SYNTHESIS AND BIOSYNTHESIS OF MIMOSINE

SYNTHESIS AND BIOSYNTHESIS

OF

MIMOSINE

BY

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SCOPE AND CONTENTS: DL-Mimosine has been synthesized by debenzylation and detosylation of the product obtained by condensation of 3-benzyloxy-4-pyrone with β-amino-α-tosylaminopropionic acid. A new method for the isolation of mimosine from Leucaena glauca Benth. is described. The biosynthesis of mimosine was studied by feeding radioactive aspartates, glycerol, glycerate and ribose to Mimosa pudica L. . Mimosine-C^{14} was isolated and partially degraded, and it was shown that the carbon-3 of aspartic acid is specifically incorporated into the pyridone ring.

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TABLE OF CONTENTS

	Page
Descriptive note	ii
Acknowledgment	iii
SECTION I: SYNTHESIS OF MIMOSINE	
PART A: MIMOSINE REVIEWED	
Introduction	1
Isolation and Characterization of Mimosine	2
The Chemistry of Mimosine	3
PART B: MIMOSINE RE-INVESTIGATED	
Introduction	11
The Isolation of Mimosine	12
The Dissociation Constants and Structure of Mimosine	14
Synthesis of Mimosine	16
Reactions of Mimosine	21
Attempts to Separate the Pyridone Nucleus from the Side Chain	22
Attempts to Isolate the Individual Carbon Atoms of Mimosine	25
SECTION II: BIOSYNTHESIS OF MIMOSINE	
PART A: THE ORIGIN OF THE PYRIDINE RING IN PLANTS	
Introduction	36
Lysine as a Possible Precursor of the Pyridine Ring	37
Tryptophan as a Possible Precursor of the Pyridine Ring	39
Nicotinic Acid as a Precursor of the Pyridine Ring of Nicotine Ricinine and Anabasine	42
Biosynthesis of the Pyridine Ring from 2-, 3- and 4- Carbon Units	43
Pyridine Dicarboxylic Acids	61
Unique Nicotinic Acid-Alkaloid Relationships	64
PART B: MIMOSINE BIOSYNTHESIS	
A. Choice of Precursors	66
B. Choice of Feeding Time	68
C. Mode of Administration	68

D. Isolation of Alkaloid 72
E. Degradation of Alkaloid 73

RESULTS AND DISCUSSION 73

EXPERIMENTAL

SECTION I: SYNTHESIS OF MIMOSINE

Isolation of Mimosine 86
The Dissociation Constants of Mimosine 87
Mimosine Synthesis 88
Preparation of Other Pyridone Derivatives 94
Attempts to Separate the Pyridone Nucleus from the Side Chain 97
Attempts to Isolate the Individual Carbon Atoms of Mimosine 101

SECTION II: BIOSYNTHESIS OF MIMOSINE

Radioactive Feeding Experiments 115
Measurement of Radioactivities 128

REFERENCES 132

SECTION 1

SYNTHESIS OF MIMOSINE

PART A

MIMOSINE REVIEWED

INTRODUCTION

The genus Leucaena belongs to the Mimosaceae, a sub-family of the Leguminosae. Leucaena glauca Benth. (Ipil-Ipil or Kao Haole) is a shrub of frequent occurrence in tropical regions: in the Hawaiian Islands and the Philippines it provides an excellent source of crude protein for animal feed. However, it has long been recognized that the plant's usefulness for this was limited due to the presence of a toxic component, to whose isolation and study considerable effort was devoted. Toxicity was reported to manifest itself in a variety of symptoms including œdema of the joints, alopecia, reduction in growth, emaciation, and in extreme cases, death.

The isolation of this toxic material, leucaenol (Mascré, 1937), was preceded by the isolation of the amino acid mimosine (I) from the sap of Mimosa pudica L. (Renz, 1936; Nienburg & Taubock, 1937). Investigation of the structure of mimosine (Renz, 1936; Nienburg & Taubock, 1937) and leucaenol (Yoshida, 1944; Adams, Cristol, Anderson & Albert, 1945; Bickel & Wibaut, 1946; Wibaut, 1946; Kostermans, 1947; Bickel, 1947, 1948; Adams, Jones & Johnson, 1947) made it likely that the two compounds were identical. Direct comparison (Kleipool & Wibaut, 1950) confirmed this identity and a synthesis of mimosine on which rested the final proof of structure, has been reported (Adams & Johnson, 1949).

Since the name "mimosine" was the first to be coined (Renz, 1936),

proof of its identity makes the names "leucaenol" (Yoshida, 1944), "leucenol" (Adams et al, 1945) and "leucaenine" (Bickel & Wibaut, 1946) redundant.

Structural investigations carried out on mimosine (I) were made difficult by the occurrence of a number of anomalous reactions which eluded explanation. A review of the chemistry of mimosine will demonstrate the salient features which make this compound unique.

ISOLATION AND CHARACTERIZATION OF MIMOSINE

Renz (1936) isolated mimosine from the exudate obtained on cutting the stems of M. pudica. The exudate was rubbed with alcohol, then extracted with 70% alcohol and the residue consisting of crude mimosine was recrystallized from water. A variety of more laborious methods was used by several workers to isolate mimosine from L. glauca seeds (Mascré, 1937; Yoshida, 1944; Bickel & Wibaut, 1946; Kostermans, 1947). Fresh finely crushed seeds were extracted with boiling water and the separated mother liquors were subjected to a series of precipitation and recrystallization operations using very large quantities of alcohol. Adams et al (1945) extracted freshly crushed seeds with 90% alcohol in a Soxhlet extractor. Yields of up to 2% mimosine were separated from freshly crushed seeds.

Renz (1936) reported that mimosine melted with decomposition at 228-9° C. and that it was laevorotary: $[\alpha]_D = -21^\circ$ (in water). Kostermans (1947) and Bickel and Wibaut (1946) agreed with these observations. Mascré (1937), however, observed mimosine to melt with decomposition at 283-7°C. and found it to be optically inactive. Adams et al (1945) were in close agreement with Mascré until further work

(Adams & Johnson, 1949) demonstrated that optically active mimosine was completely racemized by prolonged boiling (48 hours) in water. They also found that racemic mimosine formed a hemihydrate which melted with decomposition at 228-9°C. while anhydrous racemic mimosine melted at 235-6°C. (decomposition). Optically active mimosine did not form a hydrate. Their conclusions were that the melting point and optical activity of mimosine were dependent on the mode of isolation from the plant. This was further corroborated by Wibaut and Schuhmacher (1952).

THE CHEMISTRY OF MIMOSINE

A. COLOUR REACTIONS

A number of colour reactions have been reported for mimosine of which the ferric chloride test has been most widely used (Renz, 1936; Yoshida, 1944; Adams *et al*, 1945; Kostermans, 1947). Yoshida pointed out that with neutral or basic ferric chloride solution mimosine gave a red colouration, whereas a violet colour was obtained in acidic solution. Adams *et al* reported a violet colouration resulting from the reaction of mimosine with ninhydrin. These two tests for the phenolic and the amino acid function of the molecule respectively are complementary to each other, are both quite reliable and serve as an excellent combination for chromogenic sprays used in the detection of mimosine on paper chromatograms.

B. FORMATION OF DERIVATIVES

Despite the number of functional groups present in mimosine, anomalous behaviour manifested itself in the inability to form derivatives characteristic of these groups. Attempts to obtain derivatives by benzoylation, by reaction with β-naphthalenesulfonyl chloride, and by

formation of a phenylhydantoic acid all failed (Adams et al, 1945). No products were obtained from acetylation (Yoshida, 1944; Adams et al, 1945) or from catalytic hydrogenation (Adams et al, 1945; Bickel & Wibaut, 1946). The hydrochloride, hydrobromide, hydroiodide, sulfate and methyl ester dihydrochloride salts of mimosine have been isolated and characterized (Adams et al, 1945; Bickel & Wibaut, 1946; Bickel, 1948). The copper salt, and derivatives with picric acid, picrolonic acid and nitrobarbituric acid have also been prepared (Bickel & Wibaut, 1946). Some of these derivatives were difficult to obtain while all of them are of limited utility as intermediates in chemical degradations or in purification procedures.

C. PYROLYSIS OF MIMOSINE

Renz (1936) found that carbon dioxide was liberated when mimosine was strongly heated with barium hydroxide: presumably decarboxylation occurred.

Bickel and Wibaut (1946) obtained pyridine when mimosine was strongly heated with zinc dust under high vacuum. The pyrolysis product was found to be 3-hydroxy-4-pyridone (II) when pyrolysis was carried out in the presence (Adams et al, 1945) or in the absence (Adams et al, 1945; Bickel, 1947) of zinc dust.

D. METHYLATION OF MIMOSINE

(i) Methylation with Dimethylsulfate

Yoshida (1944) reported that the action of dimethylsulfate on mimosine in alkaline solution gave rise to a β-substituted acrylic acid (III). Convincing evidence or confirmation of this is lacking. Bickel and Wibaut (1946), on the other hand, reported the product from

this reaction (in almost neutral solution) to be N-methyl-3-methoxy-4-pyridone (IV) which was compared with an authentic sample and found to be identical (Wibaut & Kleipool, 1947; Bickel, 1947). Nothing is known concerning the fate of the nitrogen-attached side chain of mimosine during this reaction.

(ii) Methylation with Diazomethane

Treatment of mimosine with diazomethane according to Renz (1936) yielded a crystalline material which melted at 104°C., gave a red colouration in ferric chloride solution but was explosively unstable. Adams et al (1945) reported that essentially the same reaction gave rise to oily mixture from which no products could be isolated.

Kleipool and Wibaut (1950) treated the methyl ester dihydrochloride of mimosine with diazomethane to obtain a mixture of products. The main product melted at 165-6°C., analyzed for $C_4H_8O_3N_2$ and a Zeisel determination revealed one methoxy group: it was neutral, stable to oxidation by both acidic potassium iodide and Tollen's Reagent, and did not give any ferric chloride colouration. The results of a van Slyke nitrogen determination were negative. A second product which gave the analysis of $C_3H_7O_2N$ occurred in small amounts. Neither of these products was identified. A small quantity of 3,4-dimethoxypyridine was recovered as the picrate from the reaction mixture. The first two products were thought to be fragments arising from the pyridine ring, since treatment of 3-methoxy-4-pyridone (V) with diazomethane gave three products very similar to those obtained from mimosine, differing only in distribution. As in the case of the dimethylsulfate methylations, nothing is known as to the fate of the N-side chain of mimosine.

E. TREATMENT WITH NITROUS ACID

Yoshida (1944) attempted a diazo coupling reaction of mimosine with β-naphthol but found the test to be inconclusive. Bickel and Wibaut (1946) treated mimosine with nitrous acid but were not able to isolate any products. Kostermans (1947) claimed that mimosine and barium nitrite in sulfuric acid solution gave rise to the hydroxy acid analogue (VI) of mimosine which on recrystallization from water melted at 194°C. but no yield was reported. No further work on this reaction of mimosine has been published.

F. OXIDATIONS OF MIMOSINE

Mascré (1937) made the startling observation that mimosine reduced ammoniacal silver nitrate but did not attempt to explain this phenomenon.

Bickel and Wibaut (1946) reported that in one experiment the oxidation of mimosine with alkaline potassium permanganate gave methylamine and oxalic acid. They were unable to isolate any intermediates, or to reproduce this initial result. It was noted that 3-hydroxy-4-pyridone (II) gave rise to methylamine as the only identifiable product under the same conditions. These workers also carried out an ozonolysis reaction on mimosine but failed to isolate any products.

A reaction which was thought to be the addition of bromine to mimosine was observed to give an unidentifiable product melting with decomposition at 168°C. (Bickel & Wibaut, 1946). A more intensive study (Bickel, 1948) demonstrated oxidation to occur. The only products isolated were ammonium oxalate in less than 8% yield and α,β-diaminopropionic acid dihydrobromide in yields of about 1%.

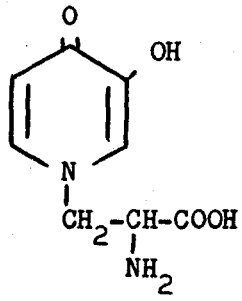
Kostermans (1947) found that mimosine, titrated with iodine in alkaline solution, gave an iodoform reaction and consumed 10.6 gram atoms of iodine per mole. Similar behaviour was observed with N-methyl-3-hydroxy-4-pyridone (VII) which consumed 8 gram atoms of iodine per mole. No reaction was observed with 3-hydroxy-4-pyridone (II) or N-methyl-3-methoxy-4-pyridone (IV); less than 1/3 gram atoms of iodine per mole was consumed in each case. Kostermans pointed out the analogous behaviour observed for ortho-diphenol and concluded that the iodoform arose from an equivalent ortho-diphenolic structure in the rings of mimosine and of N-methyl-3-hydroxy-4-pyridone. No further reports on this reaction are available.

G. MIMOSINE SYNTHESIS

The ultimate proof of the structure of mimosine rests on its unambiguous synthesis. A number of early synthetic approaches proved to be unsuccessful. It was found that 3-hydroxy-4-pyrone (VIII) or 3-methoxy-4-pyrone (IX) did not react with glycine, α,β-diaminopropionic acid, β-amino-α-bromopropionic acid (Kleipool & Wibaut, 1950) or with β-amino-α-hydroxypropionic acid (Adams & Johnson, 1949). Another approach to synthesis, which had failed, was by the Strecker route. Although condensations of 3-methoxy-4-pyridone (V) with bromoacetaldehyde diethylacetal (Bickel, 1948) and condensation of 3-methoxy-4-pyrone (IX) with aminoacetaldehyde diethylacetal (Kleipool & Wibaut, 1947) gave derivatives of the requisite aldehyde (X), further conversion of these to mimosine proved unsuccessful (Bickel, 1948; Kleipool & Wibaut, 1950).

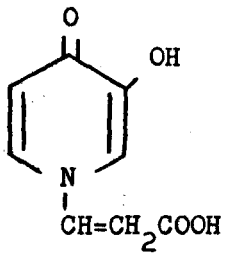
By reaction of 3-methoxy-4-pyridone (V) with α-acetamidoacrylic acid (XI) Adams and Johnson (1949) had obtained an uncharacterized adduct,

regarded as O-methyl-N-acetylmimosine (XII), which gave mimosine (I) in 25% yield on vigorous hydrolysis with hydriodic acid.

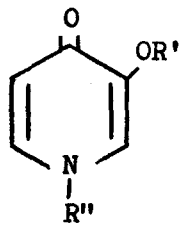


I

Mimosine



III

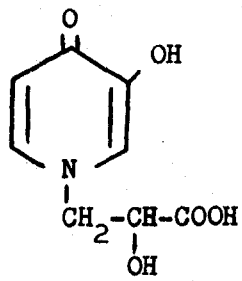


II, R=R"=H

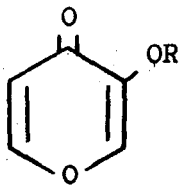
IV, R'=R"=CH_3

V, R'=CH_3, R"=H

VII, R'=H, R"=CH_3

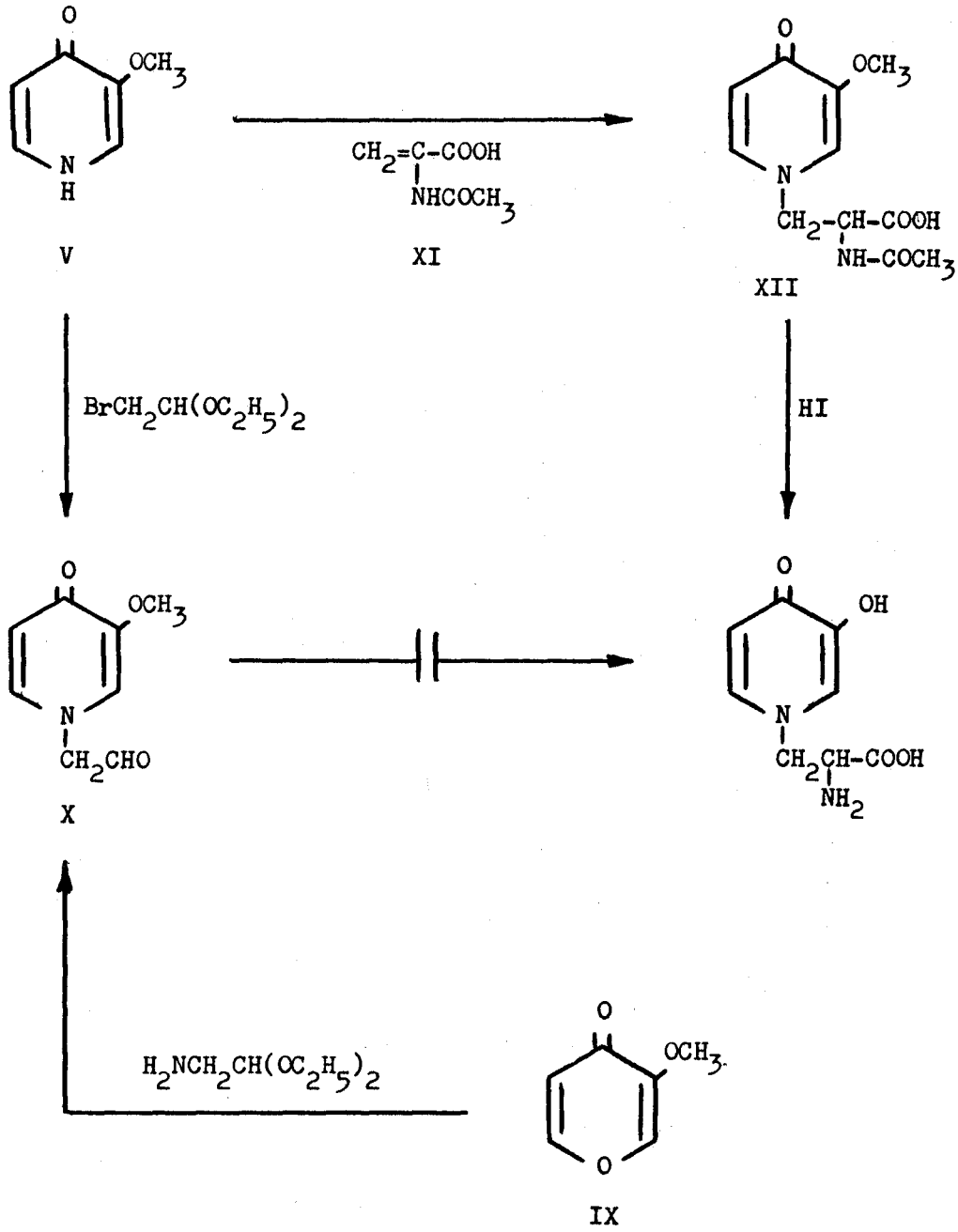


VI



VIII, R=H IX, R=CH_3

SYNTHESIS OF MIMOSINE



PART B

MIMOSINE RE-INVESTIGATED

INTRODUCTION

The primary objective of the present investigation was to gain some knowledge of the precursors used for the biosynthesis of mimosine in M. pudica plants. To establish a precursor-product relationship, specific incorporation of a labelled precursor into a product must be demonstrated. To demonstrate this conclusively, unambiguous carbon-by-carbon degradation of the labelled product is necessary. Such a degradation requires well understood reactions which lead to well characterized products in an unequivocal fashion. None of the reported reactions of mimosine was adequate for such a purpose but it appeared that re-investigation of some of these might prove of interest in connection with an acceptable degradation sequence. For these pilot studies, mimosine was required in quantities which were not conveniently obtainable from M. pudica. Chemical synthesis or isolation from L. glauca, by reported methods, were obvious alternatives as sources of mimosine for this purpose.

Attempts to repeat the reported synthesis of mimosine (Adams & Johnson, 1949; Part A, sub-section G) were disappointing. The final product of the reaction was a mixture which, on the basis of paper chromatographic analysis, did not contain mimosine but appeared to consist mainly of 3-hydroxy-4-pyridone (II) and alanine.

The extraction of mimosine from the seeds of L. glauca had been accomplished by several methods (Part A: Isolation and Characterization of Mimosine), all of which involved large volumes of solvent and the

removal from the extracts of considerable quantities of proteins and polysaccharides (Unrau, 1961). Partial hydrolysis of the latter in the course of extraction leads to gummy intractable residues from which the desired product is obtained only with great difficulty.

To obtain sufficient quantities of mimosine for the present study, more reliable methods were developed. A new synthesis and an improved method of isolation will be described. When sufficient mimosine was available, many attempts were made to find reactions suitable for a complete degradation to be used in biosynthetic studies.

THE ISOLATION OF MIMOSINE

(i) Isolation from *Mimosa pudica* L.

Renz (1936) observed a yield of 200 milligrams of mimosine from 100 young *M. pudica* plants cut in August. The age and size of the plants and the details of the method used for collecting the sap were not reported.

The most convenient method of harvesting the plants was by cutting each petiole or leaf stem individually and collecting the sap with a capillary pipette as it exuded from the fresh cut. There are many factors which influence the amount of sap which can be obtained. The best yields of sap were obtained from young healthy plants which were large and fleshy. Older woody plants, young plants in nitrogen-deficient sphagnum and small plants exuded comparatively little sap when cut. Another aspect influencing the yield appeared to be related to the moisture content of the sphagnum in which the plants were growing at the time of harvest and the atmospheric temperature and humidity during harvest. Although this relationship is not thoroughly understood, well watered plants harvested in a warm humid atmosphere appear to exude a great deal more sap than the same

plants under drier conditions. The optimal time for working with M. pudica was found to be during the warm late spring weather when fast lush growth is characterized by high sap content. Considerable variation in size was observed amongst plants grown under identically controlled conditions.

Variation in the yield of sap ranged from 250λ of sap obtained from 12 select young plants up to the same volume of sap collected from 30 older woody plants. When 250λ of sap were evaporated to dryness the yield of solid residues amounted to 38-40 milligrams. When the same quantity of sap was rubbed with alcohol and the remaining residues extracted with 70% alcohol, 10-12 milligrams of mimosine were recovered. Mimosine appeared to be the only phenolic material and the only amino acid in the sap as tested by paper chromatography. The yield of mimosine was not improved when the sap was subjected to cation exchange chromatography.

(ii) Isolation from Leucaena glauca Benth.

The difficulties involved in isolating mimosine from L. glauca seeds by reported methods have been pointed out previously (Part A: Isolation and Characterization of Mimosine; Part B: Introduction). Extraction of ground seeds gave gummy intractable residues which were subjected to a number of purification procedures. Preferential precipitation by fractional crystallization and also by the addition of soluble heavy metal salts did not meet with success. Fractionation of these residues by cation exchange chromatography enriched the mimosine content of the mixture but did not lead to an efficient separation. Attempted dialysis of these residues was not successful in recovering significant

amounts of mimosine.

Successful isolation of mimosine in high purity from L. glauca seeds was accomplished by the dialysis of the finely powdered seeds against distilled water. Although dialysis is a widely used biochemical technique, no previous report has been found where it has been applied to the isolation of alkaloids from crushed plant material. Hydrolysis of proteins and polysaccharides are minimized because there is no need to apply heat at any stage of the procedure. When the dialyzate was concentrated and allowed to stand overnight, almost pure mimosine crystallized in high yield. It was noted that yields of mimosine ranged from a maximum of 2½% obtained from freshly powdered seeds to as low as 1% from seeds which were stored for several weeks after crushing. Although there is no satisfactory explanation for this apparent deterioration, which occurs when the interior of the seed is exposed by crushing, it can be avoided by using only freshly powdered seeds.

During dialysis it was observed that a gas was evolved from the powdered seed slurry in the dialysis bag. When dialysis was carried out during the warmer weather (up to 30° C.) the gas evolution was accelerated but it appeared to have no effect on the yield or quality of mimosine isolated. This gas was characterized by its particularly foul odour but its identity and origin were not studied.

THE DISSOCIATION CONSTANTS AND STRUCTURE OF MIMOSINE

Two sets of physical measurements were made which involved all of the hydrogen atoms contained in the mimosine molecule. The first experiments consisted of potentiometric titrations in order to determine the dissociation constants of the ionizable hydrogen atoms of mimosine

while the second set of measurements consisted of the nuclear magnetic resonance spectra of mimosine in which the hydrogen atoms attached to carbon were studied.

Three dissociation constants were observed for natural mimosine (pK_1, 2.1 (COOH); pK_2, 7.2 (NH_3^+); pK_3, 9.2 (OH)). The values for pK_2 and pK_3 are in good agreement with those calculated (7.28 and 9.19 respectively) from reported potentiometric data (Bickel & Wibaut, 1946). The dissociation, pK_2, was the only one depressed in the presence of formaldehyde and must, therefore, be assigned to the α-amino group of the amino acid. Its value, unusually low for the α-amino group of an amino acid, corresponds to reported values of the dissociation constants of similar NH_3^+ groups in 1,2-diammonium derivatives (e.g., $NH_3^+CH_2CH(NH_3+)COO^-$, pK_2, 6.69 (α-NH_3^+) (Albert, 1951); $NH_3^+CH_2CH_2NH_3^+$, pK_1, 6.98 (Schwarzenbach, 1933)). The factor which lowers the basicity of the NH_2 group in these compounds is the polar effect of the proximal, charged NH_3^+ group. From the measured data, the isoelectric point of mimosine was calculated to be at pH 4.65. An aqueous solution of pure mimosine was measured to give an acidity of pH 4.65. This merely indicates that mimosine exhibits the expected zwitterion structure as far as the amino acid portion is concerned. An additional structural feature is resonance which exists in the α-pyridone nucleus. This gives rise to two equivalent resonance forms which are both necessary to represent the mimosine structure although the actual contributions of each are not known (Figure 1).

Confirmation of the mimosine structure was obtained in measuring its nuclear magnetic spectrum. Natural mimosine was dissolved in

deuterium oxide containing sodium deuteroxide and a trace of water (H_2O) which functioned as an internal reference. The spectra consisted of four signals (chemical shift in parts per million from water): (a) a triplet +1.20, +1.12, +1.03 (-CH-); (b) a doublet +0.71, +0.63 ($-CH_2-$); (c) a doublet -1.57, -1.67 (C_5-H); (d) a singlet -2.49 (C_2-H) superimposed on a doublet -2.52, -2.62 (C_6-H). The areas of the signals were in the ratio of 1:2:1:2. These results are completely compatible with the structure of mimosine.

SYNTHESIS OF MIMOSINE

The present synthesis of mimosine was based on the condensation of suitably substituted derivatives of 3-hydroxy-4-pyrone and α,β-diaminopropionic acid. It had been reported earlier that 3-hydroxy-4-pyrone or 3-methoxy-4-pyrone did not react with glycine, α,β-diaminopropionic acid, β-amino-α-bromopropionic acid or β-amino-α-hydroxypropionic acid (Part A: sub-section G). Since the crucial step in the pyrone-pyridone conversion is generally regarded as analogous to carbinolamine formation (E. Klingsberg, 1960 a), a nonprotonated amine is required as the nucleophilic reactant. It was likely that the failure of these amino acids to condense was due to their zwitterion structure, in which the amino group is protonated. No attempt had been made to control the ionic state of the reactants (Adams & Johnson, 1949; Kleipool & Wibaut, 1950). Indeed it was subsequently shown (Heyns & Vogelsang, 1954) that condensation of glycine with a number of 4-pyrone derivatives takes place only in the presence of an equimolar amount of base.

