Chemical Synthesis of Carbon-14 Labeled Ricinine and Biosynthesis of Ricinine in Ricinus communis L.[1]

K. S. YANG, R. TRIPLETT, K. S. KLOS and G. R. WALLER

Oklahoma State University, Agricultural Experiment Station, Stillwater

Ricinine (Fig. 1, formula V; 1,2-dihydro-4-methoxy-1-methyl-2-oxonicotinonitrile) is a mildly toxic alkaloid produced by the castor plant *Ricinus communis* L. Studies on the biosynthesis of ricinine have been in progress in our laboratory for several years (Waller and Henderson, 1961; Hadwiger et al., 1963; Yang and Waller, 1965). Recently Waller et al. (1965) demonstrated that 75% to 90% of ricinine-^{3}H and ricinine-8-^{14}C was degraded by the castor plant. This demonstration of metabolic activity serves to refute the earlier concepts that regarded alkaloids as by-products of a number of irreversible and useless reactions associated with nitrogen metabolism (Pictet and Court, 1907; Cromwell, 1937; Vickery, 1941). To enable us to further study the degradation of ricinine by the castor plant, alkaloid labeled with carbon-14 in the pyridine ring which possesses a high specific activity is required. This report provides detailed information on the micro-scale synthesis of ricinine-3,5-^{14}C.

The chemical synthesis of ricinine was initiated in the early part of this century by several workers in their attempts to prove the structure of the akaloid. Späth and Koller (1923) synthesized ricinine by the oxidation of 4-chloroquinoline via the intermediates 4-chloro-2-aminoquinoline-3-carboxylic acid and 2,4-dichloronicotinonitrile. Taylor and Crovetti (1956) synthesized ricinine from 3-picoline 1-oxide via the intermediates 4-nitro-3-picoline 1-oxide and 2,4-dichloronicotinonitrile. Schroeter et al. (1932) synthesized ricinine from the aliphatic compound cyanoacetyl chloride (Fig. 1). This remarkable transformation was attributed to the intermediate formation of malonamoyl chloride and cyanoketene to give 2-4-dihydroxy-6-chloronicotinonitrile (II). II was converted to 2,4-dihydroxynicotinonitrile (III) by removal of the halogen. III was converted to ricininic acid (IV) by methylating the nitrogen and IV was converted to ricinine (V) by methylating the hydroxyl group. The direct methylation of III developed by Robinson and Hook (1964) was also used in this study.

Experimental

Chemical synthesis of ricinine-3,5-^{14}C (Fig. 1)—Carbon-14 labeled ricinine was synthesized on a micro scale from sodium cyanoacetate-2-^{14}C. To convert it to the free acid one mmole (107 mg) of the salt with a total activity of 364 μc was dissolved in about 3 ml of water and passed through a Dowex 50 $\times$ 100 ion exchange column (17- $\times$ 0.9-cm). Seventeen ml of eluant was collected directly into a continuous liquid-liquid extractor. The cyanoacetic acid-2-^{14}C was extracted with ethyl ether for two days. The ether solution of cyanoacetic acid-2-^{14}C was dried with anhydrous $MgSO_4$ for several hours. To the ether solution in a 50-ml flask, 220 mg of PCl_5 was added. After all of the PCl_5 was dissolved, the ether was removed using a rotary evaporator with a water aspirator and a water bath at about 55 C. When loss of volume ceased and the solution was yellow, the flask was closed and put into the refrigerator at about 3-5 C

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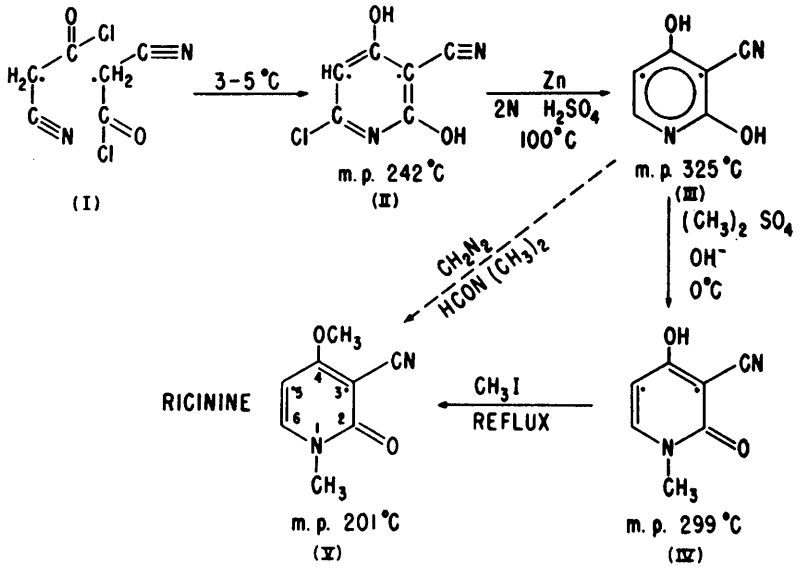

