BIOSYNTHESIS OF RICININE BY *RICINUS COMMUNIS* L.

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RICINUS COMMUNIS L.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The biosynthesis of pyridine compounds by higher plants and certain bacteria has been shown to differ from that in animals (1) and Neurospora (2,3). Many substituted pyridine compounds in animals arise from the indole nucleus of tryptophan through 3-hydroxy-anthranilic acid, while in higher plants this biological transformation does not occur (4,5). Ricinine produced in Ricinus communis L. may arise from nicotinic acid (6) which may, in turn, arise directly from small molecules.

Leete and Leitz (6) have shown that nicotinic acid-7-C^{14} is a precursor of ricinine. The ricinine formed in this experiment was labeled only in the nitrile carbon atom. This fact suggested that precursors of ricinine may also be the precursors of nicotinic acid and other pyridine compounds produced by the castor plant.

The reasons for choosing ricinine for a study of the biosynthesis of pyridine compounds are that the alkaloid is the only one found in the castor plant, it is the only cyano-substituted pyridine compound known to occur naturally, it has several other functional groups which aid in chemical degradation and it is present in larger amounts than other pyridine compounds.

The complete biosynthetic pathway for ricinine is as yet unknown. Contributions from Dubeck and Kirkwood (7), Leete (6), Waller and Henderson (8,9), Marion et al (4,14,15), Scheidt and Boeckh-Behrens (10,11) have been made in the past few years but still much remains to be done.

The lack of a suitable degradation procedure has been a serious handicap in studying the biosynthesis of ricinine until recently when several chemical methods for the degradation of the alkaloid were developed (10,12,13).

For the purpose of extending some preliminary information obtained from previous contributions (7 – 12,14) and to aid in finding new information on ricinine biosynthesis, a method which permits the individual isolation of each carbon atom except carbons two and three was employed. This method was originally developed by Boeckh-Behrens (10). It was based largely on the findings of Böttcher (13) and it was further modified in this study. It was the aim of this study to establish the labeling pattern in ricinine formed from administering carbon-14 labeled succinic acid, malonic acid, malonamide, glycerol and glycolic acid to young castor plants.

Another objective of this study was to provide additional information concerning the types of pyridine molecules which might serve in the pathway between nicotinic acid and ricinine.

CHAPTER II

LITERATURE SURVEY

Ricinine was first discovered in 1864 by Tuson (15). Evans obtained ricinine by extracting castor beans, the seed of Ricinus communis L. with boiling water, filtering, evaporating to a thick syrup, and again extracting with boiling alcohol to get pure ricinine (16).

A. Occurrence of Ricinine

Ricinine occurs in all parts of the young castor plant with an approximate yield of 0.1% of fresh weight of the whole plant (14). The bulk of ricinine located in matured castor seeds (0.18%) was found in the seed coat, with only about 0.03% being found in the kernel (13).

Weevers (17) reported that during germination in the dark the ricinine nitrogen was increased from 4 to 72 mg per 100 plants in a period of three weeks, which was a clear indication that synthesis had occured. Etiolated Ricinus seedlings were found to produce more ricinine than the normal ones (18). However, Bogdashevskaya (19) reported that the castor plant showed a reduction of ricinine content in leaves which were shaded from the light; the upper unshaded leaves of such plants produced slightly higher than normal levels of ricinine.

Ricinine extracted from young seedlings was found (17) to be located mostly in the cotyledons and hypocotyl, only traces were found in endosperm and in roots. The cotyledons contained 5-10 times as

much ricinine as did the hypocotyl (14). The roots of the castor plant are comparatively poor in ricinine content. Weevers (17) was even unable to detect ricinine in roots. However, Bogdashevskaya (20) showed that 76 percent of the total ricinine increase in the whole plants was found in roots after infiltration of 1% lysine solution for six days. She also found that ricinine increased constantly with an increase in the physiological age of the plant, but noted it declined rapidly after 20 days and then increased again after flowering.

B. Proof of Structure of Ricinine

The first study on the structure of ricinine was published by Späth and Koller (21,22). They were able to show the presence of a pyridine ring in the alkaloid.

By treating ricinine with 57% sulfuric acid, N-methyl-4-methoxy-2-pyridone and HCN were produced (23). Upon removal of the O- and N-methyl groups when ricinine was treated with caustic alkali or by Pregl's method (24), the cyano-substituted pyridone was formed, it therefore followed that ricinine was the corresponding nitrile with the structure, 3-cyano-4-methoxy-1-methyl-2-pyridone. This conclusion was reviewed (25) and confirmed by several syntheses (26-29).

The first synthesis of ricinine was by the oxidation of 4-chloroquinoline to give 4-chloroquinolinic acid which was converted through its imide into 4-chloro-2-amino-quinolino-3-carboxylic acid, from which the 2-hydroxy-derivative was prepared and converted into 2,4-dichloro-3-cyanopyridine. The 2,4-dimethoxy derivative obtained from this was converted by treatment with methyl iodide into a product identical with natural ricinine (26).

Schroeter et al (27) reported that cyano-acetyl chloride

polymerized spontaneously at 5-8°, producing a mixture in which chloro-nor-ricinine predominated. This remarkable transformation was attributed to the intermediate formation of malonamide chloride, and cyanoketene to give 6-chloro-4-hydroxy-3-cyano-2-pyridone. This product was converted to ricinine by removal of the halogen and then methylating the nitrogen and the hydroxyl group at position 4.

In 1956, Taylor et al (28,29) synthesized ricinine from 3-picoline-1-oxide through the intermediates 4-nitro-3-picoline-1-oxide, 4-nitronicotinic acid-1-oxide, 4-methoxy-nicotinic acid-1-oxide, 4-methoxy nicotinate-1-oxide, 4-methoxynicotinamide-1-oxide, 2,4-dichloro-nicotino-nitrile and 2,4-dimethoxy-3-cyano pyridine which was then heated with methyl iodide at 155° to yield ricinine.

The proven structure of ricinine is:

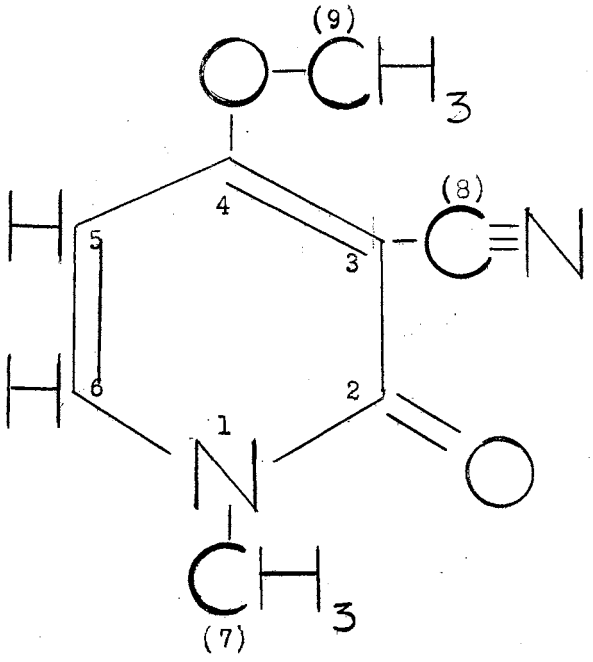

Figure 1

Structure of Ricinine
(1,2-dihydro-4-methoxy-1-methyl-2-oxonicotinonitrile)

C. Characterization of Ricinine

Ricinine is composed of C, 58.53%, H, 4.91%, N, 17.07%, O, 19.49%, is optically inactive; it melts at 201.5°C (corrected) and sublimes at 170-180°/20 m.m. (30). It is slightly soluble in water and chloroform, insoluble in ether and its solubility in cold pyridine is 2%, however, it is 34% in boiling pyridine. The absorption spectra of ricinine was reported (31) to have a maxima at 315 mμ in 95% ethyl alcohol ($E^{1\%}_{1cm}$ = 450) and 307 mμ in water (14) with a peak of less absorbence occurring at 255 mμ in both solvents. An oscillopolargraphic study of ricinine has been reported by Parrák (32) who showed that the ricinine could be detected by the position of the indentation in the oscillogram. The polarography, ultraviolet and infrared spectroscopy of ricinine have also been studied and reported by Manis et al (33).

Ricinine does not give the usual positive tests for alkaloids, but it gives some color tests such as a positive Weidel reaction (30), Fehling's solution, ferric complex and the formation of an isonitrile which can be detected when treated with 2 N NaOH and 30% H_2O_2 (34).

Ricinine forms chloride and mercuric chloride salts and gives three bromine derivatives, $C_6H_6O_2NBr$, $C_6H_7O_2NBr_2$, $C_6H_7O_2NBr_4$, and each can be characterized by their different melting points (30). Distillation in the presence of zinc dust will remove the side groups of ricinine to yield pyridine. Ricinine ($C_8H_8O_2N_2$) undergoes hydrogenation to form tetrahydroricinine ($C_8H_{12}O_2N_2$) in the presence of platinum as catalyst. Ricininic acid, a saponification product of ricinine, can be oxidized by chromic acid and sulfuric acid to yield a mixture of methylamine, oxalic acid and hydrogen cyanide (13).

Paper chromatographic separation and detection of ricinine has been reported by Robinson and Fowell (31) in which 20-100 μg of

ricinine can be detected by spraying with Dragendorf's reagent, potassium tetraiodo bismuthate. It is distinguishable from most other structurally related pyridine compounds.

The R_f values reported were 0.55 for isopropyl alcohol-toluene-acetic acid-water, 5:10:1:1 and 0.75 for t-butyl alcohol-water-acetic acid, 4:2:1.

D. Biosynthesis of Ricinine

The first literature report pertaining to the biosynthesis of ricinine in the castor plant was published in 1952 by Dubeck and Kirkwood (7). They investigated the origin of the O- and N-methyl groups of ricinine by feeding germinating castor seeds carbon-14 labeled L-methionine (methyl-C^{14}), choline (methyl-C^{14}) and sodium formate-C^{14}. Only in the case of L-methionine-C^{14} was the alkaloid appreciably labeled. It was shown that the ricinine was only labeled in the methyl groups with each group containing approximately an equal amount of the activity. These authors stated that the uniform behavior of methionine methyl as precursor of various C_1 units found in plants clearly indicated that this substance occupied a key position in the labile C_1 metabolism of higher plants. Dubeck and Kirkwood felt that the failure of formate to serve as a precursor was due to the early stage of development of Ricinus plants. Later Waller and Henderson (8) reported the incorporation of radioactivity from formate-C^{14} into ricinine and suggested that the source of labile methyl may change with growth.

Tamir and Ginsburg (35) reported that lysine-2-C^{14} hydrochloride fed to castor seedlings was incorporated into ricinine (0.01%) by the plant. They found most of the activity located at carbon 6. They also reported that α-amino-adipic acid-2-C^{14} gave rise to ricinine

labeled in carbons 2 and 6. Lysine as a precursor of ricinine was also studied by Juby and Marion (36,37) and Waller and Henderson (8). The former authors (37) used DL-lysine and found that the extent of incorporation was very low but that the activity was distributed between the nitrile, O-methyl and N-methyl carbons. Neither of these groups considered lysine to be an important precursor of the pyridine ring.

Leete and Leitz (6) and Waller and Henderson (8) showed that the pyridine ring of nicotinic acid and nicotinamide could become the α-pyridone ring of ricinine. Leete used nicotinic acid-7-C^{14} to inject 3-week-old castor bean seedling growing in vermiculite and then harvested the plant after 14 days. The ricinine was isolated, purified and treated with 57% sulfuric acid to yield N-methyl-4-methoxy-2-pyridone which contained none of the radioactivity. Thus all of the radioactivity was found to be located in the nitrile group, and it was assumed that nicotinic acid was a direct precursor of ricinine. This was confirmed by Waller and Henderson (8) using C^{14} and H^3 doubly labeled nicotinic acid as precursor.

