Metabolism of Ricinine in the Castor Plant[1, 2]

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During the early period of the twentieth century, it was generally thought that alkaloids were by-products of a number of irreversible and physiologically useless reactions associated with nitrogen metabolism (1-3). This idea was gradually discarded as an increasing number of experiments showed that alkaloids are metabolically active.

Indirect evidence for the decomposition of ricinine (II, fig 1) in the living plant was obtained by several investigators in nonisotopic experiments. Weevers (4) showed that the ricinine content decreased with increasing age of castor plants grown on nitrogen-depleted soil. Bogdashevskaya (5) showed that the content of ricinine 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 (6) reported that ricinine was rapidly utilized by castor cotyledons in the dark; however, the amount of ricinine did not decrease when nicotinic acid was present in the medium. A preliminary report (7) has been made on the degradation of ricinine in a cell-free system of young castor plants.

Tso and Jeffrey (8) were the first to use isotopic tracers to demonstrate that alkaloids are metabolically active. They fed nitrogen-15 labeled nicotine (III, fig 1), nornicotine and anabasine (IV, fig 1) to tobacco plants and found that a major portion of the alkaloids supplied was broken down. Griffith et al. (9) indicated that nicotinic acid (I) was a metabolite of nicotine. Griffith and Griffith (10) showed that transmethylation of methionine to nicotine and catabolism of the ring structures of nicotine occurs almost exclusively in root tissue of *Nicotiana rustica.*

It is well known that nicotinic acid (I, fig 1) can serve as a precursor of each of these alkaloids [fig 1; see Leete (11) for a recent review][6].

The experiments described herein were performed to determine if ricinine could be degraded by the castor plant and to observe the turnover of ricinine in different organs.

Materials and Methods

Cultural Conditions. Ricinus communis L. plants of the Cimarron variety were grown on Port clay loam soil under irrigation at either the Perkins or the Stillwater Agronomy farm. Water was supplied when needed so that the plants were never under moisture stress. These experiments were conducted over a 3 year period. The rate of growth varied considerably from year to year due to varying climatic conditions.

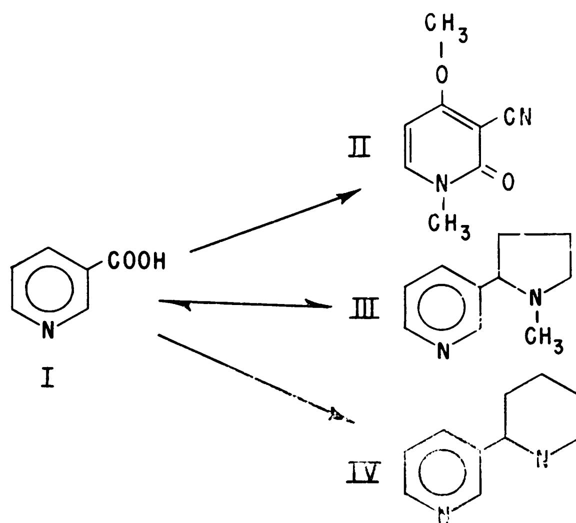

FIG. 1. Pyridine alkaloids derived from nicotinic acid.

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[3] Taken in part from a Master of Science Thesis submitted to the Oklahoma State University, November, 1963.

[4] National Science Foundation Undergraduate Research Participant, 1961.

[5] National Science Foundation Undergraduate Research Participant, 1960.

[6] In figure 7 of this review the direct conversion of quinolinic acid to nicotinic acid is shown which is not in agreement with the results which show that nicotinic acid mononucleotide is an intermediate and that free nicotinic acid is not formed directly by decarboxylation of quinolinic acid; Hadwiger, L. A., S. E. Badei, G. R. Waller, and R. K. Gholson. 1963. Biochem. Biophys. Res. Commun. 13: 466-71.

[7] Recovery of 96-99 % of the ricinine can be obtained if the fresh tissue is ground in a mortar and pestle using sand but the laboriousness of this technique made it undesirable for use in isolating ricinine in these experiments where large quantities of plant material were involved.

