# Investigations of Canavanine Biochemistry in the Jack Bean Plant, *Canavalia ensiformis* (L.) DC.

## II. CANAVANINE BIOSYNTHESIS IN THE DEVELOPING PLANT[1]

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## ABSTRACT

**The canavanine content of developing leaves of jack bean, *Canavalia ensiformis* (L.) DC., increases during leaf development. The leaf possesses the enzymes required for synthesizing canavanine by a cyclic series of reactions analogous to the ornithine-urea cycle. This reaction series involves the sequential formation of canaline, O-ureidohomoserine, and canavaninosuccinic acid.**

Canavanine is the principal nitrogen reserve compound for many leguminous plants (2). Investigations of canavanine catabolism in the developing seedling of jack bean established that the canavanine content of the leaves increased markedly during the first 13 days of growth (11). Simple translocation from the cotyledons to the developing leaves might account for the observed elevation in leaf canavanine content. On the other hand, the leaves might represent the actual site of canavanine formation; particularly as long term ontogenetic studies of the jack bean fruit failed to establish a canavanine-producing capacity for the developing seeds (12). Toward resolving this question, further ontogenetic studies were instituted to determine if leaf tissues are capable of synthesizing a sizeable canavanine pool.

The cyclic reaction sequence of Figure 1, analogous to the ornithine-urea cycle, might represent a means for synthesizing canavanine. Arginase-mediated hydrolysis of canavanine to canaline and urea was first demonstrated in studies which employed the seed of jack bean (3). Ornithine transcarbamylase mediates the formation of O-ureidohomoserine from canaline and carbamyl phosphate. This enzyme has been extensively purified from 11-day jack bean leaves (unpublished data). Significantly, free canaline has been successfully isolated from jack bean tissues (9).

An enzyme, probably argininosuccinate lyase, catalyzing the nonhydrolytic cleavage of canavaninosuccinic acid to canavanine and fumarate is present in the 11-day jack bean leaf and has been purified 530-fold from hydrated seed (15). Argininosuccinate synthetase has not been isolated previously from jack bean and substantive demonstration of its occurrence in higher plants is limited exclusively to the germinating cotyledon of pea (16, 17).

This paper reports on the formation of canavanine by developing jack bean leaves. Data are presented which suggest the possibility of a functional canaline-urea cycle in jack bean.

## MATERIALS AND METHODS

**Plant Material.** Jack bean (*Canavalia ensiformis* [L.] DC.) plants were grown as previously described (11), except that the daylight temperature was $32 \pm 1$ C, the plants were watered every 3rd day with 250 ml of distilled water, and nutrients were supplied with 0.1 N Hoagland's solution on the 7th day of growth.

**Plant Extract.** Plant extracts were prepared for canavanine determination as previously described except that all manipulations were conducted at 4 C and completed in 4 hr (12). These precautions held the spontaneous decomposition of canavanine to deaminocanavanine to less than 1% of the original canavanine level (13).

**Substrates and Assay Procedures.** L-Canavanine and L-canaline were prepared by the author (14). L-O-Ureidohomoserine was synthesized from L-canaline by chemical carbamylation with KCNO under conditions rendering the $\alpha$-$NH_2$ group unreactive (23). Canavanine (11) and canaline (14) were assayed colorimetrically. Arginine was determined by a modification (13) of the procedure of Van Pilsum *et al.* (19). O-Ureidohomoserine evaluations employed the carbamyl derivative assay of Hunninghake and Grisolia (6).

Argininosuccinate synthetase studies were conducted with citrulline instead of ureidohomoserine as the latter amino acid is not presently available as a radioactive compound. Argininosuccinate synthetase assays were predicated upon the formation of guanidino-$^{14}$C-arginine from ureido-$^{14}$C-citrulline. The reaction product was isolated with the sulfonic resin Dowex-50 in the $H^+$ form (18). Afterwards, the radioactive arginine was treated with commerically prepared arginase and urease which converted arginine to $^{14}$C-urea and finally to $^{14}$CO$_2$. The radioactive CO$_2$ was trapped in hydroxide of hyamine as previously described (11).