Juby and Marion (37) found a high percentage (93%) of activity in the cyano group of ricinine obtained from sodium acetate-1-C^{14}. Anwar et al (38) also reported that 90% of the C^{14} from acetate-1-C^{14}, glutamic acid-2-C^{14} and propionic acid-3-C^{14} was located in the nitrile carbon of ricinine. These results could best be explained by adopting the idea of Waller and Henderson (9) that succinic acid or the related four-carbon dicarboxylic acid found in the tricarboxylic acid cycle was a direct precursor of ricinine. The C_4 unit would be incorporated in such a way that one of the carboxyl groups provided the carbon for the cyano group of ricinine and the 2 and 3 positions respectively of the pyridone ring. The other carboxyl group must eventually be lost by

decarboxylation. The distribution of activity in ricinine shown for acetate and glutamate could be accounted for by the operation of the Krebs cycle with or without the glyoxalate by-pass (37). Acetate-1-C^{14} could only result in carboxyl-labeled succinate no matter how many runs are operated in the Krebs cycle and this would account for the high proportion of activity from acetate-1-C^{14}.

Feeding of succinic acid-2,3-C^{14} resulted in carbon atoms 2,3 and 8 (CN) labeled in ricinine with 38.9%, 38.3% and 20.8% respectively (12). The labeling in carbon 8 resulted from one of the carboxyl groups becoming labeled after only one operation of the Krebs cycle. This type of randomization was thought to account for the level of activity actually found in the cyano group after feeding succinic acid-2,3-C^{14}.

Succinic acid-1,4-C^{14} was reported (9, 39) to distribute the C^{14} as follows: about 85% in the cyano group and 15% in the pyridone ring.

Acetate-2-C^{14} would produce succinate labeled predominately on the methylene groups but with slight carboxyl labeling, thus the incorporation pattern would be similar to that of succinate-2,3-C^{14} (12).

If glutamic acid-2-C^{14} were involved in a transamination reaction, the resulting α-keto-glutarate-2-C^{14} would give rise to carboxyl-labeled succinate. Once again, ricinine with a high proportion of activity in the cyano group would result (37).

The labeling pattern obtained from propionic acid, which was incorporated into ricinine, suggested that this compound might give rise to succinate via β-oxidation (9).

Glycerol-1,3-C^{14} and glycerol-2-C^{14} were reported (9,36) to incorporate into ricinine to the same extent as many other precursors.

This was partly explained by its conversion to acetate through glycolysis (9).

The most recent proposal for the biosynthesis of ricinine is that made by Waller (14). He proposed the following scheme (Fig. 2) starting from simple molecules: In this pathway it was proposed that nicotinic acid was formed by joining a one-carbon compound, a two-carbon compound and a three-carbon compound by the following processes: The initial condensation of acetyl-CoA (I) with malonyl-CoA (II) could give β- keto -glutaryl-CoA (III) which would be reduced to β-hydroxy-glutaryl-CoA (IV) and then to glutaconyl-CoA (V) by losing water. Glutaconyl-CoA could undergo transamination to yield glutaconamide (VI) which condensed with formate to give N-formyl-glutaconamide (VII). Ring closure by an aldol type condensation between carbon 2 and 6 would give compound (VIII). The reduction of this compound to give the dihydropyridine derivative (IX) is followed by removal of water to give nicotinic acid. Nicotinic acid (X) is converted to nicotinamide (XI) by transamidation which is hydroxylated at position 4 to give 4-hydroxy-nicotinamide (XII) and again hydroxylated at position 2 to give 2,4-dihydroxy-nicotinamide (XIII). Compound (XIII) is converted to N-methyl-4-methoxy-3-carboxamide-2-pyridone (XIV) by two transmethylation reactions and then dehydrated to yield ricinine (XV).

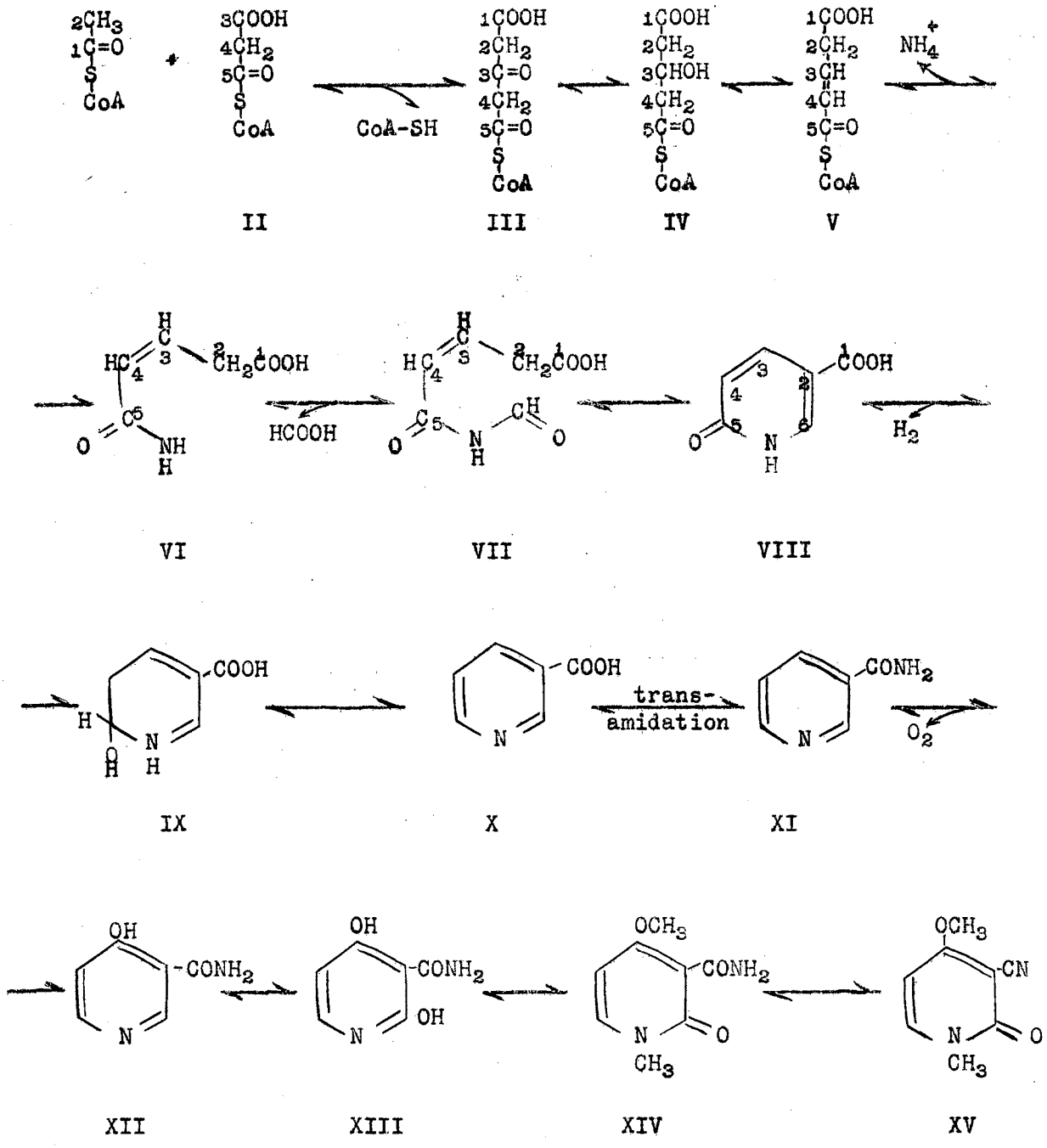

Figure 2

Proposed Pathway for the Biosynthesis of Ricinine (14)

CHAPTER III

EXPERIMENTAL METHODS AND MATERIALS

A. Growing of Castor Plants

Plants used in these experiments were grown on Port clay loam soil at the Agronomy Farm of the Oklahoma State University in Stillwater. Seeds of the Cimarron variety were planted on May 31, 1962. This group of plants was used for ricinine biosynthesis studies involving ricinine acid, ricininic acid, nicotinic acid, nicotinamide, N-methyl-nicotinamide, glycolic acid, β-alanine and glycerol.

The second group of castor plants was grown at the same location during July to the end of August in the same year. They were used for ricinine precursor studies involving labeled succinic and fumaric acids.

Experiments performed between October, 1962, and March, 1963 employed plants grown in the Horticultural Department green house. The plants were used for preparing large quantities of ricinine formed from succinic acid, fumaric acid, malonic acid and malonamide.

B. Injection of Labeled Compounds

The method of injection was that developed by Waller (14) and is as follows:

A 22 gauge hypodermic needle was inserted at the top of the second internode of a castor plant to serve as a vent. An aqueous

solution (20-200 µl usually) of the labeled compound was injected at the bottom of the internode. The hollow space within the node served to store the solution until it was completely absorbed. Absorption by the young plants occurred rapidly. It was observed that old plants bearing mature seeds, also plants beyond the tenth nodal stage, absorbed these compounds very slowly; localization of the labeled compound at the point of injection was observed one week after injection.

Injection of plants was usually made at the physiological stage of 6-9 nodes and harvested 72-144 hours following the injection.

C. Labeled Compounds Used

Small molecule as precursors: glycolic acid-1-C^{14}, glycolic acid-2-C^{14}, glycerol-1,3-C^{14}, glycerol-2-C^{14}, β-alanine-2-C^{14}, succinic acid-1,4-C^{14}, succinic acid-2,3-C^{14}, fumaric acid-2,3-C^{14}, malonic acid-1-C^{14}, malonic acid-2-C^{14} and malonamide-1-C^{14} were all purchased from reputable manufacturers in the United States. Their purity was checked by Chromatography on Whatman No. 1 paper in a suitable solvent system and then locating its radioactivity on the paper by passing it through a strip counter.+

Pyridine derivatives used as precursors were obtained from various sources. Ricinine acid ($C_8H_8O_4N$) was obtained from Dr. W. G. Robinson of the University of Michigan. He prepared ricinine acid-8-C^{14} by the *in vitro* hydrolysis (40) of ricinine-8-C^{14} which was sent to him from this laboratory obtained from ricinine biosynthesized from nicotinic acid-7-C^{14}. The specific activity of ricinine acid was 1.3×10^4 mµc/mM.

+ Radiological Service Co. Inc., Long Island City 2, New York.

Ricininic acid ($C\ H_6O_2N_2$-8-C^{14}) was synthesized from ricinine-8-C^{14} obtained from ricinine biosynthesized from nicotinic acid-7-C^{14} by refluxing ricinine with 1N NaOH for one hour (41). The resulting ricininic acid was recrystalized from boiling water. The specific activity was 171 mμc/mM.

N-methyl-nicotinamide-8-C^{14} was prepared by refluxing nicotinamide-7-C^{14} with methyl iodide for 6 hours and purified by recrystallizing from a mixed solvent of water, ethanol and ether (42). The specific activity was 9.27×10^4 mμc/mM.

Nicotinic acid-7-C^{14} and nicotinamide-7-C^{14} were obtained from New England Nuclear Corp. Boston, Mass. and were used without further purification.

D. Measurement of Radioactivity

Suitable dilutions were made of the water soluble compounds and an aliquot was counted in the Liquid Scintillation Spectrometer.+ The scintillation solvent was composed of 58.75% toluene, 39.25% absolute ethanol and 2% water. The "phosphor" was 0.5% 2,5-diphenyl oxazole and 0.02% p-bis-2-(5-phenyloxazolyl) benzene. After adjusting the proper voltage and determination of quenching effect, the system above was found to have an efficiency of 38% for carbon-14 compounds and 8.0% for tritium labeled compounds. Specific activities of ricinine precursor compounds and degradation products were determined by this method. For those having a low solubility in this solvent, the Van Slyke-Folch wet combustion method was employed (43, 44). A proper amount of solid KIO_4 was added to a known amount of sample which was

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+ Model 314 E Tri-Carb, Packard Instrument Company Inc., La Grange, Illinois.

placed in a combustion tube, then a solution of CrO_3 in H_2SO_4-H_3PO_4 (20% SO_3 content) was added. The CO_2 which was evolved was collected and counted with a Vibrating Reed Electrometer.[+] The specific activity of ricinine was also checked by wet combustion.