Administration of Labeled Compounds. 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 of the compound was injected at the bottom of the internode using a Hamilton micro syringe.

Preparation of Labeled Compounds. Two hundred mg of ricinine were labeled with tritium according to the gaseous exchange method of Wilzbach (12). The ricinine-H^3 was washed with water to remove exchangeable tritium, diluted with carrier ricinine and crystallized from water and chloroform to constant specific activity. The radiochemical purity was checked by spotting 50 to 100 μg of the ricinine-H^3 on 2.5 cm strips of Whatman No. 1 paper and developing with *t*-butyl alcohol: water: acetic acid (4:2:1) or with 85 % isopropyl alcohol. The R_F of ricinine-H^3 agreed with that of a chemically synthesized sample (13) in both systems. After developing, the paper strip was dried and cut into 2.5 × 5 cm pieces. Radioactivity measurements were made on each piece using a liquid scintillation counter. Only the radioactive spot which corresponded to ricinine was detected. The distribution of tritium activity was 8.5 % in the *O*-methyl, 24.9 % in the *N*-methyl group and the remainder was on carbons 5 and 6 of ricinine. The activity in the methyl carbons was determined according to the method of Dubeck and Kirkwood (14).

Nicotinic acid-7-C^{14} was purchased from California Biochemical Corporation and was used without further purification. Ricinine-8-C^{14} was prepared biosynthetically by isolating it from young castor plants to which nicotinic acid-7-C^{14} or nicotinamide-7-C^{14} had been administered. Both of these compounds give rise to ricinine labeled solely in the nitrile carbon (15). Its purity was checked in the same manner as was outlined for ricinine-H^3.

Measurement of Radioactivity. Radioactivity measurements were made using a Model 314 E Tri-Carb liquid scintillation spectrometer. The scintillation solvent was composed of 58.75 % toluene, 39.25 % absolute ethanol and 2 % water. This solution contained 0.5 % 2, 5-diphenyloxazole and 0.02 % *p*-bis-2-(5-phenyloxazolyl)-benzene (PPO) as phosphors. This system had an efficiency of approximately 8.0 % for tritium and 38 % for carbon^{-14} pyridinium compounds. The scintillation solvent used for measuring $^{14}CO_2$ was made of 3 ml of ethanolamine-methylcellosolve, 1:2, which was used for CO_2 absorption, and 15 ml of methylcellosolve-toluene (sulfur free), 1:2, which contained 5.5 g of PPO per liter. Ethanolamine was used because it was less quenched than the sodium hydroxide solution.

Storage of Plant Material. Whole castor plants were harvested, placed in polyethylene bags and stored at —18° until used.

Isolation of Ricinine. The procedure of Waller and Henderson (15) was used.

