Detoxification of L-Canavanine by the Tobacco Budworm, *Heliothis virescens* [Noctuidae]

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The detoxification of L-canavanine and the ability of this natural product to induce a protein able to detoxify it were investigated in the tobacco budworm, *Heliothis virescens*. The available evidence indicates that this detoxification mechanism is part of larval constitutive metabolism and not induced in response to canavanine. *H. virescens* larvae, administered 5 mg/g L-canavanine supplemented with 37.7 kBq L-[*guanidinooxy*-^{14}C]canavanine, were sacrificied 0, 1, 2, 4, 6, and 12 h postinjection. The principal products of L-canavanine degradation were [^{14}C]guanidine and [^{14}C]urea. Homoserine formation was confirmed by automated amino acid analysis. This study demonstrates that the principal detoxification pathway for L-canavanine in *H. virescens* larvae is by reductive cleavage to guanidine and L-homoserine.

INTRODUCTION

L-Canavanine, L-2-amino-4-(guanidinooxy)butyric acid, a nonprotein amino acid found in over 1500 species of leguminous plants (Bell et al., 1978), can play a seminal role in the chemical defense of these plants from herbivores (Rosenthal and Bell, 1979; Rosenthal, 1982a). This potentially toxic analogue of L-arginine can compete in virtually all metabolic reactions where arginine is the preferred substrate, including activation and aminoacylation by arginyl-tRNA synthetase. The latter reaction results in production of structurally (Rosenthal et al., 1989a) and functionally aberrant proteins (Rosenthal et al., 1989b).

With two exceptions, all insects tested to date are canavanine-sensitive insects (Rosenthal, 1977a); canavanine elicits adverse biological effects in and can be insecticidal against these insects (Rosenthal, 1977a). The exception is limited solely to canavanine-utilizing insects such as the bruchid beetle, *Caryedes brasiliensis*, and the weevil, *Sternechus tuberculatus*. These seed predators feed on a seed containing 8–9% and 6–8%, respectively, canavanine by dry weight (Bleiler et al., 1988). The principal means for canavanine metabolism by these insects is by hydrolytic cleavage of L-canavanine, mediated by arginase (EC 3.5.3.1), to L-canaline and urea (Rosenthal and Janzen, 1983). The urea is then degraded by urease (EC 3.5.1.5) to NH_3 and CO_2 (Rosenthal et al., 1982; Bleiler et al., 1988).

The tobacco budworm, *Heliothis virescens*, consumes a wide variety of plants, none of which are known to store significant canavanine. Presumably, this aggressive generalist feeder would have had only limited opportunity for long-term evolutionary adaptation to canavanine. *H. virescens* resistance to extraordinary high concentrations of dietary canavanine is demonstrated by an LC_{50} for dietary canavanine of 300 mM or nearly 40% canavanine by dry weight (Berge et al., 1986). The tobacco budworm neither excretes nor sequesters canavanine, and its weak arginase activity cannot account for the catabolism of the levels of dietary canavanine that the larvae consume (Berge et al., 1986). Therefore, *H. virescens* represents a novel third type of canavanine consumer that has been designated canavanine resistant. We were intrigued by the nature of the biochemical mechanism responsible for the canavanine resistance of this insect. These factors instigated our studies to determine how *H. virescens* is able to consume with impunity so much of this potentially lethal natural product.

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MATERIALS AND METHODS

Insect. *H. virescens* larvae, obtained from a continuous colony maintained at the University of Kentucky, were reared as described previously (Berge et al., 1986). Unless otherwise indicated, injected materials were administered by parenteral injection, using a volume equivalent to 5% of fresh body weight. The injected larvae, 200–300 mg fresh weight, were no more than 6 h into the second day of the terminal larval instar. Hemolymph samples were collected from three treated larvae by cutting a ventral proleg and transferring 15 μL of the exuding hemolymph from each larva into each of two plastic, microfuge tubes containing 500 μL of 50% (w/v) trichloroacetic acid (TCA). Each of the two tubes, representing the pooled hemolymph of the three larvae, was agitated and allowed to sit overnight at 4 °C.

