Phytochemistry, 1967, Vol. 6, pp. 1637 to 1641. Pergamon Press Ltd. Printed in England

THE ORIGIN OF THE NITROGEN ATOMS OF RICININE PRODUCED BY *RICINUS COMMUNIS* L.*

GEORGE R. WALLER and K. S. YANG

Department of Biochemistry, Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma 74074

(*Received* 26 *April* 1967)

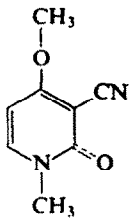
Abstract—The biosynthetic role of inorganic and organic ^{15}N labeled compounds was studied using the cyano-substituted pyridine alkaloid ricinine produced by *Ricinus communis* L. The relatively efficiency of incorporation of the compounds studied was formamide > glutamine > NH_4NO_3 > aspartate > nicotinamide (amide N) > KNO_3 > nicotinonitrile (nitrile N) > 1-methylnicotinamide (amide N) > 1-methylnicotinonitrile (nitrile N). The distribution of the ^{15}N label between the ring and nitrile nitrogen atoms of ricinine showed that the ^{15}N-pyridine compounds gave ricinine labeled only in the nitrile nitrogen whereas the simpler compounds gave the alkaloid labeled in both the ring and nitrile nitrogen atoms with the label being higher in the nitrile nitrogen during the first 4 days but being equally distributed a week after administration of the precursor.

INTRODUCTION

RICININE (I), the α-pyridone alkaloid produced by *Ricinus communis* L. (0·1–0·2 per cent fresh weight), may be derived from the pyridine ring of nicotinic acid, nicotinamide and the pyridinium moiety of the pyridine nucleotides,[1,2] metabolites which may arise directly from the condensation of small molecules such as aspartic acid and a glycerol derivative.[3–6] Feeding experiments with aspartic acid-^{15}N have shown that the ring nitrogen and the cyano nitrogen carry approximately the same nitrogen-15 abundance; however, the interpretation of these results has differed.[3,5] The role of the nitrogen atoms derived from aspartate is under further study in this laboratory and will not be reported on at this time; however, it will be shown that the nitrogen atoms may be derived from other sources. A preliminary report on the incorporation of nitrogen-15 into ricinine from $K^{15}NO_3$ and $^{15}NH_4NO_3$ was presented[7] but detailed evidence was not shown on the ditribution of the ^{15}N in ricinine formed from these compounds. This report includes studies on both inorganic and organic nitrogen compounds as precursors of ricinine.

* Supported in part by research grant GM-08624 from the National Institutes of Health, Bethesda, Maryland.

[1] G. R. WALLER and L. M. HENDERSON, *J. Biol. Chem.* **236**, 1186 (1961).
[2] G. R. WALLER, K. S. YANG, R. K. GHOLSON, L. A. HADWIGER and S. CHAYKIN, *J. Biol. Chem.* **241**, 4411 (1966).
[3] K. S. YANG and G. R. WALLER, *Phytochem.* **4**, 881 (1965).
[4] U. SCHIEDT and G. BOECKH-BEHRENS, *Z. Physiol. Chem.* **330**, 58 (1962).
[5] S. R. JOHNS and L. MARION, *Can. J. Chem.* **44**, 23 (1966).
[6] P. R. THOMAS, M. F. BARNES and L. MARION, *Can. J. Chem.* **44**, 1997 (1966).
[7] G. R. WALLER and L. M. HENDERSON, *Abstr. of Papers, Am. Chem. Soc.*, 140th National Meeting, Symposium on Biogenesis of Natural Products, Chicago, Ill., Sept., 1961, p. 30Q.



(I)

A complication that arises in the analysis of ricinine is the two nitrogen atoms, which must be differentiated one from the other. This problem may be overcome by (a) using a degradation technique that will convert one of the two nitrogen atoms, and only one, to ammonia and then analyzing the ammonia in the mass spectrometer, or (b) analyzing the alkaloid directly in a mass spectrometer. The latter procedure was used in this study.[8] This technique exploits the particular virtue of mass spectrometry for studying reaction paths in biosynthesis, which is its ability to locate a label within a molecule without prior chemical degradation.