Fig. 1. Chemical synthesis of ricinine

(· refers to position of carbon-14 label)

for one week. After spontaneous polymerization of I at this temperature the reaction mixture was treated dropwise with distilled water until a bright yellow precipitate formed. The precipitate was discarded and the supernatant liquid was allowed to stand for several days until crystals of II were formed. II was further purified by recrystallization from hot water with a 40% yield. II was dechlorinated by dissolving it in 2.5 ml 2 N H_2SO_4, adding Zn dust (0.4 g), and heating the mixture to about 100 C for 15 minutes. The product (III) precipitated as needles upon cooling and was further purified by recrystallization from hot water with a 90% yield. III was dissolved in N,N-dimethylformamide (1.0 ml) and an excess of freshly prepared diazomethane in ether solution was added dropwise at about 0 C. The ricinine-3,5-^{14}C so obtained was purified by repeated sublimation at 170 C at 2 mm Hg until a constant specific radioactivity was achieved. The identity of V was confirmed by IR and UV spectra (Yang, 1963) and by paper chromatography in three solvent systems (85% isopropyl alcohol; 1-butanol:acetic acid:water, 4:1:1; and 95% ethanol:1N ammonium acetate, 7:3).

Chemical synthesis of ricininic acid-3,5-^{14}C (IV, Fig. 2)—Ten mg of V was dissolved in 0.1 ml 1 N NaOH in a sealed tube and heated to 100 C for one hour. The mixture was neutralized and acidified with 0.5 *N* HCl. The white precipitate was collected by centrifugation and purified by recrystallization from hot water. The purity of ricininic acid-3,5-^{14}C was established by paper chromatography by comparing the R_f values with those of the authentic compound in the three solvent systems mentioned above. The yield was 90%.

Chemical synthesis of ricinine amide-3,5-^{14}C (VI, Fig. 2)—Robinson and Hook's (1964) procedure was modified for use on a micro scale. A mixture consisting of 10 mg of V and 100 mg of polyphosphoric acid (P_2O_5 +

