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THE RELATIONSHIP OF THE PYRIDINE NUCLEOTIDE CYCLE TO RICININE BIOSYNTHESIS IN *RICINUS COMMUNIS**

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Key Word Index—*Ricinus communis*, Euphorbiaceae, ricinine, pyridine, nucleotide cycle, quinolinic acid, biosynthesis

Abstract—Quinolinic acid-6-^{14}C feeding experiments were performed using *Ricinus communis* L. plants to determine the effect of selected inhibitors on the incorporation of this precursor into the alkaloid, ricinine, and into the pyridine nucleotide cycle intermediates. Administration of azaserine and azaleucine resulted in a decrease in the incorporation into ricinine and an increase in the radioactivity remaining in quinolinic acid. Administration of excess ricinine and ethionine yielded similar results. This mutual inhibition indicated an interdependency between the conversion of quinolinic acid to ricinine and to the pyridine nucleotide cycle intermediates.

INTRODUCTION

THE BIOSYNTHESIS of ricinine in *Ricinus communis* L. has been studied by a number of research groups.[1–9] In previous studies, quinolinic acid has been shown to be an efficient precursor of ricinine and of nicotinic acid mononucleotide in plants.[3,10–13] In 1956 Leete and Leitz suggested that ricinine might be formed from NAD^+ or related substituted pyridine compounds.[2] The previous evidence provided the basis for the formulation, by Waller *et al.*,[9] of the pyridine nucleotide cycle. In this formulation, the initial metabolite, quinolinic acid (QA) is converted to nicotinic acid mononucleotide by quinolinate phosphoribosyltransferase (decarboxylating); nicotinic acid mononucleotide (NaMN) is then converted

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to nicotinic acid adenine dinucleotide (desNAD$^+$) by nicotinate mononucleotide adenyltransferase (E.C 2 7 7a); nicotinic acid adenine dinucleotide is then converted to NAD$^+$ by NAD$^+$ synthetase (E C 6.3 5.1), NAD$^+$ can then be converted to nicotinamide (NaM) by NAD$^+$ glycohydrolase (E C 3.2.2.5); nicotinamide is converted to nicotinic acid (Na) by nicotinamide deamidase and nicotinic acid is converted to NaMN by nicotinate phosphoribosyltransferase (E C 2.4.2.11) Recent results have indicated that the precursors of quinolinic acid are dihydroxyacetone phosphate and aspartic acid rather than glyceraldehyde-3-phosphate and aspartic acid [14]

Several studies have been conducted which indicated that the pyridine nucleotide cycle is a necessary intermediate in the biosynthesis of ricinine in the castor plant from quinolinic acid [2,3,9] Hiles and Byerrum, however, have suggested that the biosynthesis of ricinine from quinolinic acid occurs by a separate pathway, independent of the cycle.[15] This conclusion was based on evidence showing an increase in incorporation of label into ricinine from labeled quinolinic acid in the presence of excess exogenous NAD$^+$ However, this conclusion is valid only if one assumes NAD$^+$ is an obligatory intermediate in the biosynthesis of ricinine and that exogenous NAD$^+$ can be transported across the cellular membrane intact. Recent work by Negishi and Ichiyama[16] has shown that NAD administered intravenously to the rat was extremely short lived (90% destroyed in 20 sec). Liersch *et al.*[17] have also presented evidence against such a membrane passage in perfused rat liver. Another explanation of these results is that ricinine was biosynthesized from an intermediate before NAD$^+$ in the cycle, such as NaMN or desNAD$^+$. However, the labeling experiments of Waller and Henderson indicated that the amide nitrogen of nicotinamide was incorporated into the nitrile group directly.[4] If ricinine were to arise from NaMN or desNAD$^+$, then a direct incorporation of the amide nitrogen of nicotinamide into ricinine would not be expected, but rather an incorporation from a nitrogen pool such as ammonia or glutamate Such seemingly conflicting results have left the relationship of the pyridine nucleotide cycle to ricinine biosynthesis unsolved.