E. Isolation, Purification and Identification of Ricinine

1. Isolation of ricinine (6,14)

Castor plants were cut into 1/2 to 1 inch pieces and macerated for 15 minutes with chloroform in a Waring Blendor[++], using a weight ratio of chloroform to fresh plant of 4:1. The mixture was filtered by vacuum through a sintered glass funnel (coarse porosity). The residue was returned to the blendor and extracted for 15 minutes with chloroform using a weight ratio of chloroform to fresh plant of 1:1, or a minimum volume of 50 ml. This mixture was filtered as described above. The extraction was repeated a third time when necessary. The filtrates were combined and extracted with 100 ml of 7N ammonium hydroxide. The chloroform solution was evaporated to dryness on a steam hot plate. Lipids and pigments were removed from the residue by extracting with ethyl ether for about 3 times. This was followed by another cycle of chloroform extraction and evaporation to dryness when necessary. The residue was dissolved in hot distilled water, filtered through a sintered glass funnel of medium porosity and its weight (crude yield) determined after evaporation to dryness. Most samples were about 90% pure at this stage. The ricinine in the residue was then purified to a constant specific activity either by sublimation or by repeated

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+ Applied Physics Corp., Monrovia, California.

++ Waring Products Corp., New York, New York.

recrystallization from water or redistilled chloroform.

2. Identification of ricinine

The melting point of ricinine purified as above was usually 201-202°C, which agreed with the reported value (201-201.5°C) (30). Ricinine was also identified by its ultraviolet absorption spectrum[+]. It has maximum peak wavelengths of 217, 255 and 307 mμ (Fig. 3, Appendix). The infrared spectrum of ricinine isolated in this manner agreed with that reported (Fig. 4, Appendix). The molecular weight of ricinine was determined by mass spectrometry (mass = 164).

Paper chromatography of ricinine using N-butyl alcohol-acetic acid-water, 4:1:1 gave an R_f of 0.68. Dragendorf's reagent was found to be the most useful reagent for detecting ricinine. It was prepared as follows (32): Stock solution: 80 gm $BiONO_3$ dissolved in 20-25 ml 30% HNO_3 (sp.gr. 1.18). Add this solution with stirring to a solution containing 28 gm KI and 1 ml 6N HCl in approximately 5 ml H_2O, cool in refrigeration. The solution should be orange-red in color. Dilute to 100 ml. It is stable for a few weeks if stored in a dark bottle in the refrigerator. Developer: It contains 20 ml H_2O, 2 ml Dragendorf solution, 5 ml 6N HCl and 5 ml 6N NaOH; add in this order; if $Bi(OH)_3$ does not dissolve, add a few drops of 6N HCl. Warm to room temperature before use.

F. Chemical Degradation of Ricinine

A modified procedure according to Scheidt and Boeckh-Behrens (10) was used. Dr. Erich Hecker of the Max Planck Institute for

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+ Beckman DB Spectrophotometer.

Biochemistry, Munich, Germany, kindly supplied us with a copy of Dr. G. Boeckh-Behrens dissertation in 1961.

1. Conversion of ricinine to ricininic acid

One mM (164 mg) of ricinine was dissolved in 3 ml of 1N sodium hydroxide solution, refluxed for about one hour, cooled and acidified with 6N hydrochloric acid to pH 2 (pHydrion paper). The white precipitate was collected by centrifugation (2000 rpm) for 10-15 minutes and the ricininic acid was recrystallized from boiling water. For further purification, the ricininic acid was dissolved in chloroform and evaporated to dryness followed by sublimation. The yield was approximately 90%. No attempt was made to recover the methyl alcohol liberated from the O-methyl group of ricinine.

Ricininic acid crystallizes from water as needle shaped prisms. Analysis showed C, 55.4%, H, 4.58%, N, 18.1%, and O, 21.6%[+]. Calculated values, C, 55.9%, H, 4.01%, N, 18.6% and O, 21.3%. It had a mass of 150. Ricininic acid melted at 298-299°C/ 760 m.m., was slightly soluble in water, ethanol, chloroform and ether. For the UV and IR spectra of ricininic acid see Figs. 3 and 4 (Appendix). A purchased sample of ricininic acid[++] was used for comparison.

Descending paper chromatography of ricininic acid showed an R_f value of 0.60 with the solvent system n-butanol-acetic acid-water, 4:1:1. Methyl red was used to detect the compound.

[+] Midwest Microlab Inc., Indianapolis 20, Indiana.

[++] General Biochemicals, North American Mogul Product Co. Chagrin Falls, Ohio.

2. Conversion of ricininic acid to 5,6-dihydro-ricininic acid

Preparation of 5% sodium amalgam (45): To 5.82 gm of clean metallic sodium in 20 ml toluene was added 110.7 gm of mercury by drops from a separatory funnel. The reaction was started by heating the toluene to boiling on a hot plate, followed by continuous shaking during the stage of vigorous reaction which usually lasted about 15 minutes. The excess toluene was evaporated and the sodium amalgam was brought to a molten state using an open flame. The amalgam was then solidified by pouring on porcelain and crushed into lumps for storage. It was stored at room temperature in an air-tight container.

To prepare dihydroricininic acid, 150 mg (1 mM) of ricininic acid was dissolved in 1 ml of 1N sodium hydroxide solution, 5 ml of water and 1 ml of ethanol. Four gm of sodium amalgam was added in 1 gm portions every three hours. The reaction was started at 0°C and then increased to room temperature at the end of twelve hours. The reaction mixture was then brought to pH 2 with concentrated hydrochloric acid, lustrous crystals appeared after several minutes had elapsed. The crystals accumulated in a large amount when the solution was allowed to stand overnight. They were removed by filtration and recrystallized from boiling water. Further purification could be achieved by sublimation when necessary. The yield was about 80%.

Characterization of 5,6-dihydroricininic acid: Dihydroricininic acid prepared by the above procedure melted at 240°C. It was slightly soluble in water, ethanol and chloroform, and insoluble in ether. It appeared as lustrous flat crystals from water. Its molecular composition was C, 55.8%, H, 6.46%, N, 17.6% and O, 20.5%[+], calculated

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+ Determined by Midwest Microlab Inc., Indianapolis 20, Indiana

values are: C, 55.3%, H, 5.27%, N, 18.4% and O, 21.0%. Mass spectrometric analysis showed that dihydroricininic acid had a molecular weight of 152.

The UV and IR spectra of dihydroricininic acid are shown in Figs. 3 and 4 and compared to its parent compounds, ricinine and ricininic acid. The Nuclear Magnetic Resonnance spectrum (Fig. 5) of dihydroricininic acid shows that the two newly added hydrogen atoms are located at carbon atoms 5 and 6. 5,6-dihydroricininic acid thus prepared retains its double bond between carbons 3 and 4, advantage is taken of the fact that this electron-negative linkage is easily broken thus making ring-opening of the molecule by an oxidizing agent easier.

A variation of reaction conditions may result in the formation of different hydrogenated compounds. Three different compounds have been reported by Boeckh-Behrens (10); however none of them was further studied by the author.

The 5,6-dihydroricininic acid prepared above had an R_f of 0.52 using the solvent system n-butyl alcohol-acetic acid-water, 4:1:1 (descending). It was detected by methyl red or by its absorption under ultraviolet light (2537 Å).

3. Oxidation of 5,6-dihydroricininic acid

One millimole (152 gm) of dihydroricininic acid was dissolved in 1 ml of 1N CO_2-free sodium hydroxide and 3 ml of CO_2-free water. $KMnO_4$ (1N) was added drop-wise while the mixture was shaken occasionally at room temperature. Completion of the reaction could be determined by the change of color from purple permanganate ion to brownish black, until a further drop of permanganate retains the purple color for 1-1½ hours. The titration of $KMnO_4$ was terminated when a total amount of

about 6.8 ml had been added. An excess of $KMnO_4$ was avoided for the reason that free oxidizing agent present in the mixture would cause cleavage of the molecule by another mechanism.

No isolation of compound was attempted at the end of the reaction. This intermediate was subjected to cleavage in the next step by acid hydrolysis after filtration and removal of MnO_2. A test performed for the presence of free cyanogen ion in the solution was negative.

4. Hydrolysis of the intermediate product

The product of oxidation in the last procedure was hydrolyzed with concentrated hydrochloric acid in a 50 ml three-necked reaction flask (Fig. 6). Hydrochloric acid was added by drops and the temperature was gradually increased from room temperature to 100°C by means of a water bath. Carbon dioxide evolving from the reaction was absorbed in the U-shaped tube fitted with a coarse sintered glass disc which contained 10 ml of 1N CO_2-free NaOH. Sweeping of CO_2-free air was carried out by applying water suction to the system and introduction of air filtered through NaOH solution. The reaction was completed in half an hour and the air sweeping was continued for another half hour. The system was disconnected and the reaction products were worked up separately:

a. Determination of specific activity of CO_2

The CO_2 absorbed in NaOH solution was transferred to a wet combustion tube and acidified in the Thomas Van Slyke Manometric Apparatus[+] with 85% phosphoric acid. The amount of carbon in mg was determined

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+ Arthor H. Thomas Co., Philadelphia, Pennsylvania.

