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20 April 1982

L-Canavanine, a Dietary Nitrogen Source for the Seed Predator *Caryedes brasiliensis* (Bruchidae)

Abstract. *Larvae of the bruchid beetle* Caryedes brasiliensis *(Bruchidae) develop entirely within the seed of the neotropical legume* Dioclea megacarpa. *The seed contains an appreciable concentration of* L*-canavanine, a potent antimetabolite and structural analog of* L*-arginine. This bruchid beetle uses the nitrogen stored in this toxic allelochemical as an effective dietary nitrogen source for amino acid biosynthesis.*

Maturing larvae of the bruchid beetle *Caryedes brasiliensis* (Bruchidae) develop entirely within the seed of the neotropical legume *Dioclea megacarpa* (*1*) (Fig. 1). More than 95 percent of the free amino acid nitrogen of this leguminous seed accumulates as L-canavanine, an insecticidal structural analog of L-arginine (*2*). This seed predator is distinctive in its synthesis of appreciable urease (E.C. 3.5.1.5) which makes possible effective mobilization of the nitrogen stored in canavanine via hydrolysis of urea; the latter is derived from canavanine by the action of arginase (E.C. 3.5.3.1).

$$H_2N\text{-}C(=NH)\text{-}NH\text{-}O\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \rightarrow$$

L-canavanine

$$H_2N\text{-}O\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)COOH +$$

L-canaline

$$H_2N\text{-}C(=O)\text{-}NH_2$$

urea

$$H_2N\text{-}C(=O)\text{-}NH_2 \rightarrow CO_2 + 2NH_3$$

It has been proposed that these hydrolytic reactions not only detoxify canavanine, but also make ammonia available as a dietary nitrogen source for the developing larva (*3*). If this hypothesis is correct, it would represent a significant finding since, with the possible exception of cyanide incorporation into L-asparagine (*4*), no instance is known of insects using a toxic higher plant allelochemical in amino acid synthesis. The ease with which this insect can convert canavanine to canaline and urea and the availability of ^{15}N-labeled urea have made possible an experimental verification of this hypothesis which is important to our understanding of the basic biochemical interaction between higher plants and their insect predators.

To test this hypothesis, living terminal stadium larvae (9 g, fresh weight) were washed thoroughly with deionized water to remove adhering plant material; each larva was then injected with 1 μl of 150 m*M* ^{15}N-labeled urea (*5*). The treated larvae were maintained in the dark for 48 hours at 28°C before storage at −60°C. The frozen larvae were ground for 60 seconds with 50 ml of freshly distilled acetone (Sorvall Omnimixer, full power). The larval acetone powder was collected by vacuum filtration, and reground as above for 120 seconds and dried in air overnight at 22°C. The free amino acids of the acetone powder (2.7 g) were obtained by mechanical stirring at 3°C for 20 hours with 250 ml of 50 percent aqueous ethanol containing 0.15*N* HCl. The ethanolic extract was clarified by centrifugation at 18,000*g* for 20 minutes, adjusted to *p*H 7.0 with 1*N* NaOH, and concentrated by rotary evaporation at reduced pressure. Particulate materials deposited from the extract during concentration were removed by centrifugation as above. Finally, the ethanolic extract was reduced to 35 ml, adjusted to *p*H 2.5, and filtered (Millipore GS membrane).

The clarified larval extract was placed on a column (20 by 495 mm) of Dowex-50 (H^+) and washed with 3 liters of deionized H_2O at 3°C. Amino acids were eluted fully with 1.2 liters of 0.3*N* NH_4OH

Table 1. Percent ^{15}N incorporation into the free amino acids of the larvae of the bruchid beetle *Caryedes brasiliensis*. The values presented for percent of ^{15}N incorporation were obtained by three independent determinations of the same sample. The fragment structures are based on the work of Gelpi *et al.* (*8*). The amino acids were analyzed as their *N*-TFA-*n*-butyl ester derivatives. See Fig. 2 legend for the amino acid designations.

Amino acid	^{15}N incorporation (%)	Fragment used in calculations		
		*m/e**	Relative abundance (%)	Structure
Ala	35, 33, 33	140	100	M − C_4H_9COO
Gly	18, 18, 16	126	98.9	M − C_4H_9COO
2-ABA	30, 31, 28	154	100	M − C_4H_9COO
Thr	0, 0, 0	152	33.5	$CF_3CONH{=}C{=}CHCH_3$
Ser	16, 20, 19	138	49.2	$CF_3CONH{=}C{=}CH_2$
Val	7, 5, 7	168	100	M − C_4H_9COO
3-Ala	23, –, 21	168	100	M − OC_4H_9
Leu	0, 0, 0	182	59.6	M − C_4H_9COO
Ile	0, 0, 0	182	50.5	M − C_4H_9COO
H-Ser	11, 6, 7	152	100	$CF_3CONH{=}C{=}CHCH_3$
Pro	25, 25, 25	166	100	M − C_4H_9COO
Hy-Pro	0, 0, 0	164	100	(M − C_4H_9COO) − CF_3COOH
Met	11, 9, 10	227	64.8	M − (CH_2CH − S − CH_3)
Asp/Asn	24, 22, 25	240	79.7	M − C_4H_9COO
Lys	0, 0, 0	293	14.4	M − C_4H_9COO
Glu/Gln	44, 49, 48	198	100	(M − C_4H_9COO) − C_4H_8
His	0, 0, 0	206	75.8	M − C_4H_9COO

*Ratio of mass to charge.