Several compounds have been found to inhibit the pyridine nucleotide cycle at various points Alazopeptin, [L-alanyl-(6-diazo-5-oxo)-L-norleucyl-(6-diazo-5-oxo)-L-norleucine] and 6-diazo-5-oxo-norleucine, two potent glutamine antagonists, and azaserine, a less potent glutamine antagonist, have been shown to inhibit the pyridine nucleotide cycle in animals.[18-20] Two other aza-compounds, azaleucine and azauracil, were also considered as possible inhibitors

Azaserine has been shown to be a glutamine antagonist[18] and has been shown by several other workers to inhibit the NAD$^+$ synthetase reaction in which nicotinic acid adenine dinucleotide is converted to NAD$^+$ with glutamine or ammonia as the nitrogen donor in liver[12,21,24] as well as in brain [25] Addition of excess nicotinamide before adding azaserine

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or 6-diazo-5-oxo-norleucine seemed to counteract this inhibition;[23] however, addition of glutamine after addition of azaserine or 6-diazo-5-oxo-norleucine did not counteract this inhibition. This was probably a result of irreversible inhibition by azaserine and 6-diazo-5-oxo-norleucine. Azaserine has been shown to be forty times less effective as a glutamine antagonist than 6-diazo-5-oxo-norleucine.[18] This might explain the lack of any significant accumulation of nicotinic acid adenine dinucleotide in the presence of azaserine, yet a definite accumulation was noted in the presence of 6-diazo-5-oxo-norleucine in mouse liver and mouse tumor cells.[26] Another explanation for this result could be that in mouse liver an enzyme was present which destroyed azaserine but not 6-diazo-5-oxo-norleucine.[20,27] 6-Diazo-5-oxo-norleucine also inhibited the reaction of phosphoribosyl pyrophosphate plus glutamine yielding glutamate plus 5-phosphoribosyl amine, which was catalyzed by phosphoribosyl pyrophosphate amido-transferase (E.C 2.4.2.14),[28] 6-Diazo-5-oxo-norleucine probably acted as a glutamine antagonist in this case also.

Alazopeptin has also been shown to cause inhibition of NAD^+ synthesis in mouse liver and mouse tumor cells.[26] It was shown to be as potent an inhibitor as 6-diazo-5-oxo-norleucine, and since its structure is similar to that of 6-diazo-5-oxo-norleucine it probably inhibited at the NAD^+ synthetase reaction as does 6-diazo-5-oxo-norleucine.

The similarity of all these structures lends support to the postulation that each acts as a glutamine antagonist.[21,28,29] 4-Azaleucine, an amino acid found in cultures of *Streptomyces neocaliberis*, has a structure similar to that of glutamine, 6-diazo-5-oxo-norleucine and azaserine.[28,30] Ricininic acid, or *O*-demethyl ricinine, was shown to inhibit the conversion of nicotinic acid to ricinine.[31]

The experiments described herein were designed to clarify the relationship of the pyridine nucleotide cycle to ricinine biosynthesis by inhibiting the pyridine nucleotide cycle with specific inhibitors and observing the effects on ricinine biosynthesis, then inhibiting ricinine biosynthesis and observing the effects on the biosynthesis of the pyridine nucleotide cycle intermediates.

RESULTS AND DISCUSSION

In order to investigate the biosynthesis of ricinine and of the pyridine nucleotide cycle intermediates, experiments were undertaken in which labeled quinolinic acid was injected into castor bean plants previously injected with compounds known or suspected to be pyridine nucleotide cycle inhibitors. These compounds were alazopeptin, azaserine and azaleucine. Excess ricinine and ethionine were also administered in an attempt to inhibit the biosynthesis of ricinine in its later stages and to observe any effects on the incorporation of radioactivity into the pyridine nucleotide cycle intermediates. In preliminary experiments, alazopeptin was found to be at least as effective an inhibitor as azaserine, however, further study was impossible because of the limited quantity of alazopeptin available.

In experiment 1, the concentration of the inhibitors was 45 mg/100 g fresh weight. Triplicate control, azaserine-treated and azaleucine-treated plants were allowed to metabolize

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quinolinic acid-6-^{14}C (QA-6-^{14}C) for an 11 hr period before analysis. In experiment 2, triplicate plants were treated with QA-6-^{14}C (1 μCi/plant) only, with QA-6-^{14}C plus azaserine (75 mg/100 g) or with QA-6-^{14}C plus azaleucine (75 mg/100 g) and allowed to metabolize for 20 hr In experiment 3, duplicate plants were injected with QA-6-^{14}C, with QA-6-^{14}C plus ricinine (1 mg/g) or with QA-6-^{14}C plus ethionine (40 mg/100 g) and allowed to metabolize for 20 hr. Table 1 shows the effects of these inhibitors on the incorporation of QA-6-^{14}C into ricinine

