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L-Canavanine Metabolism in Jack Bean, *Canavalia ensiformis* (L.) DC. (Leguminosae)[1]

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ABSTRACT

L-Canavanine, a highly toxic arginine antimetabolite, is the principal nonprotein amino acid of many leguminous plants. Labeled-precursor feeding studies, conducted primarily with [^{14}C]carbamoyl phosphate, and utilization of the seedlings of jack bean, *Canavalia ensiformis* (L.) DC. (Leguminosae), have provided evidence for L-canavanine biosynthesis from L-canaline via *O*-ureido-L-homoserine. This reaction pathway appears to constitute an important *in vivo* route of canavanine production. Canavanine cleavage to canaline may represent a degradative phase of canavanine metabolism distinct from the anabolic reactions described above. Thus, while these reactions of canavanine metabolism bear analogy to the mammalian Krebs-Henseleit ornithine-urea cycle, no evidence has been obtained at present for the reutilization of canaline in ureidohomoserine formation.

L-Canavanine, a nonprotein amino acid found in at least 500 species of leguminous plants (2), is a potent antimetabolite, due in large measure to its structural analogy to L-arginine. Canavanine's marked insecticidal action, its ability to disrupt many essential biochemical reactions in a wide range of prokaryotic and eukaryotic organisms, and its appreciable storage in such important tissues as the seed are consistent with its allelochemical role in plant defense against phytophagous insects and other herbivores (14).

Canavanine is a major nitrogen storage metabolite of the seed, where it can account for 95% or more of the nitrogen found in the free amino acids (15). Its mobilization and utilization in jack bean, *Canavalia ensiformis* (L.) DC. (Leguminosae), occurs via arginase (EC 3.5.3.1), which cleaves L-canavanine to L-canaline and urea (3). Urea is hydrolyzed by urease (EC 3.5.1.5) to CO_2 and NH_3; the latter, representing an important source of reduced nitrogen, is assimilated and incorporated into the amide nitrogen of asparagine (11).

Experimental evidence has been obtained in jack bean for carbamylation of canaline with carbamoyl phosphate, in a reaction catalyzed by ornithine carbamoyltransferase (EC 2.1.3.3) to yield *O*-ureido-L-homoserine, a citrulline analog (12). Argininosuccinic acid lyase (EC 4.3.2.1), the enzyme-mediating conversion of L-canavaninosuccinic acid to L-canavanine and fumaric acid, has been isolated and purified extensively from jack bean seed (16). Of the reactions depicted in Figure 1, it is the reaction fostered by argininosuccinic acid synthetase (EC 6.3.4.5), involving L-canavaninosuccinic acid formation from *O*-ureido-L-homoserine, that has received the least experimental attention.

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The enzymic reactions of the nonprotein amino acids depicted in Figure 1 result in a reaction pathway that bears similarity to mammalian urea production via the Krebs-Henseleit ornithine-urea cycle. All of the ornithine-urea cycle enzymes have been isolated from several higher plants, including jack bean (18). Attempts have been made to isolate an enzyme responsible for the formation of one of the intermediates of canavanine metabolism that is distinct from its ornithine-urea cycle counterpart. These efforts have failed (3, 9).

One can readily demonstrate all of the ornithine-urea cycle enzymes in jack bean, except for argininosuccinic acid synthetase, typically, the rate-limiting enzyme of the ornithine-urea cycle (18). The lack of appreciable argininosuccinic acid synthetase activity has prevented unequivocal *in vitro* demonstration of the role of the ornithine-urea cycle enzymes in canavanine biosynthesis. As a result, evidence was sought in jack bean for L-canavanine biosynthesis from L-canaline via *O*-ureido-L-homoserine from *in vivo* labeled-precursor feeding studies.

