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BIOSYNTHESIS OF THE PYRIDINE RING OF RICININE FROM QUINOLINIC ACID GLYCEROL AND ASPARTIC ACID*†

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Abstract—*In vivo* experiments with young *Ricinus communis L.* plants have established that quinolinic acid-2,3,7,8-^{14}C can serve as a more efficient precursor of ricinine than nicotinic acid-7-^{14}C. Chemical degradation of ricinine formed from quinolinic acid-2,3,7,8,-^{14}C, aspartate-4-^{14}C, succinate-1,4,-^{14}C and -2,3-^{14}C, glycerol-1,3-^{14}C and -2-^{14}C provided confirming evidence that carbons 2, 3, and 8 of ricinine arise from a four carbon dicarboxylic acid such as aspartate and that carbons 4, 5, and 6 arise from intact glycerol. Using aspartate -4-^{14}C,-^{15}N evidence for its role as a direct precursor of the α-pyridone ring was obtained; however, a substantial amount of the ^{15}N label was also found in the nitrile group.

INTRODUCTION

IN A preliminary communication on the role of quinolinic acid as a precursor of nicotinic acid and its derivatives in plants Hadwiger *et al.*[1] referred to *in vivo* experiments on ricinine formation by *Ricinus communis L.* from quinolinic acid which were being conducted in this laboratory. The results of these experiments are now described.

Degradation of ricinine (I, Fig. 1) by Essery, Juby, Marion and Trumbull[2] formed *in vivo* by young castor plants which had been administered glycerol-1,3,-^{14}C and glycerol-2-^{14}C showed that glycerol could be incorporated intact into carbons 4, 5, and 6. Juby and Marion[3] showed that carbon-14 labeled succinate could give rise to carbons 2, 3, and 8. Schiedt and Boeckh-Behrens,[4] using a different degradation method, found a concentration of the label in position 3 when aspartate-3-^{14}C was the precursor. We have used a modification of this latter procedure to determine the location of label in ricinine formed from carbon-14 labeled glycerol, succinic, aspartic and quinolinic acids. These results extend the preliminary findings of Waller and Henderson[5] and confirm the findings of Marion and co-workers on the role of glycerol and succinate as precursors and provide evidence for the role of aspartate as a precursor of the α-pyridone ring of ricinine. Aspartate-^{15}N can serve as a source of nitrogen for both the nitrile group and that in α-pyridone ring.

* For a preliminary account of parts of this work see *Federation Proc.* 22, 356 (1963) and the M.S. Thesis of K. S. Yang, Oklahoma State University, August, 1963.

