Experientia 43 (1987), Birkhäuser Verlag, CH-4010 Basel/Switzerland

# Full Papers

## Aberrant, canavanyl protein formation and the ability to tolerate or utilize L-canavanine

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*Summary.* L-Canavanine, 2-amino-4-(guanidinooxy)butyric acid, and L-arginine incorporation into de novo synthesized proteins was compared in six organisms. Utilizing L-[guanidinooxy$^{14}$C]canavanine and L-[guanidino$^{14}$C]arginine at substrate saturation, the canavanine to arginine incorporation ratio was determined in de novo synthesized proteins. *Caryedes brasiliensis* and *Sternechus tuberculatus,* canavanine utilizing insects; *Canavalia ensiformis,* a canavanine storing plant; and to a lesser extent *Heliothis virescens,* a canavanine resistant insect, failed to accumulate significant canavanyl proteins. By contrast, *Manduca sexta,* a canavanine-sensitive insect, and *Glycine max,* a canavanine free plant, readily incorporated canavanine into newly synthesized proteins. This study supports the contention that the incorporation of canavanine into proteins in place of arginine contributes significantly to canavanine's antimetabolic properties.

*Key words.* L-Canavanine; *M. sexta; H. virescens; C. brasiliensis; S. tuberculatus; G. max; C. ensiformis;* aberrant protein production.

### *Introduction*

L-Canavanine, a nonprotein amino acid found in hundreds of leguminous plants[1,2] is related structurally to L-arginine.

$$H_2N\text{–}C(NH_2) = N\text{–}O\text{–}CH_2\text{–}CH_2\text{–}CH(NH_2)CO_2H$$
L-canavanine

This natural product typically functions in enzyme-mediated reactions for which arginine is the preferred substrate[3]. Arginyl tRNA synthetase-mediated esterification of $tRNA^{Arg}$ to canavanine[4,5] ultimately produces structurally altered canavanyl proteins. The guanidinooxy moiety of canavanine has a $pK_a$ value of 7.01[6], substantially less than the $pK_a$ value of 12.48 for the guanidino group of arginine[7]. Since canavanine is significantly less basic than arginine, canavanine substitution for arginine in proteins can disrupt R group interactions essential for maintaining three dimensional protein conformation. Canavanine incorporation into protein not only generates an aberrant macromolecule, it can also impair its function. Evidence is mounting that the formation of aberrant, canavanine-containing proteins may be a principal basis for its antimetabolic properties in canavanine-sensitive organisms[8,9]. To evaluate this important point, two experimental approaches are being pursued. One consists of a direct examination of the effect of canavanine incorporation on the functional parameters of proteins purified from control and canavanine-treated insects. As this work is very time-consuming and progress slow, an indirect approach is also justified. This approach involves a comparative examination of canavanine-sensitive and canavanine-resistant organisms to compare canavanine incorporation into protein by these disparate groups. For, it is reasonable to assert that canavanyl protein formation carries significant biological consequences if canavanine-resistant organisms do not incorporate it into de novo-synthesized proteins while canavanine-sensitive organisms cannot avoid canavanyl protein production.

The tobacco hornworm, *Manduca sexta* (Sphingidae) is canavanine-sensitive: canavanine consumption causes a wide array of detrimental biological effects[10,11]. In contrast, the tobacco budworm, *Heliothis virescens* (Noctuidae), which does not feed on canavanine-containing plants, possesses remarkable resistance to canavanine[12]. The neotropical deciduous forest of Costa Rica is home to two insects that feed on canavanine containing seeds[13]. The bruchid beetle, *Caryedes brasiliensis* (Bruchidae) consumes seeds of *Dioclea megacarpa* (Leguminosae) which typically contain 8–9% canavanine, but can reach 13% of the seed dry weight[13]. This seed predator has adapted very effectively to its canavanine-laden food[14,15]. The other neotropical insect is a weevil, *Sternechus tuberculatus* (Curculionidae). This beetle feeds on the legume *Canavalia brasiliensis,* the seeds of which contain 6–8% canavanine by dry weight[13].

