothesis that the A-ring pre - cursor is further advanced metabolically than the B-ring pre- cursor and is thus more readily diluted. Confirmation of this theory can only be obtained, however, by degrading the molecule further and counting more carbon atoms.

The evidence available at present would thus favour the biosynthetic pathway presented in Chart IX. Schütte[19] has found an exactly analogous build-up from symmetrical intermediates in the case of the lupin alkaloids, which have six-membered instead of five-membered rings.

The aim of the present work was to examine more closely some of the degradative reactions hitherto used in these laboratories, making use of newer physical methods not previously available. In the light of new information regarding the degradation procedure, it was hoped that feeding, degrading and counting experiments would give further insight regarding pyrrolizidine biosynthesis.

CHART IX


COOH
CH·NH2
CH2
CH2
CH2NH2
Ornithine
N
COOH
(8)
H2N
O
COOH
(7)
N
⊖
(5)
CHO
CH2
CH2
CHO
(16)
OH
CH2OH
N
(15)

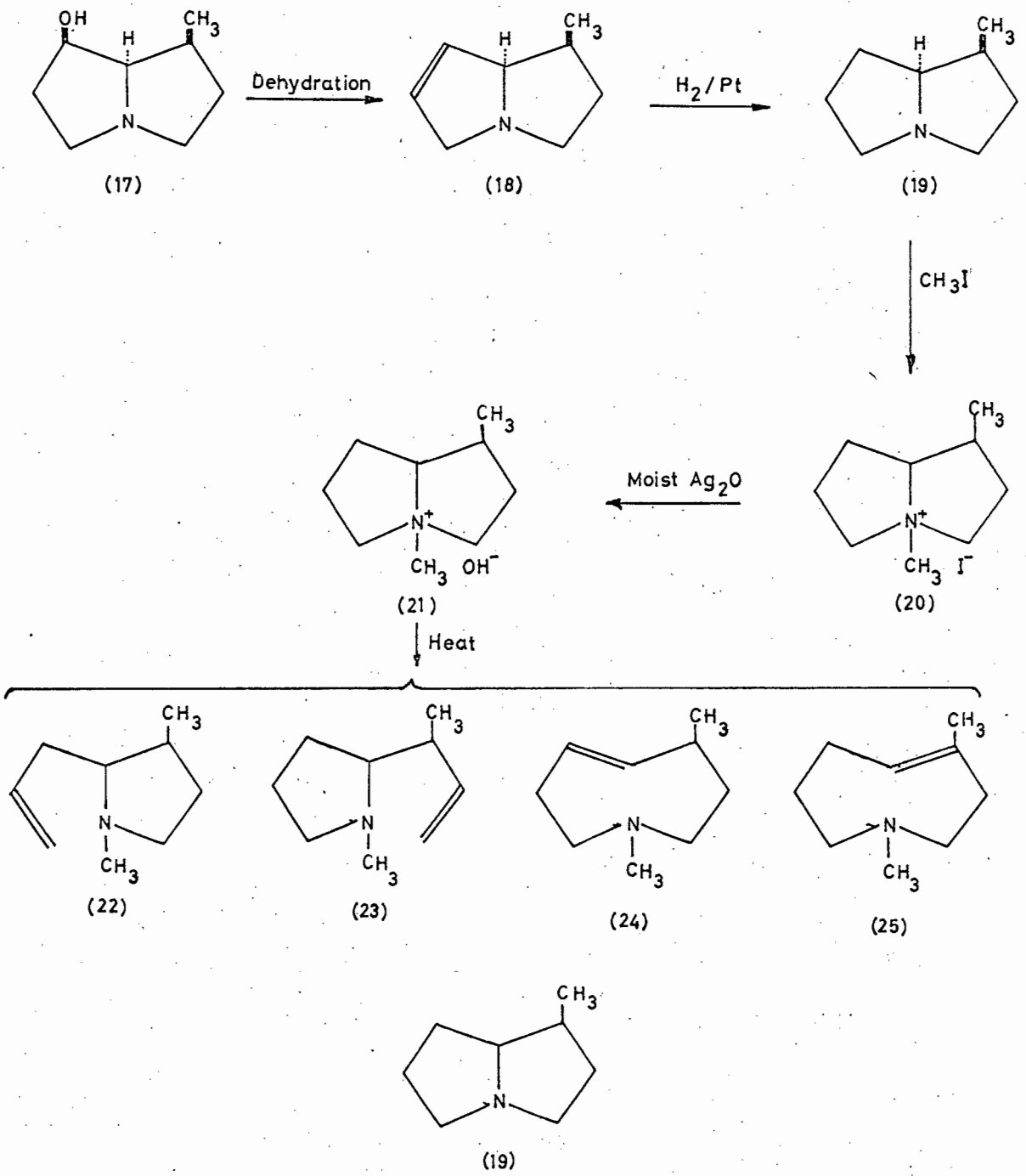
DISCUSSION

DISCUSSION

1. THE APPROACH TO THE DEGRADATION

All the previous workers in this school had considered the work of Men'shikov [20] to be the best basis for an atom by atom degradation of the pyrrolizidine moeity. Letcher attempted the Hofmann degradation on retronecanol, acetyl retronecanol, heliotridene and heliotridane [21]. He concluded that the most promising degradative procedure was to subject heliotridane (19), (Chart X), obtained by the sulphuric acid dehydration of retronecanol (17) and the subsequent hydrogenation of the heliotridene (18) formed, to the Hofmann exhaustive methylation procedure. Four methines (22 - 25) are possible from the first Hofmann reaction. The two methines (24) and (25) would not form readily because of the large rings they contain. Which of the remaining two methines is formed would depend on the relative activities of the hydrogen atoms on carbon atoms 2 and 6 of heliotridane, since the Hofmann reaction involves the elimination of a proton β to the nitrogen atom. It was argued that the inductive effect of the methyl group on ring B would lower the activity of hydrogen atoms on carbon 2 relative to those on carbon 6. Methine (22) would then be preferentially formed. Hughes [9] assumed this to hold and adapted the work of Men'shikov to a small-scale degradation of heliotridane. The results of the biosynthetic work arising from Hughes' degradation

CHART X

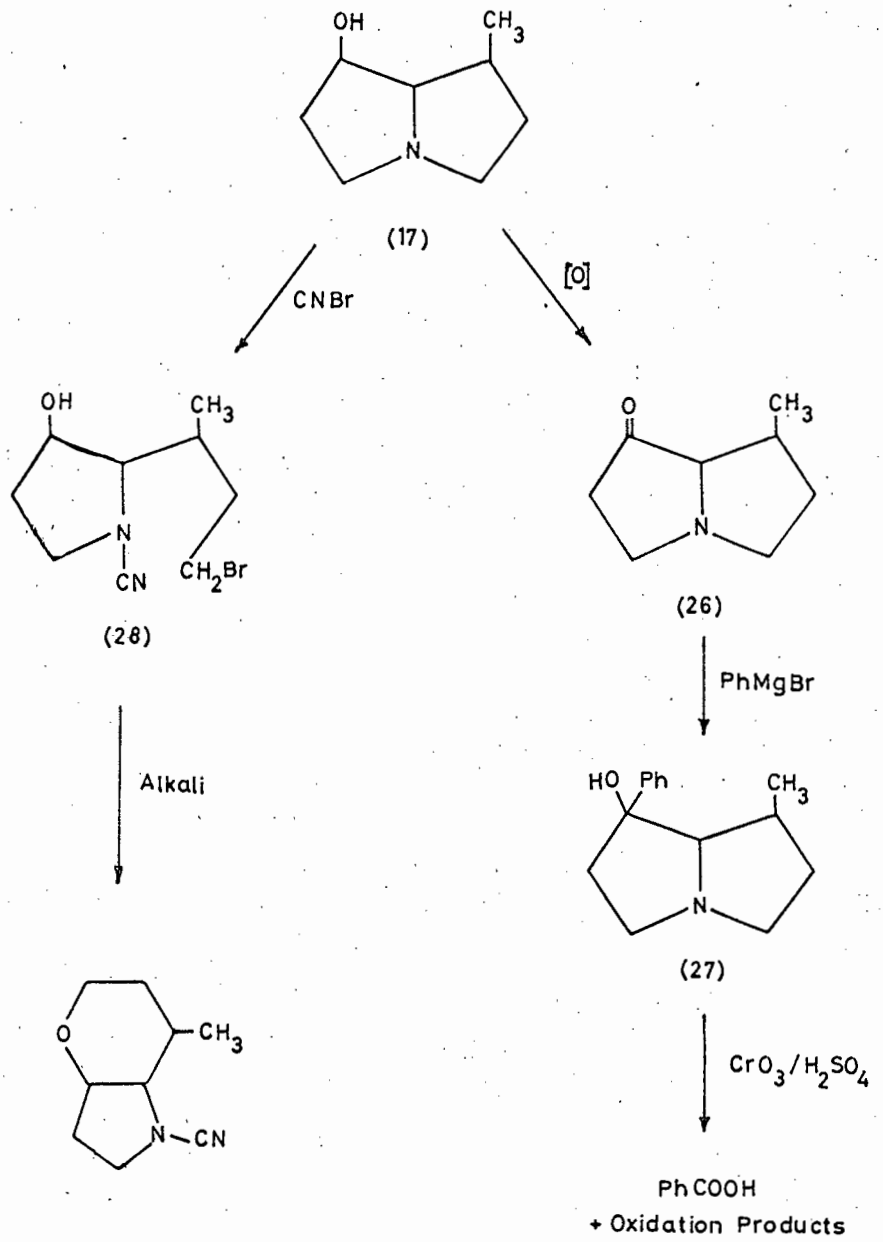

OH
H
CH3
N
(17)
Dehydration
(18)
H2/Pt
(19)
CH3I
(20)
Moist Ag2O
CH3 OH⁻
(21)
Heat
(22)
(23)
(24)
(25)
(19)

conflicted with the results of Bottomley and Geissman who approached the degradation in a different way [6]. Morgan[7] repeated the Hofmann reaction on heliotridane and obtained a mixture which he claimed had two main components. One of these was considered to be the parent heliotridane (19), arising from the elimination of methanol from heliotridane methohydroxide (21). This side reaction is well known in the Hofmann degradation [22].

It was felt by the present author that one of the reasons for the disagreement between the results of Hughes and those of Bottomley and Geissman, was the disregard by the former of the possibility of a mixture of the methines resulting from the first Hofmann reaction. The product obtained in the present work was consequently closely examined with this possibility in mind.