**Enzyme Isolation.** The buffer system consisted of 50 mM sodium tricine (pH 7.9) containing 2 mM L-aspartic acid (final pH 7.8 at 22 C). Canavanine-synthesizing enzymes were isolated by grinding 15 g of 11-day jack bean leaves with 100 ml of buffer for 25 sec with a Sorvall Omni-mixer. After expressing the resultant slurry through several layers of cheesecloth, the filtrate was centrifuged at 27,000$g$ for 15 min.

The supernatant solution was brought to 55% saturation with saturated ammonium sulfate (pH 6.7) and allowed to s·and for 30 to 45 min. Precipitated protein was collected by centrifugation as above, dissolved in 12 ml of the above buffer, and dialyzed against 100 volumes of buffer for 20 hr. The

Carbamyl
Phosphate

NH₂
|
C = O
|
NH
|
O
|
CH₂
|
CH₂
|
H—C—NH₂
|
COOH

Nitrogen
Donor

O-UREIDOHOMOSERINE

NH₂
|
O
|
CH₂
|
CH₂
|
H—C—NH₂
|
COOH

CANALINE

NH     COOH
‖        |
C—N—CH
|    H    |
HN      CH₂
|          |
O       COOH
|
CH₂
|
CH₂
|
H—C—NH₂
|
COOH

CANAVANINOSUCCINATE

NH₂
|
C = NH
|
NH
|
O
|
CH₂
|
CH₂
|
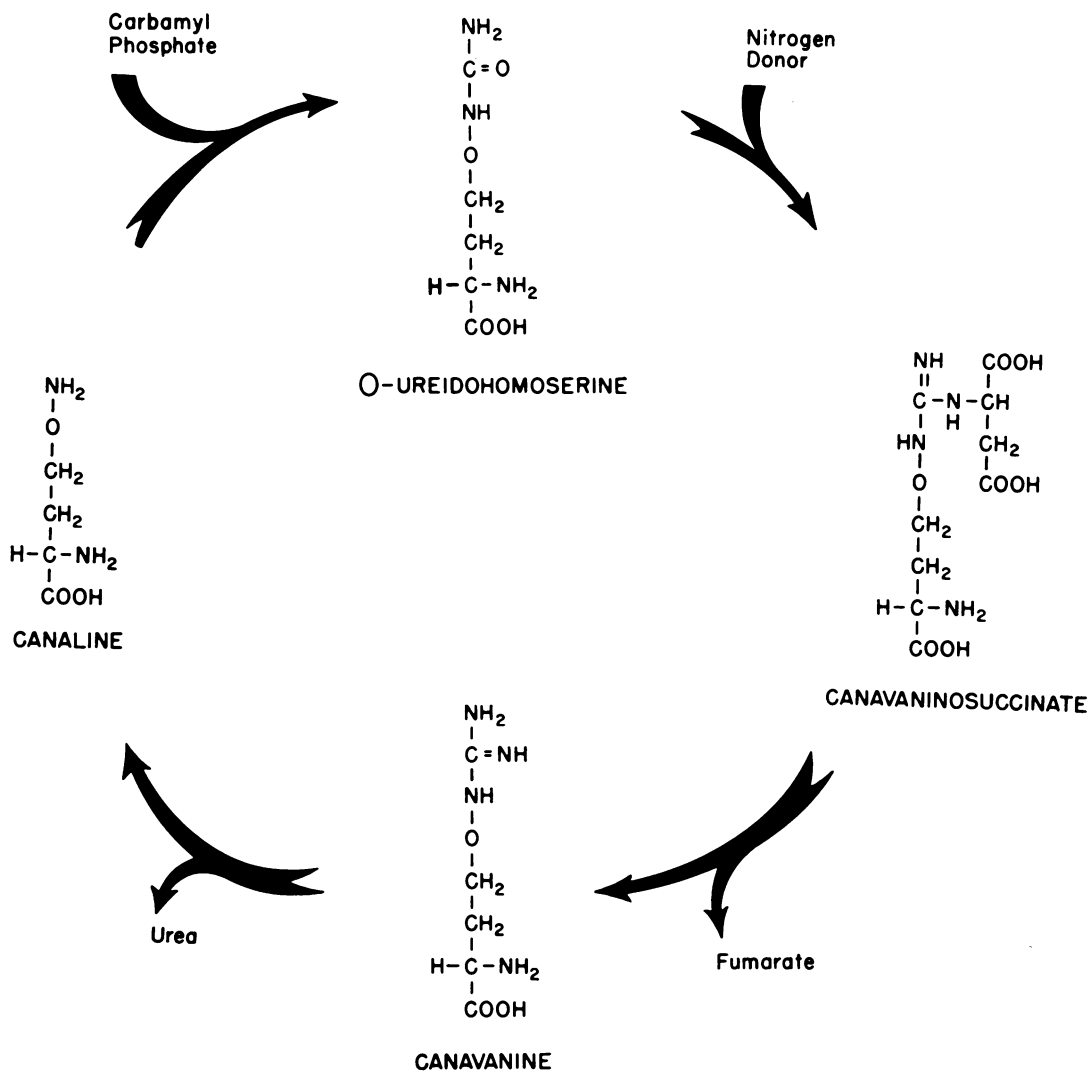H—C—NH₂
|
COOH

