METABOLISM OF RICININE IN

RICINUS COMMUNIS L.

By

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CHAPTER I

INTRODUCTION

Alkaloids have been considered as the final molecular species produced in plants. During the last decade, it has become increasingly evident that alkaloids are not the "end products of metabolism" of plants, but may be the precursors of non-alkaloidal substances of metabolic significance.

Weevers (1) showed that ricinine decreased with increasing age of castor plants grown on nitrogen depleted soil, which was a clear indication that ricinine could be metabolized by the castor plant. Waller and Nakazawa (2) showed that ricinine was rapidly utilized by sterile castor cotyledons in the dark.

Recently, Waller _et al_ (3) reported that ricinine could be metabolized to CO_2 by castor plants after administration of ricinine-8-^{14}C.

This study indicates, first, that the pyridine ring of ricinine was degraded to CO_2 by the castor plant both in the dark and in the light, second, that the ricinine content of castor leaves fell from a normal value of 6 μmoles to levels of about 0.005 μmoles per gram fresh weight as the leaves turned yellow, and third, that the rate of metabolism of ricinine in the yellow leaf was faster than that in the whole castor plant.

CHAPTER II

LITERATURE REVIEW

A. Structure and Properties of Ricinine

Ricinine, $C_8H_8O_2N_2$ (4), an alkaloid found originally in the seeds of the castor plant, Ricinus communis L., was first discovered in 1864 by Tuson (5) and was studied in more detail by Evans (6), Schulze (7), Weevers (1), Bogdashevskaya (8), Robinson (9, 10, 11), Marion (12, 13, 14, 15), and Waller (2, 3, 16, 17, 18, 19, 20, 21). Ricinine is a neutral alkaloid, crystallizes in colorless prisms, is optically inactive, melts at 200-201.5°C (corrected) and sublimes at 170-180°C at 20 mm pressure (22). Ricinine is slightly soluble in water, ethanol and chloroform. It does not form salts and does not react with the usual alkaloidal reagents; however, it does give some color reactions (23).

The ultraviolet absorption spectrum of ricinine was reported (9) to have a maxima at 320 mμ in chloroform, 315 mμ in 95 percent ethanol and 307 mμ in water. Infrared and Mass spectra of ricinine have also been studied and reported by Manis et al (24) and Waller et al (25).

Robinson and Fowell developed a paper chromatographic analysis of ricinine (9) and found that it gives a colored product when the paper is sprayed with the modified Dragendorf's reagent. The R_f values were 0.55 for isopropanol-toluene-acetic acid-water, 5:10:1:1 and 0.75 for t-butanol-water-acetic acid, 4:2:1.

Ricinine is the only cyano-substituted pyridine alkaloid known to occur naturally. The structure of ricinine (Figure 1) was proved to be 1,2-dihydro-4-methoxy-1-methyl-2-oxonicotino-nitrile by Spath and Koller (26, 27) and confirmed by several chemical syntheses (27,28,29).

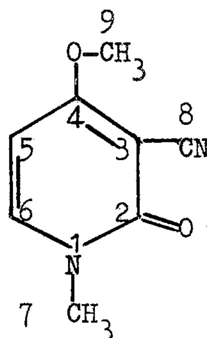

Figure 1
1,2-dihydro-4-methoxy-1-methyl-2-oxonicotino-nitrile

B. Occurrence of Ricinine

Ricinine was shown to be present in all parts of the young castor plant at levels of approximately 1 mg. per gram fresh weight (30). Bottcher (31) reported that the bulk of ricinine was located in mature castor seeds with 0.15 percent occurring in the seed coat and about 0.03 per cent in the kernel.

Weevers (1) found that, during germination in the dark, the ricinine nitrogen in 100 seeds increased from 4 to 72 mg. in three weeks, which was a clear indication that synthesis had occurred. He also reported that in younger seedlings, the cotyledons contained 5-10 times as much ricinine as did the hypocotyls and only traces were found in the roots of the castor plant.

Schulze and Winterstein (32) suggested that etiolated castor seedlings produced a higher quantity of ricinine than the normal ones. Bogdashevskaya (33) studied the formation of ricinine in whole castor

plants and found that the amount of ricinine increased constantly with plant growth, but it declined sharply after 20 days in old plants, and increased again slightly after flowering.

Waller and Nakazawa (2) reported that the ricinine content of young castor seedlings grown in sand at 30° using tap water was observed to increase 30 to 50-fold in the 2 to 5-day period following planting of the seed. Waller et al (3) have also shown that the ricinine production by individual castor plants was increased from a value of 0.1 μmole in an individual castor seed to levels of about 1.2 mmoles in 17 week old plants.

C. Biosynthesis of Ricinine

The biosynthesis of ricinine in castor plants was first reported by Dubeck and Kirkwood (34) with the use of radioisotopes. They found that the methyl carbon of methionine serves as a precursor of the O- and N-methyl groups in ricinine. Leete and Leitz (35) and Waller and Henderson (16) showed that the pyridine ring of nicotinic acid and nicotinamide becomes the α-pyridone ring of ricinine, the carboxyl carbon of nicotinic acid and the carboxamide carbon of nicotinamide becomes the nitrile carbon atom of ricinine. Yang et al (17, 36) indicated that succinate and glycerol are the primary building units for the pyridine ring and nitrile carbon atoms of ricinine, and in vivo experiments with young castor plants have established that quinolinic acid can serve as a more efficient precursor of ricinine than nicotinic acid.

Waller et al (20) have recently reported that the pyridine moieties of the pyridine nucleotides, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, and nicotinamide adenine dinucleo-

tide, were shown to be incorporated into ricinine by castor plants with an efficiency comparable to that of nicotinic acid, nicotinamide (16), and quinolinic acid (36) (Figure 2).

D. Metabolism of Ricinine

Bogdashevskaya (8) showed that ricinine content was reduced in leaves which were shaded from the light, while the upper unshaded leaves of such plants produced supernormal levels of ricinine. Waller and Nakazawa (21) reported that ricinine was most rapidly metabolized by excised cotyledons in the dark, 90% being utilized after 48 hours. However, no change in ricinine content was observed when nicotinic acid was present in the medium. They found that ricinine-8-^{14}C was metabolized in a cell-free system but the products were not identified.

Tso and Jeffrey (37) have studied the fate of nitrogen-15 labeled tobacco alkaloids in N. glauca and rustica and found that there was extensive metabolic breakdown of the radioactive alkaloids. Fairbairn et al (38, 39, 40) showed that the major alkaloids of Conium maculatum and Papaver somniferum, coniine and morphine, respectively disappeared rapidly soon after formation.

Waller et al (3) reported that ricinine-H^3 and ricinine-8-^{14}C can be metabolized by castor plants. The extent of degradation of the alkaloid varied from 75% to 95% during a period of 17 weeks.