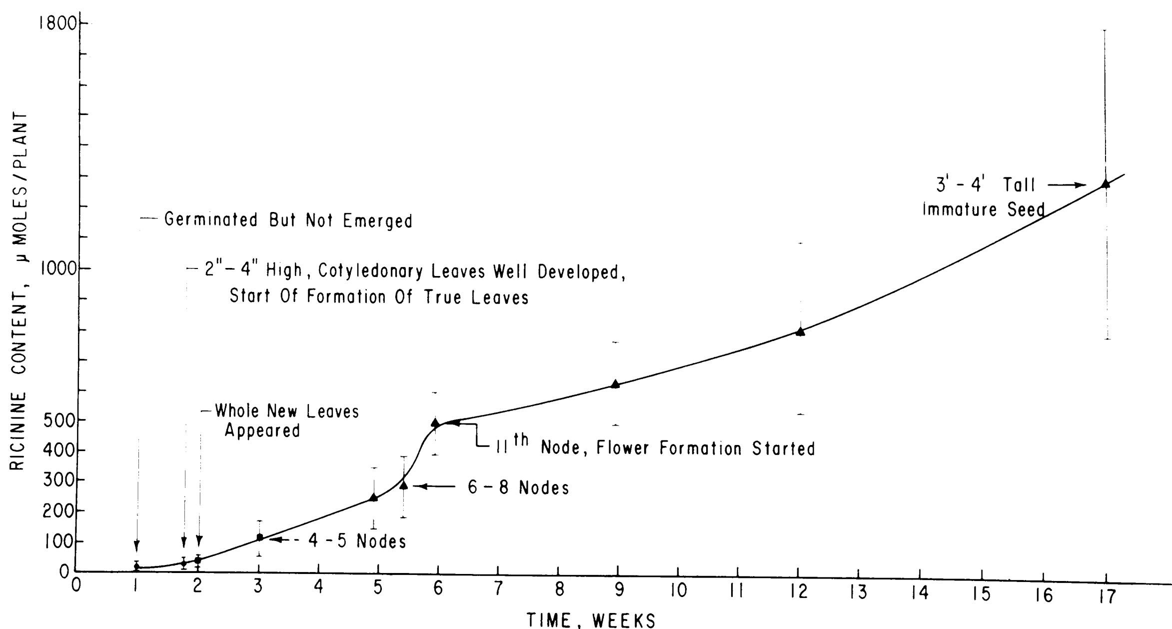

FIG. 2. Ricinine content of the castor plant at different states of development. These plants were grown at the Stillwater Agronomy farm in 1961. The physiological stages of development are noted on the graph. The entire plants were used for the isolation of ricinine. The variation in analyses are indicated by a vertical line. The number of plants used in obtaining each point is: ●, 2 lots of 20 plants each; ■, 2 lots of 5 plants each; ▲, 2 plants.

Results and Discussion

To determine the efficiency of recovery of ricinine, 2 plants were harvested immediately after administration of ricinine-H^3. The amount of ricinine recovered was 75 ± 2 %. Corrections for this loss of ricinine were made for these experiments.[7]

Figure 2 shows the ricinine production by individual castor plants. An individual castor seed contains about 0.1 μmole of ricinine and its subsequently developed plant was found to contain around 1.2 mmole at 17 weeks of age. The plant is not mature at 17 weeks of age. The mature plant is usually 5 to 7 feet tall and weighs 2 to 4 times as much as the 17 week plant. It has a woody stem which interferes with efficient extraction of ricinine. Immediately prior to flowering the observed rate of ricinine synthesis was 36 μmoles per day (fig 2; 5–6th week) which represented the highest rate in the nonflowering plants. Although this kind of experiment was not repeated in 1962 and 1963, sufficient numbers of plants were analyzed to verify the 1961 findings. There was a noticeable variation in the chronological age of the plants at the same physiological state of development during this 3-year period. The plants grew most rapidly in 1961 as is indicated by the 40 days of age required to reach the flowering stage whereas in 1963, 49 days were required. This might be expected to have an effect on the synthesis and breakdown of ricinine and a comparison of the results obtained in 1961 and 1963 confirms this hypothesis.

Figure 3 shows the rate of disappearance of ricinine-H^3, ricinine-8-C^{14} and total ricinine found in castor plants grown in 1961. A plot of the specific activity recovered (not shown) gave a curve with a similar shape to the one shown which represents the total radioactive ricinine recovered. From these results it is clear that destruction of ricinine occurred while rapid synthesis was in progress. From the ricinine-C^{14} curve it is evident that nicotinic acid is converted to ricinine and that the ricinine thus formed is degraded to the extent of about 90 % during the ensuing 11.5 weeks. The ricinine-H^3 was degraded to the extent of about 95 % during a 10-week experiment. The highest rate of metabolism occurred during the first 3 weeks following administration of ricinine-H^3 and nicotinic acid-7-C^{14}. At this stage the plants started to flower and the rate of disappearance of radioactivity from the ricinine fraction was considerably slower than before flowering.