MATERIALS AND METHODS

L-Canavanine, L-canaline, *O*-ureido-L-homoserine, and L-canavaninosuccinic acid were prepared by the author (12). The remaining biochemicals were purchased from Sigma Chemical Co. Fisher Scientific Co. provided the chemicals, except for the scintillation reagents, which were secured from Packard Instrument Co. Potassium [^{14}C]cyanate was obtained from ICN; New England Nuclear was the source for the remaining radiochemicals.

O-Ureido-L-homoserine, uniquely labeled in the terminal carbon atom constituting the ureidooxy group, was prepared by reacting 50 μCi of potassium [^{14}C]cyanate (12 μCi/μM) with a 10-fold M excess of L-canaline in 1 ml of 50 mM sodium acetate buffer (pH 5.2). Components for the synthesis of *O*-ureido-L-homoserine were reacted for 22 h at 30°C; during this time, the labeled cyanate carbamylated only the ω-NH_2 group of L-canaline. The resulting formation of *O*-ureido-L-[*ureidooxy*-^{14}C]homoserine was terminated by placing the reaction mixture on a 20- × 210-mm column of Dowex-50 (H^+) at 22°C. After washing the column with deionized H_2O until the effluent was free of radioactivity, labeled ureidohomoserine was obtained by gradient elution utilizing 1 L of N HCl and 1 L of deionized H_2O. Fractions (18 ml) were collected, and fractions 50 through 70 were pooled; HCl was removed by evaporation *in vacuo*. The final yield was 37 μCi. The elution position of the labeled ureidohomoserine coincided with that obtained in prior runs with cold, unlabeled ureidohomoserine. The purity of the radiochemical preparation was evaluated by automated amino acid analysis and collection of the spent ninhydrin reaction mixture. All of the detected radioactivity cochromatographed with the 100 nmol of carrier ureidohomoserine applied simultaneously to the column.

Growth of the Plant and Labeled Precursor Administration. The jack bean (*Canavalia ensiformis* [L.] DC. [Leguminosae]) seeds, hydrated overnight in gently flowing and aerated distilled

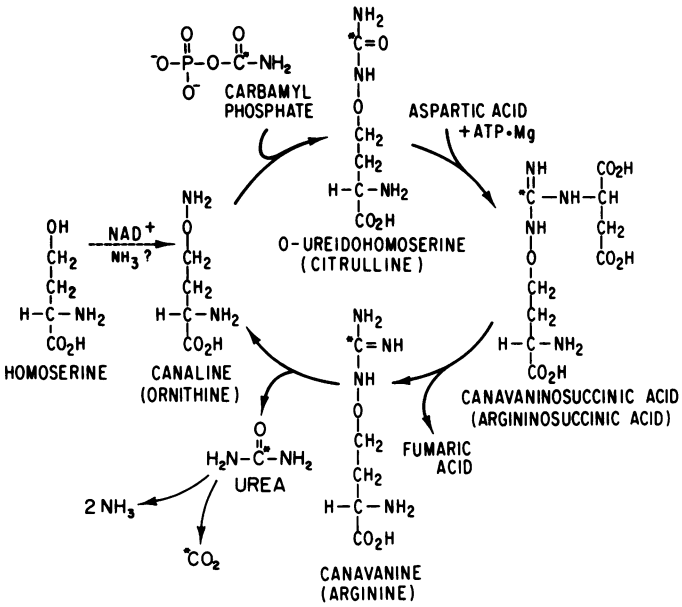

FIG. 1. Reactions of canavanine metabolism in jack bean. This reaction sequence involves a group of nonprotein amino acids bearing structural analogy to the constituents of the Krebs-Henseleit ornithine-urea cycle (the latter components are shown in parentheses). The synthetic phase of this reaction sequence from canaline to canavanine may be distinctive from the degradative phase, in which canavanine is converted to canaline and urea. The arrows indicate the predominant reaction flow; only the conversion of canavanine to canaline and urea is irreversible. The role of homoserine or one of its derivatives in providing carbon skeleton to this reaction sequence has not been firmly established. The asterisk next to various carbon atoms highlights the flow of radioactive carbon of carbamoyl phosphate through its eventual release as $^{14}CO_2$.

