Insect Biochem Vol 15, No 2, pp 315–320, 1985
Printed in Great Britain

0020–1790/85 $3 00+0 00

CELLULOSE DIGESTION IN A LEAF EATING INSECT, THE MEXICAN BEAN BEETLE, *EPILACHNA VARIVESTIS*

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(*Received* 15 *July* 1983, *revised* 12 *June and* 29 *August* 1984)

Abstract—Adult Mexican bean beetles, *Epilachna varivestis* (Mulsant) (Coleoptera Coccinellidae), are able to digest cellulose Females digest nearly three times as much cellulose and retain food in their guts three times longer than do males. There is no sexual dimorphism in enzyme activity although pH exerts a profound effect The approximate pH optima are 4 5 for C_1-cellulase, 5 5 for C_x-cellulase and 4 5–4 8 for β-glucosidase The acidic gut of beetles (pH 4 9–5 8) should permit maximum cellulase activity

Key Word Index Cellulose, C_1-cellulase, C_x-cellulase, β-glucosidase, gut pH, herbivorous, Mexican bean beetle, phytophagous insects, *Epilachna varivestis*

INTRODUCTION

Cellulose digestion has been reported for several species of wood-inhabiting and detritus-feeding insects (Lasker and Giese, 1956; Bayon and Mathelin, 1980; Taylor, 1982). Few leaf eating insects have been tested for such an ability, it has generally been assumed that they are incapable of cellulose digestion (Friend, 1958; Wigglesworth, 1972, House, 1974). Enzymatic assays have shown that while many leaf eating (phytophagous) insects, such as *Epicauta fabami* (bean blister beetle, adult), *Bombyx mori* (silkworm, larva), and *Dictyoploca naponica* (Japanese giant silk moth, larva), possess β-glucosidase (Koike, 1954, Ito and Tanaka, 1959; Mukaiyama, 1961), they lack the C_1- and C_x-cellulases necessary to initiate and carry out the hydrolysis of native cellulose (Ito and Tanaka, 1959; Feir and Beck, 1961; Khan and Ford, 1967, House, 1974). The European corn borer *Ostinia nubilalis* also lacks cellulases (Beck *et al.*, 1949) The only leaf-eating insect shown to have cellulases, the migratory locust *Shistocerca gregaria*, possesses enzymes with activity so low that nutrients may be derived from cellulose only during periods of prolonged starvation, when food is maintained in the gut for long periods (Evans and Payne, 1964)

The present study was undertaken to determine whether the Mexican bean beetle was capable of utilizing cellulose and the activity and optimum pH of the cellulases involved. Bean beetles skeletonize bean leaf tissue, yet produce liquid faeces, which seems to indicate that cellulose was being hydrolyzed

MATERIALS AND METHODS

Rearing

Mexican bean beetles were reared from egg to adulthood on lima beans, *Phaseolus lunatus*, under conditions of 15 hr light–9 hr dark, 40% r.h and 25°C