To gain a better understanding of the breakdown of labeled ricinine in the flowering plant, an experiment was conducted using plants which had just begun to flower when the ricinine-H^3 was injected. During the first week 20 % of the ricinine-H^3 was utilized. At the end of the first week, immature seeds began to appear; therefore, the plants were divided into seeds, flowers and leaf-stem portions

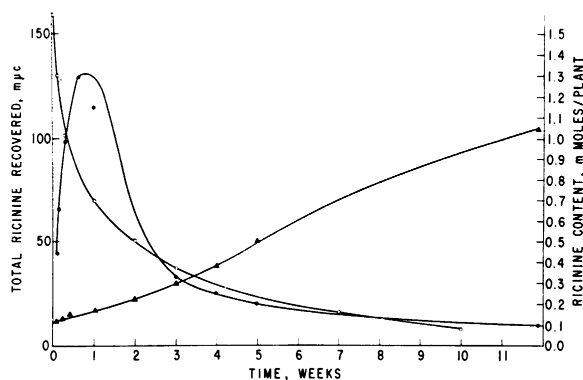

FIG. 3. Rate of disappearance of ricinine-H^3 and ricinine-8-C^{14} in castor plants. These plants were grown at the same location and time as those described in figure 2. They were 26 days of age and were at the fifth to sixth nodal stage when the labeled compounds were injected. Ricinine-H^3, 9.7 μmoles, with a specific activity of 17.0 μc/mmole was dissolved in 200 μl of distilled water and injected in a single plant. Nicotinic acid-7-C^{14}, 0.108 μmole, with a specific activity of 9.2 mc/mmole was dissolved in 10 μl of distilled water and injected in a different single plant. ○, Ricinine-H^3; ●, ricinine-C^{14}; ▲, ricinine content per plant. The entire plant was used for ricinine isolation.

for ricinine analysis. A comparison of the total ricinine-H^3 recovered is shown in figure 4A and of the total ricinine is shown in figure 4B for these parts of the plant. The roots were not analyzed in this experiment since it was determined that they contained less than 2 % of the ricinine present in the plant. In figure 4B it is shown that the increase in ricinine formation by the plant as a whole parallels that in the seed. The flowers and leaf-stem sections contribute about 5 % and 20 %, respectively, of the total ricinine at the end of the 13 weeks (actual plant age was 20 weeks since the ricinine-H^3 was administered to 7-week-old plants). Ricinine production by the flowers remained constant. The flowers were found to contain 1.07 % of the alkaloid (10.7 ± 2.0 mg/g dry wt), which was the richest source of the alkaloid in the castor plant. The ricinine content of the seed was relatively constant at a value of 7.7 ± 0.65 mg/g (0.77 % dry wt). The amount of ricinine in the leaf-stem fraction showed a slight decrease up to the ninth week and increased gradually to the end of the experiment. When the leaf-stem data are replotted to show the ricinine content per g of tissue it is evident that a marked decrease in the alkaloid per g of tissue occurred (fig 5). The decrease was from 3.8 mg/g to 1.6 mg/g and represented a net decrease of about 60 %.

The overall rate of ricinine synthesis between the twelfth and thirteenth week (actual plant age was 19 and 20 weeks) was about 2.1 mmoles or 300 μmoles per day. Although this is about 8 times as fast as the fifth to sixth week period of the preflowering plant most of the synthesis occurred in

the seed and was due simply to the fact that larger numbers of seed were formed. These castor plants have been widely used commercially because of their high production of castor oil, hence, a high number of seeds per plant is desirable because the oil yield is increased correspondingly.

In figure 4A it is shown that the total radioactivity of ricinine-H^3 decreased by 75 % during the 13-week period. Of the alkaloid remaining 20 % was found in the seeds. The accumulation of ricinine-H^3 in the seed clearly indicates that the alkaloid was transported from other tissues to the seed. It seemed unlikely that ricinine accumulated in the seed due only to translocation (see fig 4B). To establish that its synthesis also could occur in the seed the following experiment was conducted:

Excised immature castor seeds were dipped into 100 μl of distilled water containing 3.3 μmoles of nicotinic acid-7-C^{14} with a specific activity of 130 μc/mmole. Uptake was complete in 1 to 2 hours. Thirty hours later the experiment was terminated. Fifty-four μmoles of ricinine-8-C^{14} with a specific activity of 246 mμc/mmole was obtained.