A way in which the Hofmann reaction could be induced to cleave the A ring more specifically is to increase the difference in activities of hydrogen atoms on carbon atoms 2 and 6 even more than is the case in heliotridane. This would be achieved if a carbonyl function were introduced on carbon 7 of the pyrrolizidine ring A. The resulting electron-withdrawing effect on carbon 6 would make the hydrogen atoms on carbon 6 much more active relative to those at carbon 2. The Hofmann reaction on the carbonyl compound would almost certainly cause cleavage of the A ring only. Retronecanone (26) was previously prepared in low yield

CHART XI


OH
CH3
N
(17)
CNBr
[O]
OH
CH3
N
CN
CH2Br
(28)
O
CH3
N
(26)
Alkali
PhMgBr
HO
Ph
CH3
N
(27)
O
CH3
N
CN
CrO3/H2SO4
PhCOOH
+ Oxidation Products

on a large scale by the Oppenauer oxidation of retronecanol[23]. The low yield with large quantities discouraged the use of the Oppenauer oxidation on the small scale required in the present work. Recently, however, many new oxidising agents have been used successfully on the small-scale oxidation of secondary alcohols and it was thought that some of these could be fruitfully employed.

A further advantage in obtaining retronecanone from radioactive alkaloid would be that carbon 7 could then be isolated and counted. Previously the exhaustive methylation procedure had not offered a means of separately isolating carbons 7 and 8. Hughes had counted carbons 7 and 8 together and assumed no activity at carbon 7. Reaction of retronecanone(26) with phenyl magnesium bromide should result in the alcohol (27). Oxidation of (27) with chromic acid in a manner analogous to the Kuhn-Roth oxidation should then isolate carbon 7 as benzoic acid.

In the event of a mixture of methines being obtained from the usual Hofmann procedure, there is the possibility that conditions are not mild enough to allow fission of only one ring. It was decided to try the elimination under somewhat milder conditions by simply heating the heliotridane methiodide (20) with excess potassium hydroxide solution, instead of first forming the metho-hydroxide (21) with moist "silver oxide" and then heating.

Another method of accomplishing the elimination reaction which is sometimes employed with tertiary amines is the Cope pyrolysis of amine oxides. This reaction is not applicable, however, in cyclic systems where for steric reasons the β-hydrogen atom is held beyond the range of the negative oxygen[22]. Dreiding models showed this to be the case with heliotridane N-oxide.

The Emde reaction, which involves the catalytic reduction of the quaternary hydroxide of an amine with sodium amalgam or sodium in liquid ammonia, would not have been particularly beneficial in the case of the degradation of heliotridane, even if it were possible to fission one ring specifically. In this reaction, the double bond is immediately reduced, leaving a saturated compound which it would have been difficult to degrade further in an unambiguous way.

The one other method of degradation of amines, the von Braun reaction with cyanogen bromide, had previously been performed on retronecanol[24]. The product was compound (28) which, on boiling with alkali, yielded the neutral cyanamide ether (29). Thus, since the present work was directed primarily at the degradation of ring A, the von Braun reaction did not seem promising.

In view of the above, the indicated approach to the degradation, therefore, seemed to be to repeat the Hofmann reaction on heliotridane but a critical examination of the product was

essential. If a mixture of methines was obtained, the next step would be to use milder reaction conditions, eg. the excess base method. In the event of this being unsuccessful, attempts were to be made to oxidise retronecanol to retronecanone in high yield on a small scale and to do the Hofmann reaction on the latter with a view to further degradation.

Most of the modern physical methods of organic chemistry were at the disposal of the author. Of these, the most useful appeared to be the chromatographic techniques, especially preparative gas-liquid chromatography. Mass spectrometry and infrared spectroscopy as an aid to identification of compounds and measurement of purity would be used extensively, whereas the nuclear magnetic resonance spectra for the pyrrolizidine bases published in the literature [25] are very complex and would furthermore not have been very useful in the present work.

2. EXTRACTION OF THE INACTIVE ALKALOID AND CONVERSION TO RETRONECANOL

Inactive monocrotaline was extracted from seeds of *Crotalaria spectabilis* supplied by the Kilgore Seed Co.,Florida, U.S.A. The presence of the single alkaloid, monocrotaline, in the plant in relatively large quantities influenced the choice of *Crotalaria spectabilis* for this work. Another reason for this choice was that retronecanol can be obtained from monocrotaline by the single step of hydrogenolysis, whereas the conversion of retronesine to retronecanol requires an hydrogenolysis and an hydrolysis.

The extraction procedure was essentially that previously used in these laboratories. The seeds were soaked in water to soften them, and then macerated with ethanol to a slurry. This was extracted batchwise with ethanol until the extract was colourless. The extract was evaporated to dryness, taken up in water and made acid with citric acid to precipitate fats and waxes. The precipitate was centrifuged off and the centrifugate was reduced with zinc and hydrochloric acid. This reduction had been shown [26] to increase the yield of alkaloid, since the alkaloids exist partly as the N-oxide in the plant. It is noteworthy that separate extractions of the seeds with and without the reduction, yielded almost identical amounts of alkaloid. This is in contrast with the extraction of active alkaloid from the dried plant material, where the major part of the alkaloid was only extracted after the reduction.

After the reduction, the zinc was filtered off and the solution made basic. The alkaloid was then extracted from the basic solution with chloroform and recrystallised from ethanol. A 3% yield of alkaloid from the dried seeds was obtained.

Monocrotaline was hydrogenolysed in hydrochloric acid solution over Adams' catalyst to yield monocrotalic acid and retronecanol. The monocrotalic acid was extracted with ether from the acidic solution, after which the retronecanol was extracted with ether from the solution basified with sodium

hydroxide. Evaporation of the ethereal solution yielded retronecanol as crystals or as a colourless oil which crystallised on standing. Vacuum sublimation was used to purify the retronecanol, which analysed correctly for carbon, hydrogen and nitrogen, moved as a single substance on thin-layer and gas-liquid chromatography and had the correct molecular weight by mass spectrometry. A yield of 80% from monocrotaline was obtained.

3. THE DEGRADATION OF RETRONECANOL

Retronecanol was degraded to heliotridene, using sulphuric acid, and the heliotridene was reduced catalytically to heliotridane. The procedure was similar to that used by Morgan[7]. Care had to be exercised in the dehydration step to avoid excessive charring of material. Concentrated sulphuric acid was added slowly to retronecanol at a temperature of about-15° and the solution then heated at 120-125° for two hours. The resulting mixture was made basic with sodium hydroxide and heliotridene could be extracted with ether at this stage. Heliotridene was studied by thin-layer and gas-liquid chromatography (q.v.) and by mass spectrometry (q.v.). Heliotridene was shown by G.L.C. to be somewhat unstable on standing. This may be due to oxidation or to double bond migration.

Heliotridane was obtained by steam distilling the basic solution of heliotridene into dilute hydrochloric acid and hydrogenating the acid solution over Adams' catalyst.

Heliotridane was extracted with ether from the resulting solution basified with sodium hydroxide. A study of heliotridane was made with the aid of thin-layer and gas-liquid chromatography and mass spectrometry. A yield of 75% of heliotridane could be obtained from retronecanol

On addition of excess methyl iodide to heliotridane in ether solution, heliotridane methiodide precipitated immediately and could be recrystallised from acetone/ether. The hygroscopic methiodide was stored in a desiccator.

4. THE HOFMANN REACTION ON HELIOTRIDANE

The procedure for the Hofmann reaction was similar to that previously used in this school[7, 9, 21]. The heliotridane methiodide was shaken with excess moist "silver oxide", the mixture filtered and the resulting solution taken to dryness on a rotary evaporator. The methohydroxide can form the carbonate with atmospheric carbon dioxide and, since the carbonate does not decompose on heating, it is important to minimise contact of the methohydroxide with the atmosphere[7].

The methohydroxide was heated under vacuum and the volatile products collected in a special U-tube cold trap. Two layers were consistently formed, the heavier one being water. The lighter fraction was separated with a dropping pipette and was evidently immiscible with water, since no

evidence of water was found in the mixture on G.L.C.

By both thin-layer and gas-liquid chromatography (q.v.) the Hofmann products were shown to contain at least five components. All attempts to separate and purify these components on G.L.C. were abortive. Other separatory techniques were not given serious consideration since they generally require larger amounts of material than were available. Preparative thin-layer chromatography is an exception which may warrant further investigation. It was clear, however, that the normal Hofmann procedure was unsuitable as a degradative technique on the small scale anticipated with the active alkaloid. Even if the methines were separable, the yield of any given one would be too low to warrant using the reaction in the degradation, which may entail very many further steps. Uncertainty as to the actual composition of the mixture also rules out the useful employment of the whole mixture as such.

From an examination of G.L.C. data, it would seem that one of the components in the Hofmann mixture may well be heliotridane. Morgan[7] has observed a similar result and has pointed out the feasibility of finding heliotridane in the products, arising from the elimination of methanol from the methohydroxide. This abnormal reaction is frequently observed with the Hofmann procedure.

Some of the Hofmann mixture was reduced by catalytic hydrogenation and the product studied by G.L.C. (q.v.). The data are consistent with the theory that the Hofmann mixture consisted of heliotridane and the four possible methines. Two of these would be present in trace amounts, being the eight-membered cyclic methines which would form least readily. The other two methines would be present in larger amounts, being the products of fission of each of the two pyrrolizidine rings. Saturation of the four possible methines would produce three dihydro-methines and the heliotridane in the original mixture would remain unchanged. It is realised that this theory was not proved, but the available G.L.C. evidence supports the idea.

When the Hofmann procedure was modified and the heliotridane methiodide was heated with excess potassium hydroxide solution, the products obtained on vacuum distillation were identical with those obtained by the normal Hofmann procedure.

5. ATTEMPTS TO OXIDISE RETRONECANOL

As previously pointed out, the advantages in being able to oxidise the secondary hydroxyl function in retronecanol to the ketone are twofold. Firstly it is believed that the carbonyl group would direct the Hofmann elimination in a specific way and secondly the retronecanone formed on oxidation should lend itself to further degradation not hitherto performed.

The oxidation of a secondary alcohol to a ketone is usually accomplished either with a solution of the alcohol and aqueous acidic chromic acid in acetic acid or by reaction of the alcohol with aqueous acidic chromic acid as a hetrogeneous system. Alternatively, the oxidation may be effected by stirring a solution of the compound to be oxidised in benzene, methylene chloride or ether with an acidic aqueous solution of chromic acid[27].

The above reaction conditions, however, are sufficiently vigorous to effect slow oxidation of certain other functional groups, eg. amines. For this reason a milder method was sought for the present oxidation. A milder method which at least in part avoids the complications mentioned, is to use the Jones reagent[28]. An aqueous chromic acid solution, which is 8N in chromic acid, is added dropwise to an acetone solution of the compound to be oxidised. A stoichiometric amount of reagent is normally used and the reaction is run at or below room temperature. The method was unsuccessful in the present case, since only the starting material, retronecanol, was recovered from the oxidation mixture.

