THE BIOGENESIS OF THE PYRROLO(DE) PHENANTHRIDINE NUCLEUS

By

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BIOGENESIS OF THE PYRROLO(DE)

PHENANTHRIDINE NUCLEUS

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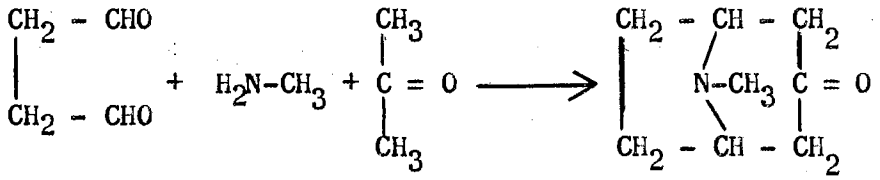
CHAPTER I

INTRODUCTION

Plant alkaloids have been of extreme interest because of their wide spectrum as physiologically active compounds. In 1877 Gerrard (1) isolated an alkaloid which he named "Narcissia" from the bulbs of Narcissus pseudonarcissus L. Oral administration of "Narcissia" caused nausea, vomiting, salivation, and purgation in animals (2).

Plants that produce alkaloids generally synthesize a number of closely related compounds. The nucleus of these alkaloids have been built up through a series of biological transformations involving condensations, methylations, decarboxylations and oxidation and reduction reactions. The amino acids or their metabolic products are undoubtedly obligatory intermediates for the synthesis of alkaloids. The first example of the chemical synthesis of an alkaloid employing biological intermediates (succinaldehyde, methyl amine, and acetone) was reported by Robinson (3). The alkaloid formed was tropinone. Later Schöph



and Lehmann (4) was able to synthesize tropinone at a pH of 7.0 to simulate physiological conditions.

Although the structure and chemical synthesis of a number of alkaloids has been reported, there is little information concerning their

biosynthetic pathways. One group of phenanthridine alkaloids that has been studied extensively is the Amaryllidaceae alkaloids. This study was initiated to find out how this type of ring was formed. To accomplish this, carbon-14 and tritium-labelled suspected precursors were administered to growing Narcissus plants. The distribution of the radioactivity in the plants and alkaloids was determined. The alkaloids were then separated by column chromotography, crystallized and degraded to determine the location of the radioactivity.

CHAPTER II

REVIEW OF LITERATURE

1. Amaryllidaceae Alkaloids

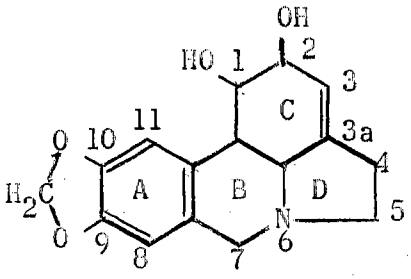
Although seventy different alkaloids have been isolated from one hundred and forty-four varieties of the Amaryllidaceae family and exist in seven ring systems, little has been done concerning the biogenetic origin of the alkaloids having the pyrrolo (de) phenanthridine nucleus. All of the alkaloids are weak bases with pK_a values of 6 to 9 . The one nitrogen present in the alkaloid is either a secondary or tertiary nitrogen. All of the alkaloids have an aromatic nucleus that is substituted with a methylenedioxy or methoxyl group(s). The number of carbon atoms varies from sixteen to twenty, depending on the ring system and the extent of substitution.

Lycorine is the most abundant and widely distributed of the alkaloids in the Amaryllidaceae family. Morishima (5) in 1897 isolated the alkaloid lycorine from Lycoris radiata Herb. Gorter (6) showed that the lycorine isolated by Morishima and the alkaloid isolated by Gerrard (1) were identical. Lycorine has been found in one hundred and eleven members of the Amaryllidaceae family.

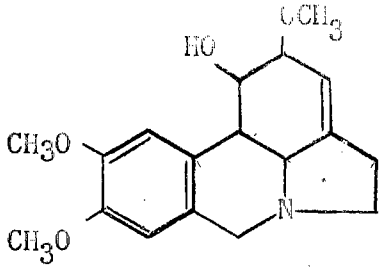
2. Proof of Structure of the Pyrrolo (de) Phenanthridine Nucleus

The alkaloids that will be studied in this thesis are lycorine (I), galanthine (II), pluviine (III), narcissidine (IV), and haemanthamine (V).

Their isolation and purification from the Deanna Durbin variety of the Narcissus plant has been described by Boit (7). They are all derivatives of the pyrrolo (de) phenanthridine nucleus except haemanthamine. This alkaloid has the 5,10-b-ethanophenanthridine nucleus.



I



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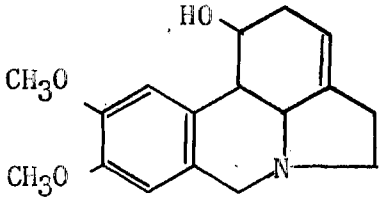
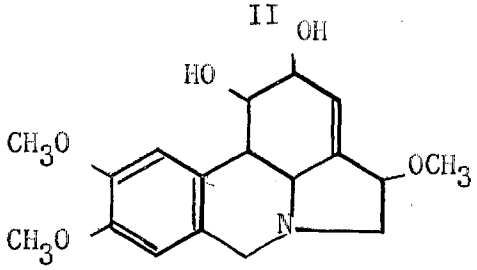
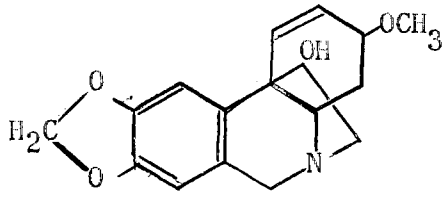


III



IV



V

A considerable amount of evidence has been accumulated to support formula (I) for lycorine ($C_{16}H_{17}NO_4$). Oxidation of lycorine with alkaline permanganate yields 4,5-methylenedioxyphthalic acid (hydrastic acid) (8). Lycorine, when distilled with zinc dust, yields a mixture of phenanthridine and substituted phenanthridines (9). Two compounds isolated after treatment with zinc are 4-methylphenanthridine and 4-ethyl-8,9-methylenedioxyphenanthridine. The later compound was confirmed by chemical synthesis. The double bond in ring C of lycorine was placed in

position 3,3a because of (1) the ease of aromatization of ring C and (2) the compound did not enolize.

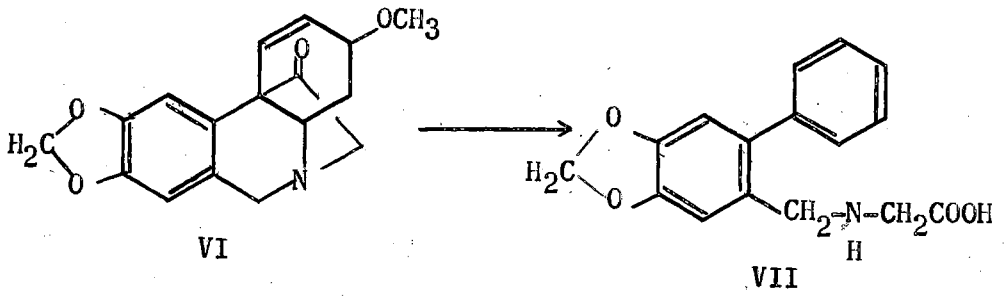
Pluviine (III), ($C_{17}H_{21}NO_3$), contains one reducible double bond, one hydroxyl group and two methoxyl groups. Pluviine gave 4,5-dimethoxyphthalic acid (m-hemipinic acid) after treatment with permanganate. The position of the hydroxyl group was determined by the formation of a red, phenolic betaine which was identical with the synthetic compound, The double bond in ring C was placed in positions 3,3a. (10).

Galanthine (II), ($C_{16}H_{23}NO_4$), differs from pluviine in that it contains one more methoxyl group. Galanthine was oxidized to m-hemipinic acid. Fales and Wildman (11) reported that the double bond was at carbon atoms-3,3a. The methoxyl group was placed on carbon atom 2 and the hydroxyl group on carbon atom 1.

Narcissidine (IV), ($C_{18}H_{23}NO_5$), contains one reducible double bond, three methoxyl and two hydroxyl groups. Since oxidation of narcissidine with permanganate yields m-hemipinic acid, it was evident that two of the three methoxyls are on the aromatic ring. Pulviine and galanthine are similar. By periodate oxidation, it was shown that the two hydroxyls (in ring C) were vicinal to each other. Treatment of narcissidine with sodium and amyl alcohol resulted in the loss of the hydroxyl group at C-2 to give pluviine (12) plus two other products. The isolation of pluviine indicated that narcissidine contains the pyrrolo(de)phenanthridine nucleus and that one hydroxyl group is located in the 1-position. Since the hydroxyls are vicinal, the second hydroxyl must be located at C-2 or C-11b. Because of the stability of narcissidine in acid and the ease of formation of the diacetyl derivative position 2 is favored. Spectral data suggest that the double bond is located between carbon atoms 3,3a. This

is analogous to the other alkaloids. The position of the aliphatic methoxyl group is not firmly substantiated. Fales and Wildman (12) have assigned the aliphatic methoxyl group to position 4. This conclusion is based on the inertness of narcissidine to potassium-t-amyl oxide and base-catalyzed elimination reactions.

