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Original article

Age-related changes in midgut ultrastructure and trypsin activity in the honey bee, *Apis mellifera* *

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Summary — Trypsin-like activity was detected in the epithelial tissue, in the fluid of the ectoperitrophic space, and within the endoperitrophic space of the midgut of adult worker honey bees, *Apis mellifera*. It was highest in free-flying bees and in caged bees fed pollen. Lower levels occurred in caged bees restricted to sucrose syrup or fed sucrose syrup in addition to either Beltsville Bee Diet or egg albumin. Levels of midgut trypsin activity were dependent on the amount of protein diet consumed. Both diet consumption and trypsin-like activity decreased as the bees aged. Ultrastructural changes in the midgut tissue accompanied this decline in enzymatic activity. In five-day-old pollen-feeding bees, the apical cytoplasm of cells in the posterior midgut contained numerous electron-opaque vesicles, and the brush border in the crypts of the distal midgut was composed of short pleomorphic microvilli. Apical discharge from the midgut cells released the opaque vesicles into the midgut lumen. However, in 30-day-old field bees, the number of opaque vesicles and the microvesiculation of the brush border were reduced. Thus, the presence of the endogenously produced endoprotease and the regional variation in cell ultrastructure suggest that the honey bee may rely on countercurrent flow to distribute enzymes and nutrients efficiently throughout the midgut.

***Apis mellifera* — midgut ultrastructure — enzymatic activity — trypsin — age**

Introduction

The exploitation of pollen as a dietary resource by the honey bee, *Apis mellifera*, is a complex process contingent on behavioral, anatomical and physiological adaptations. Although the collection, storage and consumption of pollen by honey bees have been well described in numerous publications, the function of the honey bee midgut (ventriculus) in pollen digestion remains unclear. Based on structural changes observed in pollen grains during transit through the midgut lumen, Whitcomb and Wilson (1929) designated the midgut as the primary organ of pollen digestion. Histological studies suggested that the midgut epithelia were composed of secretory and assimilatory cells and

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that these cells were involved in digestion, absorption, excretion and production of the peritrophic membrane (Snodgrass, 1956).

Zherebkin (1967) reported regional variation of some digestive enzyme activity in the honey bee midgut. Protease, diastase (amylase) and invertase (sucrase) activities were highest in the anterior portions of the midgut, but lipase activity was evenly distributed. Based on these observations, the author speculated that enzyme secretion occurred in the anterior midgut while nutrient absorption followed in the posterior regions and that the main function of the peritrophic membrane was to distribute the digestive enzymes evenly throughout the midgut.

Giebel *et al.* (1971) and Dahlmann *et al.* (1978) analyzed the proteolytic activity in the alimentary tracts of both larval and adult honey bees. Experiments using specific substrates and inhibitors indicated that two proteolytic enzymes present in the honey bee were similar in size and activity to the mammalian serine proteases, trypsin and chymotrypsin. However, the enzymes isolated were extracted from homogenates of entire larvae, pharate adult bee abdomens, and the alimentary tracts of both worker and drone honey bees of unknown age. Extracts of entire abdomens or alimentary tracts contain enzymes which may originate from honey bee tissues, dietary pollen (Stanley and Linskens, 1974), or microbial sources (Barker and Lehner, 1972; Gilliam *et al.*, 1988).

Grogan and Hunt (1979) analyzed proteases in pollen of 14 plant species which were frequently foraged by honey bees and found that all of the pollen samples contained chymotrypsin-like activity, but trypsin-like and carboxypeptidase-like activities were irregular. They then analyzed extracts from honey bee midguts and found that the chymotrypsin activity was highest in the midguts of young pollen-feeding bees and followed a seasonal pattern that coincided with periods of elevated pollen consumption (Grogan and Hunt, 1980). The trypsin activity appeared to be independent of season and pollen consumption but related to the age of the bees. Because entire midguts were analyzed, the origin of the trypsin-like protease could not be determined. With enzyme histochemistry, Peters and Kalnins (1985) demonstrated aminopeptidase activity on the peritrophic membranes and in the apical portions of honey bee midgut cells, but until recently few other digestive enzymes from the midgut tissues of the honey bee had been characterized (Peng, 1980, 1981). Moritz and Crailsheim (1987) analyzed the proteolytic activity in the midgut tissue, ectoperitrophic space, and endoperitrophic space of both pupae and imagos of worker honey bees and detected high levels of both trypsin-like and chymotrypsin-like activity that appeared to originate from the midgut tissue of adult bees. Their data corroborated the seasonal and age-dependent fluctuations in proteolytic activity observed by Grogan and Hunt (1984), but Moritz and Crailsheim (1987) considered the contribution of proteolytic activity from pollen secondary to proteases secreted by the honey bee.

