Changes in the growth and food utilization of the cabbage looper Trichoplusia Ni (Lepidoptera: Noctuidae) after consumption of an artificial diet incorporating the non-protein amino acid L-canavanine

Bradley F. Binder

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CHANGES IN THE GROWTH AND FOOD UTILIZATION OF THE CABBAGE LOOPER TRICHOPHUSIA NI (Lepidoptera: Noctuidae) AFTER CONSUMPTION OF AN ARTIFICIAL DIET INCORPORATING THE NON-PROTEIN AMINO ACID L-CANAVANINE.

A Thesis
Presented to the
Faculty of
California State
University, San Bernardino

In Partial Fulfillment
of the Requirements for the Degree

Master of Science
in
Biology

by
Bradley F. Binder

June 1985
CHANGES IN THE GROWTH AND FOOD UTILIZATION OF THE CABBAGE LOOPER *TRICHOPUSIA NI* (Lepidoptera: Noctuidae) AFTER CONSUMPTION OF AN ARTIFICIAL DIET INCORPORATING THE NON-PROTEIN AMINO ACID L-CANAVANINE.

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Approved by:

Chairman, Department of Biology Graduate Committee

Committee Member

Committee Member

Major professor
Abstract

Larvae of the fifth stadium cabbage looper, *Trichoplusia ni* (Hübner), were fed artificial diets containing 0, 10, 20, or 30 mM L-canavanine. Larval weight and developmental changes were recorded for six days. Rates of growth, food consumption, assimilation, excretion and respiration as well as approximate digestibility, percent efficiency of conversion of ingested food to body substance (ECI), and percent efficiency of conversion of digested food to body substance (ECD) were calculated. Significant (P< 0.05) reductions in larval weight gain in the groups consuming diets incorporating 20 and 30 mM L-canavanine occurred by day 1, while larvae consuming diet containing 10 mM L-canavanine remained comparable to their control counterparts until day 2. Pupation was also (P< 0.05) delayed. However, the effect of L-canavanine ingestion could be reversed if the larvae were returned to control diet after 4 days on the diet with 30 mM L-canavanine. L-canavanine ingestion resulted in decreases in the rates of feeding, growth, excretion, assimilation, and respiration, as well as reductions in the ECI and ECD. Mechanisms for the inhibitory effects of L-canavanine on larval growth and nutritional physiology are discussed.
ACKNOWLEDGEMENTS

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The completion of this manuscript was assisted by the careful review of Dr. Richard Fehn, Dr. Alexander Sokoloff, and Dr. Ruth Wilson. Special thanks to Dr. Sokoloff, for allowing me to pursue the investigation of this area of biology and for giving such helpful tips during the writing process.

Lastly, I am indebted to my wife, Sarah Sebring Binder, for her consistent emotional and financial support. Her unflagging loyalty has won my deepest love, respect, and gratitude.
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INTRODUCTION

Examination of the nutritional physiology of an insect is essential for a comprehensive evaluation of plant–insect interactions. Phytophagous insects must be able to convert plant material into usable units for energy, metabolism, and structural substances (Beck, 1972). If the insect utilizes the plant tissue efficiently there is enhanced reproduction with increased fitness and survival. However, many plants harbor secondary metabolites which will markedly deter insect feeding activity and/or alter physiological feeding parameters (Ahmed, 1983; Hedin, 1977, 1983; Rosenthal and Janzen, 1979). Insects that rely heavily on plant material as a nutrient source elude the effect of poisonous plant products by various adaptive strategies. These mechanisms include: 1) detoxification of the noxious principles to inert substances which can be metabolized with no harmful effects, 2) sequestration of toxic entities resulting in isolation from major biochemical pathways or use as a rapacious reducing component for that
insect's defense system, and 3) detection and avoidance of those plants which incorporate deleterious compounds (Blum, 1981). The consequence of devouring plant tissue containing constituents which the insect cannot avoid, sequester, or detoxify is disruption of that insect's development because of reduced food consumption or altered digestive processes. One method used to ascertain the effect of growth modifying substances and accumulate information about the physiological mechanisms of tolerance in insects is to study their quantitative nutritional physiology. This requires the measurement of animal weight gain, food consumption, and fecal output. Quantitative nutritional indices can be calculated from the above parameters which may be used to gain an understanding of nutrient source utilization by an insect.

Food quality and its effect on the nutritional physiology of insects has been described by Scriber and Slansky (1981). Slansky and Scriber (1982) presented a synopsis of insect quantitative nutritional studies in which herbivorous insects were categorized based on their mode of feeding (chewing or sucking) with subcategories identifying the useful parts of the plant for insect nutrition. Reese (1979, 1983) examined the interaction of nutrients and secondary plant compounds in artificial diets on insect growth
and development, while Beck and Reese (1976) investigated the effects of secondary plant compounds on insect metabolism. These studies illustrate the advances made on insect quantitative nutritional physiology and emphasize the need for more descriptive studies concerning insect-plant interactions.