The desired condensation of a pyrone with α,β-diaminopropionic acid requires the species $NH_2-CH_2CH(NH_3^+)COO^-$ of the latter. The

assignment of pK values (pK_2, 6.69 (α-NH_2); pK_3, 9.50 (β-NH_2)) (Albert, 1951) indicates that in aqueous solution this species is unobtainable in significant amounts. The species $NH_3^+CH_2CH(NH_2)COO^-$ which overwhelmingly predominates in the isoelectric range of the amino acid, would on condensation with 3-hydroxy-4-pyrone yield an isomer (XXVII) of mimosine. Condensation of the pyrone with the amino acid at pH > 11, i.e., with the species $NH_2CH_2CH(NH_2)COO^-$, would lead to a mixture of mimosine and its isomer (XXVII). For an unequivocal synthesis of mimosine, protection of the α-amino group was therefore required.

The selected derivative was DL-β-amino-α-tosylaminopropionic acid (XIII), which was prepared using the method of Rudinger, Poduška and Zaoral (1960) by the Hofmann rearrangement of α-N-tosylasparagine.

Condensation of the tosylamino acid with 3-methoxy-4-pyrone in the presence of an equimolar amount of sodium hydroxide gave β-(1,4-dihydro-3-methoxy-4-oxo-1-pyridyl)-N-tosyl alanine (O-methyl-N-tosylmimosine) (XIV) in 46% yield. Hydrolysis with hydrobromic and hydriodic acid under a variety of conditions gave mimosine (I), but was accompanied by considerable N-alkyl cleavage. It would appear that whereas mimosine itself is stable towards halogen acids (Adams et al, 1945; Bickel & Wibaut, 1946) derivatives of its O-methyl ether are not; this was presumably why Adams and Johnson (1949) obtained a poor yield in their synthesis and why their work could not be repeated in this laboratory. N-alkyl cleavage is also in evidence in the methylations of mimosine where the N-alkyl group is replaced by a methyl group (Part A: sub-section D). Under conditions where removal of the alanyl side chain from (XIV) was minimal, detosylation

did take place, but the ether cleavage was incomplete. The product was a mixture of mimosine (I) and its O-methyl ether (XV), fractionating of which proved to be tedious.

The difficulty was overcome by a minor variation in the route of synthesis. Condensation of DL-β-amino-α-tosylaminopropionic acid (XIII) with 3-benzyloxy-4-pyrone (XVI) gave β-(3-benzyloxy-1,4-dihydro-4-oxo-1-pyridyl)-N-tosylalanine (O-benzyl-N-tosylmimosine) (XVII) in 70% yield, which on catalytic debenzylation yielded β-(1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl)-N-tosylalanine (N-tosylmimosine) (XVIII) in yields of 80%. Reduction of the tosyl group by anhydrous hydrogen bromide and phenol in glacial acetic acid gave DL-β-(1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl)-alanine (mimosine) (I), melting at 228-30°C. (decomposition). An attempt to synthesize the L-isomer, starting with L-β-amino-α-tosylaminopropionic acid, failed, since condensation of this compound with 3-benzyloxy-4-pyrone under the prescribed conditions was accompanied by complete racemization. The melting point and infrared absorption of this synthetic material were very similar to the melting point (Adams & Johnson, 1949; Kleipool & Wibaut, 1950; Wibaut & Schuhmacher, 1952) and infrared absorption* (Adams, Jones & Johnson, 1947; Adams & Johnson, 1949; Wibaut & Schuhmacher, 1952) of samples of natural mimosine obtained from M. pudica L. and L. glauca Benth. The ultraviolet absorption curves of the synthetic and the natural

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* The observation that the infrared spectrum of a Dl-amino acid in the solid state (KBr) differs from the spectra of the individual D- and L- isomers has been repeatedly confirmed (e.g., Koegel et al, 1955).

(Adams et al, 1945; Adams et al, 1947) material were identical; identical dissociation constants and nuclear magnetic resonance spectra were also observed (c.f. the preceding section). The nuclear magnetic resonance spectra comparisons and interpretations gave unequivocal proof of identity and, incidentally, confirmation of the structure of mimosine.

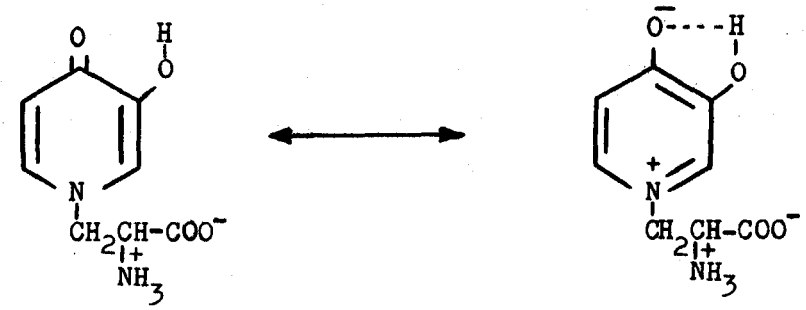
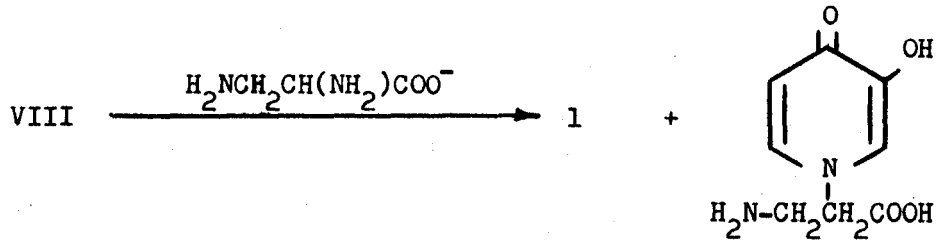
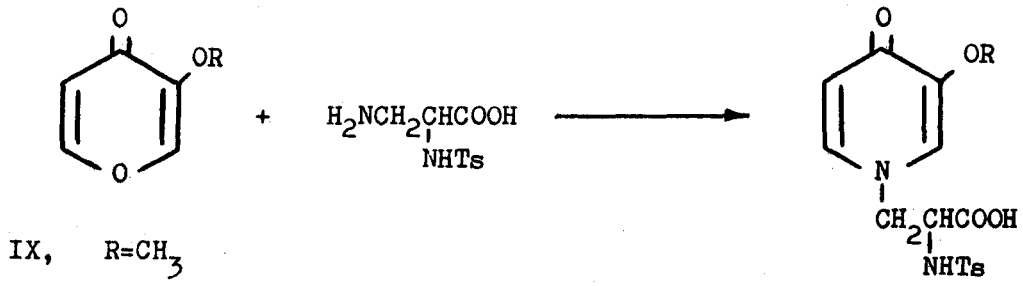


Figure 1



XXVII



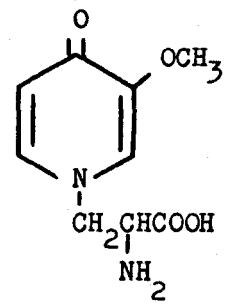
IX, $R=CH_3$

XVI, $R=CH_2C_6H_5$

XIV, $R=CH_3$

XVII, $R=CH_2C_6H_5$

XVIII, $R=H$



XV

REACTIONS OF MIMOSINE DERIVATIVES

As previously discussed (Part A: sub-section B), very little success was met in preparing derivatives of mimosine. Reactions which involved the α-amino moiety in mimosine invariably required strongly acidic reagents while the reaction products usually melted with decomposition. These products were always difficult to isolate in a state of high purity. This phenomenon would appear puzzling until it is recalled that the α-amino group is only weakly basic (Part B: The Dissociation Constants and Structure of Mimosine) and is incapable of forming salts with any but the most acidic reagents. In all probability, such derivatives as the picrate, the picrolonate and the adduct of nitrobarbituric acid are complexes rather than salts with the result that they are less stable and more difficult to isolate.

The derivative of mimosine formed in the presence of cupric ions has been prepared (Bickel & Wibaut, 1946). Attempts to obtain derivatives from the addition of lead acetate or mercuric ions to L. glauca seed extracts gave no products. Complexes rather than true salts are formed by amino acids subjected to this treatment (Greenstein & Winitz, 1961). Treatment of mimosine in aqueous solution with trichloroacetic acid failed to give products. Once again these observations are probably due to the effect of the low basicity of the α-amino group of mimosine.

Attempts to prepare derivatives involving the 3-hydroxy group of mimosine were not successful. Reactions were accompanied by degradative cleavage of the alanyl side chain (Part A: sub-section D; Part B: Synthesis of Mimosine). This phenomenon eludes explanation.

ATTEMPTS TO SEPARATE THE PYRIDONE NUCLEUS FROM THE SIDE CHAIN

In a number of reactions simple pyridone derivatives which presumably arose by cleavage of the side chain of mimosine have been isolated (Part A: sub-section C & D). Only two such reactions, the methylation of mimosine by dimethylsulfate (Part A: sub-section D(i)) and the pyrolysis of mimosine (Part A: sub-section C) have been reported in detail. The reactions studied in this section were carried out in an attempt to find an efficient method for isolating the pyridone ring of mimosine, especially by small scale reactions.

A. PYROLYSIS

The best reported yield of 3-hydroxy-4-pyridone was 35%, obtained by Bickel (1947) who distilled 2 grams of mimosine in a Woods metal bath at 200-240°C. and 1.0 mm. pressure. Bickel observed an 81% yield of crude distillate as compared with 85% obtained by Adams et al (1945) who used zinc dust catalyst with mimosine at 220-50°C. and 2 mm. pressure. Adams et al did not report any yield for the purified product.

For small scale reactions, the distillation of mimosine in the presence of zinc dust gave almost quantitative yields of crude material when carried out at temperatures under 200°C. and 0.01 mm. pressure for periods of 4-6 hours. Two successive distillations of the product at 180-190°C. and 0.01 mm. pressure followed by recrystallization from a benzene-alcohol solvent gave a maximum yield of 70% of 3-hydroxy-4-pyridone. When zinc dust was not used only an 11% yield was obtained. Aqueous extracts of the still pot residues were chromatographed on paper and showed only faint traces of 3-hydroxy-4-pyridone. No colour was observed when the chromatogram was sprayed with ninhydrin, indicating that the

amino acid side chain had been destroyed in the course of pyrolysis.

B. DECARBOXYLATION REACTIONS

Mimosine in quinoline in the presence of a copper bronze catalyst was stable to prolonged heating at 190°C. Partial decomposition to 3-hydroxy-4-pyridone was observed when mimosine alone was heated in N,N-dimethyl-p-toluidine at 190°C. for 2 hours. Total decomposition occurred when mimosine in N,N-dimethyl-p-toluidine was heated at 210°C. (under reflux) for 2 hours with a copper bronze catalyst. The reaction mixture was filtered, evaporated and distilled to give 3-hydroxy-4-pyridone in 17.6% yield. No volatile aldehydes were given off as vapours during the reaction and no aldehydes were detected in the reaction product mixture when treated with 2,4-dinitrophenylhydrazine reagent.

C. METHYLATION OF MIMOSINE

The methylation of mimosine with dimethylsulfate to yield N-methyl-3-methoxy-4-pyridone, as carried out by Bickel and Wibaut (1946), was attempted on a small scale. Despite modifications in the procedure the best yield of N-methyl-3-methoxy-4-pyridone was 11%. In no case was the reported yield of 65% obtained.

D. OXIDATION OF THE SIDE CHAIN

Mimosine was observed to be sensitive to the ninhydrin colour reaction (Adams et al, 1945). In normal ninhydrin oxidations the amino acid loses the carboxyl carbon as carbon dioxide and the amino group as ammonia to give an aldehyde; in the case of mimosine the primary reaction product would be expected to be the N-acetaldehyde analogue. This would be suitable as a starting material for further carbon-by-carbon degradation of the side chain. The aldehyde moiety could be oxidized

to a carboxyl group and decarboxylated to give carbon dioxide. The remaining carbon of the side chain would either remain attached to the nitrogen and hence be recoverable by further reaction or else be lost in a way other than by carbon dioxide formation. In the former case an N-alkyl cleavage would give the remaining carbon by itself. If the latter case occurred then two separate carbon dioxide recoveries would give direct radiocarbon measurements while the activity of the unrecovered carbon atom could be calculated by difference. Such a degradation offered the possibility of selectively recovering side chain fragments while leaving the ring intact. The possibility of an obstacle to this scheme lay in the evidence (Part A: sub-section G) which indicates that the N-aldehydopyridone (X) is unstable since only derivatives of it have been isolated.

An oxidation of mimosine was carried out on a preparative scale using stoichiometric amounts of ninhydrin. Although reaction appeared to occur readily, the large amounts of ninhydrin required and Ruhemann's purple formed in the reaction made it impossible to separate any products from the mimosine oxidation. Interference of ninhydrin products prevented qualitative assays to be carried out either by paper chromatography or by cation exchange chromatography.

From this series of reactions it emerged that the best method for obtaining the pyridone ring of mimosine in small scale preparative reactions was by zinc dust distillation under high vacuum. In each case studied, the side chain was irrecoverably lost.

ATTEMPTS TO ISOLATE THE INDIVIDUAL CARBON ATOMS OF MIMOSINE

A. REDUCTION REACTIONS OF MIMOSINE

(i) Catalytic Reductions (Hydrogenolysis)

Reports have shown that mimosine was stable to catalytic hydrogenations under moderate temperatures and pressures (Adams *et al*, 1945; Bickel & Wibaut, 1946). In confirmation of this, mimosine was now found to be completely stable in aqueous solutions in the presence of excess Raney Nickel catalyst at temperatures up to 90°C. and pressures of 1800 P.S.I. for periods of 24 hours. When mimosine was treated with a large excess of Raney Nickel catalyst at 190°C. and 2000 P.S.I. for 36 hours the reaction mixture no longer contained starting material detectable by paper chromatography. Separation of the products on a cation exchange column eluted with citrate buffers gave low recoveries of alanine and 3-hydroxy-4-pyridone. Identification of these crude products was accomplished by separating model mixtures under identical conditions and comparing the eluates by paper chromatographic analysis. Repeated experiments indicated that this catalytic cleavage was quite sensitive to temperature and pressure: since these severe conditions were not easily reproducible within a narrow range of temperature and pressure, it was thought that a less sensitive exhaustive hydrogenation might give more reproducible results. A model reaction was tested as follows:

An aqueous solution of 3-hydroxy-4-pyridone was hydrogenated at 2500 P.S.I. and 240°C. for 48 hours in the presence of a large excess of Raney Nickel catalyst. The initial solution was slightly acidic and gave a colour test with ferric chloride reagent while the product in solution gave no ferric chloride colour test and was slightly alkaline.

This was interpreted to imply complete hydrogenation.

The reduction of 3-hydroxy-4-pyridone could give rise, depending on the extent of hydrogenolysis, to a series of related products. These were expected to be one or a mixture of the following: 3-hydroxy-4-piperidone (XIX), 3,4-dihydroxypiperidine (XX), pentylamino-2,3-diol (XXI), pentylamino-3,4-diol (XXII) or pentane-2,3-diol (XXIII) and ammonia. The latter three products arise from carbon-nitrogen cleavages which are known to occur in catalytic hydrogenolysis under severe conditions (Elderfield, 1950 a).

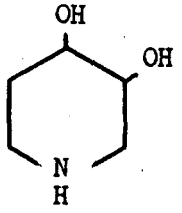
A Schotten-Bauman benzoylation was used as a means of isolating the product from dilute aqueous solution. Elemental analysis of this product was consistent with that of the tribenzoyl derivative of an amine diol having 5 carbon atoms. This product could not be separated into components by gas chromatography. The possibilities of its identity were thus limited to 3,4-dihyroxypiperidine (XX), pentylamino-2,3-diol (XXI) or pentylamino-3,4-diol (XXII). Since this method, used on a compound believed to be an intermediate in the hydrogenolysis of mimosine, gave a single product, the same procedure was used with mimosine as a starting material. For the isolation and characterization of the reduction product derived from mimosine a simplified approach was attempted. The hydrogenation reaction mixture was passed through an anion exchange column on which acidic fragments which might have arisen from the side chain of mimosine would be retained while neutral material and basic products would pass through. A Schotten-Bauman benzoylation for the recovery of the expected diol product was not attempted. Instead, the column eluate was subjected to periodate oxidation, followed by treatment with

2,4-dinitrophenylhydrazine reagent to scavenge any aldehydes produced. Since the expected product was a pentylaminodiol, the oxidation products would allow differentiation amongst the three possible products. 3,4-Dihydroxypiperidine (XX) would be expected to cleave into 3-aminopropionaldehyde and formic acid, while pentylamino-2,3-diol (XXI) would give propionaldehyde, formic acid and ammonia, whereas acetaldehyde and 3-aminopropionaldehyde would be the expected cleavage products of pentylamino-3,4-diol (XXII). On the basis of aldehyde derivative analysis by paper chromatography the only products arising from mimosine treated in this manner were the derivative of propionaldehyde and a second material which showed up as a single unidentifiable spot on the chromatogram. The absence of an acetaldehyde derivative is strong indication that 1-pentylamino-3,4-diol was not present in the original reaction mixture. This also suggests that the alanine side chain product was not further reduced to 2-aminopropanol under the severe reduction conditions. It was not possible to isolate any products on a preparative scale from these reaction mixtures.

(ii) Attempted Chemical Reductions

Mimosine was observed to be stable when treated with a large excess of sodium borohydride at room temperature over prolonged periods of time. No suitable solvents were found in which to carry out lithium aluminum hydride reductions of mimosine. A Soxhlet extraction of mimosine by anhydrous tetrahydrofuran into a still pot containing lithium aluminum hydride in suspension, led to the quantitative recovery of unchanged mimosine from the extraction thimble after the extraction had run for one week. A ninhydrin colour test on the extraction thimble showed that

mimosine had not dissolved in tetrahydrofuran in detectable amounts. Treatment of mimosine in aqueous solution with 3% sodium amalgam over extended periods of time (several days) partially converted mimosine into a mixture of ninhydrin colour positive substances which could not be identified. The chemical reduction of mimosine did not appear amenable for use in preparative reactions.



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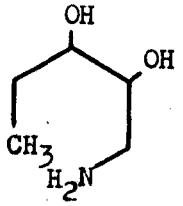
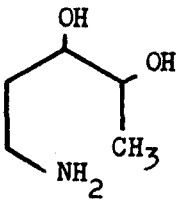
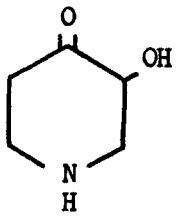


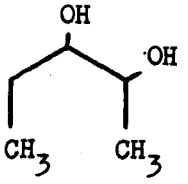
XXI



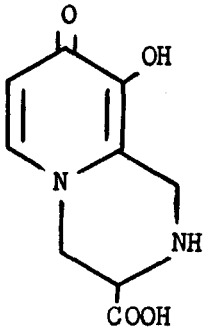
XXII



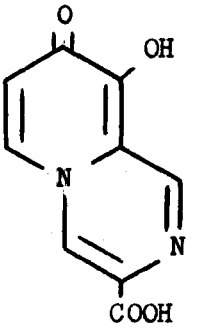
XIX



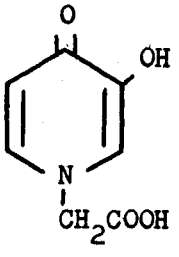
XXIII



XXIV



XXV



XXVI

B. INTRAMOLECULAR MANNICH REACTION

During the formol titration of mimosine it was observed that the solution became quite fluorescent after the addition of formaldehyde. It had been previously demonstrated (Klingsberg, 1961 b) that 3-hydroxypyridine and formaldehyde condense to give 2-hydroxymethyl-3-hydroxypyridine, and that when amines are present, a Mannich type reaction occurs. On the latter premise the expected product of mimosine and formaldehyde would be the bicyclic product (XXIV). Condensation experiments gave up to 60% yield of a material melting at 234-6°C. (decomposition), which showed an elemental analysis close to that calculated for the expected product. The discrepancies between the calculated and observed percentages in the elemental analysis could indicate hydrate formation. Discrepancies have also been observed in the elemental analysis of the adduct of tryptophan and formaldehyde which has a tendency to form a hydrate (Harvey & Robson, 1938; Harvey, Miller & Robson, 1941). Paper chromatographic comparison showed that the compound (XXIV) which was the sole product of the reaction, gave a higher R_f value than mimosine in phenol:ethanol:water solvent. The ninhydrin reaction resulted in a red colour, as compared with the violet colour obtained with mimosine.

This compound (XXIV) was of interest from the point of view of a specific degradation of mimosine. The entire side chain of mimosine with the exception of the carboxyl group is now part of a cyclic system. It was hoped that by suitable reactions a product might be obtained which contained the carbon atoms of the mimosine side chain which had hitherto been irrecoverable from degradations. Carbon-2 of the pyridone ring is common to both rings: oxidation of the pryidone portion would leave

carbon-2 in the intact ring while carbon-3 of the pyridone ring might possibly remain as a carboxyl group attached to carbon-2. From this viewpoint a number of reactions involving the mimosine-formaldehyde adduct were attempted.

Selenium dehydrogenation of this material under nitrogen gas gave a white crystalline material which immediately decomposed on exposure to air. Use of selenium as a catalyst has been reported to lead to rearrangement of carbon skeletons in a number of instances (Elderfield, 1950 b). Several attempts with various modifications to obtain a characteristic product proved fruitless. Dehydrogenation with palladium and maleic acid in aqueous solution led to the recovery of unchanged starting material. Mild dichromate oxidation destroyed the aromatic nucleus of the adduct, a conclusion based on the loss of absorption in the ultraviolet region of the spectrum. These findings demonstrated that there is no tendency of this compound to form the fully conjugated system (XXV) which could have led to a pyrazine derivative or oxidation of the 3-hydroxy-4-pyridone ring.

Methylation of the adduct (XXIV) readily occurred when allowed to react in suspension in ethereal diazomethane, but the oily product could not be purified. The product of methylation was presumably the O-methyl adduct methyl ester although there are other possibilities. Ring opening of the pyridone moiety and/or cleavage at the $-CH_2-N-$ of the pyridone nitrogen could have accompanied the reaction as was suggested in the case of mimosine (Kleipool & Wibaut, 1950; Part A: sub-section **D(ii)**). Hydrolysis in aqueous dilute sodium hydroxide gave a product, which gave a purple colour with ninhydrin and did not contain a phenolic group;

only one product was observed by the ninhydrin development of paper chromatograms. Mild dichromate oxidation of this free acid did not give a product which could be isolated. An ultraviolet absorption spectrum of the aqueous oxidation reaction mixture freed of chromium and chromate ions gave evidence of an aromatic compound different from the starting material but no further identification was possible. It became obvious that the adduct (XXIV) was not a useful intermediate in the degradation of mimosine.

C. IODOFORM REACTION

Kostermans (1947) observed that mimosine and N-methyl-3-hydroxy-4-pyridone (VII) gave the iodoform reaction (Part A: sub-section F). These two compounds along with 1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl acetic acid (XXVI) were subjected to iodoform reactions in which quantitative recoveries of iodoform were attempted. As the complexity of the N-side chain increased in this series of compounds the yield of iodoform was found to decrease ($-CH_3$, 24.3%; $-CH_2COOH$, 13.9%; $-CH_2CH(NH_2)COOH$, 12.9%). This, coupled with the fact that 3-hydroxy-4-pyridone does not give an iodoform reaction, led to the equivocal conclusions that the side chain either bears considerable influence in the reaction or contributes directly the carbon atom which appears in the iodoform.

Further examination in the case of mimosine showed that during the reaction a negligible amount of carbon dioxide was formed while oxalic acid was recovered in a yield of 34%. The reaction residues consisted of an unresolvable mixture which appeared to contain some amino acids. Acidification and steam distillation of this residue gave a distillate, a portion of which was treated with ammonium hydroxide and paper

chromatographed with ethanol:ammonia, but no products were visible on development. Since the origins of both the iodoform and oxalic acid were unknown this degradation procedure could not be used in any way.

D. OTHER REACTIONS

The reduction of ammoniacal silver nitrate by mimosine (Mascré, 1937) was carried out on a preparative scale using excessive concentrations of silver ions. Reactions at various temperatures invariably led to the destruction of the pyridone ring. Analysis of the reaction mixture by paper chromatography showed a complex mixture of amino acids and/or amines sensitive to the ninhydrin colour reaction. Recovery of products and attempted purifications by crystallization and cation exchange chromatography in each case yielded coloured resinous deposits which on paper chromatography gave a pattern comparable to that of the crude reaction mixtures.

The action of various common oxidizing agents was tested on mimosine. The test reactions were first run in dilute acidic solutions at room temperature and those which did not give extensive oxidation were re-run at higher temperatures. All reactions were monitored by paper chromatography at various intervals ranging from a few minutes up to two days. The following summary embodies the findings of this study:

Potassium permanganate and potassium dichromate destroyed mimosine completely even at room temperature and no recognizable fragments were observed. An experiment in which small successive portions of potassium permanganate solution were added and the reaction monitered after each addition, indicated that oxidation was not selective in any observable manner. Potassium ferricyanide in hot acidic solution appeared to have

no effect on mimosine. Sodium metaperiodate and hydrogen peroxide in moderate concentrations each led to the destruction of the molecule. In hot acidic solutions both reagents attacked mimosine to give a large number of reaction products. Paper chromatography and development with ninhydrin spray showed several spots characteristic of amino acids as well as colours other than violet, none of which could be matched for identification. No products were isolated.

These oxidation studies were also carried out on N-methyl-3-hydroxy-4-pyridone (VII) and 1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl acetic acid (XXVI) but the results, although similar, could not be usefully correlated with those for mimosine.

Mimosine was observed to be stable to air oxidation carried out in aqueous alkaline solutions for prolonged periods of time.

Mimosine in hot acidic solution was treated by the addition of a stoichiometric quantity of sodium nitrite solution. Analysis by paper chromatography showed that mimosine had been converted to a phenolic compound which no longer contained an amino acid moiety but no products could be isolated.

This study has presented a new and reliable synthesis of mimosine and also a much improved method of isolating mimosine from the seeds of L. glauca. Although some of the reactions which were studied gave recognizable degradation products, in almost every case the yields did not suffice for the desired purpose of an efficient small scale preparative degradation. Once this point was firmly established for each reaction, the reaction in question no longer maintained sufficient relevance

to this thesis project to merit its exhaustive study. From the reactions available the pyrolysis of mimosine in the presence of zinc dust under high vacuum appeared to be the most suitable method for the partial degradation of mimosine on a small scale. Before a complete degradation will be achieved a further investigation of the reactions of mimosine is required.

SECTION II

BIOSYNTHESIS OF MIMOSINE

PART A

THE ORIGIN OF THE PYRIDINE RING IN PLANTS

INTRODUCTION

Plant alkaloids have always been of interest to man, but only at the beginning of this century has any serious effort been made to ascertain their mode of synthesis within the plant. Georg Trier (1912) speculated that widespread substances, especially amino acids, might be metabolized in a simple manner to amines, betaines and heterocyclic alkaloids. Dreschel, discoveror of lysine, had already suggested that if lysine could be converted to δ-aminovaleraldehyde it might be a precursor of alkaloids. On these foundations, Robinson (1917) was the first to present the view that in growing plants alkaloids are derived by metabolic processes from amino acids, and in this classical work presented a large number of biogenetic schemes. The in vitro syntheses of Schöpf and Hahn carried out under "pseudophysiological" conditions helped to demonstrate the possibility of many reactions occurring under very mild conditions and dilute concentrations although enzymatic systems were not utilized. This speculative approach was helpful in structural studies of new alkaloids and in prompting experiments on living plants but very little progress was made until radioisotopic tracer techniques were introduced about a decade ago. Predicted precursors labelled with isotopic carbon, hydrogen, or nitrogen are administered to the plants and after a suitable length of time, the alkaloids are isolated, then systematically degraded to find the site of isotope incorporation.