Haemanthamine (V), ($C_{17}H_{19}NO_4$), contains one methoxyl, one methylenedioxy, and one hydroxyl group. The alkaloid when treated with dilute hydrochloric acid loses the methoxyl group. The ultraviolet spectrum remains unchanged. Since there is no change in the spectrum, the methoxyl group can not be aromatic. From the infrared spectrum and the inertness of the base to mercuric acetate and selenium dioxide, it was concluded that the nitrogen atom was part of a bridged ring system (spirane). Oxidation by chromic acid in pyridine yielded a ketone VI with a spectrum that indicated the hydroxyl group to be located in the 5 membered ring. Aromatization of ring C occured when the ketone was refluxed with potassium-t-butoxide in t-butanol.

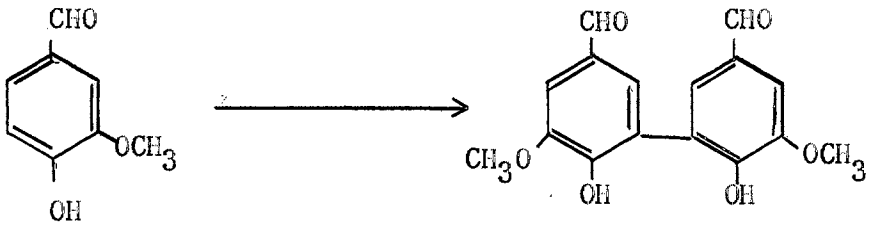


VI VII

The structure of VII was verified by a chemical synthesis (13).

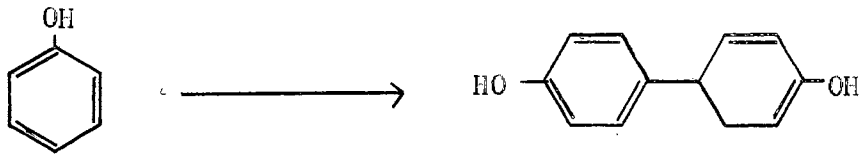
3. Proposed Mechanisms for the Biogenesis of the Pyrrolo (de) Phenanthridine Nucleus

Barton and Cohen (14) and Wenkert (15) have proposed two schemes for the biogenesis of the Amaryllidaceae alkaloids. Barton and Cohen suggested that the oxidation of structually related phenols affords radicals which are stable at room temperature. Carbon - carbon coupling due to radical pairing can be ortho - ortho, ortho - para or para - para. An example of ortho - ortho coupling is shown by the oxidation of vanillin (VIII) by potassium persulphate to yield dehyrodivanillin (IX). (16).



VIII IX

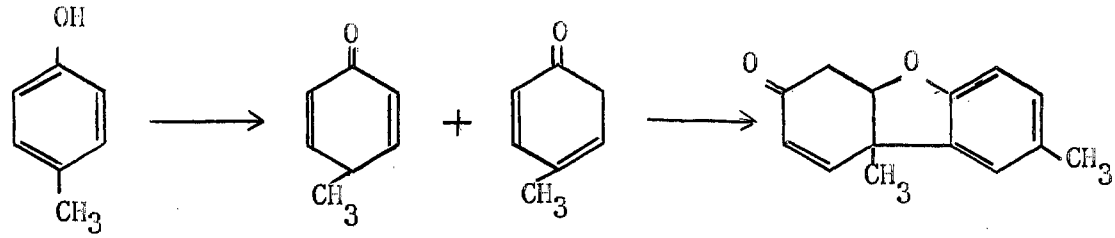
Para - para coupling is exhibited by the lead tetra-acetate oxidation of phenol (X) to give 4,4'-dihydroxydiphenyl (XI) (17).



X XI

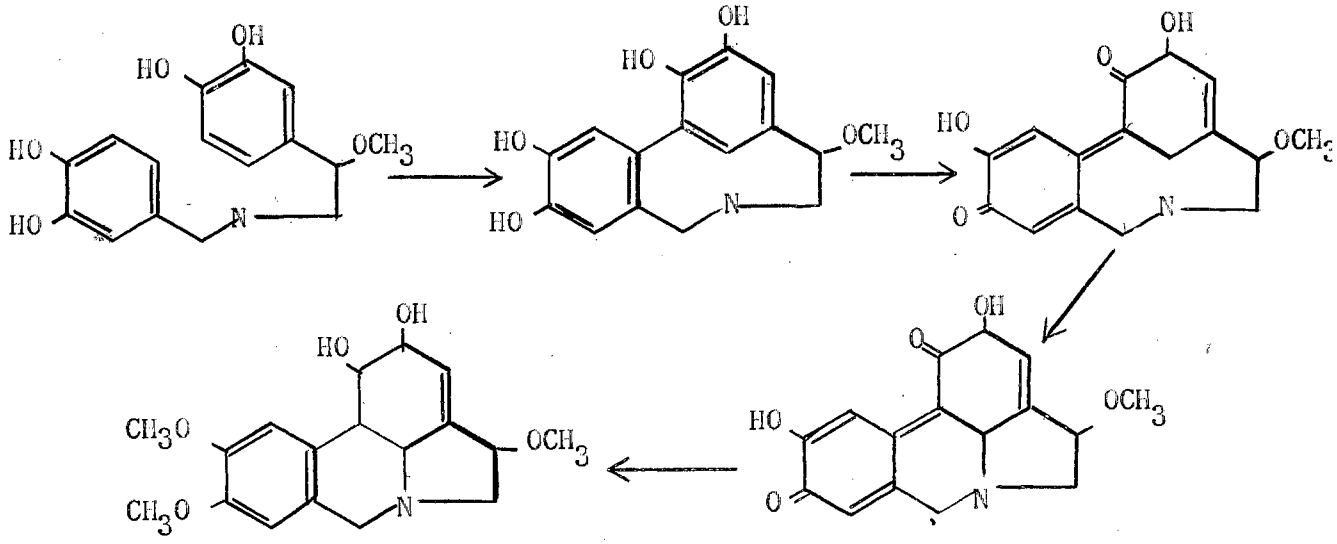
The third type of coupling, ortho - para, is best illustrated by the oxidation of para-cresol (XII) with hydrogen peroxide and ferrous

sulphate to the crystalline ketone XIII (18).



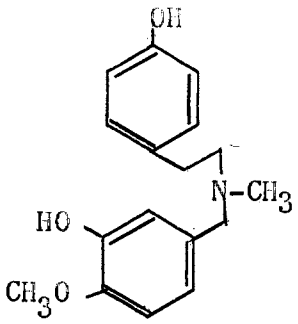
XII XIII

If the phenanthridine ring structure is going to arise by a free radical mechanism it must do so by the latter type of coupling. Using the above type of information, the biogenesis of narcissidine (IV) may be shown as follows:

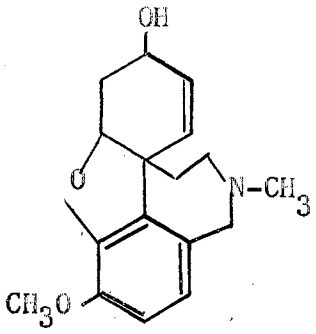


IV

Recently Barton (19) reported the synthesis of the phenol XIV labelled with carbon-14. When this was injected into King Alfred plants, some carbon-14 was found in galanthamine (XV). The position of the carbon-14 in the alkaloid was not determined. If the labelled phenol was incorporated directly, then ortho - para coupling may indeed occur in biological systems.