Urea

Fumarate

CANAVANINE

FIG. 1. The canaline-urea cycle of jack bean, *Canavalia ensiformis.*

buffer was changed twice. All operations were conducted at 4 C.

### RESULTS

**Ontogeny Studies.** The canavanine content of the developing primary leaves is illustrated in Figure 2. More than a 100% increase in leaf canavanine level occurred after cotyledon excision. Cotyledon-bearing plants exhibited a 3-fold increase in canavanine level between the 8th and 14th days of growth. More importantly, leaf assays demonstrated an increase in absolute canavanine content between the 11th and 14th growth days overwhelmingly in excess of the minute canavanine remaining in the ageing cotyledons.

Jack bean plants sustain an expected growth attenuation after losing the metabolic resources of the cotyledons. It is possible, however, to grow jack bean plants whose cotyledons—cordate, primary leaves—and even several successive trifoliate leaves have been sequentially removed, providing organ excision is delayed until the succeeding leaf is approximately 5 mm in length. Plants whose cotyledons and primary leaves were both excised nevertheless developed a first trifoliate leaf with the following canavanine content after 1, 2, and 3 weeks of growth: 17.6, 28.4, and 46.2 μmoles. Thereafter, the canavanine content of the first trifoliate leaf decreased sharply.

In a similar manner, plants were grown where as many as five successive trifoliate leaves were removed. In every case, each of the newly emerged trifoliate leaves contained appreciable canavanine.

Root ontogeny studies revealed that root tissues never developed a canavanine level comparable to that of the leaf. For example, maximum canavanine levels were obtained with 8-day roots (16.2 μmoles/root). Between the 8th and 14th growth days, the canavanine content decreased steadily to 9.3 μmoles per root. Thereafter, the canavanine level remained consistent at approximately 6 μmoles per root through the entire 24-day assay period. These studies cannot exclude the possibility of imminent translocation of canavanine from the root to the leaf.

In summary, leaves of the jack bean plant exhibit a characteristic period of elevated canavanine content varying from 3 to 5 weeks followed by declining canavanine levels. Before the aged leaf abscisses, all of the leaf canavanine will be utilized. Most significantly, even the first leaf to develop from the germinating seed accumulates appreciable quantities of canavanine.