Compounds containing acid-sensitive functions, such as acetals, ketals or basic heterocyclic nitrogen, have been oxidised by making use of Sarett's reagent[29]. This reagent is prepared by adding chromium trioxide to pyridine to form

a pyridine solution (or partial solution-partial suspension) of the complex $CrO_3.2C_5H_5N$. The alcohol to be oxidised is then added and the mixture is allowed to stand at room temperature. Several procedures for isolating the product have been reported[27]. The reaction was tried on retro - necanol, but only traces of two unidentified substances were obtained.

Recently dimethylsulphoxide has been used increasingly as an oxidising agent in organic chemistry. Mild conditions, uncomplicated work-ups and high yields with which most oxidations can be effected have elevated this technique into prominence. The Pfitzner-Moffatt method has been used to oxidise a wide variety of compounds[30]. The reaction involves addition of the alcohol substrate to a solution of dicyclohexylcarbodiimide (D.C.C.) in dimethylsulphoxide (D.M.S.O.) with phosphoric acid or pyridinium trifluoroacetate present as a proton source. The reaction conditions are nearly neutral. D.M.S.O. concentration may vary from 10 to 100% of the total solvent, an inert solvent such as benzene making up the remainder. Amines are unaffected by the conditions of the reaction.

The method was applied to retronecanol using phosphoric acid as proton source and 100% DMSO as solvent. However, only starting material was recovered.

The mechanisms of the above oxidations involves reaction at the oxygen atom of the hydroxyl group. The sterically hindered approach to this atom may explain the reluctance of retronecanol to undergo oxidation.

6. GAS-LIQUID CHROMATOGRAPHY

The instrument used was an Aerograph model A-700 "Autoprep" preparative gas chromatograph. All the columns used were of $\frac{3}{8}$" o.d. aluminium tubing. Although the apparatus was designed for optimum performance with injections of 50µl or more, a great deal of the present work was done with injection volumes of the order of 1µl, generally with fairly satisfactory reproducibility of retention times. It was felt that if good results were obtained on the analytical scale under a given set of conditions, the same apparatus could then be used on a preparative scale under very similar conditions with a minimum of discontinuity in the work.

Three different columns were prepared, each being 12 ft. long:

(a) 10% $^{W}/_{w}$ Polyethylene glycol (Carbowax) 20M on 45/60 mesh untreated Chromosorb P,

(b) 10% $^{W}/_{w}$ Polyethylene glycol (Carbowax) 4000 on 80/100 mesh acid-washed Chromosorb W,

(c) 20% $^{W}/_{w}$ Silicone G.E. SE52 on 80/100 mesh acid-washed Chromosorb W.

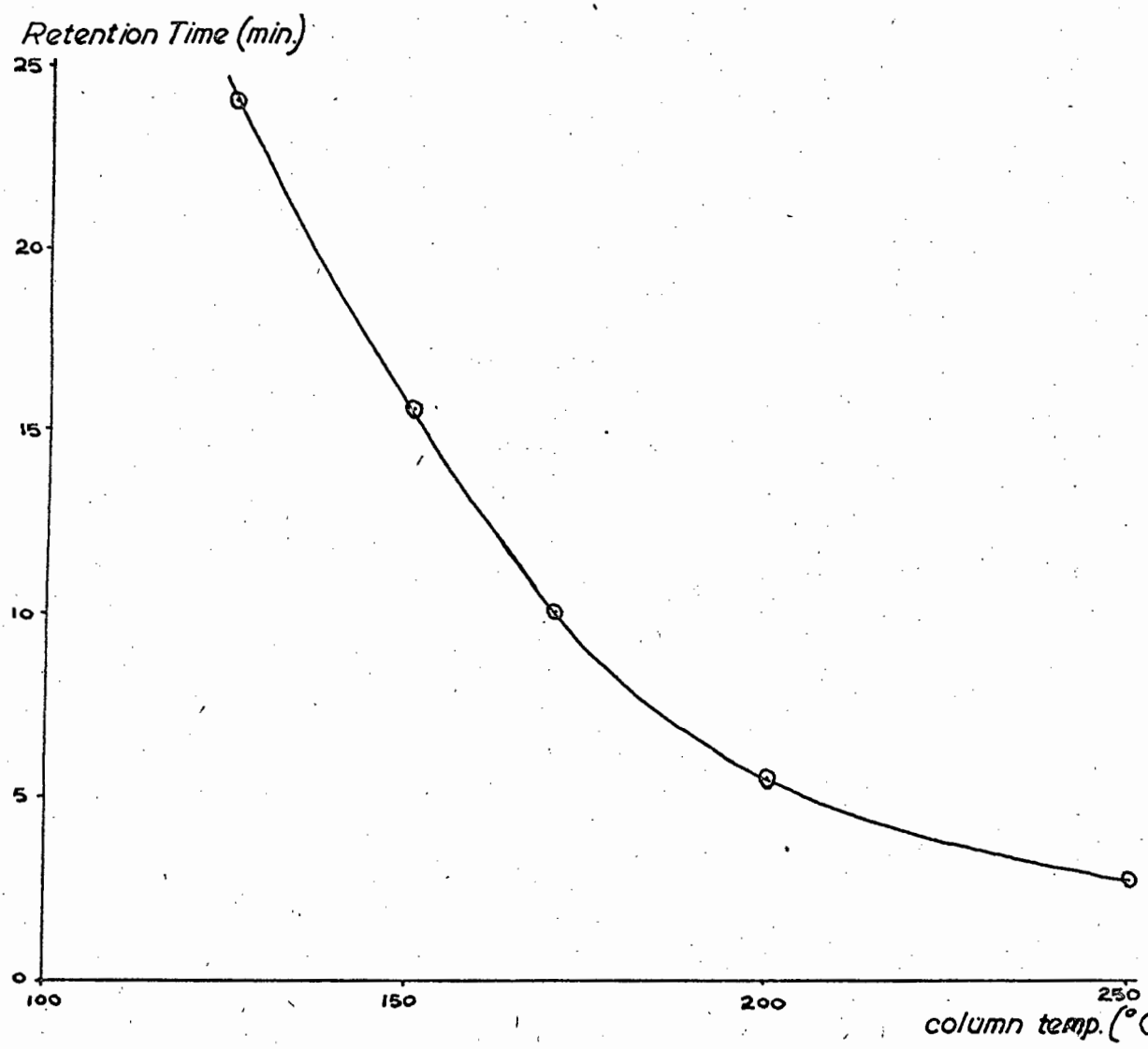
The Carbowax 20M packing was selected in view of its previously reported use for the separation of synthetic hydroxypyrrolizidines [31]. In the present work, however, it was not found to be suitable and a lower M.W. Carbowax (4000) was tried, also unsuccessfully. It was then decided to try a more universally useful packing and the Silicone G.E. SE52 column was prepared and used with some success.

(i) G.L.C. of Retronecanol, Heliotridene and Heliotridane

Retronecanol, heliotridene and heliotridane derived from monocrotaline did not move on the Carbowax columns when injected as the 10% solutions in acetone or ether, even at the maximum operating temperatures of both columns. However, on the silicone column all three compounds moved with characteristic retention times.

The variation of retention time with operating column temperature for retronecanol is shown in Fig.(i). These data were obtained on the freshly prepared silicone column. It was found, however, that as the column became older the retention times of all injected substances shortened. For retronecanol, for example, at a column temperature of 170° the retention time on the new column was 10 mins. 15 sec., whereas after two months of fairly intensive use of the column, the retention time for retronecanol was reduced to 8 min. 15 sec. For analytical work this was not a serious disadvantage, since runs done on any given day gave consistent

Fig. (i) *Variation of Retention Time with Column Temperature for Retronecanol*



results for purposes of comparison of retention times. The change in retention times were ascribed to substrate bleed from the column, especially during preparative runs.

Retronecanol moved as a single symmetrical peak when injected in acetone solution onto the silicone column.

Heliotridene, injected as the pure liquid or in ether solution, moved as a single peak when freshly prepared, but on standing for about five days a small new peak was observed which grew in relative size on allowing the heliotridene to stand for longer periods, even in the refrigerator. At a column temperature of 170° the retention time of heliotridene was 4 min. 18 sec. and that of the small peak was 5 min. The appearance of the trace of new substance may be ascribed to the instability of heliotridene, possibly owing to double-bond migration or oxidation.

Heliotridane in ether solution had a retention time of 4 min. 35 sec. at a column temperature of 170°. The substance moved as a single peak, even after prolonged standing in the refrigerator.

In view of the closely similar retention times of heliotridane and the main heliotridene peak, it was considered advisable to inject a mixture of the substances to establish a difference between them. The mixture in ether solution gave peaks with retention times of 4 min. 18 sec., 4 min. 38 sec. and 5 min. 2 sec. at a column temperature of 170°.

These corresponded to the peaks obtained from separate injections of the components of the mixture, thus verifying that the samples of heliotridane and heliotridene were different.

(ii) G.L.C. of Hofmann Reaction Products

On both the Carbowax columns the Hofmann products gave three large, poorly resolved peaks, the first and third peaks having shoulders. Thus it was suspected that the mixture had at least five components. At column temperatures of 28°, resolution on a 1µl injection scale was such that the shoulders were just resolved from the large peaks. On a 50µl scale, however, even the three large peaks were not resolved, making preparative runs impossible.

On the silicone column, the Hofmann products moved satisfactorily at temperatures of 100° or more. Below this temperature tailing increased markedly and the component with longest retention time took inordinately long to appear. The resolution was also not improved by dropping the temperature below 100°, so that this was regarded as the optimum operating temperature for the Hofmann products. A sample of the chromatograms obtained at 100° is reproduced in Fig. (ii). None of the peaks corresponded to the retention time of water. The two largest peaks were not well enough resolved to attempt preparative separation, but