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(6). The effluent was rapidly dried at reduced pressure, the residue dissolved in 10 ml of deionized H_2O, filtered (Whatman 50 paper), and stored at −60°C. The thawed insect extract was concentrated at reduced pressure to 1.5 ml and placed on a column (20 by 1350 mm) of Sephadex G-10. The column was developed with deionized water and 1.4-ml fractions were collected every 1.5 minutes; fractions 70 through 110 were pooled, concentrated at reduced pressure to 8.1 ml, and stored at −60°C (7). Amino acid analyses of the purified larval extract were conducted by automated procedures that relied on ninhydrin detection at 440 and 570 nm. The *N*-trifluoroacetyl-*n*-butyl ester derivatives were analyzed by gas chromatography–mass spectroscopy (8).

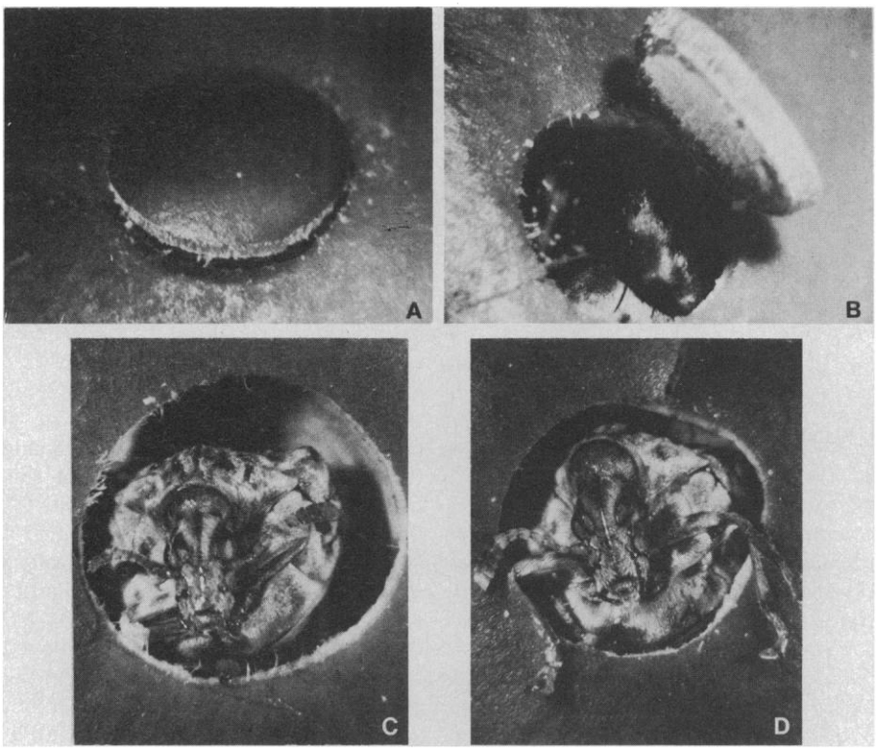

Fig. 1. (A–D) Release of the adult bruchid beetle *Caryedes brasiliensis* from the seed of *Dioclea megacarpa*. The larvae, developing within the seed, use the nitrogen of canavanine as a dietary nitrogen source. The newly formed adult emerges through an escape lid bored in the seed coat. [Photographs by James E. Mattler]