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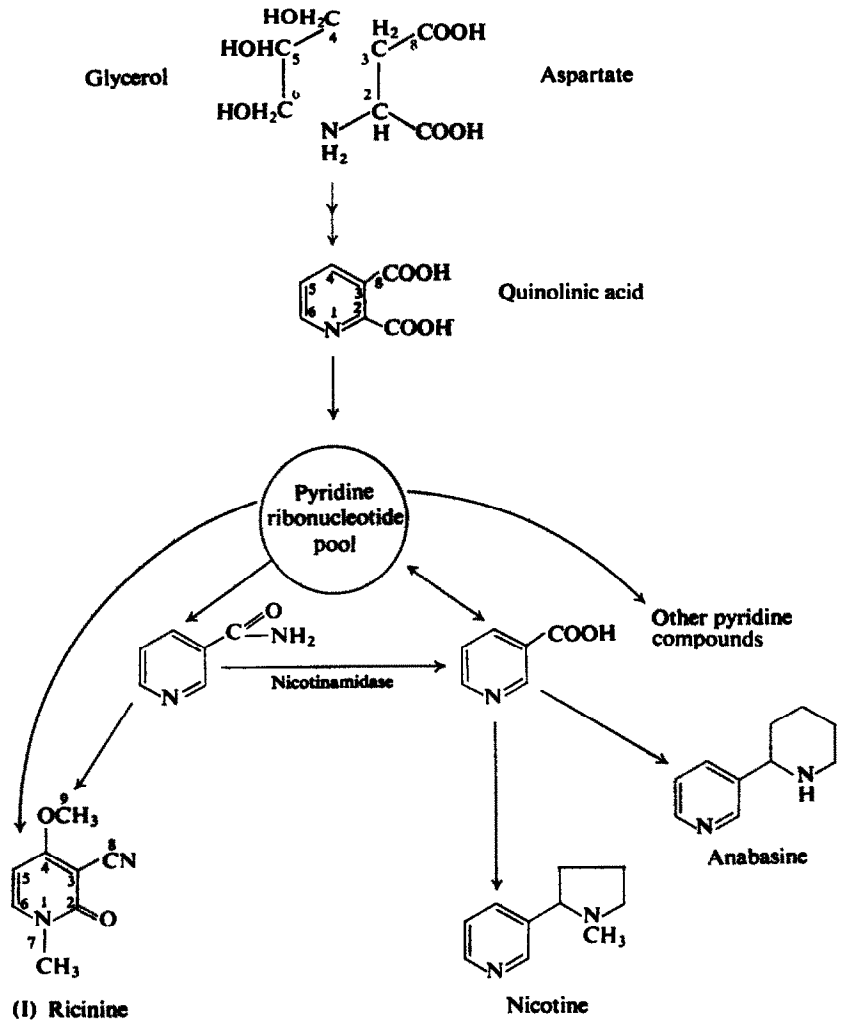

FIG. 1. PROPOSED PATHWAY FOR RICININE BIOSYNTHESIS.

RESULTS

Precursor Role of Quinolinic Acid

The results shown in Table 1 indicate that quinolinic acid is a more efficient precursor of ricinine than nicotinic acid. In the preliminary communication it was stated that quinolinic acid was about three times more efficient than nicotinic acid; however, these results were from experiments of one week duration. The technique used for studying incorporation in this report provides reliable data that is not influenced by uncontrollable agronomic conditions that sometimes occur when field-grown plants are used.[5] It can be assumed that the label is equally distributed between carbons 2, 3, 7 and 8 of quinolinic acid-2,3,7,8-^{14}C, consequently, the per cent incorporation would be increased by 25 per cent due to the loss of carbon 7 as CO_2. A value of 16·7 per cent would be used to compare with the 14·5 per cent incorporation of quinolinic acid-5-^{14}C in which no loss of label occurs. To obtain unequivocal

TABLE 1. INCORPORATION OF CARBON-14 LABELED QUINOLINIC ACID AND NICOTINIC ACID INTO RICININE*

Precursor			Ricinine		
Compound	Specific activity (μc/mmole)	Amount administered (mμmoles)	Amount isolated (μmoles)	Specific activity (mμc/mmole)	Incorporation (%)
Quinolinic acid-2,3,7,8-^{14}C	110	418	15·8	364	12·5
Quinolinic acid-5-^{14}C	431	200	20·9	223	14·5
Nicotinic acid-7-^{14}C	5200	155	18·9	4370	10·2

* The experiment duration was 96 hr following injection of precursors. Duplicate experiments indicated an error of ±0·5% in per cent incorporation. Percentage incorporation was obtained by dividing the total amount of radioactivity administered by the total amount recovered.

results on the relative efficiences of quinolinic and nicotinic acids experiments varying the amount of precursor administered were conducted and these results are shown in Fig. 2. At all levels tested quinolinic acid was a more efficient precursor than nicotinic acid. A marked toxic effect was observed on those plants that received 5 μmoles of quinolinic acid per plant and this is the reason for the dotted portion of the curve. Nicotinic acid is also toxic, but much higher concentrations are required.