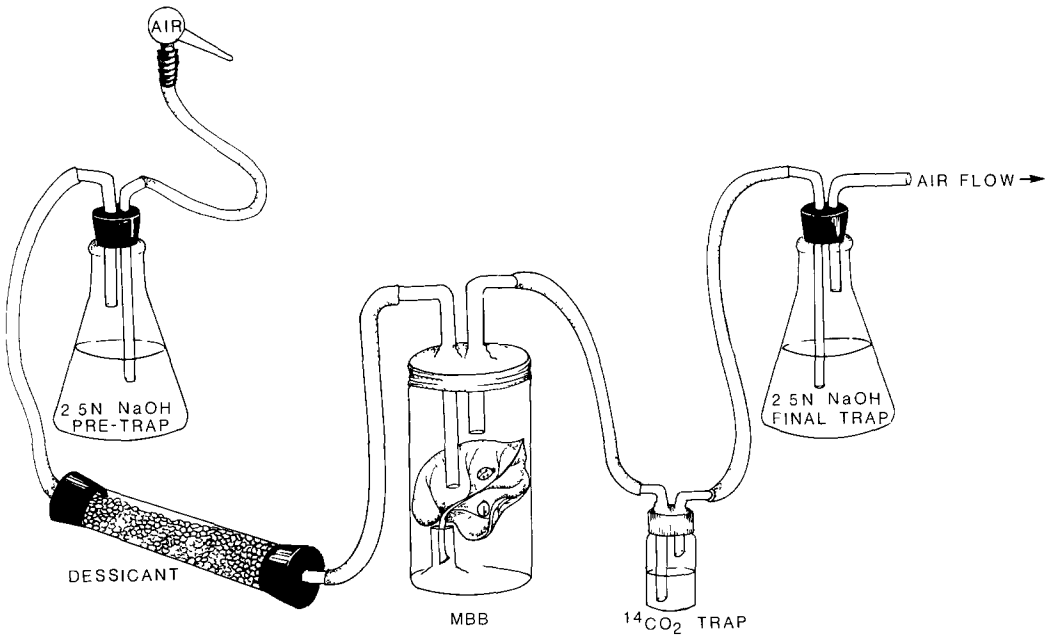

Fig 1 Apparatus for [^{14}C]cellulose degradation assay

[^{14}C]cellulose degradation

Four replicates, consisting of three 1-week-old adults per replicate, were run on each sex Beetles were starved for 12 hr, then placed in a train of flasks from which $^{14}CO_2$ could be trapped (Fig 1) Beetles were housed for the first 24 hr in small glass jars each with a lima bean leaf in a florist's water pic glued to the bottom A 3 mg mixture of [^{14}C]cellulose (New England Nuclear) and cellufil (U S Biochemicals, 1 500, sp act 7 7 μCi/mg) was placed on the leaf Expired $^{14}CO_2$ was trapped for 24 hr in 6 ml of ethanolamine–ethylene glycol monomethyl ether (1 2, v/v) trap Control experiments lacked beetles After 24 hr, the trap with $^{14}CO_2$ was replaced with a fresh vial of trap solution, the radioactive trap was shaken and 2 ml was removed and added to counting cocktail (toluene–ethylene glycol monomethyl ether 2 1, v/v and PPO, 5 5 g /l, Jeffay and Alvarez, 1961) and counted for radioactivity The uneaten bean leaves were dried (60°C), crushed and suspended in Cab-o-Sil cocktail to determine the amount of [^{14}C]cellulose not ingested (Taylor, 1982) The faeces were washed out of the small glass jar with a toluene-PPO cocktail (PPO 5 g, toluene to one litre) and counted for radioactivity The beetles were moved to clean jars with small, unlabelled bean leaves as food

After each 24 hr period, for a total of seven days, beetles were transferred to clean jars with fresh bean leaves and spent $^{14}CO_2$ traps were removed to be counted and replaced with new vials of trapping solution The remains of the leaves in used vials were dried and crushed Cab-o-Sil cocktail was added to suspend the leaves which were spotted with faeces and to dissolve any faeces adhering to the side of the vial Eggs were removed from the bean leaves, weighed, solubilized with NCS solubilizer and counted for radioactivity (Taylor, 1982) On the eighth day the digestive tracts, ovaries and fat bodies of the beetles were removed and solubilized separately, and the carcasses were dried (60°C), crushed and suspended in Cab-o-Sil cocktail A solution of NaOH in the pre-trap (Fig 1) removed CO_2 from the air to prevent the $^{14}CO_2$ trap from becoming saturated with non-labelled CO_2 The NaOH final trap prevented any $^{14}CO_2$ from being vented into the room Sub-samples of the final traps were counted for radioactivity at the end of the experiment and were found to contain no $^{14}CO_2$ Corrections were made for quenching and counting efficiency Counting rates were assayed on a Beckman model LS230 liquid scintillation counter The amount of cellulose ingested was determined by subtracting the [^{14}C]cellulose radioactivity remaining on each leaf after the initial feeding from counts of control leaves Most of the label not accounted for was probably lost as $^{14}CO_2$ during vial changes Such loss would cause an underestimation of the amount of cellulose digested Very small losses could be due to faeces squirted to marginally accessible places such as edges of gaskets, etc

After the experiment was completed it was brought to the author's attention that [^{14}C]cellulose is sometimes contaminated with other, more readily digestible compounds Subsequent purification of a sample of the [^{14}C]cellulose used in this study with ten washes each of 0 2 M HCl and 0 2 M NaOH (Leedle and Hespell, 1980), revealed that 0 91% of the counts in [^{14}C]cellulose were due to contaminants Such a small percentage would not effect the results of this study