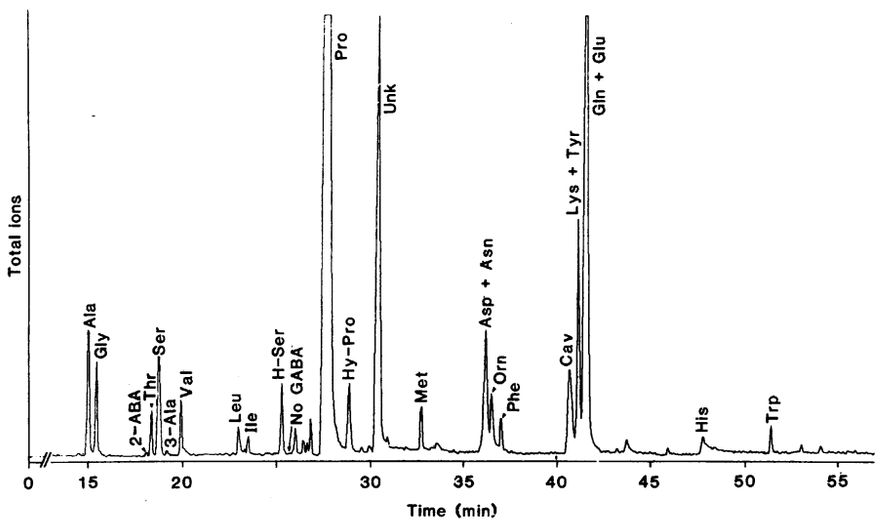

Fig. 2. Total ion chromatogram of the *N*-trifluoroacetyl-*n*-butyl ester derivatives of the free amino acids of the larvae of the bruchid beetle *Caryedes brasiliensis*. [Glass capillary column: (50 m by 0.5 mm, inside diameter), coated with OV-101; temperature from 100° to 250°C at 3° per minute; helium carrier gas flow rate, 4.5 ml per minute.] The amino acid abbreviations are: Ala, alanine; Gly, glycine; 2-ABA, 2-aminobutyric acid; Thr, threonine; Ser, serine; 3-Ala, 3-alanine; Val, valine; Leu, leucine; Ile, isoleucine; H-Ser, homoserine; Pro, proline; Hy-Pro, hydroxyproline; Unk-unknown; Met, methionine; Asp, aspartic acid; Asn, asparagine; Orn, ornithine; Phe, phenylalanine; Cav, canavanine; Lys, lysine; Tyr, tyrosine; Gln, glutamine; Glu, glutamic acid; His, histidine; Trp, tryptophan. 4-Aminobutyric acid (GABA; elution time, 25.5 minutes) may not be present in the larva since it was not detected by gas chromatography–mass spectrometry procedures that disclosed authentic material. Automated amino acid analysis disclosed a ninhydrin-positive substance with the column retention time of GABA.

Some 25 free amino acids occur in the larva of *C. brasiliensis* but the principal components are canavanine, lysine, proline, glutamic acid, glutamine, alanine, and glycine. Analysis by mass spectrometry of the ^{15}N incorporation of these larval amino acids revealed incorporation of the heavier nitrogen isotope amounting to more than 25 percent of the total for glutamic acid (or glutamine), aspartic acid (or asparagine), proline, 3-alanine, 2-aminobutyric acid, and alanine (Table 1 and Fig. 2). Lesser but readily detectable amounts of ^{15}N were found in glycine, serine, valine, homoserine, and methionine (Table 1). There was no evidence for nitrogen transfer from urea to threonine, leucine, isoleucine, lysine, histidine, and hydroxyproline (9).

The above findings are consistent with a primary role for glutamic acid and glutamine in the assimilation of urea's nitrogen by this seed predator. Aminotransferases that yield L-alanine from reaction of pyruvic acid and L-glutamic acid and that provide L-aspartic acid from oxaloacetic acid and L-glutamic acid are among the most common and catalytically active of this diverse group of enzymes in higher animals and insects (10, 11).

In addition, glutamic acid and aspartic acid can be reductively aminated with ammonia to yield their amide-containing derivatives: glutamine and asparagine (12). Insects oxidize proline to glutamic acid as a means of providing 2-oxoglutarate (via transamination) to generate energy by the reactions of the Krebs cycle (13). This reaction is known to be reversible, presumably via 4-glutamyl semialdehyde and Δ^1-pyrroline-5-carboxylic acid. Serine biosynthesis is also linked to glutamic acid since transamination of glutamic acid to 2-oxoglutarate supplies the nitrogen for 3-phosphoserine, the immediate precursor of serine. Glycine can form directly from serine in insects by an aminotransfer between glyoxylic acid and alanine (10). The appreciable ^{15}N label found in 2-amino-*n*-butyric acid (2-ABA) may, on first consideration, be surprising; however, it is generated from 2-oxobutyric acid, an oxo-containing

acid that forms readily from threonine and methionine. Homoserine, which can form either from cystathionine or more importantly by reductive deamination of canaline (*14*), is subject to a deamination that also forms 2-oxobutyric acid. While the above reactions have not been studied specifically in *C. brasiliensis*, their occurrence in insects is well documented and they represent reasonable pathways for the transfer of the ^{15}N isotope to the various amino acids of this seed-feeding insect. On the other hand, a glutamic acid-2-oxoglutarate aminotransfer is an integral aspect of leucine, isoleucine, lysine, and histidine biosynthesis. It is evident, therefore, that this nitrogen-transferring reaction does not support all the amino acid biosynthetic pathways operative in this seed predator.