Investigators once thought that all plants had equivalent nutritional parameters for the growth of any herbivorous insect (Fraenkel, 1953). That view was revised after the experiments of Waldbauer (1962), who maxillectomized larvae of the tobacco hornworm, *Manduca sexta*, to demonstrate the poor growth of the insect from consuming plant tissue with inadequate nutritional resources instead of the larval behavioral responses to plant products. Toxins or physiological inhibitors in the plant tissue which may have altered tobacco hornworm growth were not addressed. Plants containing a variety of insect phagostimulants, phagodeterrents, feeding inhibitors, and toxicants (Gordon, 1961; Beck, 1965) can impair insect growth and development, resulting from one or a combination of the following: 1) low food consumption because of deficient quantities of phagostimulants or the influence of phagodeterrents, 2) unsatisfactory digestion due to the presence of blocking metabolites or inadequate
digestive enzymes, 3) metabolite blockade of absorption or transfer sites, and 4) deficient conversion of absorbed food into body substance because of the lack of essential nutrients or the presence of blocking metabolites (Gordon, 1968b). An accurate evaluation illustrating the inhibitory influence of secondary compounds on herbivore growth can be made by removing the putative modifiers of behavior and/or growth from the plant tissue and incorporating them into an artificial diet upon which the herbivore will feed. This has been accomplished for many insect species although a comprehensive inquiry of all insects is far from complete. The use of plant secondary compounds in insect diets is a valuable tool to study insect nutritional physiology, feeding mechanisms, and to delineate plant-insect interactions.

One secondary plant compound or allelochemical (Whittaker, 1970) which is extremely toxic is the non-protein amino acid, L-canavanine sulfate. This poisonous component of legumes of the subfamily Papilionoideae is a naturally occurring structural analog of L-arginine (For review see Rosenthal, 1977). Structurally, L-canavanine (figure 1) is similar to L-arginine, except the third methylene group of arginine is replaced by oxygen (Rosenthal, 1972). L-canavanine has the dual role of a
Figure 1. The structures of L-canavanine and L-arginine.
\[
\begin{align*}
\text{NH} \\
\text{II} \\
\text{H}_2\text{N-C-NH-O-CH}_2\text{-CH}_2\text{-CH(NH}_2\text{)-COOH} \\
\text{L-canavanine}
\end{align*}
\]

\[
\begin{align*}
\text{NH} \\
\text{II} \\
\text{H}_2\text{N-C-NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH(NH}_2\text{)-COOH} \\
\text{L-arginine}
\end{align*}
\]
nitrogen storage metabolite (Rosenthal, 1970) and a defensive biochemical that protects the plant against microorganisms, molds, higher plants, and insects (Bell, 1981; Fowden et al., 1967, 1979; Rosenthal, 1977, 1979; Volcani and Snell, 1948). Current theories of L-canavanine toxicity suggest the formation of anomalous proteins during synthesis by replacement of L-canavanine in positions which would normally be occupied with arginine. (For review see Rosenthal, 1983). The mechanism of toxicity involves physiochemical differences between the two amino acids (Boyer and Marsh, 1982) leading to tertiary and/or quaternary deviations in conformation followed by disruption of the protein's metabolic function. Substitution of L-arginyl residues by L-canavanine occur in the bacteria Escherichia coli (Attias et al., 1969), in rat pars intermedia (Crine et al., 1982) and in the grasshopper Locusta migratoria migratorioides (Pines et al., 1981).

Studies concerning the effects of L-canavanine sulfate on insects have addressed the overall insecticidal activity of the amino acid on growth and development. Rehr et al. (1973) found an artificial diet containing the equivalent of 5% L-canavanine sulfate to be repellent to the armyworm Prodenia eridania; that is, there was no food consumption and 100% mortality within three days. Vanderzant and Cremos (1971)
established that L-canavanine inhibited the growth and development of the boll weevil \textit{Anthonomus grandis}. However, when arginine HCl was added to the boll weevil diet in addition to L-canavanine, the inhibitory larval growth effects of L-canavanine were diminished. Production of the adult bruchid beetle \textit{Callosobruchus maculatus} was completely prevented when dietary concentrations of L-canavanine were increased from 0.1% to 5% (Janzen et al., 1977). The beetle \textit{Tribolium castaneum} is also susceptible to growth inhibition by L-canavanine sulfate. Harry et al. (1976) detected lower arginase enzyme activity levels, retarded growth, and greater mortality in \textit{T. castaneum} fed artificial diets containing L-canavanine. The effects of the amino acid in this case were attributed to the overall stress created on the animal and not due to the direct influence of L-canavanine sulfate on the arginase system. In all of the above studies, the response of the insect to the presence of L-canavanine appears to be dose dependent. Increasing the dietary amino acid concentration of L-canavanine sulfate enhances the detrimental effect on the growth and development of the insect.

The most comprehensive study to date of the effects of L-canavanine sulfate has been on the tobacco hornworm \textit{Manduca sexta} (Dahlman and Rosenthal, 1975; and Rosenthal and Dahlman, 1975). These investigators
examined the harmful effects of L-canavanine sulfate on the growth, development, and physiology of *M. sexta*. Moreover, the intricate nutritional indices as outlined by Waldbauer (1968) and Gordon (1968b, 1972) were surveyed. Tobacco hornworm larvae in the 5th instar were sensitive to artificial diets containing L-canavanine concentrations as low as 3 mM (Dahlman and Rosenthal, 1975). Diets containing 3 mM L-canavanine produced 40% malformed pupae, while diets containing higher concentrations (11 mM and 45 mM) completely inhibited larval growth. Fourth stadium larvae fed diets with 5 mM or 10 mM L-canavanine had reduced food consumption (F), lower efficiency of conversion of ingested food to body substance (ECI), and lower efficiency of conversion of digested food to body substance (ECD) (Dahlman, 1977). Furthermore, larvae reared from egg eclosion on diets containing as little as 0.5 mM (0.009%) L-canavanine were restrained in growth and development (Dahlman, 1977). Physiological studies on *Manduca sexta* included injections of radiolabeled L-canavanine into 5th instar larvae. After 24 hours, 3.6% of the labeled amino acid was detected in non-gut larval tissue which indicated cellular incorporation of L-canavanine sulfate. Adults injected with 2, 4, and 8 mg L-canavanine/g body weight exhibited premature mortality while
administrations in excess of 8 mg L-canavanine/g body weight produced abnormal behavioral responses, muscle paralysis, and death within a few hours (Dahlin and Rosenthal, 1976).