H_2O, were grown under greenhouse conditions in flats containing a 2:1 mixture of loamy soil and perlite (pelleted styrofoam). On day 7 from the beginning of germination, while the cotyledons were still thick, fleshly, and deep green, seedlings were transplanted to individual clay pots and fertilized. On day 8, 50-μl samples of the putative precursors of canavanine were injected into the fleshy cotyledons (the storage tissues of the seed); each of two segments constituting the cotyledons received 2.5 μCi (25 μl) of ^{14}C-labeled intermediate. All treatments were replicated four times.

This injection procedure served as an excellent means for distributing labeled intermediates through the plant. The cotyledons, at this stage of seedling development, are the chief source of carbon skeleton for the metabolic reactions of the actively growing root and shoot meristems. Intact seedlings were harvested at the times indicated in the tables, washed thoroughly to remove surface radioactivity and adhering soil particles, and stored at −60°C until assayed.

Preparation of the Plant Extract. Four frozen seedlings, representing a single treatment, were cut into small pieces and ground with 150 ml of deionized H_2O on ice for 3 min at full power with a Sorvall Omni-Mixer. The plant homogenate was decanted immediately into a centrifuge bottle containing 25 ml of 50% (w/v) TCA, mixed, and allowed to precipitate for at least 15 min at 3°C. The extract was clarified by centrifugation at 18,000g for 20 min; the supernatant solution was freed of floating debris by filtration through Whatman 541 paper. TCA was removed by successive extractions with an equal volume of anhydrous ether. Finally, the plant extract was concentrated by evaporation *in vacuo* at a temperature not in excess of 30°C (this precaution minimizes cyclization of canavanine to deaminocanavanine [13]).

Isolation of the Labeled Canavanine and Arginine. The deproteinized plant extract was taken to pH 6.3 with dilute NaOH and placed on a 20- × 210-mm column of Dowex-50 (NH_4^+). After washing the resin exhaustively with deionized H_2O, canavanine was eluted with 1 L of 0.05 N NH_4OH; this resulted in the elution of more than 98% of the canavanine, while no more than 1% of the arginine emerged from the column. Arginine was eluted subsequently with 1 L of 1.5 N NH_4OH. Both samples were freed of ammonia by evaporation *in vacuo* at 30°C. The ammonia-laden, canavanine-containing effluent was collected in a salt-ice bath and evaporated *in vacuo* each time that 50 ml of effluent had accumulated. This repetitive evaporation of the chilled effluent accelerated ammonia removal and reduced cyclization of canavanine in the effluent (13). Residues of the column were dissolved in 100 mM sodium glycylglycine (containing 2 mM freshly added $MnCl_2$) at pH 7.6 for canavanine determinations and at pH 9.3 for arginine assays.

In the initial experiments, the values obtained for the canavanine and arginine content of the plant extract by enzymic hydrolyses were confirmed by collecting the spent ninhydrine after automated amino acid analysis of the plant extract. In the case of these dual determinations, the initial extract was divided into two parts. One part was processed as above to separate canavanine from arginine for enzymic analysis. The remaining material was similarly processed by ion-exchange chromatography, except that a single elution was conducted with 1.5 N NH_4OH. This removed most of the unwanted amino acids and neutral components of the plant extract. This purification procedure proved essential in avoiding overloading of the amino-acid analyzer column, while maintaining appreciable canavanine and arginine in the sample.

Amino Acid Analyses. Amino acid analyses (50-μl samples) were conducted with system designed for physiological sample analysis by Durram Chemical (Dionex Corp., Sunnyvale, CA) Sequential application of five buffers separated canavanine and arginine from the remaining amino acids and also fully resolved these two natural products. The spent ninhydrin was collected at 4-min intervals as 1.2-ml fractions; the radioactivity of each fraction was determined by counting duplicate 0.2-ml samples by liquid scintillation spectroscopy, using Bray's medium. The presence of the ninhydrin-amino acid chromogen did not affect the liquid scintillation values. Calibration with [^{14}C]canavanine or [^{14}C]arginine, labeled only in the terminal carbon atom, disclosed a consistent recovery of about 95% of the applied radioactivity. Because data obtained by automated amino acid analysis were in excellent agreement with the values obtained by enzymic hydrolyses, only the latter are reported here.

