THE ORIGIN OF CARBON ATOMS 2, 3, AND 7 OF RICININE[1]

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ABSTRACT

The isolation of carbon atoms 2 and 3 of the α-pyridone nucleus of ricinine is described. Evidence is provided to support the suggestion that succinic acid or a closely related four-carbon dicarboxylic acid provides carbon atoms 2, 3, and 7 in the biosynthesis of the ricinine molecule.

In a preliminary communication (1) evidence was presented to support proposals (2, 3) that in the higher plants the pyridine ring and related α-pyridone ring of such compounds as nicotine and ricinine (I) (see Fig. 1) may be synthesized directly from simple metabolic intermediates related to acetate, succinate, glycerol, and propionate. From the percentage activities found at the nitrile group of ricinine isolated from *Ricinus communis* L. plants that had been fed various simple labelled substances, it was suggested that succinic acid, or a closely related four-carbon dicarboxylic acid found in the Krebs tricarboxylic acid cycle, provided carbon atoms 2, 3, and 7 of ricinine. If such be the case, the four-carbon acid would be incorporated in such a way that one of the carboxyl groups (C-1) would provide the carbon for the nitrile group of ricinine (C-7), and the 2- and 3-carbon atoms would give rise to carbons 3 and 2 respectively of the pyridone ring. The other carboxyl group would be lost by decarboxylation either before or after incorporation into the pyridone ring or its precursor.

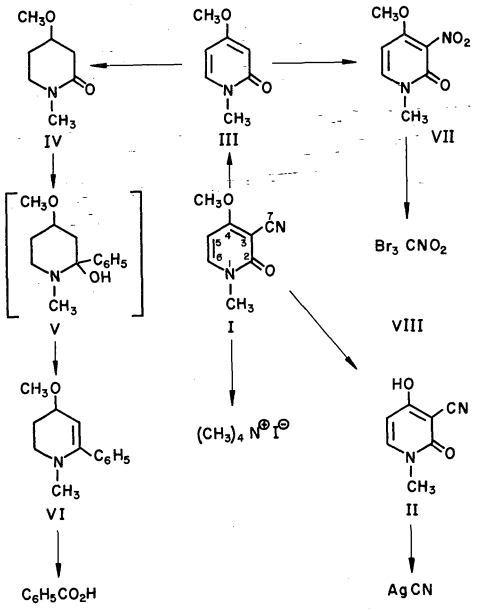

FIG. 1. Degradation schemes for ricinine.

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In order to test this suggestion it is therefore necessary to isolate carbons 2 and 3 from the remaining carbons in ricinine. Carbon atom 2 was isolated in the form of the carboxyl group of benzoic acid. Removal of the nitrile group from ricinine (I) by hydrolysis followed by decarboxylation gave rise to 4-methoxy-1-methyl-2-pyridone (III), which was reduced easily to the corresponding piperidone (IV) with hydrogen and Adams' catalyst. The action of phenyl magnesium bromide on the piperidone (IV) gave the carbinol (V), which lost the elements of water spontaneously and produced the unstable 4-methoxy-1-methyl-2-phenyl-2-piperideine (VI). The product (VI) was treated immediately with potassium permanganate, and the benzoic acid containing carbon 2 of ricinine as its carboxyl group was isolated and burnt in a combustion train. The carbon dioxide was collected as barium carbonate.

The observation (4) that nitrophenols react with calcium hypobromite to yield tribromonitromethane (bromopicrin) in which the carbon atom is the one that carried the nitro group prompted the application of this degradative procedure to the ricinine molecule. Nitration of 4-methoxy-1-methyl-2-pyridone (III) gave a mononitroderivative, the nuclear magnetic resonance spectrum of which contained two doublets centered at τ values of 3.90 and 2.55 p.p.m. with a coupling constant of 8.0 c.p.s. The product, therefore, must contain a proton on each of two adjacent carbon atoms. Accordingly, the nitro group must be located on carbon 3 and the product is 4-methoxy-1-methyl-3-nitro-2-pyridone (VII). Treatment of VII with calcium hypobromite liberated carbon 3 of ricinine as bromopicrin (VIII), which was purified by gas–liquid chromatography. The pure bromopicrin was converted by combustion to carbon dioxide, which was collected as barium carbonate.