Gut transit time

The rate at which cellulose passes through the digestive tract was determined by feeding male ($n=11$) and female ($n=8$) beetles bromcresol purple-dyed cellufil The beetles were starved for 12 hr, placed on leaves covered with dyed cellufil (2% dye in 25% alcohol, air dried) and allowed to feed for 1 hr They were then removed to fresh bean leaves to feed until purple faeces appeared

Enzyme assays

The basic procedures used were those of Taylor (1982) with slight modifications Whole guts were triturated in 0 001 M potassium phosphate buffer (pH 7 0), centrifuged (10,000 ***g***, 4°C, 20 min) and the supernatant fluid was then passed through PD-10 Sephadex columns (5-cm bed height) to give a concentration of four guts/8 ml (test for β-glucosidase at 5 pH values), 19 guts/5 3 ml (test for C_x-cellulase at 5 pH values), and 45 guts/6 6 ml (test for C_1-cellulase at 6 pH values) For each substrate, ten assays were run on each sex at each pH The substrates were suspended in 0 1 M buffers of pH 3 5 (sodium citrate), 4 5 and 5 5 (sodium acetate), 7 5 (sodium phosphate), 9 5 (sodium carbonate) and 10 5 (sodium phosphate) Controls for all enzyme assays were run with enzyme denatured by boiling in a water bath for 15 min

To determine the presence of C_x-cellulase, a 1% suspension of carboxymethyl cellulose was made in the appropriate buffer, and 0 3 ml of the solution was incubated with 0 3 ml of enzyme for 60 min at 35°C Incubation was terminated by adding 0 6 ml of 3,5-dinitrosalicylic acid reagent (Bernfield reagent) and heating the mixture in a boiling water bath for 5 min A 0 9 ml portion of water was then added, and the optical density at 540 nm determined (Bernfield, 1955)

C_1-cellulase activity was determined as above except that the microcrystalline cellulose was mixed with buffer as 50 mg/ml, incubation was for 24 hr with shaking, and incubation was terminated by rapid filtration through Celite® before Bernfield reagent was added

A 3 32 mM solution of *p*-nitrophenyl-β-D-glucoside was used to determine the presence of β-glucosidase A 0 5 ml portion of the enzyme was incubated with 0 5 ml of substrate for 15 min at 35°C Incubation was terminated by the addition of 1 ml of 1 M NH_4OH–NH_4Cl buffer (pH 9 8) and the optical density at 420 nm was determined

For the determination of the amount of protein present in each sample, the Bradford protein assay (Bradford, 1976) was used to run enzyme extracts and protein standards containing bovine albumin

Gut pH

Whole guts of fed specimens ($n=6$) and of specimens starved for 16 hr ($n=7$) were dissected out into a dry glass dish and the pH of the gut was measured with a Beetrode (W-P Instruments, 60 Fitch St, P O Box 3110, New Haven, CT 06515, U S A) and reference electrode with a fine pulled tip The Beetrode is a micro-electrode of pH sensitive wire 0 1 mm diameter by 3 mm long The gut was pierced at various points along its length by the micro-electrode and tip of the reference electrode so that an accurate, intact-gut pH could be measured

Plant tissue composition

The amount of cellulose in bean leaves was determined from greenhouse-reared *Phaseolus lunatus* by the method of Goering and Van Soest (1970)

Bacterial enumeration

Beetles were decapitated, then surface sterilized under u v light for 2 min each on dorsal and ventral body surfaces (Preliminary tests indicated that this method of sterilization was more effective than lysol, chlorox or alcohol)

Beetles were placed in sterile bowls in a laminar flow hood and the gut was dissected out using standard sterile procedures Whole guts were triturated in 10 ml of sterile 0 001 M potassium phosphate buffer (pH 7 0) Initial dilutions were shaken for 20 min on a wrist action shaker, then serial dilutions of each initial dilution were plated out on agar plates (two replica plates per dilution) Agar plates contained Skinner's cellulose medium B (Skinner, 1971) with Hoagland trace element solution, 0 5% carboxymethyl cellulose (Sigma Chemical Co), 1% agar and cycloheximide (0 1 mg/ml) to inhibit fungi All innoculated plates were incubated aerobically at 26°C, 60% r h for nine days, then plates with 30–300 colonies were flooded with 1% hexadecyltrimethyl ammonium bromide (Hankin and Anagnostakis,