TABLE 1. EFFECTS OF INHIBITORS ON THE INCORPORATION OF QA-6-^{14}C IN TO RICININE

Experiments	Inhibitor conc (mg/100 g‖)	Duration (hr)	Conc (mmoles/100 g‖)	Ricinine Incorp * (%)	Sp act (mμCi/μmole)	Isotope dilution¶
Experiment 1†						
Control	—	11	2 6	25 6 ± 2 5	1 71	25000
Azaserine	45	11	2 6	20 1 ± 9 5	0 82	53200
Azaleucine	45	11	2 6	6 5 ± 3 0	0 44	100000
Experiment 2‡						
Control	—	20	2 5	21 3 ± 4 4	2 77	15800
Azaserine	75	20	2 5	12 0 ± 1 8	1 63	26900
Azaleucine	75	20	2 4	3 2 ± 1 8	0 39	112000
Experiment 3§						
Control	—	20	3 4	35 5 ± 3 9	4 26	10288
Ricinine	100	20	5 4	5 7 ± 1 7	0 56	78035
Ethionine	40	20	4 1	8 6 ± 0 8	0 96	45521

* One μCi of quinolinic acid-6-^{14}C (sp act 43 7 μCi/μmole) was administered as a precursor
† Flowering plants 11–13 weeks old were used
‡ Non-flowering plants 8 weeks old were used
§ Non-flowering plants 6 weeks old were used
‖ Fresh plant weight
¶ The ratio of the specific activity of quinolinic acid-6-^{14}C to the sp act of ricinine

In these experiments, although the amount of ricinine remained essentially unchanged, inhibitors affected the incorporation of radioactivity into ricinine, moderately for azaserine and markedly for azaleucine. In experiment 1, azaserine did not seem to significantly change the incorporation of radioactivity into ricinine compared to the control but in experiment 2, with the level of azaserine increased by 75 mg/100 g, the reduction in incorporation was nearly 2-fold (Table 1). In experiment 1, azaleucine caused a four-fold reduction of incorporation of radioactivity into ricinine, while in experiment 2, the reduction was seven-fold These results indicated a sharp decrease in the biosynthesis of ricinine from QA

Inhibitor effects on the pyridine nucleotide cycle

Dowex 1 × 8 formate column chromatography was used to separate the pyridine nucleotide cycle intermediates [12 32 33] Several components were identified including *N*-methyl nicotinic acid and *N*-methyl nicotinamide, which eluted from the column as one peak, nicotinamide, NAD$^+$, nicotinic acid, desNAD$^+$ and QA However, except for desNAD$^+$ most of the components were present in small quantities and contained such low levels of radioactivity that inhibitor effects could not be conclusively determined

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Table 2 shows the effects of the inhibitors on the incorporation of radioactivity into desNAD$^+$ from QA-6-^{14}C. In experiments 1 and 2, azaserine caused a three-fold increase in radioactivity found in desNAD$^+$, while a two to three-fold increase in the radioactivity remaining in QA was observed. These data indicate an inhibition of the pyridine nucleotide (PN) cycle at the step of NAD$^+$ formation from desNAD$^+$, which is catalyzed by NAD$^+$ synthetase. This inhibition has been reported previously in animals,[18–20] but not in plants.

TABLE 2 INHIBITOR EFFECTS ON THE INCORPORATION OF QA-6-^{14}C INTO DesNAD$^+$

Experiments	Inhibitor conc (mg/100 g‡)	% Distribution of radioactivity* QA†	DesNAD$^+$†
Experiment 1			
Control	—	19 8 ± 3 5	2 5 ± 0 5
Azaserine	45	38 3 ± 6 6	6 2 ± 0 6
Azaleucine	45	41 2 ± 3 6	1 5 ± 0 9
Experiment 2			
Control	—	7 3 ± 2 5	1 9 ± 0·2
Azaserine	75	22 1 ± 3 0	5 4 ± 1·9
Azaleucine	75	46 4 ± 3 1	3 7 ± 0 2
Experiment 3			
Control	—	6 6 ± 2 0	—
Ricinine	100	14 3 ± 0 1	—
Ethionine	40	20 1 ± 3 4	—

* One μCi of quinolinic acid-6-^{14}C (sp act 43 7 μCi/mole) was administered as a precursor