**Canavanine Biosynthesis.** Studies of canavanine biosynthesis in jack bean leaves were preceded by experiments designed to establish the occurrence of argininosuccinate synthetase. These studies were based on the assumption that the ornithine-urea
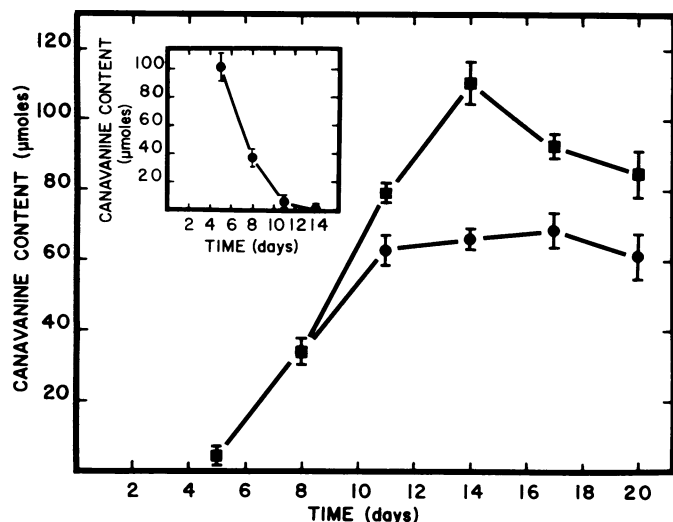
FIG. 2. Canavanine levels in jack bean leaves. On the 8th day of growth, the cotyledons were excised from half of the seedlings (●); the remaining untreated plants (■) and the excised seedlings were grown for 20 days. At the indicated intervals, plant material was harvested randomly and stored at −20 C. The canavanine content value, expressed on a per plant basis, represents the mean of five separate determinations, each performed with two plants. Canavanine content is expressed in μmoles ± SD Insert: canavanine content of the cotyledons in μmoles per plant.

cycle enzymes also mediate the canaline-urea cycle reactions. Thus, radioactive arginine formation from ureido-^{14}C-citrulline relies upon the capacity of argininosuccinate lyase to mediate the conversion of labeled argininosuccinate to guanidino-^{14}C-arginine and fumarate. Jack bean leaves also possess appreciable arginase activity which converts most of the newly-synthesized arginine to ornithine and ^{14}C-urea. It is not possible to utilize [14]CO_2 from radioactive urea to measure argininosuccinate synthetase activity, since [14]CO_2 also results from the reversible nature of ornithine transcarbamylase activity. Consequently, argininosuccinate synthetase assays were predicated upon the formation of diminutive but accurately measurable steady-state levels of arginine.

Table I illustrates the aspartic acid- and ATP-dependent production of guanidino-^{14}C-arginine from radioactive citrulline. Enhanced arginine formation, upon fumarase addition to the incubation mixture, reflects curtailed arginine attrition through its condensation with fumarate to form argininosuccinate. Citrulline-dependent arginine synthesis was monitored colorimetrically under the reaction conditions of Table I. In this manner, the apparent K$m$ for citrulline, determined at pH 7.8, was 2.2 mM.

Evidence for the operation of the cyclic reaction sequence depicted in Figure 1 was secured by two types of experiments. The first was predicated upon the direct and independent synthesis of canavanine from each of the postulated canaline-urea cycle intermediates. Studies of canavanine synthesis were commenced with O-ureidohomoserine. An equivalent quantity of O-ureidohomoserine was substituted for citrulline and reacted as described in Table I. These reaction conditions lead to the production of 0.185 μmole of arginine from citrulline and 0.125 μmole of canavanine from O-ureidohomoserine. The synthesized canavanine was isolated by ion-exchange chromatography (13) and treated with commercially prepared arginase. The resulting canaline was then identified by partition chromatography utilizing the formation of oxime type compounds between canaline and pyruvate or α-ketoglutarate (11).