1977) Zones of clearing around or under colonies (which were scraped from the plate before flooding) indicated an ability to produce C_x-cellulase, the number of colony forming units showing such clearing was enumerated

Statistics

Radioisotope data were arc-sine transformed and analyzed by Kruskal–Wallis non-parametric ANOVA

Enzymatic differences between sexes and among pHs were analyzed using the Friedman non-parametric analysis of variance Where differences were found, the Newman–Keuls multiple range test was employed Curves for figures were fitted with a cubic spline Differences in gut pH of starved and fed beetles were tested by Chi-square (Zar, 1974)

RESULTS

[^{14}C]cellulose degradation

While both male and female beetles assimilated a substantial amount of cellulose, there were significant differences between the sexes in the percentage utilization of the labelled cellulose (Table 1); females respired twice as much $^{14}CO_2$ and assimilated three times as much [^{14}C]cellulose as males. Males initially ingested far less [^{14}C]cellulose than did females and spent most of the experiment running about, presumably in search of females, rather than feeding. Because the young males were not approaching diapause, their fat bodies were very small and were not expected to have acquired much radioactive material Females, on the other hand, spent most of their time feeding, and digested nearly half of the [^{14}C]cellulose ingested Of this, two-thirds was respired while a fifth was utilized in egg production

Table 1 Distribution of label of [^{14}C]cellulose after ingestion by Mexican bean beetles

	Label distribution (%)*		
Source of label	Males	Females	Probability
CO_2	10 7±1 4	21 6±1 2	0 008
Faeces	62 9±5 9	35 9±2 7	0 008
Fat body/eggs	0 8±0 1	9 3±1 0	0 008
Carcass	1 5±0 4	1 3±0 2	NS
% Accounted for	87 8±5 1	83 1±1 4	—
% Assimilation/ingestion	17 0	47 3	—

*Values are mean ± SE

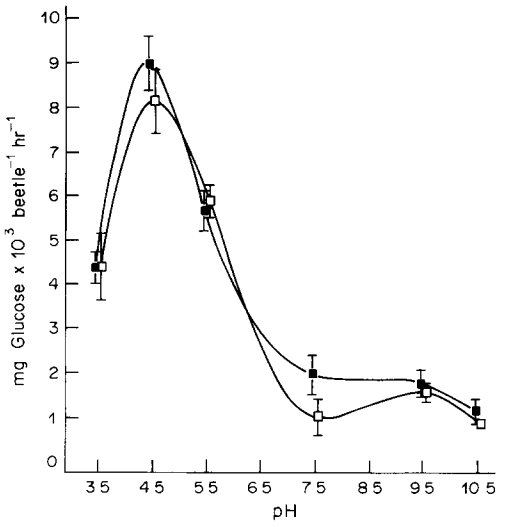

Fig 2 C_1-cellulase activity at various pH values Values are mean ± SE (■ male, □ female)

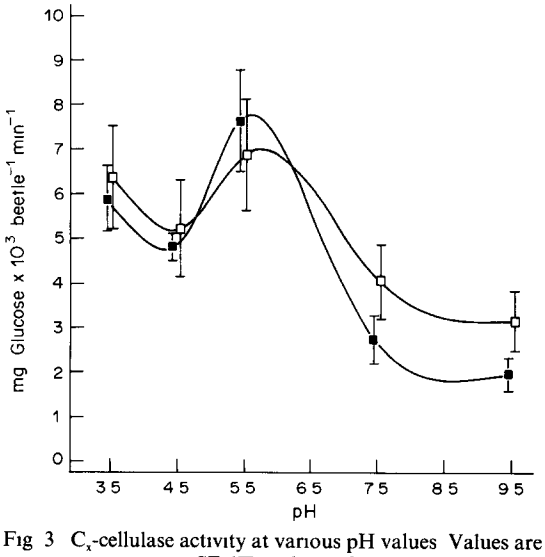

Fig 3 C_x-cellulase activity at various pH values Values are mean ± SE (■ male, □ female)

