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AVOIDANCE OF NONPROTEIN AMINO ACID INCORPORATION INTO PROTEIN BY THE SEED PREDATOR, *Caryedes brasiliensis* (BRUCHIDAE)

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Abstract—Larvae of the bruchid beetle, *Caryedes brasiliensis* (Bruchidae) have the ability to avoid significant incorporation of L-canavanine, the guanidinooxy structural analog of L-arginine, into de novo synthesized proteins. This ability is related to a highly discriminatory protein-synthesizing system which exhibits marked ability to avoid processing an array of nonprotein amino acids structurally related to arginine.

Key Words—Canavanine, *Caryedes brasiliensis*, Coleoptera, Bruchidae, *Dioclea megacarpa*, plant–insect interactions, amino acids.

INTRODUCTION

The seed of the neotropical legume, *Dioclea megacarpa* Rolfe is remarkably free of predation by insects (Janzen, 1971). This freedom from attack results in part from massive seed storage of L-canavanine, a highly toxic allelochemical and structurally analog of L-arginine that accounts for more than 95% of the nitrogen allocated to seed free amino acids (Rosenthal, 1977). Formation of structurally aberrant, canavanine-containing proteins is an important biochemical basis for the antimetabolic properties of this anginine antagonist (Rosenthal, 1982a). Bruchid beetle larvae, *Caryedes brasiliensis* (Bruchidae) develop entirely within the seed of this usually toxic legume (Figure 1). This ability is due to their capacity to avoid production of canavanyl proteins (Rosenthal et al., 1976). In this report we provide a direct

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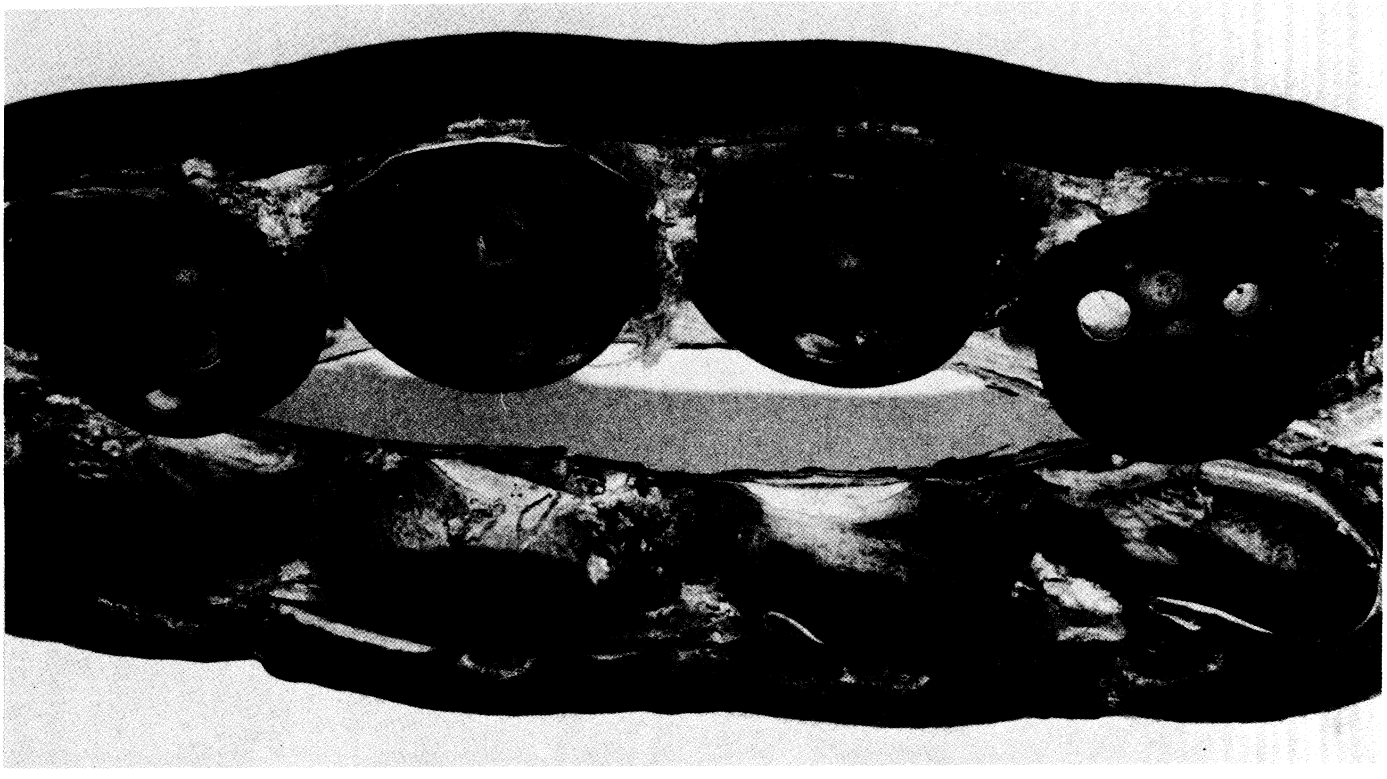


