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Biochemistry

In vitro incorporation of L-canavanine into vitellogenin of the fat body of the migratory locust *Locusta migratoria migratorioides*

(toxic nonprotein amino acids/nonprotein amino acid–insect interaction)

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ABSTRACT **L-Canavanine competes with L-arginine for incorporation into vitellogenin secreted *in vitro* by the fat body of the female locust *Locusta migratoria migratorioides*. Incorporation of L-[*guanidinooxy*-^{14}C]canavanine into vitellogenin has been established unequivocally by combined arginase and urease hydrolyses of the acid hydrolysate of antibody-precipitated canavanyl vitellogenin. Continued exposure of the fat body to canavanine decreases *in vitro* protein secretion but the proportion of canavanyl vitellogenin to native vitellogenin increases. Canavanine-mediated inhibition of fat body protein secretion is dependent on both the canavanine concentration and the arginine retention by the fat body. Canavanine replaces about 10% of the arginyl residues of canavanyl vitellogenin. The electrophoretic mobility of canavanyl vitellogenin is greater than that of native vitellogenin but the ability of this aberrant protein to react with vitellogenin antibody is unimpaired.**

Vitellogenins are female-specific extraovarian proteins synthesized by all egg-laying animals. In insects, vitellogenin is produced in the fat body, a tissue bearing many similarities to vertebrate liver. Vitellogenin is secreted into the hemolymph, transported to the developing oocytes, and sequestered ultimately as storage protein in the egg yolk (for review see ref. 1). The crucial importance of vitellogenin production and secretion to egg maturation and subsequent embryonic nutrition warrants its detailed investigation because any disruption in vitellogenesis severely impedes normal reproduction.

We have described the experimental variables for optimal synthesis and secretion by isolated locust fat body of the major plasma proteins, including vitellogenin (2, 3). We have demonstrated linear incorporation of radiolabeled amino acids into fat bodies of vitellogenic females and secretion of vitellogenin for several hours by them and that this process *in vitro* approaches the rate of synthesis *in vivo*. In this communication, we describe an investigation of the effect on vitellogenesis of L-canavanine, a nonprotein amino acid present in certain leguminous plants and highly toxic to various phytophagous insects and other herbivores (4, 5).

The principal basis for the toxicity of L-canavanine resides in its structural analogy to arginine; in some systems, this results in its activation by the arginyl-tRNA synthetase of nonproducing organisms (6, 7), and its subsequent incorporation into the nascent polypeptide chain. Several plant and animal species have been shown to incorporate canavanine into their proteins, but a specific eukaryotic protein, proven to contain canavanine, has not been isolated or studied with regard to the biological and biochemical manifestations of arginine replacement by canavanine.

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Isolated and cultured *Locusta* fat body is particularly suitable for the study of the effect of canavanine on protein synthesis under experimental conditions approximating *in vivo* protein production. Moreover, if significant canavanine is incorporated into vitellogenin, then a means is available for testing the effect of canavanine incorporation on the biological activity of the anomalous protein.

MATERIALS AND METHODS

Materials. [^{3}H]Leucine (37.9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from the Atomic Energy Commission Nuclear Research Centre (Negev, Israel); DL-[*guanidinooxy*-^{14}C]canavanine (52.3 mCi/mmol) was from Schwarz/Mann (Orangeburg, NJ). Urease (type IX, 5.26 units/mg), arginase (bovine liver, 60 units/mg), and the remaining reagents were supplied by Sigma. L-Canavanine was isolated from jack bean seeds by the method of Rosenthal (8).

Animals. *Locusta migratoria migratorioides* (R & F) were bred and maintained under crowded conditions with constant illumination. They were fed grass and flaked oats.

***In Vitro* Protein Synthesis by the Locust Fat Body.** Fat bodies (0.9–3.1 mg of protein) were removed from freshly sacrificed female locusts and washed with arginine-free incubation medium. This medium was based on the formula of Landureau and Grellet (9) except for the presence of 0.5 mM leucine and 50 mM sodium *N*-tris(hydroxymethyl)methylglycinate (Tricine), pH 7.2, and the exclusion of arginine. The fat body was divided into two portions, and each segment placed in a vial containing 0.5 ml of sterilized incubation medium.

