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STUDIES ON CYANIDE METABOLISM IN *LOTUS ARABICUS* L. AND *LOTUS TENUIS* L.*

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Abstract—Uniformly labelled L-valine-^{14}C and L-isoleucine-^{14}C serve respectively as effective precursors of the aglycone moieties of the two cyanogenic glucosides, linamarin (α-hydroxy-isobutyronitrile-β-D-glucose and lotaustralin (α-hydroxy-α-methylbutyronitrile-β-D-glucose) which occur in *Lotus arabicus* L. and *Lotus tenuis* L. Asparagine-^{14}C isolated from the tops of etiolated seedlings of these species which had been fed H^{14}CN was degraded and the data obtained provide evidence for the amide carbon atom being formed from the nitrile carbon. In the case of plants fed L-valine-U-^{14}C, the occurrence and distribution of radioactivity in asparagine-^{14}C suggests that radioactive linamarin formed from the valine breaks down slowly to yield H^{14}CN which in turn is incorporated into asparagine.

INTRODUCTION

THE WORK of Blumenthal-Goldschmidt *et al*,[1] which showed that young seedlings of a number of plant species extensively incorporate radioactivity from H^{14}CN into asparagine, prompted us to investigate whether cyanogenic glycosides can donate their nitrile group for the biosynthesis of asparagine. These compounds have a wide distribution among higher plants[2] and are normally accompanied by endogenous enzymes which can degrade the cyanogen found in the plant. The experimental results presented in this paper show that the nitrile moiety of linamarin (α-hydroxy-isobutyronitrile-β-D-glucose) provides a 1-carbon unit, undoubtedly HCN, for the biosynthesis of asparagine in *L. arabicus* and *L. tenuis*. A preliminary report of these findings has already appeared.[3] Experimental evidence for linamarin and lotaustralin (α-hydroxy-α-methylbutyronitrile-β-D-glucose) being the only cyanogenic constituents of *L. arabicus* L.[4,5] finds further support in the results obtained by feeding labeled compounds to this species. That these two cyanogenic glucosides occur in *L. tenuis* L. has been previously reported by Butler.[6]

Various biochemical, physiological and taxonomic roles have been assigned to the cyanogenic glycosides of plants. These range from the suggestion that cyanide serves as a precursor of proteins to the suggestion that these compounds are excretory products.[7] The experimental evidence concerning the function of these compounds is so meagre that one cannot attribute any definite physiological significance to these cyanogenic constituents. It is quite clear however that the cyanogenic glycosides are metabolically active rather than being inert end products of metabolism.

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RESULTS

Occurrence and Biosynthesis of Linamarin and Lotaustralin

When dry seeds of *L. arabicus* and *L. tenuis* were ground, suspended in H_2O and aerated overnight, no cyanide could be detected. However 5–7 μmoles and 13–15 μmoles/g (fresh weight) respectively of cyanogenic glycoside in *L. arabicus* and *L. tenuis* were observed in 2–3-day-old etiolated seedlings (Table 1). Thus a rapid synthesis of cyanogenic material analogous to that which occurs in sorghum seedlings[8] takes place in these species on germination.

TABLE 1. THE CYANOGENIC GLYCOSIDE CONTENT OF THE *Lotus* SPECIES

Species	Glycoside		Total
	Linamarin (μmoles/g fresh weight (% in parentheses))	Lotaustralin	
L. arabicus L.	4·3 (73)	1·6 (27)	5·9
L. tenuis L.	8·9 (60)	5·8 (40)	14·7

The tops from etiolated seedlings were extracted and the cyanogenic glucosides isolated and identified as previously described.[1, 13]

The two cyanogenic glucosides, linamarin and lotaustralin had previously been shown to account for the cyanide released from *L. arabicus* on grinding the plant material and aerating overnight.[5] Using the same procedures we confirmed[6] the presence of the same two cyanogenic glucosides in *L. tenuis*. When the tops from 5-day-old etiolated seedlings were examined for linamarin and lotaustralin, the molar ratios were approximately 7:3 and 6:4 in *L. arabicus* and *L. tenuis* respectively (Table 1). These same ratios were observed in the aerial portions of plants grown in light and were maintained over the total period of vegetative growth in both species.