Detrimental effects on tobacco hornworm larvae were ascribed to the incorporation of the amino acid into protein. The adult response, however, has recently been shown to be a neurological malfunction due to the invasion of L-canavanine into neural tissue (Kramer et al., 1978). Loss of protein activity or detrimental modifications in structural proteins lead to the observed developmental and growth anomalies of tobacco hornworm larvae. Studies of the bruchid beetle Caryedes brasiliensis, which subsists on the mature seeds of the legume Dioclea megacarpa, containing 8% L-canavanine, indicate that this beetle averts the toxic properties of L-canavanine with an arginyl-tRNA synthetase by discrimination between L-arginine and L-canavanine (Rosenthal et al., 1976). Following detection, there is breakdown of the L-canavanine to inert moieties which can be excreted. Therefore, no L-canavanine is incorporated into protein, allowing the beetle to use D. megacarpa as a dietary source even though substantial concentrations of L-canavanine exist.

Feeding responses of insects to the dietary occurrence of
L-canavanine range from acting as a complete repellent of *Prodenia eridania* (Rehr et al., 1973) to a total indifference by *Caryedes brasiliensis* (Rosenthal et al., 1976). The behavioral reactions are derived from different physiological strategies for handling the toxic non-protein amino acid. Insects not specifically equipped to identify or detoxify L-canavanine are sensitive to the dietary quantity of the amino acid. Since insects are a diverse group of animals there may be other methods, besides discrimination of the amino acid residues with arginy1 tRNA synthetase, to counteract the detrimental outcome after L-canavanine ingestion. Therefore, assessing more insect species is important; responses may be uniform for insects not indigenously exposed to L-canavanine or there could be other detoxification mechanisms currently unknown.

An economically important pest of vegetables and a suitable test animal for L-canavanine experimental studies is the cabbage looper *Trichoplusia ni*. The cabbage looper is easily reared in a laboratory, without the host plant, on an artificial diet (Ignoffo, 1963; McEwen and Hervey, 1960; Shorey and Hale, 1965). Additionally, critical morphological markers have been identified which correlate developmental and physiological events such as molting and hormone release (Jones et al., 1981; Smilowitz, 1971;
Smilowitz and Smith, 1970). These events can be carefully monitored in a laboratory, providing a developmentally synchronous group of animals for research purposes. Furthermore, there have been several studies on the development and nutritional physiology of L. ni that have focused on the effect of parasitization by the Ichneumonid parasite Hyposoter exiguae (Iwantsch and Smilowitz, 1975; Thompson, 1982b; Thompson, 1983). However, the parasite attacks primarily the early instars so there was no quantitative nutritional investigation of the later part of the cabbage looper life cycle.

The present study was conducted to determine the quantitative nutritional physiology and to examine the effects of the non-protein amino acid L-canavanine sulfate on the nutritional physiology, growth, and development of the 5th instar L. ni. Knowledge of the cabbage looper's nutritional physiology and its response to secondary compounds will promote a better understanding of insect-plant interactions and energy transformation processes in insects.
Materials and Methods

Trichoplusia ni eggs were generously provided by Mr. Gary Platner of the University of California, Riverside. Trichoplusia ni larvae were reared at 30°C, 15:9 L:D cycle, in a Sherer environmental chamber on a modified Ignoffo (1963) diet (Table 1). All experimental studies used larvae selected at the 4-5th intermolt period according to the method of Jones et al. (1981). Larvae ready to molt to the 5th instar were identified by the following criteria: 1) clearing of the gut contents, 2) larval migration to the top of the rearing cup, 3) stretching of the cuticular suture just posterior to the head capsule, and 4) head capsule slipping forward but not beyond the ocelli (Figure 4). This method provided a developmentally synchronous (±6 hours) experimental group of larvae.

Initial experiments consisted of 20 individuals fed ad libitum in 0.5 ounce Solo® cups with equal portions of modified Ignoffo diet which contained 0 mM, 10 mM, 20 mM, and 30 mM L-canavanine sulfate (Sigma Chemical Company). Animal wet weights were measured twice daily on a Torbal balance (model ET-1) through the 5th instar, pharate pupal, and pupal periods. Developmental observations were carried out through the 5th instar to adult emergence.