The standard protocol consisted of depleting the fat body of arginine by incubation in arginine-free medium for 6 hr at 30°C (after 3 hr, the tissues were transferred to fresh incubation medium for the final 3 hr). The arginine-depleted fat bodies were then placed in fresh incubation medium containing 5 μCi of [^{3}H]leucine for 3 hr. Production of secreted protein was determined by transferring 25-μl samples of the incubation medium to squares of Whatman 3MM filter paper and trapping the precipitated macromolecules according to the method of Mans and Novelli (10). The radiolabeled soluble protein retained in the fat body was evaluated by homogenizing the incubated fat body tissues in a 1-ml ground glass homogenizer. The resulting suspension (2 ml) was clarified by centrifugation at 12,000 × *g* for 10 min; floating debris was removed by filtration, and 25-μl

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samples of the supernatant solution were processed as described above.

Radioactivity was determined by toluene-based liquid scintillation spectroscopy. The term "secreted protein" denotes all proteins determined to be in the incubation medium at the time of sample withdrawal. The extent to which protein secretion into the culture medium is mediated by an active transport process as compared to simple "leakage" has not been determined.

Canavanine Incorporation Studies. Five fat body samples were depleted of arginine for 6 hr according to the standard protocol and then transferred to fresh arginine-free medium containing 2 μCi of DL-[*guanidinooxy*-^{14}C]canavanine for a final 3 hr. Secreted proteins from 2.0 ml of the pooled sample (2.5 ml) were precipitated with an equal vol of 30% (wt/vol) trichloroacetic acid after the addition of 5 mg of carrier bovine serum albumin. The precipitate was washed four times with 5% (wt/vol) trichloroacetic acid; centrifugations were at 20,000 × *g* for 15 min. The final supernatant solution lacked free canavanine. Finally, the washed pellet was hydrolyzed with 6 M HCl under nitrogen for 24 hr at 110°C; HCl was removed by lyophilization.

After the hydrolyzed proteins were dissolved in 2 ml of deionized water, one-half was applied to a 6 × 70 mm column of Dowex-50 in the NH_4^+ form. Little radioactivity appeared in the effluent obtained from the thoroughly washed resin; 0.25 M NH_4OH was used to develop the column. This procedure effectively separated arginine from canavanine (8). The first 1 ml of the effluent was discarded; the next three fractions, encompassing nearly all of the eluted radioactivity, were pooled. The pooled effluent (3 ml) was diluted 1:4 with 100 mM sodium *N*-tris(hydroxymethyl)methylglycinate at pH 7.6 and 1-ml samples were placed into 25-ml Erlenmeyer flasks. These flasks were sealed with a rubber septum supporting a small plastic center well containing a folded piece of Whatman 3MM filter paper impregnated with 5 drops of Hyamine hydroxide. A solution containing 4 mg of arginase and 4 mg of urease in the same buffer was activated for 30 min at 37°C with 2.5 mM $MnCl_2$. The metal-activated enzyme solution (1 ml) was injected into the reaction vessel which was maintained at 37°C for at least 12 hr. Then, 2 ml of 2 M HCl was injected into each flask to terminate the reaction and purged the dissolved $^{14}CO_2$ derived from the metabolism of labeled canavanine. The evolved radioactive CO_2, sequestered in the Hyamine hydroxide-laden filter paper, was measured by toluene-based liquid scintillation spectroscopy.

In this procedure, arginase mediates the hydrolytic cleavage of L-[*guanidinooxy*-^{14}C]canavanine to L-canaline and [^{14}C]urea. Urease, in turn, converts labeled urea to ammonia and $^{14}CO_2$. In this way, all of the labeled CO_2 released by enzymatic hydrolyses of the arginine-free hydrolysate could only have emanated from L-canavanine incorporated into fat body-produced protein.

Incorporation of radioactive canavanine specifically into vitellogenin was established by complexing this protein with antibody prepared against oocyte vitellin (3). Immunoprecipitation of this secreted protein was achieved by treating 0.1 ml of the pooled incubation mixture with an equal volume of the antiserum for 1 hr at 37°C. After 30 min incubation, carrier antigen was added. This mixture was kept overnight at 4°C and then centrifuged at 10,000 × *g* for 10 min. Precipitation of the vitellogenin was confirmed by gel electrophoresis of the supernatant solution. Free unlabeled arginine in the fat body was determined as described by Bieleski and Turner (11).