To determine the activity of the nitrile group, the methoxy group of ricinine was first hydrolyzed and the resulting ricinic acid (II) oxidized as previously described by Böttcher (5). The liberated hydrogen cyanide was precipitated as silver cyanide and its activity measured.[3]

The methyl groups attached to oxygen and to nitrogen were each isolated as tetramethylammonium iodide.

These methods of determination were applied to two samples of ricinine obtained from plants of *R. communis* L. that had been fed succinic acid-2,3-^{14}C and sodium acetate-2-^{14}C. Quantities of the radioactive ricinine to be used for the determinations were diluted initially with varying quantities of inactive ricinine. The results obtained are recorded in Table I, which gives the activity at each carbon atom in terms of a percentage of the total activity of ricinine.

TABLE I

Location of radioactivity in ricinine

Position		Percentage of activity of ricinine from plants fed succinic acid-2,3-^{14}C	Percentage of activity of ricinine from plants fed sodium acetate-2-^{14}C
OCH_3	(as $(CH_3)_4NI$)	0.55	0.66
NCH_3	(as $(CH_3)_4NI$)	0.58	0.65
7	(as AgCN)	19.1	20.8
2	(as $BaCO_3$ from $C_6H_5CO_2H$)	38.6	38.9
3	(as $BaCO_3$ from Br_3CNO_2)	36.9	38.3
	Total	95.7	99.3

[3]*The method of determination of the activity of the nitrile group by difference gave erratic results and was abandoned.*

The results given in Table I for ricinine obtained from plants fed succinic acid-2,3-^{14}C show that the sum of the activities of carbon atoms 2, 3, and 7 (nitrile) and the carbon atoms of the two methyl groups represents 95.7% of the total activity of the ricinine molecule. Thus, within experimental error, all the activity of the ricinine is accounted for when the plants are fed succinic acid-2,3-^{14}C. Moreover, both carbon atoms 2 and 3 are approximately equally labelled as would be expected if they were derived from carbons 3 and 2 respectively of succinic acid-2,3-^{14}C. The nitrile carbon of ricinine is derived from carbon 1 of succinic acid. This carbon becomes labelled as a result of randomization of activity in the Krebs cycle.

Feeding of sodium acetate-2-^{14}C to *Ricinus* plants gave rise to ricinine labelled similarly to that obtained from succinic acid-2,3-^{14}C. The results (see Table I) may be explained by assuming that the acetate-2-^{14}C enters the Krebs cycle with the subsequent formation of succinic acid-2,3-^{14}C. Strong experimental evidence is thus provided to support the proposal that succinic acid or a closely related four-carbon dicarboxylic acid found in the Krebs cycle is an intermediate in the biosynthesis of ricinine.

Recently, Waller and Henderson (6) have reported that most of the activity in ricinine obtained from young *Ricinus* plants fed succinate-1,4-^{14}C was located on the nitrile carbon. This is in agreement with the present observations, since the activity of carboxyl-labelled succinate will not be randomized amongst carbon atoms 2 and 3 (of succinate) as a result of the operation of the Krebs cycle.

The high extent of incorporation of such compounds as nicotinic acid and nicotinamide (7) into ricinine suggests that these compounds are closer to ricinine than succinic acid in the biosynthetic pathway. The results obtained with ricinine may well be applicable to the pyridine ring of such compounds as nicotine and the related tobacco alkaloid anabasine, since nicotinic acid has been shown (8) to be an important intermediate in the biosynthesis of nicotine. Ricinine (1) and nicotine (9, 10) isolated from the plants after feeding acetate-1-^{14}C as well as acetate-2-^{14}C to *Ricinus* and *Nicotiana* plants, respectively, show labelling patterns which are compatible with the conclusion that succinic acid (or a closely related four-carbon dicarboxylic acid) is a common precursor of the intermediate nicotinic acid.

EXPERIMENTAL

Melting points were determined on a Kofler block and are corrected. Radioactive samples were counted as thin layers on aluminum planchets in either a Radiation Counter Laboratories Inc. internal sample proportional counter or a Baird-Atomic, Inc. end window proportional counter, as indicated in the text. Appropriate corrections were made for background count and sample self-absorption. Succinic acid-2,3-^{14}C and sodium acetate-2-^{14}C were supplied by Merck and Company Ltd., Montreal.