Chemicals. L-Canavanine (free base) was isolated from acetone-defatted jack bean seeds, *Canavalia ensiformis*, by ion-exchange chromatography and purified by repetitive crystallizations (Rosenthal, 1977b). L-[*guanidinooxy*-^{14}C]Canavanine (2.15 MBq/μM) was prepared by the method of Ozinskas and Rosenthal (1986). The other radiolabeled amino acids were obtained from New England Nuclear. TS-1 tissue solubilizer was obtained from Research Products International Corp., Mount Prospect, IL. All other biochemicals were obtained from Sigma Chemical Company, St. Louis, MO.

Radiolabeled Hemolymph Proteins of Cycloheximide-Pretreated Insects. Nine larvae (three groups of three larvae each) were injected with 250 μg/g of fresh body weight cycloheximide or sterile water. After 3 h, the larvae were injected with 11.8 kBq L-[^{3}H]leucine. Four hours later, duplicate hemolymph samples (15 μL/larva) were collected from three larvae to create duplicate pooled 45-μL samples and processed as described previously.

Canavanine Clearance. Clearance data for canavanine were obtained by automated amino acid analysis of hemolymph from canavanine-treated larvae using a Dionex D-300 automat-

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ed amino acid analyzer employing a lithium citrate buffer system and ninhydrin detection at 570 nm.

H. virescens larvae were injected with 250 μg/g of fresh body weight cycloheximide or sterile water ($n = 5$). Three hours after cycloheximide treatment, each larva received 5 mg of L-canavanine/g of body weight. Hemolymph samples were collected 0, 1, 3, 6, or 12 h postinjection. Hemolymph (15 μL/larva) were taken from five larvae to create duplicate pooled 75-μL samples. The pooled samples were then treated with 50% (w/v) TCA and agitated. After sitting overnight at 4 °C, the precipitated proteins were removed by centrifugation. The supernatant solution was extracted four times with an equal volume of anhydrous ether, brought to its original volume, and assayed for canavanine ($n = 5$).

Radiolabeled Hemolymph Proteins. Twelve fifth instar larvae (four groups of three larvae each), which had ecdysed during the previous 12 h, were placed on either an artificial diet containing 150 mM canavanine or a control diet augmented with an equivalent amount of water. Following 24 h on a diet, the larvae were injected with 40.7 kBq of L-[^{35}S]methionine. Four hours later, duplicate hemolymph samples (15 μL/larva) were collected from three larvae to create duplicate pooled 45-μL samples and processed as described previously.

Larvae maintained on regular diet were used to study the acute effects of canavanine on protein incorporation. Two sets of nine larvae (three groups of three larvae each) were injected with a solution containing 40.7 kBq of L-[^{35}S]methionine and 5 mg/g of fresh body weight L-canavanine or an equivalent volume of sterile water carrying 40.70 kBq of L-[^{35}S]methionine. After 4 h, duplicate hemolymph samples (15 μL/larva) were collected from three larvae to create duplicate pooled 45-μL samples and processed as above. Hemolymph protein determinations were made by the method of Lowry et al. (1951).

The L-[^{35}S]methionine-labeled, precipitated proteins were centrifuged by using a Beckman microfuge and the supernatant solutions discarded. The precipitated proteins were washed twice with 10% (w/v) TCA, once with absolute ethanol/ether (1:1, v/v) and twice with anhydrous ether. The resulting pellets were air-dried, treated with 250 μL of TS-1 tissue solubilizer, and allowed to dissolve at 50 °C overnight. The dissolved proteins were transferred to a scintillation vial by using three 1-mL portions of Bray's scintillation medium (Bray, 1960) and counted by liquid scintillation spectroscopy (Rosenthal, 1982b).

Electrophoresis. Fifth instar larvae that had ecdysed during the previous 12 h were placed on either 150 mM canavanine or control diet. After feeding on the diets for 72 h, the larvae were sacrificed and their hemolymph, midgut, and fat body collected. The collected samples were homogenized with a glass tissue grinder in 50 mM sodium tricine buffer (pH 7.6) and clarified by centrifugation for 20 min at 12 000 rpm. Electrophoresis was carried out on the clarified samples by using 10% polyacrylamide under reduced conditions (Laemmli, 1970). The gel was stained with 0.5% (w/v) Coomassie Blue.