Despite the large number of experiments yielding the expected results, there are some alkaloids which are not derived from the predicted amino acid precursors. Most notable amongst these exceptions is the group of alkaloids which contains a substituted or modified pyridine ring.

LYSINE AS A POSSIBLE PRECURSOR OF THE PYRIDINE RING

Robinson (1917) made cursory mention that ornithine (XXVIII) and lysine (XXIX) could conceivably give rise to the pyrrolidine and the piperidine ring systems respectively. He predicted at that time that the pyridine ring of nicotine (XXX) arose from the condensation of ammonia with formaldehyde units. He soon abandoned this early prediction in favour of the view that structurally nicotine could be derived by fusion and modification of a pyrrolidine and a piperidine unit while anabasine (XXXI) would similarly be derived from two piperidine rings. Lysine (XXIX) by a number of steps, could be related to a tetrahydropyridine which Robinson (1955) suggested would be the origin of the pyridine ring in nicotine (Figure 2). This approach gained favour on the basis of a Schöpf chemical precedent (Schöpf, Komzak, Braun & Jacobi, 1948) in which 3,4,5,6-tetrahydropyridine (XXXII) dimerized at pH 8 and room temperature to give 3,4,5,6-tetrahydroanabasine (XXXIII). Experimentation demonstrated that ornithine (XXVIII) was the precursor of the pyrrolidine ring of nicotine (XXX) (Leete, 1955; Dewey *et al*, 1955) which not only strengthened Robinson's recent approach but gained support (Leete, 1956) in the idea that lysine would be the precursor of the pyridine ring of nicotine. This possibility was experimentally tested with the result that this pathway does not

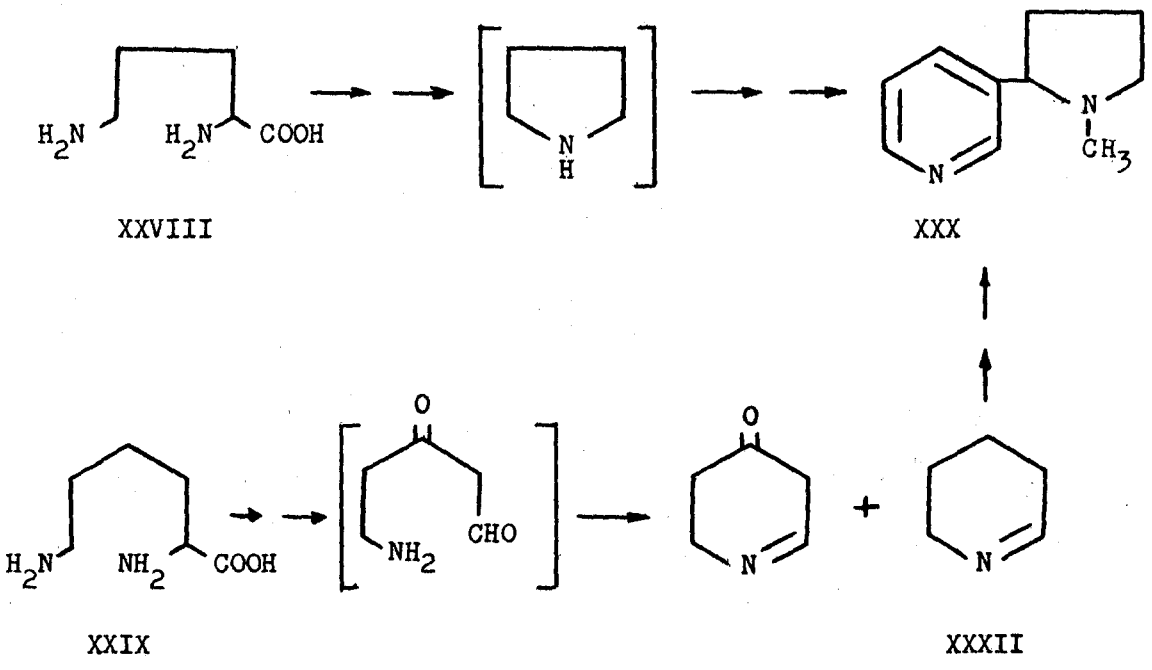
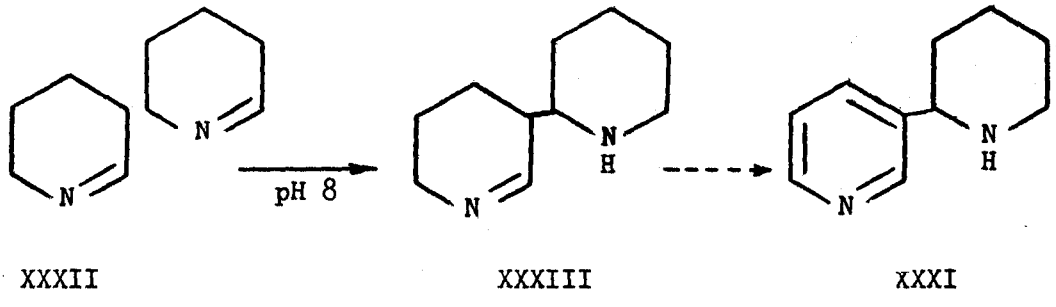


Figure 2



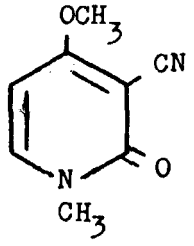
operate in higher plants. Lysine-ϵ-N^{15} was fed to N. tabacum from which the isolated nicotine was found to contain little activity. This low activity was found to be locallized in the pyrrolidine ring while the pyridine ring was found to be inactive (Bothner-By, Dawson & Christman, 1956). Although lysine-2-C^{14} is now known to be a precursor of the piperidine ring of anabasine (XXXI), Aronoff (1956) reported it not to be so. Tamir and Ginsberg (1959) reported that lysine-2-C^{14} was specifically incorporated into the α-pyridone ring of ricinine (XXXIV). Incorporation of lysine-2-C^{14} in a percentage of the same order of magnitude was demonstrated by Juby and Marion (1961) who showed that the activity in ricinine was actually randomly incorporated and the percentage of incorporation was much lower than that for several other precursors. Essery, Juby, Marion and Trumbull (1963) demonstrated unequivocally that the degradation technique used by Tamir and Ginsberg was not specific as it had been reported but gave rise to numerous artefacts. This constituted strong evidence suggesting that lysine, as such, is not incorporated into the pyridine ring.

TRYPTOPHAN AS A POSSIBLE PRECURSOR OF THE PYRIDINE RING

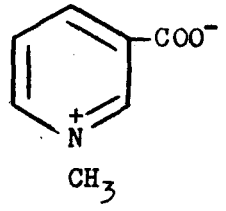
Many biochemical processes occurring in plants are often similar to or identical with those occurring in mammals or Neurospora. It is known and conclusively demonstrated that in mammals and Neurospora, tryptophan is converted by a metabolic pathway, which includes 3-hydroxyanthranilic acid and other intermediates, to nicotinic acid (Figure 3) (Dalgliesh, 1955). On this basis, tryptophan would also be predicted to be the origin of nicotinic acid and of other pyridine derivatives in plants and related microorganisms. Waller and Henderson (1961 a)

have shown, however, that tryptophan-7a-C^{14} was not incorporated into ricinine (XXXIV) and Leete (1957) isolated completely inactive nicotine (XXX) from N. tabacum which had been fed tryptophan-7a-C^{14}. Inactive nicotine from DL-tryptophan-β-C^{14} feeding was reported by Bowden (1953). The striking absence of the pathway of tryptophan to nicotinic acid in higher plants was further demonstrated by the isolation of inactive trigonelline (XXXV) as done by Aronoff (1956) after he had administered labelled 3-hydroxyanthranilic acid to soya bean leaves. Leete, Marion and Spenser (1955) obtained inactive trigonelline in an experiment in which they fed tryptophan-3-C^{14} to intact peas. These findings could merely suggest that the enzyme system required to oxidize tryptophan to kynurenine was lacking and in the event of by-passing this step by supplying an intermediate, one should observe as a likely test the conversion of anthranilic acid into the pyridine ring. Grimshaw and Marion (1958) fed anthranilic acid-1-C^{14} to N. tabacum but no activity was found in the isolated nicotine. Anthranilic acid is thus shown not to be a precursor of nicotine.

Observations by Volcani and Snell (1948) indicated that neither kynurenine nor 3-hydroxyanthranilic acid could replace nicotinic acid as a growth requirement in some species of bacteria. In other bacteria, when tryptophan was used as the only carbon source, no nicotinic acid was formed because the enzyme required for oxidizing 3-hydroxyanthranilic acid was not present (Stanier & Tsuchida, 1949). When tryptophan-C^{14} was supplied to Escherichia coli and Bacillus subtilis, inactive nicotinic acid was formed by these organisms (Yanofsky, 1954).



XXXIV



XXXV

XXXVI

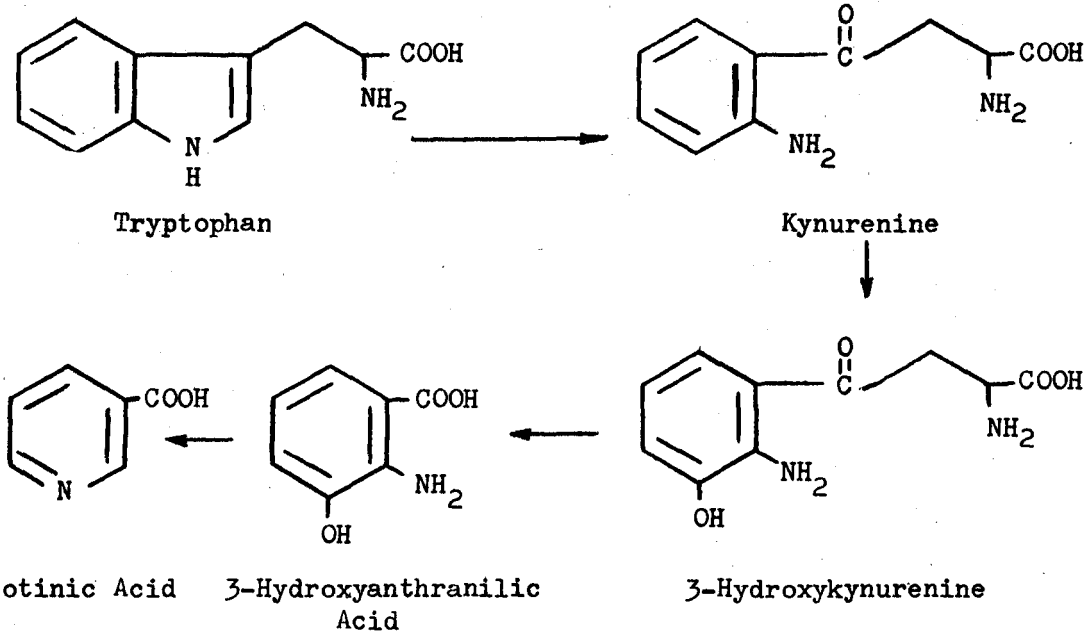


Figure 3

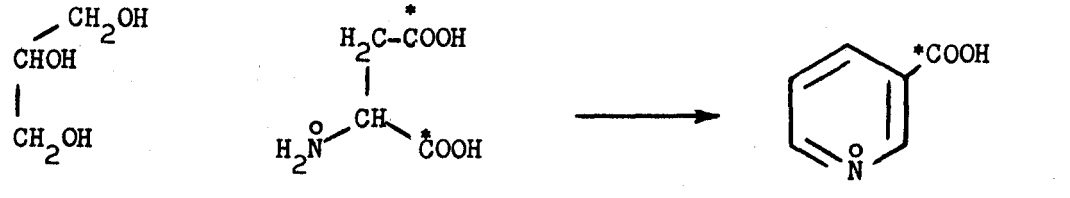


Figure 4

These data clearly indicate that in plants and some microorganisms nicotinic acid is not a metabolite of tryptophan.

NICOTINIC ACID AS A PRECURSOR OF THE PYRIDINE RING OF NICOTINE, RICININE AND ANABASINE

The earliest report of feeding labelled nicotinic acid to tobacco plants was interpreted to show that nicotinic acid-7-C^{14} was not incorporated into nicotine (Dawson, Christman & Anderson, 1953). The same precursor fed to castor plants was incorporated into ricinine which on degradation showed all of the radioactivity to be localized in the cyano group carbon (Leete & Leitz, 1957).

An extensive study carried out by Dawson, Christman, D'Adamo, Solt and Wolf (1960) showed that when nicotinic acid-2-H^3, nicotinic acid-4-H^3 and nicotinic acid-5-H^3 were separately administered to N. glauca plants, specific incorporation into nicotine occurred. In each case the percentage of incorporation was approximately the same and the tritium label in the nicotinic acid was carried intact during the conversion into nicotine where it appeared in its respective pyridine ring position. Anomalous behaviour was observed in the case of nicotinic acid-6-H^3. Although the pyridine ring of the resulting nicotine was specifically labelled in the 6-position, the radiochemical yield was only one tenth of that of the other nicotinic acid feedings. The low incorporation of nicotinic acid-6-H^3 might be indicative of an enzymatic attack on the 6-position of nicotinic acid during its conversion to nicotine, but an oxidative step is unlikely since 6-hydroxynicotinic acid-N^{15} and 1-methyl-6-oxynicotinamide-2-H^3 were not incorporated into nicotine. Precisely the identical mode of incorporation was observed for

anabasine (Solt, Dawson & Christman, 1960).

Two experiments in which doubly labelled precursors were administered to castor plants were reported by Waller and Henderson (1961 a). The ricinine isolated from the feeding of nicotinic acid-U-H^3-7-C^{14} was found to have general tritium labelling on the α-pyridone ring while all of the carbon-14 activity was found locallized in the cyano group. The feeding of nicotinamide-U-H^3-N^{15} amide yielded ricinine which contained general tritium labelling in the α-pyridone ring while all of the nitrogen-15 was found exclusively in the cyano group.

Specific incorporation of nicotinic acid into the pyridine nucleii of ricinine, nicotine and anabasine has been clearly demonstrated by these findings. With this unequivocal relationship, investigations of the origins of these, and in all likelihood other pyridine alkaloids, help to establish the pathway of nicotinic acid biosynthesis in higher plants. As a corollary to this, any studies of the origin of nicotinic acid may be pertinently applied to the modes of biosynthesis of the pyridine alkaloids.

BIOSYNTHESIS OF THE PYRIDINE RING FROM 2-, 3- AND 4-CARBON UNITS

Microrganisms not utilizing tryptophan in the biosynthesis of nicotinic acid became one focus of attention in the elucidation of an alternative biosynthetic pathway for nicotinic acid. Ortega and Brown (1960) showed that when either glycerol-1,3-C^{14} or succinate-2,3-C^{14} was present in growing E. coli cultures, effective radiochemical incorporation into nicotinic acid was observed. They also demonstrated more facile nicotinic acid production to be induced by the presence of either a 3-carbon unit such as glycerol or pyruvate, or an acid of the tricarboxylic acid cycle.

Despite the paucity of unequivocal fact, they interpreted these findings (perhaps intuitively) to suggest that in E. coli the precursors of nicotinic acid were a 4-carbon dicarboxylic acid and glycerol or one of its closely related metabolites.

A preliminary study by Mothes, Gross, Schütte and Mothes (1961) demonstrated that DL-aspartic acid-4-C^{14} administered to Mycobacterium tuberculosis gave rise to radioactive nicotinic acid which on isolation and degradation was found to contain carbon-14 exclusively in the carboxyl group. A later report (Gross, Schütte, Hübner & Mothes, 1963) on the incorporation of DL-aspartic acid-1,4-C^{14}-N^{15} showed a 48% incorporation into nicotinic acid but the relative ratio of C^{14}/N^{15} was only half of that measured in the originally doubly labelled precursor. Glycerol-1,3-C^{14} was also incorporated into nicotinic acid. This report suggests that aspartic acid, except for the loss of one carboxyl group (the carbon-1 position), is incorporated into nicotinic acid giving rise to the nitrogen atom and carbon atoms 2, 3 and 7. The other carbon atoms of the pyridine ring could come from glycerol or glyceraldehyde (Figure 4).

A large number of compounds have been tested as precursors of ricinine, nicotine and, less extensively, anabasine in order to obtain relevant information on the origin of the pyridine ring in these compounds in particular, and by inference, in plant products in general.

A. ACETATE AS PRECURSOR

Leete (1958 a) reported random incorporation of acetate-2-C^{14} into nicotine in experiments with N. tabacum while Iljin (1960) simply

reported incorporation of acetate-C^{14} into nicotine. Acetate-1-C^{14} administered to tobacco plants gave rise to only 4% of the total nicotine activity residing in the pyridine ring while acetate-2-C^{14}, which was incorporated to a greater extent, gave rise to 40% of the total nicotine activity residing in the pyridine ring, half of which was locallized at carbon-3 (Griffith & Byerrum, 1959 a, b; Griffith, Hellman & Byerrum, 1960). Dawson and Christman (1963) reported essentially the same findings.

Sodium acetate-2-C^{14}, wick fed to N. glauca, was incorporated into anabasine to the extent of 2%. Degradations on the radioactive anabasine showed 37% of the total anabasine activity to be found in the pyridine ring, located equally and almost exclusively at ring carbons 2 and 3 (Friedman & Leete, 1963).

Acetate-2-C^{14} administered to Ricinus communis L. was incorporated into ricinine to a far greater extent than acetate-1-C^{14}. In the case of acetate-1-C^{14}, at least 90% of the total ricinine activity was found in the nitrile group (Juby & Marion, 1961; Anwar, Griffith & Byerrum, 1961; Waller & Henderson, 1961 b). Juby and Marion reported that the remainder of the activity in ricinine arising from acetate-1-C^{14} was distributed between the N-methyl and O-methyl moieties. Schiedt, Boeckh-Behrens and Delluva (1962) also demonstrated that acetate-2-C^{14} was incorporated into ricinine to a greater extent than acetate-1-C^{14}. Systematic degradation of the active ricinine obtained from acetate-1-C^{14} and acetate-2-C^{14} are listed (Table 1) (Schiedt & Boeckh-Behrens, 1962). These findings are accompanied by the findings of Marion et al for acetate-2-C^{14} incorporation into ricinine (Juby & Marion, 1963; Essery, Juby, Marion & Trumbull, 1963). It must be borne in mind that the work of Schiedt

and Boeckh-Behrens is based on microanalytical determinations not nearly as refined as those of Juby and Marion, and Essery et al but the overall trends are roughly in agreement.

In accounting for these observations, the original premise must be that acetate was metabolized in the usual manner via the tricarboxylic acid cycle in which it underwent conversion to succinate, fumarate, or oxaloacetate. Each of these products, a 4-carbon dicarboxylic acid, may become incorporated into nicotinic acid by losing a carboxyl group.

PERCENT OF TOTAL RICININE ACTIVITY

Position	Acetate-1-C^{14}	Acetate-2-C^{14}	Acetate-2-C^{14} *
C-2	6	48	38.9
C-3			38.3
C-4	4	8	0.0
C-5	5	9	0.3
C-6	27	2	†
N-Me	3	8	0.65
-CN	33	12	20.8
O-Me	3	6	0.66

* Results compiled from the two papers cited: Essery et al, 1963; Juby & Marion, 1963.

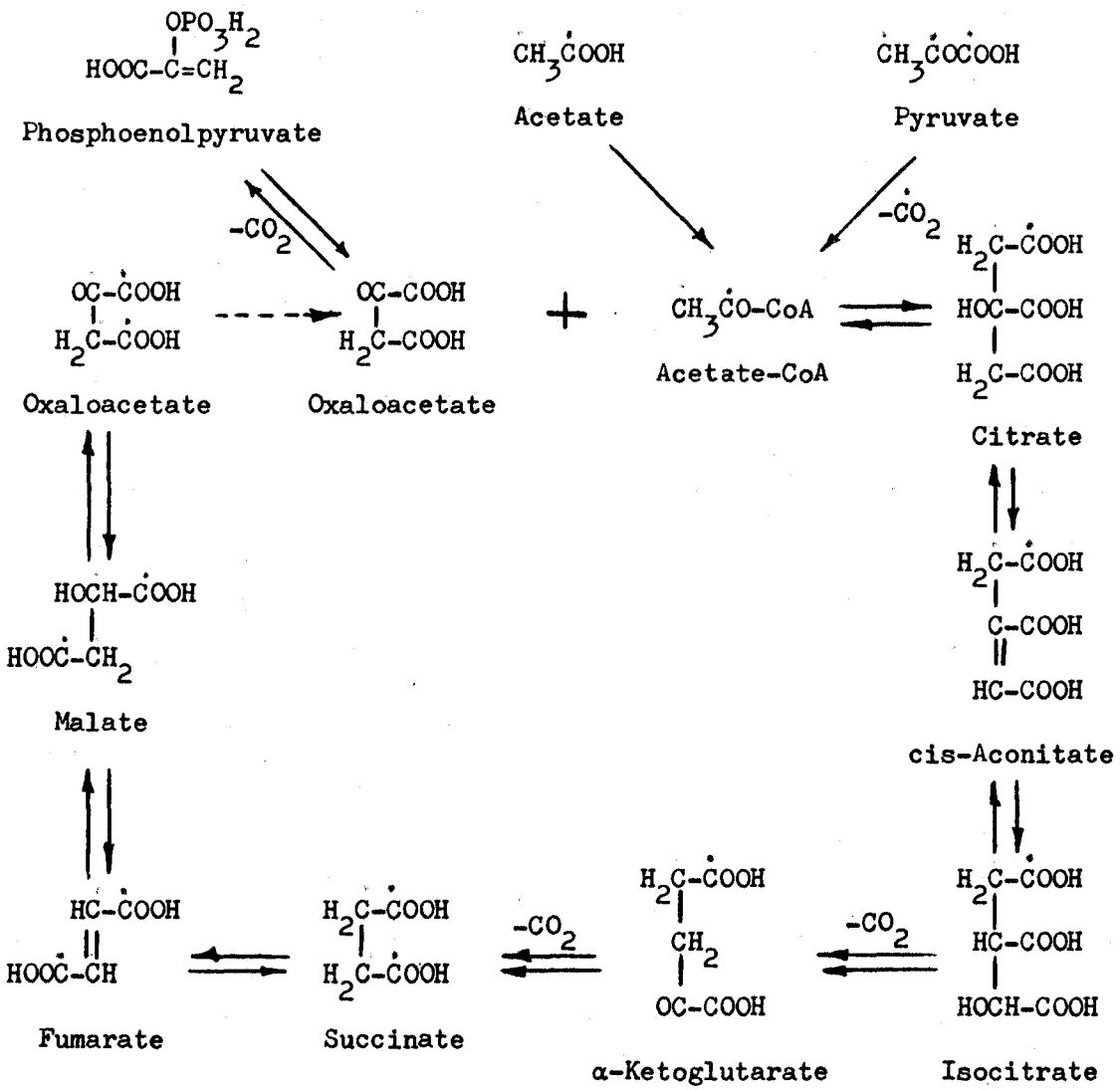
† Quantity not determined.

TABLE 1

On tracing the sequences found in tricarboxylic acid cycle, acetate-1-C^{14} gives rise to the -1,4-C^{14} species of succinate, fumarate, or oxaloacetate (c.f., Figure 5) which on the next turn of the cycle

with inactive acetate would lose all of its C^{14} as $C^{14}O_2$, or on recycling further with a molecule of acetate-1-C^{14} would be regenerated. α-Keto-glutarate-5-C^{14} would be formed as an intermediate in this process (Figure 6). This is convertible to glutamate-5-C^{14} and ornithine-5-C^{14}. Either of these can serve as precursors of the symmetrical intermediate which yields the pyrrolidine ring of nicotine (Leete, 1958 b; Leete & Siegfried, 1957; Lamberts & Byerrum, 1958; Lamberts et al, 1959). The major positions of activity in nicotine on feeding acetate-1-C^{14} would be predicted to be in carbons 2' and 5' as was indeed observed. Additional support to the concept of the symmetrical intermediate leading to the pyrrolidine ring was demonstrated by the incorporation of Δ^1-pyrroline-5-carboxylic acid-5-C^{14} (XXXVI) into nicotine. Presumably this goes via Δ^1-pyrroline-5-C^{14} to give a similar isotope distribution in nicotine as that observed with ornithine-5-C^{14} and glutamate-5-C^{14} (Krampl & Hoppert, 1961). Methyl group labelling from acetate-1-C^{14} was not only expected but observed as in the case ricinine (Juby & Marion, 1961).