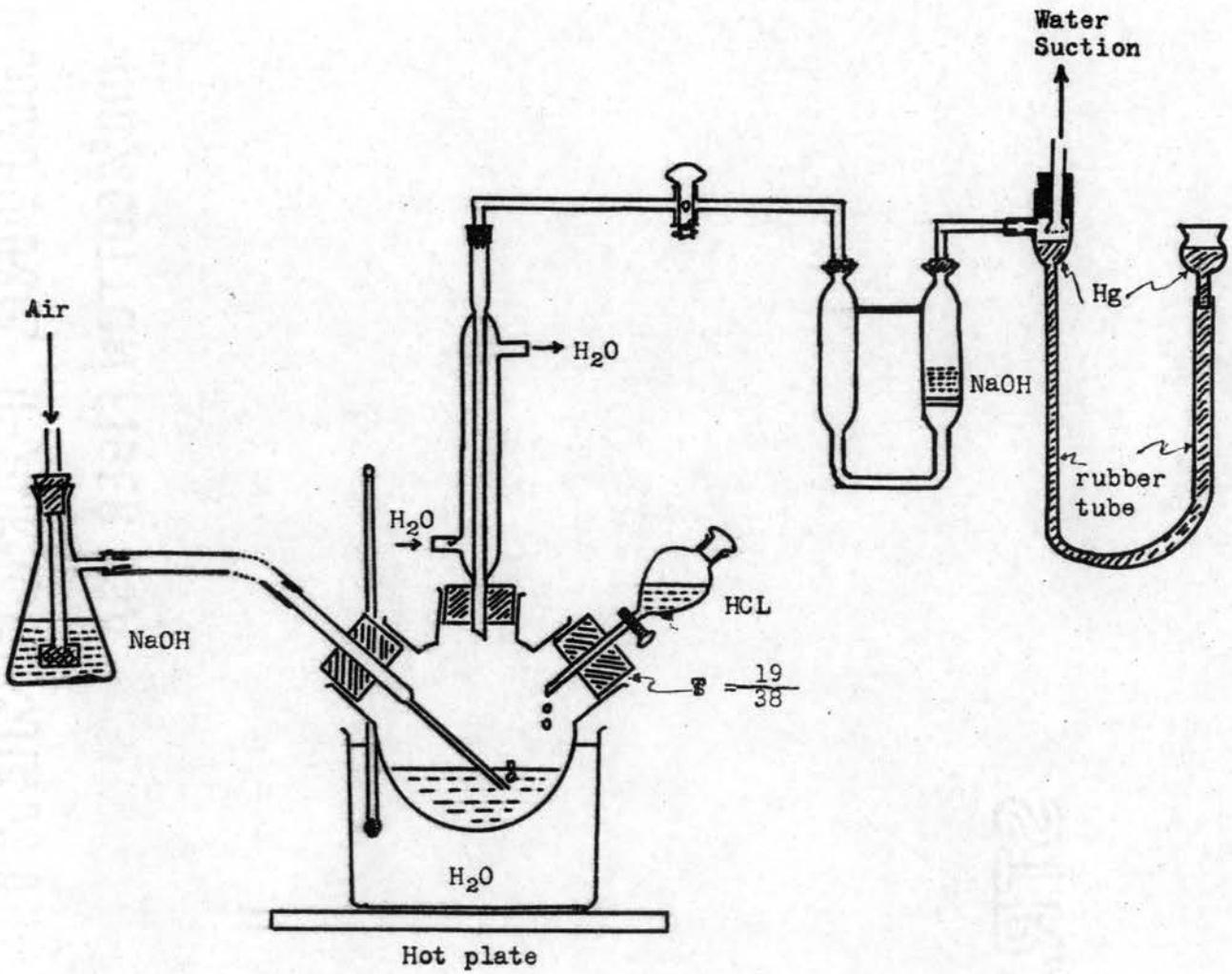

Figure 6

Apparatus for Hydrolysis of the Dihydroricininic Oxidation Intermediate Product

as follows:

$$\text{mg Carbon} = P_{CO_2} \times \text{factor}$$

where P_{CO_2} is the pressure of CO_2 corrected with blank sample, the factor was corrected according to temperature (44).

The CO_2 was transferred to an ionization chamber and counted in the Vibrating Reed Electrometer. Its specific activity was calculated as follows:

$$\text{specific activity} = \frac{\text{activity in mv/sec} \times 3.5}{\text{mMole of carbon}}, \quad (\text{mc/mM})$$

where the activity counted was corrected for background, the term 3.5 is an empirical factor used in this apparatus.

b. Separation of oxalic acid and N-methyl-β-alanine:

The reaction products left in the three-necked flask contained a mixture of oxalic acid and N-methyl-β-alanine which were separated as follows: About 30 ml of cation exchanger Dowex 50 W x 4 (20-50 mesh) was recycled with 200 ml of 2N NaOH and allowed to stand overnight. It was decanted and brought back to the hydrogen-form by treating with 2N HCl. The recycled resin was first agitated with water in a beaker and then washed into the column (1 cm x 50 ml) and backwashed with distilled water. The reaction mixture was then eluted through the column at a rate of 1 ml per minute. The eluant containing oxalic acid was collected until a total volume of 100 ml water had been eluted (eluant I).

To recover the N-methyl-β-alanine which was left on the column, 150 ml of 2.5 N HCl was allowed to pass through the column at the same rate as above, and the solution collected (eluant II).

c. Precipitation of calcium oxalate

Eluant I which contained oxalic acid was brought to pH 5 with concentrated ammonium hydroxide and precipitated by the addition of

2ml of 1N Ca $(NO_3)_2$ and 2 ml of 2.0 N NH_4Cl. The mixture was heated to about 70°C, allowed to cool, the precipitate was collected by centrifugation. The yield was about 33% with respect to the weight of dihydroricininic acid.

d. Purification of N-methyl-β-alanine

Eluant II which contained N-methyl-β-alanine was evaporated to dryness using the rotary evaporator. The residue was extracted with three 10 ml portions of 95% ethanol and again evaporated to dryness. The N-methyl-β-alanine thus obtained still contained impurities of inorganic salts and other material. Further purification was accomplished by sublimation. N-methyl-β-alanine melted at 100°C. The IR spectra of the isolated compound and of a sample of known β-alanine are shown in Fig. 7 (Appendix).

5. Treatment of N-methyl-β-alanine

For further degradation, N-methyl-β-alanine was converted to propionate through acrylic acid.

a. Methylation of N-methyl-β-alanine

The N-methyl-β-alanine was dissolved in 3 ml of 1N NaOH in a 50 ml flask, one ml of $(CH_3)_2SO_4$ (11 mM) and 280 mg (5mM) of CaO were added to the solution which was shaken occasionally at room temperature for about 12 hours. The reaction mixture was then neutralized with 25% H_2SO_4 and the solution acidified with a few drops of H_2SO_4 of the same concentration. The entire mixture was transferred to a goose necked flask for steam distillation. The distillate containing acrylic acid was collected until a total volume of 100 ml was obtained.

The presence of acrylic acid was confirmed by the acidity of the distillate (pHydrion paper) and by the reduction of $KMnO_4$. The acrylic acid was titrated with 0.01 N NaOH. The yield was about 5%

(based on N-methyl-β-alanine).

b. Precipitation of tetramethyl-ammonium salt

The steam-distillation residue containing tetramethyl-ammonium ions was neutrallized with 6N NaOH using phenolphthalein as an indicator, filtered, and the filtrate was treated with 100 ml of a 0.5% solution of Reineckate salt($NH_4[Cr(NH_3)_2(SCN)_4]$), centrifuged, the precipitate washed several times with water and the final product was recrystallized from boiling water. One of the methyl groups of the tetramethyl-ammonium Reineckate salt $(CH_3)_4N\,Cr(NH_3)_2(SCN)_4$, originated from the N-methyl group of ricinine, and its activity was determined by Liquid Scintillation counting or by the Van Slyke wet combustion method.

c. Hydrogenation of sodium acrylate

To one millimole of sodium acrylate in 100 ml of water was added 60 mg PtO_2 (Adam's catalyst) in a 500 ml shaker-flask. Hydrogen gas was introduced into the reaction system after the removal of air by applying a vacuum to the hydrogenation apparatus[+]. The flask was shaken occasionally by a mechanical device at room temperature while the hydrogen pressure was always maintained at 1 atmosphere. Completion of hydrogenation required about two hours; the product, sodium propionate, was then centrifuged to remove PtO_2 and evaporated to dryness. The yield was about 97%.

6. Degradation of propionate

First, the sodium propionate was purified by eluting through a celite-sodium sulfate column prepared by packing 5 gm of celite (which was previously thoroughly mixed with 3 ml of 0.5 N H_2SO_4) in acetone-

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+ Parr Instrument Company, Moline, Illinois.

hexane (1:10) suspension to a 0.8 by 30 cm column and anhydrous sodium sulfate was packed above the celite to a height of 5 cm. After equilibrating with 30 ml of 1% n-butanol in chloroform, the column was ready to receive the sodium propionate dissolved in 0.5 ml of 25% H_2SO_4. Propionic acid was eluted with 1% n-butanol in chloroform at a rate of 0.5-1.0 ml per minute. Five-ml fractions were collected and titrated with 0.1N CO_2-free sodium hydroxide solution. The tubes (5-10 tubes) containing sodium propionate were combined and evaporated to dryness.

To degrade sodium propionate, the flask containing 1mM sodium propionate was chilled in an ice bath and 0.6 ml of 100 per cent sulfuric acid was added by drops. Complete solution of sodium propionate was achieved by heating and the solution was chilled in an ice bath again before adding sodium azide (100 mg). The reaction was carried out in an apparatus which permitted continuous trapping of CO_2 first under vacuum at room temperature and then by heating very slowly over a period of 1 hour to 70°C. The CO_2-free air was allowed to sweep through the system for 1 hour while heating at 70°C or above. The CO_2 trapped in 10 ml of 1N NaOH was then transferred to a combustion tube for activity determination. This CO_2 corresponds to carbon 4 of ricinine.

The traps containing acid permanganate and sodium hydroxide were replaced with one containing 10 ml of 0.2N sulfuric acid. About 5 ml of 10N NaOH was slowly added to the reaction flask, and the ethylamine was then distilled into the sulfuric acid trap by heating the flask on a water bath and sweeping with air for 30 minutes.

Oxidation of ethylamine was carried out by adding 5% $KMnO_4$ in alkaline solution at room temperature for 1 hour. The reaction

mixture was then acidified with 25% H_2SO_4 and steam distilled. The acetic acid was collected, titrated with o.1 N NaOH and evaporated to dryness.

Degradation of sodium acetate proceeded exactly the same as for sodium propionate except that the methylamine was trapped using hydrochloric acid. The CO_2 collected originated from carbon 5 of ricinine. The methylamine was wet combusted to obtain the activity of carbon 6.

7. Summary of ricinine degradation

Figure 8 shows the ricinine degradation pathway used. To make a complete degradation, the preferable starting sample size of ricinine is 3 millimoles. However, one millimole can be completely degraded provided the original specific activity of ricinine is high enough (>500 mμc/mM) so that dilution of the intermediate products can be made. Certain precautions should be observed during this degradation.

Hydrogenation of ricininic acid to give dihydroricininic acid with Na-Hg is preferably carried out in alcoholic solution rather than aqueous solution. This not only increases the yield, but also gives the correct hydrogenation product, 5,6-dihydroricininic acid.

Ring opening of 5,6-dihydroricininic acid with 1N $KMnO_4$ is to be carried in such a way that an excess of permanganate must be avoided as it will also oxidize the oxalic acid. This results in a randomization of the radioactivity from carbons 2, 3, and 8 of ricinine.

Methylation of N-methyl-β-alanine increases the acidity of the reaction mixture. This may be due either to the replacement of the hydrogen atoms on the amino group of N-methyl-β-alanine by the methyl groups or to the acidity of the dimethyl sulfate. The reaction mixture