[14]C-Carbamyl phosphate and L-canaline, each at 10 mM, were substituted for O-ureidohomoserine, and the above experiment was repeated. Employment of radioactive carbamyl phosphate in the incubation mixture permitted the synthesis of oxyguanidino-^{14}C-canavanine from ureido-^{14}C-ureidohomoserine. The deproteinized incubation mixture was neutralized and placed on a 20- × 75-mm column of Dowex-50 (NH_4^+). The column was washed with water until the effluent was free of radioactivity and then developed with 0.1 N NH_4OH. The basic effluent, containing 0.115 μmole of labeled canavanine, was concentrated by rotary evaporation, titrated to pH 7.6, and treated with commercially prepared arginase and urease as previously described (11). A 96% recovery of radioactive CO_2 from canavanine-containing effluent confirmed the successful synthesis of canavanine. The identification of canavanine was identical, therefore, to the procedure used to establish the production of guanidino-^{14}C-arginine from radioactive citrulline.

Finally, canavaninosuccinic acid was synthesized enzymatically from canavanine and fumarate with purified jack bean argininosuccinate lyase (15) and converted to the stable barium salt (10). Incubation of 10 mM canavaninosuccinate with jack bean leaf extract led to the synthesis of canavanine and canaline. The reaction products were identified by colorimetric

Table I. *Detection of Argininosuccinate Synthetase in Jack Bean Leaves*

The assay mixture consisted of: L-ureido-^{14}C-citrulline, 20 mM (10,000 cpm/μmole); L-aspartic acid, 10 mM; ATP/MgCl_2, 20 mM; sodium tricine buffer at pH 7.8, 50 mM; approximately 20 mg of crude protein; and, when applicable, 0.1 mg of fumarase, 360 units/mg protein in a final volume of 3 ml.

After incubating the enzyme at 37 C for 60 min, the reaction was terminated with 2 ml of 10% (w/v) trichloroacetic acid. The deproteinized incubation mixture was extracted with ether prior to determining the guanidino-^{14}C-arginine content as described in the text. The control consisted of similarly processed enzyme but treated with trichloroacetic acid prior to incubation.

| | Guanidino-^{14}C-arginine Production |
|---|---|
| | *cpm* |
| Complete assay mixture | 1,850 |
| Minus aspartic acid | 51 |
| Minus ATP/MgCl_2 | 39 |
| Control | 27 |
| Complete assay mixture + fumarase | 2,110 |

Table II. *Canavanine Regeneration by Jack Bean Leaf Extracts*

The assay mixture contained 25 mM L-canavanine, 20 mM ATP/MgCl_2, 10 mM L-aspartic acid, leaf extract containing 8.5 mg of protein, 50 mM sodium tricine buffer at pH 7.8, and carbamyl phosphate at the indicated concentration in a final volume of 2 ml. The assay mixture was incubated for 20 min at 37 C, deproteinized with 2 ml of 25% (v/v) perchloric acid, and neutralized with 2 ml of 3.3 N KOH. Analyses of the assay mixture for canaline-urea cycle intermediates were conducted as described in the text.

| Carbamyl Phosphate | Content of Canaline-Urea Cycle Compounds | | |
| | Canavanine | Canaline | O-Ureido-homoserine |
|---|---|---|---|
| *mM* | | *μmoles* | |
| 0 | 0.0 | 48.2 | 0.0 |
| 5 | 2.4 | 42.4 | 6.3 |
| 10 | 5.6 | 35.3 | 7.8 |
| 20 | 8.9 | 28.2 | 8.6 |
| 25 | 11.1 | 25.4 | 9.1 |

