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AMMONIA UTILIZATION BY THE BRUCHID BEETLE, *Caryedes brasiliensis* [BRUCHIDAE]

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Abstract—The seed predator, *Caryedes brasiliensis* [Bruchidae] generates appreciable ammonia in its dietary use and detoxification of L-canavanine and its catabolic product, L-canaline. L-Canavanine is a toxic allelochemical of *Dioclea megacarpa* seeds, the food of the developing larvae. Bruchid beetle larvae rely upon glutamic acid dehydrogenase and glutamine synthetase to use ammonia for glutamic acid synthesis from 2-oxoglutaric acid and conversion of the former to glutamine. These reactions provide the larvae with a means for metabolically eliminating ammonia. Proline serves as a carbon skeleton source for glutamic acid formation.

Key Words—Ammonia utilization, L-canavanine catabolism, *Caryedes brasiliensis*, Coleoptera, Bruchidae.

INTRODUCTION

The bruchid beetle, *Caryedes brasiliensis* [Bruchidae] inhabits the Neotropical deciduous forests of Costa Rica where it is the sole insect predator of the seed of the leguminous vine, *Dioclea megacarpa* (Janzen, 1971). This legume is distinctive in its storage of massive amounts of the highly insecticidal nonprotein amino acid, L-canavanine (Rosenthal, 1977a); it constitutes 55% of every nitrogen atom stored in the seed and accounts for about 95% of nitrogen allocation to free amino acid production (Rosenthal, 1977b).

Caryedes brasiliensis has achieved a number of biochemical adaptations that not only enable this seed consumer to utilize canavanine as a major dietary source of nitrogen (Rosenthal et al., 1982) but also to detoxify L-canaline, the highly deleterious breakdown product of canavanine utilization (Rosenthal,

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1978). *Caryedes brasiliensis* contains arginase (Rosenthal and Janzen, 1983), and it is also a prodigious urease producer (Rosenthal et al., 1976). These enzymes mediate canavanine conversion to canaline and urea and the breakdown of the latter to carbon dioxide and ammonia:

$$H_2N-(C-NH_2)=NH-\underset{\textit{L-canavanine}}{O-CH_2-CH_2-CH(NH_2)COOH} \rightarrow$$

$$H_2N-\underset{\textit{L-canaline}}{O-CH_2-CH_2-CH(NH_2)COOH}$$

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

L-Canaline, a potent toxin to insects (Rosenthal, 1982a), is detoxified by this seed predator by converting L-canaline to L-homoserine and ammonia (Rosenthal et al., 1978). Since canaline is the predominant reaction product of canavanine catabolism, deamination of canaline increases ammonia production from canavanine by almost one half.

How does this seed consumer deal with the appreciable ammonia generated by its consumption of L-canavanine? This is an important question since ammonia can be toxic to insects, particularly terrestrial forms (Chefurka, 1965), and relatively little is known of its metabolism. *Caryedes brasiliensis* eliminates its ammonia by excretion directly into its fecal matter as the ammonium salt of organic acids. Analysis of the contribution of ammonia, urea, and uric acid to the total nitrogen of the frass disclosed that uric acid accounts for only 11% of the fecal nitrogen (Rosenthal and Janzen, 1981). Does this seed predator also eliminate ammonia by metabolic reactions, and what are these reactions? Automated amino acid analyses of the hemolymph (the circulatory fluid) of bruchid beetle larvae revealed the presence of appreciable proline and glutamine. These hemolymph analyses suggested that this seed-eating beetle might have an active glutamine synthetase (EC 6.3.1.2), an enzyme that fosters the reaciton of L-glutamic acid and ammonia to form L-glutamine (Tate and Meister, 1969). Additional removal of ammonia could be achieved by the reaction of 2-oxoglutaric acid (a tricarboxylic acid cycle intermediate) with ammonia to form L-glutamic acid. This reaction is catalyzed by glutamic acid dehydrogenase (EC 1.4.1.2). Working in concert, these reactions would provide a means of using ammonia to produce glutamic acid and then further using ammonia via glutamine formation.

METHODS AND MATERIALS

Insects. The bruchid beetle larvae used in this study were obtained from infected *D. megacarpa* seeds collected in 1983 and 1984 in Santa Rosa National Park, Guanacaste Province, Costa Rica. The remaining insects were obtained

either from colonies maintained at the University of Kentucky or were the gift of Dr. George Kennedy, University of North Carolina, or Dr. Paul Feeny, Cornell University. The hymenopterous insects were collected from feral colonies in Kentucky. All insects were stored at −60°C prior to use.

Enzyme Assays. Unless otherwise indicated, terminal stadium larvae (2–2.5 g) were ground with a mortar and pestle utilizing about 8 ml of 100 mM *N*-Tris (hydroxymethyl)methylglycine (tricine) buffer (pH 7.3) and acid-treated sea sand. After the resulting slurry was centrifuged at 18,000*g* for 15 min, floating debris was removed from the supernatant solutions by filtering over cheesecloth.