Enzyme assays

Fitting a cubic spline curve to the results of enzyme assays carried out at various pH values indicates the pH giving optimal activity for each enzyme (Fig 2)

Both males and females showed high levels of enzymatic activity, and there were no significant differences between the sexes C_1-cellulase activity in both sexes showed a sharp optimum at pH 4.5, dropping rapidly at higher and lower pH values. Optimum C_x-cellulase activity for both sexes appears to be highest at low pH values with an optimum around pH 5.5 and a smaller peak at pH 3 5 or lower (Fig 3)

β-Glucosidase has a pH optimum around 4 5–4 8, with activity dropping steeply below that pH (Fig. 4). While none of the enzymes appears to function well at pH 7 5 or above, there is measurable activity, especially for C_1- and C_x-cellulases at the more alkaline pH values

In Table 2 enzyme activities at various pH values are ranked from highest to lowest. Statistical tests indicated slight differences between sexes in the ranking of pH with maximal activity For C_1-cellulase in males, activity at pH 4.5 was significantly higher than at pH values 7.5–10.5 ($P<0.001$), 3.5 and 5 5

Table 2 Ranked differences in enzyme activity as a function of sex and pH

	Ranked pH*											
Enzyme	Males						Females					
C_1-cellulase	4 5	5 5	3 5	7 5	9 5	10 5	4 5	5 5	3 5	9 5	7 5	10 5
C_x-cellulase	5 5	3 5	4 5	7 5	9 5		5 5	3 5	4 5	7 5	9 5	
β-Glucosidase	4 5	5 5	7 5	3 5	9 5		4 5	5 5	7 5	3 5	9 5	

*Underlined variables are not significantly differently at $P<0$ 05 Relative values are given in the test

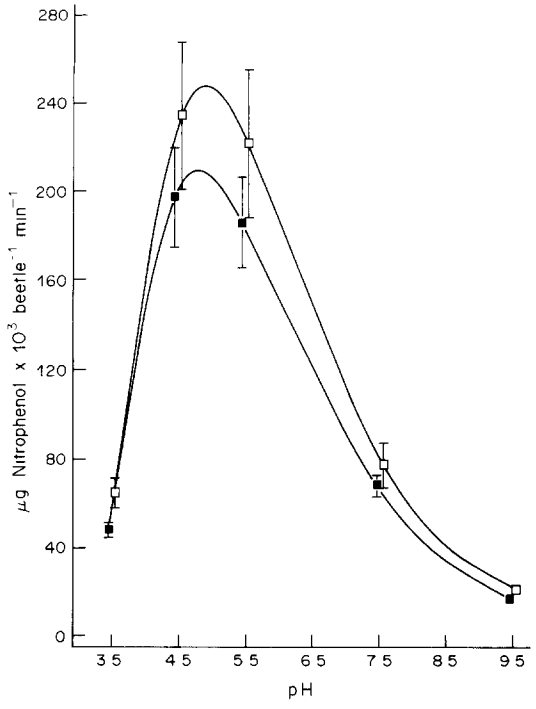

Fig 4 β-Glucosidase activity at various pH values Values are mean ± SE (■ male, □ female)

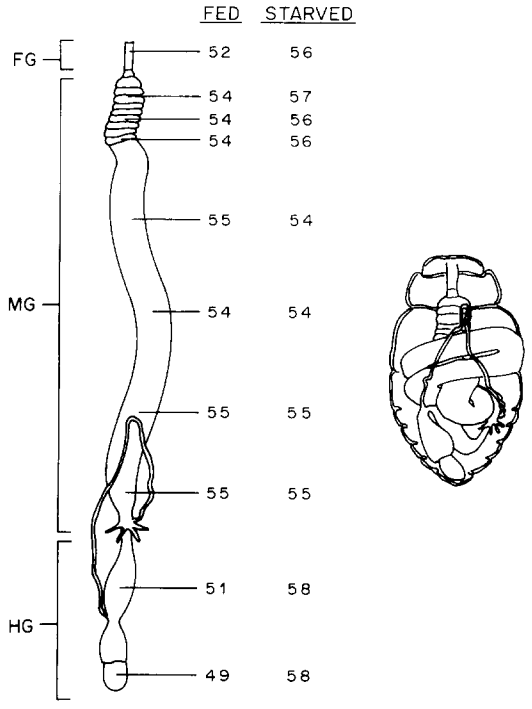

Fig 5 pH along digestive tract of fed (*P lunatus*) and starved (16 hr) Mexican bean beetles Arrangements of digestive tract in animal is shown in inset (drawn after Burgess, 1932) FG = foregut, MG = midgut, HG = hindgut

