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RICININE METABOLISM AND TRANSLOCATION IN *RICINUS COMMUNIS**

H. J. LEE and G. R. WALLER

Department of Biochemistry, Agricultural Experiment Station, Oklahoma State University, Stillwater, Okla. 74074, U.S.A.

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Abstract—A new ricinine metabolite was conclusively identified as *O*-demethylricinine (*N*-methyl-3-cyano-4-hydroxy-2-pyridone) and its interconversion with ricinine in senescent and green castor plant leaves was demonstrated. Ricinine-3,5-^{14}C administered in the yellow leaves was translocated to healthy parts of the plant, especially the growing apex. This finding indicated that translocation of ricinine may be a process performed by the plants in order to reutilize the alkaloids from the leaves which are being prepared for abscission.

INTRODUCTION

ADVANCES in our knowledge of the biosynthesis of the alkaloids have little counterpart with respect to their further metabolism. The alkaloids have generally been considered as a final product of nitrogen metabolism in the plants. The occurrence of the possible alkaloid catabolic processes has become of interest in the last decade.[1,2]

Degradation of the α-pyridone ring of ricinine to carbon dioxide by the castor bean plant had been demonstrated.[3] Recently a rapid interconversion of exogenous ricinine and *N*-demethylricinine in senescent and green castor bean plant leaves was reported.[4] The two compounds, *N*-demethylricinine and CO_2, are the only compounds which have been identified as ricinine metabolic products.

The experiments described herein were designed to identify possible new ricinine metabolites, to provide further information on the translocation of ricinine within the plant and to study the possible role of the *N*- and *O*-demethylation and methylation reactions of these alkaloids in connection with translocation.

RESULTS AND DISCUSSION

Isolation and Identification of a New Ricinine Metabolite

Detection. During the course of quantitative studies on the catabolism of ricinine in senescent leaves of the castor bean plant following injection of ricinine-3,5-^{14}C, a radioactive zone was observed at the origin of the TLC plate developed with the relatively non-polar solvent system, chloroform–methanol (5:1, v/v). Radioactivity in this non-migrating zone after development had been observed previously by Skursky;[4] however, no attempts had been made to identify the compound(s) in the zone at that time. The TLC behavior of the compound(s) in the non-polar solvent system suggested that it might be a relatively more

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[1] T. C. Tso and R. N. JEFFREY, *Arch. Biochem. Biophys.* **92**, 253 (1961).

[2] G. R. WALLER, K. S. YANG, M. R. SCOTT, F. J. GOLDBERG, J. S. MAYES and H. AUDA, *Plant Physiol.* **40**, 803 (1965).

[3] G. R. WALLER and J. L. C. LEE, *Plant Physiol.* **44**, 522 (1969).

[4] L. SKURKSY, D. BURLESON and G. R. WALLER, *J. Biol. Chem.* **244**, 3228 (1969).

polar compound than the *N*-demethylricine which was recently identified as a ricinine metabolite in senescent leaves of the castor bean plant by Skursky *et al.*[4] It was thought that this polar compound(s) could be either a cleavage product of the α-pyridone ring, one or more pyridine nucleotides, or the *O*-demethyl form of ricinine.

Isolation and purification. In order to positively identify this unknown compound(s), the silica gel zone at the origin (R_f 0·0–0·05) was removed and extracted with methanol. The methanol extract was reduced in volume, developed on TLC with a polar solvent system, EtOH–H_2O–NH_3OH (20:4:1, by vol.). A comparison of TLC plates developed with the relatively non-polar and polar solvent systems are shown in Fig. 1. The radioactive zone was eluted from the chromatogram with methanol and rechromatographed with various solvent systems. For further purification, the unknown radioactive compound was dissolved in hot methanol, crystallized twice and purified by sublimation. This new metabolite of ricinine formed white needles when crystallized from methanol and had a melting point of 279°.