The insect homogenates were assayed for glutamine synthetase activity at 37°C by the method of Rowe et al. (1970) except for omission of 2-mercaptoethanol. Zero-time samples served as the control. Glutamic acid dehydrogenase activity was monitored by evaluating the oxidation of NADH at 340 nm at 22°C. The assay mixture included 25 mM sodium 2-oxoglutarate (pH 7.3), 50 mM tricine (pH 7.3), 0.36 mM NADH, and ammonium chloride (pH 7.3) in a final volume of 1 ml. Ammonium-free samples served as the control. The apparent K_m values of Table 1 are for ammonia in the glutamic acid dehydrogenase reaction. Activity is expressed in as nanomoles product formed per minute at the indicated temperature per milligram souble insect protein. Soluble protein values were determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Analyses of the catalytic activity of these two enzymes from 16 insects in addition to *C. brasiliensis* have been conducted (Table 1). While all of the tested insects contain detectable glutamine synthetase activity, *C. brasiliensis* larvae possess twice the enzyme activity of any of the tested insects and over 40 times more than the American cockroach, *Periplaneta americana;* the saltmarsh caterpillar, *Estigmene acraea;* and the Colorado potato beetle, *Leptinotarsa decemlineata.*

Comparable examinations of the glutamic acid dehydrogenase activity indicate the disproportionately high activity of this enzyme in the larvae of *C. brasiliensis.* With the exception of *Drosophila melanogaster,* the glutamic acid dehydrogenase activity runs from 3.5 times more than in *Callosobruchus maculatus,* a closely related beetle, to 34 times more than in *Polistes* spp., a wasp. This enzyme was not detected in four of the examined invertebrates. The glutamic acid dehydrogenase assay is sufficiently sensitive to detect less than 1 nmol NAD^+ formation. The enzyme activity values of Table 1 indicate product formation per unit of time. It is not known presently if this is a reflection of the intrinsic amount of the enzyme or the rapidity with which it converts substrate to product, i.e., its turnover number.

TABLE 1. GLUTAMINE SYNTHETASE AND GLUTAMIC ACID DEHYDROGENASE OF VARIOUS INSECTS[a]

Organism	Enzyme activity		Apparent K_m
	Glutamine synthetase	Glutamic acid dehydrogenase	
	(nmol/min/mg)		(mM)
Diptera			
Drosophila melanogaster	25.4	108	600
Musca domestica	11.5	71	400
Orthoptera			
Periplaneta americana	0.8	13	190
Hymenoptera			
Vespula spp.	8.1	35	230
Polistes spp.	13.0	8	105
Lepidoptera			
Manduca sexta	10.9	49	430
Papilio polyxenes asterius	4.3	28	290
Ephestia kühniella	9.3	ND	–
Heliothis zea	10.6	ND	–
Heliothis virescens	14.9	ND	–
Hyphantria cunea	21.6	16	210
Galleria mellonella	17.2	ND	–
Estigmene acraea	1.0	21	190
Coleoptera			
Tribolium castaneum	18.6	33	245
Callosobruchus maculatus	21.2	78	240
Leptinotarsa decemlineata (adult)	1.2	48	160
Caryedes brasiliensis (adult)	12.7	62	345
Caryedes brasiliensis	49.5	272	70

[a] ND, denotes organisms lacking detectable enzymatic activity.

Another important parameter of enzyme function is indicated by the apparent Michaelis-Menten constant (K_m) of its substrate. This constant is inversely proportional to the enzyme–substrate affinity. Insectan glutamic acid dehydrogenase is characterized by a high apparent K_m for ammonia (Table 1). This enzyme clearly exhibits limited affinity for ammonia. In several instances, the K_m value was in excess of 300 mM and in *Drosophila melanogaster,* it reaches 600 mM. Except for *Polistes* spp., all of the tested insects other than *C. brasiliensis* larvae possess a K_m for ammonia on the order of 200 mM. In *Polistes* spp., although the K_m is low, enzyme activity is among the smallest of values obtained in this study. Only in the case of the larvae of the bruchid beetle are both the enzyme activity elevated and substrate affinity high. These two

factors suggest that this seed predator is competent at reacting ammonia with 2-oxoglutaric acid to form glutamic acid.

It is particularly interesting to compare these data for adult *C. brasiliensis* with those for the larvae. Adult *C. brasiliensis* are pollen and nectar feeders that do not consume canavanine-containing plant material. Adult bruchid beetles are very similar to the other tested insects both with regard to their glutamine synthetase and glutamic acid dehydrogenase activities (Table 1).

The combined action of these enzymes provides a means for moving the carbon skeleton from the reaction intermediates of the tricarboxylic acid cycle to glutamic acid and then to glutamine. However, in the event of appreciable ammonia production, these reactions can deplete the energy-producing intermediates of the tricarboxylic acid cycle. This potentially deleterious situation can be circumvented by glutamic acid formation from proline. *Caryedes brasiliensis* stores appreciable proline in its hemolymph (Rosenthal, 1983); it is a predominate component of the free amino and imino acids. Proline in the hemolymph represents a significant carbon skeleton pool for glutamic acid production without depleting tricarboxylic acid cycle intermediates. Certain insects can convert proline to glutamic acid as part of their energy-yielding reactions. Proline can be oxidized to Δ^1-pyrroline-5-carboxylic acid, spontaneously converted to glutamic acid 4-semialdehyde, and then transferred to glutamic acid. The last reaction supports insectan bioenergetics by generating reduced NAD from NAD^+.

L-[U-^{14}C]Proline (0.5 μCi/larva) was injected parenterally into 48 terminal instar larvae (3.88 g fresh weight); the larvae were sacrificed after 18 hr. The treated larvae were defatted by grinding in freshly distilled acetone and their free amino and imino acids isolated and purified by ion-exchange chromotography (Rosenthal, 1982b). The carbon-14 of the free amino and imino acids of the hemolymph was determined by automated amino acid analyses and postcolumn evaluation of the radioactivity of the various ninhydrin-positive compounds by liquid scintillation spectroscopy (Bray, 1960).

These analyses revealed that after 18 hr, 75% of the injected radioactive proline remained as proline. This limited metabolism of ^{14}C-labeled proline may result from dilution of the labeled proline by the appreciable cold proline of the hemolymph. Of the radioactive proline that was converted to amino acids, nearly 70% was found as glutamic acid and glutamine. Thus, proline does function to provide some of the carbon skeleton required for the metabolism of ammonia.

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