Growth of Ricinus Plants and Administration of the Tracers

Seeds of *R. communis* L., after removal of the outer shells, were planted just beneath the surface of moist sand contained in glass trays placed in a dark cabinet in a greenhouse kept at 20–25°. The sand was kept moist with distilled water. After 12 days the trays were brought out into the light for about 16 hours each day, and the sand kept moist with an inorganic nutrient solution containing $MgSO_4.7H_2O$ (0.85 g), KH_2PO_4 (0.14 g), $NH_4H_2PO_4$ (0.071 g), KNO_3 (0.072 g), $Ca(NO_3)_2.4H_2O$ (0.21 g), $Fe(NH_4)_2(SO_4)_2.6H_2O$ (0.036 g) per liter of distilled water. On the 22nd day of growth, when the plants had reached a height of 15–20 cm, and when the primary leaves were just emerging, an aqueous solution (total 1.0 ml) of succinic acid-2,3-^{14}C (0.85 mg) was introduced into 56 plants by means of glass capillary tubes (partly filled with the solution by capillary rise) inserted into the base of each plant stem. Uptake of the solution by each plant was usually complete within a few minutes. The succinic acid-2,3-^{14}C fed had a total activity of 9.1×10^7 d.p.m. measured on the Radiation Counter Laboratories instrument.

In a like manner an aqueous solution (0.7 ml) of sodium acetate-2-^{14}C (6.05 mg) was fed to 42 *Ricinus* plants grown under conditions similar to those described for the succinic acid feeding experiment. The acetate-2-^{14}C fed had a total activity of 3.58×10^8 d.p.m. (Radiation Counter Laboratories instrument).

Isolation of Ricinine

The tubes were left in the stems until the plants were harvested 72 hours after insertion. The wet stems and leaves were powdered by grinding in the presence of liquid nitrogen. After evaporation of the nitrogen, the ground material was extracted with absolute ethanol in a Soxhlet extractor for 12 hours. The extract was evaporated, the residue suspended in water and washed with petroleum ether (30–60°), and the aqueous solution extracted continuously for 12 hours with chloroform.

Removal of the solvent from this last extract yielded crystalline ricinine, which was purified to constant count by alternate crystallization from methanol and by sublimation at 180° and 5×10^{-4} mm.

Ricinine (415 mg), m.p. 202° (11), with radioactivity of 8000 d.p.m./mg (Radiation Counter Laboratories instrument, previously reported (1) in c.p.m./mg) was obtained from plants fed succinic acid-2,3-^{14}C, which represents an incorporation of 3.6%.

The plants fed acetate-2-^{14}C gave ricinine (353 mg), m.p. 202°, with activity of 65,000 d.p.m./mg (Radiation Counter Laboratories instrument, previously reported (1) in c.p.m./mg), which represents an incorporation of 6.6%.

Degradations of Ricinine from the Succinic Acid-2,3-^{14}C Feeding Experiment

Activities of the OMe and NMe Groups

Active ricinine (52.28 mg) from the succinic acid-2,3-^{14}C feeding experiment (8000 d.p.m./mg) was diluted with pure inactive ricinine (28.1 mg) obtained from *Ricinus* plants not fed any radioactive precursor. The two methyl groups were successively removed by a modification of the Herzig–Meyer method (12). The liberated methyl iodide in each case was trapped in 15% methanolic solutions of trimethylamine and the precipitated tetramethylammonium iodide was crystallized from methanol to constant activity. The tetramethylammonium iodide from the OMe group had an activity of 23.5 d.p.m./mg, and that from the NMe group, 24.8 d.p.m./mg, both measured on the Radiation Counter Laboratories instrument.

Determination of the Activity of the Nitrile Group

Ricinine (5.75 mg) from the feeding experiment was diluted with pure inactive ricinine (ca. 95 mg). The mixture was recrystallized from methanol. A small sample of this diluted ricinine was subjected to combustion and the resulting carbon dioxide collected in a barium hydroxide solution. The barium carbonate thus produced had an activity of 34.2 d.p.m./mg, measured on the Baird-Atomic instrument.