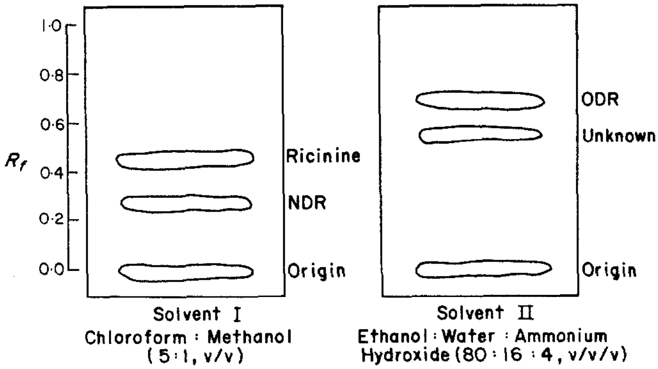

FIG. 1. SCHEMETIC DIAGRAM OF THIN LAYER CHROMATOGRAMS DEVELOPED WITH RELATIVELY NON-POLAR AND POLAR SOLVENT SYSTEMS.
NDR—*N*-demethylricinine; ODR—*O*-demethylricinine.

Identification. MS: The mass spectrum of the unknown compound was compared with mass spectrum of the authentic *O*-demethylricinine (ricininic acid) shown in Fig. 2. The mass spectrum of the unknown compound was essentially identical with that of authentic *O*-demethylricinine. Both compounds had a molecular ion, M^+150, which was their base peak. The postulated route for the formation of the relatively abundant ions are given in Fig. 3.

Decomposition reactions that can account for at least some of the prominent features could have been predicted from earlier work with similar molecules such as ricinine and *N*-demethylricinine which contain the same functional groups. The formation of similar ion species was reported[5] with α-pyridone ring compounds.

The IR spectra of *O*-demethylricinine isolated from the castor bean plant and that of standard in a KBr pellet are shown in Fig. 4. The IR spectra of the *O*-demethylricinine isolated from the castor bean plant was found to be virtually superimposable on that of the standard. Both spectra have a very broad OH stretching absorption in the region of 3·0–4·0 μ, strong =C—O stretching at 6·2 μ and a sharp ketone absorption band from 7·7 to 9·5 μ. A very sharp absorption peak at 4·5 μ indicated —C≡N stretching which is observed not only with *O*-demethylricinine but also with *N*-demethylricinine and ricinine.[4]

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[5] S. D. SASTRY, *Applications of Mass Spectrometry in Biochemistry* (edited by G. R. WALLER), p. 656, Wiley–Interscience, New York (1972).

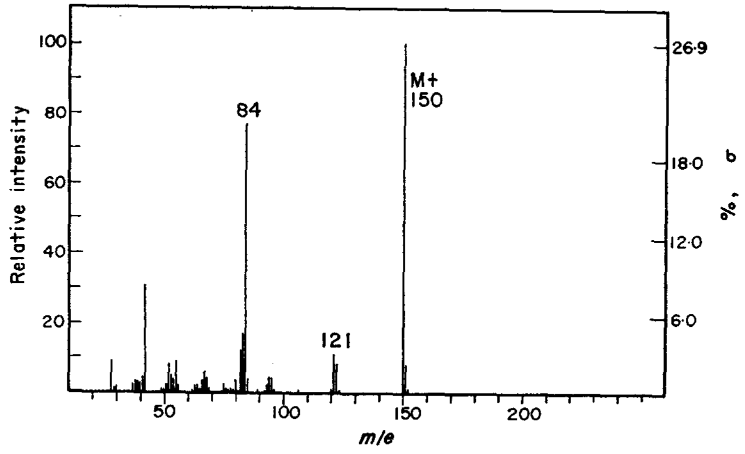

FIG. 2. MS OF *O*-DEMETHYLRICININE.

Based on these data (chromatographic, UV, IR, NMR[4] and MS) the metabolite was conclusively identified as *O*-demethylricinine (*N*-methyl-3-cyano-4-hydroxy-2-pyridone). After the unknown metabolite of ricinine had been confirmed as *O*-demethylricine, the molar extinction coefficient of the compound in water at 272 nm ($E = 7\cdot32 \times 10^3$ l. mole^{-1}) was determined and this value used for further quantitative metabolic studies.