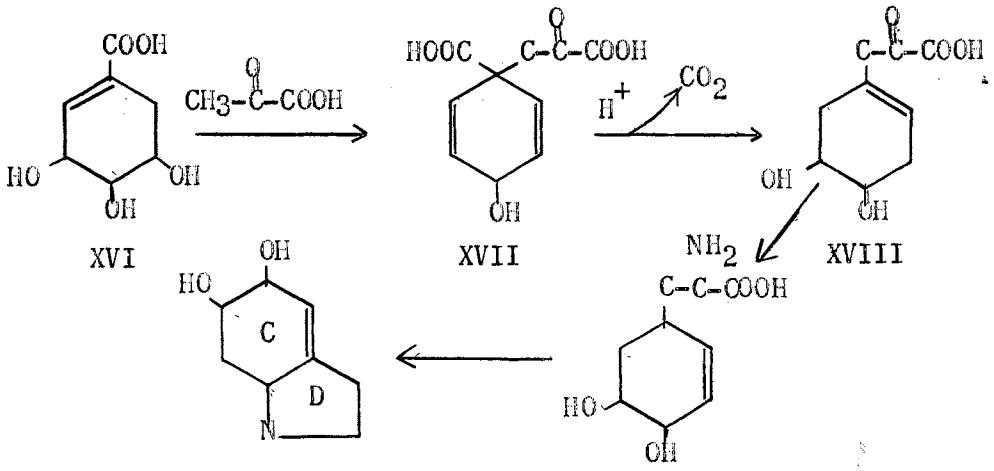


XIV



XV

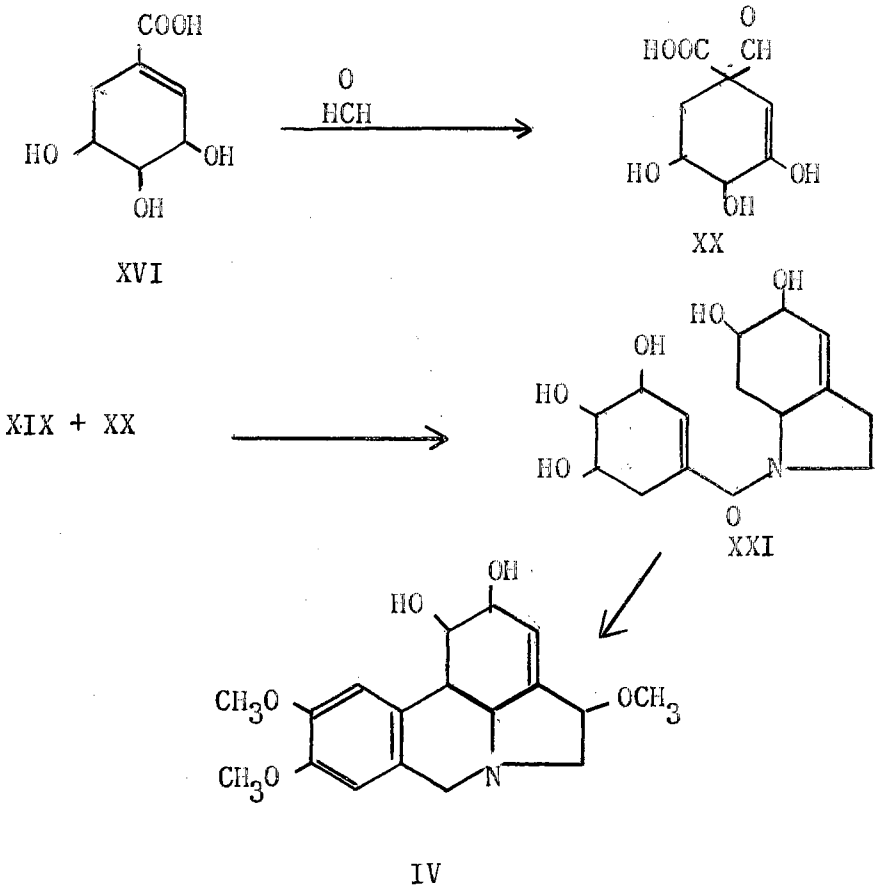
Wenkert's biogenetic scheme is based on the incorporation of shikimic acid into ring A and prephenic acid into rings C and D of narcissidine (IV). Condensation of shikimic acid (XVI) with pyruvate forms prephenic acid (XVII). The loss of one mole of carbon dioxide, results in the formation of compound XVIII. Compound XVIII by subsequent amination, oxidation(s) and reduction(s) would then give rise to rings C and D XIX.



XVI XVII XVIII XIX

Shikimic acid (XVI) condenses with formaldehyde to give compound XX. Condensations of compound XIX with XX would form an intermediate XXI structurally similar to the phenol XIV that Barton synthesized.

Compound (XXI) could be coupled and transformed as needed to give narcissidine (IV).



4. Pharmacology

Pharmacological studies of the pure Amaryllidaceae alkaloids have been studied recently. The alkaloids that have the pyrrolo (de) phenanthridine nucleus do not exhibit any analgesic properties. A number of alkaloids of this family have been screened for cardiovascular activity. Galanthine has been reported to lower the blood pressure of rabbits with experimental hypertonia at doses of 50 mg. per kilogram of body weight (20). Lycorine causes a slight decrease in blood pressure at levels of 31 mg. per kilogram of body weight. One report from Russia indicates that galanthamine has therapeutic use in poliomyelitis (19).

CHAPTER III

EXPERIMENTAL

1. Materials

DL-phenylalanine-3-C^{14} was obtained from Isotopes Specialties Company. DL-tyrosine-2-C^{14} and sodium formate-C^{14} were obtained from the Volk Radiochemical Company. Barium carbonate-C^{14} and tritium gas were obtained from Union Carbide Nuclear Company, Oak Ridge National Laboratory. Tritium-labelled noradrenalin was generously supplied by the National Institutes of Health.

Tyrosine and shikimic acid were labelled by the tritium exchange method of Wilzbach (21). Two hundred mg. of shikimic acid was dissolved in 2 ml. of water and transferred to a 10 ml. tritium-labelling ampoule. The solution of shikimic acid was taken to dryness with a rotary evaporator. The ampoule was placed into a desiccator and dried in vacuo over phosphorus pentoxide. The ampoule containing the shikimic acid was evacuated to 0.05 mm. pressure. Approximately 1 curie of tritium gas was pumped into the ampoule containing the organic compounds by means of a Toepler pump. The shikimic acid was exposed to the tritium gas for three months. After this time the tritium gas was removed by the means of a Toepler pump and transferred into another ampoule. The shikimic acid was dissolved in 5 ml. of water, transferred to a 50 ml. round bottom flask, taken to dryness and washed with 5 ml. of water 20 times to remove any exchangeable tritium. A paper chromatogram of

the crystalline shikimic acid was developed using Whatman No. 1 filter paper and irrigated in a solvent containing benzyl alcohol-tertiary butanol - isopropanol - water (3:1:1:1, v/v) (1.8 % formic acid is then added). The chromatogram was scanned by a Radiological Service Co. Automatic Windowless Chromatogram Scanner. There was a radioactive peak at the origin and a radioactive peak that coincided with a spot of authentic shikimic acid. The latter was detected by spraying with 0.5 % aqueous KIO_4 followed by benzidine. Approximately 10 mg. of the tritium-labelled shikimic acid was placed at the origin of a 9-inch sheet of Whatman No. 1 paper and irrigated with the above solvent. A half-inch strip was cut from one edge of the chromatogram and developed with KIO_4-benzidine. The material on the chromatogram coinciding with the positive spot was cut out and eluted with water. Five ml. of eluate was collected and then evaporated to dryness in a tared flask. The yield of dried residue was 5.8 mg. This was shown to be chromatographically pure when rechromatogramed with authentic shikimic acid.

Two hundred mg. of L-tyrosine was labelled by the same procedure as the shikimic acid. The tyrosine was purified by use of a Solka Floc cellulose column. The cellulose had been soaked in a mixture of butanol - pyridine - water (1:1:1 v/v) and added to a column 17 cm. x 1.1 $cm.^2$ Seven mg. of the tritium-labelled L-tyrosine was dissolved in a minimum amount of water and added to the column. The column was developed with the butanol - pyridine - water mixture. One ml. fractions were collected. The ninhydrin-positive fractions were combined and taken to dryness. The residue was dissolved in 25 ml. of water. The amount of L-tyrosine was determined by microbiological assay using Leuconostoc mesenteroides P - 60. Growth was measured by titrating the acid produced after 72 hrs.

at 37° C. (22). A quantitative ninhydrin determination was also done to determine the amount of tyrosine (23). Results with both methods were in excellent agreement.

The plants used in the following experiments were large-cupped Narcissi (N. incomparabilis Mill.), Deanna Durbin variety supplied by Peter DeJager and Sons, South Hamilton, Massachusetts.

2. Radioactivity Determinations

Analyses of the tritium compounds were made with the Automatic Packard Tricarb Scintillation Spectrometer. One-tenth ml. aliquot was added to 10.0 ml. of scintillation fluid (24) and the samples were counted for 10 minutes. The scintillation fluid was as follows: 60 ml. of toluene, 40 ml. of absolute ethanol, 0.4 g. of diphenyloxazole, and 0.02 g. of 1,3-bis-2-(5-phenyloxazolyl)-benzene. Carbon-14 analyses were performed by liquid scintillation counting and also by the wet combustion method of Van Slyke, Steele and Plazin (25). The CO_2 was collected in an ionization chamber and counted with a vibrating reed electrometer. The radioactive CO_2 was collected (as sodium carbonate) during the experiment was released by using 35 % perchloric acid. The CO_2 was collected in an ionization chamber and counted with a vibrating reed electrometer. A Count Rate Meter (Nuclear-Chicago) with a P-10 probe and Recti-Riter was used to measure the decrease in radioactivity in the photosynthesis experiments.