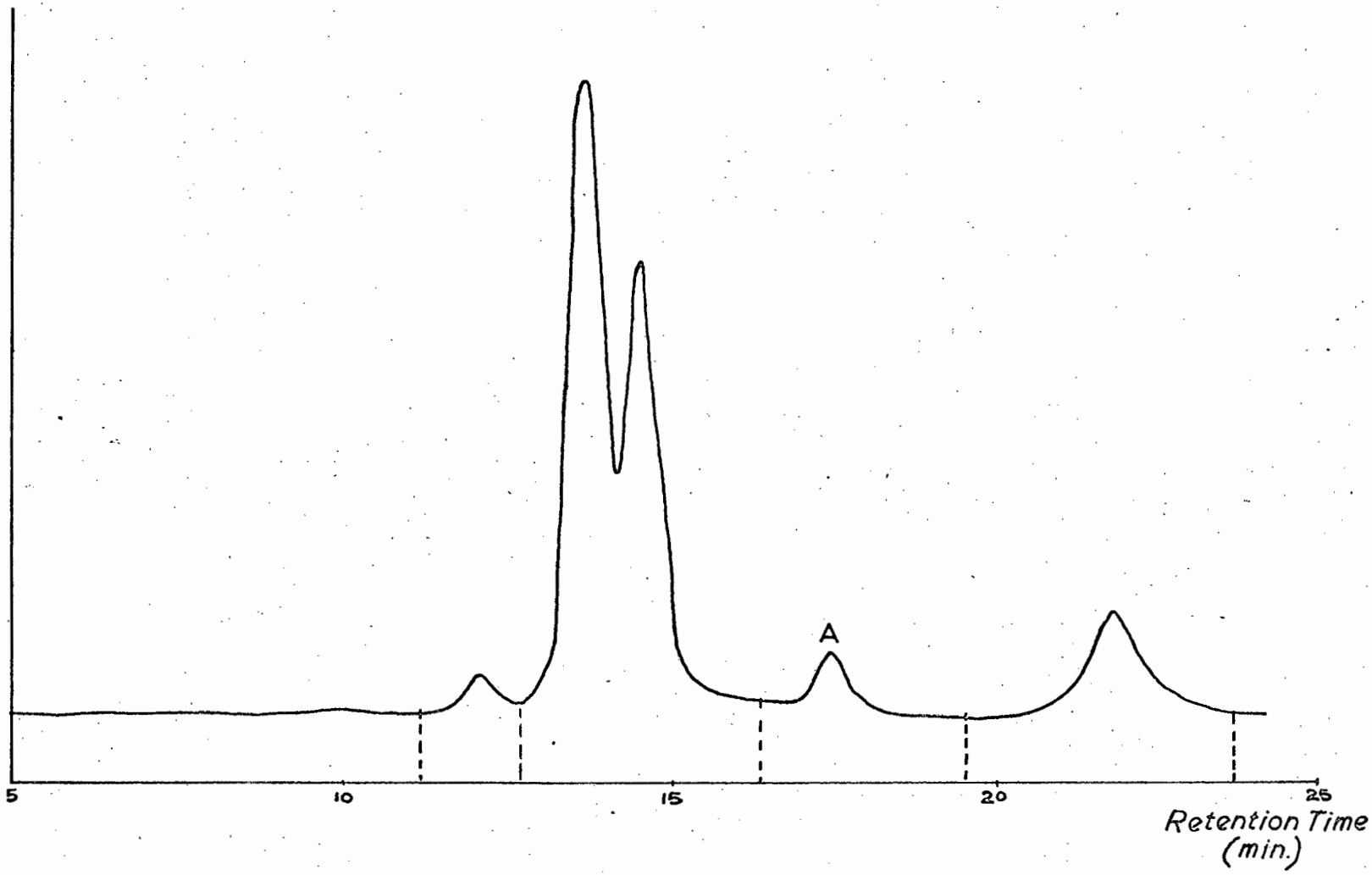


Fig.(ii) *Gas-Liquid Chromatogram of Hofmann Reaction Products*

repeated preparative runs of 50μl injections were nevertheless collected, the fractions being cut as indicated in Fig. (ii).

One microlitre aliquots of each of the four fractions were reinjected in turn. Each fraction gave the expected peak or peaks but, in addition, there were the same two new peaks from each fraction at retention times of 4 min. 55 sec. and 14 min. . Because these substances were present in all four collected fractions, it was concluded that they derived from the column packing and not as a result of decomposition of the injected Hofmann products. However, the silicone G.E. SE52, injected in toluene solution, gave no peak other than that due to toluene. The nature of the contaminating substances thus remain unknown. An attempt was made to reduce the contamination by collecting many successive 5μl injections, but, apart from the drawback that the procedure is extremely tedious, the fraction collector on the machine was not sufficiently efficient to collect such small quantities.

It was concluded that, unless a better column is found, preparative G.L.C. is unsuitable as a method of purifying and separating the Hofmann products in a small-scale degradation of heliotridane. Valuable information was, however, obtained. Hitherto the Hofmann reaction had been

thought to give at the most two products [7]. The present work confirmed the results of thin-layer chromatography in showing up five products. It is noteworthy that the retention times of heliotridane at several column temperatures from 100° to 170° on the silicone column corresponded closely to the retention times of the fourth component in the Hofmann mixture, designated peak A in Fig. (ii). Although this is no proof of identity, it is reasonable to expect heliotridane in the mixture, arising from the elimination of methanol from heliotridane methohydroxide.

(iii) G.L.C. of Hydrogenated Hofmann Reaction Products

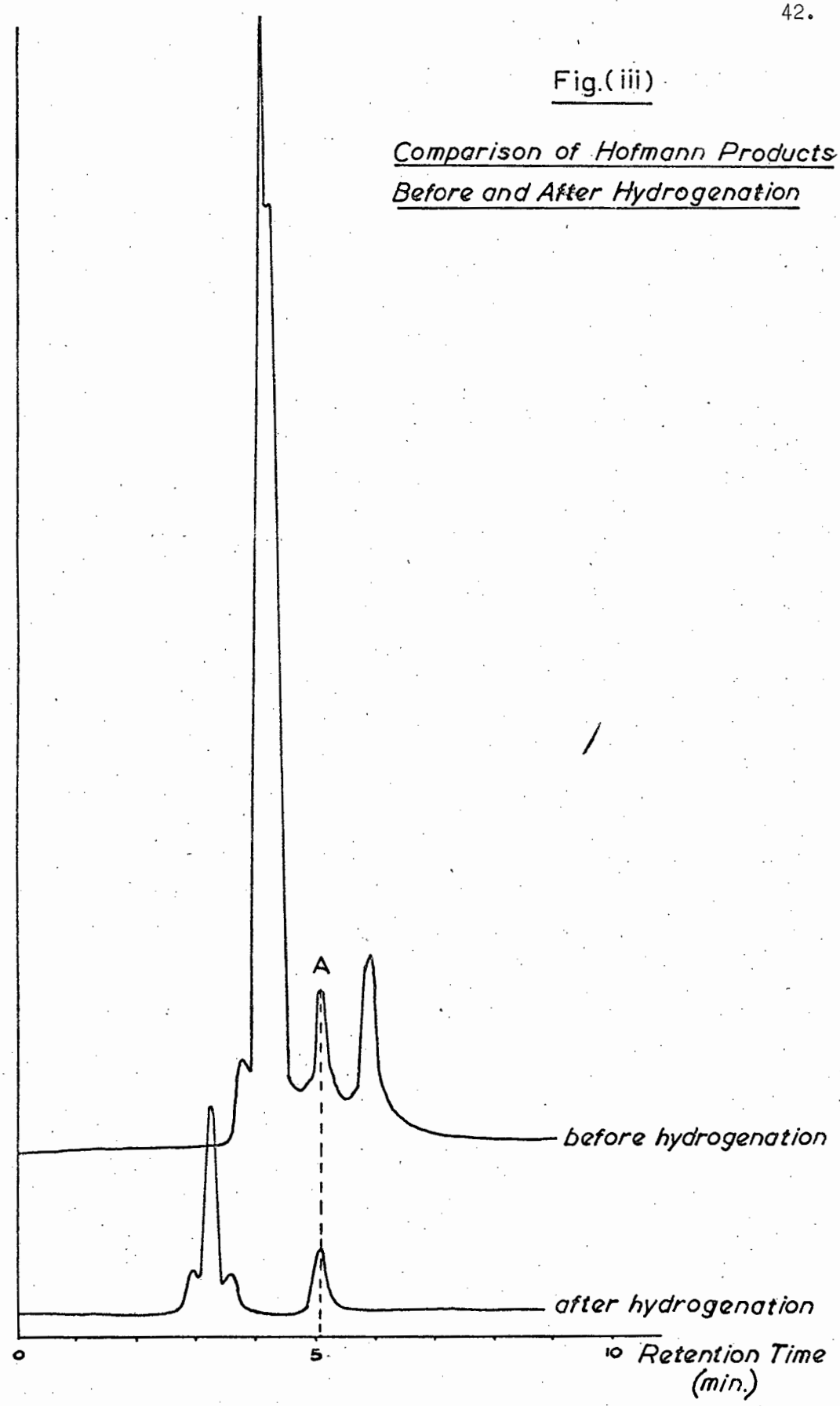
The hydrogenated Hofmann reaction products were injected at 150° onto the silicone column and the chromatogram compared with that from the Hofmann products injected at the same temperature. The traces are reproduced in Fig.(iii).

The peak labelled A in the Hofmann products corresponded closely to that produced by heliotridane at the same column temperature. This was again present in the hydrogenated products, as would be expected if it were the saturated heliotridane.

The four remaining peaks in the Hofmann products were replaced by three new peaks after the hydrogenation. This would be expected if the four possible methines were saturated on hydrogenation.

Fig.(iii)

Comparison of Hofmann Products Before and After Hydrogenation



7. MASS SPECTROMETRY

Samples of the three compounds retronecanol, heliotridane and heliotridene were submitted for mass spectrometry and the spectra analysed and rationalised. All three spectra showed clearly the expected molecular ions and, as expected, the fragmentation patterns showed definite similarities.

(i) Mass Spectrum of Retronecanol

A plot of relative intensities against mass/charge ratio for the spectrum of retronecanol is presented in Fig. (iv) and a suggested rationalisation of this fragmentation pattern is given in Chart (XII).

The molecular ion appears strongly at m/e 141. This ion, by two successive or simultaneous β-fissions, eliminates the unstable vinyl alcohol (44 mass units) to give the cyclic species of m/e 97. It is expected that, by a rearrangement, a more stable 6-membered cyclic fragment can be achieved. Elimination of a methyl radical from the latter would then give rise to the peak m/e 82. The fragments of m/e 97 and 82 have a parent-daughter relationship in view of the metastable peak appearing at m/e 69.4 (calculated by the usual formula $m^{*} = \frac{m_2^2}{m_1}$ where m^{*} is the m/e ratio of the metastable peak, m_2 is the m/e ratio of the daughter fragment 82 and m_1 is the m/e ratio of the parent 97). Peaks at m/e below 82 can be explained by