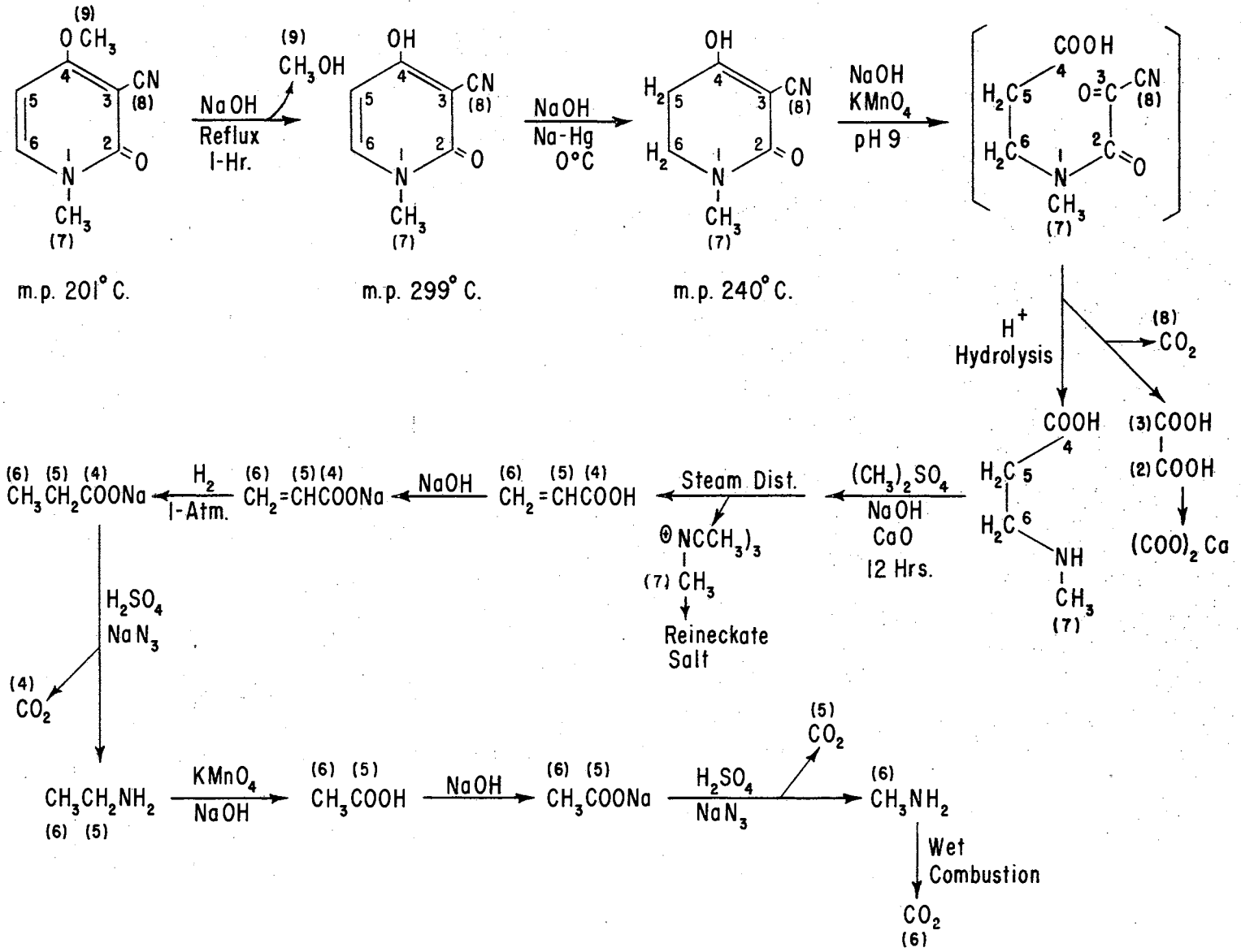


Figure 8 Degradation Scheme for Ricinine

must always be kept alkaline by occasionally adding sodium hydroxide.

The desired amount of sodium propionate to be degraded is 0.5-1.0 mMole with a total minimum activity of approximately 10 mμc.

CHAPTER IV

RESULTS AND DISCUSSION

A. Pyridine Compounds as Precursors of Ricinine

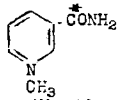
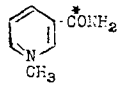
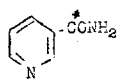
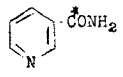
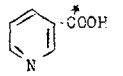
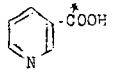
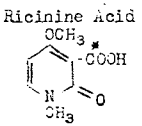
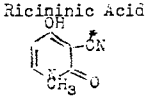
In the biosynthetic pathway from nicotinic acid to ricinine, several steps are involved, thus several intermediates might be expected to be precursors of ricinine. Pyridine compounds derived from ricinine and some synthesized from carbon-14 labeled intermediates were used in this study. The structures and the position of label (*) for the compounds used and data on the incorporation of these compounds into ricinine are shown in Table I.

Although nicotinic acid and nicotinamide have previously been shown to be the precursors of ricinine (6,8), data on their incorporation into ricinine under the conditions used for the other pyridine compounds were obtained in order that a basis of comparison for the relative efficiencies of incorporation would be available.

N-methyl-nicotinamide and ricinine acid were incorporated to a lower extent than nicotinic acid, indicating that they may be farther away from ricinine in the biosynthetic pathway, and also, that they are probably not on the direct pathway. The high dilution of ricinine acid indicated that it was not as closely related to ricinine biosynthesis as was N-methyl-nicotinamide and nicotinamide which lends more support for the role of the carboxamide group of nicotinamide being

TABLE I

INCORPORATION OF PYRIDINE COMPOUNDS INTO RICININE

Experiment Number	Precursor: Compound Injected	Precursor: Specific Activity mμc/mM	Precursor: Quantity Injected mμc	Ricinine: Yield mMole	Ricinine: Specific Activity mμc/mM	Incorporation+ %	Isotope Dilution	Experiment Duration (hours)	Physiological Stage (Nodes)
341	N-me-nicotinamide 	9.27×10^4	1860	0.44	351	8.2	264	192	9
410	N-me-Nicotinamide 	9.27×10^4	1860	0.22	522	6.1	178	96	8
342	Nicotinamide 	1.12×10^5	2240	0.39	618	10.8	181	192	9
315	Nicotinamide 	1.12×10^5	2240	0.44	535	10.4	210	96	7
412	Nicotinic Acid 	6.5×10^6	1430	0.25	970	16.9	6,700	48	8
413	Nicotinic Acid 	6.5×10^6	1430	0.46	682	22.0	9,530	96	8
411	Ricinine Acid 	1.3×10^4	18.4	0.21	11.5	13.3	1,130	96	8
415	Ricininic Acid 	1.71×10^2	2.64	0.16	19.4	123	8.8	96	8

+ Percentage Incorporation was calculated by dividing the total activity of the ricinine isolated by the total activity injected into the castor plant.

TABLE II

DEGRADATION OF RICININE FORMED FROM NICOTINIC ACID-7-C^{14}

Experiment Number	Specific Activity of Ricinine mμc/mM	C-9[+] (-O-CH_3)		C-7 (-N-CH_3)		C-8 (-CN)		C-2, C-3		C-4, C-5, C-6[++]	
		mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%
254	63.6	trace	0	trace	0	63.5	100	trace	0	0	0
412	256	trace	0	trace	0	249.0	97	trace	0	0	0
413	349	trace	0	trace	0	345.0	99	trace	0	0	0

[+] O-methyl activity was determined indirectly by subtracting the specific activity of ricininic acid from that of ricinine.

[++] Isolated as N-methyl-β-alanine.

the precursor of the nitrile group of ricinine. The high dilution of ricinine acid might also be due to metabolism of this compound by other pathways in the castor plant. This compound may be a biodegradation product of ricinine (46).

In the case of ricininic acid, it requires only one step, methylation, before it is converted to ricinine. A very high percentage of incorporation of ricininic acid into ricinine could be expected and the results obtained support this view.

Previous reports (6,8) have shown that the carboxyl carbon of nicotinic acid and the carboxamide carbon of nicotinamide were incorporated into the nitrile carbon of ricinine. This has also been confirmed in this study using an entirely different method of degradation. In the degradation procedures used the nitrile group was removed in the hydrolysis process after ring opening of the ricinine molecule. Three ricinine samples of different specific activities were degraded (Table II) and all fragments from ricinine were found inactive except the CO_2 which came from the nitrile carbon. Of importance was that this result also proved that the only CO_2 evolved during hydrolysis came from the nitrile carbon and not from carbons 2 and 3 as was originally reported (10). The improvement on the degradation procedures permits more reliance to be placed on labeling patterns where positions 2,3 and 8 of the ricinine molecule are involved.

B. Glycolic Acid as Precursor of Ricinine

Glycolic acid-1-C^{14} and glycolic acid-2-C^{14} were administered to castor plants to see if they were involved in ricinine biosynthesis. Table III shows how well this compound was incorporated into ricinine.

TABLE III

INCORPORATION OF GLYCOLIC ACID INTO RICININE

Experiment Number	Precursor Compound	Precursor Specific Activity mμc/mM	Precursor Quantity Injected mμc	Ricinine Yield mMole	Ricinine Specific Activity mμc/mM	Incorporation+ %	Isotope Dilution	Experiment Duration (hours)	Physiological Stage (Nodes)
371	Glycolic-1-C^{14}	4.91×10^5	925	0.43	10.3	0.48	4.77×10^4	48	7
372	Glycolic-1-C^{14}	4.91×10^5	463	0.17	13.0	0.48	3.78×10^4	48	7
373	Glycolic-1-C^{14}	4.91×10^5	925	0.33	20.7	0.74	2.37×10^4	96	6
374	Glycolic-1-C^{14}	4.91×10^5	463	0.28	9.0	0.55	5.46×10^4	96	8
389	Glycolic-1-C^{14}	4.91×10^5	925	0.88	3.9	0.38	1.26×10^5	48	9
390	Glycolic-1-C^{14}	4.91×10^5	925	0.70	5.9	0.45	8.3×10^4	96	9
375	Glycolic-2-C^{14}	1.41×10^6	2500	0.63	10.3	0.25	1.37×10^5	48	8
376	Glycolic-2-C^{14}	1.41×10^6	1280	0.22	396	6.77	3.56×10^3	48	6
377	Glycolic-2-C^{14}	1.41×10^6	2500	0.41	483	7.73	2.92×10^3	96	7
378	Glycolic-2-C^{14}	1.41×10^6	1280	0.25	407	7.88	3.46×10^3	96	6
391	Glycolic-2-C^{14}	1.41×10^6	2500	1.13	212	9.73	6.65×10^3	48	8
392	Glycolic-2-C^{14}	1.41×10^6	2500	0.79	120	3.7	1.18×10^4	96	9
393	H_2O	-	0	0.19	0	0	-	96	7

+ Percentage incorporation was calculated by dividing the total activity of the ricinine isolated by the total activity injected into the castor plant.

TABLE IV

DEGRADATION OF RICININE FORMED FROM GLYCOLIC ACID-2-C^{14}

Experiment Number	Specific Activity of Ricinine mμc/mM	C-9[+] (-O-Methyl) mμc/mM	%	C-7[++] (-N-Methyl) mμc/mM	%	C-8 (CN) mμc/mM	%	C-2 and C-3 mμc/mM	%	C-4, C-5, C-6[+++] mμc/mM	%
375	90.0	54.1	60.0	34.9	39.0	0.98	1.0	Trace	0	Trace	0
377	156.5	94.0	60.0	56.1	36.0	1.78	1.1	1.6	1.0	2.97	1.9

[+] O-Methyl activity was determined indirectly by subtracting the specific activity of ricininic acid from that of ricinine.

[++] N-methyl activity was calculated by taking difference of activity of ricinine and activity of other carbon atoms.

[+++] Isolated and counted as N-methyl-β-analine.

The results from these experiments indicated that the label from glycolic acid-2-C^{14} was incorporated ten times greater than that from glycolic acid-1-C^{14}. Factors which may influence the percent incorporation such as the amount of material injected and the physiological development of the plant did not appear to have any effect on the extent of incorporation.

Ricinine formed from glycolic acid-2-C^{14} was degraded (Table IV) and all carbon fragments were found inactive except the O- and N-methyl carbons. Difficulty was experienced in precisely measuring the specific activity of the tetramethyl-ammonium reineckate salt, consequently values shown for the amount of activity in the N-methyl carbon are not the result of making direct measurements.

It is well known that glycolic acid splits into two C_1 units, so that it may serve as a methyl donor.

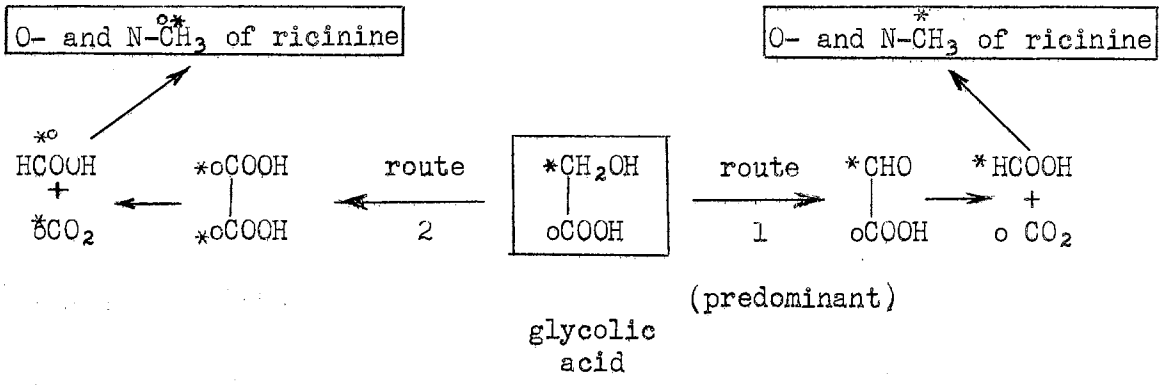


Figure 9

Proposed Glycolic Acid Metabolism in Castor Plants (47, 48, 49)

From the known metabolic pathway of glycolic acid in plant (47, 48, 49) the label from glycolic acid-2-C^{14} might be incorporated into ricinine via formic acid (route 1 and 2); while glycolic acid-1-C^{14} would lose most of its activity as CO_2, it can only be incorporated along route 2.