3. Isolation of the Alkaloids

Three weeks after the administration of the suspected precursors, the plants were washed free of dirt and cut into small sections. The roots, bulbs and leaves were kept separate. The plant material was dried

in an oven at 100° C for 24 hours and then ground to a fine powder in a Micro Wiley Mill. The crude alkaloids were extracted by a procedure similar to that of Boit (Figure 1). The alkaloids were separated on a column of alumina (Merck, #71707). Eighty g. of alumina was slurried in benzene and added to a column (185 cm x 4.3 cm^2). The crude alkaloids were dissolved in benzene and added to the column. The alkaloids were separated by adding 50 ml. portions benzene and benzene-ethylacetate (to 100 % ethylacetate by 10 % increments) followed by 100 ml. portions of ethylacetate and ethylacetate-ethanol (to 20 % ethanol). The alkaloids eluted from the alumina column were identified by paper chromtography, using Whatman No. 1 paper and irrigating with a solvent containing 50 ml. of butanol and 10 ml. of concentrated hydrochloric acid (saturated with water). The organic, water-saturated phase was used. The chromatograms after drying were sprayed with a modified Dragendorff's reagent (26). The alkaloid positive spots were bright orange. The R_f of the alkaloids from the column were in agreement with the R_f of authentic samples.

4. Root Uptake Studies with DL-Phenylalanine-3-C^{14}

Ten growing Narcissus plants, with stems 5 to 10 inches high, were placed in a desiccator (25 cm. I.D.). The bulbs were placed into holes on a polystyrene tray. The tapwater contained 2 mg. (35,600 muc.) of DL-phenylalanine-3-C^{14} plus 98 mg. of DL-phenylalanine and 0.25 % benzoic acid. Air was drawn into the desiccator by means of a vacuum pump and bubbled through the liquid medium by means of air sparges at a rate of 20 l.per minute. The air was first scrubbed free of carbon dioxide by passing through two scrubbing towers containing 200 ml. of 20 per cent

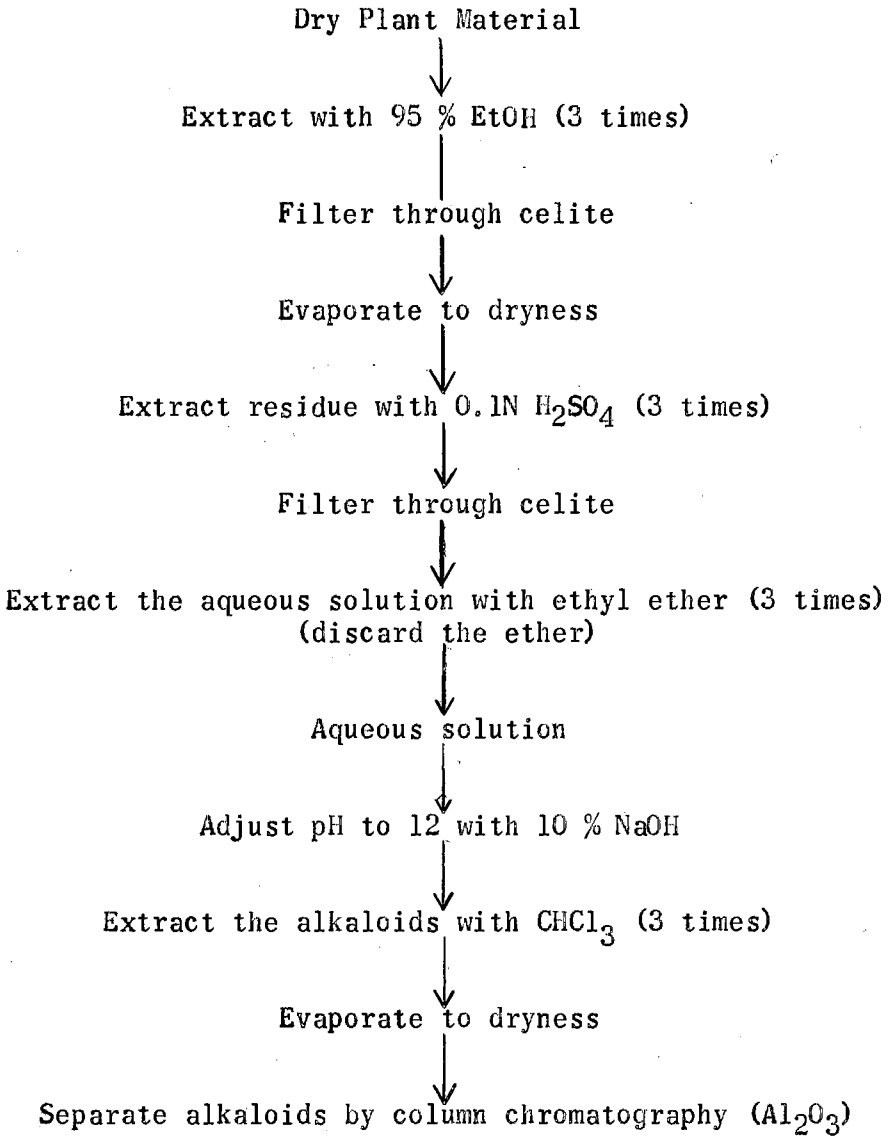


Figure 1. Outline for the Isolation of the Alkaloids from the Narcissus plants.

NaOH. The CO_2-free air was then saturated with water. The $C^{14}O_2$ in the air after leaving the desiccator was scrubbed through two towers containing 100 ml. of 15 % NaOH. The air was dried in Drierite before entering the vacuum pump. The NaOH was changed at periodic time intervals (Figure 2). Ten ml. aliquots were taken from the medium at the same time. The plants remained in the desiccator for 5 days with approximately 14 hrs. of light followed by 10 hrs. of darkness. The light was supplied by one 40 watt fluorescent bulb at a distance of 3 feet. The plants were then placed into moist pearlite for 3 additional days.

The roots, bulbs and leaves were separated and the wet material was extracted with 95 % ethanol in a Waring Blendor. The results of the experiment are described in Table I. Figure 2 shows the disappearance of DL-phenylalanine-3-C^{14} from the liquid medium and the formation of $C^{14}O_2$.

5. Incorporation of $C^{14}O_2$ into Growing Narcissus Plants

Three growing Narcissus plants were placed in the bottom of a photosynthesis chamber. Moistened pearlite was placed around the roots and 1.1 mg. (142 uc) of radioactive Na_2CO_3 was dissolved in 3 ml. of water containing two drops of phenolphthalien and added to the side arm of the chamber. The ball joints were greased and the chamber was sealed. A P-10 probe from the Count Rate Meter was placed close to a micro window in the chamber to follow the uptake of $C^{14}O_2$.

An excess of dilute sulfuric acid was injected through a rubber plug to release the $C^{14}O_2$. The side arm was then heated with warm water to accelerate the release of $C^{14}O_2$. An additional 15 mg. of Na_2CO_3, dissolved in 1 ml. of water, was injected. The light, 150 foot candles,

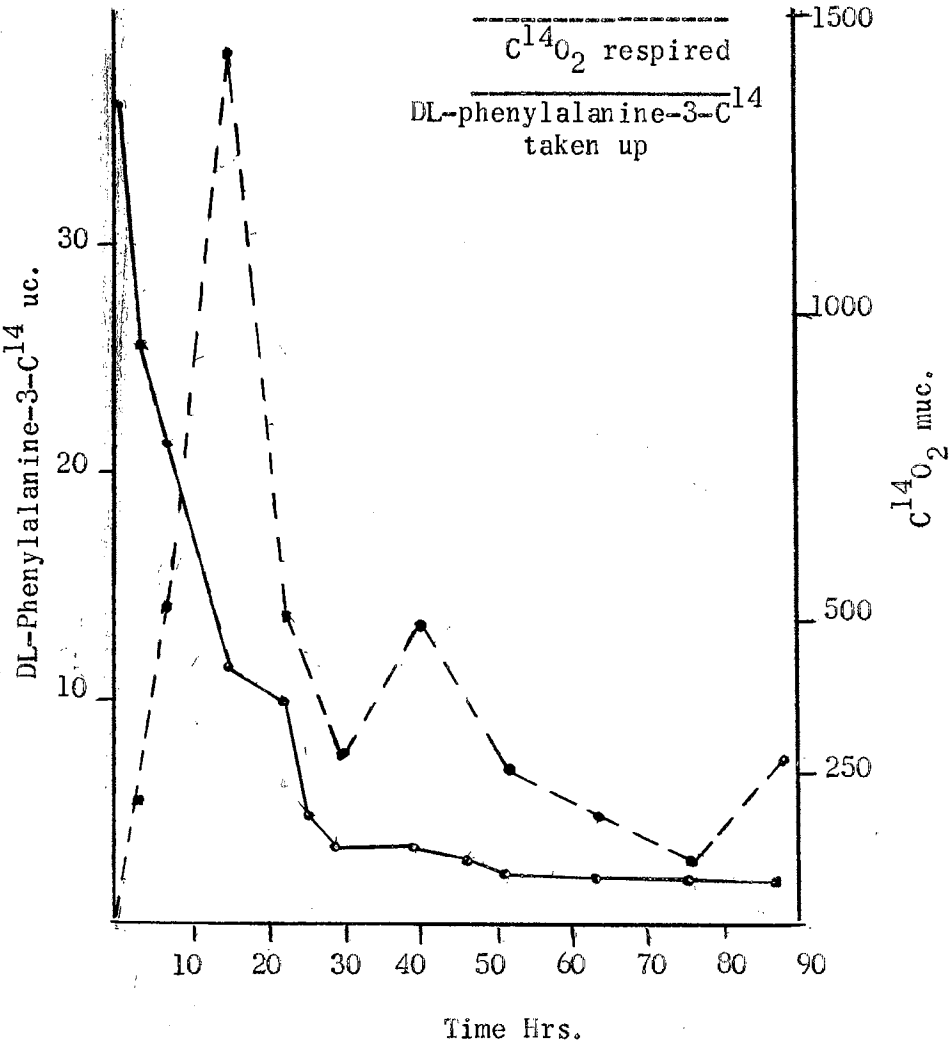


Figure 2. Uptake of DL-Phenylalanine-3-C^{14} and $C^{14}O_2$ Formation by Narcissus Plants

TABLE I

THE DISTRIBUTION OF CARBON-14 IN NARCISSUS PLANTS FROM DL-PHENYLALANINE-3-C^{14}

Total Radioactivity - 35,600 muc.