This represented 3.9 % incorporation of nicotinic acid-7-C^{14} into ricinine. It is not clear as to the amounts that were synthesized and translocated.

The loss of ricinine-H^3 and ricinine-8-C^{14} clearly indicated that this alkaloid was metabolized by the castor plant. The loss of activity of ricinine-H^3 might be due in part to an exchange of tritium from the ricinine-H^3 with hydrogen in plant cells; however, this appears to be unlikely since the disappearance of ricinine-8-C^{14} provided conclusive evidence that the alkaloid was degraded. Several attempts were made to obtain ricinine from the soil that surrounded potted castor plants which had ricinine labeled with either tritium or C^{14}. No ricinine could be recovered; however, 0.2 % of the administered radioactivity was recovered in an aqueous extract of the soil. The products excreted have not been identified.

To determine if ricinine could be degraded to

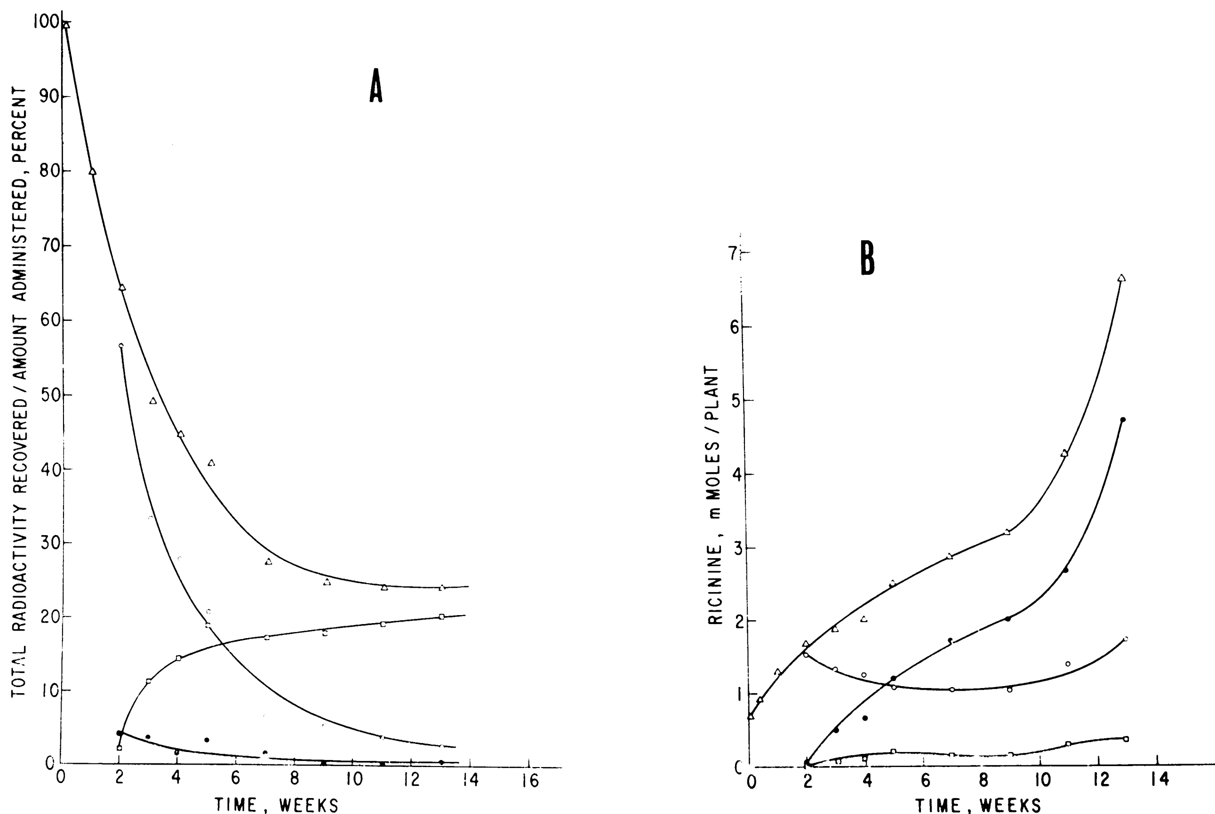

FIG. 4. A, Comparison of the rate of disappearance of ricinine-H^3 in different parts of the castor plant. B, Comparison of the rate of ricinine formation in different parts of the castor plant. These plants were grown at the Perkins Agronomy farm in the summer of 1963. Forty-nine day old plants were injected with 18.2 μmoles of ricinine-H^3 which had a specific activity of 48.5 μc/mmole. The plants were flowering and were at the tenth to eleventh nodal stage. 4A: ○, leaf stem; ●, flower; □, seed; △, total. 4B: ○, leaf stem; ●, seed; □, flower; ▲, total.

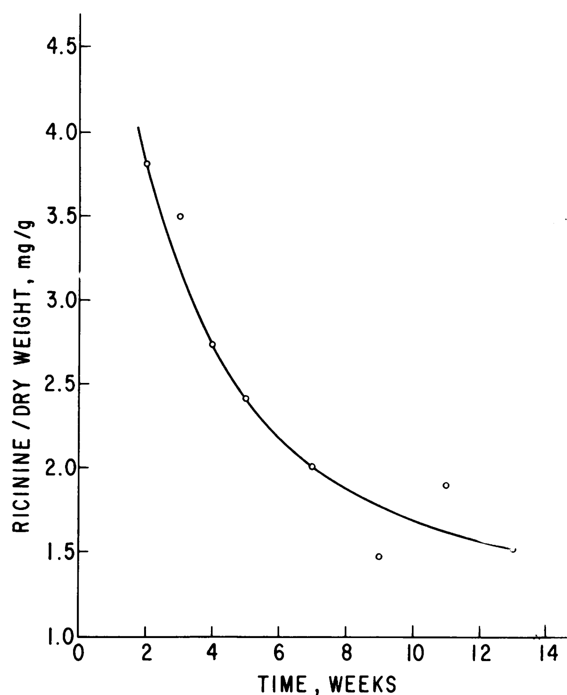

FIG. 5. The ricinine content of leaves and stems after seed formation. These were the same plants as those used for figure 4.