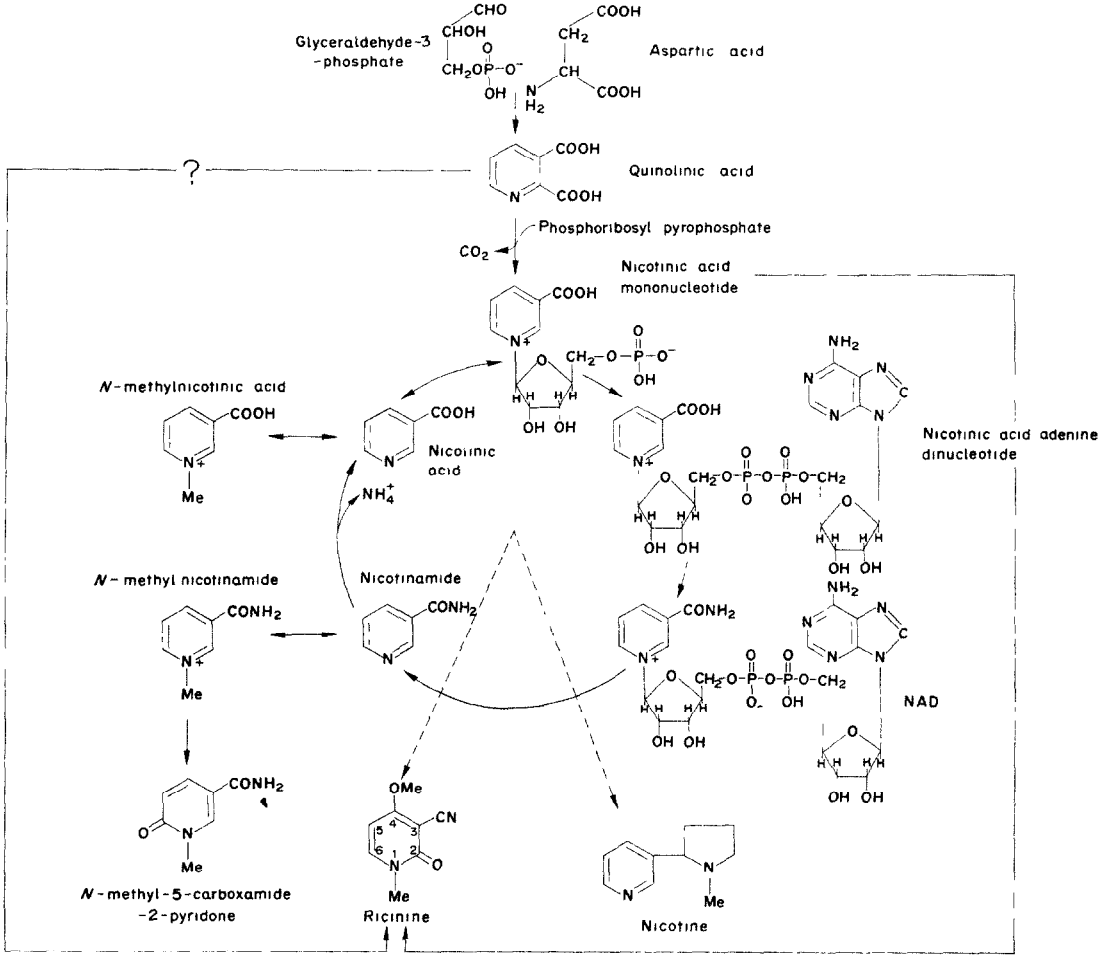
† QA = quinolinic acid. DesNAD$^+$ = nicotinic acid adenine dinucleotide

‡ Fresh plant weight.

Azaleucine also seemed to inhibit the incorporation of QA into the PN cycle intermediates. In experiment 1, azaleucine caused a two-fold increase in the radioactivity remaining in QA while in experiment 2, the increase was seven-fold. In both cases, the increase in radioactivity remaining in QA was as great or greater than the decrease in radioactivity found in ricinine. This indicates that the QA, which was inhibited from forming ricinine, was not being shunted into the PN cycle, as one would expect if the two pathways were separate. Since ricinine biosynthesis and the biosynthesis of the PN cycle intermediates were both inhibited, it is likely that the two pathways are interdependent.