Electrophoresis and Amino Acid Analysis. The electrophoretic procedures and the methods for processing the gels have been described (3). Secreted proteins were prepared for amino acid analysis by exhaustive dialysis of the incubation medium against 0.4 M NaCl. Complete removal of free canavanine was ensured by monitoring the disappearance of exogenous norleucine; dialysis was continued until norleucine was no longer detectable. Canavanine-treated fat bodies were rinsed clean of external canavanine and homogenized in 0.4 M NaCl. The resulting homogenates were clarified by centrifugation at 12,000 × *g* for 10 min and the supernatant solutions were dialyzed as described above. The dialyzed solutions were lyophilized and hydrolyzed as described above. Amino acid analyses of the various proteins were conducted with a Dionex D-502 amino acid analyzer, their commercial buffer system, and ninhydrin detection at 570 nm.

RESULTS

When depleted of arginine according to the standard protocol and then exposed to canavanine and [^{3}H]leucine, isolated fat body tissues exhibited a significantly decreased ability to synthesize and secrete [^{3}H]leucine-labeled plasma proteins. Fat-body response to canavanine was concentration dependent; 20 mM canavanine caused nearly total curtailment of protein synthesis and secretion (Fig. 1). It may be that a portion of the observed diminution in the level of secreted protein reflects the gradual loss of essential arginine during the 6 hr of arginine depletion. However, during a 9-hr experimental period at an adequate level of arginine, there was little attenuation in the ability of the fat body to produce and secrete protein *in vitro* (Fig. 2). When arginine was omitted from the culture medium, protein secretion fell and this effect was intensified over the entire experimental period (Fig. 2). The addition of canavanine to the culture medium did not exert a deleterious effect on protein secretion initially but with successive transfers to arginine-free medium its inhibitory effect on protein secretion became more fully manifested. The ability of arginine to protect the *in vitro* protein-secreting ability of the fat body against canavanine was demonstrated by the experimental finding that, after exposure of arginine-depleted fat bodies to 10 mM canavanine, protein secretion was suppressed to 10–15% of the control level. In contrast, in the presence of 5 mM arginine plus 10 mM canavanine, protein secretion fell to only 55% of the control (data

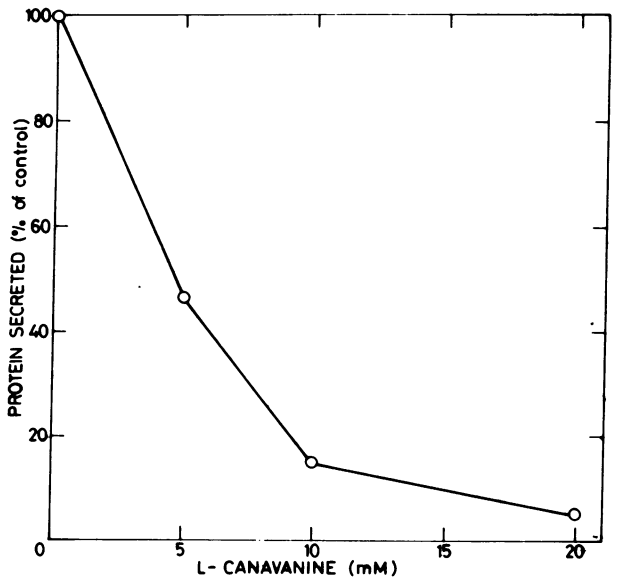

FIG. 1. Effect of canavanine on fat body production of secreted protein. The fat bodies were processed by the standard protocol for 6 hr prior to exposure to the indicated level of canavanine and 5μCi of [^{3}H]leucine for a final 3 hr. Each value is the mean of two separate determinations that gave essentially the same results.