Tissue	Wet Weight g.	Total Volume of EtOH Extract ml.	Amount of Radioactivity		
			EtOH Extract muc.	Alkaloids muc.	Percent
Leaves	163	700	700	0	0*
Roots	94	400	440	0	0*
Bulbs	591	1900	360	0	0*
Total Radio-activity			1500	0	4.2
$C^{14}O_2$ Formed			4235	0	11.9
Total Radio-activity Re-maining in Medium			2460	0	6.9

*Per cent carbon-14 found in the alkaloid fraction.

was supplied by a fluorescent bulb. The plants remained in the $C^{14}O_2$ atmosphere for 12 days. At this time, 5 ml. of 9 N NaOH was injected into the side arm to convert the $C^{14}O_2$ to $Na_2C^{14}O_3$. When the radioactivity had decreased to 1500 CPM, the chamber was flushed with air by means of a water aspirator for 2 hours. The plants were removed and the roots, bulbs and leaves were separated and dried. The alkaloids were isolated as shown in Figure 1. Table II summarizes the distribution of carbon-14 from this experiment.

6. Incorporation of Radioactive Compounds into Lycorine, Narcissidine, Pluviine, Galanthine and Haemanthamine

a. By Root Uptake

One hundred Deanna Durbin bulbs were planted in boxes and vernalized for six weeks at 4°C. After this time the plants were placed in the green house at a temperature range of 15-20°C. When the plants had grown to a height of 1 inch above the soil, thirty-two plants were removed and washed. Each plant was placed in a beaker containing 150 ml. of water and the radioactive compound (Table III). Aliquots were taken from each beaker at various times to follow the uptake of the radioactive compound. After two days the plants were removed from the beakers and replanted. At the time of replanting the radioactivity in the water had decreased 80-90 per cent. After the plants had grown for an additional 24 days at a temperature range of 23-30°C., one plant from the DL-tyrosine-2-C^{14} root uptake experiment was removed. The leaves, bulb, and roots were separated and dried in vacuo at 55°C. The alkaloids were isolated as outlined in Figure 1. Table IV summarizes the distribution of carbon-14.

b. By Injection into the Bulb

Simultaneously with the root uptake studies, the same radioactive

TABLE II

THE DISTRIBUTION OF CARBON-14 IN NARCISSUS PLANTS EXPOSED TO AN ATMOSPHERE OF $C^{14}O_2$

Total Radioactivity = 142,000 muc.

Tissue	Dry Weight g.	Total Volume of EtOH Extract ml.	Amount of Radioactivity: EtOH Extract muc.	Alkaloid muc.	Residue after EtOH Extraction muc.	Percent
Leaves	3	234	2089	0	2050	2.92
Roots	1	162	130	0	184	0.15
Bulbs	17	322	319	0	2020	2.35
Total Radioactivity			2538	0	4254	4.90

TABLE III

RADIOACTIVE COMPOUNDS ADMINISTERED TO THE NARCISSUS PLANTS

No. of Plants		Compound	Weight of Compounds*		Amount of Radio-activity	
Injec-tion	Root Uptake		Labelled	Carrier	Injected	Root Uptake
			mg.	mg.	muc.	muc.
6	8	DL-Phenylalanine-3-C^{14}	1.66	30.34	9,850	13,000
6	8	DL-Tyrosine-2-C^{14}	18.3	13.7	25,000	33,000
5	8	L-Tyrosine-H^3	3.0	30.0	16,500	26,000
6	8	Noradrenalin-H^3	0.01	32.0	10,200	13.600
6	0	Sodium Formate-C^{14}	2.04	10.0	39,000	--

*All compounds were dissolved in 16 ml. of water

TABLE IV

DISTRIBUTION OF CARBON-14 IN THE NARCISSUS PLANTS AFTER ROOT UPTAKE OF DL-TYROSINE-2-C^{14}

Total Radioactivity - 4160 muc.

Tissue	Dry Weight g.	Total Volume of EtOH Extract ml.	Amount of Radioactivity: EtOH Extract muc.	Alkaloid muc.	Residue after EtOH Extraction muc.	Percent
Leaves	2.20	348	33	0	390	10.1
Roots	2.10	335	455	0	900	32.5
Bulbs	24.40	360	51	0	430	11.6
Radioactivity Remaining in Medium						15.0
Total Recovery of Carbon-14						69.2

compounds were injected into the center of the bulbs. (Table III). The labelled compounds were injected three times at two-day intervals (0.4 ml. in the first injection and 0.3 ml. for the second and third injections). A number 23 needle and a one-half ml. syringe were used.

The plants injected with DL-tyrosine-2-C^{14} were harvested 41 days after the last injection and washed. The leaves, bulbs and roots were dried separately in an oven for 24 hours at 110°C.

The dry material was ground in a Micro Wiley Mill and the alkaloids were extracted as described in Figure 1. The chloroform extract containing the alkaloid from the bulbs was placed in the refrigerator for two days. The lycorine that crystallized was filtered, dried and weighed. (23.8 mg., m. 215-220°C.). One mg. of the lycorine was dissolved in 1 ml. of 95 per cent ethanol and the radioactivity in an aliquot was determined. The lycorine contained 19.5 muc. A paper chromtogram of the isolated lycorine was developed and compared with authentic lycorine. The alkaloid positive spot and radioactive spot had the same R_f as the authentic lycorine. The radioactivity was determined by cutting the paper chromtogram into one-half inch sections, placed in a vial containing 10 ml. of scintillation fluid and counted in the liquid scintillation spectrometer.

The remaining 22.8 mg. of lycorine was recrystallized by dissolving in 15 ml. of boiling ethanol. The yield was 6.3 mg. and the amount of radioactivity was 5.6 muc. (258 muc. per mmole). The compound melted between 254-257°C.

The chloroform filtrate from the bulbs (after removal of the lycorine) was added to the chloroform filtrate from the leaves. The lycorine in this mixture was removed by evaporating to dryness and extracting

with benzene. All of the alkaloids except lycorine are soluble in benzene. The benzene extract (313 muc.) was added to an alumina column and the alkaloids were separated as previously described. Twelve ml. fractions were collected. The distribution of the radioactivity in the fraction collected is shown in Figure 3. Tubes 132-177 (third radioactive peak) were combined and evaporated to 5 ml. The radioactive material in these tubes contained narcissidine as was shown by paper chromotography. The solution was decolorized with 50 mg. of activated carbon (Darco G-60) and filtered. The filtrate was evaporated. The yield of crude narcissidine was 163 mg. This material was recrystallized by dissolving in hot acetone followed by cooling to 0^{o}C. for 30 minutes. The mother liquor was removed and the narcissidine was dried in vacuo. The product melted between 186-196^{o}C. and weighed 9.3 mg., and contained 2.4 muc. (86 muc. per mmole). A second crop of narcissidine was isolated (15.9 mg., m. 180-193^{o}C., 11.1 muc. 232 muc. per mmole). Table V summarizes the distribution of carbon-14.

The pluviine (first peak in Figure 3), galanthine and hamanthamine (second peak in Figure 3) will be crystallized and degraded in subsequent experiments.