Canavanine toxicity and aberrant protein production can also be addressed through plant-based studies of canavanine-incorporation into protein. The jack bean, *Canavalia ensiformis* (Leguminosae) stores 3.5% canavanine by dry weight in the seed, whereas the soy bean, *Glycine max* (Leguminosae) a canavanine-free plant, provides a control organism for plant-based studies of canavanine incorporation into proteins. To evaluate the relationship of aberrant protein synthesis to an organism's ability to tolerate or utilize canavanine, canavanyl protein production has been compared in these six organisms.

### *Materials and methods*

*Materials. Caryedes brasiliensis* and *Sternechus tuberculatus* were field collected in Santa Rosa National Park, Costa Rica during the winter of 1984–1985. Terminal instar larvae were obtained from infested *Dioclea megacarpa* and *Canavalia brasiliensis* seeds, respectively. *Manduca sexta* and *Heliothis virescens* were secured from continuous colonies maintained at the University of Kentucky. Terminal, fifth instar larvae that had ecdysed one

day previously were used in all experiments. Insects were reared as described previously[10,12]. *Canavalia ensiformis* and *Glycine max* were grown under greenhouse conditions from hydrated seeds for 10 or 14 days, respectively[16].

L-[Guanidinooxy-$^{14}$C]canavanine (58 μCi/μmol) was prepared by the method of Ozinskas and Rosenthal[17]. L-[Guanidino-$^{14}$C]Arginine (55 μCi/μmol] was obtained from New England Nuclear, Boston, Mass. L-Canavanine, as the free-base, was isolated from acetone-defatted jack bean seeds[18]. The TS-1 tissue solubilizer was obtained from Research Products International Corp., Mount Prospect, Il. All remaining biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

*Methods. Canavanine and arginine administration.* In *C. brasiliensis* and *S. tuberculatus* larvae, the average canavanine and arginine concentration was determined by automated amino acid analysis of deproteinized whole-body larval extracts. The canavanine to arginine ratio dictated the ratio of the administered dose of L-[guanidinooxy-$^{14}$C]canavanine and L-[guanidino-$^{14}$C]arginine in that the specific activity of each amino acid was set equal. The same procedure was used for *M. sexta* and *H. virescens* except that deproteinized hemolymph was used to determine endogenous arginine and sufficient carrier canavanine was provided to these canavanine-free larvae such that, at the time of injection, both the specific activity and molar concentration of larval canavanine and arginine were equalized. All compounds were administered by parenteral injection after relaxing the *M. sexta* larvae by exposure to 4 °C and *H. virescens* larvae to carbon dioxide. At the indicated time, the treated larvae were sacrificed and samples stored at −60 °C.

The average canavanine content of *Canavalia ensiformis* was determined by automated amino acid analysis of the deproteinized extract of 10-day-old plants minus the cotyledons. This value (25 μmol/plant) determined the carrier canavanine provided to 14-day-old *Glycine max* in comparing canavanine incorporation into plant proteins. L-[guanidinooxy-$^{14}$C]canavanine (1.5 μCi/plant) was administered 1–2 mm below the cotyledons, the seed storage organ, by direct injection into the stem. The cotyledons were removed prior to injection to minimize canavanine degradation. At 7 h post injection, the treated plants were collected and stored at −60 °C.

*Preparation of biological extracts.* Whole-body extracts of *Caryedes brasiliensis* and *Sternechus tuberculatus* were prepared as described previously[14]. In contrast, it was feasible to analyze separately the hemolymph and various body tissues of *Manduca sexta* and *Heliothis virescens*. The latter group was anesthetized, drained of hemolymph, and sliced open. Hemolymph was added to an equal volume of 50% trichloroacetic acid (TCA) solution. The fat body was removed from the body wall and each was rinsed with physiological saline. All tissues were mechanically homogenized, precipitated with TCA and prepared as described earlier[12]. Details on the preparation of whole-plant extracts were presented elsewhere[19]. In all cases, the radiolabeled proteins of the biological extract were precipitated and then washed three times with 10% TCA (w/v), followed once each with absolute ethanol-anhydrous ether (v/v,1:1), and anhydrous ether. The precipitated proteins were collected by centrifugation at 12,000 × g for 12 min, and dried in vacuo at 45–50 °C overnight. Trichloroacetic acid-precipitated materials (1–4 mg) were dissolved in 0.3 ml commercially prepared TS-1 tissue solubilizer and the radioactivity determined by liquid scintillation spectroscopy[20].