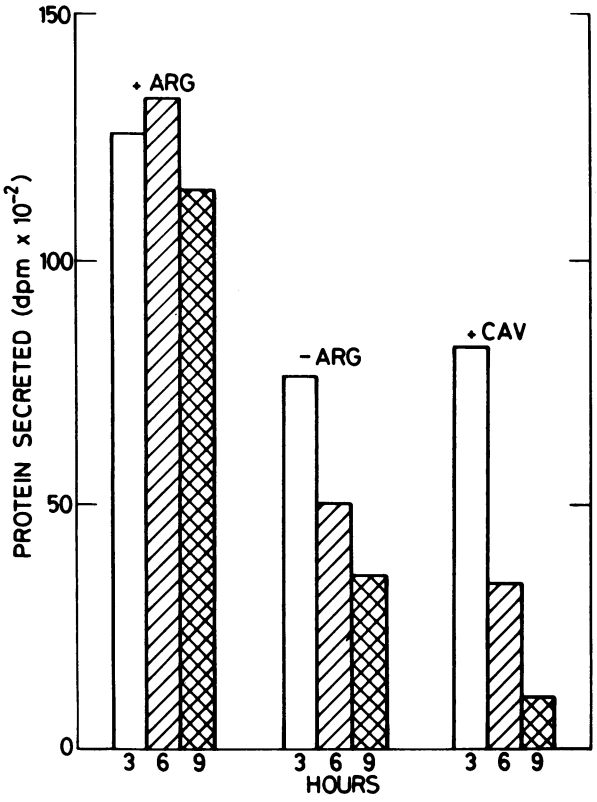

FIG. 2. Influence of arginine depletion and canavanine on fat body protein secretion. The fat body samples were maintained in standard incubation media containing 5 μCi of [^{3}H]leucine and supplemented with 5 mM arginine or 10 mM canavanine or lacking arginine. After 3 and 6 hr, the fat bodies were transferred to appropriate fresh radioactive medium. The time on the abscissa denotes the hour at the beginning of which the samples were removed for determining protein production. The data presented are typical of several determinations that gave essentially the same results.

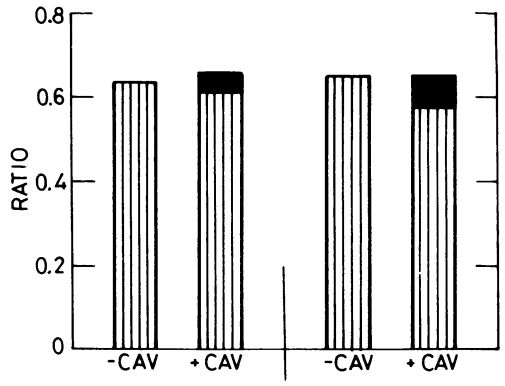

FIG. 3. Amino acid ratios and canavanine treatment. The ratios of arginine to valine (striped bars) and of canavanine to valine (black bars) were determined from the hydrolysate of the protein secreted by fat bodies maintained on medium containing 10 mM canavanine but free of arginine (+CAV) or on control medium (−CAV). Two separate experiments are shown.

not shown). These experimental findings suggest that canavanine functions as an arginine antagonist, competing directly with it in the metabolic reactions that culminate in protein production and secretion.

The possibility that canavanine is incorporated into the proteins of locust fat body was evaluated experimentally (Table 1). It is germane that a significant increase in canavanine incorporation into secreted protein occurred as arginine depletion proceeded. Assay of the arginine pool of the fat body revealed that this amino acid was depleted rapidly and was almost undetectable after 9 hr of incubation in arginine-free medium. The possibility exists that canavanine is metabolized by the fat body and that metabolic transfer of the labeled carbon atom of canavanine to a protein amino acid occurs. This possibility was discounted by analyzing the constituent proteins retained in the fat body, those secreted into the medium, and vitellogenin of the culture medium that had been precipitated specifically with antivitellin. These samples were hydrolyzed individually and the various protein hydrolysates were subjected to ion exchange chromatography to separate canavanine from arginine and to concurrent arginase and urease treatment. This coupled enzyme assay revealed that >95% of the radioactivity of the protein hydrolysate was evolved as $^{14}CO_2$. Thus, >95% of the radioactive carbon in all of the examined fat body protein emanated uniquely from canavanine.