To make certain that the metabolite had actually been produced by the castor bean plant and was not an artifact or formed as the result of microbial action, the following two experiments were conducted. In the first, ricinine (10 mg) was added to a fresh homogenate

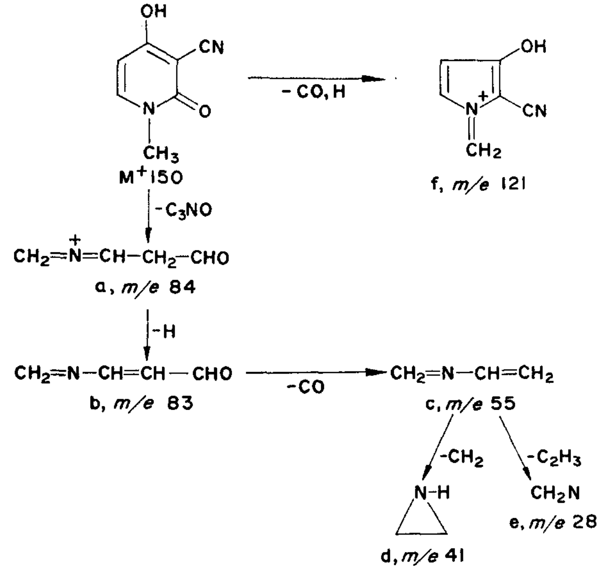

FIG. 3. PROPOSED PARTIAL FRAGMENTATION OF *O*-DEMETHYLRICININE.

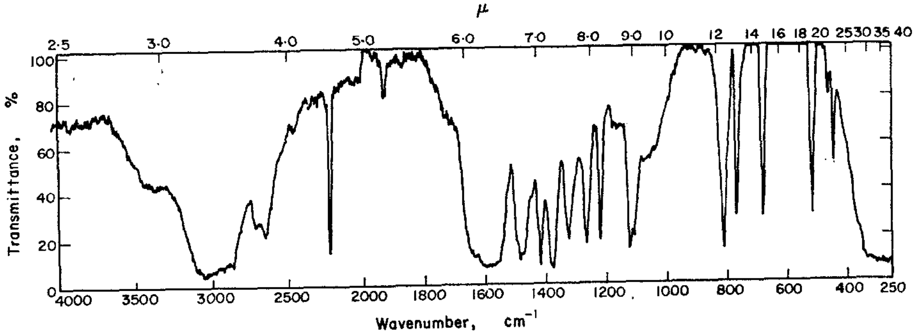

FIG. 4. IR SPECTRA OF *O*-DEMETHYLRICININE.

of two green leaves (10 g) and ricinine and its metabolites were isolated as described. The green leaves were chosen for this experiment since it was known that they did not have demethylating activity[4] even though they contained a significant amount of endogenous ricinine (1 % dry wt.). The recovery of ricinine was 94 % and practically no metabolite of ricinine was detected. In the second, a short term experiment under semisterile conditions as described by Skursky *et al.*[4] was carried out to eliminate the possible demethylation by microorganisms. Ricinine (10 mg) in a sterile solution was injected into two yellow leaves (15 g) which had been previously washed with 1 % aq. sodium hypochlorite solution and rinsed with sterile water. The recovery and conversion data after 3 hr of injection is shown in Table 1. These two short term experiments provided convincing evidence that *O*-demethylricinine in the yellow leaves of the castor bean plant was a normal metabolite of ricinine.

Interconversion of Ricinine and o-Demethylricinine in Excised Senescent and Green Leaves

The formation of *N*-demethylricinine, *O*-demethylricinine and carbon dioxide from different amount of administered ricinine in excised senescent castor bean plant leaves is summarized in Fig. 5. A major drawback to studying the metabolism of ricinine has been the difficulty in synthesizing high specific activity labelled ricinine. Large amounts of ricinine were administered in previous work,[4] but the metabolism of ricinine using different dosage levels had not been done. In this study, three levels of ricinine, 34·5, 84 and 143 μg/g fresh wt., were administered to senescent leaves. These results (Fig. 5) suggest that ricinine inhibited the *O*- and *N*-demethylating reactions. They also indicated that senescent leaves limit the rate of demethylation activity. The low extent of *N*-demethylating activity obtained after the administration of the high level of ricinine agreed with previous work.[4] The results showed that majority of the administered ricinine could be converted to its demethylated forms at low dosage level which is comparable to the normal physiological levels. The approproximate disappearance rate of ricinine obtained by extrapolating the ricinine curve in Fig. 5 is 3·8 μg/g fresh wt./day. The low yield of CO_2, 0·2–0·4 %, suggested that intensive degradation of the α-pyridone ring does not occur in the yellow leaves, therefore, these results confirmed earlier findings.[3]