evaluation of the discriminatory capacity of the protein-synthesizing system of this insect and address the question of whether it provides broad-spectrum resistance to nonprotein amino acid incorporation into protein.

METHODS AND MATERIALS

Insects. The bruchid beetles used in this study were obtained from infected *Dioclea megacarpa* seeds collected in December, 1981 in Santa Rosa National Park, northwestern Guanacaste Province, Costa Rica. Tobacco hornworms, *Manduca sexta (L.)*(*Sphingidae*), were obtained from a colony maintained at the University of Kentucky.

Radioactive Amino Acids. The radioactive arginine analogs were prepared from ornithine, 5-hydroxylysine, lysine, canaline, 2,3-diaminopropionic acid, and 2-4-diaminobutyric acid by reaction with [^{14}C]*O*-methylisourea. This incorporated a radioactive ω-carbon atom into these compounds to create: [*guanidino*-^{14}C]arginine, [*guanidino*-^{14}C]5-hydroxyhomoarginine, [*guanidino*-^{14}C]homoarginine, [*guanidinooxy*-^{14}C]canavanine, [*guanidino*-^{14}C]2-amino-3-guanidinopropionic acid, and [*guanidino*-^{14}C-amino-4-guanidinobutyric acid, respectively (Figure 2).

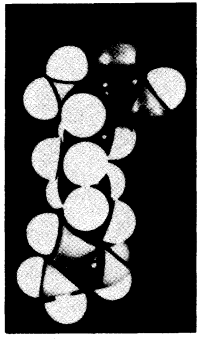
The free amino acids were reacted with excess CuO to form a copper salt protecting the α-NH_2 group from guanidination. Full details on the preparation of both [^{14}C]*O*-methylisourea from [^{14}C]$BaCO_3$ via labeled cyanamide and the various labeled amino acids have been published (Rosenthal *et al.*, 1983).

Amino Acid Administration. The amount of labeled canavanine or arginine injected into each bruchid beetle was adjusted to equalize the specific activity of each amino acid. Measurement of the free canavanine and arginine of these insects over the course of the experimental protocol revealed a lack of appreciable alteration in the relative pool size of these free amino acids.

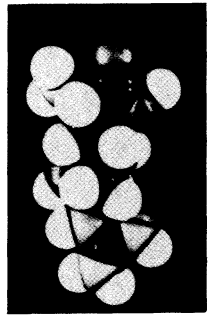
Unlike the bruchid beetle, terminal instar tobacco hornworm larvae grow significantly during 18 hr. In order to avoid dilution of the radioactive arginine provided to these larvae, the time from injection of the amino acid to insect grinding was reduced to 3 hr. Since terminal instar tobacco hornworm larvae do not synthesize canavanine, comparable initial specific activity for arginine and canavanine was achieved by providing carrier canavanine; this compound does not inhibit protein synthesis (Rosenthal, 1982a).

Analysis of Radioactive Proteins. Incorporation of radioactive amino acid into de novo synthesized insect protein was evaluated by injecting the

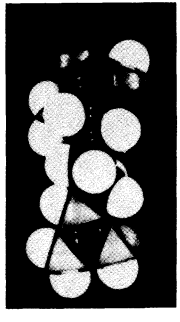
FIG. 1. Infected *Dioclea megacarpa* seeds. The matured fruit was opened to reveal the heavily infected seeds. The exit holes as well as several adult *Caryedes brasiliensis* can also be seen.