An independent demonstration of canavanine incorporation into proteins secreted into the culture medium by the fat body or retained as soluble intercellular protein was obtained by direct amino acid analysis of the protein hydrolysate. In this way, the presence of canavanine in the protein hydrolysate was dem-

Table 1. Canavanine incorporation into fat body-secreted proteins as a function of arginine depletion time

Depletion time, hr	[^{14}C]Canavanine incorporation into secreted proteins*
0	0.426 ± 0.035
3	0.540 ± 0.039
6	0.645 ± 0.018

Five fat bodies were processed and evaluated. The depletion time denotes the experimental period that the fat bodies were maintained in arginine-free medium prior to a 3-hr exposure to [^{14}C]canavanine.
* The values are the mean and range of three separate determinations. Incorporation of [^{14}C]canavanine into secreted proteins is expressed as nmol/mg of soluble protein.

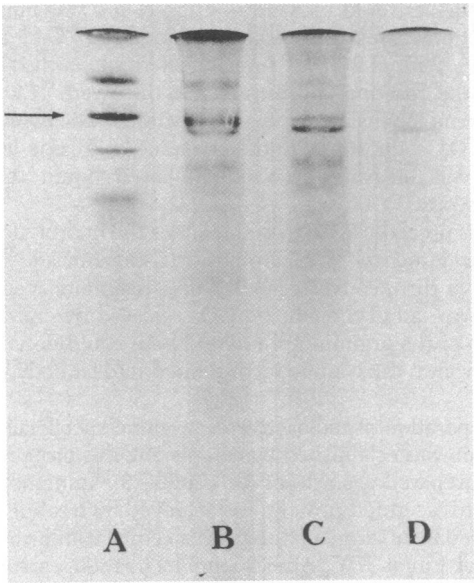

FIG. 4. Polyacrylamide gel electrophoresis of secreted fat body proteins. Lane A, protein pattern from fat bodies maintained on arginine-free incubation medium for 3 hr at 30°C; lanes B, C, and D, from comparable materials maintained on 10 mM canavanine for 3, 6, and 9 hr, respectively (lane C, transfer to fresh incubation medium after 3 hr; lane D, transfer after 3 and 6 hr). In each instance, the incubation media from five fat bodies were pooled (2.5 ml) and 0.1 ml was applied to the gels. Arrow, position of vitellogenin.

onstrated only when the fat body was exposed to canavanine. The ratios of arginine to valine and of canavanine to valine were calculated from the results of amino acid analyses of the hydrolysate of proteins secreted either by canavanine-treated or control fat bodies. The loss in arginine content relative to valine was exactly compensated for by the additional canavanine found in the canavanine-containing proteins (Fig. 3). Exposure of the cultured fat body to 10 mM canavanine resulted in about a 10% replacement of the arginine residues by canavanine. Much less canavanine, about 1%, could be detected in the soluble proteins retained by the fat body (data not shown).

Discovery of the replacement of arginine by canavanine in fat body-secreted proteins instigated an examination of the electrophoretic pattern of canavanine treated proteins of the fat body (Fig. 4). Treatment with canavanine for 3 hr resulted in the diminution of the vitellogenin band and the appearance of a novel band of greater electrophoretic mobility that is unique to canavanine-treated fat bodies. A second transfer to fresh, arginine-free, canavanine-containing medium intensified this effect (in lane C, the novel band is stained more densely than vitellogenin itself). The two successive transfers and 9-hr incubation with canavanine greatly diminished protein production, an effect anticipated by the deleterious action of canavanine on protein synthesis. Nevertheless, only the novel band representing canavanyl vitellogenin is discernible in lane D. These gels are evidence of a discernible physiochemical alteration in a eukaryotic protein associated with canavanine incorporation into the protein. Preliminary evidence indicates that canavanyl vitellogenin is immunoreactive with antivitellin, but the details of this crossreaction have not yet been examined.