L-[*guanidinooxy*-^{14}C]Canavanine Treatment and Sample Preparation. *H. virescens* larvae were injected with 37.7 kBq of [^{14}C]canavanine with sufficient cold carrier to provide 5 mg/g of fresh body weight. The dosed animals were sacrificed after 0, 1, 2, 4, 6, or 12 h ($n = 5$). The 24-h treatment group was placed in a 30-mL metabolic cage immediately after injection, and respiratory [^{14}C]CO_2 was collected. Air, drawn from the cage at a rate of 6 mL/min under vacuum, was conveyed into a CO_2 trap containing 5 mL of methoxyethanol and 2-aminoethanol [2:1 (v/v)]. A small sample of trapping solution (100 μL) was counted by liquid scintillation spectroscopy. Since only trace amounts of [^{14}C]CO_2 (<0.01% of the administered dose) were detected after 24 h, CO_2 collection was not preformed for all other treatment groups. All treated larvae were frozen and stored at −60 °C until processed. Oral exudate regurgitated by the treated larvae was collected by rinsing the metabolic cages repeatedly with water (final volume 50 mL); 500-μL samples of the water rinses were counted by liquid scintillation spectroscopy.

The frozen larvae were ground in 20 mL of 50 mM sodium tricine, pH 7.6, treated with an equal volume of 50% (w/v) TCA, and refrigerated overnight at 4 °C. These precipitated samples were centrifuged at 12000*g* for 20 min and the pellets removed. Following centrifugation, the supernatant solution was extracted four times with an equal volume of anhydrous ether, and the pH was adjusted to 7.6 with 1 N NaOH. All analyses were conducted in triplicate.

Analysis of L-[*guanidinooxy*-^{14}C]Canavanine and [^{14}C]-Urea. L-[*guanidinooxy*-^{14}C]Canavanine and [^{14}C]urea were measured by treating appropriate samples with an excess of jack bean leaf arginase–urease (Rosenthal and Thomas, 1985). The evolved $^{14}CO_2$, derived from guanidinooxy-labeled canavanine via the sequential action of arginase and urease, was trapped in hydroxide of hyamine and counted by liquid scintillation spectroscopy. A comparable sample was treated only with jack bean urease; the resulting $^{14}CO_2$ was trapped as above and measured to determine the [^{14}C]urea. The difference in the $^{14}CO_2$ values obtained from the above assays provided the $^{14}CO_2$ attributed *solely* to [^{14}C]canavanine catabolism (Rosenthal and Thomas, 1985). All analyses were carried out in duplicate.

Determination of [^{14}C]Guanidine. The supernatant solutions from the L-[*guanidinooxy*-^{14}C]canavanine-treated larvae were adjusted to pH 3.5 with 1 N HCl, and 1 mL was applied to a 7.5 × 25 mm Dowex 50(NH_4^+) column and washed with 5 bed volumes of deionized H_2O. The column, treated with 10 bed volumes of 50 mM NH_4OH to remove any [^{14}C]canavanine, was then developed with 10 bed volumes of 200 mM ammonia. The latter removed any L-[*guanidino*-^{14}C]arginine that may have formed from L-[*guanidinooxy*-^{14}C]canavanine by transamidination. The 200 mM effluent fraction was treated with jack bean leaf arginase–urease and any radioactive CO_2 collected to determine the amount of L-[*guanidino*-^{14}C]arginine. Finally, the column was treated with 5 bed volumes of 2 N NaOH to remove [^{14}C]guanidine. The final eluant was taken to neutrality with 1 N HCl, diluted to 50 mL, and counted by liquid scintillation spectroscopy. The identification of [^{14}C]guanidine was confirmed by assaying the spent effluent from a Dionex D-300 automated amino acid analyzer in which the run was conducted without ninhydrin. The spent effluent, collected at 2-min intervals, was assayed by using the diacetyl reaction (Bonas et al., 1978) and liquid scintillation spectroscopy.