C. Three-Carbon Compounds as Precursors of Ricinine

Compounds used in these experiments were β-alanine-1-C^{14}, glycerol-1-C^{14}, malonic acid-1-C^{14}, malonic acid-2-C^{14} and malonamide -1-C^{14}. Percentages of incorporation of radioactivity from these compounds into ricinine are shown in Table V. Distributions of the carbon-14 label in ricinine formed from glycerol, malonate and malonamide are shown in Table VI.

Glycerol-1-C^{14} was found to contribute some activity to each carbon atom of ricinine except for carbon 5; glycerol-2-C^{14} contributed its activity mostly to the O- and N-methyl carbons, to carbons 2 and 3, and to carbon 5. It may be concluded that glycerol is incorporated into ricinine by three routes. One is without cleavage where the whole molecule can be incorporated into carbons 4,5 and 6 of ricinine; the other two routes are that glycerol cleaves into C_1 and C_2 units before it is incorporated into ricinine. Based on known pathways of glycerol metabolism this might proceed according to the scheme shown in Figure 10. About 50% of the total activity of glycerol (1-C^{14} and 2-C^{14}) was found in carbons 2,3 and 8. The conclusion is that route 2 is predominent over the other routes. This labeling pattern is different from that reported by Marion et al (36). They have shown that about 40-50% of the activity was located in carbons 4, 5 and 6, with the rest of it in the O- and N-methyl carbons and the nitrile carbon.

TABLE V

INCORPORATION OF THREE-CARBON COMPOUNDS INTO RICININE

Experiment Number	Precursor			Ricinine		Incorporation %	Isotope Dilution	Experiment Duration (hours)	Physiological Stage (Nodes)
	Compound	Specific Activity mμc/mM	Quantity Injected mμc	Yield mMole	Specific Activity mμc/mM				
379	β-Alanine-1-C^{14}	2.80×10^5	1400	0.26	10.3	0.19	2.72×10^4	48	6
380	β-Alanine-1-C^{14}	2.80×10^5	1400	0.39	3.52	0.10	7.95×10^4	96	7
381	β-Alanine-1-C^{14}	2.80×10^5	2800	0.36	12.0	0.15	2.33×10^4	48	7
382	β-Alanine-1-C^{14}	2.80×10^5	2800	0.30	20.0	0.22	1.4×10^4	96	7
367	Glycerol-1,3-C^{14}	3.76×10^6	4930	0.55	37.7	0.42	1.0×10^5	48	9
368	Glycerol-1,3-C^{14}	3.76×10^6	2960	0.27	39.6	0.36	9.5×10^4	48	7
369	Glycerol-1,3-C^{14}	3.76×10^6	4930	0.62	37.3	0.47	1.0×10^5	96	10
370	Glycerol-1,3-C^{14}	3.76×10^6	2960	0.41	34.0	0.46	1.1×10^5	96	8
466++	Glycerol-1,3-C^{14}	——	100,000	0.83	262.0	0.22	——	192	7-9
467+	Glycerol-2-C^{14}	——	50,000	0.68	297.0	0.41	——	192	7-9
461+	Malonate-1-C^{14}	4.37×10^6	100,000	0.80	89.3	0.072	4.9×10^4	144	9-11
464+	Malonate-1-C^{14}	4.37×10^6	100,000	0.91	22.6	0.021	1.93×10^5	144	9-11
462+	Malonate-2-C^{14}	3.24×10^6	100,000	1.04	139	0.15	3.33×10^4	144	9-11
465++	Malonate-2-C^{14}	3.24×10^6	10,000	1.28	20.0	0.25	1.62×10^5	144	9-11
463+++	Malonamide-1-C^{14}	6.1×10^6	50,000	0.47	152	0.14	4.0×10^4	144	9-11

\+ 10 plants grown in green house

++ 20 plants grown in green house

+++ 5 plants grown in green house

TABLE VI

DEGRADATION OF RICININE FORMED FROM GLYCEROL, MALONATE AND MALONAMIDE

Experiment Number	Precursor	Specific Activity of Ricinine mμc/mM	C-9[+] (-O-CH_3)		C-7[++] (-N-CH_3)		C-8 (CN)		C-2 and C-3		C-4		C-5		C-6	
			mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%
466(1)	Glycerol-1-C^{14}	262.0	26.0	10.0	26.0	10.0	41.7	16.0	88.8	34.0	(21.4, C-4, C-5, C-6[+++])					
466(2)	Glycerol-1-C^{14}	358.0	42.0	11.7	42.0	11.7	41.6	11.0	118.0	33.0	49.5	13.8	0	0	53.3	14.9
467	Glycerol-2-C^{14}	297.0	60.0	20.2	60.0	20.2	31.5	10.6	124.0	41.8	0	0	65.0	22.0	0	0
471	Malonate-1-C^{14}	63.1	9.6	15.2	9.6	15.2	10.5	16.7	11.0	17.5	—	—	—	—	—	—
472	Malonate-2-C^{14}	71.0	15.4	21.7	15.4	21.7	7.5	10.5	21.7	30.5	—	—	—	—	—	—
473	Malonamide-1-C^{14}	6.65	1.62	24.4	1.62	24.4	1.0	15.0	1.75	26.3	—	—	—	—	—	—

[+] O-Methyl activity was determined indirectly by subtracting the specific activity of ricininic acid from that of ricinine.

[++] N-methyl activity was assumed to be equal to O-methyl activity (7.14).

[+++] Isolated and counted as Na-propionate.

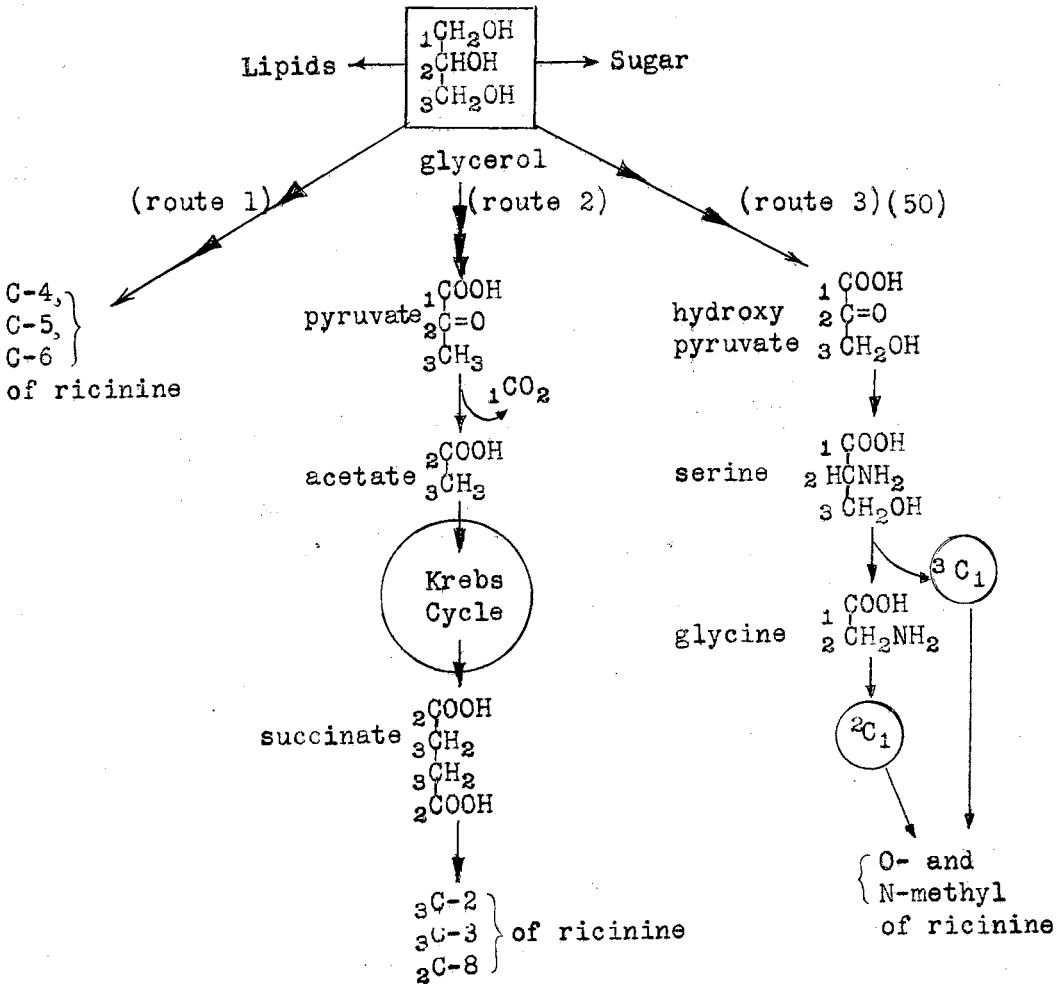


Figure 10

Proposed Glycerol Metabolism in Castor Plants

Malonate and malonamide may be incorporated into ricinine in the same way as glycerol ; the distribution of radioactivity of each carbon atom of ricinine falls into approximately the same pattern as for glycerol; however, the propionate did not contain a sufficient amount of radioactivity to be completely degraded.

D. Four-Carbon Dicarboxylic Acids as Precursors of Ricinine

Two dicarboxylic acids, succinic acid-1,4-C^{14}, succinic acid-2,3-C^{14} and fumaric acid-2,3-C^{14} were used in this study. These metabolites found in the Krebs cycle were reported (14) to have a high percentage of incorporation into ricinine. Labeling patterns of succinic acid-1,4-C^{14} and succinic acid-2,3-C^{14} have recently been reported (9,12). Fumaric acid-2,3-C^{14} would be expected to give the same labeling pattern.

In this study, the experiments were carried out with increasing duration of time. The results show (Table VII) that the best incorporation is 48-hours after administration of these compounds. After that time, the percentage of incorporation falls greatly, which may be due partly to the breakdown of ricinine (46).

Results of degradation are shown in Table VIII. In the case of succinic acid-1,4-C^{14}, all carbon atoms are found to be inactive except carbons 2,3 and 8, with most of the activity being in carbon 8, which agrees with earlier reports (9, 12, 14). In the case of succinic acid-2,3-C^{14} and fumaric acid-2,3-C^{14}, activities were also found in carbons 2,3 and 8, with most of the activity in carbons 2 and 3.

The incorporation of four-carbon dicarboxylic acids into ricinine may proceed through aspartic acid, based on the fact that this amino acid can be incorporated into ricinine (10).