In experiment 3, excess ricinine administration caused a six-fold reduction in incorporation into ricinine and a two-fold increase in the radioactivity remaining in QA. In the presence of ethionine, a four-fold reduction of incorporation into ricinine was observed and a three-fold increase in the radioactivity in QA. It would be expected that blockage of the later stages of ricinine biosynthesis by ethionine and excess ricinine would cause shunting of QA into other pathways branching from the PN cycle. *N*-methylnicotinic acid and *N*-methylnicotinamide are found in plants in much larger amounts than the PN cycle intermediates and are probably storage forms of the pyridinium moiety. The biosynthesis of these compounds results from methylation of the two cycle intermediates, as shown in Scheme 1, nicotinic acid and nicotinamide, respectively. One might expect increases in the incorporation of radioactivity into these compounds in the presence of excess ricinine and ethionine, and this was observed. The total radioactivity found in these compounds was

8% in the presence of excess ricinine, 7% in the presence of ethionine and 4% in the control plants. The ether extracts of the various plants contained less than 1% of the administered radioactivity and this indicated negligible metabolism of pyridinium compounds resulting in the formation of ether soluble products occurred

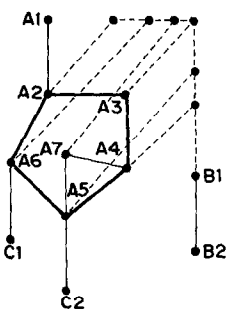


SCHEME 1 THE PYRIDINE NUCLEOTIDE CYCLE AND ITS RELATION TO PYRIDINE ALKALOID BIOSYNTHESIS

Although the results of these experiments indicate an interdependency between the PN cycle and ricinine biosynthesis, the details concerning the order of intermediates in ricinine biosynthesis have not been elucidated The conflicting evidence[9,15] concerning which PN cycle intermediate is the most immediate precursor of ricinine may best be explained by a more complicated relationship between the cycle and ricinine biosynthesis One possible relationship is the metabolic grid concept, which is defined as a series of parallel reactions in which analagous transformations occur, but at different rates, thus a compound may be converted to a product by several different parallel pathways.[34] The proposed metabolic grid, as shown in the structure, in which several of the PN cycle intermediates enter

[34] BU'LOCK J D (1965) in *The Biosynthesis of Natural Products*, pp 81-82 McGraw-Hill New York

into the ricinine biosynthesis pathway, explains the similarity of radioisotopic incorporation results when each of the PN cycle intermediates (carbon-14 labeled) were fed.[9] The report that a 10-fold excess of NAD^+ caused an increase in the incorporation of radioactivity into ricinine from QA-6-^{14}C[15] does not necessarily mean that ricinine was biosynthesized from QA independent of the PN cycle, but can be readily explained as a shunting of radioactivity from QA-6-^{14}C into ricinine through the first two cycle intermediates, nicotinic acid mononucleotide (NaMN) and desNAD$^+$, since excess NAD^+ blocks the cycle at the step of NAD^+ formation from desNAD$^+$. It has also been reported that QA is a better precursor of ricinine than the PN cycle intermediates;[15] however, this conflicts with other results.[9] The possibility remains that ricinine is biosynthesized from QA by two pathways, one through the cycle and one independent of it. However, this seems less likely, since inhibition of the conversion of QA to the PN cycle intermediates by azaserine and azaleucine also inhibited incorporation into ricinine. If there were a major cycle-independent pathway from QA to ricinine, then one would expect to see little change in the incorporation into ricinine upon inhibition of the PN cycle.



Ricinine biosynthesis

A1 = Quinolinic acid
A2 Nicotinic acid mononucleotide
A3 Nicotinic acid adenine dinucleotide
A4 Nicotinamide adenine dinucleotide
A5 Nicotinamide
A6 Nicotinic acid
A7 Nicotinamide mononucleotide
B1 *N*-Demethyl ricinine
B2 Ricinine
C1 *N*-Methyl nicotinic acid
C2 *N*-Methyl nicotinamide

——— Pyridine nucleotide cycle
----- Postulated reaction sequence

A metabolic grid proposed for ricinine biosynthesis

Further studies using carbon-14 labeled PN cycle intermediates as ricinine precursors in the presence of the inhibitors azaserine, azaleucine, alazopeptin and DON should be carried out to investigate further the proposed relationship between the PN cycle and ricinine biosynthesis.