Acetate-2-C^{14} would give rise to -2,3-C^{14} labelled succinate, fumarate, or oxaloacetate on its first turn through the tricarboxylic acid cycle. These on a further turn with inactive acetate would become equally labelled in positions 1, 2, 3 and 4 (Figure 7). On further turns with inactive acetate, activity from positions 1 and 4 would be lost more quickly than from positions 2 and 3. Increased labelling would occur at the latter positions relative to the former on successive turns. This would lead to less activity in the C-2' and C-5' than in the C-3' and C-4' positions of the pyrrolidine ring and most of the pyridine ring activity at C-2 and C-3. In ricinine only a little activity



Labelled Acetate and Pyruvate in the Tricarboxylic Acid Cycle

(dotted line indicates start of second cycle)

Figure 5

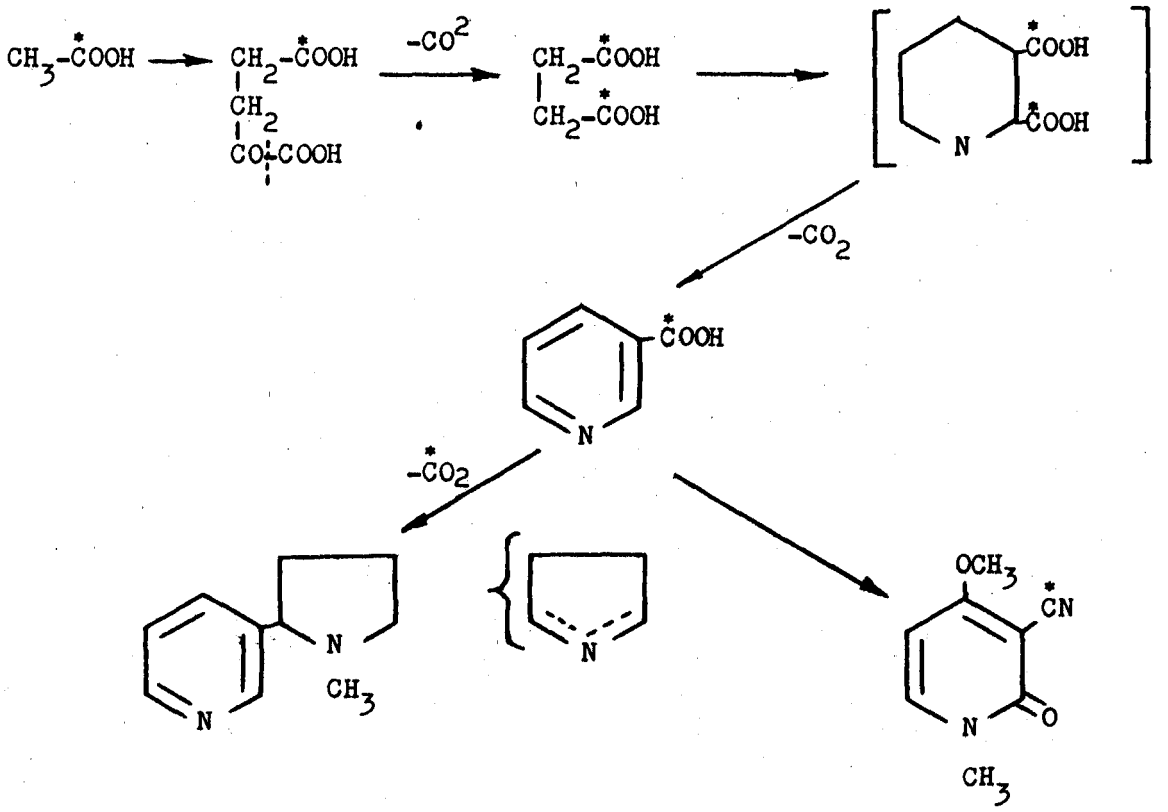


Figure 6

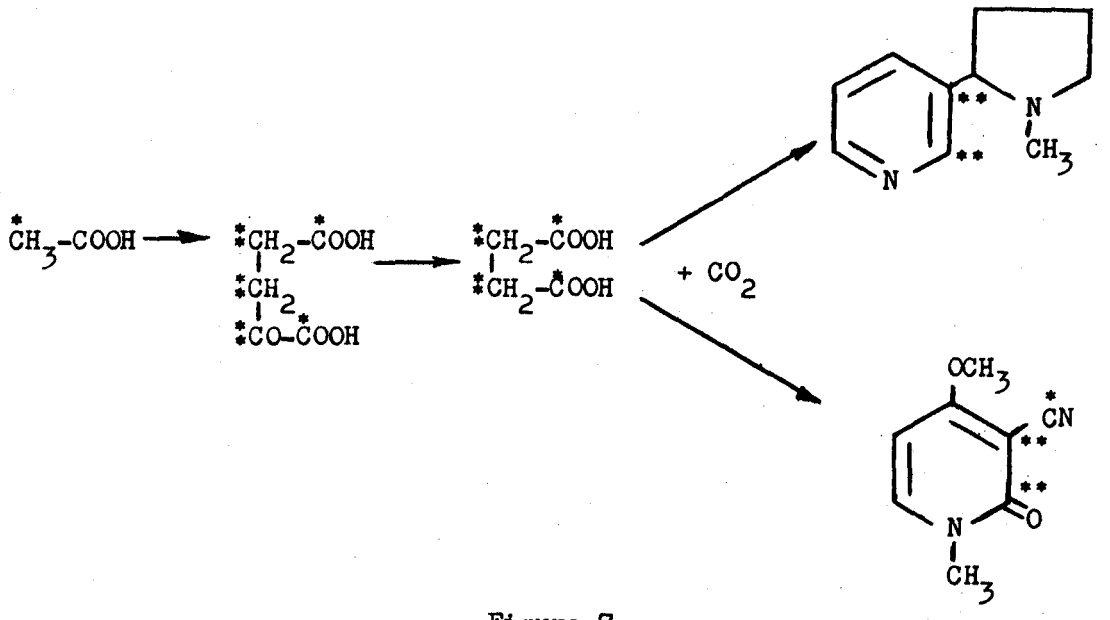


Figure 7

would be expected in the methyl groups, some in the cyano group and the bulk of the activity at C-2 and C-3 of the α-pyridone ring. As pointed out this is entirely in agreement with available experimental results.

B. SUCCINATE AS PRECURSOR

Juby and Marion (1961) reported that the incorporation of succinate-2,3-C^{14} into ricinine by castor plants gave an activity distribution identical with that obtained from the incorporation of acetate-2-C^{14}. This was demonstrated unequivocally in later work and is fully in accord with the involvement of the tricarboxylic acid cycle.

Waller and Henderson (1961 b) reported that administration of succinate-2,3-C^{14} gave rise to ricinine labelled exclusively in the α-pyridone ring. Succinate-1,4-C^{14} was a less efficient precursor than its 2,3-homologue and when fed to flowering castor plants gave rise to radioactive ricinine, 75% of whose total activity resided in the pyridone ring while 25% was found in the nitrile group. This anomalous situation was clarified (Waller & Henderson, 1961 c) by the observation that in non-flowering plants this precursor gave rise to 85% of the total ricinine activity located in the nitrile group with only 15% of the activity in the ring as would be expected. Furthermore an editor's note accompanies this reference with assurances that the authors have supporting unpublished data which indicate that a different metabolic pathway is available for ricinine synthesis in flowering plants.

Griffith and Byerrum (1963) reported that in nicotine

the pyridine ring activity arising from succinate-2,3-C^{14} was almost identical with that arising from acetate-2-C^{14}. In both cases between 70% and 80% of the pyridine ring activity was distributed virtually equally between carbons 2 and 3.

Christman and Dawson (1963) showed that succinate-2-C^{14} and fumarate-2-C^{14} were incorporated into nicotine in almost identical amounts with roughly equal isotopic distribution between the pyridine and pyrrolidine rings. Succinate-1-C^{14} was incorporated only one-tenth as well with only about 14% of the total nicotine activity located in the pyridine ring and the remainder in the pyrrolidine ring.

C. ASPARTATE AS PRECURSOR

Griffith, Hellman and Byerrum (1962; c.f., Chem. & Eng. News, 1961) demonstrated aspartic acid-3-C^{14} to be a precursor of nicotine (giving a radiochemical yield of 0.28%) in tobacco plants. Degradations showed the activity to be distributed approximately equally between the pyridine and pyrrolidine rings with the actual amounts being somewhat dependent on the time lapse between precursor administration and nicotine isolation. About one half of the pyridine ring activity was located at carbon-3. The pyrrolidine ring labelling can be explained if aspartate is converted to oxaloacetate which participates in the tricarboxylic acid cycle to give rise to α-ketoglutarate. α-Ketoglutarate could then go into the pyrrolidine ring by a pathway in which glutamic acid is an intermediate. In a two hour experiment the 2' and 5' positions were labelled almost exclusively with little or no randomization. Interpretation of the activity distribution in the pyridine ring indicated that after two hours, aspartic acid-3-C^{14} could have converted either to

succinate or to fumarate before incorporation.

Christman and Dawson (1963) reported that DL-aspartic acid-3-C^{14} was incorporated into nicotine more efficiently than L-aspartic acid-2,3-C^{14}. In these two cases and in the case of aspartic acid-N^{15}, the isotope was found to be distributed almost equally between the pyridine and the pyrrolidine rings. DL-Aspartic acid-4-C^{14} showed virtually no incorporation into nicotine.

L-Aspartic acid-2,3-C^{14}, succinate-2-C^{14}, and fumarate-2-C^{14} gave almost identical percentages of incorporation into nicotine with very similar distribution of activity between the two rings (Christman & Dawson, 1963). Aspartate nitrogen was incorporated into nicotine (Christman & Dawson, 1963) while aspartate nitrogen and an accompanying labelled carbon atom have been shown to incorporate as a unit into nicotinic acid (Gross *et al*, 1963). In the light of this evidence, the suggestion of Griffith *et al* (1962), that aspartate converts to either succinate or fumarate before it is incorporated into the pyridine ring of nicotine, does not seem as likely as the reverse situation. Marion's group (Essery *et al*, 1963) are presently studying the origin of the nitrogen atom in ricinine; this may help clarify the issue.

Juby and Marion (1962) have suggested that on the basis of all the available evidence to that date there is every indication that carbons 2 and 3 of the ricinine ring originate from the same source as carbons 2 and 3 of the pyridine ring of nicotine. Schiedt and Boeckh-Behrens (1962) reported the incorporation of aspartate-3-C^{14} into ricinine with 40% of the total ricinine activity found at carbon 3 while no activity was found at carbon 2. There appears to be no logical explanation for the

complete absence of activity at carbon 2 except perhaps there may have been an error in the analysis because the total analysis only accounts for 83% of the total activity.

D. PYRUVATE AS PRECURSOR

The incorporation of labelled pyruvate into nicotine has been observed to give radiochemical yields in the following order: pyruvate-3-C^{14} > pyruvate-2-C^{14} > pyruvate-1-C^{14} (Griffith & Byerrum, 1959 a). For the most part it appeared that pyruvate was metabolized to acetate before it was incorporated into nicotine but this did not appear to be an exclusive route for pyruvate utilization. Pyruvate-3-C^{14} showed a relatively higher incorporation than the acetates while pyruvate-1-C^{14} was observed to give rise to radioactive nicotine. The latter case could also arise from a reversal of glycolysis where pyruvate is converted to glycerol.

E. GLYCEROL AS PRECURSOR

On feeding glycerol-1,3-C^{14} to tobacco plants, the resulting nicotine showed a much greater percentage of the total activity located in the pyridine ring than when acetate-2-C^{14} was fed (Griffith *et al*, 1960). Glycerate-3-C^{14} was incorporated into nicotine to a greater extent than acetate-2-C^{14} but not as well as glycerol (Griffith & Byerrum, 1962). Christman and Dawson (1963) reported that in the incorporation of glycerol-1-C^{14}, 50% of the total nicotine activity was located in the pyridine ring as compared with 43% for the incorporation of glycerol-2-C^{14}. Griffith *et al* (1962) found that glycerol-2-C^{14} gave rise to 90% of the total nicotine activity being localized in the pyridine ring when the time lapse between precursor administration and nicotine harvest

was 2 hours but underwent diminution to 57% when the time interval was 7 days. In the two hour experiment, the low activity in the pyrrolidine ring had the same isotopic distribution as that obtained from acetate-1-C^{14}. This suggests that the small amount of glycerol entering the pyrrolidine ring goes by way of the tricarboxylic acid cycle and was almost completely metabolized to acetate. The distribution of the pyrrolidine ring activity after 7 days was rationalized on the assumption that glycerol was metabolized to α-ketoglutarate which could arise from glycerol either through glycolysis and acetate to the tricarboxylic acid cycle or through a carboxylation of pyruvate or phosphoenolpyruvate to the tricarboxylic acid cycle (Mazelis & Vennesland, 1957). This explanation was modified slightly in a later report in suggesting that glycerol entered glycolysis by phosphorylation and oxidation to dihydroxyacetone phosphate. In long term experiments it was considered that in addition to this, part of the glycerol was converted to acetate and part to oxaloacetate (as mentioned before) through a carboxylation of phosphoenolpyruvate or pyruvic acid before being utilized in pyrrolidine ring synthesis (Wu, Griffith & Byerrum, 1962). Glycerol-1,3-C^{14} and glycerol-2-C^{14} were incorporated into nicotine to about the same extent while glycerate-3-C^{14} went in lesser amounts (Griffith et al, 1962; Griffith & Byerrum, 1962). This constitutes strong evidence in favour of glycerol or a closely related metabolite being a major source of carbon for at least part of the pyridine ring and that glycerol participates as a 3-carbon unit, probably giving rise to carbons 4, 5 and 6 of the pyridine ring. In direct contrast, Dawson and Christman (Chem. & Eng. News, 1961) reported that glycerol-1,3-C^{14} was incorporated into nicotine only half as well as glycerol-2-C^{14} in experiments with

sterile root cultures from Nicotiana plants. They interpreted these data to mean that only two carbons from glycerol were utilized in the biosynthesis of the pyridine ring, presumably carbons 5 and 6. Their most recent report (Christman & Dawson, 1963) still favours this idea.

Waller and Henderson (1961 b) found glycerol-1,3-C^{14} more efficient than glycerol-2-C^{14} when used as a precursor of ricinine in castor plants. This could be due to the conversion of glycerol to acetate through glycolysis in which glycerol-1,3-C^{14} and glycerol-2-C^{14} give rise to acetate-2-C^{14} and acetate-1-C^{14} respectively.

Juby and Marion (1961) noted that glycerol-1-C^{14} was a better precursor of ricinine than glycerol-2-C^{14} with activity in the methyl groups and nitrile moiety accounting for about 45% of the total ricinine activity in both cases. Further work (Essery et al, 1963) demonstrated that glycerol-1-C^{14} was incorporated into ricinine to give high centres of activity at ring carbons 4 and 6 with low activity at carbon 5. Glycerol-2-C^{14} gave a very high percentage of the total ricinine activity at carbon 5 and very low levels at carbon 4 and 6. From the combined results it is readily seen that the combined activities of carbons 2 and 3 hardly amount to 14% of the total ricinine activity in either instance. The randomization of activity especially in the methyl and nitrile groups indicated considerable breakdown of the glycerol molecule into simpler units before incorporation into ricinine. The striking distribution of activity in the α-pyridone ring, however, leaves little doubt as to why these researchers proposed that carbons 4, 5 and 6 were derived directly from the three carbon unit of glycerol or a closely related metabolite. There was a slight inequality of labelling between the C-4 and C-6 positions

resulting from glycerol-1-C^{14}. This suggested that the precursor could be unsymmetrically derived not only from glycerol but also from some of its fragmentary metabolites. The authors indicated that work is in progress which utilizes shorter duration experiments in order to minimize extensive glycerol breakdown and thus eliminate widespread randomization of isotopic distribution. Such an observation would corroborate the already convincing accumulation of evidence.

F. GLUTAMATE AS PRECURSOR

Glutamate-2-C^{14} metabolized by castor plants gave rise to radioactive ricinine of which 90% of the total activity was found in the cyano moiety (Anwar et al, 1961). Schiedt and Boeckh-Behrens (1962) reported only 55% of the total ricinine activity was located in the cyano group, but the essential point of agreement was maintained. This can be rationalized on the assumption that glutamate-2-C^{14} underwent transamination to α-ketoglutarate-2-C^{14} which in turn gave rise to carboxyl labelled succinate (c.f., Figure 6).

G. PROPIONATE AS PRECURSOR

The incorporation of the labelled propionic acids into nicotine was observed to give radiochemical yields of the following order:

propionate-2-C^{14} > propionate-3-C^{14} >> propionate-1-C^{14}

(Griffith et al, 1960; Wu et al, 1962; Griffith et al, 1962). The distribution of activity for propionate-2-C^{14} was the same as for acetate-2-C^{14} while the distribution of activity for propionate-3-C^{14} was identical with that of acetate-1-C^{14}. Since propionate-3-C^{14} was not utilized directly for pyridine ring synthesis in nicotine (Griffith et al, 1962), this eliminated the earlier idea (Griffith et al, 1960) that it could

have been utilized as a 3-carbon unit (i.e., β-alanine or a similar intermediate). The authors observed that propionate could be converted to acetate by a β-oxidative pathway which could also be applicable in the case of ricinine.

Anwar et al (1961) administered propionate-3-C^{14} and propionate-1-C^{14} to castor plants. The active ricinine isolated in both cases showed 90% of the total activity to be located in the nitrile group. A possible explanation involves the β-oxidation of propionate to malonate followed by decarboxylation to acetate.

$$\overset{3\ 2\ 1}{CH_3CH_2COOH} \longrightarrow \overset{3\ 2\ 1}{HOOCCH_2COOH} \longrightarrow \overset{2\ 1,3}{CH_3COOH}$$

Waller and Henderson (1961 b) observed that the three labelled propionates were incorporated into ricinine by non-flowering castor plants in the same relative order as these compounds were incorporated into nicotine. In flowering plants all three labelled propionates are incorporated approximately equally and in very low percentages. These authors suggested that propionate gave rise to succinic acid via the β-oxidative pathway reported by Giovanelli and Stumpf (1957) (Figure 8). By this pathway activity from

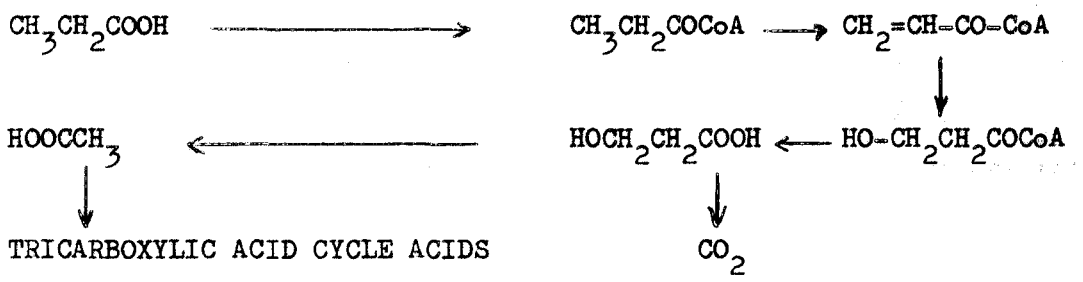


FIGURE 8

propionate-1-C^{14} would not enter the tricarboxylic acid cycle while propionate-2-C^{14} and propionate-3-C^{14} would give rise to acetate-2-C^{14}

and acetate-1-C^{14} respectively. The conversion of propionate to succinate via methyl malonic acid (Flavin, Ortiz & Ochoa, 1955; Flavin & Ochoa, 1957) was not considered to be involved in the light of the evidence favouring the initial conversion to acetate.

H. β-ALANINE AS PRECURSOR

The greater incorporation of β-alanine-2-C^{14} than of β-alanine-1-C^{14} into ricinine was shown by Waller and Henderson (1961 b). In nicotine about 50% of the total activity resulting from β-alanine-2-C^{14} feedings was found in carbon 3 of the pyridine ring (Griffith & Byerrum, Chem. & Eng. News, 1961) while an additional 25% was found at carbon 2 (Dawson & Christman, Chem. & Eng. News, 1961). This could be interpreted as arising from the conversion of β-alanine to either propionate or malonate before it was incorporated into the pyridine ring. By way of malonate, β-alanine-2-C^{14} would give rise to acetate-2-C^{14} which is incorporated more readily than acetate-1-C^{14} which would arise from either β-alanine-1-C^{14} or β-alanine-3-C^{14}.

Dawson and Christman (ibid.) suggested that β-alanine was incorporated into nicotine by way of citrate. A recent report (Christman & Dawson, 1963) showed that the incorporation of β-alanine into nicotine occurs in the order: β-alanine-2-C^{14} > β-alanine-3-C^{14} >> β-alanine-1-C^{14}. The incorporation of β-alanine-2-C^{14} into the pyridine ring amounts to 30-40% of the total nicotine activity while it is less than 1% for β-alanine-3-C^{14}. This is indeed similar to the observed behaviour of the propionates.

I. OTHER COMPOUNDS AS PRECURSORS

Dawson and Christman (Chem. & Eng. News, 1961; Christman & Dawson, 1963) considered citrate a precursor of carbons 2, 3 and 4 of the pyridine ring on the grounds that carbons 2 and 3 of succinate, fumarate, aspartate, and carbon 2 of acetate and malonate were incorporated into the pyridine ring much more readily than their carboxyl groups. From the data on β-alanine-2-C^{14} incorporation, carbon 3 in the pyridine ring accounted for one half of this ring's activity while carbon 2 accounted for one quarter. If the citric acid hypothesis is correct, the remainder of the activity should be found in carbon 4.

D-Ribose-1-C^{14}, D-glucose-1-C^{14}, sodium formate-C^{14} and glycine-2-C^{14} have been fed to Ricinus communis L. (Waller and Henderson, 1961 a). Citrate-1-C^{14}, glucose-1-C^{14}, glucose-6-C^{14}, glycine-1-C^{14}, mevalonic acid-2-C^{14} and β-hydroxy-β-methylglutaric acid-β-C^{14} were fed to excised root cultures of Nicotiana plants (Christman & Dawson, 1963). In each case the percentage of incorporation into the pyridine ring was very low.

In summary, it has been observed that acetate-2-C^{14} was incorporated into the pyridine ring more readily than acetate-1-C^{14}. In the efficiencies of the incorporations of the pyruvates, propionates, β-alanines and, for that matter, of all other precursors discussed in this section (with reservations in the case of glycerol) there is a relationship readily explained by invoking the pathway of the tricarboxylic acid cycle. Until recently a great number of conflicting observations and interpretations left an open question as to whether the five carbons of the pyridine ring are (1) derived from the three carbons of glycerol and two carbons of a dicarboxylic acid of the tricarboxylic acid cycle or

are (2) derived from two of the carbons of glycerol and three carbons from citrate. Two groups (Griffith et al, 1960; Juby and Marion, 1961) favoured the former approach while two other groups (Waller & Henderson, 1961 b; Dawson & Christman, Chem. & Eng. News, 1961) preferred the latter. As noted earlier in the present review, the most recent publications of several groups showed by partial degradation of nicotine (Griffith & Byerrum, 1963) and by the total degradation of ricinine (Juby & Marion, 1963; Essery et al, 1963) that the pyridine ring does indeed arise by the first of the two pathways mentioned.

From considerations of the 2, 3 and 4 carbon precursors, it would appear that the most consistent results for incorporation of precursors into the pyridine ring can be illustrated as shown (Figure 9).

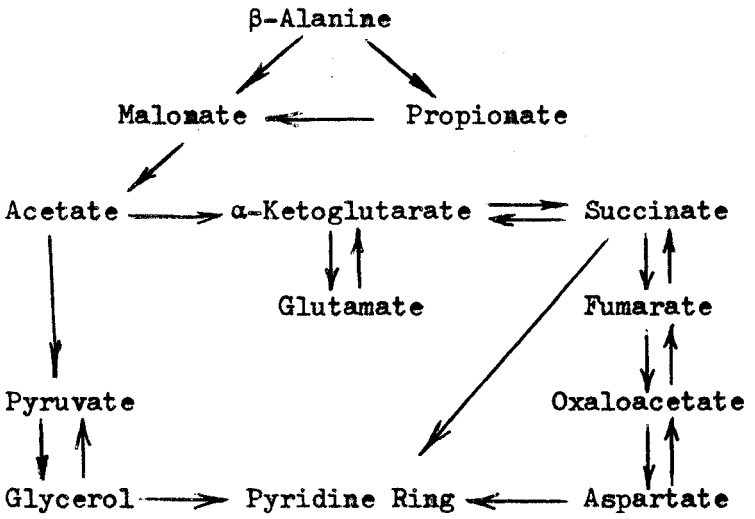


FIGURE 9

The plant alkaloid mimosine (I) is a substituted pyridone structure which bears the salient features of the pyridine nucleus under discussion. If there is a common pathway for the biosynthesis of the pyridine ring and its oxidized modifications in all pyridine type alkaloids as has been suggested (Griffith et al, 1960; Waller & Henderson, 1961 a; Juby

& Marion, 1963), the observations and conclusions presented in this section should not only be applicable to, but be entirely parallelled by, the biosynthesis of the pyridine ring of mimosine.

PYRIDINE DICARBOXYLIC ACIDS

In mammals and Neurospora nicotinic acid is metabolically derived from tryptophan. The nature of some of the intermediates on this biosynthetic route is still under discussion. Henderson (1949) suggested that quinolinic acid (pyridine-2,3-dicarboxylic acid) was a true intermediate between 3-hydroxyanthranilic acid and nicotinic acid. This suggestion was criticized and evidence was presented which appeared to indicate that quinolinic acid was a by-product rather than an obligatory intermediate of nicotinic acid biosynthesis. Mehler (1956) reported that, enzymatically 3-hydroxyanthranilic acid actually gave rise to pyridine-2-carboxylic acid, and that quinolinic acid was formed by non-enzymatic reactions. However, Wilson and Henderson (1960) established that in developing chick embryo quinolinic acid is enzymatically converted into nicotinic acid while control experiments showed that this reaction does not take place non-enzymatically. Experiments with 3-hydroxyanthranilic acid-H^3 and quinolinic acid-H^3 also indicated that quinolinic acid is an intermediate in the conversion of 3-hydroxyanthranilic acid to nicotinic acid.

Compelling evidence favours the theory that in certain microorganisms and in plants, nicotinic acid is built up from 3- and 4- carbon units and that it is an intermediate in the biosynthesis of more complex pyridine derivatives. However, there are other reports which merit some discussion.

Abdel Kader and Fawzy (1960) claimed that the tryptophan pathway for the biosynthesis of nicotinic acid operates in broad bean plants.

Experimental details of this report were not available but apparently no radioisotope experiments were carried out. Wilson and Henderson (1961) stated that Xanthomonas pruni could utilize either 3-hydroxanthranilic acid or quinolinic acid in place of nicotinic acid as a growth factor, and these precursors, when labelled were incorporated into nicotinic acid. This was presented as further evidence that the tryptophan pathway for nicotinic acid was operative in this bacterial species. Lingens (1960) reported that Lactobacillus arabinosus was able to survive in a medium in which cinchomeronic acid (pyridine-3,4-dicarboxylic acid) replaced nicotinic acid as nutrient. This report was not accompanied by experimental details. Since the reactivity of the 2- and 4- position of the pyridine ring are very similar, decarboxylation at the 4-position is not difficult to visualize in the light of quinolinic acid utilization.

A number of interesting points arise from this brief review. Although this has not been demonstrated, there is the possibility that quinolinic acid or a reduced quinolinic acid derivative could be an intermediate in the 3 plus 4 condensation for nicotinic acid biosynthesis. In no instance has the claim been made that 3-hydroxanthranilic acid is the only precursor of quinolinic acid. Another pyridine dicarboxylic acid whose biosynthesis has been studied is 2,6-dipicolinic acid (pyridine-2,6-dicarboxylic acid). This compound is produced in large quantities in certain sporulating bacteria. Although dipicolinic acid has not been implicated in any way in a pathway to nicotinic acid, its biosynthetic origin is of direct relevance to a discussion of the origin of the pyridine ring system.

In an early report Perry and Foster (1955) claimed that 2,6-diaminopimelic acid was incorporated into pyridine-2,6-dicarboxylic acid

in the spores of Bacillus cereus (Mycoides). This was followed by the observations of Gilvarg (1956) that resting cells of a mutant (26-26) of Escherichia coli exhibited increased excretion of 2,6-diaminopimelic acid when incubated with glucose or pyruvic acid together with a nitrogen source. Aspartic acid was more effective than ammonia for this purpose. A more intensive study of the biosynthesis of pyridine-2,6-dicarboxylic acid in Bacillus megaterium (Martin & Foster, 1958) established a number of compounds as precursors for this system but did not clarify the role of 2,6-diaminopimelic acid. One definite finding of this study was that the tryptophan-3-hydroxyanthranilic acid pathway for nicotinic acid operative in Neurospora and in mammals did not constitute a route to dipicolinic acid. The most efficient precursors of this product were found to be glutamic acid, aspartic acid, alanine, serine and proline. The known metabolic relationships of several efficient precursors (glutamate, aspartate and oxaloacetate) led to the conclusion that intermediates of the tricarboxylic acid cycle participated in the biosynthesis of the product. The direct participation of aspartic acid (including nitrogen) as a unit was suggested as an attractive possibility. Further relationships also indicated that the combination of 3- and 4- carbon units to form the pyridine ring was a likely occurrence. Two alternative routes to the product, based on the established incorporation pattern were suggested, the first from aspartate and pyruvate, the second from alanine and oxaloacetate. Although glycerol was not tested, one of its known metabolites, serine, was observed to be incorporated efficiently into pyridine-2,6-dicarboxylic acid.

These precursors utilized for the biosynthesis of nicotinic acid and of picolinic acid are thus very similar. This high degree of coincidence

may be indicative of an extremely close similarity in the biosynthesis of the pyridine dicarboxylic acids and of nicotinic acid. Indeed, it would not be surprising if, on clarification of these similarities, it were found that the pyridine ring arises from a common origin and that there is some direct relationship or mode of interconversion amongst the pyridine dicarboxylic acids and nicotinic acid.

UNIQUE NICOTINIC ACID-ALKALOID RELATIONSHIPS

Throughout investigations of alkaloid biosynthesis in intact plants there arises the problem of the variability of the plant material. The constancy of alkaloid content and rates of formation within individual plants in a given experiment and the variation from experiment to experiment has never been investigated. A further uncertainty concerns the reversibility of the late stages of the biosynthetic pathway immediately preceding the final elaboration of the structure of the alkaloids. It is also unknown whether artefacts arise from the addition of foreign materials to the plant or from the increase beyond normal amounts of materials which are already present.