Many, perhaps even all, of the metabolic capacities reflected in the catabolism and detoxification of canavanine and canaline by *C. brasiliensis* larvae (*14*) may emanate from the enzymes synthesized by the prokaryotic flora of the insect rather than the insect itself.

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5. [1,3-$^{15}N_2$]Urea (99 percent ^{15}N) was purchased from Stohler Isotope Chemical Co., Azusa, Calif. Its purity and ^{15}N content were confirmed by mass spectrometry.
6. Stirring the resin overnight at 3°C with a large excess of 0.5*N* ammonia failed to yield additional amino acids.
7. Automated amino acid analysis of pooled fractions 1 to 35, 36 to 69, 111 to 125, and 126 to 140 failed to reveal the presence of appreciable ninhydrin-positive material.
8. Automated amino acid analysis was conducted with the lithium citrate physiological buffer system of Durram Chemical Co. (Dionex Corp.). The process involves the sequential application of five buffers and requires 5 hours to complete. The amino acid derivatives used for gas chromatography–mass spectrometry were prepared by the method of D. L. Stalling and C. W. Gehrke [*Biochem. Biophys. Res. Commun.* **22**, 329 (1966)]. Mass fragment identification was based on the work of E. Gelpi *et al.* [*J. Chromatogr. Sci.* **7**, 604 (1969)] and R. E. Summons *et al.* [*Anal. Chem.* **46**, 582 (1974)]. Gas chromatography–mass spectrometry analyses were conducted with a Hewlett-Packard model 5985-A mass spectrometer operated in the electron impact mode with an accelerating voltage of 70 eV and source temperature of 150°C.
9. The small relative abundance of mass spectral fragments containing nitrogen prevented the determination of the ^{15}N content of phenylalanine, tyrosine, and tryptophan. Ornithine and canavanine were not assayed since neither ^{15}N-labeled standards for relevant mass spectral literature exist for the compounds. ^{15}N-Labeled canaline was not assayed since either during derivatization or the actual gas chromatography–mass spectrometry process it was degraded.
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20 October 1981; revised 22 February 1982

Cholinergic Agonists Induce Vectorial Release of Serotonin from Duodenal Enterochromaffin Cells

Abstract. *Serotonin-containing enterochromaffin cells in the rabbit duodenal mucosa span the tissue contacting both the luminal and serosal sides. When the serosal surface is stimulated with carbachol in vitro, serotonin is secreted on the serosal side but not the mucosal side. Carbachol added to the luminal side is ineffective. Atropine but not hexamethonium blocks the effect of carbachol. Acetylcholine on the serosal surface also stimulates serotonin release on the serosal side. These findings indicate that enterochromaffin cells possess on their serosal surfaces muscarinic receptors that mediate vectorial release of serotonin when activated by cholinergic agonists.*

The enterochromaffin cells in the gastrointestinal mucosa contain a large proportion of the body's store of serotonin (*1*). In spite of this, little is known about the regulation of these cells or the precise role of the serotonin they release. The enterochromaffin cells typically span the mucosa. In general, the apical pole of each cell borders the intestinal lumen and is striated with microvilli (*2*). The polymorphous secretory granules that contain the amine are most commonly found in the widened base of the cell but occasionally are found in the apical portion (*3*). Presumably, dietary stimuli induce responses in the apical portion (*4*) and endocrine or nerve-derived factors induce responses in the basal portion (*5*). In vivo studies indicate that the cells have the ability to secrete serotonin from their apical or basal sides, leading to increases in either luminal (*4*) or blood (*5*) concentrations of serotonin. We have been studying the biochemical control mechanisms in these cells in vitro. We report that enterochromaffin cells in the rabbit duodenum appear to contain muscarinic receptors which, when activated, lead to serotonin secretion on the vascular side of the mucosa.

Duodenal tissue was obtained from New Zealand White rabbits and the longitudinal and circular muscle layers were removed. The pieces of mucosa were mounted in modified Ussing chambers so that the serosal and mucosal surfaces could be incubated with separate solutions of Ringer bicarbonate. Test solutions could then be added to either or both sides of the preparations so that the vectorial release of serotonin could be studied (*6*). Preliminary studies showed that the spontaneous release of serotonin to the serosal side decreased and became stable during the first 30 minutes of incubation (Fig. 1). The spontaneous release of serotonin to the mucosal side was initially higher and more variable but also stabilized at a low level after 30 minutes.

The solutions bathing both sides of the tissue were then removed and replaced with control or drug-containing test solutions. Replacing the bathing solutions with control solutions caused some increase in the release of serotonin to the mucosal side, but release to the serosal side was not altered (Fig. 1). Since most of the serotonin released into the mucosal bathing chamber was removable by low-speed centrifugation, it probably was attributable to exfoliated enterochromaffin cells. When carbachol ($>10^{-6}M$) was added to the serosal side there was a dramatic increase in the

0036-8075/82/0723-0355$01.00/0