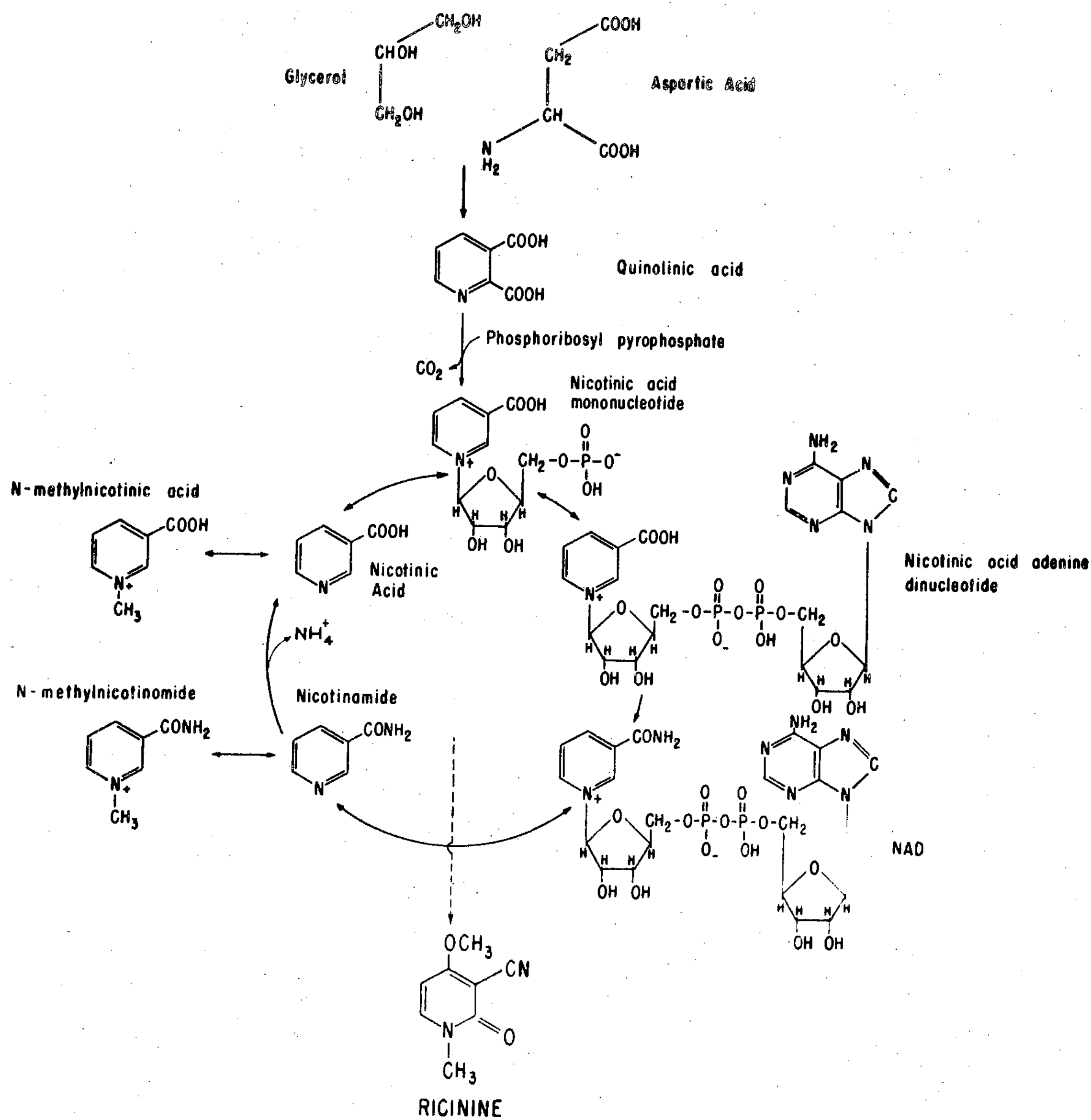

Figure 2. The pyridine nucleotide cycle and proposed biosynthetic pathway for ricinine (20).

CHAPTER III

EXPERIMENTAL METHODS

A. Conditions for Growing Castor Plants

The first group of plants used in this study were grown on port clay loam soil at the Agronomy Farm of Oklahoma State University in Perkins. Seeds of the Cimarron variety were planted on April 19, 1966. These plants were used for the estimation of ricinine production, the biodegradation of ricinine, the isolation and determination of radioactive ricinine studied.

A second and third group of castor plants were grown in soil in the Plant Growth Chamber* in the Biochemistry Department. The Plant Growth Chamber conditions were: 15 hours in light at $90 \pm 2^{\circ}F$ and 9 hours in dark at $80 \pm 2^{\circ}F$. These plants were used for the determination of ricinine formation and the biodegradation of ricinine.

B. Labeled Compounds Used

Ricinine-3,5-^{14}C was synthesized on a micro scale from sodium cyanoacetate-2-^{14}C, according to the procedure of Yang et al (41). The purity was checked by ascending chromatography on Whatman No. 1 paper in three different solvent systems and its radioactivity on the paper was located using a Nuclear-Chicago 4π Actigraph III paper strip counter.

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*Model PGW-60 Plant Growth Chamber, Percival Refrigeration and MFG. Co., Boone, Iowa.

The R_f values of the ricinine-3,5-^{14}C were 0.75 in 85% isopropanol, 0.89 in 60% n-propanol and 0.85 in 95% ethanol-1 M ammonium acetate, 7:3 v/v, pH 5.0. It had a specific activity of 360 µc/mmole. As determined by paper chromatography with 85% isopropanol, the ricinine is sometimes contaminated with traces of ricininc acid (0.5%).

Quinolinic acid-2,3,7,8-^{14}C was synthesized by condensing glycerol and aniline-U-^{14}C (18). It had a specific activity of 50.7 µc/mmole.

C. Administration of Labeled Compounds

The method of injection used for the first group of plants was developed by Waller (30). A 22 gauge hypodermic needle was inserted at the top of the internode of a castor plant to serve as a vent. An aqueous solution of the labeled compound was injected at the bottom of the internode. The solution was completely absorbed within a few minutes after injection. The administration technique used for the second and the third group of plants were usually made with a micro syringe in the upper part of the stem and the desired amount of labeled compound was injected. Uptake of 50 µl of solution was usually completed in fifteen minutes.

D. Harvest and Storage of Plant Materials

Most castor plants were harvested and processed immediately. A few were placed in polyethylene bags and stored at -18°C until processed.

E. Measurement of Radioactivity

All measurements of radioactivity in this study were made either by direct counting of the compounds in a Liquid Scintillation

Spectrometer [a, b] or by using a Nuclear-Chicago 4π Actigraph III paper strip counter, which had an efficiency of approximately 25% for carbon-14. The scintillation solvent was composed of 58.75% toluene, 39.25% absolute ethanol and 2% water. The phospher was 0.5% 2,5-diphenyloxazole (PPO) and 0.02% p-bis-2-(5-phenyloxazolyl) benzene (POPOP). This system had an efficiency of about 38% for carbon-14 compounds in instrument [a] and 64% in instrument [b]. For the determination of the $^{14}CO_2$ activity, ethanolamine-ethylene glycol monomethyl ether (methylcellosolve), 1:2 v/v was used to trap the $^{14}CO_2$ it evolved, and methylcellosolve-toluene (sulfur free), 1:2 v/v which contained 5.5 grams of PPO per liter was used for scintillation cocktail. The ratio of ethanolamine-methylcellosolve-toluene were made of 1:7:10 (42).

F. Isolation of Ricinine

Roots were removed from the harvested plants, weighed, frozen with liquid nitrogen, ground to a fine powder with a mortar and pestle, and extracted five times with chloroform using a weight ratio of chloroform to fresh plant of 3:1. The chloroform solution was washed with a ratio of 5:1 of 7.4 N ammonium hydroxide, then evaporated to dryness on a steam hot-plate. Lipids and pigments were removed from the residue by treatment with ethyl ether. The residue remaining after extraction was dissolved in hot water and transferred into a sublimater and dried in an oven at 110°C overnight. The residue was sublimed at about 170°C, 20 mm Hg pressure for 1.5 to 2 hours. Ricinine on the

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[a] Model 314 E Tri-Carb, Packard Instrument Co, Inc., La Grange, Illinois.

[b] Model 3314/3950 Tri-Carb, Packard Instrument Co, Inc., La Grange, Illinois.

cold finger of the sublimater was dissolved in hot water and transferred to a vial, and dried in an oven at 110°C. Purification was achieved by repeated sublimation. The melting point of ricinine purified as above was usually 201°C.

G. Measurement of Respiratory $^{14}CO_2$ From Castor Plant

Carbon-14 labeled ricinine or quinolinic acid were administered to a young castor plant by injection into the stem. The plant was immediately placed in a closed chamber. The entering air was passed through with 1N NaOH so that carbon dioxide-free air was present in the chamber. The outlet of the chamber was connected by glass tubing joined by small sections of rubber tubing to a series of four test tubes (150 X 15 mm) as shown in Figure 3. The first tube was cooled to -78°C, and was used for trapping the volatile compounds. The second and third tubes each contained 10 ml of ethanolamine in methylcellosolve, 1:2 v/v, and were used for collecting the respiratory CO_2. The final tube contained 10 ml of saturated barium hydroxide, which was used as an indicator; the lack of precipitation in the saturated barium hydroxide indicated that all the carbon dioxide was quantitatively trapped by the ethanolamine in methylcellosolve.