A number of investigations have attempted to throw some light on these questions, but no conclusive information is available. From nitrogen balance studies, Dawson (1940) and Mothes (1928) arrived at the conclusion that nicotine does not serve as a nitrogen source for protein synthesis in the tobacco plant. Dawson did, however, report an incomplete recovery of nicotine when this compound was administered to tobacco plants. He suggested that the alkaloid was metabolized in the leaves in some unknown manner. Tso and Jeffrey (1956) observed the disappearance of nicotine from *N. glutinosa*, presumably due to formation of nornicotine. Leete and

Bell (1959) observed extensive metabolic breakdown of nicotine-2,5-C^{14} (only 6% recovered) and of nicotine-methyl-C^{14} (only 1% recovered) in N. tabacum. Radioactive choline was isolated from the nicotine-methyl-C^{14} feeding and degradation indicated that 90% of the activity appeared in the methyl groups of choline. They concluded that nicotine thus acts as a methyl donor in tobacco plants. Leete and Bell further observed that there was high initial loss of activity from both labelled nicotines with little subsequent decrease in the activity after 1 week. This is compatible with the hypothesis that metabolism of the radioactive nicotine occurs in the roots, which are the main site of nicotine synthesis in N. tabacum, and little further metabolism occurs after the nicotine has been translocated to the leaves.

Ring labelled nicotine-C^{14} injected into growing tobacco plants gave rise to labelled nicotinic acid (Griffith et al, 1960). Solt, Dawson and Christman (1960) pointed out that in N. glauca nicotinic acid was incorporated into nicotine and anabasine through a pathway which in part was common to both alkaloids. When ring labelled nicotine-C^{14} was metabolized by these plants, the recovered nicotine showed 60% of the original pyridine ring labelling but only 43% of the original pyrrolidine ring labelling. Some radioactivity was found in nornicotine, but, surprisingly, none in anabasine. In a recent review, Johnson (1963) expressed doubt that nicotine metabolism was functional.

As early as 1933, Weevers reported the disappearance of ricinine (presumably through metabolism) in castor plants during growth on nitrogen depleted soil. Waller and Nakazawa (1963) have stated that they have unpublished evidence showing that tritium labelled ricinine administered

to castor plants gives rise to tritium labelled nicotinic acid. They reported that ricinine was metabolized in sterile excised cotyledons of castor plants cultured in the dark and a sparing action on ricinine was observed when nicotinic acid was added. On these foundations they suggested that there exists a metabolic vitamin-alkaloid relationship not previously found in a plant system.

Although definite conclusions would be premature, this summary of observations illustrates that the study of the biosynthesis of two particular alkaloids has brought to light a number of far-reaching effects. It is probable that besides establishing the origins of the pyridine alkaloids in plants, which at the present stage of development of the field is the main object of all investigations, new facts may emerge from biosynthetic studies which could throw light on the functions of these alkaloids in the plant and show their relationship with other plant material.

MIMOSINE BIOSYNTHESIS

A. CHOICE OF PRECURSORS

The purpose of this study was to establish whether or not an anabolic similarity exists between mimosine in Mimosa pudica L. and other pyridine alkaloids occurring in higher plants.

If nicotinic acid were a precursor of the pyridone ring of mimosine, it would have to suffer decarboxylation on route to the product. Investigation of the possible incorporation of the nicotinic acid nucleus would have been of considerable value in this study. Ring labelled nicotinic acid-C^{14} is not available, however, and incorporation of precursors of nicotinic acid was therefore investigated.

Aspartic acid and glycerol were selected, both of which are known

precursors of nicotinic acid, the common intermediate on the route to nicotine, anabasine and ricinine (Figure 4). Activity from any of the radiomers of glycerol would be expected in momisine if this were formed *via* nicotinic acid and the latter were formed by the 3 plus 4 pathway. Glycerol-1,3-C^{14} was the chosen compound. To test the possibility of non-symmetrical or partial incorporation of a fragment related to glycerol, glyceric acid-3-C^{14} was also selected. Aspartic acid was chosen as a representative of a 4-carbon dicarboxylic acid precursor. Of the three radiomers tested, only aspartic acid-3-C^{14} would be expected to supply activity to the mimosine nucleus if this were to arise *via* nicotinic acid. Activity from aspartic acid-1-C^{14} and -4-C^{14} would not be expected to enter the nicotinic acid nucleus by the 3 plus 4 route (Figure 4), unless *de novo* synthesis of precursor from carbon dioxide were rapid. It was hoped that the results of incorporation of the three radiomers of aspartic acid would be complementary and lead to an unequivocal conclusion.

A biogenetic scheme for mimosine has been put forward, based entirely on the formal structural similarity of the alkaloid with ribose and with α,β-diaminopropionic acid, an amino acid found in certain *Mimosa* species. According to this scheme the pyridone ring of mimosine is postulated to arise in a manner bearing no similarity whatsoever to the origin of the pyridine ring in those alkaloids whose biosynthesis has been reviewed in this thesis. This biogenetic hypothesis first put forward by Gmelin (1959) and adopted by Leete (1960) postulates the elaboration of the intact carbon chain of ribose into mimosine (Figure 10). Ribose-1-C^{14}, radioactivity from which would, according to this scheme, enter the mimosine nucleus, was also chosen for investigation.

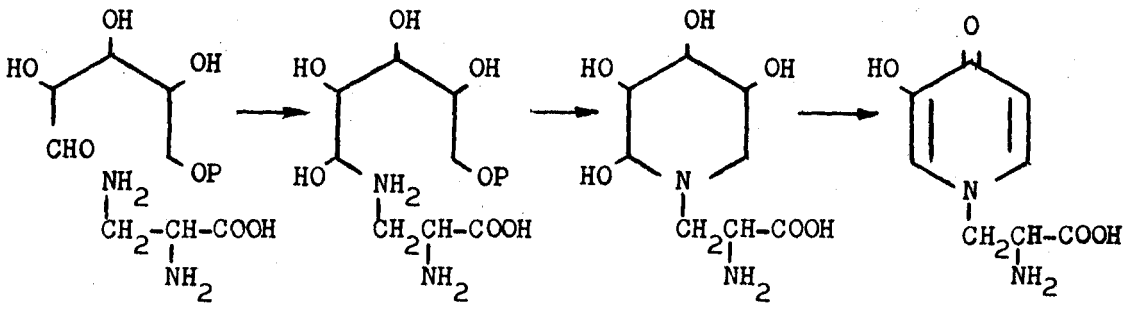
B. CHOICE OF FEEDING TIME

No mimosine appears to be present in the seeds of M. pudica nor in the cotyledons after germination. As soon as the first leaf (consisting of leaflets) is formed, sap obtained by cutting the stems is observed to contain small quantities of a phenolic amino acid whose R_f value corresponds to that of mimosine. The amino acid appears to be localized in the sap throughout the development of the plant. Plants from 7 to 11 weeks old yield the substance in quantitites sufficient for isolation. Older plants become woody and yield only small quantities of sap.

To determine whether mimosine biosynthesis was taking place, plants 7-11 weeks old were allowed to grow in an atmosphere of carbon dioxide-C^{14} in several experiments and in each case the isolated mimosine showed a consistently significant incorporation of carbon-14. Mimosine biosynthesis was thus taking place in M. pudica plants at a stage of development 7 to 11 weeks from the germination of the seeds. Such plants were used for all further experiments.

C. MODE OF ADMINISTRATION

The carbon dioxide-C^{14} control experiments were carried out in a glass chamber large enough to hold a 5-inch pot containing 6 Mimosa plants. The chamber (Figure 11) consisted of a glass cylinder with an inside diameter of 14 cm. and a height of 65 cm. A flange on the open end was ground smooth to form a seal when the cylinder was placed on a plate glass footing. The cylinder was fitted with an external duct containing a thin metal strip which could be vibrated by means of an electromagnet operating on an A.C. line. This served as a circulatory system. A shunt for introducing carbon dioxide-C^{14} and an outlet system to withdraw a sample of the atmosphere



Carbinolamine Mimosine

Figure 10

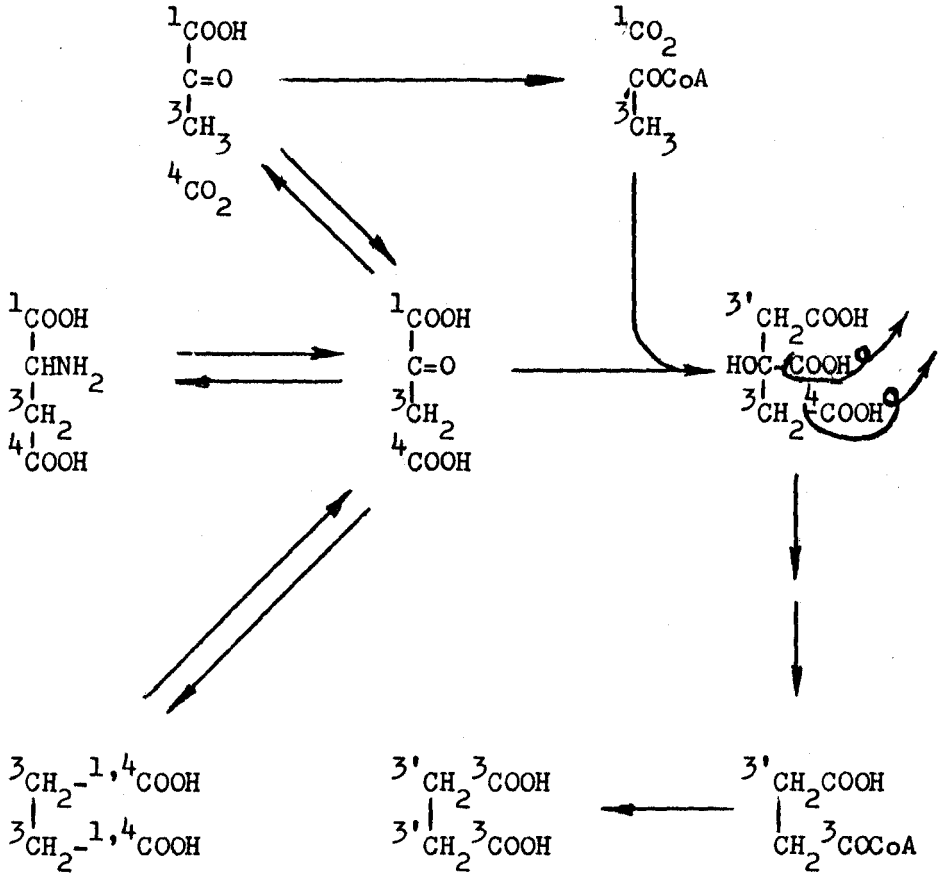
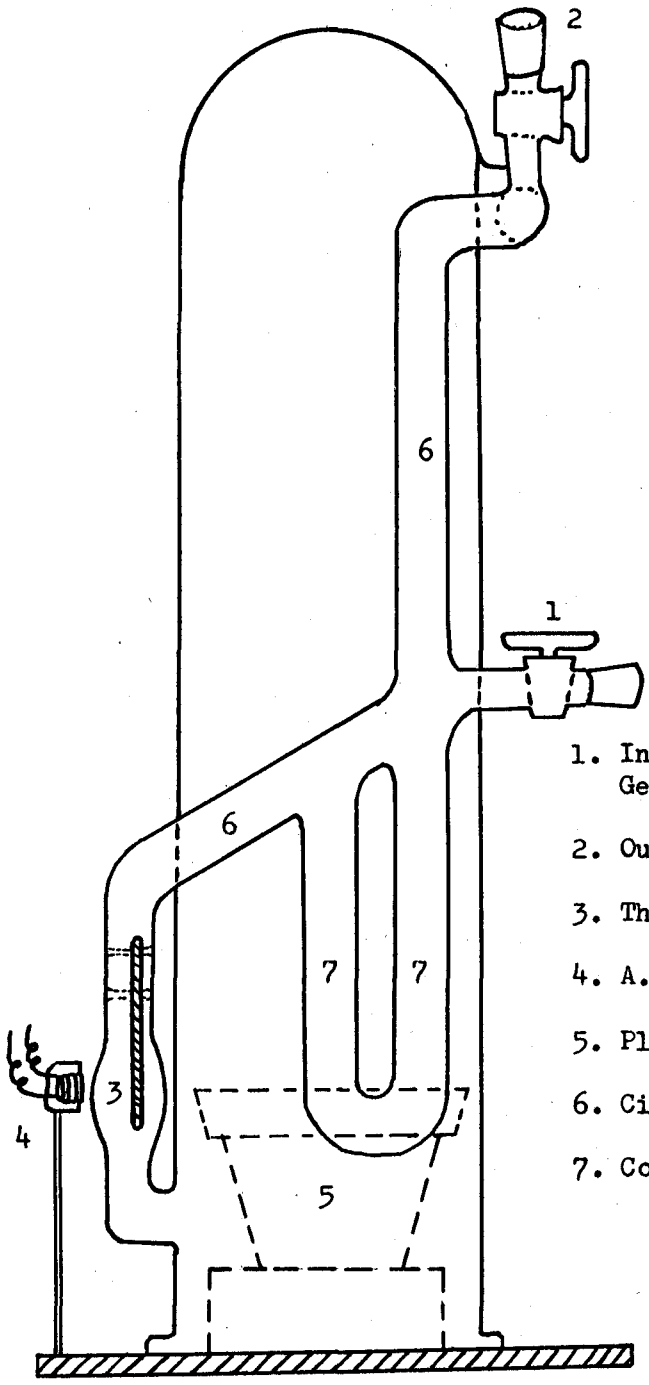


Figure 14



1. Inlet from Carbon Dioxide-C^{14} Generating Vessel
2. Outlet for Gas Sampling
3. Thin Steel Strip (Vibrator)
4. A.C. Electromagnet
5. Plant Pot
6. Circulatory Duct
7. Condenser Shunt

GROWTH CHAMBER FOR CARBON DIOXIDE-C^{14} EXPERIMENTS

Figure 11

FIGURE 12

within the chamber for carbon dioxide-C^{14} assay were also attached.

All other radioactive precursors were administered to the Mimosa plants by the "wick" method (Comar, 1955) (Figure 12). The precursor in aqueous solution was measured into small receptacles which were anchored in the soil near each plant. An ordinary sewing needle was threaded with a double strand of unmercerized cotton and passed through the stem of the plant. The ends of the thread were immersed in the receptacle and the solution of radioactive material was thus able to enter the plant by capillary action.

The time interval between initial administration of the labelled compound and the time of harvest was 2 days in the case of all feeding experiments but two. Three days were allowed for the uptake of ribose-1-C^{14} which for some unexplained reason was inordinately slow. One of the two aspartic acid-3-C^{14} feeding experiments was carried out over a 2-week interval. In each case, after all of the radioactive solution was taken up, the receptacles were repeatedly refilled with distilled water to "wash" residual activity into the plant. During the time interval of each feeding, the receptacles were never allowed to become totally dry. After harvesting the wicks were extracted to determine residual radioactivity which had not been taken up by the plants.

D. ISOLATION OF ALKALOID

Mimosine was isolated from the exudate obtained by cutting the stems of Mimosa plants. The recovery of mimosine was carried out as previously described (Section I: Isolation of Mimosine; sub-section (i)). From 7 to 15 milligrams of mimosine was obtained from each experiment. For final purification the crude radioactive mimosine was diluted about

fivefold with a known weight of inactive mimosine and recrystallized from water. In each case, the radiopurity of the original sample was checked by thin layer chromatography and its specific activity was calculated from the activity of the diluted sample.

E. DEGRADATION OF ALKALOID

The diluted mimosine obtained from each feeding experiment was distilled with zinc dust under vacuum by the optimum procedure outlined previously (Section I: Reactions of Mimosine; sub-section A). 3-Hydroxy-4-pyridone obtained from the distillate was recrystallized and its specific activity was determined.

RESULTS AND DISCUSSION

In all, seven feeding experiments were conducted. In each of these the same number of plants was used under conditions which were reproduced as closely as possible, particularly with respect to the size of the plants (7-11 weeks old) and the duration of feeding (2 days). Feeding time was different (3 days) in the ribose-1-C^{14} experiment, as previously mentioned (c.f. Mode of Administration), and in one of the two aspartic acid-3-C^{14} experiments (2 weeks) for reasons which will be fully discussed in their proper turn. Labelled mimosine was isolated from each of the experiments. Table 2 records the yield of the product obtained from each of the experiments. To conserve material, the values for the specific activity of the original mimosine samples were not measured directly, but were calculated from the values obtained after the original samples were diluted with carrier and recrystallized. The calculated values given in Table 2 for the specific activity of the original mimosine are therefore only approximate since they are based on the assumption those samples were chemically pure. Due

TABLE 2

PRECURSOR		PRODUCT	
	TOTAL ACTIVITY ADMINISTERED μc	YIELD OF MIMOSINE MGMS.	SPECIFIC ACTIVITY x 10^{-3} cpm $mmole^{-1}$ FOR CRUDE MIMOSINE
Aspartic Acid-3-C^{14} (2 Day)	100	12.9	129.3 ± 2.5
Ribose-1-C^{14}	50	5.6	62.3 ± 1.5
Aspartic Acid-1-C^{14}	100	9.7	37.8 ± 0.7
Glycerol-1,3-C^{14}	100	6.5	34.3 ± 0.8
Aspartic Acid-3-C^{14} (14 Day)	100	7.4	25.1 ± 0.4
Calcium Glycerate-3-C^{14}	100	14.0	19.6 ± 0.4
Aspartic Acid-4-C^{14}	100	11.0	12.5 ± 0.1

TABLE 3

PRECURSOR	SPECIFIC ACTIVITY x 10^{-3}	COUNTS $MINUTE^{-1}$ $MMOLE^{-1}$	%
	PURIFIED MIMOSINE	PURIFIED 3-HYDROXY-4-PYRIDONE	RING ACTIVITY / TOTAL ACTIVITY
Aspartic Acid-3-C^{14} (2 Day)	23.50 ± 0.46	21.25 ± 0.39	90.4 ± 2.4
Aspartic Acid-3-C^{14} (14 Day)	2.786 ± 0.058	2.434 ± 0.041	87.4 ± 2.6
Glycerol-1,3-C^{14}	3.176 ± 0.074	2.097 ± 0.062	66.0 ± 1.7
Aspartic Acid-1-C^{14}	5.147 ± 0.108	3.382 ± 0.103	65.7 ± 2.4
Aspartic Acid-4-C^{14}	1.880 ± 0.017	1.074 ± 0.045	57.1 ± 2.5
Ribose-1-C^{14}	4.605 ± 0.113	2.519 ± 0.059	54.7 ± 1.9
Calcium Glycerate-3-C^{14}	3.821 ± 0.086	1.787 ± 0.091	46.7 ± 2.6
Random 5/8			62.5

to the variability of intact biological material, comparison of the specific activities of samples obtained from parallel biosynthetic experiments with different precursors is never more than a guide to the order of magnitude of incorporation. For the same reason radiochemical yields are only rough guides to the relative efficiency of incorporation of different precursors. In the present instance the yield of sap from which mimosine was isolated is dependent on a number of variables, some of which are not fully understood. Although the yield of mimosine from the sap has been observed to be quite constant, there is no assurance that the amount of sap actually collected from individual plants represents a constant fraction of the amount available for collection. The values for the specific activity of the crude mimosine may give a semi-quantitative indication of precursor efficiency. These values have no observeable relationship with the amount of mimosine recovered. Two of the values cannot be used even for such a qualitative comparison, since in one case the time, in the other the amount of radioactivity, differed from that used in all other experiments. The lower specific activity of mimosine from the 2-week experiment with aspartic acid-3-C^{14} could have been due to long term dilution after a short period of efficient incorporation. The experimental plants were young, healthy and rapidly growing during the feeding. After the labelled precursor had been metabolized, later synthesis of inactive mimosine would lead to the dilution of the active mimosine previously formed, and hence to the lower specific activity. The ribose-1-C^{14} feeding was carried out with 50 μc of precursor and although it might be tempting to double the observed value of the specific activity of mimosine derived from this experiment for comparison with the other values, this would not necessarily be valid.

Although the uptake of the total ribose-1-C^{14} dose was slow, the plants were growing for a period of 3 days in contact with the radioactive precursor. This variation in time makes comparison of the ribose result with the other results uncertain.

The order in which the precursors are listed in Table 2 may, with reservation, be considered as a qualitative indication of precursor efficiencies. There is evidence that several separate pools of the same amino acids exist in at least one microorganism (Smith et al, 1961). Differences in pool sizes and ease of entry into any given pool are variables which govern the extent of radioactive labelling. Unless pathways, pool sizes and numbers are known, radiochemical yield cannot serve as a direct measure for comparison of precursor efficiencies.

In a partial degradation, the pyridone ring of each mimosine sample was isolated as 3-hydroxy-4-pyridone and its specific activity was measured and compared with that of the intact molecule. The results are listed in Table 3 in the order of decreasing ring activities.

Since 5 of the 8 carbon atoms of mimosine are located in the pyridone ring, the ring should contain 5/8 or 62.5% of the total radioactivity when incorporation of the label is completely random. If more than 62.5% of the activity from a given precursor is found in the ring, such a precursor would appear to be specificially incorporated into the ring. A precursor yielding less than 62.5% activity in the ring would similarly appear to be preferentially incorporated into the side chain. The higher the percentage activity of the ring is above this value, the more specific is the incorporation into the ring, while values decreasingly lower than 62.5% signify higher side chain specificity.

Table 3 shows that only aspartic acid-3-C^{14} incorporation exhibits a very marked specificity and this is in favour of the ring. This result is even more striking when comparison is made between the 2-day and 14-day feedings and contrasted with the aspartic acid-1-C^{14} and aspartic acid-4-C^{14} feedings. The 2-day aspartate-3-C^{14} feeding illustrates the high specificity (90%) of a short term experiment while the 14-day experiment, which was carried out to test the persistence of label in the product over a longer period, showed that the specificity (87%) decreased little during this long term in which randomization of label through turnover and participation of minor pathways might be expected to take place. The contrasting values for aspartate-1-C^{14} (66%) and aspartate-4-C^{14} (57%) show only small divergence from the 62.5% value for complete randomization.

It can be considered that there is a highly specific ring incorporation of carbon-14 from aspartate-3-C^{14}, showing that aspartic acid or a close relative serves as a direct precursor of the ring, but non-specific incorporation when the label is in the 1- or 4- position of aspartate. Both carboxyl groups of aspartate are evidently lost in the course of the elaboration of the aspartate molecule to mimosine.

These results can be rationalized on the basis of the known metabolic pathways of aspartic acid. These arguments are based mainly on the known metabolic interconversions of the Calvin cycle, the tricarboxylic acid cycle and several other related metabolic pathways. Transamination of aspartate yields oxaloacetate which is an active metabolic intermediate. It can either participate in the tricarboxylic acid cycle or undergo decarboxylation to pyruvate. Pyruvate can either be converted to phosphoglycerate or decarboxylate to acetyl co-enzyme A and carbon dioxide. Acetyl CoA formed

in this way could re-enter the tricarboxylic acid cycle and be eventually metabolized to carbon dioxide. Aspartate-3-C^{14} *via* oxaloacetate-3-C^{14} could give rise to pyruvate-3-C^{14} which in turn would yield phosphoglycerate-3-C^{14} or acetyl CoA-2-C^{14} and inactive carbon dioxide. The oxaloacetate-3-C^{14}, en route through the tricarboxylic acid cycle would give rise to succinate-1,4-C^{14} on the first turn of the cycle which would complete the first turn as oxaloacetate-1,4-C^{14}. Succinate-2,3-C^{14} could also arise through conversion *via* malate through fumarate. On the second turn inactive succinate or succinate-U-C^{14} would be formed. The latter would then lose carbon dioxide-C^{14} on further turns of the cycle. Acetyl CoA-2-C^{14} enters the cycle by condensing with oxaloacetate to give rise to succinate-2,3-C^{14} which, by way of succinate-U-C^{14} eventually cycles out of the system as carbon dioxide.

Aspartate-4-C^{14}, similarly converted to oxaloacetate-4-C^{14} loses its labelling very readily either by decarboxylation to inactive pyruvate or in the first turn of the tricarboxylic acid cycle where inactive succinyl CoA is formed by the decarboxylation of α-ketoglutarate-1-C^{14}. Aspartate-1-C^{14} gives oxaloacetate-1-C^{14} which can give rise to pyruvate-1-C^{14} which is further converted to phosphoglycerate-1-C^{14} or to inactive acetyl CoA and carbon dioxide-C^{14}. In the tricarboxylic acid cycle the oxaloacetate-1-C^{14} label is lost as carbon dioxide-C^{14} on formation of inactive α-ketoglutarate. Succinate-1,4 could arise *via* malate through fumarate but route reversal would lead to carbon dioxide-C^{14} loss in the first complete turn of the tricarboxylic acid cycle.

The specific ring incorporation of aspartate-3-C^{14} and the nearly random incorporation of asparate-1-C^{14} and aspartate-4-C^{14} can be accommodated

by (i) the direct incorporation of aspartate or of a closely related dicarboxylic acid into the ring so that carbons 2 and 3 of aspartate yield carbons 2 and 3 (or 5 and 6) of the ring or (ii) the conversion to acetate followed by participation in the tricarboxylic acid cycle. Complete metabolic conversion to carbon dioxide followed by de novo synthesis of ring precursor is eliminated automatically since aspartate-3-C^{14} exhibited such high ring specificity.

The incorporation of aspartate into mimosine as an acetyl unit is eliminated because aspartate-3-C^{14} and glycerate-3-C^{14} would both be expected to give rise to acetyl CoA-2-C^{14} via pyruvate-3-C^{14}. Table 3 shows glycerate-3-C^{14} to exhibit preferential side chain incorporation. Furthermore, the acetyl CoA-2-C^{14} participation in the tricarboxylic acid cycle would be expected to condense with some of the oxaloacetate-3-C^{14} arising from aspartate-3-C^{14}, and as previously pointed out, randomization via carbon dioxide-C^{14} would accompany formation of intermediates in the cycle. This could possibly lead to a specific labelling pattern superimposed on a random labelling distribution which would be less specific than what has been observed. Aspartate and glycerate are thus incorporated into mimosine in a different manner. This finding would appear to eliminate an acetate pathway for incorporation of aspartate into mimosine.

The results in Table 3 cannot completely eliminate the possibility that a 4-carbon acid intermediate of the tricarboxylic acid cycle is the intermediate for aspartate incorporation. The trend observed in these values offers some indication against this possibility. If aspartate were indeed to participate in the tricarboxylic acid cycle by way of oxaloacetate prior to incorporation into mimosine, the loss of carbon dioxide-C^{14} from both

aspartate-1-C^{14} and aspartate-4-C^{14} would be complete by the time a 4-carbon dicarboxylic acid would be formed. Assuming that the radioactive label is lost from these two precursors before ring synthesis starts, a closer similarity than that observed in the results for aspartate-1-C^{14} (66%) and aspartate-4-C^{14} (57%) would be expected. This argument is reinforced by the close correspondence of the results of the two aspartate-3-C^{14} feedings, which despite a large time difference still gave values within 3% of each other. This indicates that reproducibility of result is to be expected if the same pathway operates for radioactive incorporation. Since the supporting experimental evidence shows that the only major pathways available for incorporation of aspartate-1-C^{14} and aspartate-4-C^{14} into mimosine are <u>via</u> carbon dioxide-C^{14}, a simple rationalization of this can be found in considering aspartate to enter pyridone ring synthesis as an intact unit. If indeed aspartate is incorporated into some structure which gives rise to a nicotinic acid intermediate and progresses to the final mimosine structure, the labelled carboxyl group from aspartate-1-C^{14} would be lost on the route to nicotinic acid synthesis whereas the labelled carboxyl group of aspartate-4-C^{14} is detached in a later step, between nicotinic acid and mimosine. If nicotinic acid is an intermediate, then carbon dioxide-C^{14} would become available for incorporation earlier from aspartate-1-C^{14} than from aspartate-4-C^{14} and lead to random incorporation of labelled carbon dioxide into mimosine from 1-C^{14} more readily than from 4-C^{14}. This is indeed found. If carbon dioxide-C^{14} release occurs after ring formation then <u>de novo</u> synthesis of other fragments at the site of alkaloid synthesis could allow for incorporation of carbon-14 into side chain fragments which in turn become attached to some of the inactive rings.