To determine the relative contribution of L-[guanidinooxy-$^{14}$C]canavanine or L-[guanidino-$^{14}$C]arginine to the total $^{14}$carbon content, samples of the radiolabeled proteins of the six test organisms were acid hydrolyzed. The radiolabeled hydrolysate was subjected to ion-exchange chromatography to separate L-[guanidinooxy-$^{14}$C]canavanine from L-[guanidino-$^{14}$C]arginine[21]. The $^{14}$carbon content of these amino acids was then deter-

Table 1. Incorporation of L-[guanidinooxy-$^{14}$C]canavanine or L-[guanidino-$^{14}$C] arginine into de novo-synthesized proteins

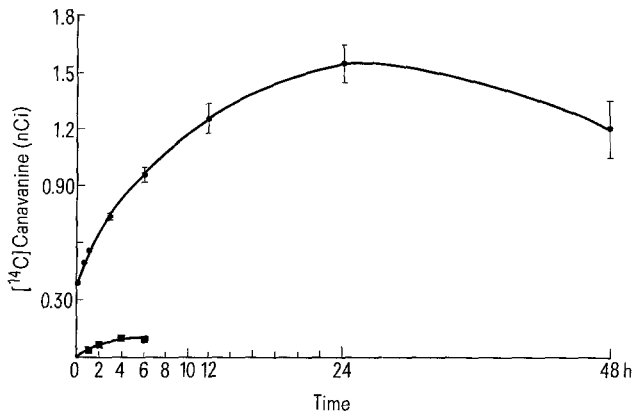
| Insect | Amino acid | Amino acid incorporation (% of administered dose) | | |
|---|---|---|---|---|
| | | Hemolymph | Fat body | Body wall |
| *Manduca sexta* | Arginine | 17.2 | 1.37 | 0.91 |
| | Canavanine | 3.0 | 0.51 | 0.43 |
| *Heliothis virescens* | Arginine | 0.75 | – | – |
| | Canavanine | < 0.01 | < 0.01 | < 0.01 |

The larvae received 3.0 μCi of $^{14}$C-labeled amino acid with 1 mg carrier L-canavanine/g fresh b. wt in *Manduca sexta* or 5 mg/g in *Heliothis virescens*. *Manduca sexta* hemolymph was collected 24 h post-injection while comparable samples were taken from *Heliothis virescens* after 4 h. Each value is the mean of 2 independent determinations.

Table 2. Comparative incorporation of L-[guanidinooxy-$^{14}$C]canavanine and L-[guanidino-$^{14}$C]arginine into larval proteins

| Insect | $^{14}$C-arginine to $^{14}$C-canavanine incorporation ratio |
|---|---|
| Canavanine-utilizing | |
| *Caryedes brasiliensis* | 365:1* |
| *Sternechus tuberculatus* | > 500:1 |
| Canavanine-resistant | |
| *Heliothis virescens* | 75:1 |
| Canavanine-sensitive | |
| *Manduca sexta* | 3.3:1 |

Radiolabeled proteins were obtained from whole larval extracts as described in the text. Each value is the mean of two independent determinations. * Data of Rosenthal (1983).




Incorporation of L-[guanidinooxy$^{14}$C]canavanine into *de novo*-synthesized hemolymph proteins. Each value for *Manduca sexta* (●) represents the mean and standard error of 3 samples, while *Heliothis virescens* (■) represents 2 samples. The labeled hemolymph proteins were collected and processed as described in the text.

Experientia 43 (1987), Birkhäuser Verlag, CH-4010 Basel/Switzerland

mined by a radiometric assay involving enzymatic hydrolyses that mediate the release of $^{14}$carbon as $^{14}CO_2$; the latter is trapped quantitatively[21].