When *O*-demethylricinine-3,5-^{14}C was administered to excised green leaves, 38·7 % was methylated in 2 days. This conversion percentage is higher than the reported value of 12·3 %

TABLE 1. FORMATION OF *N*-DEMETHYLRICININE AND *O*-DEMETHYLRICININE FROM RICININE BY SEMI-STERILIZED YELLOW LEAVES

Compound	Isolated (mg)
Ricinine*	7·6
N-Demethylricinine	1·35
O-Demethylricinine	0·2
Total	9·15

* Duration of experiment was 3 hr: 10 mg of ricinine were fed.

obtained with use of the whole plant.[6] Skursky *et al.* obtained about 48·7% incorporation into ricinine after administration of *N*-demethylricinine-G-^{14}C. These two experimental results show that the interconversion of ricinine and *O*-demethylricinine is not as active as the interconversion of ricinine and *N*- methylricinine in the castor bean plant. Nowacki recently showed an interesting result that *O*-demethylricinine inhibited the biosynthesis of ricinine from nicotinic acid-7-^{14}C.[7] A schematic diagram summarizing the extent of the demethylation of ricinine and the methylation of the demethylated forms of ricinine is given in Fig. 6. Even though the demethylation processes of some alkaloids have been demonstrated and some of the methylated products have been identified, the physiological or metabolic significance of this process is totally unknown, much like the nature of the alkaloids themselves.

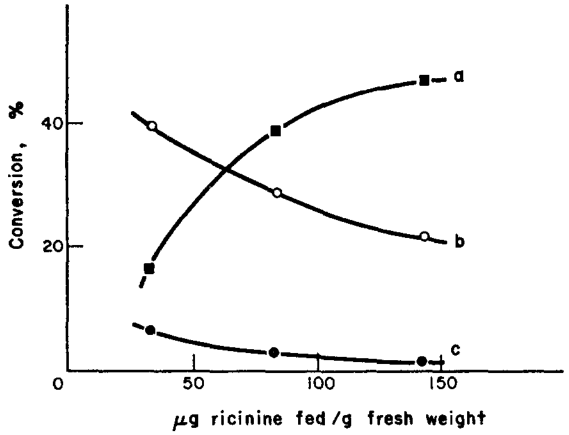

FIG. 5. FORMATION OF *N*-DEMETHYLRICININE AND *O*-DEMETHYLRICININE FROM RICININE-3,5-^{14}C ADMINISTERED IN THE YELLOW LEAVES AFTER 48 hr.
a—Ricinine; b—*N*-Demethylricinine; c—*O*-Demethylricinine. About 0·2% of the radioactivity fed was recovered as CO_2.

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[6] K. S. YANG, *Biosynthesis of Ricinine by* Ricinus communis *L.*, M. S. Thesis, Oklahoma State University, Stillwater, Oklahoma (1963).

[7] E. NOWACKI and G. R. WALLER, *Abh. Deutch. Acad. Wiss. Berlin.* In press (1972).

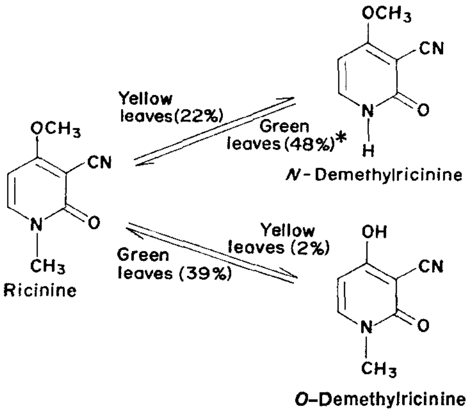

FIG. 6. METHYLATION AND DEMETHYLATION REACTIONS INVOLVING THE ALKALOID RICININE IN THE CASTOR BEAN PLANT LEAVES.

* Value of Skursky at a similar precursor level. Conversion percentages are based on the results obtained when 1 mg of precursor was administered to one leaf of about 5 g.