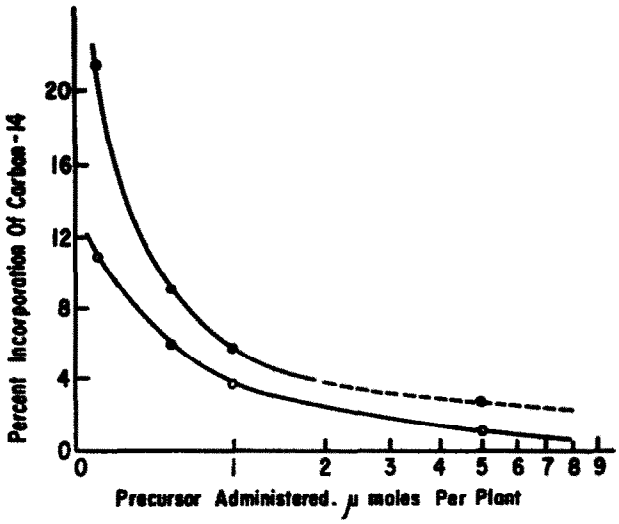

FIG. 2. EFFECT OF PRECURSOR CONCENTRATION ON PER CENT INCORPORATION OF CARBON-14 INTO RICININE.

The duration of the experiments was 96 hr following administration of the compounds. ●-Quinolinic acid-2,3,7,8,^{14}C, specific activity, 148 μc/mmole: ○-Nicotinic acid -7-^{14}C, specific activity, 106 μc/mmole. The results are presented in a semi-logarithmic plot. No correction for loss of carbon 7 of quinolinic acid was made.

In the modification of the Schiedt and Boeckh-Behrens[4] procedure for degrading ricinine it was necessary to establish that the CO_2 released upon hydrolysis of the 5,6-dihydro-*N*-methyl-4-methoxy-3-cyano-2-pyridone came only from carbon 8 (see Fig. 1). The results

shown in Table 2 were obtained from degrading ricinine-8-^{14}C formed biosynthetically from nicotinic acid-7-^{14}C and indicate that 97 per cent of the radioactivity from carbon 8 was recovered as CO_2 in this procedure. This modified degradation procedure was used throughout this study since it was possible to distinguish between the amount of label in carbon 8 and in carbons 2 and 3. Results from the degradation of ricinine formed from quinolinic acid-2,3,7,8-^{14}C presented in Table 2 show that the label was located in carbons 2, 3 and 8.

Precursor Role of Aspartate, Succinate and Glycerol

Results from using DL-aspartate-4-^{14}C-^{15}N as a precursor are shown in Table 3. Aspartate can serve as a source of both the α-pyridone ring nitrogen and the nitrile nitrogen in an approximate 3:2 ratio. The percent incorporation of ^{15}N into the α-pyridone ring and of ^{14}C into the nitrile carbon (Table 2) are approximately equal.

The labeling pattern of ricinine formed from succinate-1,4-^{14}C, succinate-2,3-^{14}C, glycerol-1,3-^{14}C and glycerol-2-^{14}C is shown in Table 2. Data on the incorporation efficiencies of these compounds have been published.[5]

DISCUSSION

The results presented above clearly establish the role of quinolinic acid as a more effective precursor of ricinine than nicotinic acid. In this transformation carbon 7 of quinolinic acid is lost as CO_2 and carbons 2, 3 and 8 become carbons 2, 3 and 8 of ricinine as is shown in Fig. 1. Quinolinic acid can also serve as a precursor of the pyridine ring of nicotine produced by *Nicotiana tabacum*.[6]