The bulbs injected with tritium-labelled noradranalin and DL-phenylanine-3-C^{14} were harvested 41 days after the last injection. The alkaloids were isolated by the procedure as described in Figure 1. The alkaloids from the DL-phenylalanine-3-C^{14} experiment have not been separated by column chromtography. Table VI summarizes the distribution of carbon-14 from the DL-phenylalanine-3-C^{14} experiment. The alkaloids in the residue, from the chloroform extract of the noradrenalin experiment, were extracted with benzene. The benzene-soluble alkaloids were then

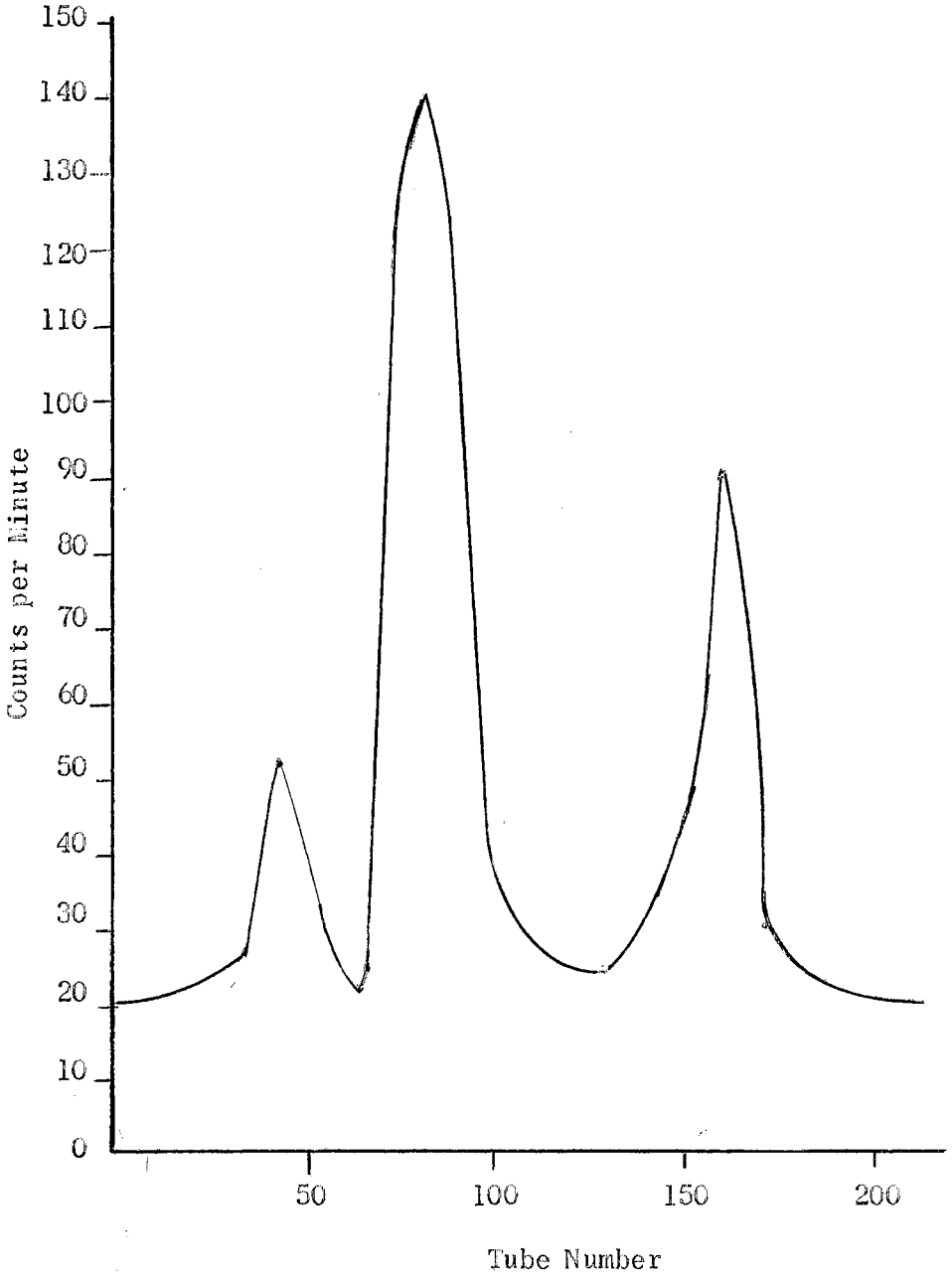


Figure 3. Separation of the Alkaloids from the Narcissus Plants Injected with DL-tyrosine-2-C^{14}

TABLE V

DISTRIBUTION OF CARBON-14 IN THE NARCISSUS PLANTS AFTER THE INJECTION OF DL-TYROSINE-2-C^{14}

Total Radioactivity 20,800 muc.*

Tissue	Dry Weight g.	Total Volume of EtOH Extract ml.	Amount of Radioactivity: EtOH Extract muc.	Alkaloid muc.	Residue After EtOH Extraction muc.	Percent
Leaves	16.5	635	1235	135	3060	21.3
Roots	14.0	630	0	0	185	0.9
Bulbs	129.5	1433	1768	207	10590	60.3
Total Recovery of Carbon-14						82.5

*Based on five plants. See Table III

TABLE VI

DISTRIBUTION OF CARBON-14 IN THE NARCISSUS PLANTS AFTER INJECTION OF DL-PHENYLALANINE-3-C^{14}

Total Radioactivity - 9,850 muc.

Tissue	Dry Weight g.	Total Volume of EtOH Extract ml.	Amount of Radioactivity: EtOH Extract muc.	Alkaloid muc.	Residue After EtOH Extraction muc.	Percent
Leaves	26.2	438	440	--	1900	23.8
Roots	26.8	359	17	0	0	0.0
Bulbs	163.2	1062	1029	90	90	61.5
Total Recovery of Carbon-14						85.3

separated on an alumina column. All of the fractions that were counted had essentially no radioactivity. The crude lycorine remaining after the extraction with benzene was filtered off and dried. The product weighed 40 mg. and melted between 200°-225°C. Table VII summarizes the distribution of the tritium-labelled compound.

The plants injected with sodium formate-C^{14} and tritium-labelled shikimic acid and L-tyrosine were harvested 41 days after the last injection. The alkaloids will be isolated and the position of the labelling determined.

7. Degradation Procedures

a. Lycorine

The radioactive lycorine isolated from the Narcissus plants after the injection of DL-tyrosine-2-C^{14} was degraded according to the scheme in Figure 4.

Lycorine (I) (137 mg., 0.48 mmole) was dissolved in boiling methanol (20 ml.). The radioactive lycorine (6.5 mg. 0.02 mmole) in methanol (2 ml.) was added to this solution. This was then treated with a twenty-five fold excess of methyl iodide (1.8 g., 12.5 mmole). The mixture was refluxed on a steam bath for one and one-half hours, cooled to room temperature and evaporated to dryness. The lycorine methiodide (XXII) was washed with ethyl ether (3 times with 5 ml. portions) to remove the unreacted lycorine and methyl iodide. The compound was dried in vacuo. The yield of the α - and β -methiodides was essentially quantitative (224 mg., 0.52 mmole) (27).

The lycorine methiodide (XXII) was subjected to the Hofmann exhaustive methylation. Lycorine methiodide (110 mg. 0.250 mmoles) was dissolved in water (1 ml.). This was treated with a one and one-half fold excess

TABLE VII

DISTRIBUTION OF TRITIUM IN NARCISSUS PLANTS AFTER INJECTION OF NORADRENALIN-H^3

Total Radioactivity - 10,200 muc.

Tissue	Dry Weight g.	Total Volume of EtOH Extract ml.	Amount of Radioactivity		
			EtOH Extract muc.	Alkaloids muc.	Percent
Leaves	30.7	640	882	--	8.6
Roots	22.3	415	76	0	0.0
Bulbs	161.9	1190	1341	50	13.6