## Results

*Insect-based studies.* Larvae of the canavanine-sensitive tobacco hornworm, *Manduca sexta* readily incorporate L-[guanidinooxy-$^{14}$C]canavanine into de novo-synthesized hemolymph proteins (fig). $^{14}$C-Canavanine-labeled hemolymph protein content increases for 24 h after canavanine administration, but declines gradually during the subsequent day. Canavanyl hemolymph protein production exceeds that depicted in the figure since the larvae possess an active system for preferential degradation of aberrant proteins; removal of canavanyl hemolymph proteins by larval *Manduca sexta* is 2–4 times faster than the breakdown of their normal counterparts[20,22]. In contrast, formation of [$^{14}$C]canavanine-labeled hemolymph proteins by *Heliothis virescens* larvae is maximized within 4 h and there is considerably less canavanine incorporated into its proteins relative to *Manduca sexta* (fig.).

To compare more fully the ability of *Manduca sexta* and *Heliothis virescens* larvae to assimilate canavanine into protein relative to arginine, three further protein pools were examined. *M. sexta* placed 17.2% of the administered L-[guanidino-$^{14}$C]arginine and 3.0% of the L-[guanidinooxy-$^{14}$C]canavanine into newly formed hemolymph proteins (table 1). Thus, under conditions in which the larval concentration of each amino acid is equalized, the ratio of incorporated canavanine to arginine is 1:5.7. Similar evaluations of the newly produced proteins of the fat body and body wall (the thoracic musculature and integument) reveal an incorporation ratio of 1:2.7 and 1:2.1, respectively (table 1). These analyses establish that *Manduca sexta* larvae can effectively utilize canavanine in place of arginine in de novo protein synthesis.

Comparable data obtained with *Heliothis virescens* present a strikingly different picture. Analysis of larval hemolymph proteins indicates a canavanine to arginine incorporation ratio in excess of 1:75. Moreover, the amount of canavanine incorporated into de novo-synthesized protein of the fat body and body wall is so minute as to be just detectable (table 1). It is apparent that the canavanine-resistant larvae of *Heliothis virescens* fail to produce significant amounts of canavanine-containing proteins.

It is reasonable to hypothesize that an insect that has adapted to the consumption of significant dietary canavanine would exhibit an ability to circumvent canavanyl protein production, if such aberrant protein formation carries significant deleterious biological effects. A direct comparison of whole-body, larval extracts of *Manduca sexta, Heliothis virescens, Caryedes brasiliensis,* and *Sternechus tuberculatus* discloses that canavanine-resistant and -utilizing larvae have a pronounced ability to avoid canavanyl protein production (table 2). Overall, only 1 molecule of canavanine is fixed into protein for every 365 molecules of arginine for the proteins of *Caryedes brasiliensis*[23]. *Sternechus tuberculatus,* an insect discovered recently to consume canavanine-containing seeds, has an even greater discriminatory capacity. The canavanine to arginine incorporation ratio is not more than 1 to 500 and it may approach 1 to 1000[13]; it is not feasible to measure accurately such minute levels of canavanine incorporation[21]. Thus, the canavanine to arginine incorporation ratio for *Heliothis virescens,* a canavanine-resistant but non-utilizing herbivore, is appreciably less than that observed with canavanine-consuming insects but still significantly greater than the ratio noted with the non-canavanine-adapted *Manduca sexta.*

*Plant-based studies. Glycine max* does not store detectable canavanine[9]; administration of canavanine to 14-day-old plants results in appreciable labeling of de novo-synthesized proteins (table 3). A concentration-dependent formation of $^{14}$C-labeled protein is observed over the range of 10–50 µmoles. Treated soy bean readily incorporate canavanine into newly formed proteins (table 4). Nearly 25 nmoles of L-[guanidinooxy-$^{14}$C]canavanine are present in each mg of soluble protein obtained from canavanine-treated plants. This compares to less than 0.2 nmoles $^{14}$C-labeled canavanine/mg soluble protein for comparable materials from jack bean (table 4). Therefore, under these experimental conditions, soy bean plants incorporate at least 125 times more radioactive canavanine into protein than that observed with jack bean plants.