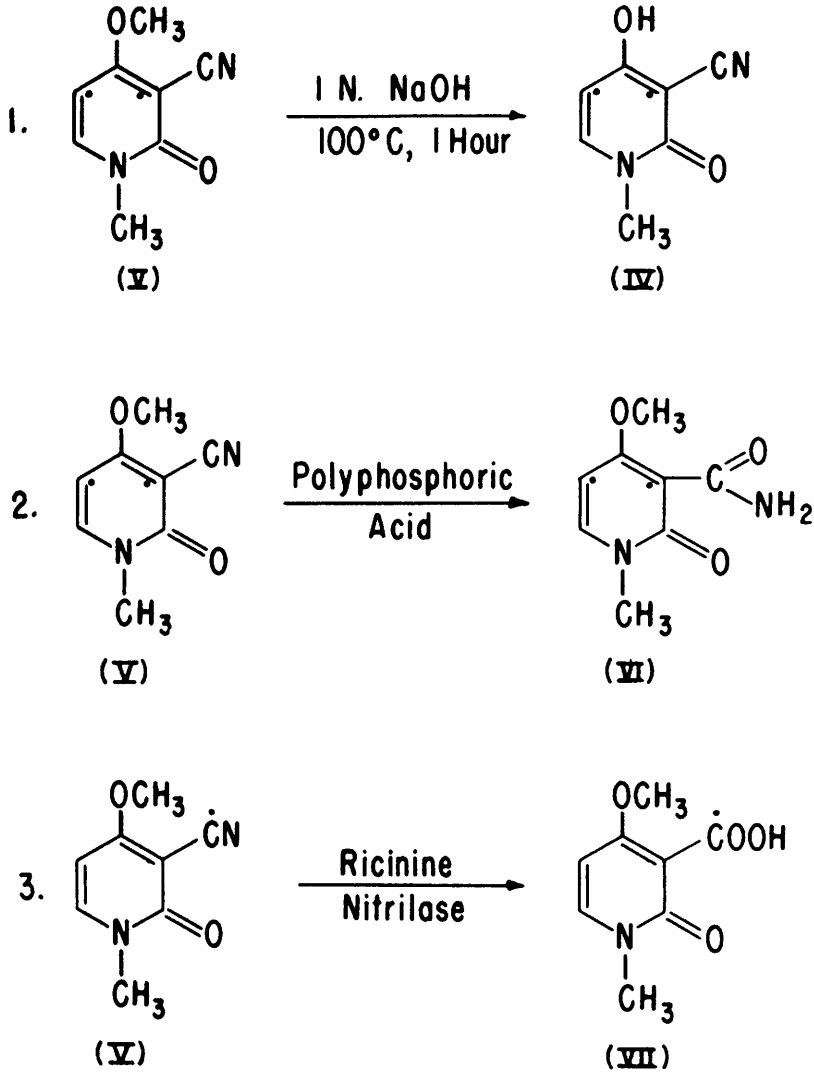

Fig. 2. Chemical synthesis of carbon-14 labeled ricinine derivatives

phosphoric acid, 1:1) was heated at 107 C for 1.5 hours. The reaction mixture was chilled in an ice bath and neutralized to pH 6 with saturated $Ba(OH)_2$ solution and then spotted on Whatman No. 1 filter paper for preparative chromatography. The ricinine amide-3,5-^{14}C spot on the paper after ascending development with 1-butanol:acetic acid:water (4:1:1) was recovered by elution with water and checked for purity in the other two solvent systems previously mentioned. The yield was 40%.

Ricinine acid-8-^{14}C (VII, Fig. 2)—Robinson and Hook (1964) reported that the enzyme ricinine nitrilase from a species of *Pseudomonas* catalyzes the conversion of ricinine into ricinine acid. The ricinine acid-8-^{14}C was prepared by these investigators from ricinine-8-^{14}C supplied from this laboratory.

Determination of radioactivity—Liquid scintillation counting (Tricarb, Model 314, Packard Instrument Co., LaGrange, Ill.) was employed for carbon-14 labeled ricinine and compounds appreciably soluble in the scintillation solvent which was composed of 58.75% toluene, 39.25% ethanol, and 2.0% water. The phosphor was 0.5% 2,5-diphenyloxazole and 0.02% *p*-bis-(5-phenyl-2-oxazolyl) benzene. For detecting radioactive compounds on paper chromatograms a paper strip counter (Nuclear Chicago Actigraph III 4 π Scanner) was used.

Biosynthesis of ricinine—The methods used in the biosynthesis of ricinine were those reported by Yang and Waller (1965).

Results and Discussion

Chemically synthesized ricinine-3,5-^{14}C (V) with a specific activity of 567.0 μc/mmole was prepared. The radiochemical yield of V was found to be 20-25%. The compounds (III, IV, VI, VII) for biosynthetic studies were radiochemically pure after paper chromatography in three solvent systems. Studies on the biodegradation of V by *Ricinus communis* L. are in progress in this laboratory.

Recent evidence obtained by Yang and Waller (1965) from *in vivo* experiments with young *Ricinus communis* L. plants indicated that ricinine is probably derived from aspartic acid and a glycerol derivative. Quinolinic acid, which probably is the first compound produced from the condensation of four-carbon and three-carbon units, can serve as an efficient precursor of ricinine. Recent unpublished work from the laboratory showed that the pyridine moieties of the pyridine nucleotides (nicotinic acid mononucleotide, desamido NAD$^+$ and NAD$^+$) serve as efficient precursors of ricinine. However, establishment of a complete biosynthetic pathway is still dependent on the discovery of the "nearest precursors". Some compounds were synthesized from ricinine-3,5-^{14}C to determine if they were more efficient than the known precursors (Fig. 2). Results of these biosynthesis studies using carbon-14 labeled compounds (III, IV, VI VII) as precursors are shown in Table I. The extent of incorporation of each of these precursors was low in comparison with that of nicotinic acid (10%) or quinolinic acid (15%) when identical experimental conditions were used (Yang and Waller, 1965). Thus it is concluded that among the compounds studied, ricininic and ricinine acids can be converted to ricinine by the castor plant but that their conversion efficiency is so low that neither can be considered a "nearest precursor". It is of interest to note that ricininic acid is nearly four times as efficient as ricinine acid. Ricinine amide and 2,4-dihydroxynicotinonitrile were not incorporated into ricinine, and therefore are not involved in the biosynthesis of the alkaloid.

TABLE I. INCORPORATION OF CARBON-14 LABELED COMPOUNDS INTO RICININE*

Compound	Precursor		Ricinine		Incorporation
	Specific Activity μc/mmole	Amount Administered mμc	Specific Activity mμc/mmole	Amount Isolated mμc	%
Ricininic acid-3,5-^{14}C (IV)	567.0	15.0	75.5	1.5	10.0
Ricinine acid-8-^{14}C (VII)	1.56	3.36	2.93	0.094	2.8
Ricinine amide-3,5-^{14}C (VI)	567.0	74.7	0	0	0
2,4-Dihydro-nicotinonitrile-3,5-^{14}C (III)	567.0	41.7	0	0	0

*The experiment duration was 96 hours following injection of precursors. Percentage of incorporation was calculated by dividing the total amount of radioactivity isolated by the total radioactivity administered.

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