Further experiments were conducted to determine the effect of
Table 1. Modified Igoffo (1963) Diet*

<table>
<thead>
<tr>
<th>Wet Ingredients</th>
<th>Amount (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>500 boiling</td>
</tr>
<tr>
<td></td>
<td>275 cool</td>
</tr>
<tr>
<td>Methyl-P-hydroxy benzoate</td>
<td>9 15% w/v in 95%EtOH</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>9 0.1g/ml</td>
</tr>
<tr>
<td>4 M KOH</td>
<td>45</td>
</tr>
<tr>
<td>Formaldehyde solution</td>
<td>4 0.1g/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dry Ingredients</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>20</td>
</tr>
<tr>
<td>Casein</td>
<td>32</td>
</tr>
<tr>
<td>Sucrose</td>
<td>32</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>27</td>
</tr>
<tr>
<td>Wesson's salts</td>
<td>9</td>
</tr>
<tr>
<td>Alphacel</td>
<td>4.5</td>
</tr>
<tr>
<td>Vanderzant's vitamin mix</td>
<td></td>
</tr>
<tr>
<td>fortified with ascorbic acid</td>
<td>20</td>
</tr>
</tbody>
</table>

* The diet was prepared by heating agar to boiling in 500 ml water. All wet ingredients were mixed in except choline chloride. All dry ingredients, except the vitamins, were added. Cool water was added to reduce the diet mixture to 60°C. The vitamins and choline chloride were added to the cool mixture, blended, and the resulting solution poured into the rearing cups.

† Diet ingredients were purchased from ICN Nutritional Biochemicals, Cleveland, Ohio.
L-canavanine sulfate on the nutritional physiology of *L. ni*. The experimental protocol is the same as described above. An additional 20 larvae were weighed, oven dried, and weighed again to establish initial larval dry to wet weight ratios. Gravimetric determinations, after a 2.5 day feeding period, of the dry weight gain, dry food consumption, and dry feces production allowed calculations to be made of the nutritional rates and indices (Table 2 and 3) for insects as outlined by Gordon (1968, 1973) and Waldbauer (1968) respectively. All values in Table 2 are expressed in mg x g⁻¹ x day⁻¹ while the values in Table 3 are presented as percent efficiency of food utilization.

Weight gain was calculated from the measured dry weight of the larvae at the end of the feeding period minus the arithmetic product of the initial larval wet weight and larval dry to wet ratio. Food consumption was calculated similarly, the arithmetic product of the initial diet wet weight and the diet dry to wet ratio minus the remaining dry diet at the end of the feeding period. Feces production was measured directly as the dry weight of the feces at the end of the feeding period. All dry weights were measured on a Torbal balance (model ET-1) after oven drying at 65°C for 72 hours.
Table 2. Quantitative nutritional rates* after Gordon (1973).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Arithmetic calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Food consumption rate</td>
<td>( F = \frac{\Delta F}{(W_e \times t)} )</td>
</tr>
<tr>
<td></td>
<td>Where: ( \Delta F ) = dry weight of food consumed during</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the feeding period (mg)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Growth rate</td>
<td>( G = \frac{\Delta G}{(W_e \times t)} )</td>
</tr>
<tr>
<td></td>
<td>where: ( \Delta G ) = dry weight gain of the insect during the</td>
<td></td>
</tr>
<tr>
<td></td>
<td>feeding period (mg)</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>Excretion rate</td>
<td>( S = \frac{\Delta S}{(W_e \times t)} )</td>
</tr>
<tr>
<td></td>
<td>where: ( \Delta S ) = dry weight of the feces produced during</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the feeding period (mg)</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>Respiration rate or the weight loss through metabolic oxidation</td>
<td>( O = \frac{{(\Delta F-\Delta S)-\Delta G}}{(W_e \times t)} )</td>
</tr>
<tr>
<td>A</td>
<td>Assimilation rate</td>
<td>( A = \frac{\Delta F-\Delta S}{(W_e \times t)} )</td>
</tr>
<tr>
<td>W_e</td>
<td>Exponential mean weight of the insect during the feeding period where:</td>
<td>( W_e = \frac{\Delta W}{\ln(W_f+\Delta W)} \times \frac{1}{d_f+d_i/2} )</td>
</tr>
<tr>
<td></td>
<td>( \Delta W ) = insect dry weight change during the feeding period</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( W_f ) = final dry insect weight</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( W_i ) = initial dry insect weight</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( d_f ) = final dry/wet insect ratio</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( d_i ) = initial dry/wet insect ratio</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( t ) = feeding period (2.5 days)</td>
<td></td>
</tr>
</tbody>
</table>

* All rates are expressed in mg x g\(^{-1}\) x day\(^{-1}\).
Table 3. Quantitative nutritional indices* after Waldbauer (1968).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Arithmetic calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.C.I.</td>
<td>Efficiency of conversion of ingested food to body substance</td>
<td>$(ECI) = (G/F) \times 100$</td>
</tr>
<tr>
<td>E.C.D.</td>
<td>Efficiency of conversion of digested food to body substance</td>
<td>$(ECD) = (\Delta G / (\Delta F - \Delta S)) \times 100$</td>
</tr>
<tr>
<td>A.D.</td>
<td>Approximate digestibility</td>
<td>$(AD) = (\Delta F - \Delta S / \Delta F) \times 100$</td>
</tr>
<tr>
<td>E.C.C.I.</td>
<td>Efficiency of conversion of caloric ingestion to body substance</td>
<td>$(ECCI) = (\Delta G / \Delta Cal) \times 100$</td>
</tr>
</tbody>
</table>

*All values are expressed as percent efficiency of food utilization.
A 2.5 day experimental period was designated to assess all groups prior to the onset of pupation. Because control larvae (0 mM) begin their developmental changes in anticipation of pupal formation at day 3, it was necessary to end the experiment before metamorphosis commenced. The developmental changes at pupation consist of a dramatic weight loss, cessation of feeding, larval migration, and cocoon spinning. Nutritional parameters measured following any metamorphic changes would yield results which are inconsistent with the actual feeding and growth behavior of the animal.