In the event that $^{14}$C-labeled canavanine does not equilibrate fully with endogenous canavanine, 25 µmoles of carrier canavanine were also injected into jack bean plants. Exogenously supplied canavanine did not significantly effect radioactive canavanine incorporation into proteins.

Table 3: Incorporation of L-[guanidinooxy-$^{14}$C]canavanine into de novo synthesized proteins of *Glycine max*

| Canavanine (µmoles) | Canavanine incorporation (nmol/mg soluble protein) |
|---|---|
| 0 | 0.20 |
| 10 | 12.1 |
| 25 | 28.2 |
| 50 | 47.2 |

Soy bean plants were injected with 1.5 µCi L-[guanidinooxy-$^{14}$C]-canavavine with the indicated amount of carrier canavanine. Each treatment used 3 plants that were pooled for a single determination; the values are the mean of 2 independent determinations.

Table 4. Comparative incorporation of L-[guanidinooxy-$^{14}$C]canavanine into *Canavalia ensiformis* and *Glycine max*

| Plant | $^{14}$C-Canavanine incorporation (nmol/mg soluble protein) |
|---|---|
| *Canavalia ensiformis* | 0.2 |
| *Glycine max* | 24.7 |

Each treatment used 3 jack bean or 6 soy bean plants that were pooled for a single determination; the values are the mean of 2 independent determinations.

## Discussion

Canavanine and arginine incorporation into newly formed proteins of six test organisms have been compared. These analyses reveal that canavanine-utilizing insects and canavanine-storing plant do not accumulate substantive aberrant, canavanyl proteins. This property is also exhibited by *Heliothis virescens,* a canavanine-resistant insect, but to a lesser extent than that noted with *Caryedes brasiliensis* and *Sternechus tuberculatus.* In contrast, the canavanine-sensitive larvae of *Manduca sexta*

and the canavanine-free *Glycine max* readily incorporate canavanine into de novo-synthesized proteins and both organisms accumulate appreciable anomalous canavanyl proteins.

The above findings provide consistent evidence to support the view that canavanine consumption and/or resistance is associated with the lack of canavanyl proteins. This property may result from an ability to avoid canavanyl protein production or from the capacity to degrade preferentially aberrant proteins. Anomalous insect proteins are degraded 2–4 times faster than their normal counterparts[20]. This differential degradation rate cannot account for the minute amount of canavanyl proteins found in *Caryedes brasiliensis* or *Sternechus tuberculatus*[8]. It is far more reasonable to ascribe the lack of canavanyl proteins to diminished canavanyl protein synthesis.

Studies of the relationship of canavanine to protein synthesis in *Manuca sexta* larvae reveal that canavanine not only fails to inhibit incorporation of [$^3$H]leucine into de novo-synthesized proteins but rather increases the amount of [$^3$H]leucine-containing proteins[20]. Thus, canavanine-utilizing insects must selectively control canavanine-dependent aspects of protein formation. An ability to curtail canavanine-dependent protein production would most likely result from an arginyl-tRNA synthetase possessing an aminoacylating activity that is discriminatory between canavanine and arginine. Alternatively, canavanyl-tRNA$^{Arg}$ may form, but this novel metabolite may be effectively degraded prior to mRNA translation or it may exhibit a diminished ribosomal binding capacity. Thus, while alternatives exist, it is most reasonable to propose that canavanine-utilizing insects possess an arginyl-tRNA synthetase that can distinguish between canavanine and arginine, and thereby avoid canavanine acylation without curtailing arginine-dependent protein production.