($P < 0\ 005$) Activity at pH 5.5 was significantly higher than at 7 5–10 5 ($P < 0\ 005$), while pH 3 5 showed greater activity than 9 5, 10 5 ($P < 0.025$) and 7.5 ($P < 0\ 01$) In females a similar trend was apparent with activity at pH 4 5 being significantly higher than at pH values 7.5–10 5 ($P < 0\ 001$), 3 5 ($P < 0.005$) and 5 5 ($P < 0\ 025$). pH 5 5 ranked next ($P < 0\ 005$ for differences with pH values 7 5–10 5), while pH 3 5 showed higher activity than pH values 7 5 and 9 5 ($P < 0\ 005$) and 10.5 ($P < 0\ 01$)

Only males showed significant differences among pH values in C_x-cellulase activity with pH 5.5 being greater than pH values 7 5 and 9 5 ($P < 0\ 05$) Although the large variance makes the pH at 5.5 not statistically significant for females, it seems probable, based upon the shape of the curve, that this pH is nearly optimum for this enzyme

β-Glucosidase activity in males indicated that pH 4 5 and 5 5 showed significantly higher activity than other pH values tested (all $P < 0\ 005$) In females pH 4 5 and 5 5 showed significantly greater activity than pH 9.5 ($P < 0\ 005$) and pH 3 5 and 7 5 ($P < 0\ 01$)

Extracts for C_1-cellulase and C_x-cellulase contained 48–110 ($\bar{x} = 67$) μg protein/ml and for β-glucosidase extracts contained 8–32 ($\bar{x} = 23$) μg protein/ml

Gut pH

Whether beetles were fed or starved, the whole gut tended toward acidic pH (4 9–5.6), although in fed animals the pH in the foregut and hindgut was slightly less acidic (Fig 5). Differences between sexes and between fed and starved insects, however, were not statistically significant

DISCUSSION

Mexican bean beetles prefer feeding on beans of the genus *Phaseolus* and on the soy bean, *Glycine max*. While other aspects of bean beetle nutrition have been studied (LaPidus *et al*, 1963, Nayer and Fraenkel, 1963, Kogan, 1972), the digestion of cellulose has not been examined In fact, an artificial diet developed for rearing Mexican bean beetles includes cellulose as a non-nutritive substrate to provide bulk (Kogan, 1971)

The activity of C_1-cellulase, C_x-cellulase and β-glucosidase were similar in male and female beetles indicating that both sexes possess the same capacity to digest cellulose However, females assimilated nearly three times as much [^{14}C]cellulose as males which is probably due to male beetles retaining cellulose in their digestive tracts for only 2 25–3 50 hr, whereas females retained cellulose for 8.75–12 hr Such a disparity between enzyme activity and cellulose consumption has also been found in the cockroach *Periplaneta americana*, in which males have higher cellulase levels than females, but only adult, egg-laying females consume filter paper (Wharton *et al*, 1965)

The ability to digest cellulose would be a function, not only of the presence of cellulolytic enzymes, but also of a gut pH that allows these enzymes to function at their maximum rate The pH range of the gut of the Mexican bean beetle is near the optimum range for the cellulolytic enzymes found and therefore should permit maximum cellulose digestion Cellulases found in other arthropods are, like bean beetle cellulases, most active at acidic pH values (Lasker and Giese, 1956, Newcomer, 1956) and the gut pH of cellulose-digesting invertebrates, such as the snail *Helix pomatia* (Myers and Northcote, 1958) and the slug *Arion ater* (Evans and Jones, 1962), are acidic Invertebrates

which are unable to digest cellulose, such as *Tipula abdominalis*, and some caddisflies and stoneflies, often have a neutral to alkaline gut (Martin *et al.*, 1980, 1981a, 1981b).