H. Determination of Radioactivity of the Degradation Products Formed in Castor Plants From Ricinine-3,5-^{14}C

1. Paper Chromatography

The harvested plants were frozen with liquid nitrogen, ground to a fine powder with a mortar and pestle, and were extracted five times with 10 times the weight of the sample in 95% ethanol at 40°C. The plant extracts were centrifuged, the supernatants combined and evaporated to 5 ml volume on the steam hot-plate. An aliquot of this solu-

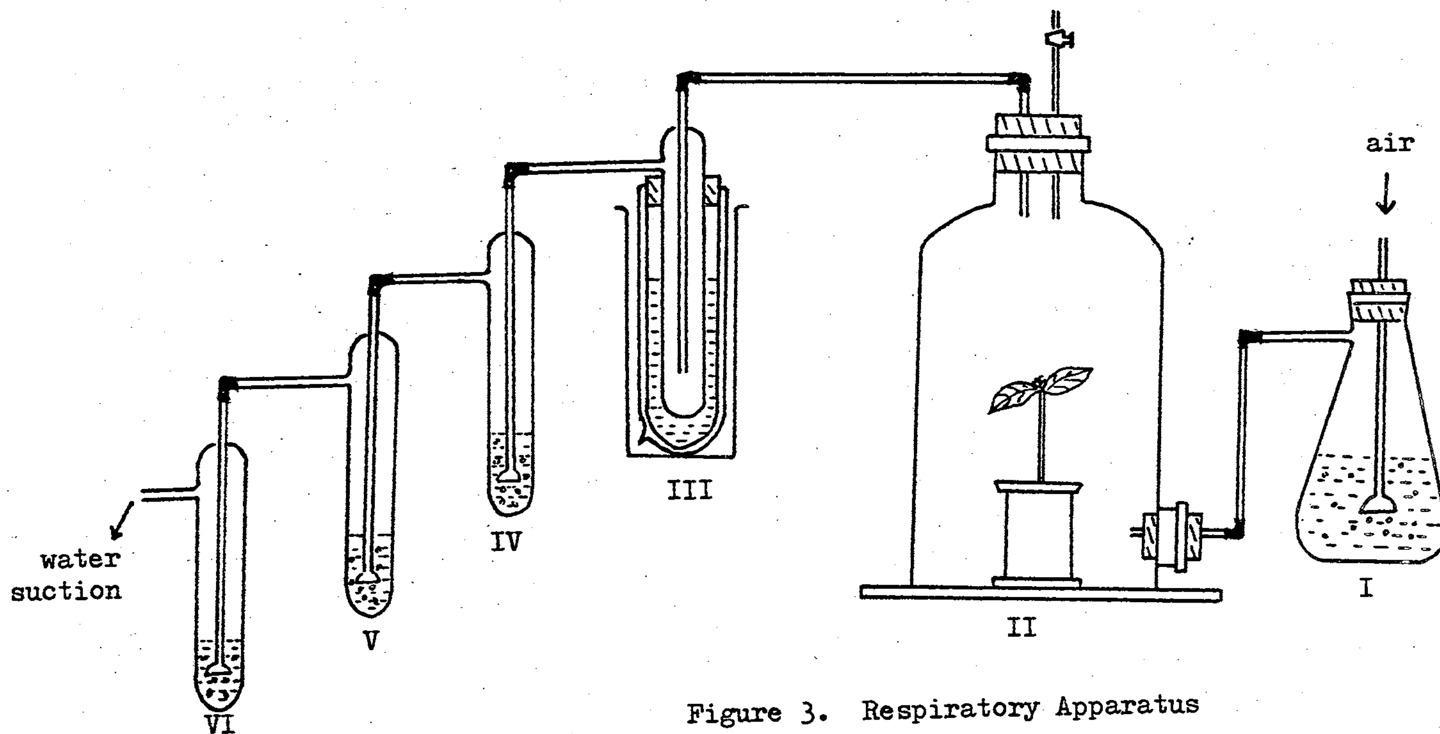

Figure 3. Respiratory Apparatus

I. NaOH (1N)
II. Respiratory Chamber
III. Absorption Trap (# 1)
IV. Absorption Trap (# 2): 10 ml Ethanolamine-methylcellosolve
V. Absorption Trap (# 3): 10 ml Ethanolamine-methylcellosolve
VI. Absorption Trap (# 4): 10 ml Saturated barium hydroxide

tion (100 µl) was spotted on Whatman No. 1 paper strips, and ascending paper chromatography was carried out in five different solvent systems: 85% isopropanol, 60% n-propanol, n-butanol-acetic acid-water (4:1:1), n-butanol saturated with 3% ammonium hydroxide and 95% ethanol-1M ammonium acetate (7:3 v/v), pH 5.0. After developing and drying, the radioactivity on the chromatograms was located with a Nuclear-Chicago 4π Actigraph III paper strip counter. Alternatively, the paper strips were cut into 1 to 2 cm pieces, placed in a counting vial with 10 ml of scintillation solvent, and counted by Liquid Scintillation Spectrometry.

2. By electrophoresis

An aliquot of plant extracts prepared in part H-1, 100 µl, was placed on Whatman No. 1 paper strips, 20 cm from one end. One end of the paper was dipped in the buffer (pyridine-acetic acid-water, 50:50: 1900 v/v, pH 4.8. The excess buffer was removed by blotting between two sheets of filter paper. This procedure was repeated to wet the paper on the other end. The paper strip was placed in the electrophoresis tank in the proper position, and a voltage of 2500 volts through a distance of about 40 cm for 60 minutes was applied. The paper was removed from the tank, dried, and the radioactivity determined using the counter.

3. DEAE-sephadex G-25 column chromatography

A two milliliter aliquot, prepared as described in part H-1, was applied to a DEAE-sephadex G-25 column (0.7 X 12 cm). A linear gradient was set up of 0 to 0.4 M NH_4HCO_3. The flow rate was 1 ml/min.. Fractions of 1 ml each were collected, and 0.05 ml aliquots were counted in a liquid scintillation spectrometer.

4. Ion exchange column chromatography

Two milliliters of plant extract were put on a Dowex 1 (8% cross-linked) column (1.5 X 32 cm). The column was eluted with deionized distilled water, followed by ammonium formate buffer, pH 4.0 of increasing molarity, 0.05M to 0.5M. The flow rate was about 1 ml/min.. Fractions of 10 ml were collected, and 0.2 ml aliquots were counted in a liquid scintillation spectrometer. Aliquots of each 10 ml fraction were assayed for absorbancy at 340 mμ to 200 mμ using the Beckman DB Spectrophotometer. The fractions which had the same ultraviolet absorption peak were combined and evaporated under vacuum to 1 ml. One-tenth ml of this solution was spotted on a 4 cm Whatman No. 1 paper strip and ascending chromatography was carried out in 85% isopropanol and 60% n-propanol. The radioactivity on the chromatograms was located with a Nuclear-Chicago 4π Actigraph III paper strip counter.

CHAPTER IV

RESULTS AND DISCUSSION

A. Biodegradation of Ricinine in Ricinus communis L.

The results of a time course study of administration of ricinine-3,5-^{14}C to a series of castor plants are shown in Table I. Equal amounts of radioactivity (155 mμc) were injected into each plant at six weeks of age. The whole plants (except the root) were used for the isolation of ricinine. Time intervals selected for harvest ranged from zero time to six months. One month following injection of ricinine-3,5-^{14}C, flowers and immature seeds began to appear (a normal development since it also was observed for the control plants); therefore the plants were divided into leaf, flower and seed for subsequent ricinine isolation. The zero time sample was harvested immediately after injecting the ricinine-3,5-^{14}C and 90% of the radioactivity was recovered in the ricinine sample. The 10% loss might be due to experimental error or ricinine bound to protein or carbohydrate. Fifty-two percent of the administered ricinine-3,5-^{14}C was recovered during the first 24 hours of the experiment and only 21% of the radioactivity remained after one month.