TABLE 2. INCORPORATION OF L-VALINE-U-^{14}C AND L-ISOLEUCINE-U-^{14}C INTO LINAMARIN AND LOTAUSTRALIN BY SEEDLINGS OF *Lotus* SPECIES

Compound administered			HCN released		Per cent incorporated
Compound	(Counts/min)	Percentage uptake	Amount (μmoles)	(Counts/min)	
A. *Lotus arabicus* L.					
L-valine-U-^{14}C	$1·3 \times 10^6$	98	0·7	6870	2·7
L-isoleucine-U-^{14}C	$2·3 \times 10^5$	92	0·8	510	1·0
B. *Lotus tenuis* L.					
L-valine-U-^{14}C	$1·3 \times 10^6$	97	2·5	24,400	9·7
L-isoleucine-U-^{14}C	$2·3 \times 10^5$	90	2·5	5090	8·5

Specific activities of the valine and isoleucine administered in these experiments were 200 and 14·3 μc/μmole respectively; 0·005 μmole of labeled valine and 0·07 μmole of labeled isoleucine were fed to 0·12 g each of *L. arabicus* seedlings and the same amounts were administered to 0·20 g each of *L. tenuis* seedlings.

[8] T. AKAZAWA, P. MILJANICH and E. E. CONN, *Plant Physiol.* **35**, 535 (1960).

Studies on the biosynthesis of the cyanogens in these species readily revealed that uniformly labeled L-valine-^{14}C and L-isoleucine-^{14}C are extensively incorporated into the aglycone moieties of linamarin and lotaustralin respectively. In a typical experiment (Table 2) the administration of L-valine-U-^{14}C in tracer concentration (0·005 μmole) to twenty etiolated seedling tops (0·12 g fresh weight) of *L. arabicus* for 20 hr showed an incorporation of 2·7 per cent into linamarin. Under similar conditions, the incorporation of valine into linamarin in *L. tenuis* was 9·7 per cent.

Similar results were obtained from the incorporation of L-isoleucine-U-^{14}C into lotaustralin. For example, when 0·07 μmole of L-isoleucine-U-^{14}C was fed to twenty seedling tops of *L. tenuis* weighing 0·2 g for 20 hr, the percentage conversion of carbon-14 was found to be 8·5 (Table 2).

H^{14}CN *Feeding*

When etiolated seedling tops of these two species were exposed to H^{14}CN (5 or 10 μc) for 24 hr, radioactivity was incorporated into asparagine (Table 3). These members of the

TABLE 3. THE INCORPORATION OF H^{14}CN AND L-VALINE-U-^{14}C INTO ASPARAGINE IN *Lotus* SPECIES

Compound administered		Asparagine-^{14}C isolated		
Compound	Counts/min	Amount (μmoles)	Counts/min	Per cent incorporation
A. *Lotus arabicus* L.				
H^{14}CN	$1·2 \times 10^7$	33·2	$1·4 \times 10^5$	1·2
L-valine-U-^{14}C	$7·3 \times 10^6$	43·7	$1·1 \times 10^5$	1·3
B. *Lotus tenuis* L.				
H^{14}CN	$5·9 \times 10^6$	46·7	$1·5 \times 10^5$	2·6
L-valine-U-^{14}C	$7·5 \times 10^6$	45·3	$2·8 \times 10^4$	0·4

The specific activities of H^{14}CN and L-valine-U-^{14}C were 7·94 μc/μmole and 200 μc/μmole respectively; 1·26 μmoles of H^{14}CN and 0·025 μmole of L-valine-U-^{14}C were administered to 1·21 g of *L. arabicus* seedling tops. 0·63 μmole of H^{14}CN and 0·025 μmole of L-valine-U-^{14}C were administered to 1·25 g of *L. tenuis* seedling tops.