analyses and confirmed by paper chromatography employing four solvent systems (v/v): phenol-water (4:1), $R_F$ 0.65, 0.95; n-butanol-glacial acetic acid-water (4:1:5), $R_F$ 0.19, 0.65; methyl ethyl ketone-acetone-water (80:4:12), $R_F$ 0.05, 0.48; and ethanol-glacial acetic acid-water (65:1:34), $R_F$ 0.69, 0.91. The $R_F$ data for canavanine and canaline respectively agreed remarkably with the values obtained for authentic samples of these amino acids. The identity of the canavanine spot was also verified by spraying with buffered pentacyanoammonioferrate, a reagent which produces a magenta-colored spot with canavanine at pH 7. Thus, independent synthesis of canavanine form O-ureidohomoserine, canaline, and canavaninosuccinate was demonstrated in jack bean leaf extracts.

Evidence for the operation of the reaction pathway of Fig. 1 was also secured by cyclic regeneration of canavanine. As shown in Table II, when carbamyl phosphate was omitted from the incubation mixture, all of the canavanine was degraded to canaline; neither canavanine nor O-ureidohomoserine could be found in the incubation mixture. Addition of carbamyl phosphate to the incubation mixture caused a decrease in canaline concentration while elevating the level of O-ureidohomoserine. Of greater importance, however, a marked enhancement in the steady-state level of canavanine occurred.

These results indicate that as carbamyl phosphate became increasingly available, a greater proportion of the canaline formed by canavanine hydrolysis reacted to form O-ureidohomoserine. The O-ureidohomoserine, in turn, served as a substrate for canavanine production. Thus, as the carbamyl phosphate level rose, more of the degraded canavanine was regenerated by the activities of the canaline-urea cycle enzymes.

Carbamyl phosphate can function as a carbamylating agent converting enzyme residues such as lysine to homocitrulline (5). The observed increase in canavanine content with elevated carbamyl phosphate concentration might reflect carbamyl phosphate-mediated inactivation of arginase, but separate experiments clearly demonstrated that even 30 mM carbamyl phosphate did not impede jack bean arginase activity under the conditions described in Table II.

## DISCUSSION

Fowden (4) has suggested that the metabolic pathways culminating in the syntheses of certain nonprotein amino acids might reflect subtle alteration in the genome responsible for directing the formation of crucial amino acids. Most plant species possess the ornithine-urea cycle enzymes, thereby providing a mechanism for the biosynthesis of arginine, an essential building block for a myriad of plant products. Thus, the canaline-urea cycle may have its origin as a metabolic pathway which evolved by modification of a preexisting means for the production of arginine.

In a similar vein, Bell (1) has discussed some of the rationales for the occurrence of certain "uncommon" amino acids such as canavanine in members of the Leguminosae. Bell contends that while these unique amino acids function primarily as storage metabolites they may also provide an adaptive advantage to the plant; for example, to render the plant less susceptible to attack by various animals and lower plants. Indeed, the capacity of canavanine to inhibit bacterial growth (20), yeast and algal development (21), tissue cultures of human (8) and other mammalian cell lines (7) lends credence to this concept.

Canavanine production is observed to commence with the very first leaf to emerge from the developing embryo. Yet if canavanine is synthesized to serve solely as a storage metabolite, then its synthesis at a much later time in the development of the jack bean plant seems more appropriate. Effective

conservation of the nitrogen resources of the plant would be better achieved if canavanine production was coincident with flower development and anthesis. On the other hand, if canavanine also functions as a type of allelochemic (22), then its synthesis by the newly developing seedling is more easily understood.

Finally, the data presented in this paper establishes the capacity of the ornithine-urea cycle enzymes to metabolize sequentially the canaline-urea cycle intermediates. The results, however, do not prove that the cyclic reaction series of Figure 1 is the sole or even the principal in vivo mechanism for canavanine production. Studies in progress have demonstrated that jack bean leaves can transfer radioactive carbon from simple metabolites such as bicarbonate to canavanine. Work is underway to isolate and identify the radioactive intermediates and to determine if a functional canaline-urea cycle occurs in jack bean leaves.

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