The remainder of the diluted active ricinine was hydrolyzed with dilute aqueous potassium hydroxide to the corresponding hydroxy compound, ricinic acid (II), which was oxidized with chromic acid according to the procedure described by Böttcher (5). The liberated hydrogen cyanide was collected in a solution of silver nitrate acidified with nitric acid. The precipitated silver cyanide (35 mg) had an activity of 77.0 d.p.m./mg (Baird-Atomic instrument).

Dilution of Active Ricinine

All the following experiments were carried out with active ricinine from the succinic acid-2,3-^{14}C feeding which was diluted with pure, inactive ricinine by a factor of approximately 11. The active ricinine was co-crystallized with the inactive sample. A small sample of the diluted ricinine was converted by combustion to carbon dioxide, which was converted to barium carbonate as usual. The barium carbonate, counted on the Baird-Atomic instrument, had an activity of 56.0 d.p.m./mg.

Removal of the Nitrile Group from Ricinine

The diluted ricinine (550 mg) was hydrolyzed and decarboxylated (13) with 57% sulphuric acid solution to 4-methoxy-1-methyl-2-pyridone (350 mg) (III). The product was purified by sublimation at 80° and 5×10^{-4} mm and had m.p. 114–116°.

Reduction of 4-Methoxy-1-methyl-2-pyridone to the Corresponding Piperidone (IV)

A solution of the pyridone (III) (195 mg) in methanol (25 ml) was shaken in an atmosphere of hydrogen with Adams' catalyst at room temperature and under pressure of a small head of mercury, until no further uptake of hydrogen was observed. The 4-methoxy-1-methyl-2-piperidone (IV) was worked up in the usual manner and distilled, b.p. 60–64° at 1 mm. In the ultraviolet the oily product showed end absorption only. Found: C, 58.83; H, 9.29; N, 9.63. Calc. for $C_7H_{13}O_2N$: C, 58.72; H, 9.15; N, 9.78%.

4-Methoxy-1-methyl-2-phenyl-2-piperideine (VI)

Phenyl magnesium bromide was prepared in dry ether (25 ml) in the usual manner using magnesium (400 mg) and bromobenzene (2.62 g). A portion (5 ml) of the ethereal solution of the Grignard reagent was added to 4-methoxy-1-methyl-2-piperidone (170 mg) dissolved in dry ether (6 ml) and the reaction mixture was heated under reflux for 16 hours. The complex was decomposed with dilute hydrochloric acid, the aqueous layer saturated with sodium carbonate, and the mixture shaken. The ether layer was removed and the aqueous layer re-extracted exhaustively with chloroform. The ether and chloroform extracts were combined, the solvents removed, and the residue subjected to a short-path distillation at 12 mm. The fraction distilling at 80–120° (air-bath temperature) was collected. It consisted of a colorless oil that rapidly turned red-brown on exposure to air. The product showed a maximum at 247 mμ (ϵ = 4000) in its ultraviolet absorption spectrum. An infrared spectrum (liquid film) of the product showed no O—H stretching absorption, but contained strong absorption in the 1580–1640 cm^{-1} region, indicative of unsaturation, together with strong C—H deformation bands in the 700–800 cm^{-1} region. The product failed to form either a crystalline hydro-

chloride or a satisfactory picrate. It was not further characterized, but used immediately in the next step.

Oxidation of the Piperideine (VI) to Benzoic Acid

The distilled piperideine was heated under reflux with potassium permanganate (2.0 g) in water (40 ml) for 2 hours. Ethanol was added to the cooled mixture to destroy the excess permanganate, and the filtered solution was acidified with concentrated hydrochloric acid. Extraction with ether gave an extract which on evaporation left a solid residue (80 mg). This residue was purified to constant count by crystallization from water and sublimation, after which it consisted of a colorless crystalline product, m.p. 121–122°, either alone or in admixture with an authentic specimen of benzoic acid. Comparison of infrared spectra confirmed the identity. A sample of the active benzoic acid was converted by combustion to carbon dioxide, which by collection in a barium hydroxide solution was obtained as barium carbonate, with an activity of 24.7 d.p.m./mg (Baird-Atomic instrument).