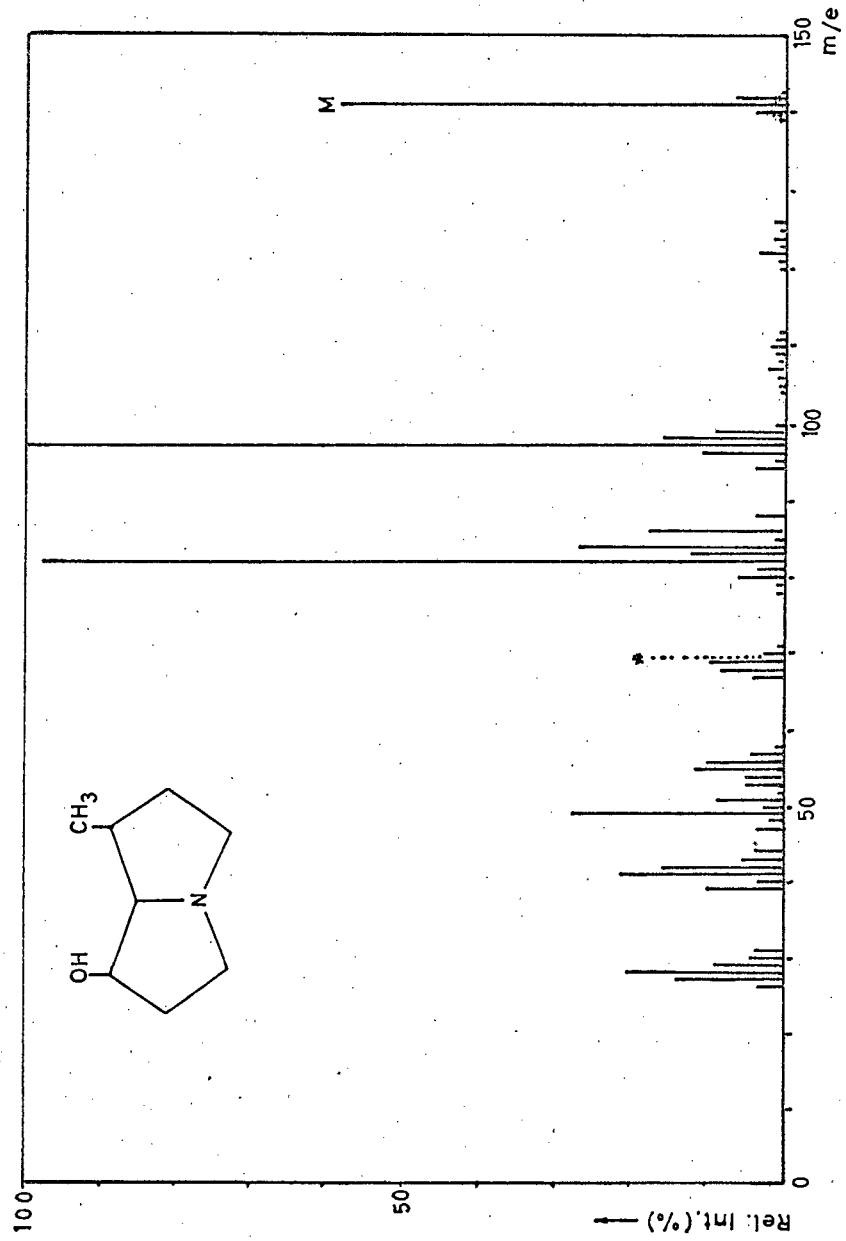
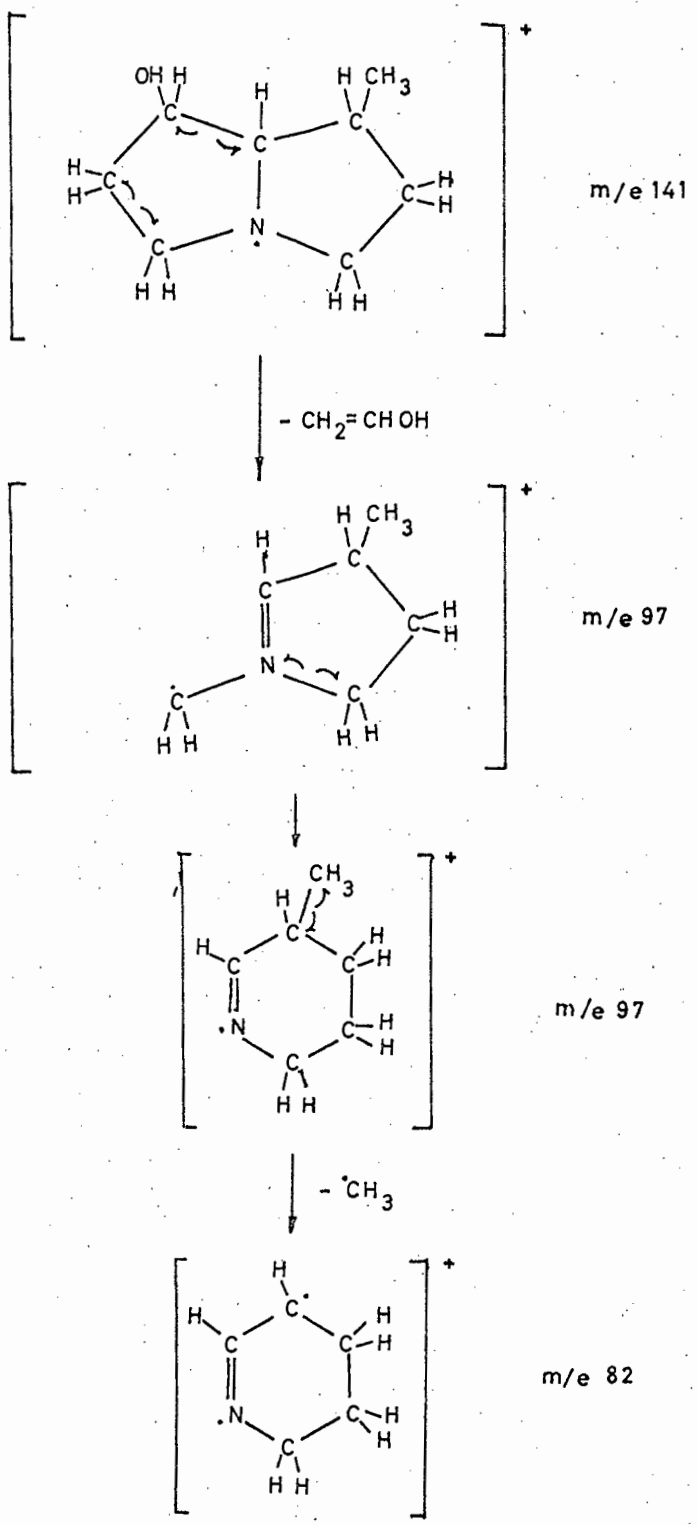


Fig.(iv) Mass Spectrum of Retronecanol

CHART XII



further fragmentation of the m/e 82 ion.

(ii) Mass Spectrum of Heliotridane

The mass spectrum is presented in Fig.(v) and the suggested fragmentation sequence is given in Chart (XIII). The molecular ion appears at m/e 125. To account for all the peaks, two competing fragmentation processes are invoked. The first is exactly analogous to that suggested for retronecanol, the only difference being the elimination of ethylene in place of vinyl alcohol in the first step. Again a metastable peak at m/e 69.5 relates the peaks at m/e 97 and 82.

In the second, apparently more favourable, fragmentation sequence, the initial loss of a methyl radical gives rise to the peak at m/e 110. Two subsequent successive or simultaneous β-fissions eliminates the radical C_2H_3, leaving a 5-membered cyclic fragment of m/e 83, which could be stabilised by rearrangement to the 6-membered cyclic species. Further peaks in the spectrum can arise from fragmentation of the species of m/e 82 or 83.

(iii) Mass Spectrum of Heliotridene

To rationalise the mass spectrum of heliotridene, Fig. (vi), a slightly different initial breakdown is invoked, Chart (XIV). The molecular ion appears at m/e 123. The stepwise elimination of two hydrogen radicals by β-fissions gives rise to the fairly stable fragment m/e 121. A further

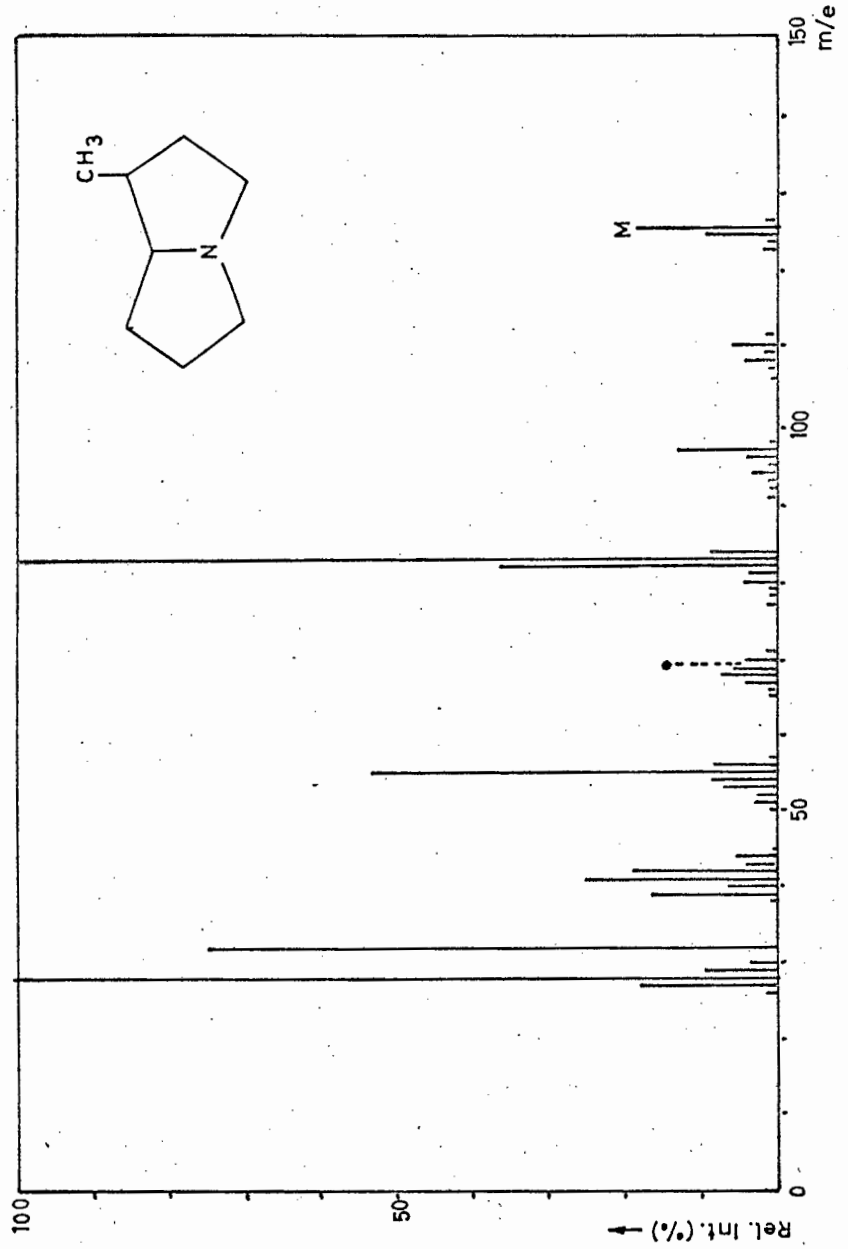
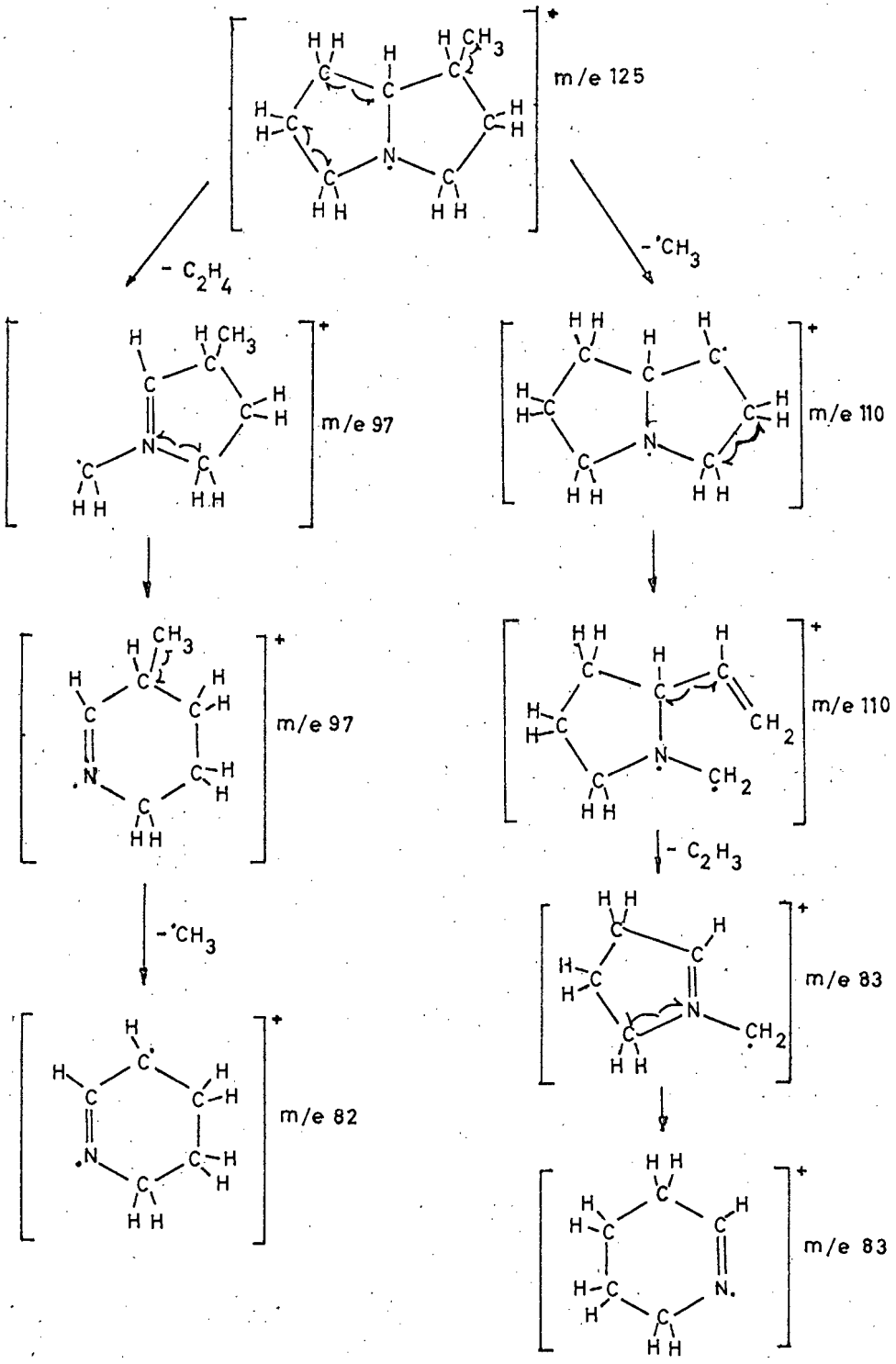