CO_2, 5 μc of ricinine-8-C^{14} was injected into a castor plant which was immediately placed in a dark closed system to prevent photosynthesis and to promote catabolism. Carbon dioxide-free air was passed through the system and the respiratory CO_2 was trapped in ethanolamine-methylcellosolve for 28 hours. The results showed that approximately 2.5 % of the radioactivity from ricinine-8-C^{14} was lost as respiratory $^{14}CO_2$. The route of formation of $^{14}CO_2$ is not known. A plausible intermediate might be *N*-methyl-4-methoxy-2-pyridone which would be formed if a direct cleavage of the nitrile group occurred. In vitro experiments with ricinine-8-C^{14} (7) failed to yield any *N*-methyl-4-methoxy-2-pyridone and attempts to isolate this compound from castor plants have been unsuccessful. Chromatographic examinations of aqueous and ethanol extracts failed to show any formation of radioactive nicotinic acid. It is possible that ricinine may be broken down into small molecules by a pathway excluding nicotinic acid. Further work on the identification of the metabolic breakdown products is in progress.

Summary

Ricinine-H^3 and ricinine-8-C^{14} can be metabolized by castor plants (*Ricinus communis* L.). The extent of degradation of the alkaloid varied from 75 to 95 %. Synthesis of ricinine occurred continually during these experiments. It was shown that ricinine could be transported to the seeds and that in situ synthesis also occurred. The results indicated that no net destruction of ricinine-H^3 occurred in the seed. At 20 weeks of age the ricinine in the seed accounted for about 75 % of the total alkaloid in the plant. In post-flowering plants there was a 60 % decrease in the amount of ricinine per g of leaves and stems but the ricinine content of the flowers and seeds remained relatively constant.

Acknowledgments

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