It is proposed (Figure 11) that aspartic acid (I) condenses

TABLE VII

INCORPORATION OF FOUR-CARBON DICARBOXYLIC ACIDS INTO RICININE

Experiment Number	Precursor			Ricinine		Incorporation+ %	Isotope Dilution	Experiment Duration (hours)	Physiological Stage (Nodes)
	Compound	Specific Activity μc/mM	Quantity Injected μc	Yield mMole	Specific Activity μc/mM				
426	Succinic Acid-1,4-C^{14}	5000	1770	0.25	3.25	0.045	1,540,000	12	8
427	Succinic Acid-1,4-C^{14}	5000	1770	0.31	9.07	0.16	550,000	24	10
428	Succinic Acid-1,4-C^{14}	5000	1770	0.23	28.0	0.36	179,000	48	7
429	Succinic Acid-1,4-C^{14}	5000	1770	0.12	27.2	0.18	184,000	96	7
430	Succinic Acid-2,3-C^{14}	8750	1100	0.16	3.3	0.048	2650,000	12	8
431	Succinic Acid-2,3-C^{14}	8750	1100	0.18	51.5	0.84	1700,000	24	7
432	Succinic Acid-2,3-C^{14}	8750	1100	0.095	142	1.22	615,000	48	6
433	Succinic Acid-2,3-C^{14}	8750	1100	0.21	82.5	1.55	107,000	96	9
434	Fumaric Acid-2,3-C^{14}	6100	2600	0.29	4.67	0.051	1300,000	12	9
435	Fumaric Acid-2,3-C^{14}	6100	2600	0.19	93.3	0.67	65,500	24	7
436	Fumaric Acid-2,3-C^{14}	6100	2600	0.115	457	2.0	13,300	48	6
437	Fumaric Acid-2,3-C^{14}	6100	2600	0.105	241	1.36	25,300	96	6
438	H_2O	—	0	0.17	0	0	—	96	6

+ Percentage incorporation was calculated by dividing the total activity of the ricinine isolated by the total activity injected into the castor plant.

TABLE VIII

DEGRADATION OF RICININE FORMED FROM FOUR-CARBON DICARBOXYLIC ACIDS

Experiment Number	Precursor	Specific Activity of Ricinine mμc/mM	C-9[+] (-O-CH_3)		C-7 (-N-CH_3)		C-8 (-CN)		C-2 and C-3		C-4,C-5,C-6[++]	
			mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%	mμc/mM	%
162	Succinic Acid-1,4-C^{14}	23.4	0	0	0	0	18.1	77.3	2.44	10.4	—	—
469	Succinic Acid-2,3-C^{14}	135.0	Trace	0	Trace	0	20.9	15.5	62.6	46.5	Trace	0
468	Fumaric Acid-2,3-C^{14}	214.0	0	0	0	0	30.6	14.3	104	48.7	4.2	2.4

[+] O-Methyl activity was determined indirectly by subtracting the specific activity of ricininic acid from that of ricinine.

[++] Isolated and counted as N-methyl-β-alanine.

with a three-carbon unit (II) to give rise to the piperidine derivative, compound III, which is then dehydrated and dehydrogenated to form quinolinic acid (IV) which is converted to nicotinic acid (V) by decarboxylation (51). Further steps in the biosynthetic pathway from nicotinic acid to ricinine have been proposed (14); however, according to the results of this study, ricininic acid (VI) may be the last intermediate in the pathway.

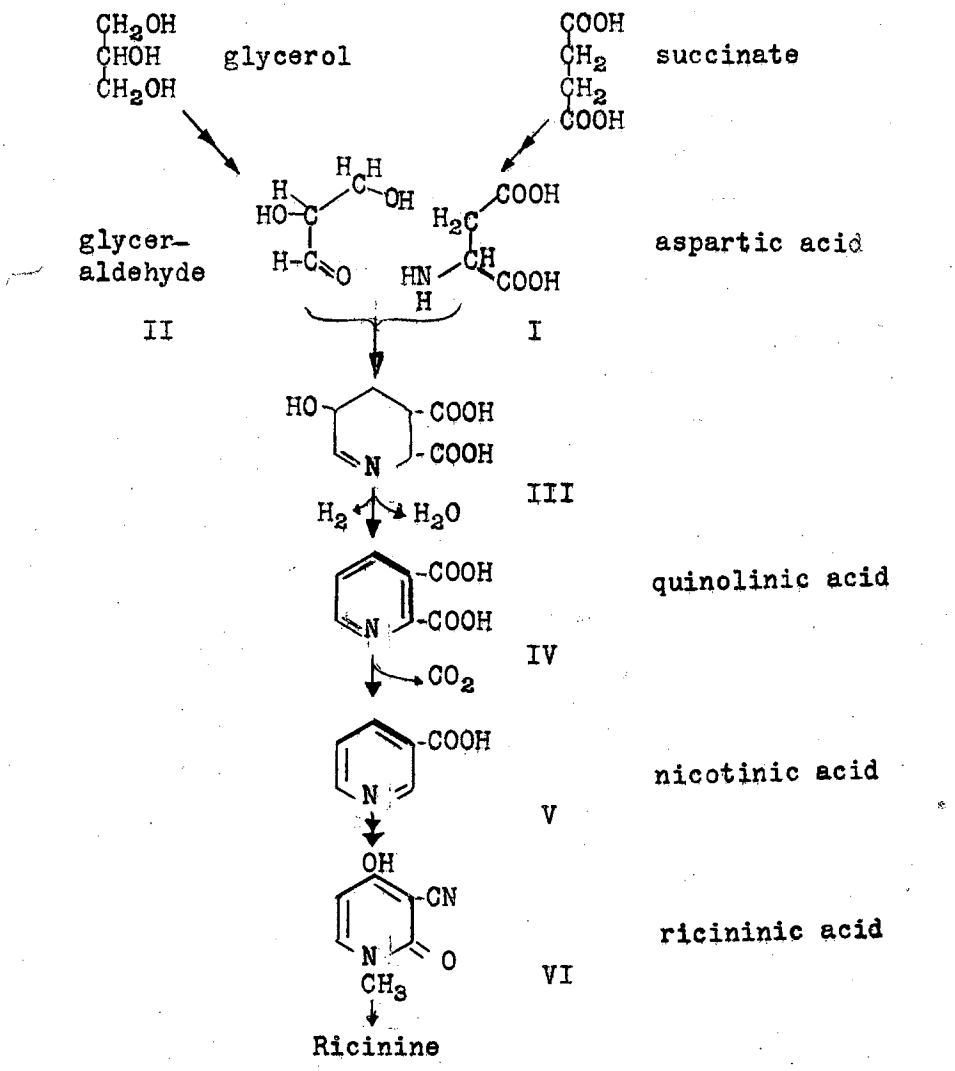


Figure 11

Proposed Pathway for Ricinine Biosynthesis

CHAPTER V

SUMMARY

A number of selected pyridine compounds and small molecules, most of them known precursors of ricinine, were studied in an attempt to provide more knowledge on the biosynthesis of the pyridine nucleus in plants.

The extent of incorporation of these compounds into ricinine was established and the ricinine formed from nicotinic acid, glycolic acid, glycerol, malonate, malonamide, succinate and fumarate was chemically degraded to determine the amount of radioactivity located in each carbon atom of the alkaloid.

The result from degradation of ricinine formed from nicotinic acid-7-C^{14} showed that all of the activity in the alkaloid was found in the nitrile carbon atom. This agreed with previous reports.

Degradation of ricinine formed from glycolic acid showed that this compound served only as a methyl donor since all of the activity was found in the O- and N-methyl carbons.

Activity was found to spread over each carbon atom of ricinine formed from glycerol. This was explained by the labeling pattern which showed that, a) glycerol was cleaved into smaller fragments, which in turn contributed to the methyl carbons, the nitrile carbon and carbons 2, 3 and 8 of ricinine by being metabolized via known pathways, and

b) carbons 4, 5 and 6 of the alkaloid originated from glycerol which had not been cleaved into smaller units.

Malonate and malonamide showed approximately the same extent of incorporation into ricinine as did glycerol. Activity from these compounds was found to be distributed in every carbon atom of ricinine. A labeling pattern similar to that for glycerol was proposed.

Succinic and fumaric acids were found to contribute only to carbons 2, 3 and 8 of the ricinine molecule. These data confirm and extend the reports of other scientists working in this field. It may be concluded that succinate and glycerol are the primary building units for the pyridine ring and nitrile carbon atom of ricinine.

Among the pyridine compounds as probable direct precursors of ricinine, ricininic acid was found to be quantitatively incorporated.

The complete pathway of ricinine biosynthesis may soon be elucidated by using similar approaches and by studying the in vitro metabolism of these and structurally related compounds.

A preliminary report on this research has been made (52).