Evaluation of the Position of the Transferred Label. The transfer of radioactive carbon from the precursor compounds into the terminal carbon atom of canavanine and arginine was determined by simultaneous treatment with arginase and urease and measurement of the evolved $^{14}CO_2$ (6). The procedure is predicated upon arginase-mediated conversion of L-[*guanidino*-^{14}C]arginine and L-[*guanidinooxy*-^{14}C]canavanine to L-ornithine and L-canaline, respectively, with the concomitant production of [^{14}C]urea. The latter substance is hydrolyzed by urease to ammonia and $^{14}CO_2$; the labeled material can be captured completely in hydroxide of hyamine and quantitated by liquid scintillation spectroscopy. This procedure is noteworthy since a stoichiometric release of $^{14}CO_2$ from a labeled substance, in a reaction that is arginase- and urease-dependent, establishes unequivocally that the radioactivity emanated from arginine and/or canavanine.

RESULTS

Injection of [^{14}C]carbamoyl phosphate into the cotyledons was followed by appreciable labeling of both the arginine and canavanine contained within the TCA-treated seedling extract. Analysis for these amino acids at 6, 12, and 24 h after labeled precursor

Table I. *Formation of L-[Guanidinooxy-^{14}C]Canavanine and L-[Guanidino-^{14}C]Arginine from [^{14}C]Carbamoyl Phosphate by 8-Day-Old, Intact Jack Bean Seedlings*

Each of four plants received 5 μCi of [^{14}C]carbamoyl phosphate. The radioactivity of the plant extract is based upon the water-soluble activity after TCA treatment and extraction. ^{14}C-Incorporation values are based upon data from enzymic hydrolyses of the indicated basic amino acids isolated from the TCA-treated plant extract.

Time	Radioactivity in Extract	^{14}C-Incorporation[a]		Arginine: Canavanine
		Canavanine	Arginine	
h	*dpm* × 10^{-6}	*% total soluble radioactivity*		*ratio*
6	4.37	7.3	19.8	2.71
12	3.56	6.1	15.1	2.48
24	1.98	4.4	9.8	2.23

[a] Values are expressed on a per plant basis.

Table II. *Incorporation of Labeled Precursors into the Terminal Carbon Atom of Canavanine and Arginine*

Each of four plants received 5 μCi of labeled precursor and, when applicable, 25 μmol of L-canaline. The plants were harvested after 12 h.

Treatment	Radioactivity in Extract	^{14}C-Incorporation[a]	
		Canavanine	Arginine
	dpm × 10^{-6}	*% total soluble radioactivity*	
[^{14}C]Carbamoyl phosphate	5.1	6.7	19.2
[^{14}C]Carbamoyl phosphate + canaline	5.6	9.9	18.3
[^{14}C]Ureidohomoserine	7.8	63.7	

[a] Values are expressed on a per plant basis.

administered revealed that 27.1, 21.2, and 14.2%, respectively, of the total water-soluble radioactivity of the plant extract was sequestered in canavanine and arginine (Table I). It is known that carbamoyl phosphate converts to cyanate which can carbamylate canaline chemically (1). To ascertain whether the introduction of label into these basic amino acids occurred nonenzymically, parallel [^{14}C]cyanate-feeding studies were conducted. [^{14}C]Cynanate failed to produce significant arginine or canavanine in treated jack bean seedlings. Demonstration of the role of certain ornithine-urea cycle enzymes in canavanine biosynthesis is rendered much more difficult by the failure of jack bean seedlings to accumulate detectable pools of canaline, ureidohomoserine, or canavaninosuccinic acid; presumably, these secondary metabolites turn over rapidly in the developing plant. This factor has precluded both the isolation, after labeled precursor administration, of these nonprotein amino acids and the determination of the sequential transfer of a given radioactive carbon atom through these metabolic reaction intermediates. Evidence for diminished canavanine formation from ureidohomoserine, due to canavaninosuccinic acid loss via anhydride or other derivative formation, was not obtained. This loss is known to occur with argininosuccinic acid (10).