An estimation of larval caloric consumption was made in triplicate (Figure 4) by the combustion of dry diet aliquots in a Parr "bomb" calorimeter. Values are reported as the mean of three trials. The "bomb" was composed of a metal bucket, a stirrer, a thermometer, 2000 grams of water and, a metal cylinder which contained the reactants. Experiments were performed according to the methods described by the manufacturer's specification and Shoemaker et al. (1981). Dry diet samples were introduced into the bomb under an atmosphere of pure oxygen (200 p.s.i.). Temperature changes were recorded for 35 minutes after initiation of combustion. Prior standardization of the bomb with benzoic acid revealed
that the liberation of heat equivalent to 1350 calories was required to alter the bomb temperature 1°F. Temperature differences (final-initial) after the combustion of each sample indicate the caloric content per milligram dry diet as described below:

\[(T_{\text{final}} - T_{\text{initial}}) \times 1350 \text{ cal} = \text{cal/diet sample}\]

\[\text{cal/diet sample} \times \text{diet sample/mg dry diet} = \text{cal/mg dry diet}\]

Appropriate adjustments for the combustion of nitrogen and wire were also included in the above calculation. Larval caloric consumption is expressed as the efficiency of conversion of caloric ingestion into body substance.
RESULTS

Effect of L-canavanine sulfate on growth

Incorporation of L-canavanine sulfate into the artificial diet of the 5th instar larval Trichoplusia ni had severe effects on the ensuing growth of the animals (figure 2). Increasing the dietary concentrations of L-canavanine sulfate resulted in lower rates of larval wet weight gain as compared to control (0 mM) individuals. Concomitant with lower wet weight gain were reductions in larval maximal weight and a delay in attaining the maximal weight. Specifically, larvae reared on diets with no L-canavanine sulfate reached a maximal weight of 260 mg at day 2.5 while larvae reared on diets with 10 mM, 20 mM, and 30 mM L-canavanine sulfate reached 255 mg at day 3, 197 mg at day 4, and 136 mg at day 5 respectively. In addition, significant reductions in larval weight gain occurred in groups consuming diets incorporating 20 and 30 mM L-canavanine by day 1 (P<0.05), while larvae consuming diet containing 10 mM L-canavanine remained comparable to their control counterparts until day 2 (P<0.05). When larvae reared on diets with 30 mM L-canavanine were returned to control diet after 4 days (figure 3), growth and weight gain were similar to control larvae. However, the maximal larval weight did not attain the maximal weight acquired by the control larvae; instead, onset of pupation occurred when the
Figure 2. Mean larval/pupal body weights of *I. ni* reared on diets containing 0, 10, 20, and 30 mM concentrations of L-canavanine sulfate. Diet concentrations: □, 0 mM; +, 10 mM; ◦, 20 mM; Δ, 30 mM. Solid symbols indicate pupae.
Figure 3. Mean larval/pupal body weights of *L. ni* reared on diets containing 0, 20, 30 mM concentrations of L-canavanine sulfate and then returned to control diet at day 4. Diet concentrations: □, 0 mM; +, 20 mM; ◆, 30 mM; Δ, 30 mM/replaced to control diet at day 4. Solid symbols indicate pupae.
rapidly growing larvae maintained an equivalent current weight of the control insects.

**Effect of L-canavanine sulfate on development**

In addition to a delay in maximal weight attainment as described above there was a dramatic hindrance on developmental progression by all larvae raised on diets containing L-canavanine sulfate. Pupal formation was significantly delayed or prevented as the dietary concentration of L-canavanine sulfate was increased (Table 4). Some larvae matured to pupae regardless of the concentration of L-canavanine; however, nearly all larvae consuming diet with the 30 mM concentration of the amino acid were prevented from pupation. These were malformed larvae/pupae because of melanization prior to actual pupal formation. Deviations of developmental progression when 5th instar larvae are grown on diets with different concentrations of L-canavanine sulfate are illustrated in Figures 5-7.

Larvae reared on control diet after 4 days of growth on diet with 30 mM L-canavanine sulfate, promptly progressed to the pupal stage (Table 5 and Figure 8).

Adult emergence was dramatically reduced due to the effects of L-canavanine. Pupae reared from control and 10 mM dietary levels of
Table 4. Delay and reduction in the frequency of pupation of *Trichoplusia ni* when reared on control and experimental diets containing L-canavanine sulfate.

<table>
<thead>
<tr>
<th>Time to pupation (Days)</th>
<th>Number of pupae at each dietary L-canavanine sulfate concentration (mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>4.0</td>
<td>6</td>
</tr>
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</tr>
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<td>6.5</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

Mean time to pupation (days) 4.4 5.0 6.3 7.0

Sample size 17 18 16 15

Percent pupation 88 89 63 7

Percent adult emerged 65 61 6 ---

* Pupation time for animals at all diet concentrations (0 mM, 10 mM, 20 mM, 30 mM) were significantly different statistically (P << 0.001) from each other except the 20 mM and 30 mM comparison as determined by the Kruskal-Wallis test (Conover, 1980).
Table 5. Delay and reduction in the frequency of pupation of *Trichoplusia ni* when reared on control and experimental diets containing L-canavanine sulfate for different periods of time.