Bacteria able to produce C_x-cellulase are present in the digestive tract of the Mexican bean beetle, but in such low numbers (600–1750 colony forming units cultured per beetle) and with such moderate enzymatic activity, that they could not account for the high levels of enzymatic activity found in the gut This is in marked contrast to the 10^5–10^8 C_x-cellulase producing bacteria per gut which were cultured from the guts of cockroaches where cellulases are often of microbial origin (Cruden and Markovetz, 1979). It is very unlikely that strict anaerobic cellulolytic bacteria are present in the bean beetle digestive tract, since the guts of such small animals are aerobic (Gillot, 1980). It is therefore probable that cellulose digestion in the Mexican bean beetle is not due to microorganisms.

Cellulose digestion should contribute substantially to meeting the energy needs for egg production and maintenance in female Mexican bean beetles. Females weighed 48.91 ± 1 58 mg ($\bar{x}$ ± standard error, $n = 10$) and each laid a clutch of 45–60 ($\bar{x} = 53$) eggs, weighing 14.32 ± 0.61 mg per clutch ($n = 7$) every two days for about five weeks. Thus they utilized the equivalent of nearly 30% of their body weight for egg production every two days, hence their energy needs are quite high. Lima beans contain 21% (dry wt) cellulose (Table 3), so an ability to digest this polysaccharide

Table 3 Percentage composition of fibre in *P. lunatus* leaves*

Soluble non-cell wall†	48 0 ± 2 7
Hemicellulose	26 7 ± 0 7
Cellulose	21 4 ± 2 0
Lignin	2 9 ± 0 1
Ash	1 0 ± 0 2

*Values are mean ± SE for 5–0 5g dry weight samples †Includes such readily digestible nutrients as proteins, sugars, starches, etc

raises the amount of readily digestible plant material from 48 to 58% (whether hemicellulose can be digested is not known). The increased efficiency in metabolizing bean plants because of an ability to digest cellulose means that individuals, especially females, can minimize time spent in foraging. Two potential effects of this are the avoidance of predators (Plummer and Landis, 1932, Howard and Landis, 1936) and, perhaps more importantly, the ability to remain in suitable microclimates for relatively long periods (Bernhardt and Shepard, 1978).

Mexican bean beetles are believed to have evolved on the plateau of southern Mexico, an area characterized by moderate temperatures and high rainfall (Marcovitch and Stanley, 1930) As might be expected of an organism that evolved under such a regime, high temperature and low relative humidity have adverse effects on such life history traits as adult longevity, time of laying the first egg mass, percentage of beetles ovipositing, percentage egg hatch, fecundity, and adult and larval survival (Kitayama *et al.*, 1979, Lockwood *et al.*, 1979; Sprenkel and Rabb, 1981) Increased efficiency in food plant utilization, however, should result in low exposure to adverse conditions otherwise encountered in extensive foraging, and, in the case of females, more time spent in microhabitats suitable for the development of their eggs This could be of particular value in the southwestern United States where summers are characterized by high temperatures, low relative humidities and high solar radiation (Miller, 1930, Douglas, 1933).

One might expect that phytophagous insects would have been selected to use their host plants efficiently, and that an abundant, energy-rich constituent of leaves such as cellulose would be utilized. Omnivores, detritivores and wood feeders, however, have been the primary trophic groups tested for an ability to digest cellulose Of the thousands of leaf eating insects only three, *Bombyx mori* (silkworm), *Ostinia nubialis* (European corn borer) and *Schistocerca gregaria* (desert locust), have been tested for cellulases (Beck *et al.*, 1949; Ito and Tanaka, 1959; Evans and Payne, 1964; for results of these studies see Introduction section). Yet it has been assumed that phytophagous insects cannot utilize the cellulose of their host plants and that any cellulose added to artificial diets adds only bulk and serves no nutritive function Clearly, there are insufficient data to support such conclusions The finding that Mexican bean beetles do digest cellulose suggests that leaf-eating insects are capable of cellulose digestion and that this trait should be looked for in other species of phytophagous insects

Acknowledgements—I thank F W. Taylor and C S Crawford for review of the manuscript This work was supported in part by NSF grant DEB-8104698 to F W Taylor and UNM research allocations grants to R G Cates and F W Taylor

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