The appreciable time-dependent diminution in the label contained within canavanine and arginine is consistent with their role in providing nitrogen for the developing plant. As indicated by the data of Table I, the ratio of arginine to canavanine fell over the course of the experimental period. This may result from a lack of precursors for canaline synthesis relative to that for ornithine or from a relatively greater demand by the growing plant for arginine relative to that for canavanine. Enzymic hydrolyses of the radioactive canavanine and arginine, generated *in vivo* from [^{14}C]carbamoyl phosphate, with arginase and urease revealed that virtually all of the radioactivity was contained in the terminal guanidinooxy and guanidino groups, respectively. This is exactly the isotope pattern expected from the postulated pathway for canavanine formation involving carbamoyl phosphate (Fig. 1).

Addition of 25 μmol of L-canaline to the labeled carbamoyl phosphate prior to injection increased by 48% the radioactivity of the *in vivo*-formed canavanine after 12 h, as compared to [^{14}C] carbamoyl phosphate alone (Table II). This finding suggests that canaline may be deficient in the intact plant. (Canaline is in direct competition for carbamoyl phosphate with ornithine in arginine production and with aspartic acid in pyridmidine synthesis.) Once again, enzymic analyses of the Table II samples showed that virtually all of the radioactive carbon of canavanine transferred from carbamoyl phosphate occurred exclusively to the guanidinooxy moiety of canavanine. These findings are in full accord with the contention that canaline, in conjunction with carbamoyl phosphate, supports the metabolic reactions of Figure 1, which are responsible for canavanine biosynthesis.

Greater canavanine production would be expected from a labeled presursor that is not subject to the reactions competing directly for carbamoyl phosphate and that resides closer to the actual formation of canavanine. In order to supply such a precursor, a method was developed for the synthesis of *O*-ureido-L-[*ureidooxy*-^{14}C]homoserine. This precursor, uniquely labeled in the terminal carbon atom (see Fig. 1, asterisk), must transfer its label solely to the guanidinooxy moiety of canavanine, if the proposed reactions are responsible for the formulation of L-[*guanidinooxy*-^{14}]canavanine. Injection of radioactive ureidohomoserine into the cotyledons resulted in a 9-fold increase in the radioactivity transferred to canavanine as compared to carbamoyl phosphate itself (Table II). Enzymic hydrolyses of the isolated radioactive canavanine released essentially all of the radioactivity as $^{14}CO_2$.

A possible alternate route for canavanine production involves a transamidination reaction in which L-arginine serves as a guanidino group donor to L-canaline. Administration of L-[*guanidino*-^{14}C]arginine either alone or in combination with 25 μmol of L-canaline failed to produce detectable [^{14}C]canavanine. This observation is consistent with an earlier finding by O'Neal (9). Glutamic acid, glutamine, urea, bicarbonate, and acetate were unable to support canavanine synthesis. Some labeling (less than 1%) of canavanine occurred from aspartic acid and asparagine, but this was not judged to be significant.