Nitration of 4-Methoxy-1-methyl-2-pyridone

The pyridone (III) (140 mg) was dissolved in concentrated sulphuric acid (1.0 ml) and the solution cooled in an ice-water bath. A solution of fuming nitric acid (0.2 ml) in concentrated sulphuric acid (2.0 ml) was added to the pyridone solution and the mixture allowed to stand at room temperature for 20 minutes. It was neutralized with aqueous sodium hydroxide and extracted several times with chloroform. The 4-methoxy-1-methyl-3-nitro-2-pyridone (VII) produced was purified by recrystallization from benzene, m.p. 172–173°. Found: C, 45.75; H, 4.35; N, 15.39. Calc. for $C_7H_8O_4N_2$: C, 45.65; H, 4.38; N, 15.21%. A nuclear magnetic resonance spectrum (60 Mc) of the product (VII) in $CDCl_3$ using tetramethylsilane as an internal reference showed signals at τ values of 6.48 and 6.08 p.p.m. and two doublets centered at 3.90 and 2.55 p.p.m. with $J = 8.0$ c.p.s. The nitropyridone gradually decomposed when exposed to light.

Preparation of Tribromonitromethane (VIII) from 4-Methoxy-1-methyl-3-nitro-2-pyridone

A paste was prepared from calcium hydroxide (10 g), water (40 ml), both at 0°, and bromine (3.3 ml). The nitropyridone (VII) (150 mg) was well mixed with the paste. The mixture was gently warmed for several minutes, then strongly heated until about 6.0 ml of distillate had been collected. The crude tribromonitromethane (bromopicrin) (200 mg) was separated by centrifuging, washed with distilled water, and distilled over a short path at 12 mm and an air-bath temperature of 60–70°. The distilled product was purified in two batches by gas–liquid chromatography. A 2 m long, 0.635 cm diameter silicone grease column was used at 80°, with the helium carrier gas at a pressure of 25 p.s.i., giving a flow rate of approximately 150-cc/min. A retention time (peak maximum) of about 36 minutes was observed for the bromopicrin. The bromopicrin (70 mg) was collected in a small U-tube cooled with an ice–water mixture. An infrared spectrum of the purified bromopicrin showed absorption in good agreement with reported absorption maxima (14). The spectrum was also superimposable upon a spectrum obtained from bromopicrin prepared from picric acid by a similar procedure (15). Gas–liquid chromatography of a small sample of the purified bromopicrin showed only one component with a retention time almost identical with that obtained for authentic bromopicrin. The pure bromopicrin was converted by combustion to carbon dixoide, which was collected in a barium hydroxide solution as barium carbonate. The barium carbonate had an activity of 165.5 d.p.m./mg (Baird-Atomic instrument).

Degradations of Ricinine Obtained from the Acetate-2-^{14}C Feeding Experiment

Activities of the OMe and NMe Groups

The O-methyl and N-methyl groups were isolated as described for ricinine from the succinic acid-2,3-^{14}C feeding experiment. The active ricinine (30.4 mg) was first diluted with pure inactive ricinine (48.6 mg). The tetramethylammonium iodide from the OMe group had an activity of 135.0 d.p.m./mg and that from the NMe group, 132.5 d.p.m./mg, both measured on the Radiation Counter Laboratories instrument.

Determination of the Activity of the Nitrile Group

Ricinine (29.2 mg) from the feeding experiment was diluted with pure inactive ricinine (83.5 mg). The nitrile group was isolated as silver cyanide as before. The silver cyanide had an activity of 4300.0 d.p.m./mg (Radiation Counter Laboratories instrument).

Degradations to Isolate Carbon Atoms 2 and 3

Active ricinine was diluted approximately 20 times by the usual method. A small sample of the diluted ricinine was converted by combustion to carbon dioxide and collected as barium carbonate, which had an activity of 229.0 d.p.m./mg (Baird-Atomic Instrument).

By methods outlined in Fig. 1 and described above, carbon atoms 2 and 3 were isolated as the carboxyl group of benzoic acid and as bromopicrin respectively. Both derivatives were converted by combustion to carbon dioxide, which was collected in a barium hydroxide solution as barium carbonate. The barium carbonate from C-2 had an activity of 71.7 d.p.m./mg, and that from C-3, 701.0 d.p.m./mg (Baird-Atomic instrument).

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