As shown in Table I, the formation of ricinine increased approximately two-fold during the one month period following administration of ricinine-3,5-^{14}C. However, the isotope dilution for the isolated ricinine increased from a value of 229 in the zero time sample to a

TABLE I

RECOVERY OF RICININE-3,5-^{14}C FROM WHOLE FIELD GROWN CASTOR PLANTS

Plant No.	Duration of Exp.	Total Activity Injected mμc	Fresh Weight of plant gm	Ricinine Isolated					Recovery	Isotope Dilution
				mg	mmole	mg per gm Fresh wt.	Total Activity Recovered mμc	Specific Activity mμc/mole	%	
P-1	0 hours	155	13.4	14.63	0.09	1.09	139.5	1570	90.3	229
P-2	1 "	155	14.85	17.50	0.11	1.18	122.0	1150	78.8	313
P-3	2 "	155	17.4	17.16	0.11	0.98	96.0	914	62.0	395
P-4	4 "	155	18.1	16.4	0.10	0.91	81.8	818	52.7	440
P-5	24 "	155	17.4	18.8	0.12	1.08	80.4	700	51.9	514
P-6	1 week	155	61.0	23.5	0.14	0.39	66.0	461	42.5	780
P-7	1 month	155	82.5	33.8	0.21	0.41	33.1	160	21.5	2250
P-8*	16 weeks	155								
Leaf			6.2	7.0	0.043	1.13	0.18	4.3	—	8.35×10^4
Flower			6.5	3.1	0.019	0.48	0.11	5.3	—	6.8×10^4
Seed			6.8	3.2	0.02	0.47	0.24	12	—	3.0×10^4
P-9*	20 weeks	155								
Leaf			4.3	4.1	0.025	0.95	0.07	2.8	—	12.8×10^4
Flower			4.2	2.3	0.014	0.55	0.024	1.7	—	21.2×10^4
Seed			4.1	2.0	0.012	0.49	0.14	11.5	—	31.2×10^4
P-10*	24 weeks	155								
Leaf			3.6	2.9	0.018	0.80	0.006	0.3	—	120×10^4
Flower			3.4	1.5	0.09	0.44	0.001	0.1	—	360×10^4
Seed			3.5	1.6	0.01	0.46	0.01	1	—	36×10^4

* Does not represent total weight and is only a representative sample.

value of 2250 in the one month sample, a ten-fold increase. This 8-fold difference between net ricinine synthesis and dilution of the administered ricinine-3,5-^{14}C clearly indicates that metabolism has occurred and is in good agreement with the loss of 79% of the ricinine-3,5-^{14}C administered during the same time period.

Due to the large size of the plants, one month following ricinine-3,5,^{14}C administration (10 weeks actual age) it was more convenient to use only representative tissue samples for ricinine analysis. In Table I it is shown that the specific activity of ricinine in the seed is much greater than that in the leaf and flower. This result indicated that ricinine could be translocated from the lower part of the stem and leaf to the seed. From the high isotope dilution of ricinine obtained in this experiment it is strongly evident that ricinine was: (a) metabolized by the castor plant (1,2,3) and (b) translocated to a site where the alkaloid concentration is highest. The ricinine-3,5-^{14}C was degraded to the extent of about 90% during a metabolism period of 24 weeks.

B. <u>In Vivo Conversion of Ricinine-3,5-^{14}C to $^{14}CO_2$ by the Castor Plant</u>

1. Dark Conditions

To obtain a better understanding of the breakdown of ricinine-3,5-^{14}C in the castor plant, the $^{14}CO_2$ released by a plant that had been injected with the alkaloid was collected and analyzed. The percent of radioactivity released as $^{14}CO_2$ was found to be a linear function of time (Figure 4). About 5.5% of the administered ricinine-3,5-^{14}C was evolved as respiratory $^{14}CO_2$ during the 168 hours of duration of the experiment. As shown in the Figure, a rapid degradation of

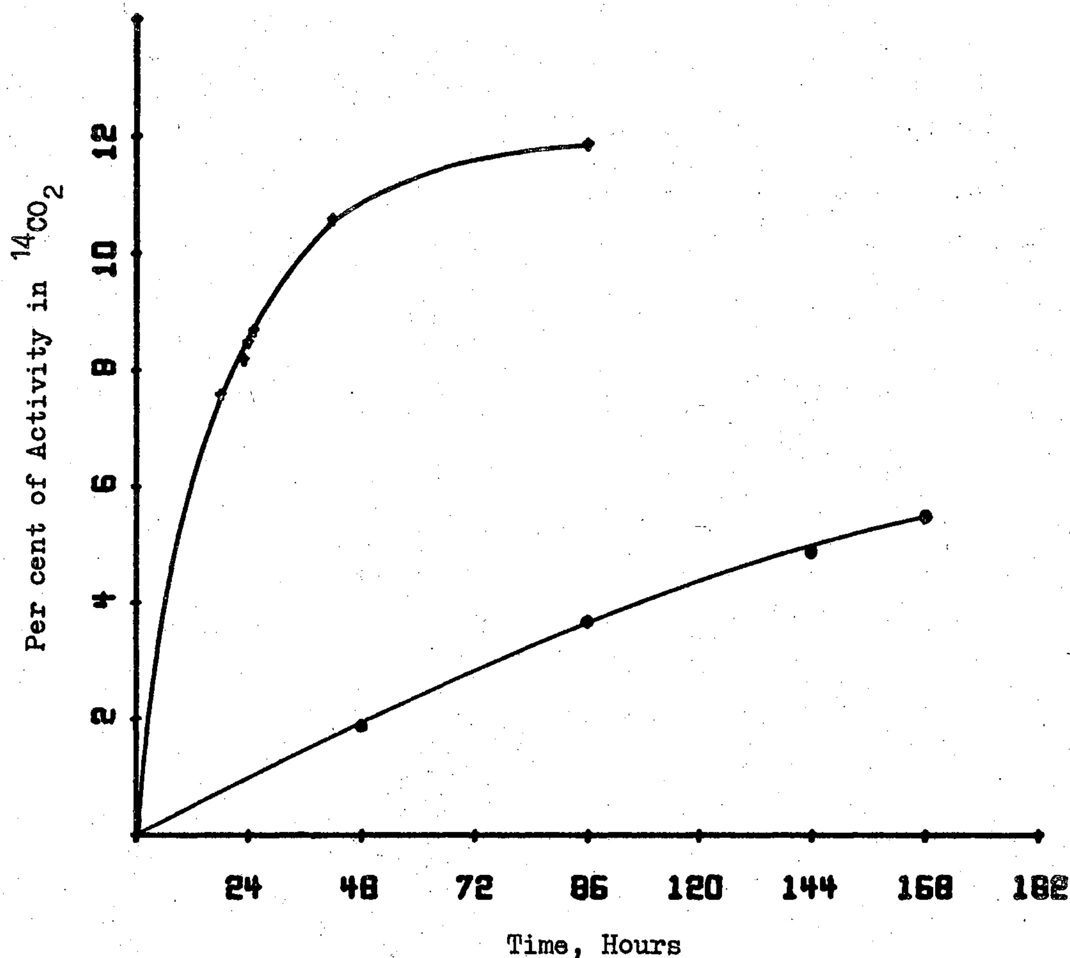

Figure 4. Comparison of $^{14}CO_2$ Released by Castor Plants in the dark After Injection of Quinolinic Acid-2,3,7,8-^{14}C and Ricinine-3,5-^{14}C.

These plants were grown in the Plant Growth Chamber. They were three weeks old when the labeled compounds were injected. Quinolinic acid-2,3,7,8-^{14}C, 1.86 μmoles with a specific activity of 50.7 μc/mM was dissolved in 20 μl of distilled water and injected into a single plant. Ricinine-3,5-^{14}C, 0.56 μmoles with a specific activity of 360 μc/mM was dissolved in 200 μl of distilled water and injected into a different single plant. After the labeled compound was injected into a castor plant, the plant was immediately placed in a dark closed Bell jar. Carbon dioxide-free air was passed through the Bell jar and the respiratory CO_2 was trapped in ethanolamine-methylcellosolve solution. ●: ricinine-3,5-^{14}C; +: Quinolinic acid-2,3,7,8-^{14}C.

quinolinic acid-2,3,7,8-^{14}C to $^{14}CO_2$ was also observed up to 42 hours, after which time no further increase was observed. A total of 12% of the radioactivity from quinolinic acid-2,3,7,8-^{14}C was released as respiratory $^{14}CO_2$ during a period of 96 hours. This result suggested that degradation of the pyridine ring of ricinine might be expected to proceed through some of the same intermediates as in the degradation of quinolinic acid. A difference is that the ricinine molecule might be much more stable than quinolinic acid, because of its functional groups.