The canavanine-storing jack bean catabolizes canavanine via arginine-utilizing enzymes. These enzymes are distinct in their high affinity for and rapid turnover with canavanine relative to the same enzymes of the canavanine-free soy bean[4]. Thus, the evolution of an active site able to respond differentially to and thereby discriminate between L-arginine and L-canavanine may be a key factor in animal and plant adaptation to canavanine. The above contention predicts that a canavanine-resistant insect that does not use this nonprotein amino acid nor presumably is adapted to it, would nevertheless avoid aberrant, canavanyl protein production. Discovery of the canavanine-resistant larvae of *Heliothis virescens* provided the first test system to evaluate this point. Detailed analysis of arginine- and canavanine-dependent de novo protein synthesis disclosed the appreciable ability of this insect to avoid canavanyl protein production. *Heliothis virescens* has a pronounced ability to detoxify canavanine; the half-life of parenterally injected canavanine (5 mg/g fresh larval weight) is 135 min[12]. Its rapid canavanine catabolism may contribute to or account for its limited canavanyl protein synthesis.

In summary, it is proposed that the formation of aberrant, canavanyl proteins is avoided or circumvented in organisms that use, produce and store, or are resistant to this potentially toxic nonprotein amino acid. Those organisms that are either not adapted to canavanine or are sensitive to its potentially deleterious biological effects produce appreciable canavanyl protein. Until a canavanine-resistant organism is found that also produces significant canavanyl proteins, the available evidence fully supports the contention that the incorporation of canavanine into proteins carries adverse biological effects.

Acknowledgments. The authors acknowledge gratefully support by the National Science Foundation (grant PCM-82-19721), and the National Institutes of Health (grant DK-17322).

1 Bell, E. A., Lackey, J. A., and Pohill, R. M., Biochem. Ecol. System *6* (1978) 201.
2 Van Etten, C. H., Kwolek, W. F., Peters, J. E., and Barclay, A. S., J. Agric. Fd Chem. *15* (1967) 1077.
3 Rosenthal, G. A., Q. Rev. Biol. *52* (1977) 155.
4 Downum, K. R., Rosenthal, G. A., and Cohen, W. S., Pl. Physiol. *73* (1983) 965.
5 Mitra, S. K., and Mehler, A. H., J. biol. Chem. *242* (1967) 5490.
6 Boyar, A., and Marsh, R. E., J. Am. chem. Soc. *104* (1982) 1995.
7 Greenstein, J. P., and Winitz, M., Chemistry of the Amino Acids. John Wiley & Sons, Inc., New York 1961.
8 Rosenthal, G. A., J. chem. Ecol. *12* (1986) 1145.
9 Rosenthal, G. A., and Dahlman, D. L., Experientia *38* (1982) 1034.
10 Dahlman, D. L., and Rosenthal, G. A., J. Insect Physiol. *22* (1976) 265.
11 Rosenthal, G. A., and Dahlman, D. L., Comp. Biochem. Physiol. *52A* (1975) 105.
12 Berge, M. A., Rosenthal, G. A., and Dahlman, D. L., Pest. Biochem. Physiol. *25* (1986) 319.
13 Bleiler, J. A., Rosenthal, G. A. and Janzen, D. H., Ecology 1987 (in press).
14 Rosenthal, G. A., and Dahlman, D. L., and Janzen, D. H., Science *192* (1976) 256.
15 Rosenthal, G. A., J. chem. Ecol. *9* (1983) 1353.
16 Rosenthal, G. A., Pl. Physiol. *69* (1982) 1066.
17 Ozinskas, A. J., and Rosenthal, G. A., Bioorg. Chem. *14* (1986) 157.
18 Rosenthal, G. A., Analyt. Biochem. *77* (1977) 147.
19 Rosenthal, G. A., and Rhodes, D. L., Pl. Physiol. *76* (1984) 541.
20 Rosenthal, G. A., and Dahlman, D. L., Proc. natn. Acad. Sci. USA *83* (1986a) 14.
21 Rosenthal, G. A., and Thomas, D. A., Analyt. Biochem. *147* (1985) 428.
22 Rosenthal, G. A., and Dahlman, D. L., J. Insect Biochem. (1986b) in review.
23 Rosenthal, G. A., Sci. Am. *249* (1983) 164.

0014-4754/87/050558-04$1.50 + 0.20/0