RESULTS AND DISCUSSION

Table 1 shows the incorporation of nitrogen-15 into ricinine from various labeled precursors and Table 2 shows the location of the labeled nitrogen atoms in ricinine. A comparison of the precursor role of inorganic nitrogen ($^{15}NH_4NO_3$ and $K^{15}NO_3$) showed that ammonium nitrogen is a better precursor than nitrate nitrogen. The extent of incorporation of nitrogen-15 from $K^{15}NO_3$ varied threefold in different experiments while little difference was observed when $^{15}NH_4NO_3$ was the precursor. These results support the view[9] that ammonia, the product of nitrogen fixation, is incorporated into organic molecules in preference to nitrate. Nitrogen-15 from each of these simple molecules was found in both atoms of ricinine but the predominant amount was always located in the nitrile nitrogen.

The role of formamide is of considerable interest since it was the most efficient precursor found in this study. Its incorporation was rapid as indicated by the 0·27 per cent value obtained after 12 hr and 0·86 per cent after 24 hr, but it fell off rapidly showing only a 30 per cent further increase in 24–96 hr. The ricinine-labeling pattern (Table 2) shows all of the label located in the nitrile nitrogen after 12 hr, about 25 per cent in the ring and 75 per cent in the nitrile nitrogen at 24 and 48 hr and equal distribution after 96 hr. Formamide[10] is an efficient nitrogen source for oat, wheat and millet plants. It may also be toxic[11] to plants in large amounts; however, no sign of toxicity in the *Ricinus communis* L. plants used in this study was observed. Although the significance of the rapid incorporation of nitrogen-15 into the nitrile nitrogen is not clear, the nitrogen-15 probably becomes part of the amino nitrogen pool and is transferred to the nicotinic acid derivative to form the nicotinamide derivatives[1–3] which serve as precursors of ricinine. It should be pointed out that whereas nitrate, ammonia, glutamine, aspartate and other forms of organic nitrogen are endogeneous constituents, the natural occurrence of formamide is not known in plants. Therefore, in contrast with the other forms of organic nitrogen supplied, formamide-^{15}N will probably not be subject to dilution by endogenous material.

[9] G. R. WALLER, R. RYHAGE and S. MEYERSON, *Anal. Biochem.* **16**, 277 (1966).
[10] R. M. ALLISON and R. H. BURRIS, *J. Biol. Chem.* **224**, 351 (1957).
[11] B. E. BROWN and F. R. REID, *Soil Sci.* **43**, 341 (1937).
[11] G. E. BLACKMAN, *J. Exptl Botany* **3**, 1 (1952).

TABLE 1. INCORPORATION OF NITROGEN-15 FROM LABELED PRECURSORS INTO RICININE

Precursor				Ricinine			
Compound injected	Duration of experiment (hr)	^{15}N excess (%)	Amount injected (μmoles)	Amount isolated (μmoles)	^{15}N excess (%)	*Incorporation (%)	Isotope dilution
$K^{15}NO_3$	48	31·0	198·0	273·0	0·02	0·18	1,550
	96	31·0	198·0	258·0	0·06	0·51	510
$^{15}NH_4NO_3$	48	32·0	46·0	210·0	0·20	5·7	160
	96	32·0	46·0	189·0	0·23	5·9	139
$HCO^{15}NH_2$	12	20·0	1,480·0	359·0	0·27	0·66	74
	24	20·0	860·0	310·0	0·86	1·67	43
	48	20·0	1,230·0	243·0	1·03	2·08	19
	96	20·0	1,480·0	314·0	1·23	2·65	16
L-Glutamine-^{15}N (amide)	24	97·0	20·0	131·0	0·05	0·68	1,940
	48	97·0	20·0	65·0	0·23	1·54	422
	96	97·0	20·0	147·0	0·30	4·60	324
	168	97·0	20·0	92·0	0·49	4·65	198
Nicotinamide-N (amide N)	48	30·9	20·0	246·0	0·18	5·4	230
	96	30·9	20·0	271·0	0·24	8·9	172
1-Methylnicotinamide-^{15}N (amide N)	96	30·9	15·0	158·0	0·03	1·0	1,030
Nicotinonitrile-^{15}N (nitrile N)	96	30·9	15·0	219·0	0·02	1·0	1,550
1-Methylnicotinonitrile-^{15}N (nitrile N)	96	30·9	15·0	276·0	0·02	1·2	1,550