Leete and Leitz[7] proposed that NADH might serve as an intermediate in ricinine biosynthesis; however, their proposal had not been supported. With the demonstration that nicotinic acid mononucleotide can be formed from quinolinic acid and PRPP without proceeding through nicotinic acid in *E. coli* by Andreoli *et al.*[8] and in plants by Hadwiger *et al.*[1] it becomes necessary to reconsider the proposal of Leete and Leitz. The pyridine nucleotides are implicated as precursors of ricinine (Fig. 1) because of the higher efficiency of quinolinic acid as a precursor and because free nicotinic acid is not an intermediate in nicotinic acid mononucleotide formation. Experiments to determine if the pyridine nucleotides can serve as precursors are in progress. This possibility makes it necessary to re-evaluate the report of Waller and Henderson[9] that the carboxamide group of nicotinamide undergoes intramolecular dehydration to form the nitrile group of ricinine. It now appears that at least two pathways may exist and these are shown in Fig. 1. This hypothesis proposes separate routes for the precursor roles of quinolinic acid (which involves the pyridine nucleotides as intermediates) and nicotinamide. Free nicotinamide could be formed by the action of NAD^+ nucleotidase. Newell and Waller[10] have presented evidence for the occurrence of nicotinamidase in castor plants. The nicotinic acid thus formed may be utilized for synthesis of the pyridine nucleotides, nicotine and anabasine. The significance of this route in which both intact nicotinamide and nicotinic acid derived from nicotinamide are used is further demonstrated by the finding that ^{15}N also appears in the α-pyridone ring of ricinine when the

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TABLE 2. DISTRIBUTION OF ISOTOPE IN RICININE FORMED FROM VARIOUS PRECURSORS

Precursor	Specific activity of ricinine (mμc/mmole)	O-CH_3 (C-9) (mμc/mM)	(%)	N-CH_3 (C-7) (mμc/mM)	(%)	C-8 (mμc/mM)	(%)	C-2 C-3 (mμc/mM)	(%)	C-4 (mμc/mM)	(%)	C-5 (mμc/mM)	(%)	C-6 (mμc/mM)	(%)
Nicotinic acid-7-^{14}C	256·0	0	0	0	0	249·0	97·3		trace	0	0	0	0	0	0
Quinolinic acid-2,3,7,8-^{14}C	241·0	0	0	0	0	47·4	19·7	166·0	69·0	0	0	0	0	0	0
D,L-Aspartate-4-^{14}C	61·5	0	0	0	0	59·5	97·0	0	0	0	0	0	0	0	0
Succinate-2,3-^{14}C	135·0	trace	trace	trace	trace	20·9	15·5	62·6	46·5	*	*	*	*	*	*
Succinate-1,4-^{14}C	23·4	0	0	0	0	18·1	77·3	2·5	10·4	0	0	0	0	0	0
Glycerol-1,3-^{14}C	358·0	42·0	11·7	35·8	10·0	41·6	11·0	118·0	33·0	49·5	13·8	0	0	53·3	14·9
Glycerol-2-^{14}C	297·0	60·0	20·2	51·1	17·2	31·5	10·6	124·0	41·8	0	0	65·0	22·0	0	0

* A low recovery of *N*-Me-β-alanine was obtained. Carbon-14 assays on this compound showed a trace of activity from the ricinine labelled with succinate 2,3-^{14}C.

TABLE 3. D,L-ASPARTIC ACID-4-^{14}C-^{15}N* AS A PRECURSOR OF RICININE

Precursor			Ricinine							
					^{15}N Excess		Incorporation			
Specific activity (μc/mmole)	^{15}N excess (atom %)	Amount administered (μmoles)	Amount isolated (μmoles)	Specific activity (μc/mmole)	α-Pyridone ring (atom %)	Nitrile nitrogen (atom %)	^{15}N in α-pyridone (%)	^{15}N in nitrile nitrogen (%)	^{14}C into ricinine (%)	Ratio of percent incorporation of α-pyridone ring-^{15}N to carbon-14
129·0	45·0	12·5	14·0	0·49	0·09	0·06	0·46	0·30	0·43	$\frac{0·46}{0·43} = 1·07$