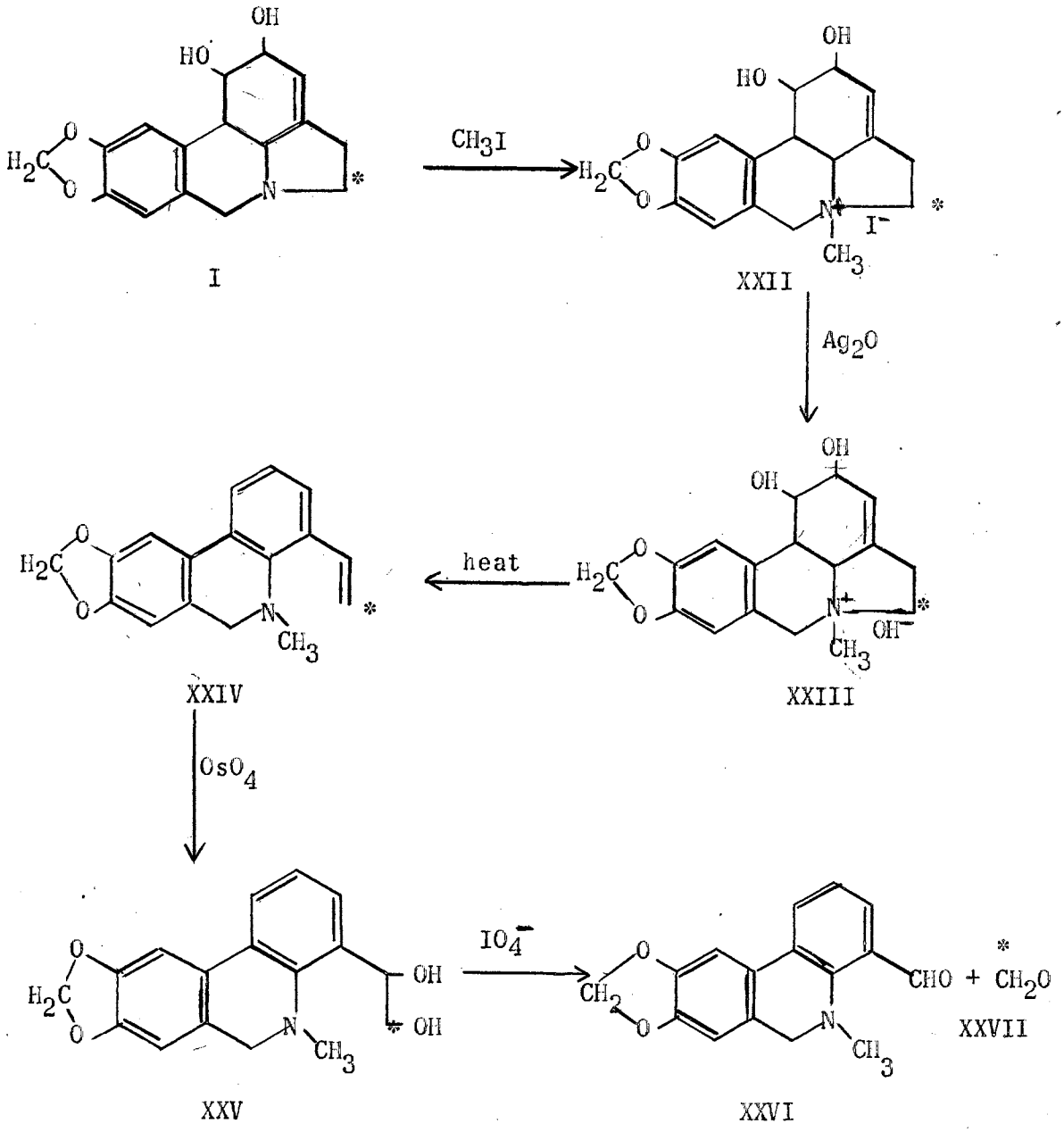


Figure 4. Degradation of Lycorine-5-C^{14}

of freshly prepared silver oxide. The mixture was stirred ten minutes on a steam bath and the insoluble silver oxide and silver iodide was removed by centifugation. The supernatant was decanted. The precipitate was washed with water (2 times with 2 ml. portions). The washings were combined with the original supernatant and evaporated in a 100 ml. round bottom flask. The residue in the flask was heated in an oil bath at 150°C for 30 minutes. The elimination of water, as indicated by frothing, is typical of the Hofmann exhaustive methylation reaction. The decomposition product XXIII was extracted with ethyl ether (2 times with 25 ml. portions) and the remaining residue was heated again for 4 hours. At this time the residue was extracted again with ethyl ether (2 times 10 ml. portions). The ether extracts were combined and evaporated. The yield of anhydrolycorine methine (XXIV) was 35 mg., (0.130 mmole). The yield was 52 percent.

The glycol-osmate-pyridine-complex of the anhydrolycorine methine was prepared by dissolving the methine (35 mg., 0.130 mmole) in ether (5 ml.) and pyridine (0.1 ml.) and cooled to -70°C. Osmuim tetroxide (37 mg., 0.143 mmole) in ethyl ether (3 ml.) was added dropwise to the solution of the methine over a period of 5 minutes. The dropping funnel was washed once with ethyl ether (2 ml.). The mixture was stirred for an additional 30 min. at -70°C. and then allowed to come to room temperature (about 30 min.). The reaction mixture was then stirred for another 2 hours. The insoluble glycol-osmate-pyridine complex was filtered and washed with ethyl ether (3 times with 5 ml. portions). The glycol (XXV) was formed by dissolving sodium sulfite (40 mg., 0.44 mmole) and potassium carbonate (12.5 mg., 0.09 mmole) in 50 per cent ethanol (8 ml.). The insoluble osmate complex was added to the sulfite-carbonate

solution. The mixture was stirred for 30 minutes and the glycol was extracted with ethyl ether (3 times with 10 ml. portions). The ether was evaporated. The yield at the glycol was 16 mg. (0.053 mmole, 41 per cent). Carbon atom-5 was oxidized to formaldehyde (XXVII) by oxidation of the glycol with sodium periodate. The glycol was dissolved in 95 per cent ethanol (1 ml.) and 20 per cent sulfuric acid (0.2 ml.). One ml. of sodium periodate (15 mg., 0.07 mmole in water) was added. After two and one-half hours the formaldehyde was removed by steam distillation. The distillate was collected in four fractions (13 ml., 14 ml., 15 ml., and 28 ml. volumes). Formaldehyde was determined by the method of Boyd and Logan (29). The first two fractions contained all of the formaldehyde (1000 ug., 0.033 mmole). The yield of formaldehyde was 62 per cent. One hundred ug. of formaldehyde was converted to the dimedone derivative. After one hour the derivative was removed by centrifugation and the amount of formaldehyde in the supernatant was determined. The yield of the dimedone was 52 per cent. The amount of radioactivity in the dimedone was then determined (Table VIII). Another aliquot of the formaldehyde was converted to the dimedone. The derivative was dried over night in vacuo. The melting point was 187-188°C. A mixed melting point with the authentic derivative showed no depression. Table VIII summarizes the results of the conversion of carbon atom-5 of lycorine to formaldehyde.

b. Narcissidine

The radioactive narcissidine (26 mg.) from the tyrosine-2-C^{14} experiment was added to carrier narcissidine (70 mg.). The narcissidine was then dissolved in acetone (5 ml.), filtered and crystallized. The yield of the crystalline product was 58 mg. (m. 186-196°C.). Narcissidine methiodide was formed by dissolving narcissidine (58 mg., 0.175

TABLE VIII

CONVERSION OF CARBON ATOM-5 OF LYCORINE TO FORMALDEHYDE

Compound	mg.	mmole	% Yield	muc./mmole
Lycorine	137	0.50	—	258
Lycorine methiodide	224	0.52	10.8	—
Lycorine methohydoxide	—	—	—	—
Anhydrolycorinemethine	70	0.26	50	—
Glycol of Lycorine	32	0.106	41	—
Formaldehyde (carbon-atom 5)	2.0	0.066	62*	288
Dimedone derivative of formaldehyde	10.2	0.034	52**	195

*Based on the colorimetric determination of the formaldehyde in the distillate.

**Based on the formaldehyde remaining in the supernatant after centifugation of the dimedone derivative.

mmole) in methanol (1 ml.). A twenty-five fold excess of methyl iodide (610 gm., 4.38 mmole) was added and the mixture was refluxed for 4 hours. The methanol and methyl iodide was removed in vacuo and the narcissidine was washed with ethyl ether (3 times with 5 ml. portions) and dried to yield narcissidine methiodide (77 mg., 0.170 mmole). The yield was essentially quantitative.

The narcissidine methiodide was dissolved in carbon dioxide free water (4 ml.) and treated with one and one-half fold excess of freshly prepared silver oxide. The mixture was heated on a steam bath with stirring and then centifuged. The supernatant was decanted. The residue was washed with water (2 times with 1 ml. portions). The supernatants were combined and sodium hydroxide (235 mg.) was added. The mixture was refluxed for 2 hours and allowed to stand for 12 hours at room temperature. The solution was then extracted with ethyl ether (3 times with 10 ml. portions.) There was no methine in the ether extracts. Therefore, subsequent degradations for the isolation of formaldehyde from carbon atom-5 of narcissidine were not possible.

CHAPTER IV

RESULTS AND DISCUSSION

There have been two schemes reported for the biogenesis of the Amaryllidaceae alkaloids. The first scheme was that proposed by Barton and Cohen (14). These authors suggested that the phenanthridine alkaloids arise by oxidation of phenolic compounds. These intermediate oxidation products would then undergo radical pairing (ortho - para) and eventually result in the formation of lycorine, narcissidine, haemanthamine, galanthine and pluviine. Wenkert (15) proposed that the Amaryllidaceae alkaloids arise from shikimic and prephenic acid and that the aromatic amino acids were not directly involved. His proposal involved carbon-carbon condensations via ionic mechanisms. Although neither scheme is entirely satisfactory, the possibility that phenylalanine, tyrosine or noradrenalin could serve as direct precursors for the phenanthridine ring of the Amaryllidaceae alkaloids seemed quite reasonable. Suhadolnik and Chenoweth (30) and Suhadolnik and Winstead (31) reported that phenylalanine and m-tyrosine were excellent precursors for the formation of a similar heterocyclic ring system that required the cyclization of the aliphatic side chain. Another factor that prompted the use of these compounds was the fact that partial enzymatic reductions of the aromatic ring had also been reported (31). This would allow phenylalanine, tyrosine or noradrenalin to serve as the precursor for ring C of the phenanthridine alkaloids. This hypothesis would not eliminate these compounds as pre-

cursors for rings A and B.