It is of interest that the demethylated form of ricinine is practically absent in the green leaves and that very small amounts of ricinine and *N*-demethylricinine are present in the yellow leaves.[4] The very small amounts of ricinine and *N*-demethylricinine found in the yellow leaves might be due to the incopmplete process of senescence since naturally detached leaves were found to be void of both compounds. The fact that: (a) absence of both ricinine and its metabolites in the yellow leaves, (b) the methylation and demethylation reactions occur in the green leaves and yellow leaves respectively, and (c) the α-pyridone ring of ricinine is not intensively degraded in the yellow leaves, supported the conclusion that ricinine and/or *N*-demethylricinine or *O*-demethylricinine in the yellow leaves are translocated from the senescent tissue to other parts of the plant.

Demethylation and Translocation of Ricinine

Table 2 shows the demethylation and translocation of ricinine-3 5-^{14}C administered to a yellow leaf attached to the lower part of the stem of a castor bean plant. The castor bean plant used in this experiment was 4-months-old, 60 cm high and grown in a pot in the greenhouse. A plant possessing a similar appearance and similar conditions was selected for a duplicate experiment. The radioactivity in ricinine and in its demethylated forms were found to be highest in the yellow leaf where ricinine was injected. *N*-Demethylricinine-3,5-^{14}C was found in all parts of the plant except in the root. The second highest total recovery of radioactive ricinine and *N*-demethylricinine was in the stems; however, the recovery of radioactive ricinine per gram of fresh plant weight and that of *O*-demethylricinine were high in the growing apex and stems respectively.

To gain a better understanding of the process of demethylation and translocation of ricinine-3,5-^{14}C administered to a yellow leaf, an experiment was conducted using castor bean plant cuttings with one yellow and one green leaf (Fig. 7). Castor bean plants 4-months-old grown in pots in the green house were selected; the distance between the yellow and green leaves on the cuttings varied from 15 to 20 cm but the plants were otherwise similar in appearance. The degree of senescence also varied; i.e. the extent of yellowing of the leaves was 80–90%. A time course study on the accumulation of ricinine and its demethylated

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN THE CASTOR BEAN PLANTS FED WITH RICININE-3,5-^{14}C TO THE YELLOW LEAVES

Plant part	Isolated compound Ricinine	 *N*-Demethylricinine dis/min × 10^{-2} (%)	 *O*-Demethylricinine	Total
One yellow leaf (4 g)	64·42 (14·1)	31·60 (9·6)	6·26 (1·9)	84·28 (25·6)
Three green leaves (13 g)	8·88 (2·7)	1·65 (0·5)	—	10·53 (3·2)
Stems (18 g)	38·85 (11·8)	21·40 (6·5)	3·95 (1·2)	64·19 (19·5)
Growing apex (5 g)	17·45 (5·3)	3·62 (1·1)	—	21·07 (6·4)
Roots (15 g)	3·62 (1·1)	—	—	3·62 (1·1)
Total	115·22 (35·0)	58·27 (17·7)	10·21 (3·1)	183·69 (55·8)

The radioactivity was the average of two experiments. Duration of experiment was 48 hr. The plants used were grown in the green-house. Ricinine-3,5-^{14}C (2 mg) with a total radioactivity of 3·292 × 10^4 dis/min was administered. Percentage of the incorporation was determined by dividing the total radioactivity administered by the total amounts recovered.

forms in the stems, and the yellow and green leaves following the administration of ricinine-3, 5-^{14}C to a yellow leaf of these castor bean plant cuttings is shown in Table 3.