This in effect would imbalance the randomization effect in favour of the side chain and is compatible with the observed results.

The aspartate-1-C^{14} ring labelling (65.7 ± 2.4%) is very similar to that observed for glycerol-1,3-C^{14} (66.0 ± 1.7%). On the basis of this result alone, randomization through carbon dioxide-C^{14} formation should be considered. Glycerol-1,3-C^{14} can give rise to pyruvate-1,3-C^{14} which in turn would give acetate-2-C^{14} and carbon dioxide-C^{14}. Acetate-2-C^{14} *via* the tricarboxylic acid cycle would give succinate-2,3-C^{14}, oxaloacetate-2,3-C^{14} and eventually carbon dioxide-C^{14}. This would imply preferential ring incorporation superimposed on fairly extensive randomization from carbon dioxide-C^{14} incorporation. This by itself could be reasonable and compatible with the result observed. There is, however, the observation for calcium glycerate-3-C^{14} which by the same reasoning should give an even greater amount of acetate-2-C^{14} and much less carbon dioxide-C^{14} (which could only arise from repeated turns of the tricarboxylic acid cycle). This would imply that calcium glycerate-3-C^{14} would give an even higher specificity for ring labelling but the observed value (46.7 ± 2.6%) favours the side chain to an appreciable extent. The relationship between glycerol and glycerate is very close

Glycerol-P ⇌ Dihydroxyacetone-P ⇌ Glyceraldehyde-P ⇌ Glycerate-P

so that a unitized incorporation of either species with very few intervening intermediates makes an appealing suggestion. Conversion of glycerate-3-C^{14} *via* the carboxylation of pyruvate-3-C^{14} to oxaloacetate-3-C^{14} with further conversion to aspartate-3-C^{14} by transamination and aspartate-U-C^{14} by participation in the tricarboxylic acid cycle before transamination is unlikely since this would result in a high degree of ring labelling. The

consideration of a 5-carbon unit as a direct precursor of the ring is immediately discarded, not only on the basis of the conclusions drawn from the aspartate feedings but also because ribose-1-C^{14} shows a slight preference for side chain incorporation. These considerations are all indicative of the utilization of a 3-carbon unit in the biosynthesis of mimosine.

Participation of ribose-1-C^{14} in the Calvin cycle would give rise to phosphoglycerate-3-C^{14}. An alternative metabolic route for the utilization of ribose-1-C^{14} would be to glyceraldehyde-3-C^{14}-3-phosphate by way of xylulose-5-phosphate and by sedoheptulose-7-phosphate. This product is one step closer to glycerol than is glycerate so that this route could give rise to a slightly greater amount of glycerol-3-C^{14} than would be formed through the exclusive route of glycerate-3-C^{14}. This interepretation is compatible with observed results since the specificity of ribose-1-C^{14} is similar to that of glycerate-3-C^{14} but also falls intermediate between glycerol-1,3-C^{14} and glycerate-3-C^{14}. The possibility that ribose is an immediate precursor of the ring of mimosine as suggested by Gmelin (1959) and Leete (1960) is thus precluded.

Further consideration into the utilization of glycerol and glycerate makes it likely that glycerol could possibly enter the pyridone ring as such or as a closely related metabolite. Glycerate may be incorporated into the ring as such but the available pathway to serine (Smith, Bassham & Kirk, 1961) (Figure 13) through the non-reductive amination of 2-phosphoglycerate makes it an attractive choice for a side chain precursor.

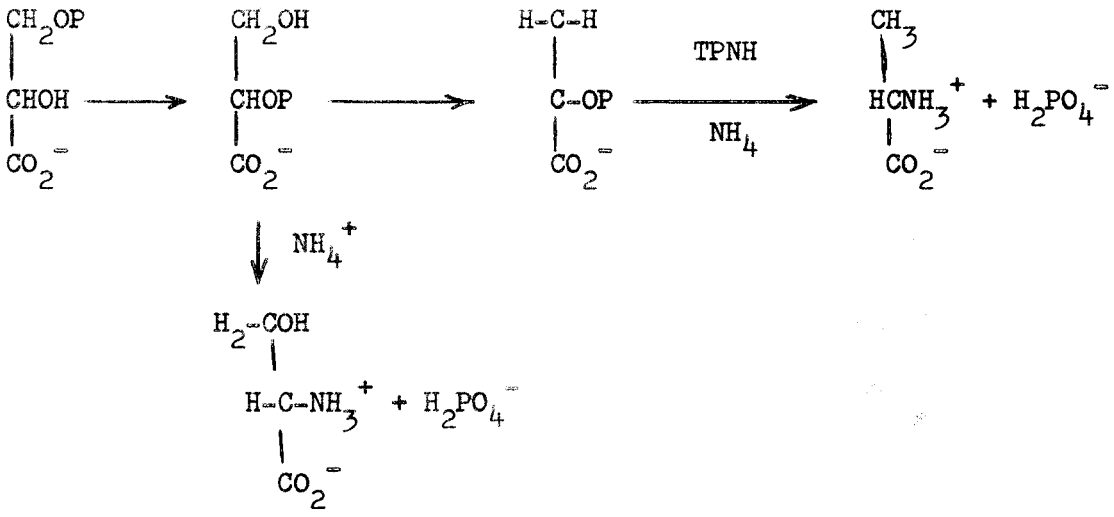


FIGURE 13

It would seem that a reduced metabolite of glycerate would favour ring incorporation while glycerate itself is a closer precursor of the side chain. The obvious test for helping to clarify this issue is the further study of mimosine biosynthesis in which serine-C^{14} would be tested for side chain specificity.

The glyoxylate shunt was reviewed as a possible metabolic pathway since isocitrate could give rise to succinate and glyoxylate, a 2-carbon unit which appears to be metabolized to formate and carbon dioxide. Griffith and Byerrum (1958) found that ribose-1-C^{14} gave rise to glycine-2-C^{14} in certain metabolic experiments which they interpreted as arising <u>via</u> glyoxalate. Glycerol-1,3-C^{14} <u>via</u> ribose-U-C^{14} and glycerate-3-C^{14} <u>via</u> ribose-1,5-C^{14} would give rise to glycine-U-C^{14} and glycine-2-C^{14} respectively while carbon dioxide-C^{14} metabolism would give glycine-U-C^{14}. The utilization of glycine in this study did not appear relevant since no scheme could be formulated in which glycine or glyoxalate could be incorporated without being metabolized to single carbon units first.

The incorporation of carbon dioxide-C^{14} occurs *via* the Calvin cycle in which the initial step is the carboxylation of ribulose-1,5-diphosphate by carbon dioxide-C^{14}. This immediately leads to 3-phosphoglycerate-1-C^{14} which undergoes a variety of reactions in which 3-phosphoglycerate-U-C^{14} results after one turn of the cycle. After this initial entry of carbon-14, random labelling rapidly permeates all of the major pools within the plant which are characterized by high turnover.

In summary, the labelled carbon atom in aspartic acid-3-C^{14} is a highly specific precursor of the pyridone ring of mimosine. Supporting evidence found in the literature makes it likely that incorporation probably occurs as an entire aspartate unit, but there is still a possibility that incorporation is preceded by the conversion of aspartate to a 4-carbon dicarboxylic acid of the tricarboxylic acid cycle. Interpretations placed on the other results favour the incorporation of aspartate-1-C^{14} labelling and aspartate-4-C^{14} labelling to occur via carbon dioxide-C^{14} incorporation. Ribose-1-C^{14} appears to be incorporated *via* glycerate-3-C^{14} which favours side chain incorporation, perhaps as a serine unit. Glycerol-1,3-C^{14} appears to be a better precursor of the pyridone ring than glycerate-3-C^{14} but the specificity of incorporation is very low. The labelling pattern of the major metabolites derived from aspartate, which has been referred to in this discussion, is summarized in Figure 14. If further work were to be carried out, it is suggested that the efficiency of serine-C^{14} as a precursor of the mimosine side chain should be tested.

EXPERIMENTAL

SECTION I: SYNTHESIS OF MIMOSINE

ISOLATION OF MIMOSINE

(i) Isolation from M. pudica (c.f., Renz, 1936)

The exudate from freshly cut green stems and petioles of Mimosa was aspirated into a lambda pipette. A sample (250 µl) was rubbed with ethanol (5 ml), when crude mimosine (12 mg) precipitated, which after treatment with charcoal and recrystallization from water melted at 224-226° (decomp.). A sample of exudate (250 µl) was evaporated to dryness in a vacuum desiccator to give a brown residue (39 mg) which was dissolved in water, decolourized with charcoal and yielded mimosine, melting at 221-223° (decomp.)

A sample of exudate (250 µl) was dissolved in phthalate buffer (pH 4, 0.2 M) (3 ml), and applied to a cation exchange column (Dowex 50W-X4) in the hydrogen form. Water eluted a fluorescent material which was not further investigated. Mimosine was displaced with ammonia (0.02 M). The eluate was concentrated *in vacuo* and dried in a vacuum desiccator over concentrated sulfuric acid, yielding mimosine (9 mg), melting at 225-226° (decomp.).

(ii) Isolation from L. glauca

Freshly crushed ripe seeds were pulverized to a fine powder in a ball mill. The powdered seeds (40 g) were slurried in distilled water (150 ml) then poured into a dialysis bag (length 35 cm, cross section 3 cm) which was then completely immersed in distilled water (1 ℓ per bag; a total of 5 ℓ). After dialyzing 24 hours the dialyzate was concentrated at reduced pressure to approximately 200 ml, when almost pure mimosine crystallized. Mimosine (5.0 g, 2.5%), melting at 227-228° (decomp.) after recrystallization

from water was obtained from 200 g of seeds. Further dialysis of the seeds resulted in only insignificant recoveries of mimosine.

When the seeds were not freshly crushed the yield of mimosine was 0.61 g (1.2%) from 50 g of seeds. This dialysis was carried out in water (3 x 1 ℓ). After 3 changes of external solvent the dialyzate showed only a weakly phenolic reaction.

THE DISSOCIATION CONSTANTS OF MIMOSINE

Recrystallized mimosine (11.17 mg, .000564 moles) was dissolved in distilled water (3 ml) which had previously been vigorously boiled to remove dissolved carbon dioxide. A very fine bubbler was affixed in the titration beaker so that scrubbed nitrogen gas could agitate the solution and also provide a protective layer of gas over the solution. The pH readings were taken with a Beckman Zeromatic pH meter fitted with microelectrodes. The meter reading for the pH of the solution was allowed to come to equilibrium before the titration was started. The pH of a pure mimosine solution (aqueous) was observed to be pH 4.65, presumably the isoelectric point. The solution was first titrated with 0.1001 N sodium hydroxide (0.1 ml per addition) up to a pH 11.55. The basic solution was then titrated with 0.1095 hydrochloric acid (0.1 ml per addition) down to pH 2. The pH meter reading was recorded for each addition of acid or base. From a plot of these results (equivalents of acid and equivalents of base as the abscissa and pH readings as ordinate) after water corrections were applied, the pK values were determined as the pH at half titration of each dissociation.

A second titration was carried out similarly with mimosine (10.47 mg, .00053 moles) dissolved in distilled water (3 ml) to which 10% formaldehyde

solution (0.4 ml) neutralized to pH 7, had been added. This formol titration was carried out by first titrating the solution with 0.1095 M hydrochloric acid to pH 2.9, then with 0.1001 M sodium hydroxide up to pH 11.40.

Two other titrations were carried out; mimosine (10.54 mg, .000533 moles) in a simple titration and mimosine (12.02 mg, .000608 moles) in a formol titration.

Dissociation constants were found: pK_1, 2.1 (-COOH); pK_2, 7.2 (α-NH_3^+); pK_3, 9.2 (phenolic-OH). Only pK_2 was depressed in the presence of formaldehyde, and on this basis it was assigned to the α-amino group.

MIMOSINE SYNTHESIS

PREPARATION OF PYRONE DERIVATIVES

1. 3-Hydroxy-4-pyrone (VIII)

3-Hydroxy-4-pyrone, melting at 116-118°, was prepared (Bickel, 1947) in 66% yield by pyrolysis of anhydrous meconic acid and was purified by sublimation at 110° and 10^{-3} mm pressure.

2. 3-Methoxy-4-pyrone (IX)

3-Methoxy-4-pyrone, melting at 93-95°, was obtained in 84% yield by methylation of 3-hydroxy-4-pyrone with diazomethane in ether solution (Bickel, 1947). It was purified by distillation at 5×10^{-3} mm and 100-110°.

3. 3-Benzyloxy-4-pyrone (XVI)

A mixture of 3-hydroxy-4-pyrone (2.24 g, 0.02 mole), potassium iodide (0.30 g, 0.00019 mole), anhydrous potassium carbonate (2.67 g, 0.019 mole), and benzyl chloride (2.70 g, 2.45 ml, 0.022 mole) in dimethylformamide (100 ml) was heated for 8 hours on the steam bath with continuous stirring. The hot mixture was filtered, the residue washed repeatedly with ethanol, the combined filtrates evaporated to dryness, and the residual

solid exhaustively extracted with ether. The extract was dried (Na_2SO_4) and concentrated to yield crystals of 3-benzyloxy-4-pyrone (3.30 g, 81%), melting at 84-85° after recrystallization from ether. (Found: C, 71.0; H, 5.0. $C_{12}H_{10}O_3$ requires: C, 71.3; H, 5.0 %.)

Preparation of L- and DL-β-amino-α-tosylaminopropionic acid (XIII)

This was prepared according to Rudinger, Poduška and Zaoral (1960) from L- and DL-α-N-tosylasparagine respectively.

PREPARATION OF PYRIDONE INTERMEDIATES OF MIMOSINE

1. β-(1,4-Dihydro-3-methoxy-4-oxo-1-pyridyl)-N-tosylalanine (XIV) (O-Methyl-N-tosylamimosine)

3-Methoxy-4-pyrone (1.39 g, 0.011 mole) in water (10 ml) was added to a solution of DL-β-amino-α-tosylaminopropionic acid (2.58 g, 0.01 mole) in 0.1 M sodium hydroxide (100 ml, 0.01 mole). The mixture was heated for 3 hours on the steam bath and then concentrated to a volume of 50 ml, when the pH, originally above 11, had dropped to pH 9. The pH was adjusted to pH 7 by dropwise addition of concentrated hydrochloric acid and the solution was allowed to stand at 5° for several hours. Unreacted tosylamino acid (0.31 g, 0.0012 mole) was filtered off and the pH of the filtrate was adjusted to pH 2 with concentrated hydrochloric acid. Crystallization of the product started almost immediately and was complete after 12 hours at 5°, yielding DL-β-(1,4-dihydro-3-methoxy-4-oxo-1-pyridyl)-N-tosylalanine (1.51 g, 47%), melting at 200-201° (decomp.) after recrystallization from water. (Found: C, 52.7; H, 5.1; N, 7.6; S, 9.0. $C_{16}H_{18}N_2O_6S$ requires: C, 52.5; H, 5.0; N, 7.7; S, 8.7 %.)

Condensation of 3-methoxy-4-pyrone with L-β-amino-α-tosylaminopropionic acid in place of the DL-compound was accompanied by racemization,

also yielding optically inactive condensation product.

2. β-(3-Benzyloxy-1,4-dihydro-4-oxo-1-pyridyl)-N-tosylalanine (XVII) (O-Benzyl-N-tosylmimosine)

3-Benzyloxy-4-pyrone (2.24 g, 0.011 mole) in ethanol (15 ml) was mixed with a solution of DL-β-amino-α-tosylaminopropionic acid (2.58 g, 0.01 mole) in 0.1 M sodium hydroxide (100 ml, 0.01 mole). The mixture was warmed and shaken until homogeneous, and the solution was heated on the steam bath under reflux for 8 hours. Concentrated hydrochloric acid (3 ml) was added with rapid stirring and the solution allowed to stand at 5° overnight, when β-(3-benzyloxy-1,4-dihydro-4-oxo-1-pyridyl)-N-tosylalanine (3.25 g, 74%) melting at 203-5° (decomp.) after recrystallization from ethanol, was obtained. (Found: C, 59.5; H, 5.3; N, 6.3; S, 7.4. $C_{22}H_{22}N_2O_6S$ requires: C, 59.7; H, 5.0; N, 6.3; S, 7.2 %.)

3. β-(1,4-Dihydro-3-hydroxy-4-oxo-1-pyridyl)-N-tosylalanine (XVIII) (N-Tosylmimosine)

O-Benzyl-N-tosylmimosine (1.00 g, 0.0023 mole) in dimethylformamide (100 ml) was shaken 45 hours at room temperature under hydrogen at 18 p.s.i. in the presence of 5% palladium on charcoal (1.00 g), the catalyst having been prehydrogenated at 25 p.s.i. in ethanol (50 ml) at room temperature for 3 hours. The reaction mixture was heated and filtered and the catalyst exhaustively washed with hot ethanol. The filtrate was evaporated to dryness and the residue crystallized from ethanol, yielding β-(1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl)-N-tosylalanine (0.64 g, 80%) melting at 203-5° (decomp.). (Found: C, 51.0; H, 4.8; N, 7.8; S, 9.3. $C_{15}H_{16}N_2O_6S$ requires: C, 51.1; H, 4.6; N, 8.0; S, 9.1 %.)

4. β-(1,4-Dihydro-3-methoxy-4-oxo-1-pyridyl)-alanine (O-Methylmimosine)

O-Methyl-N-tosylmimosine (XIV) (0.50 g, .0014 moles) was dissolved in glacial acetic acid (10 ml) containing phenol (0.50 g, .0053 moles). The solution was saturated with anhydrous hydrogen bromide at room temperature and then incubated in a stoppered flask at 65° for 4 days. The reaction mixture was cooled to 0°, diluted with dry ether (400 ml) and allowed to stand at 5° for several hours until precipitation was complete. The mixture was decanted and the residue washed several times with ether then crystallized from ethanol to give a yield of 0.16 g (42%) crude product (hydrobromide salt). This product was dissolved in water, the solution was basified with ammonia, repeatedly evaporated to dryness and the product was finally recrystallized from water. β-(1,4-Dihydro-3-methoxy-4-oxo-1-pyridyl)-alanine so obtained started to decompose at 170° with gas evolution and finally melted with discolouration at 184°.

PREPARATION OF MIMOSINE

1. β-(1,4-Dihydro-3-hydroxy-4-oxo-1-pyridyl)-alanine (I) (DL-Mimosine) from N-tosylmimosine (XVIII)

The tosyl derivative (XVIII) (0.40 g) was dissolved in sufficient glacial acetic acid (approximately 25 ml) to give a homogenous solution. Phenol (0.40 g) was added and the solution was saturated at room temperature with dry hydrogen bromide and left at 60-65° in a stoppered flask. The reaction mixture was repeatedly monitored by ascending paper chromatography (phenol-ethanol-water, 3:1:1) and incubation was continued until the phenolic spot corresponding to N-tosylmimosine (R_f 0.78) failed to appear and only that corresponding to mimosine (R_f 0.27) was observed on development

of the chromatogram with ferric chloride solution. This generally required 2-3 days but in some runs additional hydrogen bromide was passed into the solution after 3 or 4 days in order to complete the reduction. The cooled solution was diluted with dry ether (400 ml) and allowed to stand at 5° until the separation of mimosine hydrobromide was complete. The supernatant liquid was decanted and the residue repeatedly extracted with ether and then dissolved in water. The aqueous solution was basified with concentrated ammonium hydroxide and evaporated to dryness under reduced pressure. The remaining solid was repeatedly moistened with water and evaporated to dryness under reduced pressure to remove excess ammonia, and finally dissolved in hot water, decolourized with charcoal, and allowed to crystallize, yielding DL-mimosine (0.10 g, 45%) melting at 222-225° (decomp.). For analysis a sample was recrystallized from boiling water and the product filtered from the hot solution to give anhydrous DL-mimosine, melting at 228-230° (decomp.). (Found: C, 48.7; H, 5.4; N, 14.1. Calculated for $C_8H_{10}N_2O_4$: C, 48.5; H, 5.1; N, 14.1 %.)

2. Mimosine (I) from O-Methyl-N-tosylmimosine (XIV)

O-Methyl-N-tosylmimosine in 48% hydrobromic acid boiled under reflux for 2 hours did not give rise to any easily isolable products. A paper chromatographic analysis revealed that the product mixture contained mimosine, O-methylmimosine, 3-hydroxy-4-pyridone and alanine. A second experiment was conducted in which the O-methyl-N-tosylmimosine was treated as above except that samples were taken at ½ hour intervals for paper chromatographic analysis. Only N-tosyl removal was observed after ½ hour while considerable N-tosyl and O-methyl removal accompanied by N-alkyl cleavage (giving rise to 3-hydroxy-4-pyridone) occurred after 1 hour. Although the

mimosine spot did not appear to increase in intensity, extensive N-alkyl cleavage was in evidence after 1½ hours. Almost identical behaviour was observed when 47% hydriodic acid was used except that more extensive N-alkyl cleavage seemed to occur after 1 hour's heating.

Solutions of 6 M sodium iodide with acid concentrations of 0.1, 0.3, 0.4, 0.5, 0.7 and 1 M hydriodic acid respectively were used as cleavage reagents. In each case the preparations were refluxed for 1 hour and then passed through a Dowex 50W-X4 (200-400 mesh) cation exchange resin column. The column was thoroughly washed with water then eluted with 0.1 M ammonia. The eluates were paper chromatographed and in each case the results were very similar to those obtained from treatment with 47% hydriodic acid. When mimosine was subjected to these treatments in control experiments, analysis by paper chromatography revealed only a mimosine spot accompanied by a second "mimosine" spot which developed on application of either ninhydrin or ferric chloride sprays. The R_f value of this spot was slightly lower than that of mimosine and its intensity varied with each experiment. Another control in which mimosine and p-toluenesulfonic acid in 0.4 M hydriodic acid was boiled under reflux for 2 hours showed no change occurring in mimosine as observed by paper chromatographic analysis.

From these experiments it appeared that the optimum yield of mimosine would be obtained by boiling under reflux for 1 hour a solution of O-methyl-N-tosylmimosine in 48% hydrobromic acid.

O-Methyl-N-tosylmimosine (0.250 g, .00068 moles) in 48% hydrobromic acid (3 ml) was boiled under reflux for 1 hour. The reaction mixture was then concentrated under vacuum and mild heating (under 100°) to yield a slightly moist solid residue. The residue was dissolved in water (5 ml)

and loaded on a Dowex 500W-X4 (200-400 mesh) cation exchange resin column (2 cm diameter x 15 cm length). After thorough washing with water, the column was eluted with 1 M ammonia and the column eluates were evaporated to dryness. The residues were repeatedly dissolved in water and evaporated to dryness to remove as much ammonia as possible. The residues were then dissolved in phenol-ethanol-water, 3:1:1, (5 ml) and loaded on a cellulose column (Whatman cellulose powder, standard grade) (1 cm diameter x 28 cm length). The column was eluted with phenol-ethanol-water, 3:1:1, and each fraction (0.5 ml) was analyzed by paper chromatography. The fractions observed to contain mimosine were combined and concentrated. After standing at 5° for several hours, mimosine (18 mg, 12%) crystallized at 224-225° with decomposition.

In further experiments, the residues obtained from cation exchange chromatography eluates were fractionally crystallized from phenol-ethanol-water, 3:1:1, to give mimosine but only in yields of approximately 4-5%.

PREPARATION OF OTHER PYRIDONE DERIVATIVES

1. 3-Benzyloxy-4-pyridone

3-Benzyloxy-4-pyrone (2.05 g, .011 mole) was dissolved by warming and shaking in a solution of ethanol (10 ml) in 6% aqueous ammonia (100 ml). The solution was heated under reflux on a steam bath for 6 hours, and concentrated to a volume of approximately 75 ml. After standing overnight at 5° the reaction mixture was filtered to yield 3-benzyloxy-4-pyridone (1.54 g, 71%) melting at 194-196°.

2. 3-Hydroxy-4-pyridone (II)

Bickel (1947) obtained 3-hydroxy-4-pyridone (II) by condensation of 3-methoxy-4-pyrone with ammonia, followed by demethylation with HCl in

a sealed tube. It was found to be more convenient to debenzylate 3-benzyloxy-4-pyridone by reductive cleavage.

3-Benzyloxy-4-pyridone (1.75 g, .0087 mole) in ethanol (100 ml) was shaken 48 hours at room temperature under hydrogen 35 p.s.i. in the presence of 5% palladium on charcoal (1.75 g), the catalyst having been prehydrogenated at 40 p.s.i. in ethanol (50 ml) at room temperature for 2 hours. The reaction mixture was heated and filtered and the catalyst exhaustively washed with hot ethanol. The filtrate was evaporated to approximately 5 ml and while hot, ether was added until the solution became turbid. The solution was allowed to stand overnight at 5° to complete crystallization. 3-Hydroxy-4-pyridone (0.52 g, 54%) was obtained in fine crystals melting with decomposition at 234-236° (c.f., Bickel (1947) 238-239° (decomp.)).

3. N-Methyl-3-benzyloxy-4-pyridone

3-Benzyloxy-4-pyrone (1.00 g, .00496 mole) was suspended in 10% aqueous methylamine (40 ml, .129 mole) and ethanol (5 ml). The mixture was warmed and shaken until homogeneous, and the solution was heated on the steam bath under reflux for 6 hours. The solution was evaporated to give an oily residue which was dissolved in ethanol, decolourized with charcoal, evaporated to dryness and distilled at 170-180° and 4 x 10^{-3} mm. The distillate was redistilled to yield N-methyl-3-benzyloxy-4-pyridone (0.731 g, 69%), melting at 193-195°. (Found: C, 72.7; H, 6.2; N, 6.5; O, 15.1. $C_{13}H_{13}NO_2$ requires: C, 72.5; H, 6.1; N, 6.5; O, 14.9 %.)

4. N-Methyl-3-hydroxy-4-pyridone (VII)

N-Methyl-3-hydroxy-4-pyridone has been synthesized by Bickel (1947) from 3-methoxy-4-pyrone *via* N-methyl-3-methoxy-4-pyridone. It was found

more convenient to start with 3-benzyloxy-4-pyrone.

N-Methyl-3-benzyloxy-4-pyridone (0.604 g, .0028 mole) was dissolved by stirring it into a suspension of 5% palladium on charcoal (1 g) in ethanol (50 ml) which had been prehydrogenated at 25 p.s.i. for 1 hour at room temperature. The mixture was shaken for 45 hours under hydrogen at 22 p.s.i. and room temperature, and was then filtered and evaporated to dryness. The residues were crystallized from a methanol-ether mixture to yield N-methyl-3-hydroxy-4-pyridone (0.350 g, 45%) melting at 225-7° (c.f., 226-8° (Bickel, 1947)).