1. Root Uptake Studies

In an initial experiment, DL-phenylalanine-3-C^{14} was added to water into which the roots of growing Narcissus plants were immersed. The results of this study are shown in Figure 2 and Table I. Although there was a rapid uptake of DL-phenylalanine-3-C^{14}, with little $C^{14}O_2$ formation, no radioactivity was in the isolated alkaloids. The translocation of the phenylalanine-3-C^{14} to the leaves was also very small (1.97 per cent). The lack of incorporation of phenylalanine-3-C^{14} into the alkaloids could be explained as follows: (1) the precursor was not translocated to the site of alkaloid synthesis, (2) the experiment was terminated too rapidly, (3) phenylalanine is not hydroxylated in this plant, (4) the plants are not synthesizing the alkaloids.

Recently, Kursanov (32) discussed the transport of organic substances in plants. The lack of translocation of the phenylalanine from the roots to the leaves could be attributed to the age of the plant, the temperature at which the experiment was conducted or the inability of the amino acids to enter the conducting strands. Glycine, threonine, alanine, serine and glutamic acid have been reported to be more readily distributed in the plant than proline or γ-aminobutyric acid.

Since the initial study on the incorporation of phenylalanine into the alkaloids was negative, the experiment was repeated with the exception that younger plants were allowed to absorb the precursors by root uptake (Table III). After three days the plants were removed from the water and allowed to grow in soil for an additional 41 days. The results of the experiment for DL-tyrosine-2-C^{14} are shown in Table IV. Although

10.1, 11.6 and 32.5 per cent of the radioisotope was found in the leaves, bulbs and roots respectively, no radioactivity was present in the alkaloids. It appears then that tyrosine or its radioactive biological products are translocated to the bulbs and the leaves from the roots, but can not serve as a precursor for the alkaloids under the conditions of this experiment.

2. Distribution of Carbon-14 from $C^{14}O_2$ Experiment

Since the phenylalanine-3-C^{14} and tyrosine-2-C^{14} were not incorporated into the alkaloids by root uptake it became necessary to know if the alkaloids were being synthesized at this stage of plant development. To determine if the alkaloids were being formed, an experiment was designed in which three plants were grown in an atmosphere of $C^{14}O_2$. The results of this experiment are shown in Table II. Again it can be seen that the alkaloids were not radioactive. Tso, Jeffrey, and Sorokin (33) recently reported a similar experiment with Nicotiana plants in an atmosphere of $C^{14}O_2$. The alkaloids isolated after eleven days were radioactive. In view of these results, it appears that the alkaloids in the Narcissus plants are being synthesized from preformed organic molecules. This is quite plausible since the Narcissus plant has a large carbohydrate reserve in the bulb.

3. Distribution and Labelling Pattern of Radioactive Compounds Injected into the Bulbs

Since $C^{14}O_2$ and root uptake studies did not yield radioactive alkaloids, it appeared that the suspected precursors (Table III) had to be injected into the center of the growing bulb. From the results presented in Tables V and VI it is evident that DL-phenylalanine-3-C^{14} and DL-

tyrosine-2-C^{14} are incorporated into the Amaryllidaceae alkaloids. The alkaloids isolated from the bulbs from the DL-phenylalanine-3-C^{14} experiment contained 1.0 per cent of the radioactivity administered. Noradrenalin-H^3 (Table VII) was also injected since it is structurally related to narcissidine and since it has been recently isolated from plant materials (33). Although the alkaloid extract from the bulbs contained some radioactivity (0.5 per cent), the narcissidine isolated after separation on an alumina column was not radioactive. These findings tend to eliminate noradrenalin as a direct precursor for rings C and D of narcissidine. In view of these findings Carbon atom-4 of narcissidine is probably hydroxylated after the heterocylic ring is formed. The results of the experiment presented in Table V show that one per cent of the radioactivity from the DL-tyrosine-2-C^{14} was incorporated into the alkaloids isolated from the bulbs. The per cent of radioactivity incorporated into the alkaloids isolated from the bulbs from the phenylalanine and tyrosine experiments were identical (1 per cent). Approximately the same amount of radioactivity (DL-tyrosine-2-C^{14} experiment) was incorporated into the alkaloids isolated from the leaves (0.84 per cent).

The alkaloids from the tyrosine-2-C^{14} experiment were further purified by crystallization of the lycorine followed by separation on an alumina column. The distribution of the radioactivity in the fractions collected is shown in Figure 3. The alkaloid in the first radioactive peak was pluviine, the second peak was galanthine and haemanthamine and the last peak was narcissidine. The specific activity of the lycorine was 258 muc. per mmole. This represents a 382 fold dilution (based on the L-tyrosine). If tyrosine-2-C^{14} were incorporated directly into rings A and B of lycorine and narcissidine, the radioactivity would reside in carbon atom-11-C. If the amino acid were the precursor for rings C and

D, the radioactivity would reside in carbon atom-5. To determine if the radioactivity were in carbon atom-5, lycorine was degraded such that carbon atom-5 was isolated as formaldehyde. The remaining portion of the molecule is ether soluble and can be isolated and the amount of radioactivity determined. The scheme for the degradation of lycorine is shown in Figure 4. The results shown in Table VIII indicate that all of the radioactivity resides in carbon atom-5 of lycorine. If the aliphatic side chain is incorporated directly along with the hydroxylated benzene ring, it appears that tyrosine can serve as the carbon skeleton for rings C and D of the pyrrolo(de)phenathridine ring.

No radioactivity remained in the aldehyde moiety (XXVII). To prove that tyrosine is incorporated directly into lycorine, tyrosine-H^3 was injected into 6 plants. The plants have been harvested and dried. The alkaloids have not been isolated.

The narcissidine from the tyrosine-2-C^{14} experiment was to be degraded the same way as the lycorine. However, the conversion of the narcissidine methohydroxide to the methine did not take place. This may be attributed to the fact that there is a methoxyl group on carbon atom-4. Since the Hofmann exhaustive methylation requires the elimination of a proton on the carbon Beta to the nitrogen atom, it may be that the methoxyl group on the β-carbon atom of narcissidine has a marked effect on preventing the elimination of this β-proton.

The results on the incorporation of DL-tyrosine-2-C^{14} into rings C and D of lycorine lend support to the biosynthetic scheme proposed by Barton and Cohen (14). More recently Battersby, Binks and Wildman (35) have reported that tyrosine-2-C^{14} was incorporated into lycorine by Twink plants. The radioactivity was expressed such that lycorine had an

activity of 1.0 and the formaldehyde from carbon atom-5 had an activity of 0.96. No information was given as to the administration of the radioactivity, the mg. administered, the muc. administered, the dilution or the yield of compounds.

CHAPTER V

SUMMARY

1. In this study, the biogenesis of four alkaloids possessing the pyrrolo (de)phananthridine nucleus is investigated. To accomplish this, radioactive compounds were administered to the Deanna Durbin variety of Narcissus plants.

2. When the labelled precursors (DL-phenylalanine-3-C^{14} and DL-tyrosine-2-C^{14}) were taken up by the roots, there was no incorporation of radioactivity into the alkaloids.

3. When intact plants were grown in an atmosphere of $C^{14}O_2$ for eleven days, only 2.8 per cent of the $C^{14}O_2$ was incorporated into the ethanol soluble extract. None of the radioactivity resided in the alkaloids.

4. One per cent of the radioactivity was incorporated into the alkaloids when DL-phenylalanine-3-C^{14} and DL-tyrosine-2-C^{14} were injected into the bulbs of the Narcissus plants.

5. When the alkaloids from the DL-tyrosine-2-C^{14} experiment were separated by crystallization and column chromotography, lycorine, pluviine, galanthamine, haemanthamine and narcissidine were radioactive. Lycorine had a specific activity of 258 muc. per mmole. This represents a 382 fold dilution. Narcissidine had a specific activity of 180 muc. per mmole. This represents a 450 fold dilution.

6. Lycorine-C^{14} was degraded and carbon atom-5 was isolated as formaldehyde was converted to the dimedone derivative and had a specific activity

of 195 muc. per mmole. The aldehyde remaining after steam distillation of formaldehyde was not radioactive.

7. The degradation of narcissidine using the same procedure employed for lycorine (Hofmann exhaustive methylation) was not successful. This may be attributed to the methoxyl group on carbon atom-4 of narcissidine which is absent in lycorine.

8. Noradrenalin-H^3 was not incorporated into narcissidine.

9. If tyrosine is incorporated intact into rings C and D of lycorine, this would be another example involving aromatic amino acids in which the benzene ring is partially reduced.

10. The location of the radioactivity in the pyrrolo(de)phenanthridine alkaloids isolated from plants injected with DL-phenylalanine-3-C^{14}, L-tyrosine-H^3, formate and shikimate will be determined.

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