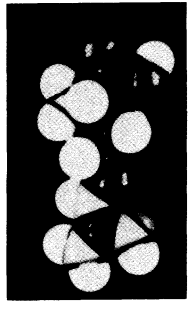


HOMOARGININE



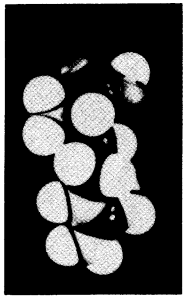
5-HYDROXYHOMOARGININE

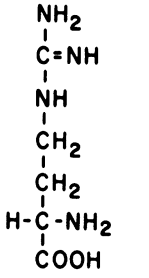




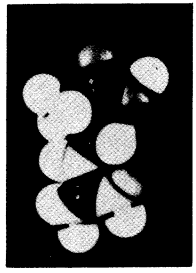
ARGININE

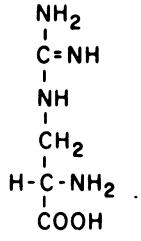
CANAVANINE





2-AMINO-4-GUANIDINOBUTYRIC ACID





2-AMINO-3-GUANIDINOPROPIONIC ACID

FIG. 2. CPK space-filling model of L-arginine and some of its structural analogs.

appropriate radioactive compound into the body of final instar larvae. The treated larvae were ground with 25 ml of 50 mM tris HCl buffer (pH 9.5) and kept at 37°C for 30 min to discharge amino acid-bound tRNA. After centrifugation at 13,000 *g* for 3 min to remove cellular debris, the supernatant solution was decanted into 6 ml of 50% (w/v) trichloroacetic acid. The insoluble materials were allowed to precipitate for 30 min at 3°C and then collected by centrifugation at 19,000 *g* for 15 min.

Trichloroacetic acid-precipitated materials were freed of unincorporated radioactive amino acids by four successive extractions with 25 ml of 5% (w/v) trichloroacetic acid and then processed once each with absolute ethanol-anhydrous ether (1:1, v/v) and anhydrous ether. After each extraction, the acid-insoluble materials were collected by centrifugation as above. The final pellet was air-dried, ground very finely, and dried in vacuo at 80°C. Protein incorporation values for the various amino acids were secured by acid hydrolysis of the radioactive protein and automated amino acid analysis coupled with liquid scintillation spectroscopy (Bray, 1960).

Procedures for automated amino acid analysis of the protein hydrolysate and evaluation of the spent ninhydrin-amino acid complex for its labeled carbon content have been thoroughly described (Rosenthal, 1982b).

Analysis of Acid Hydrolysate. Canavanine or arginine in the acid hydrolysate was isolated by ion-exchange chromatography. These radioactive amino acids were then subjected to combined enzymic cleavage with arginase and urease and the subsequent capture of the labeled, ω-carbon as $^{14}CO_2$. This procedure results in a stoichiometric conversion of L-[*guanidino*-^{14}C]arginine or L-[*guanidinooxy*-^{14}C]canavanine to $^{14}CO_2$ and unequivocally establishes the level of these amino acids (Rosenthal, 1982b).

RESULTS AND DISCUSSION

A comparative evaluation was made of the placement of L-[*guanidino*-^{14}C]arginine and L-[*guanidinooxy*-^{14}C]canavanine into the proteins of the bruchid beetle and the tobacco hornworm, *Manduca sexta* (Sphingidae). Of the labeled amino acids administered to the bruchid beetle, a considerable portion was diverted into respiratory $^{14}CO_2$, movement into non-amino acid pools, or fixation into various amino acids (Figure 3). Enzymatic evalutation of arginine and canavanine from the acid-hydrolyzed proteins of the bruchid beetle revealed incorporation of 347 nCi of arginine, from the 445 nCi of labeled material found in all protein amino acids, but only 0.95 nCi of canavanine (Figure 3). Thus, the ratio of labeled arginine to canavanine incorporated into the proteins of the bruchid beetle larvae was 347:0.95 or 365:1. This value provides a quantitative measure of the relatively poor ability