* The percent incorporation was calculated by dividing the total amount of ^{15}N recovered by the total amount of ^{15}N injected into the plant.

TABLE 2. DISTRIBUTION OF NITROGEN-15 IN BIOSYNTHESIZED RICININE*

Precursor	Duration of experiment (hr)	^{15}N excess in ricinine (%)	^{15}N excess in nitrile nitrogen (%)	^{15}N excess in α-pyridone ring (%)	^{15}N excess in nitrile (%)
$K^{15}NO_3$	96	0·03	0·02	33	67
$^{15}NH_4NO_3$	48	0·20	0·025	12	88
	96	0·23	0·08	35	65
$HCO^{15}NH_2$	12	0·27	(−0·01)	0	100
	24	0·86	0·23	27	73
	48	1·03	0·20	20	80
	96	1·23	0·52	42	58
L-Glutamine-^{15}N (amide N)	48	0·23	0·05	22	78
	96	0·30	0·06	20	80
	168	0·49	0·22	46	54
Nicotinamide-^{15}N (amide N)	96	0·24	(−0·01)	0	100
1-Methylnicotinamide-^{15}N (amide N)	96	0·03	0·0	0	100
Nicotinonitrile-^{15}N (nitrile N)	96	0·02	0·0	0	100
1-Methylnicotinonitrile (nitrile N)	96	0·02	0·0	0	100

* The percent ^{15}N excess in ricinine and in the fragment ion m/e 83 was determined in the manner described under Experimental.

Incorporation of nitrogen-15 from L-glutamine-^{15}N (amide N) is of about the same order of magnitude as the incorporation of nitrogen from DL-aspartic acid-^{15}N.[3] A predominant amount of label from this compound was found in the nitrile nitrogen up to 96 hr, but, after 168 hr (1 week), an equal amount of labeling was found in both the ring and nitrile nitrogen atoms. It was somewhat surprising that a more rapid equilibration of the amide nitrogen with the amino nitrogen pool was not observed. Glutamine is probably the immediate nitrogen donor to form the amide of nicotinamide and therefore higher labeling in the nitrile nitrogen at short times after feeding would be expected.

Nicotinamide-^{15}N (amide N), 1-methylnicotinamide-^{15}N (amide N), nicotinonitrile-^{15}N (nitrile N) and 1-methylnicotinonitrile-^{15}N (nitrile N) were incorporated into ricinine and all of the nitrogen-15 label was located in the nitrile nitrogen. The efficiency of nicotinamide-^{15}N as a precursor was considerably higher than that of the other compounds and was of the same order of magnitude as was obtained with nicotinamide-7-^{14}C.[1,3] The extent of incorporation of the other nitrogen-15 labeled pyridinium compounds was of the same order of magnitude as that obtained with the corresponding carbon-14 labeled compounds.* These data support the results previously presented on the occurrence of the pyridine nucleotide cycle and its relationship to the biosynthesis of ricinine in *R. communis*.[2]

The data presented on the distribution of nitrogen-15 in ricinine, show that the rates of synthesis of the nitrile group and that of the pyridine ring differ. This is to be expected since the pyridine nucleus is formed before changes occur in the carboxyl group attached to carbon-3. All nonpyridine compounds studied were capable of providing nitrogen in both the

* Unpublished results obtained in our laboratories on the biosynthesis of ricinine from nicotinonitrile-7-^{14}C and 1-methylnicotinonitrile-8-^{14}C have provided results comparable to those shown in Table 1 for the corresponding ^{15}N labeled compounds.

ring and nitrile positions; however, the labeled pyridinium compounds provided nitrogen only for the nitrile group. The high efficiency of formamide as a nitrogen donor is noteworthy but further studies will be required to clarify its role in plant metabolism.