2. Light Conditions

To establish that ricinine could be metabolized under the normal conditions, an experiment was conducted by maintaining the plant under average day (16 hours) and night (8 hours) conditions. The results are shown in Figure 5. The increase of respiratory $^{14}CO_2$ was nearly linear with respect to experiment duration. A total of 6.6% radioactivity was recovered in $^{14}CO_2$ during the period of 156 hours. It is suggested that ricinine metabolism by the castor plant under normal conditions was relatively close to its metabolism in the dark. The percent of activity released as $^{14}CO_2$ in the dark was higher than that in the light during the first 3 days, after which time a higher recovery of respiratory $^{14}CO_2$ in the light was observed. This might indicate that placing the plant in the dark promotes catabolism; thus the rate of ricinine metabolism in the castor plant was faster during the first 3 days that the plant was kept in the dark. However, after a longer time in the dark, photosynthesis had stopped, and the plant had reach abnormal physiological conditions, so that the rate of metabolism became slower. Based on this information it is clearly indicated that ricinine is not an "end product of metabolism" and that the pyridine ring of ricinine

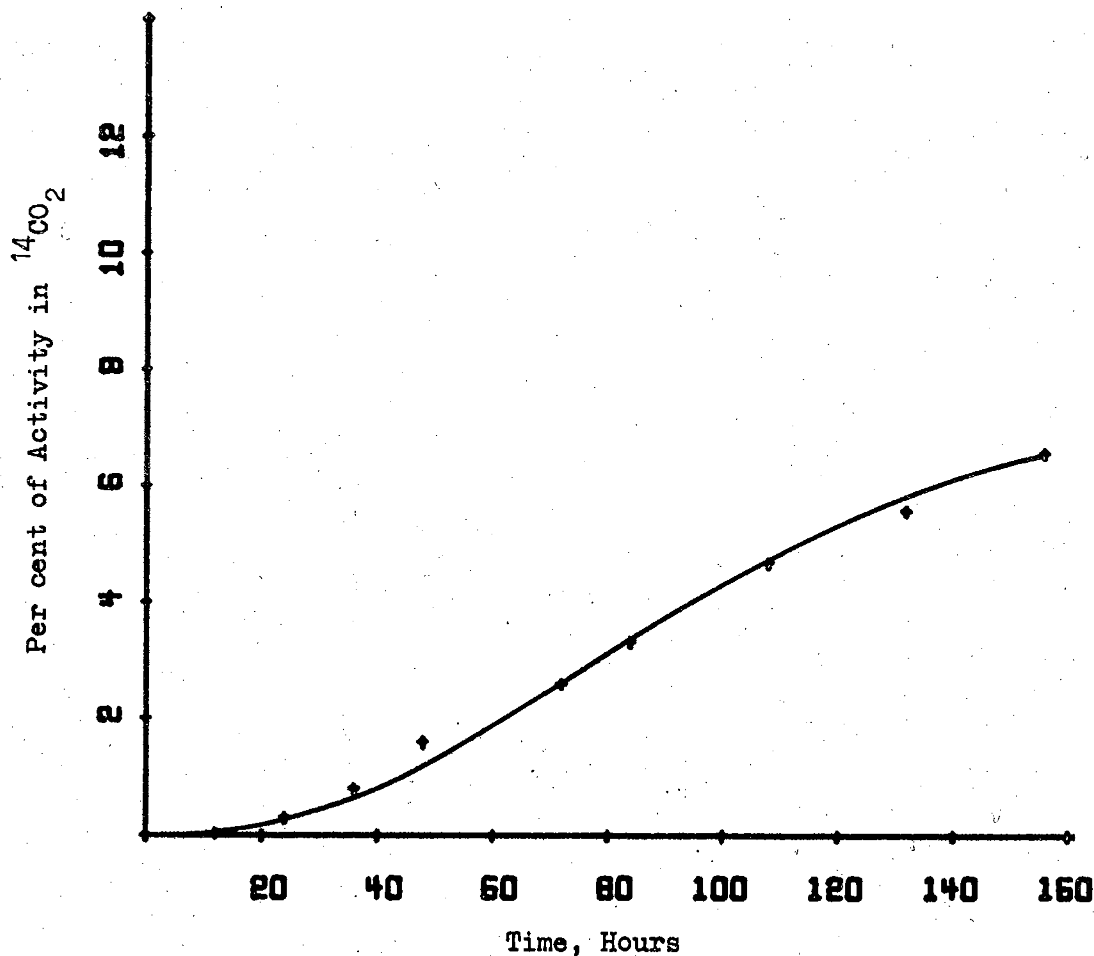

Figure 5. Time Course Study of $^{14}CO_2$ Released by Castor Plant in the light after Injection of Ricinine-3,5-^{14}C.

This plant was grown at the Perkins Agronomy Farm. Ricinine-3,5-^{14}C, 0.8 µmoles with a specific activity of 360 µc/mM and dissolved in 200 µl of distilled water was injected into a plant which was immediately placed in a closed system. Conditions are the same as described in Figure 4 except for 16 hrs. in normal laboratory light (day time) and 8 hrs. in dark (night).

was degraded to CO_2 by a series of slow reactions.

3. Isolation and Identification of Intermediates Formed in Castor Plants from Ricinine-3,5-^{14}C

Two ml of plant extract prepared in part H-1 were applied to a Dowex 1-formate column (1.5 X 32 cm) ; the column was eluted with water, followed by ammonium formate buffer, pH 4.0. The results are shown in Figure 6. Fractions 4-5, 7-9, and 32-38 contained radioactivity and also contained material which had an ultraviolet absorption at 260 mμ. A total of 0.4%, 0.2%, 0.43%, 35.5% and 0.85% of the fed radioactivity was found in fraction 4-5, 6, 7-9, 32-38, and 60-70 respectively. In an attempt to understand more about the compounds present in these various fractions, each fraction was assayed for absorbancy at 340 mμ to 200 mμ using the Beckman DB Spectrophotometer. The results are presented in Figure 7. The ultraviolet absorption spectrum of fraction 4-5 was shown to have a maxima at 265 mμ in water, it decolorized neutral potassium permanganate solution, and saturated bromine water instantly, showed a broad absorption band in the infrared spectrum (KBr pellet) at 2.7 to 3.7 μ, 5.8 to 6.5 μ, 7.1 to 7.4 μ, and 8.7 to 10.1 μ and no molecular ion was observed in its mass spectrum; however, ions of relatively high intensity were found at m/e = 144, 126, 105, 101, 85, 73, 72, 61, 60, 55, 46 and 17. Based on this information it is suggested that the unknown compound from fraction 4-5 contains a conjugated double bond and that it may be a carboxylic acid bound to a sugar. Fraction 32-38 gave a maxima ultraviolet absorption at 307 mμ and 255 mμ which permitted its positive identification as ricinine. The intermediates in the other fractions were not studied.