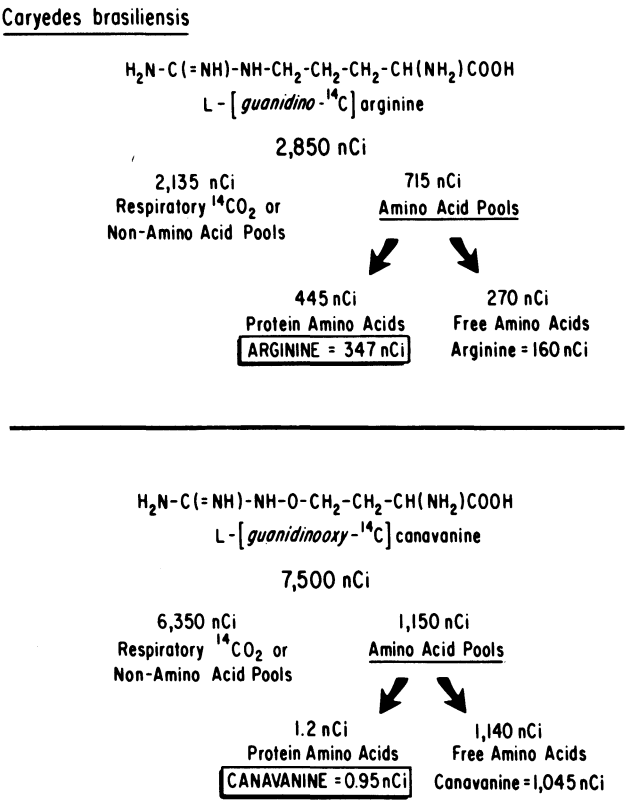


FIG. 3. Incorporation of labeled amino acid into soluble insect protein. Each terminal instar bruchid beetle larva (3.98 g fresh weight, $N = 30$) was administered 95 nCi arginine or 250 nCi canavanine and left for 18 hr. Newly ecdysed fifth stadium larvae of the tobacco hornworm (9.2 g fresh weight, $N = 3$) received 2500 nCi of L-[*guanidino*-^{14}C]arginine or L-[*guanidinooxy*-^{14}C]canavanine for 3 hr.

of canavanine as compared to arginine to support protein formation in this canavanine-resistant insect.

Comparable evaluations were made of the radioactive proteins of the larvae of the tobacco hornworm, an insect that does not feed on any canavanine-containing plant and which is highly susceptible to the deleterious action of canavanine. These determinations revealed an arginine to canavanine ratio of 5.6:1. These values provide a dramatic indication of bruchid beetle ability relative to that of the tobacco hornworm to avoid canavanyl protein formation. Such indirect evaluations are necessary since direct determinations with canavanine of the appropriate kinetic parameters

TABLE 1. INCORPORATION OF ARGININE AND CERTAIN OF ITS STRUCTURAL ANALOGS INTO SOLUBLE INSECT PROTEIN

Substrate[a]	^{14}C incorporation (pCi/mg soluble protein)	
	Manduca sexta	*Caryedes brasiliensis*
Arginine	20,278	12,393
Canavanine	3,611	34
Homoarginine	264	<5
5-Hydroxyhomoarginine	480	<5
2-Amino-4-guanidinobutyric acid	262	<5
2-Amino-3-guanidinopropionic acid	112	<5

[a]Arginine and canavanine were administered to the tested larvae as described in Figure 3; the remaining substrates were provided as indicated for canavanine.

of bruchid beetle arginyl tRNA synthetase is not feasible due to the minimal activity of canavanine as a substrate (Rosenthal et al., 1976).

Protein incorporation values for a series of structural analogs of arginine (Figure 2) were also tested with these insects. Tobacco hornworm larvae fixed each of the tested compounds into de novo synthesized proteins (Table 1). Both the immediately higher and lower arginine homolog, namely homoarginine and 2-amino-4-guanidinobutyric acid, were incorporated to an equivalent degree while the smallest of the tested molecules, 2-amino-3-guanidinopropioninc acid, was assimilated to a lesser extent. The presence of a hydroxyl group on the penultimate carbon of homoarginine increased its presence in protein relative to that of homoarginine (Table 1). Canavanine, which is the most toxic of the tested arginine analogs, is also most effectively assimilated into protein; this finding is consistent with existing evidence that canavanine toxicity is related to abnormal protein formation (Rosenthal, 1982a). In systems that fail to aminoacylate canavanine, this nonprotein amino acid is not demonstrably toxic (Rosenthal, 1982a).

Evaluation of the tested arginine analogs for their assimilation into bruchid beetle protein disclosed the acutely discriminatory nature of this insect's protein-synthesizing system (Table 1). The ability of this bruchid beetle to distinguish molecules structurally akin to arginine confers the biological benefit of a general resistance to the incorporation of such nonprotein amino acids into newly synthesized proteins.

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