5. 1,4-Dihydro-3-benzyloxy-4-oxo-1-pyridyl acetic acid

3-Benzyloxy-4-pyrone (1.016 g, .00505 mole) in ethanol (7 ml) was mixed with a solution of glycine (0.375 g) in 0.102 M sodium hydroxide (50 ml, .0051 mole). The mixture was warmed and shaken until homogeneous, and the solution was heated on the steam bath under reflux for 8 hours. Concentrated hydrochloric acid (1.0 ml) was added with rapid stirring and the solution allowed to stand at 5° overnight, when 1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl acetic acid (0.964 g, 72.5%), melting at 230-232° (decomp.) after recrystallization from acetic acid and water, was obtained. (Found: C, 65.0; H, 5.3; N, 5.2; O, 24.7. $C_{14}H_{13}NO_4$ requires: C, 64.9; H, 5.1; N, 5.4; O, 24.7 %.)

6. 1,4-Dihydro-3-hydroxy-4-oxo-1-pyridyl acetic acid (XXVI)

1,4-Dihydro-3-benzyloxy-4-oxo-1-pyridyl acetic acid (0.800 g, .00304 mole) in dimethylformamide (40 ml) was shaken 25 hours at room temperature under hydrogen at 35 p.s.i. in the presence of 5% palladium on charcoal (0.800 g), the catalyst having been prehydrogenated at 40 p.s.i. in ethanol (50 ml) at room temperature for 2 hours. The reaction mixture was heated and filtered and the catalyst exhuastively washed with hot ethanol. The

filtrate was evaporated to dryness and the residue crystallized from acetic acid and water, yielding 1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl acetic acid (.306 g, 59%), melting at 283-285° (decomp.). (Found: C, 49.5; H, 4.4; N, 8.2; O, 38.0. $C_7H_7NO_4$ requires: C, 49.7; H, 4.2; N, 8.2; O, 37.8%.)

ATTEMPTS TO SEPARATE THE PYRIDONE NUCLEUS FROM THE SIDE CHAIN

A. PYROLYSIS

(i) Pyrolysis in the Presence of Zinc Dust

Mimosine (88.6 mg, .45 mmole) was mixed with zinc dust (90 mg) and distilled at .003 mm pressure and 190-195° for 6 hours. The distillate thus obtained was redistilled at .003 mm and 175°. The product was recrystallized from benzene-ethanol to give 3-hydroxy-4-pyridone (35 mg, 70%), melting at 235-237° (decomp.). This was the method used for the degradation of the radioactive samples.

(ii) Pyrolysis in the Absence of Zinc Dust

Mimosine (100 mg, .50 mmole) was heated in vacuum at 200-205° and .003 mm pressure for 6 hours. The sublimate thus obtained was redistilled at 185° and .003 mm pressure. The distillate was crystallized from benzene-ethanol to yield 3-hydroxy-4-pyridone (6.3 mg, 11.2%), melting at 235-237° (decomp.).

In both cases the infrared spectra of the pure products were identical with that of authentic 3-hydroxy-4-pyridone. Product purity was checked by paper chromatography.

After each experiment, the still pot residues were extracted with boiling water and the extracts analyzed by paper. The only substance to appear was 3-hydroxy-4-pyridone in trace amounts.

B. DECARBOXYLATION REACTIONS IN SOLVENTS

Mimosine was heated in quinoline in the presence of a copper-bronze catalyst at 190° for 4 hours. Unchanged mimosine was recovered quantitatively. Analysis of the reaction mixture by paper chromatography showed only mimosine to be present.

When mimosine was heated at 190° for 2 hours in N,N-dimethyl-p-toluidine, analysis of the mixture by paper chromatography showed the formation of traces of 3-hydroxy-4-pyridone.

Mimosine (200 mg, .0010 mole) and a copper-bronze catalyst mixed in N,N-dimethyl-p-toluidine (2 ml) were boiled under reflux for 2 hours under a gentle stream of nitrogen gas, introduced into the reaction mixture by a fine bubbler. The condenser outlet was fitted with a trap containing an alcoholic solution of 2,4-dinitrophenylhydrazine and sulfuric acid. No carbonyl compounds were detected in the trap at the end of the reaction. The black reaction mixture was diluted with ethanol (10 ml) and filtered while hot. A small portion, tested with 2,4-dinitrophenylhydrazine solution, gave no derivative. The filtrates were evaporated to dryness under vacuum and the remaining residues were distilled at 180° and .002 mm pressure. Some difficulty was encountered in separating droplets of residual N,N-dimethyl-p-toluidine from the crystalline distillate. The crystalline distillate was recrystallized from benzene-ethanol to yield 3-hydroxy-4-pyridone (19.6 mg, 17.6%), melting at 235-237° (decomp.), identical in infrared spectrum and paper chromatography with an authentic specimen.

C. METHYLATION OF MIMOSINE WITH DIMETHYLSULFATE

(c.f., Bickel & Wibaut, 1946.)

Mimosine (300 mg, .0015 mole) was dissolved in water (10 ml), and

the solution placed in a 3-necked flask equipped with 2 dropping funnels, a condenser and a magnetic stirrer. Dimethylsulfate (2 ml, .021 mole) was added dropwise over a period of ½ hour while the temperature was maintained at 70°. The pH was controlled at pH 7 by the dropwise addition of 30% sodium hydroxide (3 ml) throughout the course of the reaction. The mixture was heated at 100° for 2½ hours in order to complete this initial reaction. After a further addition of 30% sodium hydroxide (3 ml) and dimethylsulfate (2 ml) the reaction mixture was boiled under reflux for 18 hours. This addition was repeated. Initially, trimethylamine was liberated from the boiling mixture but near the end of the second treatment with dimethylsulfate this was no longer detected. The reaction mixture was cooled, adjusted to pH 10 and filtered. The filtrates were extracted with amyl acetate for 18 hours in a liquid-liquid extractor. The amylacetate solution was concentrated to dryness and the residue was extracted with benzene but nothing was recovered from the benzene extracts. The extracted residue was crystallized from amyl acetate to yield N-methyl-3-methoxy-4-pyridone (25 mg, 11.6%), melting at 92-93°. The infrared spectrum was identical with that of an authentic sample.

According to Bickel and Wibaut (1946), mimosine (3 g) gave N-methyl-3-methoxy-4-pyridone (65% yield) melting at 92.5% by essentially the same procedure.

Variations of the procedure after the initial methylation of mimosine in further experiments gave only poorer results.

D. OXIDATION OF THE SIDE CHAIN

NINHYDRIN OXIDATION OF MIMOSINE

Mimosine (20 mg, .1 mmole) was dissolved in water (20 ml) and the

solution adjusted to pH 2 by the dropwise addition of 0.1 M hydrochloric acid. Ninhydrin (triketohydrindene hydrate) (42 mg, .24 mmole) was added to the solution which was then heated with stirring on the steam bath for 15 minutes. A deep violet colour developed and the pH of the solution rose to pH 4. When the pH was re-adjusted to pH 2 by the addition of hydrochloric acid, the colour faded and the solution remained colourless on further heating although the pH rose slightly. Saturated sodium bisulfite solution (4 ml) was added to the cooled solution (a deep orange colour developed) which was then allowed to stand at 5° for several hours. The solution became colourless but no precipitation occurred until concentration to a volume of 5 ml was carried out. Paper chromatograms of the precipitate did not reveal any products when sprayed with 1% ferric chloride. The filtrates were adjusted to pH 8 by addition of solid sodium carbonate. A solution of hydroxylamine (500 mg) in water (5 ml) adjusted to pH 8 by the addition of solid sodium carbonate was added to the filtrates and the resulting solution was allowed to stand at 5° overnight. A voluminous white precipitate formed. The precipitate could not be characterized in any way except that it did not give any coloration with ferric chloride solution. The filtrates gave violet colorations with ferric chloride but did not yield any products when extracted with ether and with benzene. The aqueous layer from the extraction was adjusted to pH 4 with hydrochloric acid and loaded onto a cation exchange column (Dowex 50W-X4 (200-400 mesh) in the H^+ form). Material which gave a violet coloration with ferric chloride solution was readily eluted from the column with water, but the eluate contained so little material that it could not be characterized any further. When the cation exchange column was finally washed free of this material, two pink bands had appeared

at about 1/4 and ½ of the distance down from the top of the column but were removed together on elution with 0.05 M ammonia. The ammonia eluate was evaporated to dryness to give a small amount of residue which gave a violet coloration in ferric chloride solution. No products could be resolved from this eluate either by crystallization or by paper chromatography developed with ferric chloride spray.

ATTEMPTS TO ISOLATE THE INDIVIDUAL CARBON ATOMS OF MIMOSINE

A. REDUCTION REACTIONS OF MIMOSINE

(i) CATALYTIC REDUCTIONS (HYDROGENOLYSIS)

(a) Partial Hydrogenolysis of Mimosine

Mimosine (100 mg, .50 mmole) dissolved in water (50 ml) was hydrogenated in the presence of Raney Nickel catalyst (15 mg) at 90° and 1800 p.s.i. pressure for 24 hours. Only unchanged mimosine was observed in the reaction mixture on analysis by paper chromatography. The ultraviolet absorption spectrum showed that at least 95% of the mimosine was unchanged.

Mimosine (103 mg, .52 mmole) dissolved in water (50 ml) was hydrogenated at 190° and 2000 p.s.i. for 36 hours in the presence of Raney Nickel catalyst (40 mg). Analysis of the reaction mixture by paper chromatography showed only one spot when developed with ferric chloride spray. This coincided with the spot of 3-hydroxy-4-pyridone. Several violet spots (presumably amino acids) developed on spraying with ninhydrin. One of these spots coincided with alanine.

A model mixture of alanine (12.5 mg) and 3-hydroxy-4-pyridone (17.5 mg) in water (2 ml) was applied to a cation exchange column (Dowex 50W-X4 (200-400 mesh) in the H^+ form) (2 cm diameter x 10 cm length). Elution with 0.2 M citrate buffer at pH 3.25 (8 fractions of 40 ml) followed by 0.2 M

citrate buffer at pH 4.25 (6 fractions of 40 ml) gave alanine in fraction 5 and 3-hydroxy-4-pyridone in fraction 11. The reaction mixture from the mimosine hydrogenation was chromatographed in the identical manner as the model experiment. Each fraction emerging from the column was analyzed by paper chromatography. Only alanine and 3-hydroxy-4-pyridone were observed to emerge (these were in fractions 5 and 11 respectively). Fractions 5 and 11 were each passed through a separate cation exchange column to remove the citrate buffer. Alanine and 3-hydroxy-4-pyridone respectively were eluted from their columns with .05 M ammonia. The eluates were evaporated to dryness giving alanine (3.7 mg, 6%) and 3-hydroxy-4-pyridone (18.9 mg, 34%) respectively. Identification was confirmed by paper chromatography.

(b) Complete Hydrogenolysis of 3-Hydroxy-4-pyridone

3-Hydroxy-4-pyridone (100 mg, .9 mmole) in water (50 ml) was hydogenated in the presence of Raney Nickel catalyst (30 mg) at 2500 p.s.i. and 240° for 48 hours. The initial solution was acidic to neutral litmus paper and gave a violet coloration with neutral ferric chloride solution while the reaction mixture was basic and gave no ferric chloride coloration. The mixture was filtered free of catalyst and the filtrate was shaken with benzoyl chloride (1 ml) with the dropwise addition of 40% sodium hydroxide solution to maintain the mixture at pH 10. After 1 hour this mixture was extracted three times with ether (3 x 40 ml). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The residue was distilled at 150° and 0.01 mm pressure to give a solid condensing at the warm end of the condenser and an oily liquid collecting further away from the still pot. The solid was recrystallized from methanol-water, gave a melting point of 120-121° and a mixed melting point with benzoic

acid 120-121°. The liquid was redistilled at 130° and 0.01 mm pressure to give further traces of benzoic acid and a very oily liquid which cooled to a resinous clear solid and was recovered as a white powder by scraping it from the condenser. Analysis with a Fisher-Gulf gas chromatography unit Model 300 with a 7-foot Silicon 550 column containing 0.6% oleic acid showed only a single product. The compound was introduced as an ether solution, flash evaporated at 195° and carried by helium gas. The single product emerged 39 minutes after introduction when run through the column at 160°. The compound, thought to be a dibenzoylbenzamide of an aminopentane-diol melted indistinctly at 87° with the characteristics of a paracrystalline state persisting up to 100°. (Found: C, 72.2; H, 6.0; N, 3.5. $C_{26}H_{25}N_2O_5$ requires: C, 72.4; H, 5.8; N, 3.3; O, 18.5 %.)

(c) Complete Hydrogenolysis of Mimosine

Mimosine (102 mg, .51 mmole) in water (50 ml) was hydrogenated in the presence of Raney Nickel catalyst (60 mg) at 2500 p.s.i. and 250° for 36 hours. The reaction mixture was filtered, the catalyst washed with water and the combined filtrates applied to a Dowex 2-X8 (200-400 mesh) anion exchange column (in the OH^- form; 2 cm diameter x 10 cm length). The effluent from the application and subsequent water washes were collected (approximately 160 ml).

The column was eluted successively with 3% acetic acid, 10% acetic acid and finally with 4% hydrochloric acid. The first stage gave a white powdery material (24.8 mg, 24%), the second stage gave a similar product (15.6 mg, 15%) while the third stage gave a brown resinous material (4.7 mg, 4.5%). Accurate melting points could not be obtained for any of these products and only the third product, which appeared to be mainly alanine, could be

analyzed by paper chromatography.

The initial column eluate (160 ml) was adjusted to pH 4 by the dropwise addition of 0.1 M sulfuric acid. The solution was then treated with 0.1 M sodium metaperiodate solution (10 ml) and allowed to stand at room temperature for 1 hour. A 3% solution of 2,4-dinitrophenylhydrazine solution* (1 ml) was added with stirring and immediate turbidity was observed. More 2,4-dinitrophenylhydrazine was added after 10 minutes and again after 3 hours. The mixture was allowed to stand at 5^{o} overnight. The precipitates were filtered from the mixture and a sample of the precipitate recrystallized from ethanol. Both the crude and the recrystallized precipitates were analyzed by reverse phase descending paper chromatography as described by Hattori (1956). The samples were applied to Whatman No. 1 filter paper and allowed to dry. The paper was then sprayed with tetralin (about 0.75 ml per 100 cm^2) and, while moist with tetralin, developed by the descending technique with methanol-acetic acid-tetralin-water, 10:1.5:1:1.4. After drying, the papers were examined under ultraviolet light. A spot coinciding with that of the derivative of propionaldehyde was present in both samples. A great deal of 2,4-dinitrophenylhydrazine was present and also a very intense spot with an R_f value lower than propionaldehyde which could not be identified. Further purification of the precipitate by recrystallization did not appear to affect the relative amounts of the three products present. The reference samples used consisted of a model mixture of 2,4-dinitrophenylhydrazine and its hydrazones of formaldehyde, acetaldehyde and propionaldehyde.

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* 3% $\frac{w}{v}$ 2,4-dinitrophenyl hydrazine in a solution of alcohol (65% $\frac{v}{v}$), sulfuric acid (14% $\frac{v}{v}$) and water.

(ii) ATTEMPTED CHEMICAL REDUCTIONS

(a) Sodium Borohydride

Mimosine (100 mg, .00050 mole) was dissolved in .008 M sodium hydroxide in 60% methanol solution (50 ml) and treated with sodium borohydride (100 mg, .03 mole). After 2 hours effervescence had ceased. A quantitative ultraviolet absorption spectrum of the solution showed no change in the mimosine concentration. Repeated additions of sodium borohydride (50 mg, .015 mole) every 2 hours for an 8-hour period failed to lead to any change.

(b) Lithium Aluminum Hydride

Mimosine (100 mg, .50 mmole) in a paper thimble was placed in a Soxhlet extractor in which the still pot was charged with anhydrous tetrahydrofuran and lithium aluminum hydride (214 mg). The extraction was carried out under the protection of a calcium chloride drying tube and after 48 hours an additional amount of lithium aluminum hydride (312 mg) was added to the still pot. After one week, the extraction was stopped and recovery of unchanged starting material from the thimble yielded mimosine (96 mg, 96%).

(c) Sodium Amalgam

Mimosine (201 mg, .00102 mole) was dissolved in .02 M sodium hydroxide solution (100 ml). 3% Sodium amalgam (2 gm) was added at suitable intervals so that the solution, which was agitated by a magnetic stirrer, continually effervesced. The reaction mixture was maintained at 37° and monitored every 12 hours by paper chromatographic analysis. A number of compounds were observed to slowly increase in concentration as reduction progressed. These compounds were characterized by violet colorations at low R_f values on paper chromatograms developed with ninhydrin spray. After one week a relatively large amount of mimosine remained intact. None of the products could be

identified or isolated on a preparative scale. Attempts at quantitative recovery of unchanged mimosine were subject to high losses because of the accumulation of large concentrations of sodium hydroxide in the reaction mixture.

B. INTRAMOLECULAR MANNICH REACTION

(i) PREPARATION OF 1,3,4,8-TETRAHYDRO-3-CARBOXY-9-HYDROXY-8-OXO-2-H-PYRIDO (1, 2 a) PYRAZINE (XXIV) (MIMOSINE-FORMALDEHYDE CONDENSATION PRODUCT)

Mimosine (100 mg, .50 mmole) was dissolved in 0.10 M hydrochloric acid (5 ml) and 10% aqueous formaldehyde solution (0.16 ml) was added. The solution was allowed to stand at 38° for 20 hours during which time a green fluorescence developed. The solution was cooled, adjusted to pH 5.0 by the dropwise addition of dilute sodium hydroxide solution, and allowed to stand at 5° overnight. The product, presumably (XXIV) (62.4 mg, 60%), melting at 234-236° (decomp.) after recrystallization from water, was obtained. (Found: C, 48.2; H, 5.4; N, 12.1; O, 33.0. $C_9H_{10}N_2O_4.H_2O$ requires: C, 47.4; H, 5.3; N, 12.3; O, 35.0 %). Prolonged drying did not lead to an improvement in the analysis.

(ii) CHROMIC ACID OXIDATIONS OF THE CONDENSATION PRODUCT (XXIV)

(a) Oxidation in Boiling Solution

Condensation product (XXIV) (20 mg, .14 mmole) in water (2 ml) was brought to the boil and 10% potassium dichromate solution (3 ml) pre-mixed with glacial acetic acid (0.6 ml) was added and the solution boiled for 1 minute. On addition of the acidic reagent solution the fluorescence of the reaction mixture was lost and a turbid orange-brown coloration developed. Paper chromatographic analysis using ferrous sulfate and ninhydrin sprays

failed to show the presence of any pyridine-2- or pyrazine-2- carboxylic acid and amino acids respectively. The reaction mixture was treated with sulfur dioxide to reduce the remaining dichromate and then adjusted to pH 10 with 2 M sodium hydroxide solution to precipitate chromium hydroxide. The solution was filtered and despite the fact that the filtrates appeared to have a pale blue fluorescence, no products were observed by ultraviolet spectroscopy. It appeared that total destruction of the molecule had occurred.

(b) Oxidation at Room Temperature

In a second experiment procedure (a) was modified. The condensation product (XXIV) (20 mg, .14 mmole) was dissolved in water (2 ml), the solution allowed to cool to room temperature, the acid dichromate solution was added and the mixture left to stand ½ hour at room temperature. Although the chromium-free filtrates appeared to exhibit a weak yellow-green fluorescence, no products were detected.

(iii) DEHYDROGENATION OF THE CONDENSATION PRODUCT (XXIV)

(a) Dehydrogenation with Selenium

The condensation product (XXIV) (35.9 mg, .17 mmole) and powdered selenium (222 mg) were mixed in a small vial. The vial was placed at the bottom of a very long test tube (1 cm diameter x 50 cm length) which was fitted from the top with a long probe which allowed nitrogen gas to be introduced around the vial. The closed end of the tube was immersed into a Wood's metal bath. Heating was started at 220° and increased to 280° over a period of 45 minutes, and to 310° over the next hour. White crystals formed on the wall of the tube just above the level of the metal bath surface, but on cooling and exposure to air these crystals decomposed to a grey filmy solid. Repeated attempts failed to yield any products which could be identified

by ultraviolet or infrared spectroscopy or by paper chromatography.

(b) Attempted Catalytic Dehydrogenation with Maleic Acid

Condensation product (XXIV) (10.3 mg, .047 mmole), maleic acid (17.9 mg, .15 mmole), palladium black (4.4 mg) and water (0.3 ml) were sealed in a small tube which was placed in a protective container and heated vigorously on a steam bath for 5 hours. On cooling, the tube was opened and the pale yellow solution was filtered free of the catalyst. The ultraviolet spectrum of the reaction mixture was identical with that of the starting material showing no change had occurred.

(iv) METHYLATION OF PRODUCT XXIV WITH DIAZOMETHANE

Condensation product (XXIV) (62 mg, .28 mmole) was suspended in methanol (25 ml) and a 2% ethereal diazomethane solution (20 ml) was added portionwise. The solution was maintained at 5° throughout the 3-hour period of treatment, then allowed to warm up to room temperature during which time a pink coloration developed and faded. The mixture was evaporated to a volume of 2 ml and allowed to stand at 5° for several hours but no precipitates formed. Analysis by paper chromatography in which the chromatograms were examined under ultraviolet light showed only one product. This apparently fully methylated product gave an R_f value of 0.91 as compared with 0.56 for the starting material. Only a faint brown coloration appeared on ninhydrin development while ferric chloride spray gave rise to a pale yellow spot. Further purification could not be accomplished.

The entire product from the methylation reaction was evaporated to an oily residue, mixed thoroughly with 0.1 M sodium hydroxide solution (5 ml) and heated on a steam bath for 2 hours. On analysis by paper chromatography only one product was observed. This product was observed on

the chromatogram under ultraviolet light at an R_f value of 0.70, gave a red coloration with ninhydrin spray but only a pale yellow spot with ferric chloride spray. The reaction mixture was evaporated to an oily residue but no products could be separated.

A small portion of this hydrolysis residue (20 mg) was heated for 1 minute with potassium dichromate acetic acid (as previously described for the original condensation product (XXIV)). After the reaction mixture was freed of interfering ions, an ultraviolet absorption spectrum showed that a product different from the unoxidized material had formed but further identification was not possible.

C. IODOFORM REACTION

Stock Iodine Solution. Iodine (2.5 g) and potassium iodide (5 g) were dissolved in distilled water (20 ml) which had been vigorously boiled to liberate dissolved carbon dioxide.

Stock 11% Sodium Hydroxide (low in carbonate). Sodium hydroxide pellets (60 g) were dissolved in water (50 ml) at 60° then allowed to stand at room temperature for several hours. The mixture, which had partially crystallized, was vacuum filtered through a Buchner funnel and the filtrates diluted with carbonate-free distilled water (350 ml). The solution was assayed by titration (giving a value of 11% sodium hydroxide) and stored in a bottle which was vented through an Ascarite tube.

IODOFORM REACTION ON MIMOSINE

Mimosine (100 mg, .50 mmole) in 11% sodium hydroxide (4 ml) was treated with stock iodine solution (6 ml). A yellow precipitate formed immediately but the reaction mixture was allowed to stand at room temperature for 1 hour. The mixture was extracted three times with chloroform

(3 x 10 ml); the combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under vacuum. The residues were recrystallized from methanol-water to give iodoform (36 mg, 9%).

The aqueous solution was freed of hypoiodite by bubbling sulfur dioxide gas into the solution until the solution attained pH 6. Calcium hydroxide (100 mg, .00135 mole) was added to the solution which, after vigorous stirring, was allowed to stand at 5^{o} for 1 hour. The precipitate was carefully recovered by centrifugation and water washed twice.

The combined supernatant liquids were acidified with concentrated sulfuric acid (0.3 ml) and steam distilled until 25 ml of distillate had been collected. A portion of this distillate (5 ml) was basified with 5 M ammonia (0.5 ml) and concentrated to a volume of $\frac{1}{2}$ ml. Analysis of this mixture was carried out by paper chromatography. The chromatograms were run in ethanol-ammonia, 100:1, and developed with a spray consisting of bromphenol blue in citric acid solution. Any carboxylic acids present would appear as blue spots on a yellow background. No products were detected.

Analysis of the still pot residue from the steam distillation by paper chromatography gave several violet coloured spots when developed with ninhydrin spray. None of these could be identified nor could any product be isolated by ion exchange chromatography.

The calcium salt precipitate was carefully degraded with concentrated hydrochloric acid (1 ml) so that a quantitative recovery of carbon dioxide could be carried out. The reaction mixture was boiled and gently flushed with nitrogen gas while carbon dioxide was collected in a trap containing 11% sodium hydroxide solution (5 ml). When the reaction was complete, the trap solution was treated with 10% barium chloride solution (4 ml). The

resulting precipitates were quantitatively recovered to yield barium carbonate (102.5 mg). A control reaction with no mimosine was run under identical conditions to give barium carbonate (96 mg). The net yield of barium carbonate from the iodoform reaction was 6.5 mg (6.5%).

The solution remaining in the reaction vessel after the carbonate determination was adjusted to pH 5.5 and allowed to stand at 5° overnight. The precipitate was quantitatively recovered to give calcium oxalate (22 mg, 34%).

COMPARATIVE IODOFORM REACTIONS

(i) MIMOSINE (I)

Mimosine (10 mg, .05 mmole) in 11% sodium hydroxide (3 ml) was treated by the dropwise addition of stock iodine solution (3 ml) at room temperature. After thorough mixing the turbid mixture was allowed to stand for ½ hour and was then extracted three times with chloroform (3 x 5 ml). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under vacuum. The residues were crystallized from methanol-water to yield iodoform (2.5 mg, 12.9%), melting at 118-119°.

(ii) 3-HYDROXY-4-PYRIDONE-N-ACETIC ACID (XXVI)

3-Hydroxy-4-pyridone-N-Acetic acid (11.5 mg, .068 mmole) in 11% sodium hydroxide (3 ml) was treated by the dropwise addition of stock iodine solution (3 ml) at room temperature. After thorough mixing the turbid mixture was allowed to stand for ½ hour and was then extracted three times with chloroform (3 x 5 ml). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under vacuum. The residues were crystallized from methanol-water to yield iodoform (3.7 mg, 13.9%), melting at 118-119°.

(iii) N-METHYL-3-HYDROXY-4-PYRIDONE (VII)

N-Methyl-3-hydroxy-4-pyridone (10 mg, .08 mmole) in 11% sodium hydroxide (3 ml) was treated by the dropwise addition of stock iodine solution (3 ml) at room temperature. After thorough mixing, the turbid mixture was allowed to stand for ½ hour and was then extracted three times with chloroform (3 x 5 ml). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under vacuum. The residues were crystallized from methanol-water to yield iodoform (7.6 mg, 24.2%), melting at 118-119°.

D. OTHER REACTIONS OF MIMOSINE

OXIDATION WITH AMMONIACAL SILVER NITRATE

Mimosine (50 mg, .25 mmole) was dissolved in 0.1% ammoniacal silver nitrate solution (10 ml) and allowed to stand at 50° for three hours during which time a dark grey precipitate formed. Additional 0.1% ammoniacal silver nitrate (10 ml) was stirred into the reaction mixture which was then allowed to stand at 50° overnight. A monitoring test by paper chromatography showed only faint traces of mimosine when developed with ferric chloride spray while an identical amount developed with ninhydrin spray gave a number of poorly resolved violet coloured spots. Saturated sodium chloride solution was added dropwise to the reaction mixture until no turbidity was observed on further addition of salt solution to a sample of the mixture which had been clarified by centrifugation. The precipitate was removed and the filtrate was concentrated *in vacuo* to a volume of 2 ml. This residue was applied to a Dowex 50W-X4 (200-400 mesh) cation exchange column (in the H^+ form) which was washed with water to remove anionic material then eluted with 0.5 M ammonia. The ammonia effluent gave an analysis by paper chromatography

identical with that of the crude reaction mixture. Further attempts at separation by cation exchange chromatography and fraction crystallization were fruitless.