Fig.(v) Mass Spectrum of Heliotridane

CHART XIII



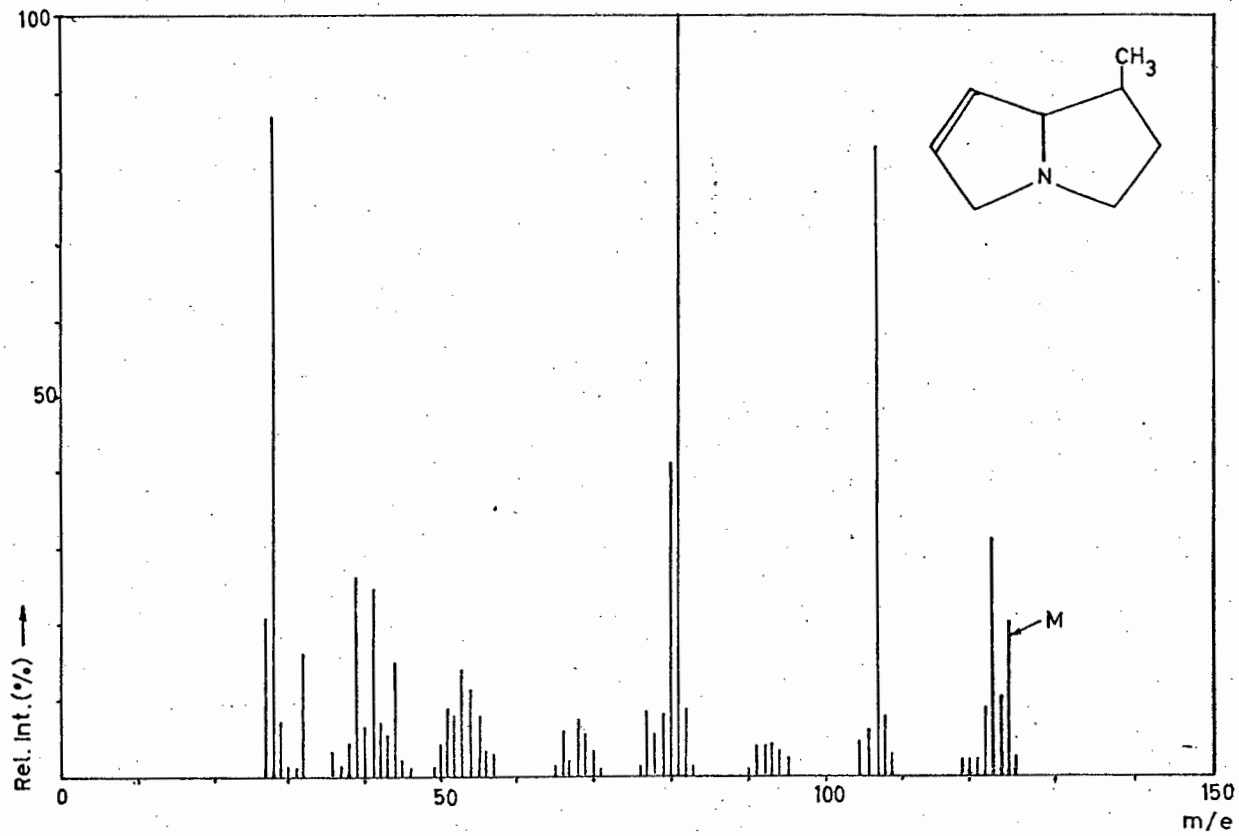
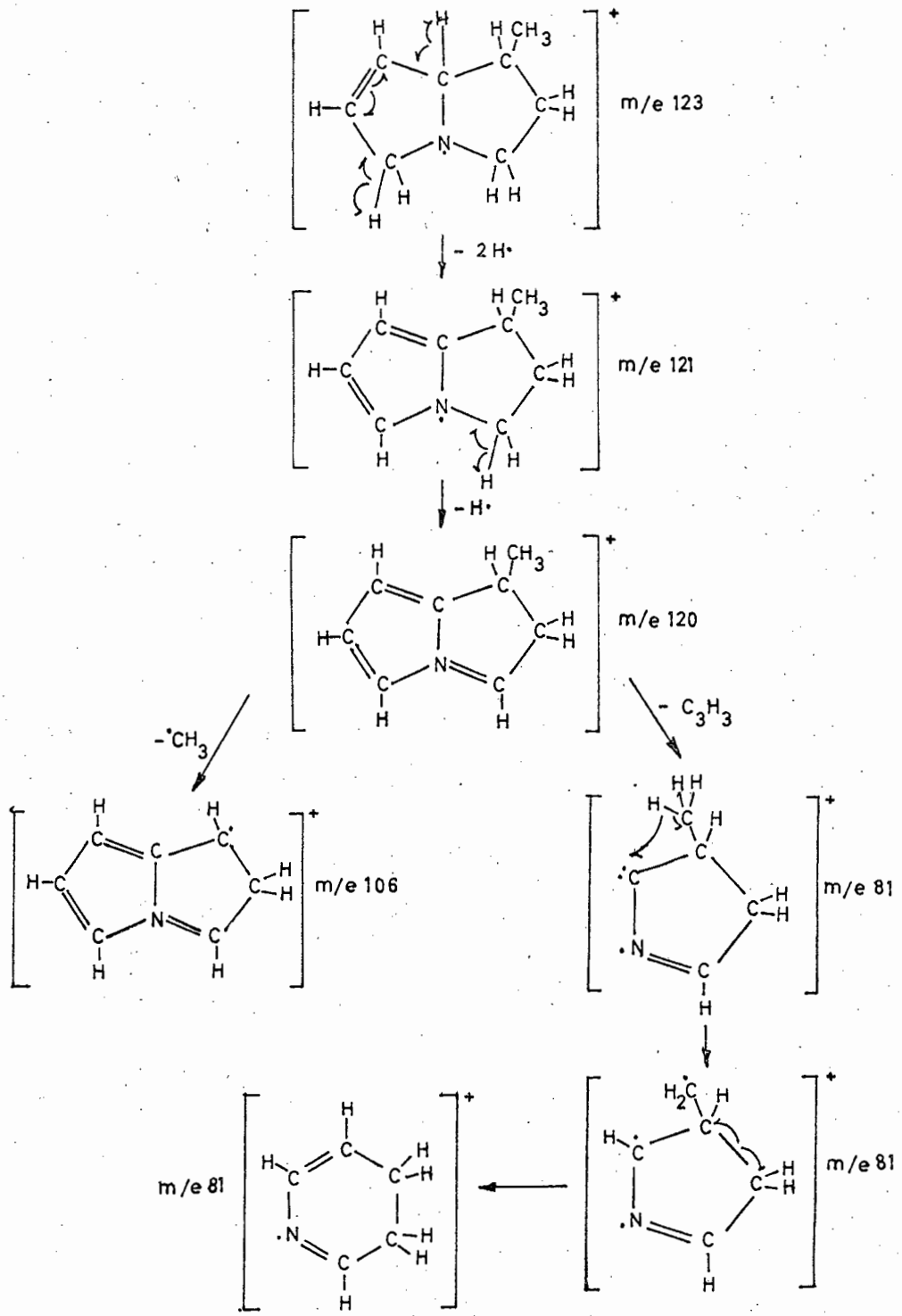


Fig.(vi) Mass Spectrum of Heliotridene

CHART XIV



β-fission and elimination of another hydrogen radical produces the fragment m/e 120, which has extensive conjugation of the double bonds. The loss of a methyl radical from the m/e 120 fragment gives rise to the peak at m/e 106.