Homoserine Analysis. Samples for homoserine analysis were lactonized and processed on Dowex 50(NH_4^+) as described by Rosenthal and Berge (1989). All homoserine determinations were conducted by automated amino acid analysis.

RESULTS

H. virescens is effective in avoiding the adverse biological effects of L-canavanine consumption (Berge et al., 1986). It achieves this because of its ability to catabolize this potentially deleterious natural product. Possession of this ability raises the question of whether its metabolic capacity to detoxify canavanine is part of its constitutive metabolism or rather is induced in response to larval exposure to canavanine.

To distinguish between these two possibilities, three lines of experimental evidence were pursued. First, we analyzed the canavanine clearance rate for parenterally injected canavanine, using larvae that were maintained on a 150 mM canavanine containing diet for 48 h as compared to larvae maintained on a canavanine-free diet (Table I). These analyses revealed that canavanine cleared from the canavanine-fed larvae with a $t_{1/2}$ of 138 min. This was the same $t_{1/2}$ value as larvae that were not exposed to canavanine. We reasoned that if the metabolic capacity to detoxify canavanine was inducible, then the larvae exposed to canavanine for 48 h should have had an enhanced ability to catabolize this nonprotein amino acid relative to the control larvae.

In a similar vein, we compared the canavanine clearance rate in control larvae with organisms whose ability to synthesize proteins was impeded by treatment with 250 μg of cycloheximide (per gram of fresh larval weight). Parallel studies of similarly treated larvae disclosed that

Table I. Canavanine Clearance Rate for *H. virescens* following 48 h on Canavanine-Containing or Canavanine-Free Diets[a]

treatment	$t_{1/2}$ for canavanine clearance, min
canavanine-free diet	133 ± 4
150 mM canavanine diet	138 ± 6

[a] Each value is the mean ± SEM. See text for additional experimental details.

Table II. Effect of Inhibiting de Novo Protein Synthesis on Canavanine Clearance by *H. virescens*[a]

treatment	$t_{1/2}$ for canavanine clearance, min
sterile water	119 ± 5
cycloheximide	121 ± 4

[a] Each value is the mean ± SEM. See text for additional experimental details.

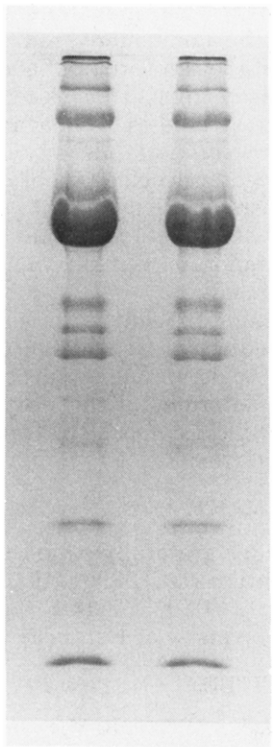

Figure 1. Electrophoretic profiles of *H. virescens* hemolymph proteins from larvae exposed to control diet (A) or 150 mM canavanine diet (B) for 72 h.

cycloheximide inhibited nearly 80% of the L-[^{3}H]leucine-labeled protein production noted with the control animals. Under these experimental conditions, the $t_{1/2}$ for canavanine clearance for the cycloheximide-treated larvae was 121 min; this value was the same for the control insects (Table II). Thus, neither significant inhibition of larval protein synthesis nor long-term exposure to canavanine affected the rate at which *H. virescens* catabolized canavanine.

Finally, gel electrophoretic analysis of extracts of the gut, fat body, and hemolymph of *H. virescens* larvae maintained on the 150 mM canavanine containing diet for 3 days did not disclose a novel protein band resulting from canavanine consumption (Figure 1). Administration of L-[^{35}S]methionine to comparable canavanine-treated animals failed to indicate de novo synthesis of a novel protein produced uniquely by the canavanine-treated larvae.

Table III. Incorporation of L-[^{35}S]Methionine into Hemolymph Proteins by *H. virescens* Larvae Exposed to Dietary Canavanine[a]

treatment	[^{35}S]methionine/mg of hemolymph protein, Bq
control diet	213.8 ± 20.8
150 mM canavanine	209.5 ± 21.5

[a] Each value is the mean ± SEM. See text for additional experimental details.