* This is a mixture of aspartic acid-4-^{14}C and -^{15}N, not a doubly labeled compound. Percentage of incorporation was determined by dividing the total amount of radioactivity by the total amount isolated and by dividing the total amount of ^{15}N administered by the -^{15}N in the α-pyridone ring and the nitrile nitrogen atoms respectively.

biosynthesis experiment using nicotinamide-^{15}N (amide N) is allowed to proceed for a week.* The amide nitrogen can be incorporated into the ring when longer times are used and intramolecular dehydration of the carboxamide group of nicotinamide to yield the nitrile group also occurs. It is likely that the ^{15}N (amide N) in the ring arises from the action of nicotinamidase followed by labeling of aspartate with ^{15}N which could then be utilized in the formation of quinolinic acid and the other pyridine compounds as shown in Fig. 1.

The mechanism of formation of quinolinic acid remains to be established. The present studies on the labeling pattern of ricinine from glycerol-1,3-^{14}C, glycerol-2-^{14}C, succinate-1,4-^{14}C, succinate-2,3-^{14}C and aspartate-4-^{14}C-^{15}N serve as confirming evidence for the occurrence of a reaction between a 3-carbon compound related to glycerol and a 4-carbon compound related to aspartate in the formation of pyridine compounds by plants[2–4, 11–15] and bacteria.[16, 17] The results shown in Table 2 offer evidence that glycerol is utilized intact in forming carbons 4, 5 and 6 and that succinate and aspartate give rise to carbons 2, 3 and 8 of ricinine.

The ratio of percent incorporation of ^{14}C (carbon 8) and ^{15}N (α-pyridone ring) of 1·07 provides evidence for the direct incorporation of aspartate into carbons 2, 3 and 8 of ricinine. It is suggested that the pathway for aspartate proceeds via quinolinic acid as a precursor (Fig. 1). The ^{15}N found in the nitrile group (approximately 40 per cent) presumably arises via an ammonia pool. The evidence presented here for the role of aspartate as a precursor of ricinine is not contradictory to the earlier findings of no incorporation of aspartate-^{3}H by Waller and Henderson.[9] This result would be expected since there are no hydrogen atoms on carbons 2 and 3 of ricinine. The role of aspartate as a direct precursor of nicotinic acid was reported by Gross *et al.*[17] who found that nicotinic acid formed from aspartate-1,4-^{14}C-^{15}N by *Mycobacterium tuberculosis* had a ^{14}C to ^{15}N ratio of 0·46 (0·5 theoretical value). Incorporation of aspartate-^{15}N into the pyridine ring of nicotine by excised root cultures was reported by Christman and Dawson;[11] however, since, equal amount of the label also appeared in the pyrrolidine ring their interpretation was that the nitrogen atoms in both rings arose via an ammonia pool rather than from aspartate directly.

Known pathways of metabolism can be used in interpreting some of the randomization of label which occurred from using glycerol-1,3-^{14}C, glycerol-2-^{14}C and succinate-2,3-^{14}C as precursors but the finding of radioactivity in carbons 2 and 3 of ricinine from succinate-1,4-^{14}C is not so readily explained. A plausible explanation for this labeling pattern is based on the formation of malonate from oxalocetate, a reaction which has been found to occur in bush bean roots by de Vellis *et al.*[18] Aspartate was also demonstrated to be a source of malonate; however, oxalocetate was the immediate precursor. Waller and Henderson[19]

* In an experiment of one week duration 20·7 μmoles of nicotinamide-7-^{14}C, ^{15}N (specific activity, 121·0 μc/mmole; ^{15}N excess, 28·6%) was administered to a young castor plant. The ricinine isolated (specific activity, 1·4 μc/mmole; ^{15}N excess, 0·123%)[5] was converted to *N*-methyl-4-methoxy-2-pyridone (specific activity, 0 μc/mmole; ^{15}N excess, 0·05%) which was found to contain 22% of the total ^{15}N. By difference the ^{15}N content of the nitrile group is 0·118 per cent which gives a ^{14}C:^{15}N nitrile ratio of 1·17.