<table>
<thead>
<tr>
<th>Time to pupation (Days)</th>
<th>Number of pupae at each dietary L-canavanine sulfate concentration (mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>4.0</td>
<td>7</td>
</tr>
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<td>11</td>
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</tr>
<tr>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

Mean time to pupation (Days) 4.3 6.4 7.0 7.3

Sample size 19 18 19 20

Percent pupation 100 72 5 100

Percent adult emerged 63 6 --- 55

* Pupation time for animals at all diet concentrations (0 mM, 20 mM, 30 mM, 30 mM/control) were significantly different statistically (P<0.001) from each other except the 20 mM and 30 mM groups or the 30 mM and 30 mM/control as determined by the Kruskal-Wallis test (Conover, 1980).

† Larvae were reared on 30 mM L-canavanine diet for 4 days then transferred to control diet for the remainder of the experiment.
Figure 4. Bubble-head fourth instar larvae used to initiate developmental and nutritional studies. Note the head capsule slipping forward and the stretching of the cuticular suture just posterior to the head capsule which is characteristic of molting larvae: A, dorsal view; B, lateral view.
Figure 5. Larval growth and development inhibition, after three days, resulting from the consumption of diets containing 0, 10, 20, and 30 mM L-canavanine sulfate: A, control animal just prior to pupation illustrates normal growth and development; B, animal feeding on diet containing 10 mM L-canavanine illustrates the 12 hour delay in development; C, animal feeding on diet containing 20 mM L-canavanine illustrates the inhibition of weight gain and the developmental delay; D, animal feeding on diet containing 30 mM L-canavanine illustrates the severely retarded growth and development.
Figure 6. Larval and pupal growth and development inhibition, after six days, resulting from the consumption of diets containing 0, 10, 20, and 30 mM L-canavanine sulfate: A, illustrates the normal pupal development of the control animals; B and C, illustrates the pupal development of animals reared on diet containing 10 or 20 mM L-canavanine respectively; D, illustrates the inhibition of pupal development of animals reared on diet containing 30 mM L-canavanine.
Figure 7. Pupal weight and development inhibition, after nine days, resulting from the consumption of diets containing 0, 10, 20, and 30 mM L-canavanine sulfate: A, illustrates the normal pupal development of the control animals; B and C, illustrates the pupal development of animals reared on diet containing 10 or 20 mM L-canavanine respectively; D, illustrates the malformed pupae resulting from the consumption of diets containing 30 mM L-canavanine.
Figure 8. Reversal of larval growth and development inhibition, after nine days, resulting from the consumption of diets containing 30 mM concentrations of L-canavanine sulfate and then return to control diet on day 4: A, pupal formation following larval return to control diet after four days of compulsory consumption of 30 mM L-canavanine diet; B, malformed pupae following larval consumption of diets incorporating 30 mM L-canavanine.
L-canavanine produced approximately the same percentage of adults (Table 4). When the concentration of the amino acid was increased to 20 mM there were drastic reductions in the number of adults eclosed. Higher dietary concentrations of L-canavanine (30 mM) prevented all developmental progression to the adult. However, adult emergence from larvae grown on 30 mM L-canavanine levels and then transferred to control diet after 4 days was comparable to control emergence rates (Table 5). This illustrates that the impaired growth, development, and adult eclosion of L. ni due to the presence of L-canavanine sulfate is temporary and can be reversed.

**Effect of L-canavanine sulfate on nutritional physiology**

The observed larval growth suppression when larvae are reared on artificial diets incorporating L-canavanine sulfate are also manifested in the larval nutritional parameters (Table 6). There were successive decreases in the rates of growth (G), food consumption (F), assimilation (A), respiration (O), and excretion (S) with increasing concentrations of L-canavanine sulfate. The efficiency with which the larvae converted ingested (ECI) and digested (ECD) food to body substance was similar for larvae fed either control diet or 10 mM L-canavanine diet. However, larvae fed higher concentrations of the amino acid could not convert food to body
Table 6. Effect of dietary L-canavanine sulfate concentration on the nutritional physiology of *Trichoplusia ni* reared on artificial diet.

<table>
<thead>
<tr>
<th>Nutritional Parameter (mg x g⁻¹ x day⁻¹)</th>
<th>Dietary L-canavanine concentration (mM)¹</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G</em></td>
<td></td>
<td>160±4</td>
<td>115±2</td>
<td>50±3</td>
<td>3±2</td>
</tr>
<tr>
<td><em>F</em></td>
<td></td>
<td>558±14</td>
<td>400±6</td>
<td>211±8</td>
<td>61±8</td>
</tr>
<tr>
<td><em>A</em></td>
<td></td>
<td>333±8</td>
<td>235±3</td>
<td>120±4</td>
<td>30±6</td>
</tr>
<tr>
<td><em>O</em></td>
<td></td>
<td>173±4</td>
<td>120±2</td>
<td>70±2</td>
<td>30±5</td>
</tr>
<tr>
<td><em>S</em></td>
<td></td>
<td>225±7</td>
<td>166±4</td>
<td>91±4</td>
<td>30±3</td>
</tr>
<tr>
<td>Indices</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.C.D.(%)</td>
<td></td>
<td>48±1</td>
<td>49±1</td>
<td>41±1</td>
<td>11±6</td>
</tr>
<tr>
<td>E.C.I.(%)</td>
<td></td>
<td>29±1</td>
<td>29±1</td>
<td>24±1</td>
<td>5±4</td>
</tr>
<tr>
<td>A.D.(%)</td>
<td></td>
<td>60±1</td>
<td>59±1</td>
<td>57±1</td>
<td>54±4</td>
</tr>
<tr>
<td>E.C.C.I.(%)</td>
<td></td>
<td>7±1</td>
<td>7±1</td>
<td>5±1</td>
<td>1±1</td>
</tr>
</tbody>
</table>


¹ Means ± S.E. All groups (n = 20) are significantly different (P << .001) except A.D. and E.C.C.I. (not tested) based on ANOVA tests.
substance as readily, which is indicated by a lower ECI and ECD. In contrast, there was no difference in the approximate digestibility (AD) between all groups.