The complete breakdown of the unsubstituted ring in the m/e 120 fragment produces elimination of a C_3H_3 radical, giving a fragment of m/e 81. Hydrogen radical transfer and rearrangement within the m/e 81 fragment will produce a species similar to those invoked in the rationalisation of the spectra of heliotridane and retronecanol.

8. FEEDING EXPERIMENTS AND EXPERIMENTS ON ACTIVE ALKALOID

The Crotalaria spectabilis plants were grown from seeds in a sheltered position out of doors. The seed was sown in August (Spring) and the plants were fed and harvested in February (Summer). Seeds sown in boxes in a glass-house did not thrive.

The labelled ornithine was fed into the plants by injection using glass capillaries. In this way a much higher incorporation of radioactivity in the alkaloid is obtained than when the labelled substances are fed via the roots of the plant. The method also has the advantages of being quick and convenient, and bacterial breakdown of the precursor in the hydroponic solution need not be considered. The method suffers from the disadvantage, however, that the sudden change in precursor concentration could lead to the

plant adopting an abnormal metabolism. There is also the danger that the extensive damage caused to the plant could affect the metabolism or leave the plant susceptible to attack by diseases.

The Crotalaria spectabilis plants showed no signs of rotting at the wounds and no other signs of abnormality were apparent.

Five days after feeding, the plants were harvested, dried and the active monocrotaline extracted in essentially the manner previously described for the seeds. The high yield (878 mg.) and relatively high activity (1.261×10^6 c.p.m./mMole) obtained from feeding ten plants with 0.5m Curie of active ornithine, justified the choice of Crotalaria spectabilis plants and confirmed that ornithine is a good precursor for the alkaloid.

A portion of the active monocrotaline (150 mg.) was diluted to about 300 mg. with inactive material. This step was justified in view of the high activity of the alkaloid. Retronecanol was obtained from the diluted alkaloid as previously described, and a Kuhn-Roth oxidation of the C - CH_3 group using chromic acid was performed. Pyrolysis of the resulting barium acetate gave acetone and barium carbonate, each of which was utilised to give the percentage activity at carbons 1 and 1'.

The findings of the present work are in complete agreement with those of Morgan for Senecio sceleratus and of Bottomley and Geissman for S. douglasii. The carbon atoms 1 and 1' had 28.6% of the total retronecanol activity, whilst C1' had 21.5% and C1 had 5.2% of the total retronecanol activity.

It is thus confirmed that, in Crotalaria spectabilis as in the two species mentioned above, a symmetrical intermediate is involved in the build-up of ring B. This once again conflicts with the results of Hughes who worked with Senecio isatideus. Whilst it is conceivable that the metabolism is different in S. isatideus from that in the other three species, this is thought unlikely in view of the fact that retrorsine is the major alkaloid in both S. isatideus and S. sceleratus. The alternative explanation is that Hughes' results are unreliable. This is possible when one considers the extremely low activities at which he worked.

EXPERIMENTAL

1. EXTRACTION OF INACTIVE MONOCROTALINE

Crotalaria spectabilis seeds (500 g.) were immersed in water and allowed to soften overnight. The water was decanted and the seeds were homogenised with ethanol in a Waring Blendor to a thin slurry. The slurry was boiled under reflux for 1 hour and the plant material then allowed to settle. The liquid was filtered off, fresh ethanol added to the residue and the mixture again refluxed for one hour. This extractingprocess was repeated until a total of five fresh aliquots of alcohol had been used and the extract was colourless. The bulked ethanol extract was flash-evaporated nearly to dryness and the residual ethanol removed on a rotary evaporator under reduced pressure.

The light-brown mass was taken up in water (500 ml.) and acidified to a pH of 2-3 using citric acid. The solution was cooled for 12 hours in a refrigerator to consolidate the precipitated fats and these were then centrifuged off. After decanting the clear reddish liquid, the solids were washed with dilute citric acid solution and again centrifuged. The combined centrifugates were extracted with several 50 ml. aliquots of ether to remove the last traces of fats and oils.

Concentrated hydrochloric acid (20 ml.) and zinc dust (10 g.) was then added to the aqueous solution and the mixture

vigorously stirred for one hour in order to reduce any N-oxide present to the free amine. After filtration, the aqueous solution was made alkaline with concentrated ammonia solution and the resulting dark-brown liquid extracted with four 100 ml. aliquots of chloroform.

The chloroform solution was taken to dryness under reduced pressure on a rotary evaporator and the residual crude monocrotaline recrystallised from ethanol (15 g., m.p. 198-200°, Yield 3.0%).

2. HYDROGENOLYSIS OF MONOCROTALINE AND PURIFICATION OF RETRONECANOL

Monocrotaline (2.0 g.) was dissolved in 0.1 M hydrochloric acid (65 ml.) and Adams' catalyst (0.2 g.) was suspended in the solution. The mixture was shaken for 15-17 hrs. under hydrogen at a pressure of 45 p.s.i.g. in a Parr bomb. The catalyst was filtered off and the aqueous solution continuously extracted with ether for 12 hours to remove monocrotalic acid.

The aqueous fraction remaining after ether extraction was made alkaline with sodium hydroxide and again continuously ether extracted for 12 hours. The ethereal solution of retronecanol was dried over sodium hydroxide pellets and then evaporated to dryness under reduced pressure. The retronecanol remained either as a white crystalline mass or as a colourless

oil which solidified on standing. The solid was purified by vacuum sublimation (80°C, 0.1 mm. Hg). Resublimation produced clean crystals (0.7g., m.p. 90-91°, 80% yield) which slowly turned brown in sunlight but which were stable in a dessicator in the dark. The material, dissolved in acetone, moved as a single spot on unactivated alumina thin layer plates using ethyl acetate as developer and iodine as colouring agent. Gas-liquid chromatography and mass spectrometry (q.v.) confirmed the purity and identity of retronecanol.

A microanalysis gave:- C:67.2%, H:10.5%, N:9.8%

calculated for $C_8H_{15}NO$:- C:68.0%, H:10.7%, N:9.9%.

3. CONVERSION OF RETRONECANOL TO HELIOTRIDANE METHIODIDE

Retronecanol (1.5g.) was cooled to -15°C in a flask by means of an ice/hydrochloric acid freezing mixture. Concentrated sulphuric acid was introduced dropwise down the wall of the flask and allowed to cool to the flask temperature before coming into contact with the crystals of retronecanol. When all the crystals had been treated in this way (about 3 ml. of acid was needed) excess sulphuric acid (6 ml.) was added, a reflux condenser fitted to the flask and the flask heated in an oil bath at 120-125°C for 2 hours. Higher oil bath temperatures produced excessive charring of material. The flask, still with the condenser on, was then cooled in ice. Clean ice (40 g.) and then excess 33% sodium hydroxide (50 ml.) at 0°C was added slowly

via the reflux condenser. During some experimental runs, the heliotridene was ether extracted (5x50 ml. aliquots) from the mixture at this stage. The ethereal solution, after drying over sodium hydroxide pellets, was evaporated under reduced pressure to yield heliotridene as a mobile, colourless oil (1.05 g. 80% yield from retronecanol). A picrate of the oil, formed in and recrystallised from ethanol, melted at 220-221°C (Letcher reports m.p. 220°C, Konovalova reports m.p. 224°C) G.L.C. and mass spectra confirmed the purity and identity and G.L.C. revealed the instability of heliotridene on standing.

During those experimental runs in which heliotridene was not isolated, the alkaline solution obtained on addition of sodium hydroxide was steam distilled into 2M hydrochloric acid (30 ml.) until 100 ml. of distillate had collected. This acidified distillate was reduced with hydrogen in a Parr bomb at 45 p.s.i.g. over Adams' catalyst for 12-15 hours. The catalyst was then filtered off and the solution made alkaline with sodium hydroxide. The basic solution was well shaken out with ether (6 x 50 ml. aliquots), the ethereal solution dried over sodium hydroxide pellets and the solution concentrated carefully under reduced pressure on a rotary evaporator. Heliotridane (1.0 g., 75% yield from retronecanol) remained as a mobile, colourless oil. The picrate was prepared in and recrystallised from ethanol, m.p. 233-235°C (Menshikov reported

m.p. 233-235°C). Gas-liquid chromatographic and mass spectral studies of the material were conducted and the infrared spectrum was consistent with the structure of heliotridane. The oil, kept in a refrigerator, was found to be unchanged after several months.

To a solution of heliotridane (1.0g.) in dry ether (10 ml.) was added an excess (3 ml.) of methyl iodide. An immediate white precipitate of heliotridane methiodide formed. The ether was decanted and the crystals taken up in pure acetone (10 ml.). The solution was poured into excess ether (70 ml.) to reprecipitate the methiodide. This purification was repeated several times until the ether was no longer discoloured after precipitation of the methiodide (2.0g., 70% yield from retronecanol). A metho-picrate of heliotridane crystallised out of ethanol in needles, m.p. 277-279°C (Morgan reports m.p. 276-278°C). The crystals of heliotridane methiodide were found to be hygroscopic and were kept in a vacuum dessicator. Solutions of the methiodide did not move on either silica or alumina thin layer plates using a range of solvent systems and no peaks were obtained on the gas-liquid chromatograph.

4. (i) <u>The Hofmann Reaction on Heliotridane</u>

Heliotridane methiodide (2.0g.) was dissolved in water (2 ml.) in a 50 ml. flask. Excess moist, alkali-free silver oxide (ex 1.0g. silver nitrate) was added to the solution of

heliotridane methiodide and the mixture was vigorously shaken for 1 hour. The solution of heliotridane meth-hydroxide was filtered off, the filter paper being washed with a minimum of distilled water and care being taken to minimise direct contact of the solution with the air. The water was then removed from the solution on a rotary evaporator (60 mm./40°C), leaving a brown gum of heliotridane meth-hydroxide.

A 10 ml. flask containing the gum was fitted with a still-head and a specially designed U-tube trap which was cooled in liquid nitrogen. The heliotridane meth-hydroxide was distilled at 0.5m.m./120°C for 2 hours, after which time only a dark, solid residue remained in the flask. Several different experimental runs were conducted using distilling temperatures from 80°C to 180°C and pressures from 0.5 mm. Hg to 50 mm. Hg, but all these experiments resulted in the same products and the best yield was obtained under the conditions described above.