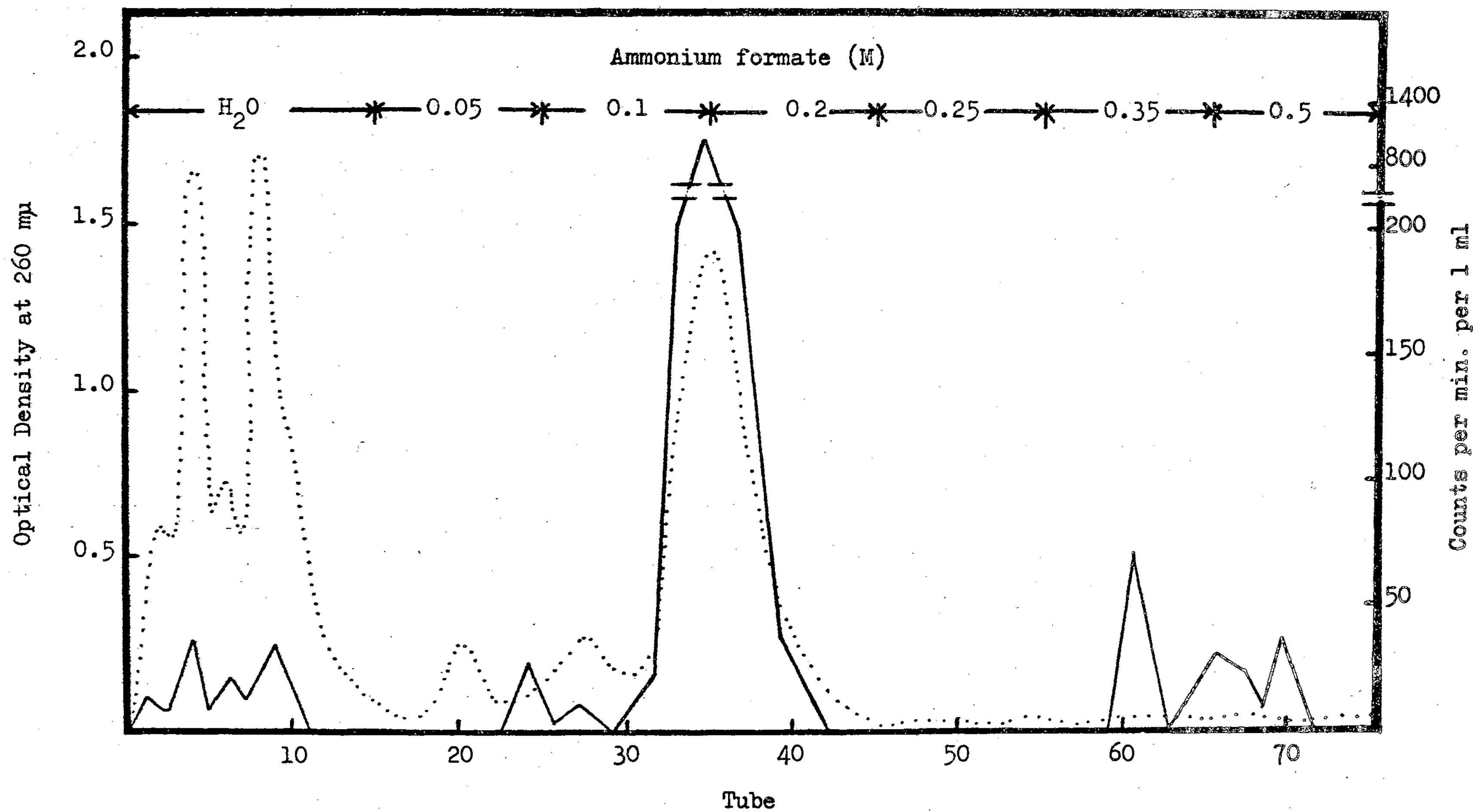

Figure 6. Column Chromatography of the Intermediates on a Dowex 1-formate Column.

—— indicates radioactivity of the eluates; ····· indicates optical density at 260 mμ.

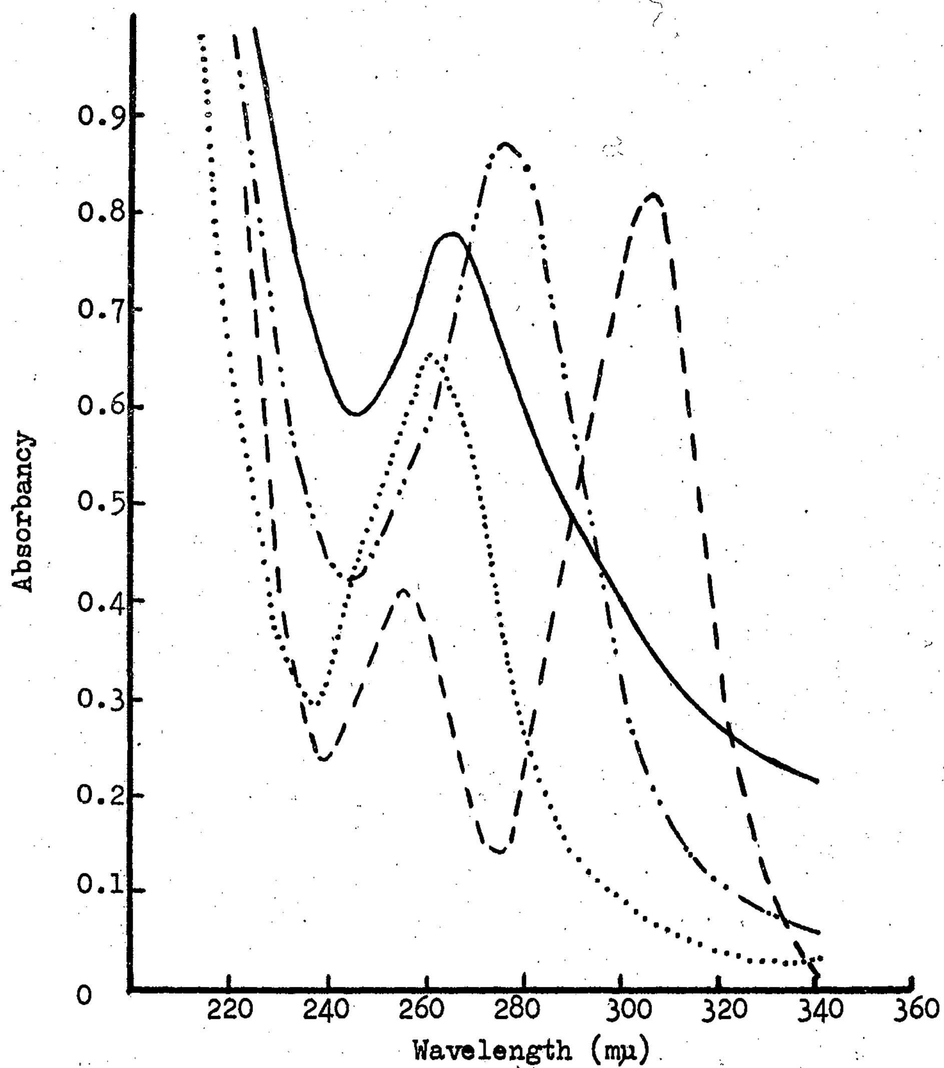

Figure 7. Ultraviolet Spectra of Selected Fractions from Dowex 1-formate Column Chromatography.

Column chromatography in fraction 4-5,———; fraction 6, -·-·-·-; fraction 7-9,·····: in water, fraction 32-38, - - - - -: in Ammonium formate buffer, 0.1-0.2 M.

C. <u>Content of Ricinine in the Castor Plant</u>

1. Young Castor Plants Which Were Grown in the Plant Growth Chamber

As shown in Figure 8, the ricinine content of young castor plants increased with the development of the plant from a value of 12 µmoles per plant in 6 days old to a value 51 µmoles per plant in 3 weeks plants. After this time, it remained relatively constant. Based on the ricinine content per gram fresh weight it is seen that the 2 weeks old plant was highest in its content of ricinine, after which time it fell rapidly.

2. From Field Grown Castor Plants

The ricinine content of castor plants which were grown in the field was different than those grown in the Plant Growth Chamber. Figure 9 shows that the ricinine content in the whole plant increased with development of the plant, but on the basis of alkaloid per gram fresh weight it decreased with time. This might be interpreted to indicate that the plant growth and ricinine formation are not mutually dependent on each other. After 16 weeks development the plant was divided into green leaf, flower, seed and yellow leaf for ricinine isolation. In Figure 9 it can be seen that the green leaf contained the highest amount of ricinine per gram fresh weight; the flower and the seed are next to the green leaf with both fractions containing almost the same amount of ricinine per gram fresh weight. In the yellow leaf the ricinine content was very low; approximately 0 to 0.005 µmoles per gram fresh weight was observed. The ratio of ricinine in green leaves to yellow leaves was about 1,000 to 1,400.