EXPERIMENTAL

Materials Plants Castor bean plants (*Ricinus communis*), of the Cimarron variety were grown in port clay loam soil at the Agronomy farm of Oklahoma State University in Stillwater during the summer of 1971 The plants varied in age from 6 to 12 weeks during the experiments (weight 75–200 g)

Inhibitors DL-4-Azaleucine, azaserine and azauracil were purchased from Calbiochem, Los Angeles, California. riciniinic acid was obtained from General Biochemicals, Chagrin Falls, Ohio and alazopeptin was obtained as a gift from Lederle Laboratories, Pearl River, New York

Radioactive compounds Quinolinic acid-6-^{14}C (sp act of 43 7 mCi/mmole) was purchased from Amersham/ Searle Corporation, Arlington Heights, Illinois It was subjected to paper chromatography using 1 M NH_4OAc, 95% EtOH (3 7, v/v) as solvent, and the radioactivity located using a Nuclear Chicago Actigraph III Model 1002 4π Chromatogram Scanner The results indicated a radiochemical purity greater than 99%

Chemical reagents Solvents and chemical reagents were of analytical reagent grade unless otherwise noted Non-radioactive authentic pyridine nucleotide cycle compounds were purchased from Sigma Chemical Company, St Louis, Missouri, Biochemical Research Company, Los Angeles, California, Nutritional Biochemical Corporation, Cleveland, Ohio or Merck and Company, Rahway, New Jersey Dowex 1 × 8 chloride form, 200-400 mesh, was purchased from J T Baker Chemical Company, Phillipsburg, New Jersey, BioRad AG 1 × 4 chloride form, 200-400 mesh, was purchased from Calbiochem Los Angeles, California Both were converted to the formate form prior to use

Methods: Administration of inhibitors and labeled compounds Inhibitors were injected into the hollow internodular stem section of the castor bean plants using a micro syringe The labeled compounds (1 μCi) were administered into the stem section by the same technique, approximately 1 hr after injection of the inhibitors The plants were samples at varying time periods up to 20 hr

Isolation of metabolites Plants were cut into small pieces and blended with 300 ml 80% MeOH at 50-60° to a coarse slurry The extract was filtered and the residue re-extracted until colorless The residue was dried ground, weighed and analyzed for radioactivity by wet combustion and gas counting The combined extracts were evaporated to dryness and taken up in 150 ml of H_2O This phase was extracted with 3 × 100 ml Et_2O, the Et_2O phase was dried, weighed and counted by liquid scintillation spectrometry The aqueous phase was evaporated to near dryness, taken up in 10 ml H_2O 2 ml aliquots were then analyzed by TLC on silica gel HF in $CHCl_3$ MeOH (5 1) Ricinine was eluted with MeOH and purified to constant specific activity, while the remainder of the silica gel from the plate was extracted 5× with 50% MeOH Quantitative analysis was made by measuring the absorbancy at 255 and 307 mm[35] and the radioactivity determined The extract was then evaporated to near dryness taken up in 10 ml H_2O and analyzed on either a Dowex 1 × 8 of BioRad Ag 1 × 4 formate column

Anion exchange column chromatography Analysis of the ricinine-free phase was achieved by column chromatography using a 1 4 × 50 cm Dowex 1 × 8 or BioRad AG 1 × 4 formate column [32 33] Components were eluted using a stepwise HCOOH conc gradient, from H_2O to 3 M acid (80 ml/hr) 6 ml fractions were analyzed by UV spectrophotometry at 260 nm and counted Reference compounds were subjected to column chromatography to determine their elution volumes for identification purposes

PC Column chromatography fractions containing radioactive peaks were combined and dried by lyophilization The residues were dissolved in MeOH or H_2O, spotted on Whatman No 3 paper strips along with reference compounds Representative strips of each residue were chromatographed in 1 M NH_4OAc 99% EtOH (3 7) and isobutyric acid conc NH_4OH H_2O (66 1 7 33 by vol)

Measurement of radioactivity Each anion-exchange column fraction was analyzed for radioactivity by placing 2 ml of each fraction in 10 ml of Bray's scintillation solution[36] and counting on a Model 3320 Packard TriCarb Scintillation Spectrometer The carbon-14 remaining in the plant residues were determined by the wet combustion procedure of Van Slyke *et al*[37] with subsequent counting of the carbon dioxide with a vibrating reed electrometer

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