On thawing out the contents of the U-tube trap, two colourless mobile liquids separated into well-defined layers. The lower layer turned anhydrous copper sulphate blue and was shown by G.L.C. to consist mainly of water with trace amounts of the upper layer. The upper layer, designated the Hofmann reaction products, had a characteristic strong,

fishy odour. The liquid formed a picrate in ethanol but recrystallisation of the crystals produced picrates melting over wide temperature ranges. Thin-layer chromatography of the liquid on unactivated alumina using methanol as developing liquid and iodine as colouring agent revealed the presence of three major and two minor components in the mixture. G.L.C. studies on the mixture were conducted. The mixture was unchanged after long periods (up to one month) in the refrigerator and heating the liquid at 125°C for 2 hours at atmospheric pressure also left it unchanged (as evidenced by infrared spectroscopy, thin layer chromatography and G.L.C.). The liquid had a boiling point (by Siwoliboff's method) of 165 - 166°C. A pine splint moistened with hydrochloric acid was not reddened by the Hofmann reaction products, thus pointing to the probable absence of pyrroles in the mixture.

(ii) Hydrogenation of the Hofmann Reaction Products

A small quantity (200 mg.) of the Hofmann reaction product was taken up in an excess (5ml.) of 1M. hydrochloric acid. Adams' catalyst (100 mg.) was added to the solution and the mixture shaken for 8 hours in a Parr bomb under hydrogen at a pressure of 45 p.s.i.g. The catalyst was filtered off and the solution made alkaline with sodium hydroxide. The alkaline solution was shaken out with ether (4 x 15 ml.) and the ether solution was dried over

sodium hydroxide pellets. The ether was taken off on a rotary evaporator, leaving a drop of a clear, mobile oil. An ethereal solution of this hydrogenated product was subjected to G.L.C. studies.

(iii) The Excess Base Modification of the Hofmann Reaction

Heliotridane methiodide (1.0g.) was dissolved in excess 50% aqueous KOH solution (10 ml.). The resulting solution was taken to a light brown gum on a rotary evaporator and distilled in the special apparatus described in section 4(i) at 0.5 mm./120^{o} for two hours. The products, collected in the U-tube trap, were shown by thin-layer and gas-liquid chromatography to be identical to those obtained when the Hofmann reaction was carried out in the usual way.

5. GAS - LIQUID CHROMATOGRAPHY

An Aerograph model A-700 "Autoprep" preparative gas chromatograph was used. The detector was of the catharometer type and the recorder was a Honeywell-Brown instrument having a range of 0-3m.volts. Hydrogen was used as carrier gas but helium could be used without greatly changing retention times. A carrier gas flowrate of 200 ml./min. was used throughout. The injector, detector and collector of the instrument was maintained at 25 - 50^{o} above the column temperature.

Three columns were prepared :

(a) A 12ft., $\frac{2}{8}$" o.d. aluminium column was packed with 10% ^{w}/w polyethylene glycol (Carbowax) 20M on 45/60 mesh untreated Chromosorb P. The column was conditioned at 190° for 50 hours and its operating temperature limit was 170°.

(b) A 12ft., $\frac{2}{8}$" o.d. aluminium column was packed with 10% ^{w}/w polyethylene glycol (Carbowax) 4000 on 80/100 mesh acid-washed Chromosorb W. The column was conditioned at 150° for 40 hours and the operating temperature limit was 130°.

(c) A 12ft., $\frac{3}{8}$" o.d. aluminium column was packed with 20% ^{w}/w Silicone G.E. SE52 on 80/100 mesh acid-washed Chromosorb W. The column was conditioned at 280° for 50 hours and had an operating temperature limit of 250°.

In the present work, retention was taken as the time from injection to the time at the apex of the peak.

6. ATTEMPTED OXIDATION OF RETRONECANOL

(i) The Sarett Oxidation

Sarett's reagent was made up by slowly adding chromium trioxide (3.1g.) to pyridine (30 ml.) at 10°C and shaking the bright yellow suspension well. To 3 ml. of this reagent was added slowly and with stirring a solution of retronecanol (250 mg.) in pyridine (4 ml.), the flask being kept at 10°C. The mixture was then left to stand overnight at room temperature.

Ether (50 ml.) was added, the mixture shaken well and then filtered through a Celite pad. The ethereal filtrate was dried over sodium hydroxide pellets and the ether was removed on a rotary evaporator. The brown, fairly viscous residue smelt strongly of pyridine and gave an infrared spectrum almost identical with that of pyridine. An ethereal solution of the product on the gas-liquid chromatograph gave a peak with retention time corresponding to that of pyridine and small peaks representing trace amounts of other unidentified substances. The amounts were insufficient for preparative gas-liquid chromatography. Thin layer chromatography on silica plates using ethyl acetate as developer and iodine as colouring agent showed the isolated product to be mainly pyridine with traces of two other substances.

(ii) The Jones Oxidation

The Jones reagent was prepared by adding concentrated sulphuric acid (2.3 ml.) slowly to a solution of chromium trioxide (2.67 g.) in water (4 ml.) and making up to 10 ml. with distilled water. This solution was 8N w.r.t. oxygen.

The Jones reagent was added dropwise to a solution of 1.0g. of retronecanol in 10 ml. of A.R. acetone which had been distilled over potassium permangate. The solution initially became cloudy, but eventually, on addition of a single drop, cleared to an orange colour which indicated the end point of the oxidation. The solution was made just alkaline to litmus

with sodium hydroxide and extracted with ether (4 x 20 ml.). The ethereal extract was dried over sodium hydroxide pellets and the ether removed on a rotary evaporator. The residual oil solidified to white crystals (0.8g.) on standing. The crystals were shown by melting point, thin-layer and gas-liquid chromatography and infrared spectroscopy to be identical with an authentic sample of retronecanol, the starting material.

(iii) The Pfitzner-Moffatt Oxidation

Crystalline, 100% orthophosphoric acid was prepared by adding to dry phosphorous pentoxide (14.2 g.) the calculated amount of water (5.4 ml.). The resulting viscous liquid solidified on standing overnight.

Dicyclohexylcarbodiimide (1.236g., 6m moles.)and crystalline phosphoric acid (0.294 g., 3m moles) was dissolved in 10 ml. of dried dimethylsulphoxide (twice distilled and stored over a molecular sieve). To this mixture was added 0.282g.(2m moles) of retronecanol dissolved in 2 ml. of dimethylsulphoxide, care being taken to exclude moisture.

The mixture was shaken for 8 hours, during which time a white precipitate of dicyclohexylurea formed. Water (10 ml.) was then added to destroy excess reagent and the precipitate was filtered off. The filtrate was made basic with lithium hydroxide, filtered again to remove lithium phosphate formed and the oxidation product was then extracted with ether

(5 x 20 ml. portions). The ether extract was dried over sodium hydroxide pellets and taken down on a rotary evaporator, leaving a colourless oil which solidified to white crystals (0.205 g.) on standing. This substance was shown by melting point, infrared spectroscopy and thin-layer and gas-liquid chromatography to be identical with an authentic sample of the starting material, retronecanol.

7. FEEDING METHODS AND EXPERIMENTS ON ACTIVE ALKALOID

Seeds of Crotalaria spectabilis were sown in August in a sheltered position out of doors after soaking overnight in water. In February, ten of the plants (about 9" tall) were fed with 8.6 mg. of [5- ^{14}C]-ornithine hydrochloride having a total activity of 0.5 m.Curie. The active amino acid was dissolved in about 2 ml. of distilled water. The solution was drawn up into glass capillary tubes about 0.5 mm. diameter sharpened at one end, and the pointed ends of the capillaries were pushed into holes made in the plant stems with a thick sterilised needle. Each plant carried up to three such capillaries for five days with no apparant deleterious effects. A daily check was made on the capillaries to ensure that the plants were absorbing the solution and those that were not emptying were removed and rinsed out. The washings were drawn up into new capillaries which were again placed in the plant stems.

Five days after the initial feeding, the plants were uprooted and the whole plants dried at about 60° overnight. Monocrotaline was extracted from the dried, crushed plant material in the manner previously described for the crushed seeds (Section 1), except that a Soxhlet extractor was used for the initial ethanol extraction. The monocrotaline was recrystallised from ethanol (yield 878 mg.).

Monocrotaline (11.73 mg.) was combusted in a stream of oxygen and the carbon dioxide was collected as barium carbonate by flushing the gas through barium hydroxide solution. Portions of the dried, finely ground barium carbonate were weighed into special sample bottles and suspended, together with 0.4g. of Cab-O-Sil, in 10 ml. of scintillator liquid (composition : 3g. PPO, 300 mg. POPOP, 1 l. toluene). These samples were counted on a Beckman three-channel automatic liquid scintillation spectrometer.

Activity : 400 c.p.m. /mg. barium carbonate

1.261×10^6 c.p.m./ mMole monocrotaline.

Active monocrotaline (150.6mg.) was diluted with inactive monocrotaline (151.3 mg.) and the active retronecanol (52.0mg. resublimed) extracted as previously described.

Retronecanol (4.8 mg.) was combusted to carbon dioxide and collected and counted as barium carbonate.

Activity : 305.4 c.p.m./mg. barium carbonate

481×10^3 c.p.m./m Mole retronecanol

(i) Kuhn-Roth Oxidation of Retronecanol and Determination of Relative Activities of C(1) and C(1').

Retronecanol (40.7 mg.) was refluxed with oxidant (8 ml. of 4N chromic acid and 2 ml. of concentrated sulphuric acid) for one and a half hours at 160 - 170°. The solution was then steam distilled in Kjeldahl-type apparatus and the acetic acid (in about 20 ml. distillate) was collected in a flask cooled in ice. The solution was titrated with standard barium hydroxide using phenolphthalein as indicator.

The volume of 0.0493N. barium hydroxide required was 4.56 ml. This corresponds to a 77.9% yield of acetic acid from retronecanol.

The barium acetate solution was taken to dryness under reduced pressure and the residue dissolved in 2ml. of water. This solution was then evaporated to dryness in a weighed platinum boat under an infrared lamp.

A portion of the barium acetate (about a quarter) was transferred to another platinum boat and magnesium sulphate (10 mg.) was added. The mixture was dissolved in a few drops of water and again evaporated to dryness. The resulting magnesium acetate was combusted and the carbon dioxide collected and counted as barium carbonate.

Activity : 348.4 c.p.m./mg. barium carbonate

137.4×10^3 c.p.m./mMole acetic acid

i.e. 28.6 % of the total activity of retronecanol

The remaining barium acetate was pyrolysed at 450 - 500° C to give barium carbonate and acetone. The latter was combusted directly to carbon dioxide and collected and counted as barium carbonate

Activity : 394.2 c.p.m./mg. barium carbonate

232.7×10^3 c.p.m./mMole acetone

The barium carbonate residue from the pyrolysis was acidified with lactic acid and the carbon dioxide evolved was flushed into barium hydroxide solution with nitrogen. The regenerated barium carbonate was collected and counted.

Activity : 131.6 c.p.m./mg.barium carbonate

25.9×10^3 c.p.m./mMole barium carbonate

i.e. 5.4 % of the total activity of retronecanol is in C(1).

From the activities of the acetone and the residual barium carbonate, the activity of C(1') was calculated as 103.4×10^3 c.p.m./m Mole. i.e. 21.5 % of the total activity of retronecanol is in C(1').

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