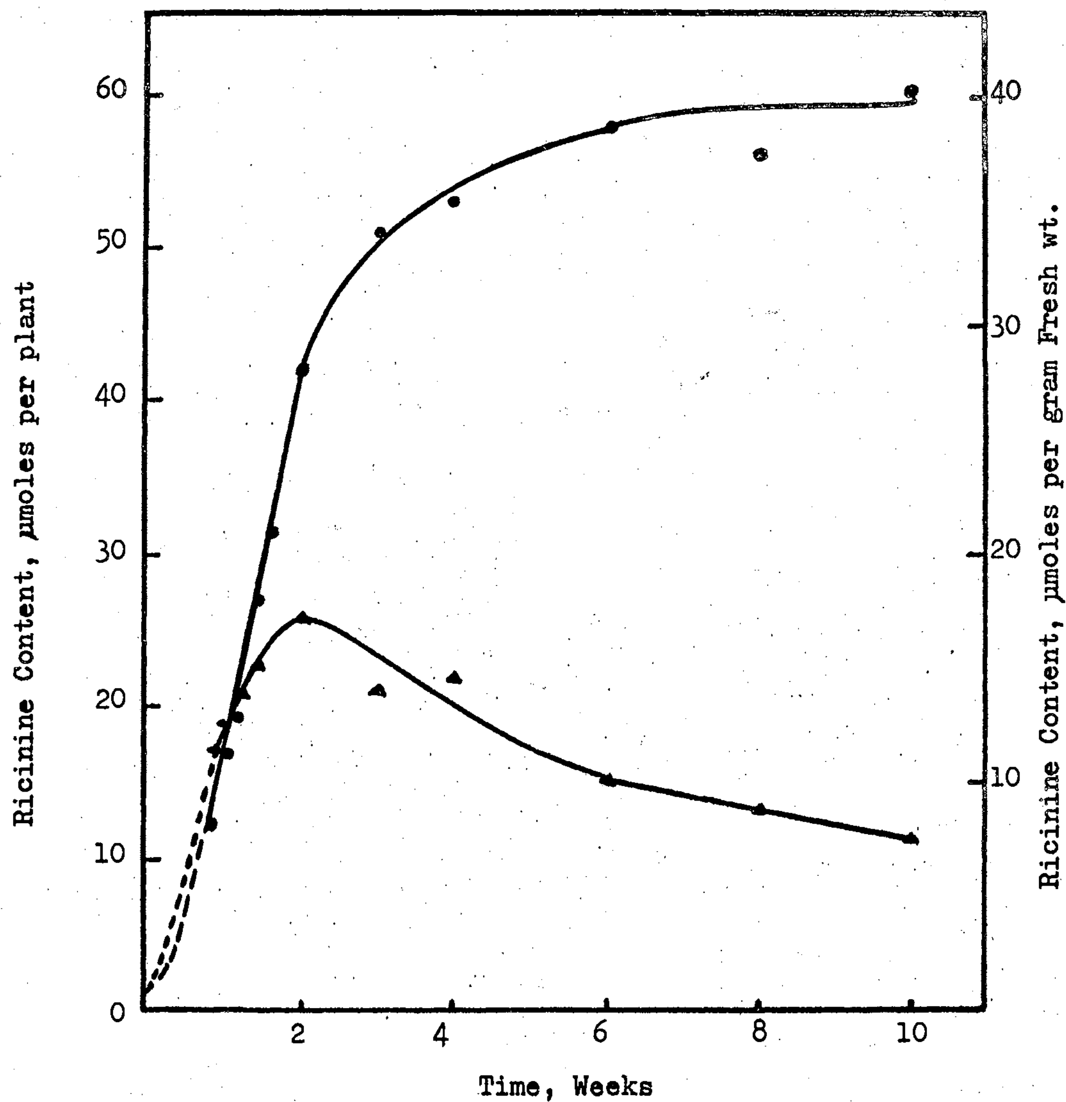

Figure 8. Ricinine Formation by Young Castor Plants.

These plants were grown in the Plant Growth Chamber. The whole plant (without root) was used for the isolation of ricinine. Each point obtained represented an average obtained from using 2 plants. ●, ricinine content, μmoles per plant. ▲, ricinine content, μmoles per gram fresh weight.

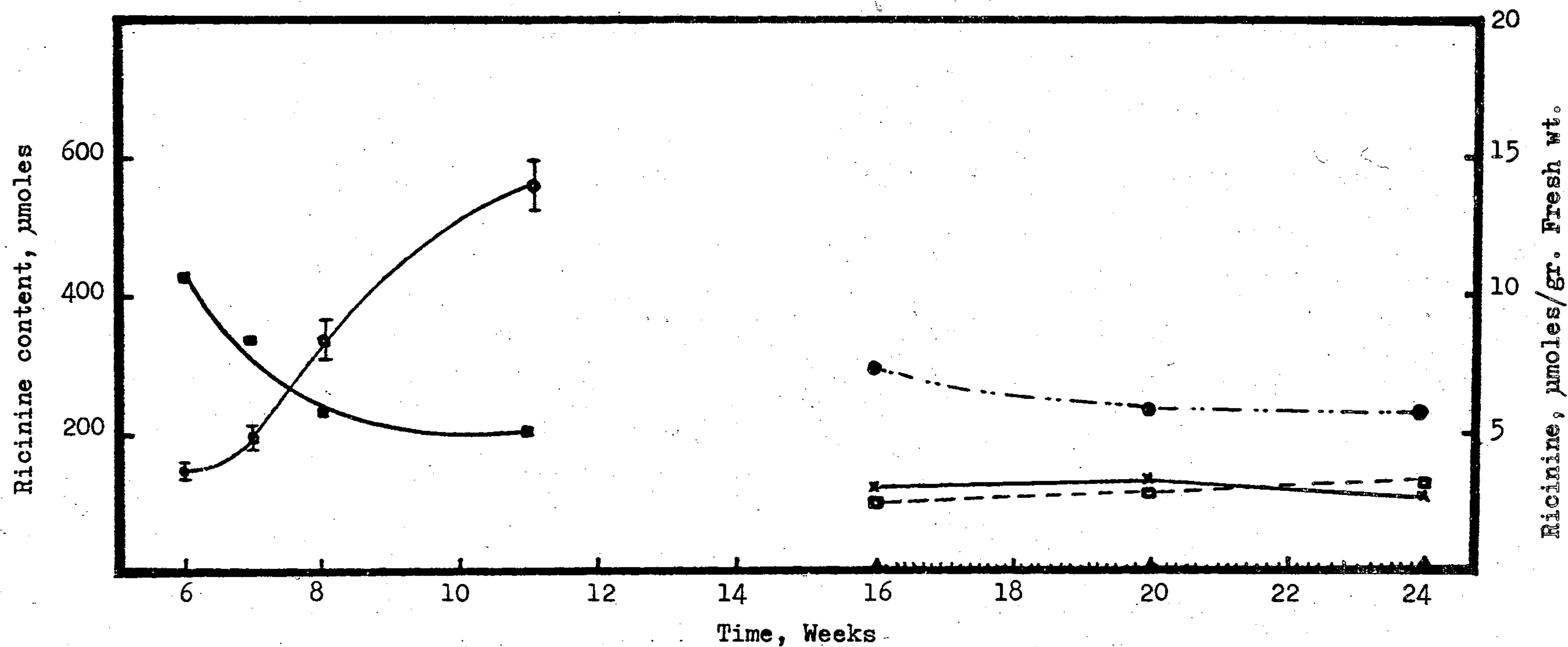

Figure 9. Ricinine Content of the Castor Plant.

The variation in analyses are indicated by a vertical line. O-total ricinine, µmoles per plant; ■-total ricinine, µmoles per gram fresh weight; ●-ricinine in green leaves, µmoles per gram fresh weight; x-ricinine in flower, µmoles per gram fresh weight; □-ricinine in seed, µmoles per gram fresh weight; △-ricinine in yellow leaves, µmoles per gram fresh weight.