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reported that malonate-1,3-^{14}C and malonate-2-^{14}C could serve as precursors of ricinine. The ricinine thus formed has been partially degraded. The distribution of the label in ricinine formed from (*a*) malonate-1,3-^{14}C was 30% in carbons 7 and 9, 17% in carbon 8, 22% in carbons 2 and 3 and the remainder in carbons 4, 5 and 6 and (*b*) malonate-2-^{14}C was 42% in carbons 7 and 9, 11% in carbon 8, 30% in carbons 2 and 3 and the remainder in carbons 4, 5 and 6. Using these data it is possible to interpret the labeling pattern in ricinine from succinate-1,4-^{14}C. The mechanism by which malonate is incorporated into ricinine is not clear, but based on the incomplete ricinine labeling patterns presented it is predicted that several routes may be involved. The best known is the decarboxylation reaction described by Hatch and Stumpf[20] but there are at least ten different metabolic pathways[18] by which malonate can be formed by plants and it is likely that some of these are reversible. If the α-decarboxylation reaction described by de Vellis *et al.*[18] is reversible then the distribution of label from succinate-1,4-^{14}C could be explained. Plants can form carbon-14 labeled acids of the Kreb's cycle from malonate-1,3-^{14}C as demonstrated by Giovanelli and Stumpf[21] and from malonate-2-^{14}C as demonstrated by Young and Shannon.[22] The incorporation of $H_2{}^{14}CO_3$ into the α-pyridone ring at carbon 2 was shown by Schiedt and Boeckh-Behrens.[4]

EXPERIMENTAL

Labeled Compounds Used

Succinate-1,4-^{14}C (5·0 mc/mmole) and -2,3-^{14}C (8·75 mc/mmole) were purchased from California Corporation for Biochemical Research. Glycerol-1,3-^{14}C (3·76 mc/mmole) and -2-^{14}C (7·0 mc/mmole) were purchased from New England Nuclear Corporation. DL-aspartate-4-^{14}C (0·95 mc/mmole) and -^{15}N (45·0 atom % ^{15}N excess) were purchased from Schwarz Bio Research. Quinolinic acid-2,3,7,8-^{14}C and -5-^{14}C were prepared according to the procedure reported by Gholson *et al.*[23] using aniline-U-^{14}C and glycerol-2-^{14}C purchased from New England Nuclear Corporation. These compounds were checked for radiochemical purity by paper chromatography in the following solvents: *n*-butanol:acetic acid:water (4:1:1); 85% isopropanol; 95% ethanol:1 N ammonium acetate (7:3) and only one spot corresponding to the particular compound studied was found. Each compound was dissolved in distilled water prior to administration to the castor plants.

Cultural Conditions

Castor seeds of the Cimarron variety were germinated in sand at 30°. After germination plants of uniform size were selected and placed under Gro-Lux lamps (Model F-40-GRO; Sylvania Electric Products, Inc., Salem, Massachusetts) at 25° for one week before administering the labeled compounds.

Administration of Labeled Compounds

A 22-gauge hypodermic needle was used to make an opening in the stem about halfway between the surface of the sand and the leaves. Using a Hamilton micro syringe 20–40 μl of the dissolved compound was placed at the opening on the stem. The liquid was completely absorbed within a few minutes. This was followed by several applications of distilled water.

[20] M. D. Hatch and P. K. Stumpf, *Plant Physiol.* **37**, 121 (1962).
[21] J. Giovanelli and P. K. Stumpf, *Plant Physiol.* **32**, 498 (1957).
[22] R. H. Young and L. M. Shannon, *Plant Physiol.* **34**, 149 (1959).
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Isolation of Ricinine

A modification of the published procedure was used.[9] The major modification consisted of homogenizing the washed plant with a mortar and pestle. The alkaloid was then extracted and purified in a manner similar to the published method.