Table IV. Incorporation of L-[^{35}S]Methionine into Hemolymph Proteins by *H. virescens* Larvae Given Parenterally Injected Canavanine[a]

treatment	[^{35}S]methionine/mg of hemolymph protein, Bq
sterile water	208.1 ± 20.8
5 mg/g canavanine	213.3 ± 10.4

[a] Each value is the mean ± SEM. See text for additional experimental details.

Exposure of larvae of the tobacco hornworm, *Manduca sexta*, to canavanine resulted in a marked stimulation of protein synthesis (Rosenthal and Dahlman, 1986). Administration of L-[^{3}H]leucine, 30 min after providing canavanine, increased the amount of radiolabeled protein by 67% (Rosenthal and Dahlman, 1988). This observation was confirmed in larvae of the meat-eating fly, *Phormia terranovae*, where canavanine caused a 37% increase in the level of tritiated leucine-containing proteins (Rosenthal et al., 1989a). These experiments with canavanine-sensitive insects stand in sharp contrast to the [^{35}S]-methionine-feeding studies with *H. virescens*. The latter experiments failed to show any canavanine-mediated enhancement in protein synthesis. As a result, we examined both the effect of dietary canavanine and canavanine provided by parenteral injection on the production and accumulation of [^{35}S]methionine-labeled proteins. Neither canavanine provided in the diet (Table III) nor that provided by parenteral injection (Table IV) affected the level of radiolabeled hemolymph protein. Thus, this evidence suggested that in *H. virescens* larvae canavanine did not affect protein synthesis.

To elucidate the metabolic disposition of canavanine in this generalist herbivore, we analyzed the catabolism of L-[*guanidinooxy*-^{14}C]canavanine. Parenteral injection of L-[*guanidinooxy*-^{14}C]canavanine, provided with 5 mg/g (fresh body weight) carrier L-canavanine, confirmed the pronounced ability of *H. virescens* to metabolize this non-protein amino acid (Table V). The zero-time samples contained 100% unmetabolized canavanine, whereas over 91% of the administered radiolabeled canavanine was metabolized within 12 h.

Sequential hydrolyses of L-[*guanidinooxy*-^{14}C]canavanine by arginase and urease release stoichiometric amounts of [^{14}C]CO_2. Assay of [^{14}C]CO_2 formation by canavanine-treated larvae established that only trace amounts of L-[*guanidinooxy*-^{14}C]canavanine were degraded by the $^{14}CO_2$-producing reactions of the above hydrolytic pathway. Assay of the arginase and urease of *H. virescens* indicated that the larvae possess weak arginase activity but virtually no urease activity. The absence of urease prevents the functioning of this hydrolytic pathway and undoubtedly accounts for the paucity of [^{14}C]CO_2. After injection of canavanine, the larvae typically regurgitate a small volume of brown liquid, apparently in response to canavanine injection and handling. This oral exudate was combined with the larval extract in determining the metabolic disposition of the administered canavanine. This material

Table V. Metabolism of L-[*guanidinooxy*-^{14}C]Canavanine by *H. virescens*[a]

fraction or substance	% ^{14}C recovered				
	1 h	2 h	4 h	6 h	12 h
canavanine	81.8 ± 0.6	67.1 ± 3.4	48.2 ± 1.3	32.1 ± 1.7	8.6 ± 5.6
guanidine	14.0 ± 2.0	22.8 ± 3.8	43.5 ± 6.4	53.5 ± 6.5	72.3 ± 8.3
urea	1.6 ± 0.4	3.7 ± 0.3	7.2 ± 0.5	7.6 ± 1.2	8.7 ± 1.0
respiratory $^{14}CO_2$					0 ± 0.0
total ^{14}C recovered	97.9	94.0	99.3	93.6	90.1

[a] Each value is the mean ± SEM where $n = 3$. See text for additional experimental details.

did not result from leakage at the injection site since the wound was sealed immediately with beeswax.