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APPENDIX

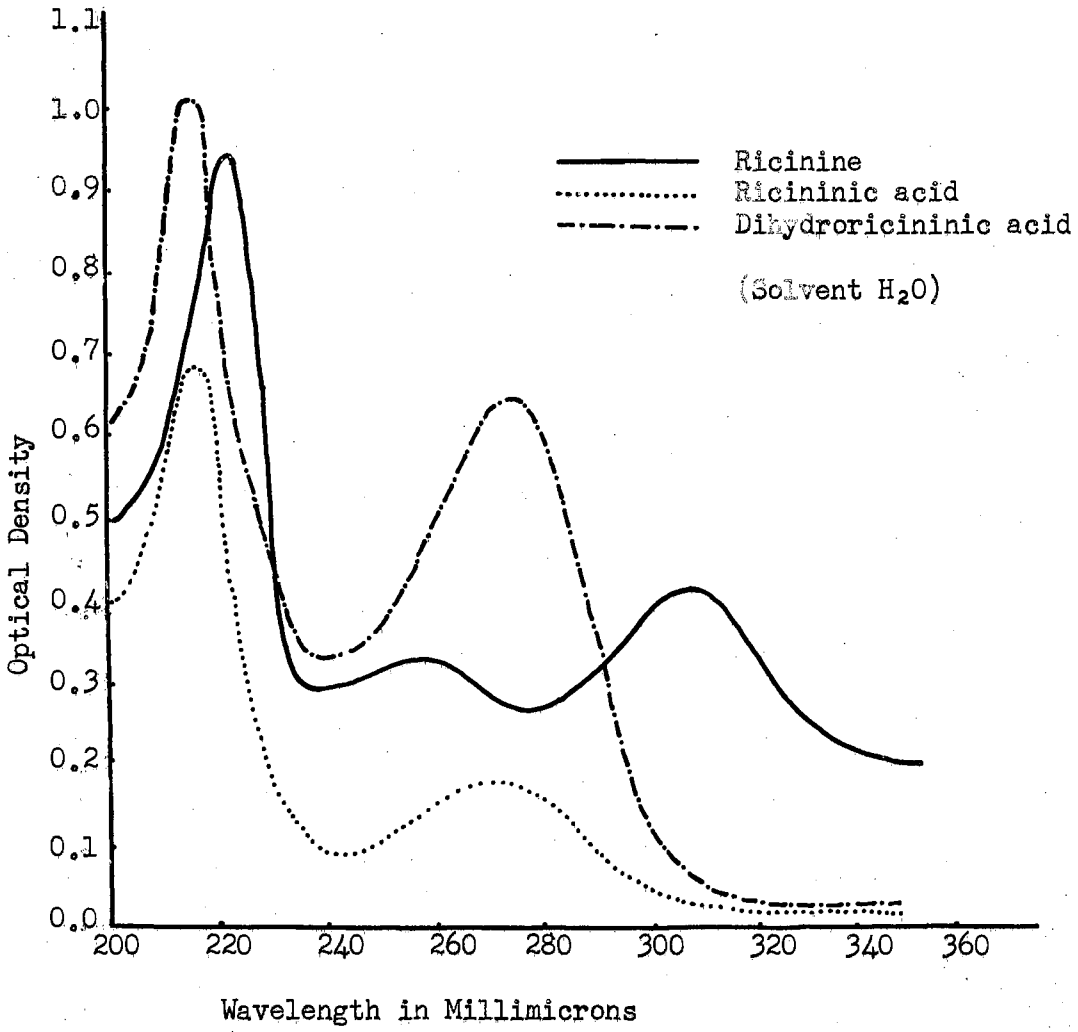

Figure 3

Ultraviolet Spectra of Ricinine, Ricininic Acid and Dihydroricininic Acid

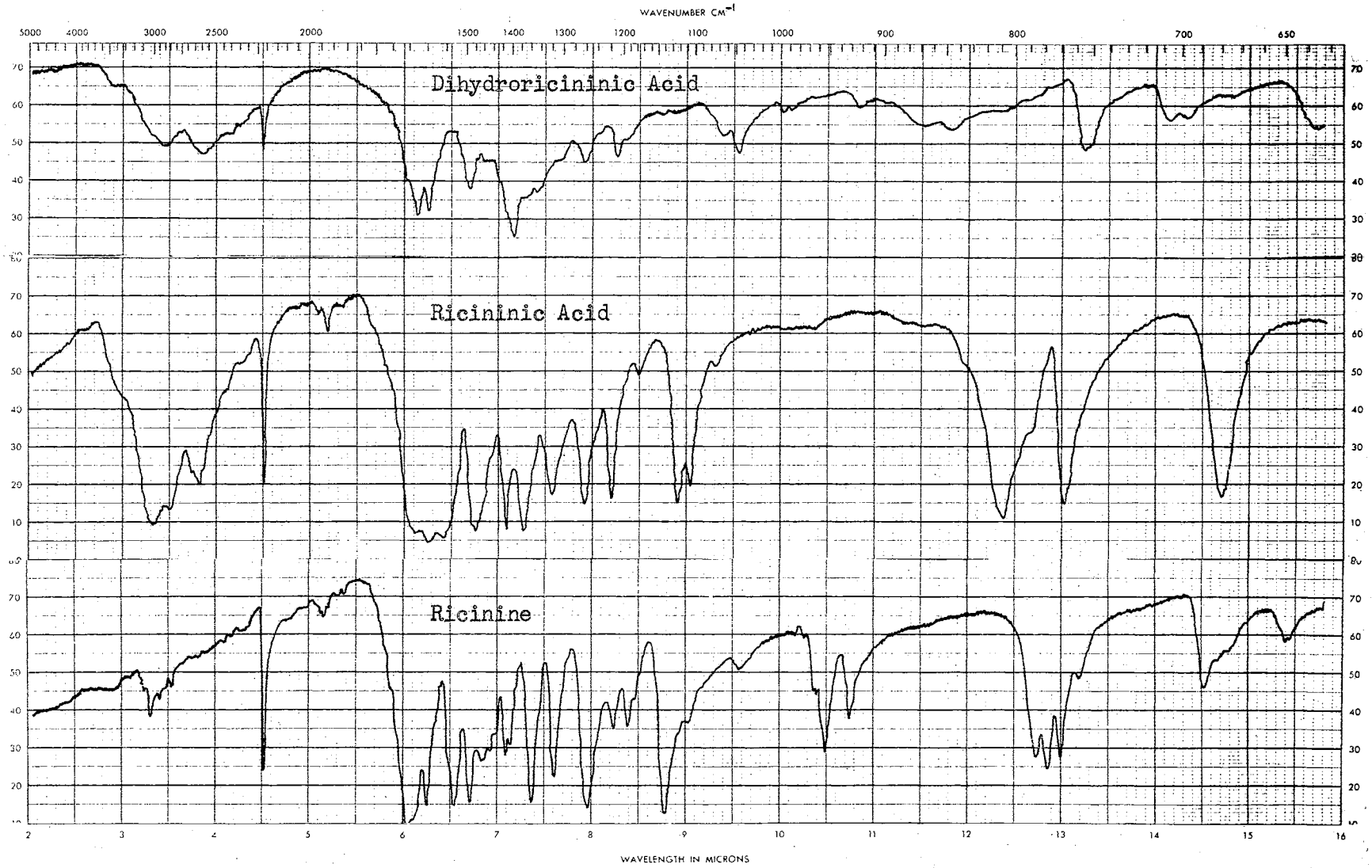

Figure 4

Infrared Spectra of Ricinine, Ricininic Acid and Dihydroricininic Acid (KBr pellets)

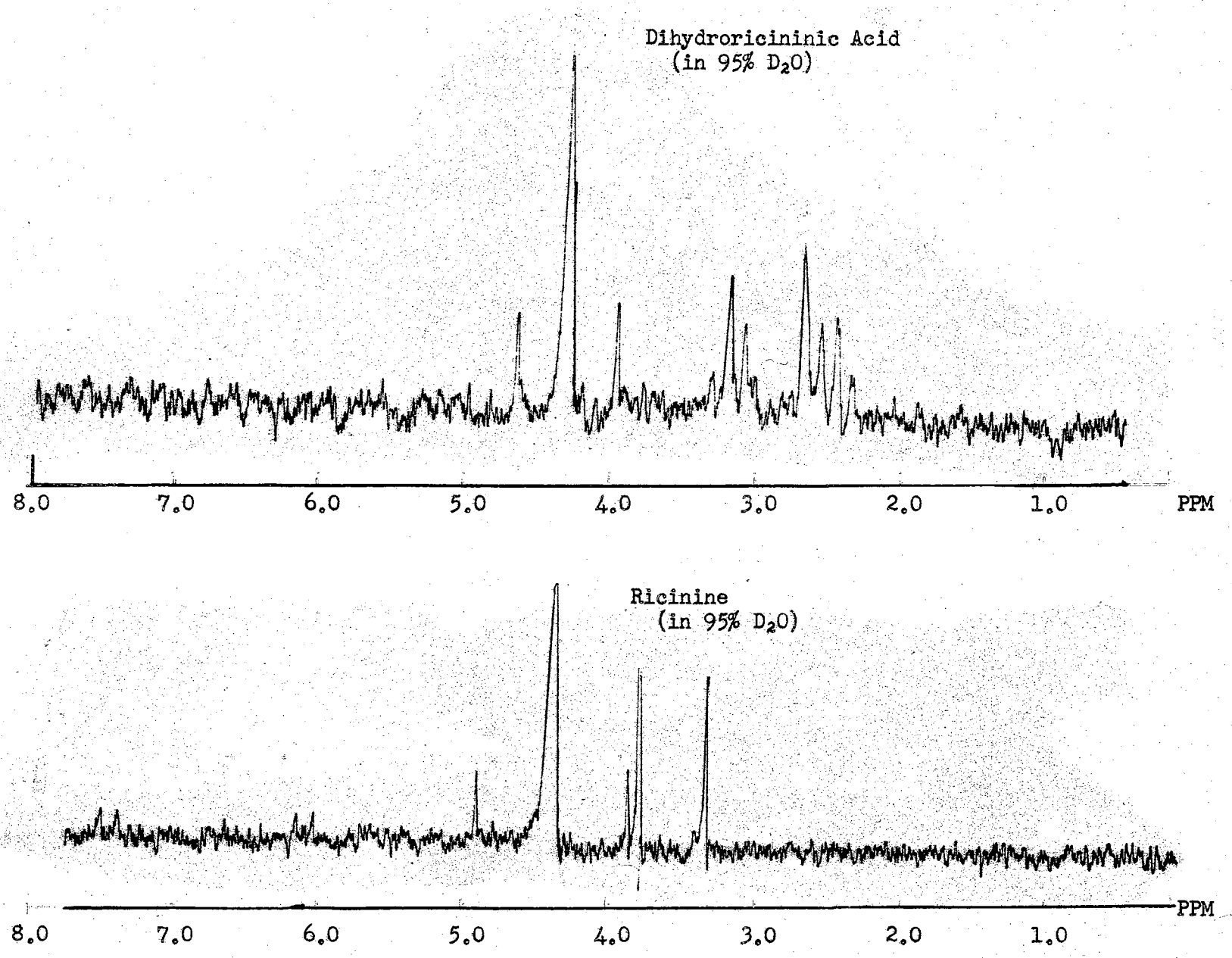

Figure 5

Nuclear Magnetic Resonance Spectra of Ricinine and Dihydroricininic Acid

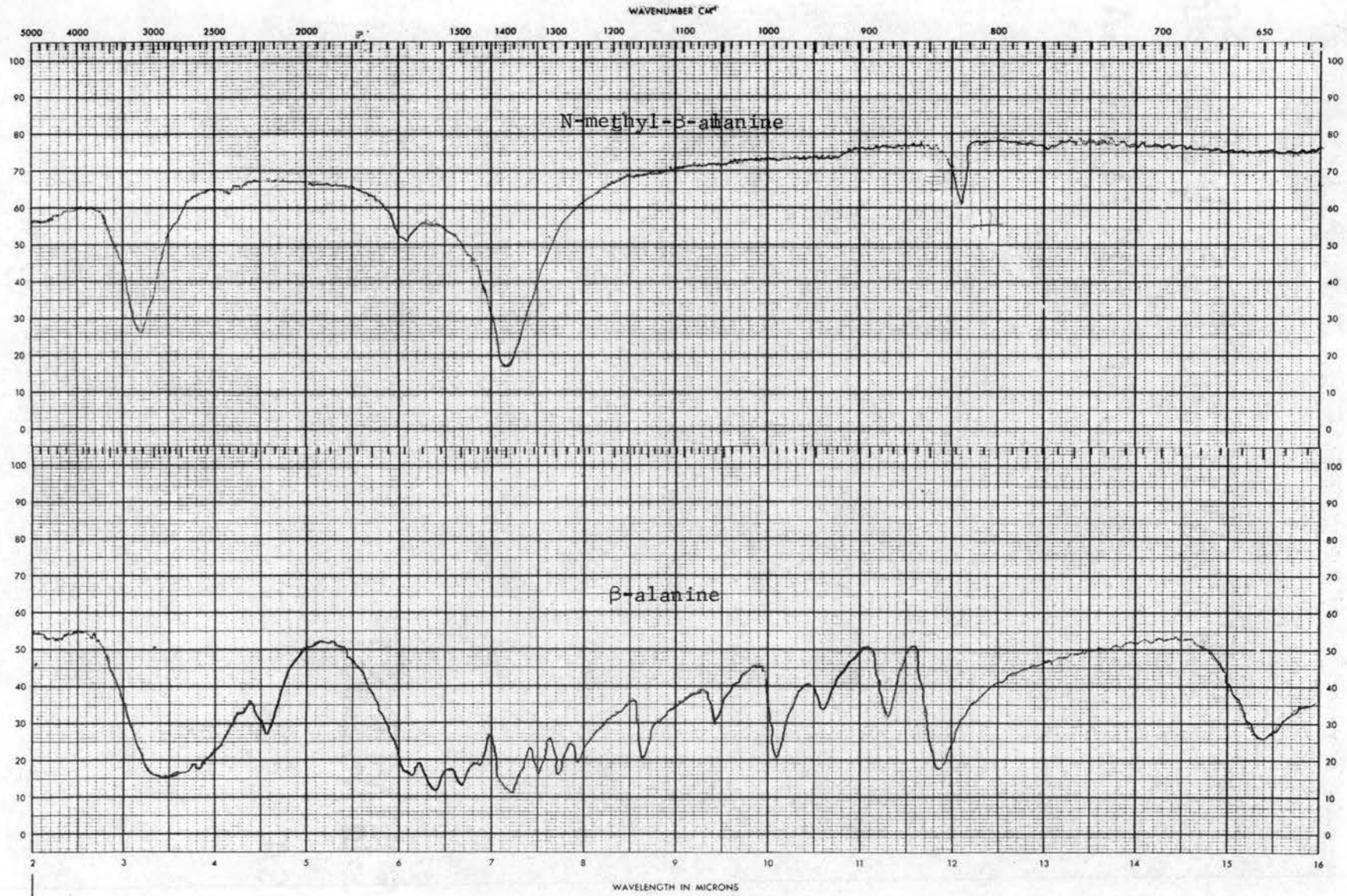

Figure 7 Infrared spectra of N-methyl-β-alanine and β-alanine (KBr pellets)

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