Degradation of Ricinine

A modification of the procedure of Schiedt and Boeckh-Behrens[4] was used. Ricinine (I, Fig. 1) was refluxed with 2 N NaOH for 1 hr followed by acidification to pH 3 and *N*-methyl-4-hydroxy-3-cyano-2-pyridone (II) was recrystallized to constant specific activity (m.p. 299°). The difference between the specific activity of I and II is the specific activity of carbon 9 of ricinine. II was hydrogenated to give the corresponding 5,6 dihydro compound, III, which had the same specific activity. The oxidation of III as reported[4] was carried out by adding an excess of CO_2-free 1 N KM_nO_4. CO_2 and oxalate were formed from carbons 2, 3 and 8 but randomization of the label occurred. This step was modified as follows: one mmole of III was dissolved in 20 ml of 0·1 N NaOH and 1 N $KMnO_4$ was added dropwise while shaking until a faint pink color persisted for one hour. This mixture was centrifuged to remove MnO_2. The supernatant fluid was decanted and the residue washed thoroughly with water. The combined aqueous solutions were hydrolyzed with 6 N HCl (1 ml/mmole I) and the CO_2 collected as Na_2CO_3. The CO_2 was found to originate solely from carbon 8. The hydrolysate which contained oxalic acid (IV) and *N*-methyl-β-alanine (V) was passed through a Dowex-50 ion-exchange column in the H^+ form at pH 2·5. Oxalic acid was eluted with water and *N*-methyl-β-alanine with 2·5 N HCl. The oxalic acid was precipitated as calcium oxalate and combusted to CO_2 to obtain the amount of radioactivity located in carbons 2 and 3. The *N*-methyl-β-alanine was subjected to exhaustive methylation. Acrylic acid (VI) was recovered by steam distillation. The tetra methyl ammonium salt (carbon 7) formed was precipitated as the reineckate and recrystallized to constant specific activity. VI was hydrogenated at 1 atm. using PtO_2 as the catalyst to give propionic acid (VII). VII was degraded stepwise according to Schmidt and Phares[24] to give the radioactivity located in carbons 4, 5 and 6. Determinations of the radioactivity in the *O*- and *N*-methyl carbons (7 and 9) were also made independently using the modified method of Pregl suggested by Dubeck and Kirkwood[25] with results which agreed with those obtained with the Schiedt and Boeckh-Behrens[4] procedure. This degradation can be carried out on 1 mmole of ricinine.

Isotope Analysis

Carbon-14 analyses were made either by the wet combustion procedure of Van Slyke *et al.*[26] with subsequent counting of the CO_2 with the vibrating reed electrometer or by counting the compound directly in a liquid scintillation spectrometer (Tricarb, Packard Instrument Company, LaGrange, Illinois).

Nitrogen-15 analyses for ricinine formed from aspartate-4-^{14}C-^{15}N were made on an Atlas CH4 mass spectrometer equipped with high mass double collectors and a measuring bridge by measuring the isotopic excess for the parent ion ($P=m/e$ 164) and for a fragment ion ($F=m/e$ 82). It was established through the use of appropriately labeled biologically synthesized ricinine samples that m/e 82 contained one of the methyl carbons, carbons 4, 5 and 6 of the α-pyridone ring and the nitrogen atom, but not the nitrile nitrogen atom.

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The reproducibility of ^{15}N measurements by this technique was $\pm 0{\cdot}01\%$. (This mass spectrometric technique was recently developed by the author (G.R.W.) in Dr. Ragnar Ryhage's Laboratory of Mass Spectrometry, Chemistry I, Karolinska Institute, Stockholm, Sweden. The complete procedure is to be published shortly.)

Nitrogen-15 analyses for the ricinine experiment described in the footnote on p. 886 were performed by Mr. Seymour Meyerson using a modified Consolidated 21-103 Analytical Mass Spectrometer.[27]

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