EXPERIMENTAL

Growth of plants and isolation of ricinine. The cultural conditions, administration of labeled compounds and procedure for isolation of ricinine are the same as those previously published.[12] The plants were harvested at appropriate times following injection of the precursor, and ricinine was isolated from the *whole* plant.

Preparation of ^{15}N-compounds

Nicotinamide-^{15}N (amide N) and nicotinonitrile-^{15}N (nitrile N). Nicotinamide-^{15}N (amide N) containing 30·9% ^{15}N excess in the amide nitrogen was synthesized from nicotinic acid and $^{15}NH_3$ by direct amidation of the acid *in vacuo* in a sealed vessel by the procedure of Pike and Shane.[13] To convert nicotinamide to nicotinonitrile, the procedure reported by Camps[14] was employed. The nicotinamide-^{15} and P_2O_5 were distilled at 30 mm Hg pressure. The nicotinonitrile-^{15}N upon cooling crystallized to a snow-white solid and was purified by distillation at ordinary pressure.

1-methylnicotinamide-^{15}N (amide N) and 1-methylnicotinonitrile-^{15}N (nitrile N). One ml of methyl iodide was added to 1 mmole of nicotinamide-^{15}N and the mixture was refluxed at 50° for 6 hr. The mixture was dissolved in a minimum of hot water and recrystallized from ethanol and ether as 1-methylnicotinamide iodide. It was further purified by repeated recrystallization from the same solvents: m.p. 205–6° uncorr.

1-methylnicotinonitrile-^{15}N (nitrile N) was prepared by methylating the nicotinonitrile-^{15}N with methyl iodide in a sealed tube at 100° and purified by the same method employed for 1-methylnicotinamide-^{15}N. (m.p. 203–204° uncorr.).

Preparation of formamide-^{15}N. The $^{15}NH_3$ was generated from $^{15}NH_4NO_3$ and continuously swept with a stream of N_2 gas through 38·0 mmole of methyl formate cooled to −78°. The system was flushed with N_2 gas for an additional hour. The vessel containing the methyl formate and liquid $^{15}NH_3$ was sealed under vacuum, and then allowed to warm gradually to 45° and held there for 1½ hours. The tube was opened and the unreacted methyl formate and NH_3 were immediately removed by use of a water aspirator. The purity of the formamide was confirmed by determining its refractive index and its molecular weight (unlabeled species has a mass of 45, labeled species has a mass of 46 by mass spectrometric analysis).

Other nitrogen-15 labeled compounds. Glutamine-^{15}N (amine) with 97% atom excess of ^{15}N was purchased from Merck, Sharp and Dohme of Canada.

Nitrogen-15 analyses. The mass spectrometric method of Waller *et al.*[8] was used. The labeled ricinine samples were analyzed directly in an Atlas CH4 mass spectrometer equipped with high mass double collectors. This technique permits the amount of ^{15}N in the nitrile and ring nitrogen atoms to be determined.

Acknowledgements—We are deeply indebted to Dr. Ragnar Ryhage for valuable discussions and for making available the Atlas CH4 mass spectrometer and other facilities in his laboratory. We wish to express our appreciation to Mr. Sune Melkersson and Mr. Sten Wikstrom for competent technical assistance.

[12] G. R. Waller and L. M. Henderson, *Biochim. Biophys. Res. Commun.* **5**, 5 (1961); **6**, 398 (1961).
[13] E. F. Pike and R. S. Shane, U.S. Patent 2,412,749 (1956).
[14] R. Camps, *Arch. Pharm.* **240**, 366 (1902).