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN ASPARAGINE-^{14}C

Compound	Asparagine-^{14}C isolated		
	Asparagine (counts/min)	Amide carbon atom (counts/min)	Carboxyl carbon atom (counts/min)
A. *Lotus arabicus* L.			
H^{14}CN	13,080	10,440	1,660
L-valine-U-^{14}C	7,850	5,990	740
B. *Lotus tenuis* L.			
H^{14}CN	14,350	11,760	1,630
L-valine-U-^{14}C	11,250	7,870	2,180

The radioactivity in the asparagine carboxyl and amide carbon atoms was determined by decarboxylation with *N*-bromosuccinimide.[23] The samples were counted in Bray's solution[24] in the Packard Liquid Scintillation Counter at an efficiency of 45–55 per cent.

Lotus family therefore metabolize $H^{14}CN$ in the same manner as has been observed for numerous other plants.[1] When the asparagine-^{14}C formed from $H^{14}CN$ was degraded to determine the distribution of radioactivity in the molecule, the amide carbon atom contained 80 per cent or more of the radioactivity in the molecule. The carboxyl-C accounted for only 11–13 per cent of the activity (Table 4).

Uniformly Labeled L-valine-^{14}C Feeding

During our study of the incorporation of L-valine-U-^{14}C into linamarin by etiolated seedling tops of the *Lotus* species we observed that asparagine also became labeled. As shown in Table 3 when seedling tops of *L. arabicus* were fed 5 μc of uniformly labeled L-valine-^{14}C, as much radioactivity was incorporated into asparagine as when $H^{14}CN$ was administered. The incorporation by *L. tenuis* tops was not as great. When the distribution of radioactivity in the purified asparagine-^{14}C formed from the amino acid was determined (Table 4), the distribution of labeled carbon was similar to that obtained in plants fed $H^{14}CN$ in that the amide carbon atom contained the major amount (70 or 76 per cent) of the radioactivity in the asparagine.

DISCUSSION

The ability of valine and isoleucine to serve as precursors of linamarin and lotaustralin respectively in both *L. arabicus* and *L. tenuis* is in agreement with earlier observations on the cyanohydrin group of cyanogenic glycosides being the result of amino acid metabolism.[9, 10, 11] In addition, the data suggests the operation of a biosynthetic pathway for the formation of linamarin and lotaustralin in *Lotus* species which is identical with that in *Trifolium repens* L.,[12] *Osteospermum jucundum* Norlindh,[6] and *Linum usitatissimum*.[13] In this pathway the α-carbon and nitrogen atoms of the amino acid become the nitrile group of the glycoside while the remainder of the aglycone derives from the remainder of the amino acid.

The ability of *Lotus* species to incorporate $H^{14}CN$ into the amide carbon atom of asparagine extends the list of higher plants known[1, 14, 15] to accomplish this process. A more significant aspect of this observation pertains to a possible metabolic role for the cyanogenic glycosides. A similarity in the labeling pattern of radioactive asparagine isolated from plants fed uniformly labeled L-valine-^{14}C to that isolated from plants exposed to $H^{14}CN$, suggests that the nitrile moiety of linamarin can provide the $H^{14}CN$ for the biosynthesis of asparagine. Presumably this route for the biosynthesis of asparagine is limited to those plants containing cyanogenic compounds.

The distribution of radioactivity in asparagine-^{14}C produced in plants fed $H^{14}CN$ and L-valine-^{14}C deserves further comment. Blumenthal-Goldschmidt *et al.*[1] observed that 98 per cent of the activity in asparagine produced from $H^{14}CN$ in *Sorghum vulgare* is present in the amide carbon atom and suggested that the other three carbon atoms are derived from serine. Recently Nigam and Ressler[16] have provided experimental evidence in support of this suggestion. It would thus appear that any radioactivity found in the carboxyl carbon

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atom of asparagine is incorporated by an indirect route. One possibility which readily suggests itself is the deamination of asparagine to aspartic acid which, by subsequent conversion to fumaric acid, would randomize the label in the two carboxyl groups. Such a sequence would have to be reversible and need to occur during the course of feeding experiments lasting 24–40 hr. No experiments were performed to validate this assumption but radioactivity in aspartic acid was observed when some plants were given large amounts of $H^{14}CN$.