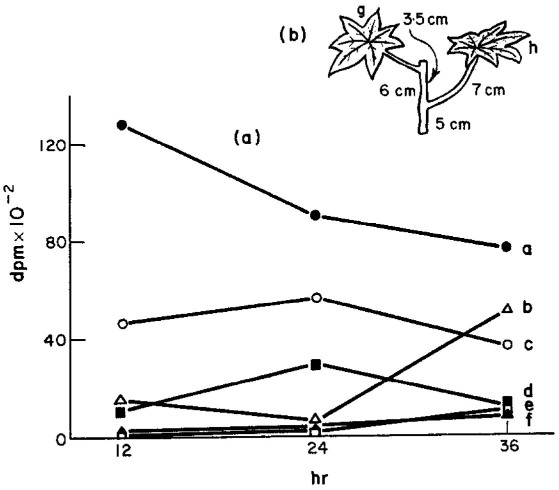

FIG. 7. DEMETHYLATION AND TRANSLOCATION OF RICININE-3,5-^{14}C FROM THE YELLOW LEAF TO THE ADJACENT GREEN LEAF OF THE CASTOR BEAN PLANT CUTTINGS (A), AND SCHEMATIC REPRESENTATION OF THE CASTOR BEAN PLANT CUTTINGS WITH ONE GREEN AND ONE YELLOW LEAF (B). a—Ricinine in the yellow leaves; b—Demethylated form of ricinine in the yellow leaves; c—Ricinine in the stems; d—Demethylated form of ricinine in the stems; e—Demethylated form of ricinine in the green leaves; f—Ricinine in the green leaves; g—Green leaf; h—Yellow leaf.

TABLE 3. DEMTHYLATION AND TRANSLOCATION OF RICININE-3,5-^{14}C FROM YELLOW LEAF TO GREEN LEAF OF THE CASTOR BEAN PLANT CUTTINGS

Exp. No.	Isolated compound	Plant part			Total	%
		Green	Yellow	Stem		
		dis/min × 10^{-2}				
	Ricinine	2·6	129·3	47·3	179·2	54·4
I	*N*-Demethylricinine	1·3	14·8	12·3	28·6	8·7
(12 hr)	*O*-Demethylricinine	—	0·1	0·8	0·9	0·3
	Total	3·9	174·2	60·4	208·7	63·4
	Ricinine	4·5	90·8	56·9	152·2	46·2
II	*N*-Demethylricinine	1·5	2·8	28·5	62·8	19·1
(24 hr)	*O*-Demethylricinine	0·4	2·0	1·1	3·5	1·1
	Total	6·4	95·6	86·5	218·5	66·4
	Ricinine	8·6	78·4	36·8	123·8	37·6
III	*N*-Demethylricinine	7·3	52·1	8·5	67·9	20·6
(36 hr)	*O*-Demethylricinine	1·5	2·0	0·3	3·8	1·2
	Total	17·4	132·5	45·6	195·5	59·4

The plants used were grown in the green-house. Ricinine-3,5-^{14}C (2 mg) with total radioactivity 3·292 × 10^4 dis/min was administered. Percentage of incorporation was determined by dividing total radioactivity administered by the total amounts recovered.

The recovery of radioactive ricininine and its demethylated forms in the green leaves were always very low compared to that found in the stems of the yellow leaves where ricinine was administered. This result suggested that the translocation of ricinine and/or its demethylated forms are reduced when the preferred translocation site organ is removed. The highest concentration of radioactive ricinine per gram of fresh plant weight was found in the growing apex except the yellow leaf where ricinine-3,5-^{14}C was administered. In the yellow leaves, radioactive ricinine was decreased and that of its demethylated forms were increased as the time elapsed (Fig. 7).

Interpretations of the above two results were complicated by the fact that the radioactivity ratio between ricinine and its demethylated forms did not necessarily indicate the ratio of translocated forms since the demethylated form of ricinine upon arrival to the green leaf from the yellow leaf may undergo methylation. The results showed that ricinine administered in the yellow leaves was translocated to healthy parts of the plant, especially the growing apex, where ricinine is actively synthesized. The result supported the idea that the ricinine translocation process might be a salvage operation performed by the plant in order to reutilize ricinine from the leaves which are being prepared for abscission. This is a broad concept of the traditional source-sink relationship;[8] the translocation of ricinine is a reutilization or saving-type process. Another possible speculation for the phenomenon may be that the demethylation reaction, which is generally believed to make a compound more metabolically active, in the yellow leaves of the castor bean plants may represent a process whereby the vital precursors for the compounds in the pyridine nucleotide cycle are maintained within the yellowing leaves. This process might inhibit the progression of senescence.

The finding that both ricinine and its demethylated forms are present and the ratios between them in stems and yellow leaves are not strikingly different, indicates that there is no preferred form of translocation between them.

[8] A. S. CRAFTS, *Translocation in Plants*, p. 147, Holt, Rinehart & Winston, New York (1961).