A canavanine-treated larva produced only a few small fecal pellets that were desiccated and contained only a minute amount of radiolabeled canavanine. Fecal pellets, collected for 48 h after canavanine administration, contained less than 2% of the injected [^{14}C]carbon and only a trace of L-[*guanidinooxy*-^{14}C]canavanine.

The basic fraction associated with L-arginine in the 1–12-h samples possessed 0.5% of the total [^{14}C]carbon. Treatment with arginase and urease failed to produce any [^{14}C]CO_2, thereby eliminating even trace transamidination activity of L-canavanine to L-arginine as a mode of canavanine metabolism.

The primary degradation product of L-[*guanidinooxy*-^{14}C]canavanine was [^{14}C]guanidine. Within the 12-h experimental group, [^{14}C]guanidine accounted for 72% of the metabolized L-[*guanidinooxy*-^{14}C]canavanine (Table V). Independent analysis of homoserine catabolism by *H. virescens* established that this amino acid was cleared with a $t_{1/2}$ of 191 ± 18 min (mean ± SEM, where $n = 2$), about 60 min longer than that for canavanine. The difference in the $t_{1/2}$ value between canavanine and homoserine permitted homoserine to accumulate in the larval hemolymph. Automated amino acid analysis disclosed the presence of homoserine in canavanine-treated larvae. Guanidine, however, was a metabolic end product.

DISCUSSION

H. virescens, while a major agricultural pest, normally does not attack such agronomically important canavanine-containing plants as clover, alfalfa, *Lespedeza*, and numerous trefoils. Yet, it exhibited a remarkable ability to tolerate levels of dietary or injected canavanine that would be lethal to canavanine-sensitive insects (Dahlman and Rosenthal, 1975). Analyses of canavanine detoxification in *H. virescens* indicated that this insect was drawing upon a metabolic detoxification capacity that was part of its constitutive metabolism and not induced in response to canavanine exposure. The presence of this metabolic detoxification capacity might enable this aggressive herbivore to process other potential toxicants and contributed to its ability to forage over a diversified array of higher plants (Lincoln, 1972).

Analysis of L-[*guanidinooxy*-^{14}C]canavanine catabolism by *H. virescens* larvae indicated that the principal pathway for L-canavanine was by reductive cleavage to L-homoserine and guanidine. This insect did not employ arginase- and urease-mediated sequential hydrolytic cleavages that would produce canaline and urea and then ammonia and carbon dioxide. Insects that eat canavanine-containing seeds, such as the bruchid beetle, *C. brasiliensis*, and the weevil, *S. tuberculatus*, have active arginase and pronounced urease activity (Bleiler et al., 1988). These insects rely upon these enzymes to mobilize the nitrogen stored in the guanidinooxy moiety of canavanine and utilize the released nitrogen to support their amino acid metabolism (Rosenthal, 1983). Likewise, plants that produce canavanine utilize arginase as their preeminent means for canavanine catabolism (Rosenthal et al., 1988). There is no evidence that any higher plant is capable of cleaving reductively L-canavanine to L-homoserine and guanidine (Rosenthal and Berge, 1989). In a similar manner, the rat also enjoys a marked ability to catabolize canavanine (Thomas and Rosenthal, 1987). Given a 2 mg/g dose ip of L-[*guanidinooxy*-^{14}C]canavanine, this mammal will excrete 75% of the administered radiolabeled carbon as urea; only 5% of the recovered radiolabeled carbon is in the form of [^{14}C]guanidine (Thomas and Rosenthal, 1987).

In sharp contrast, studies of canavanine catabolism in *Streptococcus faecalis* established that these prokaryotes rely upon reductive cleavage of canavanine to homoserine and guanidine (Kihara et al., 1957). Prior to our study, this reductive metabolic pathway had not been shown to be of importance in canavanine metabolism by eukaryotic organisms.

We are assessing presently the enzyme system responsible for reductive cleavage of canavanine in *H. virescens* larvae to determine its substrate specificity. It is important to determine if this enzyme can serve the insect in its chemical defense strategy against a broad spectrum of plant toxins.

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