Variations in the amount of silver nitrate and in the degree of heating used made little difference in the reaction and no products were isolated.

OTHER OXIDATION REACTIONS

The reaction of a number of other oxidizing agents with mimosine (I), 3-hydroxy-4-pyridone-N-acetic acid (XXVI) and N-methyl-3-hydroxy-4-pyridone (VII) was studied. The reagents tested were potassium dichromate, potassium permanganate, sodium metaperiodate, potassium ferricyanide and hydrogen peroxide.

The first series of reactions was carried out in the following manner:

The organic test material (10 mg) was dissolved in 0.1 N sulfuric acid (1.0 ml) and water (2 ml). Oxidizing reagent in 0.100 M solution (1 ml) was added with stirring and the reaction mixture was then allowed to stand at room temperature. Samples were taken for paper chromatographic analysis at 20-minute intervals for the first 3 analyses, hourly for the next 2, every second hour for the next 2 and when no reaction was apparent, every 12 hours thereafter up to 2 days. Duplicate chromatograms were run so that development could be carried out with ninhydrin and with ferric chloride sprays.

The results showed that both potassium dichromate and potassium permanganate destroyed the ring in each of the 3 test compounds within the first 20 minutes of reaction. The other reagents showed no effect on any of the test compounds, even after 2 days.

In order to test whether selective oxidation occurred, mimosine (10 mg)

in 0.1 N sulfuric acid (1 ml) and water (2 ml) was treated by the portion-wise addition (0.1 ml) of .05 M potassium permanganate solution (1 ml). Samples for analysis were removed after each addition. Development of the chromatograms showed a complex pattern of violet ninhydrin spots to appear. Further addition of oxidizing agent intensified, but did not change the product pattern. This indicated the complete absence of selectivity in this reaction.

A second series of reactions was carried out with the weaker oxidizing reagents (i.e., sodium metaperiodate, hydrogen peroxide and potassium ferricyanide).

Test material (10 mg) in 0.1 N sulfuric acid (1 ml) and water (2 ml) was mixed with 9.1 M oxidizing reagent solution (1 ml) and boiled for 1 minute. Chromatographic samples were taken and after boiling for an additional 5 minutes another set of samples were taken.

Potassium ferricyanide did not react with any of the test compounds while sodium metaperiodate and hydrogen peroxide led to the destruction of each of these materials. The chromatographic patterns and colorations observed for the latter two reagents were very similar with all three test compounds. The chromatograms from the peroxide test were faint but when the reaction mixture was again boiled with excess peroxide the similarities with the periodate oxidation were very striking. When these chromatograms were sprayed with ninhydrin, pale blue, yellow and brown as well as several violet coloured spots were observed. The products could not be identified nor could any of them be isolated when the reaction was repeated on a preparative scale.

TREATMENT OF MIMOSINE WITH NITROUS ACID

Mimosine (500 mg, .0025 mole) was dissolved in 0.1 N sulfuric acid (30 ml) and heated to 95°. Sodium nitrite (260 mg, .0025 mole) in water (5 ml) was added through a dropping funnel. The stem of the funnel was submerged below the reaction mixture so that none of the evolved gases could escape during the addition which lasted 30 minutes. The solution was filtered hot, concentrated in vacuum to a volume of 5 ml and allowed to stand overnight. No products separated but chromatographic analysis revealed the presence of 3-hydroxy-4-pyridone as the only detectable product.

PAPER CHROMATOGRAPHY

Except where otherwise mentioned, all reaction mixtures and plant extracts were monitored by ascending paper chromatography on Whatman No. 1 paper, using phenol-ethanol-water, 3:1:1, as the solvent. Spots were developed with ferric chloride (1%) and/or ninhydrin (3% in acetone). Before development the papers were dried for 1 hour at 80° to ensure complete removal of phenol.

Under these conditions the following R_f values were found: α,β-diaminopropionic acid, R_f 0.16; mimosine, R_f 0.27; alanine, R_f 0.44; O-methylmimosine, R_f 0.61; 3-hydroxy-4-pyridone, R_f 0.70; N-tosylmimosine, R_f 0.78; 3-hydroxy-4-pyrone, R_f 0.83.

SECTION II: BIOSYNTHESIS OF MIMOSINE

RADIOACTIVE FEEDING EXPERIMENTS

1. CARBON DIOXIDE-C^{14}

Six Mimosa plants (3 seven weeks old and 3 eleven weeks old) were transplanted into sphagnum contained in a 5-inch glass pot and allowed to grow undisturbed for 2 days. The pot containing the plants was then placed into a sealed glass chamber (Figure 11). Carbon dioxide-C^{14} was introduced by decomposing a sodium carbonate solution (70 μc, 10 ml) with concentrated sulfuric acid (1 ml) in a reaction vessel venting into the glass chamber. A small vibrating metal strip enclosed in an external duct and activated by an A.C. electromagnet maintained atmospheric circulation within the chamber. A period of 1 hour was allowed for the carbon dioxide-C^{14} to spread throughout the system. A gas sample (100 ml) was removed from the chamber and the level of radioactivity as measured with a reed electrometer was found to be .0595 μc/100 ml. After the plants were allowed to grow for 3 hours in the noonday sunshine another gas sample (100 ml) was withdrawn and measurement showed an activity of .0154 μc/100 ml. This corresponds to a disappearance of 74% of the activity. Since some of the carbon dioxide-C^{14} could have been absorbed into the moist sphagnum, the plants were allowed to remain growing in the chamber for 48 hours. The plants were then harvested by collecting the sap in two lots keeping the sap of the younger and older plants separate.

Samples of sap were separated on paper chromatograms which were sectioned. Each section was extracted with .01 M hydrochloric acid, the extracts were evaporated to 0.5 ml and plated for radioactive counting.

This procedure revealed significant activity in the mimosine fractions from plants of both ages.

In each case mimosine was precipitated from the sap with ethanol and further purified by recrystallization from ethanol-water.

Mimosine (2 mg) thus obtained from the older plants (11 weeks old) was observed to melt at 224-225° (decomp.). The radioactivity of this sample was found to be (1.37 ± .40) x 10^3 counts $minute^{-1}$ $mmole^{-1}$. Mimosine (1 mg) obtained from the young plants (7 weeks old) was measured to have an activity of 4.39 (± .11) x 10^3 counts $minute^{-1}$ $mmole^{-1}$. A repeat experiment confirmed these findings.

2. ASPARTIC ACID-3-C^{14} (2 DAY)

A. MIMOSINE-C^{14}

Aspartic acid-3-C^{14} (1.96 mg, total activity 100 μc, specific activity 6.8 mc $mmole^{-1}$) was dissolved in water (10 ml). For radio-assay, a sample of this solution (100 λ) was diluted to 10 ml and 10 λ portions of the diluted solution, i.e., 10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 450.4 (± 4.6) x 10^5 counts $minute^{-1}$.

The solution (10 ml) containing the aspartic acid-3-C^{14} was administered equally to Mimosa plants (7 weeks old) by the "wick" method (Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 2 days and mimosine (12.9 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the wicks were extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml.

Samples (10 λ each) of this solution were assayed for residual radioactivity. A total of 98.8 (± 1.3) x 10^4 counts $minute^{-1}$ or 2.2% of the original activity had not been absorbed by the plants.

A sample of the isolated mimosine (100 μg) was assayed for radiopurity by thin layer chromatography. The chromatogram with a mimosine and aspartic acid reference sample was run in ethanol-water, 4:1, on Silica G. The reference sample was developed with ninhydrin spray and the rest of the chromatogram was sectioned with respect to the fractions observed in the reference. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the mimosine sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which was similarly applied to the thin layer chromatographic plate but not chromatographed.

Crude mimosine (11.5 mg) was diluted with inactive pure mimosine (51.7 mg) and crystallized from ethanol-water. Mimosine-C^{14} (60.1 mg), specific activity 23.50 (± 0.46) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be 129.3 (± 2.5) x 10^3 counts $minute^{-1}$ $mmole^{-1}$.

B. 3-HYDROXY-4-PYRIDONE-C^{14}

Diluted mimosine-C^{14} (56.5 mg, .285 mmole, specific activity 23.50 (± 0.46) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (70 mg) at 200-205° and .01 mm pressure for 4 hours. The distillate was redistilled at 180° and .01 mm pressure and again at 170° and .01 mm presure to give 3-hydroxy-4-pyridone-C^{14} (20.8 mg, 66%). 3-Hydroxy-4-

pyridone-C^{14} (20.8 mg, 66%). 3-Hydroxy-4-pyridone-C^{14}, specific activity 21.25 (± 0.39) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. Thus it was demonstrated that 90.4 ± 2.4 % of the mimosine activity was located in the pyridone ring.

3. ASPARTIC ACID-3-C^{14} (14 DAY)

A. MIMOSINE-C^{14}

Aspartic acid-3-C^{14} (1.96 mg, total activity 100 μc, specific activity 6.8 mc $mmole^{-1}$) was dissolved in water (10 ml). For radio-assay, a sample of this solution (100 λ) was diluted to 10 ml and 10 λ portions of the diluted solution, i.e., 10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 470.0 (± 6.5) x 10^5 counts $minute^{-1}$.

The solution (10 ml) containing the aspartic acid-3-C^{14} was administered equally to 20 Mimosa plants (7 weeks old) by the "wick" method (Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 14 days and mimosine (7.4 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of the Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the "wicks" were extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml. Samples (10 λ each) of this solution were assayed for residual radioactivity. A total of 50.5 (± 1.6) x 10^4 counts $minute^{-1}$ or 1.1% of the original activity had not been absorbed by the plants.

A sample of the isolated mimosine (50 μg) was assayed for radiopurity by thin layer chromatography. Aspartic acid and mimosine references

and the sample were run in ethanol-water, 4:1, on Silica G plates. The references were developed with ninhydrin spray and the rest of the chromatogram was sectioned with respect to fractions observed in the references. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which was similarly applied to the thin layer plate but not chromatographed.

Crude mimosine (4.0 mg) was diluted with inactive pure mimosine (55.8 mg) and crystallized from ethanol-water. Mimosine-C^{14} (53.6 mg), specific activity 2.786 (± 0.058) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be 25.1 (± 0.4) x 10^3 counts $minute^{-1}$ $mmole^{-1}$.

B. 3-HYDROXY-4-PYRIDONE-C^{14}

Diluted mimosine-C^{14} (50.1 mg, .25 mmole, specific activity 2.786 (± 0.058) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (58 mg) at 195-200° and .005 mm pressure for 6 hours. The distillate was redistilled at 180° and .005 mm pressure and again at 175° and .005 mm pressure to give 3-hydroxy-4-pyridone (16.2 mg, 58%). 3-Hydroxy-4-pyridone-C^{14}, specific activity 2.434 (± 0.041) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. This corresponds to 87.4 ± 2.6 % of the mimosine activity.

4. ASPARTIC ACID-1-C^{14}

A. MIMOSINE-C^{14}

Aspartic acid-1-C^{14} (2.98 mg, total activity 100 μc, specific activity

4.5 mc mmole^{-1}) was dissolved in water (10 ml). For radio-assay, a sample of this solution (100 λ) was diluted to 10 ml and 10 λ portions of the diluted solution, i.e., 10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 354.5 (± 5.8) x 10^5 counts minute^{-1}.

The solution (10 ml) containing the aspartic acid-1-C^{14} was administered equally to 20 Mimosa plants (7 weeks old) by the "wick" method (Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 2 days and mimosine (9.7 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of the Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the "wicks" were extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml. Samples (10 λ each) of this solution were assayed for residual radioactivity. A total of 98.2 (± 2.6) x 10^4 counts minute^{-1} or 2.8% of the original activity had not been absorbed by the plants.

A sample of the isolated mimosine (50 μg) was assayed for radiopurity by thin layer chromatography. Aspartic acid and mimosine references and the sample were run in ethanol-water, 4:1, on Silica G plates. The references were developed with ninhydrin spray and the rest of the chromatogram was sectioned with respect to fractions observed in the references. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which

was similarly applied to the thin layer plate but not chromatographed.

Crude mimosine (8.6 mg) was diluted with inactive pure mimosine (52.8 mg) and crystallized from ethanol-water. Mimosine-C^{14} (47.9 mg), specific activity 5.147 (± 0.108) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be 37.8 (± 0.7) x 10^3 counts $minute^{-1}$ $mmole^{-1}$.

B. 3-HYDROXY-4-PYRIDONE-C^{14}

Diluted mimosine-C^{14} (46.6 mg, .235 mmole, specific activity 5.147 (± 0.108) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (49 mg) at 195-200° and .01 mm pressure for 4 hours. The distillate was redistilled at 180° and 0.01 mm pressure and again at 175° and 0.01 mm pressure to give 3-hydroxy-4-pyridone (12.9 mg, 50%). 3-Hydroxy-4-pyridone-C^{14}, specific activity 3.382 (± 0.103) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. This corresponds to 65.7 ± 2.4 % of the mimosine activity.

5. ASPARTIC ACID-4-C^{14}

A. MIMOSINE-C^{14}

Aspartic acid-4-C^{14} (6.34 mg, total activity 100 μc, specific activity 2.1 mc $mmole^{-1}$) was dissolved in water (10 ml). For radio-assay, a sample of this solution (100 λ) was diluted to 10 ml and 10 λ portions of the diluted solution, i.e., 10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 251.1 (± 4.5) x 10^5 counts $minute^{-1}$.

The solution (10 ml) containing the aspartic acid-4-C^{14} was administered equally to 20 Mimosa plants (7 weeks old) by the "wick" method

(Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 2 days and mimosine (11.0 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of the Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the "wicks" extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml. Samples (10 λ each) of this solution were assayed for residual radioactivity. A total of 28.4 (± 0.4) x 10^4 counts $minute^{-1}$ or 1.1% of the original activity had not been absorbed by the plants.

A sample of the isolated mimosine (50 μg) was assayed for radiopurity by thin layer chromatography. Aspartic acid and mimosine references and the sample were run in ethanol-water, 4:1, on Silica G plates. The references were developed with ninhydrin spray and the rest of the chromatogram was sectioned with respect to fractions observed in the references. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which was similarly applied to the thin layer plate but not chromatographed.

Crude mimosine (9.2 mg) was diluted with inactive pure mimosine (51.6 mg) and crystallized from ethanol-water. Mimosine-C^{14} (44.7 mg), specific activity 1.880 (± 0.017) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be 12.5 (± 0.1) x 10^3 counts $minute^{-1}$ $mmole^{-1}$.

B. 3-HYDROXY-4-PYRIDONE

Diluted mimosine-C^{14} (41.7 mg, .21 mmole, specific activity 1.880 (± 0.017) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (52 mg) at 195-200° and .004 mm pressure for 4 hours. The distillate was redistilled at 180° and .004 mm pressure and again at 175° and .004 mm pressure to give 3-hydroxy-4-pyridone (18.5 mg, 80%). 3-Hydroxy-4-pyridone-C^{14}, specific activity 1.074 (± 0.045) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. This corresponds to 57.1 ± 2.5 % of the mimosine activity.

6. GLYCEROL-1,3-C^{14}

A. MIMOSINE-C^{14}

Glycerol-1,3-C^{14} (0.614 mg, total activity 100 μc, specific activity 15 mc $mmole^{-1}$) was dissolved in water (10 ml). For radio-assay, a sample of this solution (100 λ) was diluted to 10 ml and 10 λ portions, i.e., 10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 382.8 (± 7.8) x 10^5 counts $minute^{-1}$.

The solution (10 ml) containing the glycerol-1,3-C^{14} was administered equally to 20 Mimosa plants (7 weeks old) by the "wick" method (Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 2 days and mimosine (6.5 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of the Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the wicks were extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml. Samples (10 λ each) of this solution were assayed for residual radioactivity.

A total of 110.6 (± 2.4) x 10^4 counts $minute^{-1}$ or 2.9% of the original activity had not been absorbed by the plants.

A sample of the isolated mimosine was assayed for radiopurity by thin layer chromatography. Reference samples of mimosine and glycerol and the radioactive sample were run on Silica G plates in ethanol-water, 4:1. The reference sample was developed by first spraying with a potassium periodate solution, then with a borate buffered starch solution. The rest of the chromatogram was sectioned with respect to the fractions observed in the reference mixture. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the mimosine sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which was similarly applied to the thin layer chromatographic plate but not chromatographed.

Crude mimosine (5.8 mg) was diluted with inactive pure mimosine (56.4 mg) and crystallized from ethanol-water. Mimosine-C^{14} (50.7 mg), specific activity 3.176 (± 0.074) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be (34.3 ± 0.8) x 10^3 counts $minute^{-1}$ $mmole^{-1}$.

B. 3-HYDROXY-4-PYRIDONE-C^{14}

Diluted mimosine-C^{14} (47.8 mg, .24 mmole, specific activity 3.176 (± 0.074) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (61 mg) at 195-200° and .01 mm pressure for 4 hours. The distillate was redistilled at 180° and .01 mm pressure and again at 170° and .01 mm pressure to give 3-hydroxy-4-pyridone-C^{14} (23.9 mg, 89%). 3-Hydroxy-4-

pyridone-C^{14}, specific activity 2.097 (± 0.062) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. Thus it was demonstrated that 66.0 ± 1.7 % of the mimosine activity was located in the pyridone ring.

7. CALCIUM GLYCERATE-3-C^{14}

A. MIMOSINE-C^{14}

Calcium glycerate-3-C^{14} (5.8 mg, total activity 50 μc, specific activity 2.13 mc $mmole^{-1}$) was dissolved in water (10 ml). For radio-assay, a sample of this solution (100 λ) was diluted to 10 ml and 10 λ portions, i.e., 10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 268.1 (± 3.9) x 10^5 counts $minute^{-1}$.

The solution (10 ml) containing the calcium glycerate-3-C^{14} was administered equally to 20 Mimosa plants (7 weeks old) by the "wick" method (Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 2 days and mimosine (14.0 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the "wicks" were extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml. Samples (10 λ each) of this solution were assayed for residual activity. A total of 276.1 (± 3.2) x 10^4 counts $minute^{-1}$ or 10.3% of the original activity had not been absorbed by the plant.

A sample of mimosine (50 μg) was assayed for radiopurity by thin layer chromatography. A reference mixture of mimosine, calcium 3-phosphoglycerate and glyceric acid were run alongside the samples on a Silica G plate in ethanol-water, 4:1. The reference was developed with potassium periodate followed by borate buffered starch sprays and the rest of the

chromatogram was sectioned with respect to the fractions observed in the reference. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the mimosine sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which was similarly applied to the thin layer plate but not chromatographed.

Crude mimosine (12.6 mg) was diluted with inactive pure mimosine (51.7 mg) and crystallized from ethanol-water. Mimosine-C^{14} (61.5 mg), specific activity 3.821 (± 0.086) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be 19.6 (± 0.4) x 10^3 counts $minute^{-1}$ $mmole^{-1}$.

B. 3-HYDROXY-4-PYRIDONE-C^{14}

Diluted mimosine-C^{14} (53 mg, .268 mmole, specific activity 3.821 (± 0.086) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (67 mg) at 195-200° and .002 mm pressure for 4 hours. The distillate was redistilled at 180° and .002 mm pressure and again at 170° and .002 mm pressure to yield 3-hydroxy-4-pyridone-C^{14} (24.6 mg, 83%). 3-Hydroxy-4-pyridone-C^{14}, specific activity 1.787 (± 0.091) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. Thus it was demonstrated that 46.7 ± 2.6 % of the mimosine activity was located in the pyridone ring.

8. RIBOSE-1-C^{14}

A. MIMOSINE-C^{14}

Ribose-1-C^{14} (3.48 mg, total activity 50 μc, specific activity 2.16 mc

mmole^{-1}) was dissolved in water (10 ml). For radio-assay a sample of this solution (100 λ) was diluted to 10 ml and 20 λ portions, i.e., 2×10^{-5} of the original activity, were plated in triplicate. The total activity of the sample was found to be 212.2 (± 4.5) x 10^5 counts minute^{-1}.

The solution (10 ml) containing ribose-1-C^{14} was administered equally to 20 Mimosa plants (7 weeks old) by the "wick" method (Section II: Mimosine Biosynthesis: C. Mode of Administration) (Figure 12). The plants were harvested after 3 days and mimosine (5.6 mg) was isolated from the sap (Section II: Mimosine Biosynthesis: D. Isolation of Alkaloid).

After harvesting was completed, the small glass feeding receptacles were repeatedly rinsed and the "wicks" were extracted three times with distilled water. The combined washings and extracts were made up to a volume of 100 ml. Samples (10 λ each) of this solution were assayed for residual radioactivity. A total of 1.85 (± 0.03) x 10^5 counts minute^{-1} or 0.9% of the original activity had not been absorbed by the plants.

A sample of mimosine (50 μg) was assayed for radiopurity by thin layer chromatography. References of ribose and mimosine and the sample were run on Silica G plates in ethanol-water, 4:1. The references were developed with a potassium periodate spray followed by spraying with a borate buffered starch indicator. The rest of the chromatogram was sectioned with respect to the fractions observed in the references. The absorbant from each section was scraped onto a planchette and its radioactivity determined. No radiochemical impurities were detected in the mimosine sample by this method. The radioactivity found in the section containing the mimosine corresponded to that of a sample of the original preparation which was similarly applied to the thin layer plate but not chromatographed.

Crude mimosine (4.6 mg) was diluted with inactive pure mimosine (56.7 mg) and crystallized from ethanol-water. Mimosine-C^{14} (56.7 mg), specific activity 4.605 (± 0.113) x 10^3 counts minute^{-1} mmole^{-1}, was obtained. On the assumption that the original mimosine sample contained no radiochemical impurities, its specific activity was calculated to be (62.3 ± 1.5) x 10^3 counts minute^{-1} mmole^{-1}.

B. 3-HYDROXY-4-PYRIDONE-C^{14}

Diluted mimosine-C^{14} (50.7 mg, .256 mmole, specific activity 4.605 (± 0.113) x 10^3 counts $minute^{-1}$ $mmole^{-1}$) was distilled in the presence of zinc dust (55 mg) at 195-200° and .003 mm pressure for 4 hours. The distillate was redistilled at 180° and .003 mm pressure and again at 170° and .003 mm pressure to give mimosine-C^{14} (22.7 mg, 80%). 3-Hydroxy-4-pyridone-C^{14}, specific activity 2.519 (± 0.059) x 10^3 counts $minute^{-1}$ $mmole^{-1}$, was obtained on crystallization from benzene-ethanol. Thus it was demonstrated that 54.7 ± 1.9 % of the mimosine activity was located in the pyridone ring.

MEASUREMENT OF RADIOACTIVITIES

A. EQUIPMENT

Radioactivity was determined on solid samples of finite thickness. The equipment used consisted of an Automatic Sample Changer (Nuclear Chicago Model C115) connected to a Model 181B Scaler and a Model C111B Printer Timer. The detection system consisted of a Modified D48 gas-flow sample detector (for 1 1/4" samples) operating under Q Gas (98.7% helium and 1.3% butane) and surrounded by a guard detector tube. Cosmic ray background which actuates the guard tube is eliminated in a coincidence circuit so that only sample radiation is counted.

B. SAMPLES

Solid samples (200-1000 μg) of material to be counted were weighed directly onto tared aluminum planchettes (31 mm diameter (1 1/4"); area 7.545 cm^2) and dissolved in 1 N ammonia (0.2 ml) followed by 10% sucrose solution (0.05 ml). The solution in the planchette was overlaid with a thin lens paper disk (which exactly fit the planchette) to ensure even spreading

and evaporated to dryness under a heat lamp. The sucrose served to bind the paper to the planchette. The samples were heated under the lamp for ½ hour after they appeared dry, cooled and again weighed to determine the total weight of the material in the planchette. Each sample was prepared in triplicate and counted for 1000 counts for at least three readings (more if large variations in time readings were observed). The planchette holders for each sample were counted for background (100 counts) in the respective position in the automatic sample changer where the readings were taken.

C. CALCULATIONS

The complete calculations for a single sample and derived results for its duplicate will be presented as an example of how radioactivities were calculated. The example chosen is the aspartic acid-3-C^{14} (2 day) feeding experiment. The calculations will be carried through to show how confidence limits were retained in the final result.

Diluted Purified Mimosine-C^{14}

	Planchette 2236	Planchette 2337	Units
Mimosine sample weight	.700	1.177	mg
Total sample weight	11.62	11.78	mg
Sample density	1.54	1.56	mg cm^{-2}
Self absorption factor*	.808	.806	

The samples were counted five times for 1000 counts and the backgrounds counted for 100 counts four times. The first step in data treatment was to ascertain a count rate with confidence limits derived from standard deviation (σ) calculations.

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* Tolbert & Siri (1960).

	Time for 1000 counts	x	x^2
1.	8.56	- .08	.0064
2.	8.90	+ .26	.0676
3.	8.65	+ .01	.0001
4.	8.55	- .09	.0081
5.	8.55	- .09	.0081
	TOTAL 43.21		.0903

$$\text{AVERAGE} = \frac{43.2}{5} = 8.64 \qquad \sigma^2 = \frac{.0903}{5-1} = .0226$$

$$\text{COUNT RATE} = \frac{1000}{8.64} \pm \frac{1000}{8.64}\sqrt{\frac{.0226}{8.64^2}}$$

$$= 115.74 \pm 2.01 \text{ counts minute}^{-1}$$

The background was determined in the same manner and for this sample was found to be 2.764 ± 0.266 counts minute^{-1}.

The reading for the radioactive sample was corrected for background in the following manner:

$$115.74 - 2.76 \pm \sqrt{2.01^2 + 0.266^2}$$

$$= 112.98 \pm 2.03 \text{ counts minute}^{-1}$$

Correction is now applied for weight and self-absorption.

$$(112.98 \pm 2.03)\left[\frac{198.12 \text{ mg mmole}^{-1}}{1.177 \text{ mg} \times 0.808}\right] \text{ counts minute}^{-1}$$

$$= 2.347\ (\pm 0.052) \times 10^4 \text{ counts minute}^{-1} \text{ mmole}^{-1}$$

The corresponding value derived from planchette 2236 was

$$2.353\ (\pm\ 0.086) \times 10^4 \text{ counts minute}^{-1} \text{ mmole}^{-1}.$$

The mean specific activity for these duplicate readings was found:

$$\frac{2.347 + 2.353}{2} \pm \frac{\sqrt{.052^2 + .086^2}}{2} \times 10^4 \text{ counts minute}^{-1} \text{ mmole}^{-1}$$

$$= 2.350\ (\pm\ 0.050) \times 10^4 \text{ counts minute}^{-1} \text{ mmole}^{-1}.$$

Purified 3-Hydroxy-4-pyridone-C^{14}

Duplicate samples of 3-hydroxy-4-pyridone gave a mean average of $2.125\ (\pm\ 0.039) \times 10^4$ counts minute^{-1} mmole^{-1}. This was expressed as a percentage of the total mimosine activity.

$$\left[\frac{2.125}{2.350} \pm \frac{2.125}{2.350}\sqrt{\frac{.050^2}{2.350^2} + \frac{.039^2}{2.125^2}}\right] \times 100$$

$$= 90.41\ (\pm\ 2.42)\ \%.$$

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