EXPERIMENTAL

Plants. The castor bean plants used were of the Cimarron variety grown on port clay loam to the Agronomy farm of the Oklahoma State University in Stillwater, Oklahoma. Plants 4–6 months-old which were planted on 30 May 1970 were used for these experiments. The other groups of the plants planted in 30 June 1970 were grown in pots with a mixture of clay loam soil and vermiculite at the green house of the Horticulture Department, Oklahoma State University, Stillwater, Oklahoma. The yellow leaves used were the senescent ones (from 80 to 90% of the leaf was yellow) which were on the lower part of the stalk.

Labelled compounds used. Ricinine-3,5-^{14}C was synthesized on a micro scale from Na cyanoacetate-2-^{14}C, according to the procedure described by Yang and Waller.[9] The synthesized ricinine was purified by preparative TLC using $CHCl_3$–MeOH (5:1, v/v). The constant specific activity of the ricinine used was $1{\cdot}78 \times 10^4$ dis/min/mg.

O-Demethylricinine-3,5-^{14}C was isolated from the yellow castor bean plant leaves fed with ricinine correspondingly labelled by the procedure described in the following section. The constant specific radioactivity of this compound was $1{\cdot}12 \times 10^4$ dis/min/mg.

Chemical reagents. Solvents and chemical reagents were of analytical reagent grade and solvents were redistilled using glassware. *O*-Demethylricinine (ricininic acid) was purchased from General Biochemicals, Chagrin Falls, Ohio and recrystallized from MeOH.

Administration of labelled compounds. The administration technique used for plants[10] was made with a micro syringe in the petioles near the yellow leaves and the desired amount of labelled compound in 5% MeOH was injected slowly.

Isolation and purification. Fresh plant material was weighed, frozen with liquid N_2, homogenized with Virtis '23' omnimixer in 80% MeOH. This extraction procedure with methanol was repeated until the remaining material was free of soluble pigments. The organic solvent was removed from the pooled extracts by evaporation at room temp. and reduced pressure. The remaining aqueous solution was freed of lipid and pigments by extraction with light petroleum. The aqueous phase, which contained ricinine and the metabolites of interest, was evaporated to dryness. The light petrol extract and the plant debris were not further examined. The dry residue from the aqueous portion of the extract was extracted with boiling MeOH, the volume was reduced, and this solution was then used for preparative TLC. The plates, 0·75 mm thick, were made from SiO_2 pH 254 + 366, Merck. The solvent systems were: (I) $CHCl_3$–MeOH (5:1, v/v), (II) EtOH–NH_4OH–H_2O (80:4:16, by vol.). Solvent system I and II were used for separation of the relatively nonpolar compound, ricinine and *N*-demethylricinine, and relatively polar unknown compounds respectively. The radioactive and/or UV quenching zones of interest on TLC plates was taken out, put in a 2 × 15 cm column and extracted with sufficient volume of MeOH. The extract was usually rechromatographed for further purification. Isolated compounds were crystallized in MeOH and sublimed. The purity of the isolated compounds was checked with ascending paper chromatography using *iso*PrOH–toluene–HOAc–H_2O (5:10:1:1, by vol.) solvent system.

Measurement of radioactivity. The radioactivity on the TLC chromatograms was located with a Nuclear Chicago 4 π Actigraph-III strip counter. The radioactivity of the purified compounds was measured using toluene–MeOH cocktail with Packard Model 3320 Liquid Scintillation Spectrometer. The respiratory $^{14}CO_2$ from castor bean plants was trapped with the ethanolamine in methylcellosolve as described by Waller and Lee.[3] A portion of CO_2 trapping solution (3 ml) was added to scintillation solution (15 ml) which was composed of methylcellosolve and toluene (1:2, v/v) with 5·5 g of PPO per liter.

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[9] K. S. Yang and G. R. Waller, *Phytochem.* **4,** 881 (1965).

[10] G. R. Waller, D. S. Yang, R. K. Gholson, L. A. Hadwiger and S. Chaykin, *J. Biol. Chem.* **241,** 4411 (1966).

Key Word Index—Ricinus communis; Euphorbiaceae; ricinine; *O*-demethylricinine; alkaloid metabolism.