D. Metabolism of Ricinine in Yellow Leaf of a Castor Plant

It was shown by the previous experiment that the ricinine content in the yellow leaf was at a negligible level. This suggested that the yellow leaf might play an important role in the metabolism or in the transportation of ricinine. In Table 11 it is shown that a total of 12.2% of radioactivity (ricinine-3,5-^{14}C recovered plus 0.5% that remained in solution) was recovered as ricinine during the 45 hours experiment duration. In another experiment, 10.54% of radioactivity (ricinine-3,5-^{14}C recovered plus 10% that remained in solution) was recovered at the end of the 56 hours; however, only a small amount of respiratory $^{14}CO_2$ was recovered. A total of 13.3% of activity was recovered in the water soluble fraction in the first experiment and 16.4% of activity was found in the second experiment. More experiments are necessary to verify these preliminary results. It suggested that (a) the rate of metabolism of ricinine in the yellow leaf was faster than that in the whole castor plant and (b) the alkaloid ricinine might be completely metabolized to non-alkaloidal substances or translocated to the other tissues before the leaf turned yellow and subsequently fell from the living plant.

TABLE II

TIME COURSE STUDY OF $^{14}CO_2$ RELEASED FROM YELLOW LEAF OF A CASTOR PLANT IN A RICININE-3,5-^{14}C SOLUTION

Experiment No.	Experiment Duration Hours	$^{14}CO_2$ Recovered		Ricinine-3,5-^{14}C Recovered		
		mμc	%	mg.	mμc	%
1*	21	0.15	0.16			
	45	0.21	0.24	0.16	10.5	11.7
2**	4	0.04	0.04			
	25	0.18	0.16			
	56	0.37	0.32	0.03	0.56	0.54

* 90 mμc Ricinine-3,5-^{14}C was dissolved in 0.5 ml of water. The test tube was rinsed three times with 0.5 ml water. 99.5% of the administered radioactivity was taken up by the leaf.

** 114 mμc Ricinine-3,5-^{14}C was dissolved in 0.5 ml of water. The test tube was rinsed three times with 0.5 ml water. 90% of the administered radioactivity was taken up by the leaf.

CHAPTER V

SUMMARY

Ricinine-3,5-^{14}C was used to study the metabolism of ricinine in *Ricinus communis* L..

In a time course study, ricinine-3,5-^{14}C was administered to a series of castor plants and the radioactivity recovered in the ricinine samples showed a decrease with an increase in time. It was observed that ricinine was metabolized by the castor plant and translocated to the seed where the alkaloid concentration was highest.

The *in vivo* conversion of ricinine-3,5-^{14}C to respiratory $^{14}CO_2$ by castor plants both in the dark and in the light showed that the pyridine ring of ricinine was completely degraded.

It was also found that the ricinine content in each tissue differs; in the yellow leaf there was only a negligible amount of ricinine found. By using ricinine-3,5-^{14}C, preliminary results indicated that the rate of metabolism of ricinine in the yellow leaf was faster than that in the whole castor plant.

Based on the results observed in this study, it is concluded that the alkaloid ricinine is metabolically active and that it is degraded to CO_2 by cleavage of the pyridine ring. The intermediate compounds involved in its metabolism are not known.

BIBLIOGRAPHY

1. Weevers, T., Rec. trav. botan. neerland, 30, 336 (1932).

2. Waller, G. R., and Nakazawa, K., Plant Physiol., 38, 318 (1963).

3. Waller, G. R., Tang, M. S., Scott, M. R., Goldberg, F. J., Mayes, J. S., and Auda, H., Plant Physiol., 40, 803 (1965).

4. Maquenne, L., and Philippe, L., Compt. rend., 138, 506 (1904).

5. Tuson, R. V., J. Chem. Soc., 17, 195 (1864).

6. Evans, T., J. Am. Chem. Soc., 22, 39 (1900).

7. Schulze, E., Chem. Soc. Abstr., i, 42 (1898); ii, 112 (1905).

8. Bogdashevskaya, O. V., Dokalady Akad. Nauk. S. S. S. R., 99, 853 (1954).

9. Robinson, T., and Fowell, E., Nature, 183, 833 (1959).

10. Robinson, T., Phytochemistry, 4, 67 (1965).

11. Robinson, T., and Cepurneek, C., Phytochemistry, 4, 75 (1965).

12. Juby, P. F., and Marion, L., Can. J. Chem., 41, 117 (1963).

13. Essery, J. M., Juby, P. F., Marion, L., and Trumbull, E., Can. J. Chem., 41, 117 (1963).

14. Johns, S. R., and Marion, L., Can. J. Chem., 44, 23 (1966).

15. Thomas, P. R., Barnes, M. F., and Marion, L., Can. J. Chem., 44, 1997 (1966).

16. Waller, G. R., and Henderson, L. M., J. Biol. Chem., 236, 1186 (1961).

17. Yang, K. S., and Waller, G. R., Phytochemistry, 4, 881 (1965).

18. Yang, K. S., Gholson, R. K., and Waller, G. R., J. Am. Chem. Soc., 87, 4184 (1965).

19. Hadwiger, L. A., Badiei, S. E., Waller, G. R., and Gholson, R. K., Biochem. Biophys. Res. Communun., 13, 466 (1963).

20. Waller, G. R., Yang, K. S., Gholson, R. K., Hadwiger, L. A., and Chaykin, S., J. Biol. Chem., 241, 4411 (1966).

21. Waller, G. R., and Nakazawa, K., Fed. Proc., 21, 467 (1962).

22. Manske, R. H. F., and Holmes, H. L., The Alkaloids, Vol. 1, Academic Press inc., Publishers, New York, p. 206 (1950).

23. Kirk, R. E. and Othmer, D. F., Encyclopedia of Chemical Technology, Vol. 1, The Interscience Encyclopedia, Inc., New York, p. 468, (1947).

24. Mannis, V., Horak, M. Brabence, J., and Santavy, Acta. Univ. Palackianae Olomucensis, 20, 21 (1960). via C. A., 55, 7762i.

25. Waller, G. R., Rhyage, R., and Merson, S., Anal. Biochem., 16, 277 (1966).

26. Spath, E., and Tschelnitz, E., Monatsh, 42, 251 (1921).

27. Spath, E., and Koller, G., Ber., 56 (B), 880 (1923).

28. Schnoeter, G., Seidler, C., Salzbacher, M., and Kanitz, R., Ber., 65, 432 (1932).

29. Taylor, E. C., and Crovetti, A. J., J. Am. Chem. Soc., 78, 214 (1956).

30. Waller, G. R., Biosynthesis of the Alkaloid Ricinine in the Castor Plant Ricinus Communis L., Ph. D. Dissertation, Oklahoma State University, Stillwater, Oklahoma (1961).

31. Bottcher, B., Ber., 51, 673 (1918).

32. Schulze, E., and Winterstein, E., Z. Physiol. Chem., 43, 211 (1904).

33. Bogdashevskaya, O. V., Dokalady Akad. Nauk. S. S. S. R., 82, 1001 (1952).

34. Dubeck, M. and Kirkwood, S., J. Biol. Chem., 199, 307 (1952).

35. Leete, E., and Leitz, F. H. B., Chem. and Ind., London, 1572 (1927).

36. Yang, K. S. Biosynthesis of Ricinine by Ricinus Communis L., Masters Thesis, Oklahoma State University, Stillwater, Oklahoma (1963).

37. Tso, T. C., and Jeffrey, R. N., Arch. Biochem. Biophys., 80, 46 (1959).

38. Fairbairn, J. W., and Suwal, P. N., Phytochemistry, 1, 38 (1961).

39. Fairbairn, J. W., and Wassel, G., Phytochemistry, 3, 253 (1964).

40. Fairbairn, J. W., Paterson, A., and Wassel, G., Phytochemistry, 3, 577 (1964).

41. Yang, K. S., Triplett, R., Klos, K. S., and Waller, G. R. Proc. Okla. Acad. Sci., 46, 142 (1966).

42. Jeffay, H., and Alvarez, J. Anal. Chem., 33, 612 (1961).

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