**Diet caloric determinations**

Three replicate combustions were made on dry control diet samples to determine the dietary caloric content (figure 9). Since the dietary addition of L-canavanine sulfate represents less than 1% of the total dry weight of the diet, differences due to diets containing 10, 20, and 30 mM concentrations of L-canavanine sulfate were assumed to be below the level of detection of the assay. Larval caloric consumption was calculated from the dry food consumed by each larvae times the caloric content (4.4 Cal/mg) of dry diet. The mean larval caloric consumption parallels the mean larval food consumption, diminishing with greater concentrations of dietary L-canavanine sulfate. The efficiency of conversion of caloric ingestion to body substance (ECI) follows the ECI; it is similar for larvae fed either control (0 mM) or 10 mM L-canavanine diet but decreases when larvae are fed either 20 mM or 30 mM canavanine diet.
Figure 9. Bomb calorimeter temperature changes during the combustion of control (0 mM) diet: Δ, replicate 1; ◆, replicate 2; +, replicate 3, illustrate the consistent temperature change when similar amounts of diet are burned in different replicates.
DISCUSSION

Observations on the growth, development, and nutritional physiology of *L. ni* indicate that the physiological processes responded to different dose concentrations of L-canavanine sulfate incorporated into their diets. Fifth stadium cabbage looper larvae consuming diets containing 20 or 30 mM L-canavanine weighed significantly less than their control counterparts by day 1, while larvae fed a diet including 10 mM L-canavanine remained comparable to controls until the second day. Maximum differences in weight occurred by day 2.5. Pupal formation was also significantly delayed when dietary concentrations of L-canavanine were increased. Furthermore, larvae fed diets containing 10 mM canavanine were developmentally delayed but adult emergence was similar to that of control animals. Most larvae ingesting diets containing higher concentrations of L-canavanine never emerged as adults. Moreover, the rates of food consumption (*F*), growth (*G*), assimilation (*A*), respiration (*R*), excretion (*S*), percent efficiency of conversion of ingested food to body substance (*ECI*), and the percent efficiency of digested food to body substance (*ECD*) decreased with increased L-canavanine consumption. Similar deleterious results were observed for the growth, development, and nutritional physiology of
Manduca sexta (Dahlman, 1977) and (Dahlman and Rosenthal, 1975, 1976), Anthonomus grandis (Vanderzant and Cremos, 1971), and Tribolium castaneum (Harry et al., 1976) following the consumption of diets incorporating L-canavanine. These insects as well I. ni may have gained weight more slowly and required more time to attain metamorphic changes, after the ingestion of L-canavanine, because of anomalous protein formation by substitution of L-canavanine for L-arginine (Rosenthal, 1983). The concept of anomalous protein formation has been supported by Pines et al. (1981) who demonstrated in vitro L-canavanine incorporation into the vitellogenin protein of the migratory locust Locusta migratoria migratorioides and by Dahlman and Rosenthal (1976) who verified in vivo integration of L-canavanine into Manduca sexta larval non-gut proteins. Further tests will be required to determine if L-canavanine is also assembled into I. ni larval proteins.

While there was no apparent avoidance or other behavioral inhibition of the cabbage looper larvae to diets containing L-canavanine, larval food consumption was dramatically reduced in those groups exposed to dietary L-canavanine. Likewise, Manduca sexta larvae maintained on artificial diet containing L-canavanine also displayed reduced food consumption.
(Dahlman, 1977). However, Prodenia eridania larvae were completely repelled by artificial diets with high concentrations of L-canavanine (Rehr, 1973) indicating that the amino acid was a phagodeterrent to that insect. Reduced L. ni food consumption does not seem to be a direct behavioral response to the presence of L-canavanine as that observed in Prodenia eridania but possibly a result of attenuated physiological processes. In other words, the larval digestive and metabolic systems are not capable of using the food efficiently for growth and development which may contribute, via a feedback mechanism, to a reduced food consumption. L-canavanine is known to be a metabolic inhibitor of enzymes such as alcohol dehydrogenase, lactic acid dehydrogenase, β-glucosidase as well as enzymes for arginine metabolism (Rosenthal, 1977). Altered enzyme activity, due to anomalous protein formation or the direct interference of L-canavanine moieties, could lead to a serious loss of biological function of all cells. It appears that L-canavanine may have an effect on insects at many levels including behavioral and physiological.

Trichoplusia ni larvae returned to the control diet after ingesting diet with L-canavanine for four days exhibited remarkable resurgence in growth and development. Because anomalous proteins are degraded more rapidly
than normal proteins in human diploid fibroblasts (Fong and Poole, 1982) it
is possible that rapid degradation of aberrant proteins is true of insects as
well. If so, then anomalous proteins incorporating canavanine would quickly
be destroyed, replaced by new proteins without L-canavanine, returning
metabolic functions, food consumption, and growth to normal in L. ni larvae.