The seed predator, *Caryedes brasiliensis*, detoxifies L-canaline by a reductive deamination that produces L-homoserine and ammonia (17). This observation is compatible with the report of Töpfer *et al.* (19) that the legume, *Caragana spinosa*, can convert radioactive canavanine (isotopically undefined) to labeled homoserine with the intermediate formation of canaline. In his study of canavanine degradation in jack bean, Johnstone (5) claimed homoserine as a metabolic product of canavanine degradation by the developing plant. These findings led to the working hypothesis, incorporated into Figure 1, that amination of homoserine may result in canaline formation either with ammonia or with an amino acid nitrogen donor, such as glutamine or asparagine. Injection of L-[*U*-^{14}C]homoserine was followed by appreciable incorporation of radioactivity into the basic amino acid fraction of jack bean; examination of this radioactivity revealed, however, that most of the label resided in L-lysine, not in L-canavanine, although some radioactive canavanine was produced. In a typical labeled-precursor feeding study, with [^{14}C]homoserine, that spanned 12 h, 2.9% of the administered carbon occurred in lysine but only 0.7% occurred in canavanine. This production of radioactive lysine probably represents the conversion of homoserine to aspartic semialdehyde, which is then transformed to lysine via 2,3-dihydropicolinic acid and LL- or *meso*-2,6-diaminopimelic acid. Labeled aspartic acid administration culminated in appreciable labeling of the lysine fraction of the plant.

DISCUSSION

The leguminous plant *Canavalia ensiformis* is but one of several higher plants known to possess the enzymes and intermediates of the mammalian Krebs-Henseleit ornithine-urea cycle. Higher plants do not synthesize urea for the excretion of extraneous nitrogen, and urea that is formed is subject typically to rapid hydrolysis to CO_2 and ammonia by urease, a widely distributed higher plant enzyme. Nevertheless, the ornithine-urea cycle reactions have significance in higher plants, since they are the principal means for the production and storage of arginine and, in some plants, citrulline as well. Watermelon, *Citrullus vulgaris*, the original source of citrulline, is an example of a plant that accumulates appreciable levels of this secondary product. Information is not available presently on reactions, external to this cycle, which involve either citrulline or argininosuccinic acid. Ornithine is also an important plant constituent, since it is a proline precursor, can serve as a source of glutamic acid, and functions in polyamine synthesis (18). In essence, the reactions of the ornithine-urea cycle are of paramount importance in higher plant production of citrulline and arginine and constitute a vital aspect of the synthetic reactions of ornithine metabolism.

In addition to the above reactions, certain leguminous plants, such as jack bean, also synthesize canavanine and in a manner that bears striking similarity to arginine biosynthesis (Fig. 1). Jack bean mediates a reaction sequence from canaline that culminates in canavanine biosynthesis, and this is most appropriately viewed as the synthetic phase of canavanine metabolism. The combined hydrolytic actions of arginase and urease mobilize the nitrogen of the guanidinooxy moiety of canavanine by the sequential transfer to canaline and urea, and eventually to CO_2 and ammonia (11). These reactions are best viewed as the degradative phase of canavanine metabolism. Since experimental evidence has not yet been obtained for the reutilization of canaline in ureidohomoserine formation, these reactions cannot presently be considered to represent a higher plant cyclic reaction sequence.

The failure of radioactive homoserine to label canavanine was disappointing, since it is wholly reasonable to propose that homoserine provides the required carbon skeleton for canaline and canavanine formation by simple amination of homoserine. On the other hand, homoserine supports a multitude of higher plant biosynthetic reactions that complete effectively for his nonprotein amino acid. Homoserine *per se* is not always physiologically active in higher plant metabolic reactions. For example, *O*-phospho-L-homoserine, but not L-homoserine, supports the formation of cystathionine and homocysteine (4), and *O*-acetyl-L-serine, but not L-serine, is the alanyl side-chain source for many heterocyclic, nonprotein amino acids (7). Finally, higher plants are readily able to segregate or compartmentalize their metabolic reactions (8). For example, there is considerable evidence that exogenously supplied ornithine infiltrates metabolic reactions that are distinctive from those supported by internal ornithine. Exogenous ornithine is processed as catabolic ornithine rather than being part of its synthetic reactions (18). This dichotomy may also apply to homoserine administered to the cotyledons and also to canaline, itself. These factors may account for the limited transfer of labeled carbon from homoserine to canavanine observed in this investigation.

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