Finally, our studies support the recent observations of Butler[6] that the distribution of lotaustralin is closely similar to linamarin, and that the latter is preferentially synthesized in species of several genera. These appear however to be marked varietal differences in the relative amounts of the two cyanogenic glucosides. For example, in *L. tenuis* Waldst. et Kit. ex Willd. from New Zealand the linamarin and lotaustralin occur in the ratio of 6 to 94, while in *L. tenuis* L. (creeping bird's foot trefoil) var. narrow leaf the ratio is 60 to 40.

EXPERIMENTAL

Seeds of *Lotus arabicus* L. were supplied by M. Villax, Rabat, Morocco. Seeds of *Lotus tenuis* L. (creeping bird's foot trefoil) var. narrow leaf were purchased from F. F. Smith and Co. Sacramento, California. Etiolated seedlings which were utilized for administration of labeled compounds were grown as follows: the seeds were soaked overnight in tap water and spread on moist cheese-cloth mounted on inverted, perforated, plastic baskets. The baskets were placed in a beaker containing dilute mineral solution[17] and the beaker was then placed in a light-proof box at 28°. Under these conditions uniform germination and growth occurred.

The procedures for feeding uniformly labeled L-valine-^{14}C and L-isoleucine-^{14}C (New England Nuclear Corporation), as well as the extraction, chromatographic isolation and identification of linamarin and lotaustralin have been previously outlined.[13] The HCN released, following the hydrolysis of cyanogenic glucosides with linamarase, was estimated by the method of Aldridge.[18] Linamarase was purified approximately twenty-fold from linseed meal by the method of Coop.[19] Quantitative determination of glucose released on paper chromatograms was made by Wilson's method.[20]

The $H^{14}CN$ was administered as follows: 2–3-day-old etiolated seedling tops weighing 0·5–0·75 g were placed in a 5 ml beaker containing 2 ml distilled water. This beaker and another one containing $Na^{14}CN$ (5 or 10 μc) in 0·1 N NaOH were placed on the porcelain plate in a 2·5 l. desiccator. After addition of a slight excess of mineral acid to release the $H^{14}CN$, the desiccator was immediately sealed and left for 24 hr.

The asparagine-^{14}C was isolated from plants fed $H^{14}CN$ and L-valine-^{14}C by passing the extraction solutions (80% ethanol) through an ion exchange column (Dowex AG 50 Wx–8, 20–50 mesh, H^+ form). The amino acids were then eluted with 0·4 M NH_4OH and purified chromatographically on paper with three different solvents: (*a*) butan-1-ol: acetic acid: water (12:3:5, by vol.), (*b*) phenol: water (80:20, by vol.), (*c*) methanol: pyridine: water (160:40;8, by vol.) as solvents. A final purification was carried out by paper electrophoresis using pyridine: acetic acid: water (25:1:225 by vol., pH 6·4).[21] Purified asparagine-^{14}C was

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enzymatically hydrolyzed to aspartic acid-^{14}C with a *Pseudomonas* enzyme which exhibits asparaginase activity.[1,22]

The distribution of carbon-14 in the asparagine-^{14}C and aspartic acid-^{14}C was determined by decarboxylation of the carboxyl groups of the two compounds with *N*-bromosuccinimide.[23] The radioactivity in the amide-carbon atom was calculated by subtracting the activity in the carboxyl group of asparagine from the radioactivity in both carboxyl groups of the aspartic acid derived from the asparagine by the *Pseudomonas* enzyme. The $^{14}CO_2$ liberated was collected in 0·5 ml 1 M potassium hydroxide in the center well of Warburg vessels and determined by the method of Bray[24] in a Packard Liquid Scintillation Counter, Model 314EX with a counting efficiency of 45–55 per cent. Asparagine was estimated spectrophotometrically by ninhydrin[25] and manometrically by *N*-bromosuccinimide.[23]

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