Anomalous protein formation could account for all reductions in the
observed L. ni nutritional parameters with increasing L-canavanine
concentrations except approximate digestibility (AD) which remains
constant. Essential nutrients are carried across the gut tissue by
proteinaceous membrane transport systems (Cioffi, 1984) and should be
affected by anomalous protein formation. A severe reduction in approximate
digestibility (AD) in addition to the observed reductions in the rates of
growth (G), food consumption (F), assimilation (A), respiration (O), and
excretion (S), the percent efficiency of conversion of ingested food (ECI) and
digested food (ECD) to body substance would be expected if all
proteinaceous systems were affected. However, if inhibition were
occurring within the gut, excluding the gut membrane, approximate
digestibility might remain steady regardless of the L-canavanine
concentration. In that case an elevated efficiency of conversion of ingested
and digested food to body substance might occur to compensate for the lower food consumption as reported for *L. ni* parasitized by *Hyposoter exiguae* (Thompson, 1982b). This was not observed in *L. ni* treated with L-canavanine, suggesting a more complex interaction between L-canavanine and cabbage looper larvae. Determination of *in vivo* L-canavanine uptake across gut membrane would yield pertinent information on the nature of transport systems and nutrient absorption in stressed *L. ni* larvae. Moreover, estimates of gut enzyme activity for carbohydrate, lipid, and protein digestion would identify some of the locations of L-canavanine action. These areas need to be addressed to gain a complete understanding of L-canavanine-insect interactions.

Deviant protein formation may be the major characteristic of L-canavanine toxicity (Rosenthal, 1977); however, there are other curious observations when insects are exposed to L-canavanine: fifth stadium *Manduca sexta* larvae fed artificial diets with 5:1 and 10:1 ratios of L-arginine to L-canavanine (where L-canavanine concentrations were 2.5 mM and 5.0 mM respectively) attained almost twice the weight of comparable control larvae (Dahlman and Rosenthal, 1976). L-canavanine enhancement of the normal diuretic hormone response in the locust *Locusta migratoria*
migratorioides was followed by an increase in Malpighian tubule secretion and a drop in haemolymph volume (Rafaeli and Applebaum, 1982). Continuous motor activity was induced one to two hours after injection of L-canavanine into adult *Manduca sexta* moths (Kammer et al., 1978). L-canavanine conversion to L-canaline and urea by arginase is the predominant catabolic pathway (Rosenthal, 1970) and L-canaline's biological effects are even more harsh than L-canavanine. Enzymes possessing associations with pyridoxal phosphate (vitamin B₆) were inhibited because of L-canaline–pyridoxal phosphate complexes (Rosenthal, 1983). These results suggest alternative mechanisms for the toxicity of L-canavanine in addition to anomalous protein formation.

Recent evidence indicates that urea and uric acid may also play a role in insect growth and development: *Drosophila melanogaster* larvae exposed to high dietary levels of waste products such as uric acid and urea experienced delayed development which may have resulted from intoxication (Botella et al., 1985). Perhaps a similar effect is prevailing in *L. ni*: intoxicating influences of excess urea from the catabolism of L-canavanine may have reduced the food consumption and growth rate while leaving the membrane transport systems intact. Approximate digestibility would then
remain constant as verified (Table 6). Botella et al. (1985) demonstrated an association between overcrowded rearing conditions with increases in waste products and delays in development. Delays in \textit{I. ni} larval growth and production of malformed pupae are similar when reared on diets incorporating L-canavanine and in overcrowded rearing conditions (Binder, unpublished observations). \textit{Trichoplusia ni} larvae may be responding to gut lumen increases in urea from the catabolism of L-canavanine. The above observations exemplify the various physiological manifestations after infiltration of L-canavanine into the systems of insects. Additional experimental evidence is needed to identify alternate schemes for the observed deleterious effects of L-canavanine on cabbage looper larvae.

The mechanisms of the metabolic action of the non-protein amino acid L-canavanine and other secondary plant compounds could be one or a combination of the following: 1) modification or disruption of DNA replication, RNA transcription, and protein synthesis, 2) rearrangement of active or passive membrane transport processes, 3) enzyme inhibition or activation, and 4) blocking or enhancing receptor sites for chemical transmitters (Robinson, 1979). L-canavanine may cause anomalous protein production in \textit{I. ni} and also have other metabolic interactions. All aspects
of the mode of action of L-canavanine should be addressed to give a complete physiological and behavioral scheme of L-canavanine–T. ni interactions. Further research on T. ni–canavanine interactions will certainly elucidate these alternate mechanisms and improve an understanding of secondary plant compound–insect interactions.

SUMMARY

Trichoplusia ni larvae reared on artificial diets containing L-canavanine sulfate exhibited lower rates of weight gain and delays in development as compared to their control counterparts. Moreover, there were reductions in all quantitative nutritional rates and indices, except approximate digestibility, due to the presence of dietary L-canavanine. Observations indicate that the animal's growth, development, and nutritional physiology is more severely inhibited when dose concentrations of L-canavanine were increased. However, the inhibition from ingestion of L-canavanine can be reversed if the T. ni larvae are returned to control diet. These observations illustrate the complex nature of L-canavanine–T. ni interactions and the need for more experimental studies on this topic.
References


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