DISCUSSION

These findings provide substantive evidence for the ability of canavanine to disrupt markedly insect protein synthesis. It is the most sensitive system thus far discovered for studying the fundamental basis for canavanine-mediated inhibition of eukaryotic protein synthesis. Detailed studies of canavanine's biological effects have been conducted with only two eukaryotic organisms: the aquatic microphyte *Lemna minor* and the tobacco hornworm *Manduca sexta* (12–16). In both systems, canavanine was shown to curtail DNA metabolism and to be a potent inhibitor of RNA metabolism but had no discernible effect on protein production. Thus, isolated *Locusta* fat body represents a uniquely suitable system for investigating the mechanism of canavanine-mediated disruption of protein synthesis.

It is germane that canavanine is able to disrupt essential reactions of both DNA and RNA metabolism and that this arginine analogue is a potent inhibitor of RNA metabolism in *M. sexta* (5). This fact creates the additional possibility that curtailment of certain reactions of nucleic acid by canavanine may contribute to the observed diminution in vitellogenin secretion.

The production of canavanine-containing vitellogenin makes available a major eukaryotic protein whose primary structure has been altered by the addition of this nonprotein amino acid. This change is manifested by differential electrophoretic mobility. Canavanyl vitellogenin retains its immunoreactivity with antivitellin but other functional characteristics of this protein may have been altered. For example, it is of basic and practical interest to determine whether canavanyl vitellogenin is recognized to the same extent as native vitellogenin by the plasma membrane of the developing oocyte (a requisite to its preferential sequestration as egg yolk protein) and whether it is resistant to degradation to the same extent as is native vitellogenin.

The latter point is particularly noteworthy because studies with *M. sexta* have resulted in the consistent finding that although a detectable level of radioactive canavanine is incorporated into newly synthesized proteins, no evidence for preferential degradation of these canavanyl proteins is observed. This stands in contradistinction to a large body of evidence that anomalous proteins created by canavanine inclusion are degraded preferentially by the producing organism (17, 18). The ability to produce canavanyl vitellogenin will permit direct examination of the question of insect capacity to recognize and degrade aberrant proteins effectively.

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1. Hagedorn, H. H. & Kunkel, J. G. (1979) *Annu. Rev. Entomol.* **24**, 475–505.
2. Harry, P., Pines, M. & Applebaum, S. W. (1978) *Arch. Biochem. Biophys.* **191**, 325–330.
3. Harry, P., Pines, M. & Applebaum, S. W. (1979) *Comp. Biochem. Physiol.* **63B**, 287–293.
4. Janzen, D. H. (1975) in *Evolutionary Strategies of Parasitic Insects and Mites*, ed. Price, P. W. (Plenum, New York), pp. 154–186.
5. Rosenthal, G. A. (1977) *Q. Rev. Biol.* **52**, 155–178.
6. Allende, C. C. & Allende, J. F. (1964) *J. Biol. Chem.* **239**, 1102–1106.
7. Mitra, S. K. & Mehler, A. H. (1967) *J. Biol. Chem.* **242**, 5490–5494.
8. Rosenthal, G. A. (1977) *Anal. Biochem.* **77**, 147–151.
9. Landureau, E. M. & Grellet, P. (1972) *C. Hebd-R. Seances Acad. Sci. Ser. D* **274**, 1372–1375.
10. Mans, R. J. & Novelli, G. D. (1961) *Arch. Biochem. Biophys.* **94**, 48–53.
11. Bieleski, R. L. & Turner, B. A. (1966) *Anal. Biochem.* **17**, 278–293.
12. Dahlman, D. L. & Rosenthal, G. A. (1975) *Comp. Biochem. Physiol.* **51A**, 33–36.
13. Dahlman, D. L. & Rosenthal, G. A. (1976) *J. Insect Physiol.* **22**, 265–271.
14. Rosenthal, G. A. & Dahlman, D. L. (1975) *Comp. Biochem. Physiol.* **52A**, 105–108.
15. Rosenthal, G. A., Gulati, D. K. & Sabharwal, P. S. (1975) *Plant Physiol.* **56**, 420–424.
16. Rosenthal, G. A., Gulati, D. K. & Sabharwal, P. S. (1976) *Plant Physiol.* **57**, 493–496.
17. Goldberg, A. L. & Dice, J. R. (1974) *Annu. Rev. Biochem.* **43**, 835–869.
18. Goldberg, A. L. & St. John, A. C. (1976) *Annu. Rev. Biochem.* **45**, 747–803.