The induction of digestive enzyme synthesis in the insect midgut may be regulated by both direct secretagogue response and hormonal control (Applebaum, 1985). Following synthesis, some enzymes such as peptidases and other hydrolases may remain bound to the microvilli and dedicated to terminal digestion at the brush border (Ferreira and Terra, 1980). But others, such as the endoproteases, must be secreted into the midgut in a way that allows for luminal activity but prevents autolytic digestion of the tissues responsible for their secretion. The process of

protein secretion occurs by one of two routes : constitutive secretion occurs in cells where protein is secreted as fast as it is synthesized and regulated secretion occurs in cells where protein is synthesized and stored in specialized vesicles prior to release (Palade, 1975; Kelly, 1985). Historically, the frequent observation of apical extrusions from insect midgut cells and the absence of obvious storage vesicles in histological preparations suggested that digestive enzymes were elaborated *via* constitutive secretion and released to the gut lumen through holocrine discharge (Snodgrass, 1956; Wigglesworth, 1972). But Khan and Ford (1962) found that holocrine discharge of the midgut epithelial cells increased in starved *Dysdercus faciatus* and was accompanied by reduced enzyme (α-glucosidase) activity; the midgut of feeding insects contained fewer apical extrusions and more enzyme. De Priester (1971) suggested that apical extrusions in histological preparations of the posterior midgut of *Calliphora erythrocephala* were merely artifacts resulting from hypotonic fixation. Recent ultrastructural examination of the insect alimentary tract has revealed accumulations of electron-dense vesicles in specialized midgut cells of numerous insects (for a review see Bignell *et al.*, 1982). Cyclic fluctuations of midgut proteolytic activity have been correlated with the accumulation and secretion of these vesicles (Lehane, 1976; Houk and Hardy, 1982; Berner *et al.*, 1983). This work and morphometric analyses of the cells involved in the cyclic release of these vesicles suggest that some insect digestive enzymes may be elaborated *via* regulated secretion and merocrine discharge (Lehane, 1987).

The purpose of the present work was to determine the influence of age and diet on trypsin-like activity in the honey bee midgut and on the ultrastructural morphology of the midgut epithelial cells. We aimed to correlate biochemical and histological events to gain a better understanding of the process of trypsin secretion in the honey bee midgut. Since enzymes in the endoperitrophic space of pollen-feeding bees may originate from exogenous sources, caged bees were given artificial diets, and our analyses focused on endogenous enzyme activity in the ectoperitrophic space and the tissues of the midgut epithelium.

Material and Methods

Bees

Combs with healthy brood containing pharate adult worker honey bees were collected from free-flying colonies headed by naturally-mated sister queens. The adult bees were allowed to emerge in a swarm box maintained at 27 °C and 30% relative humidity (RH). Twenty-four h following comb collection, bees which had emerged were either collected directly into cages by use of gentle vacuum or were marked individually on the thorax with a spot of quick drying enamel and then promptly returned to the colony from which the comb was obtained. Marked bees of a known age could then be rapidly collected from within a normal developmental niche throughout the extended foraging season (February—October) in the Sonoran desert. The effect of aging on enzyme secretion in the marked bees in the colony and in bees receiving control diets in small laboratory cages was compared.

Four 6" (15.5 cm) x 6" (15.5 cm) x 2" (5.5 cm) steel and screen mesh cages each containing 120 1-day-old honey bees were maintained at 27 °C and 30% RH and supplied with water and 50% sucrose syrup (w/v). Bees in 3 of the 4 cages were provided with protein diets *ad libitum*. A removable polyethylene flask stopper at the front of the cage held a known amount of test diet which was renewed every other day. Weight change was recorded as a measure of diet consumption. Duplicate stoppers containing the diet were weighed simultaneously. One stopper had been placed in an

empty cage to detect any loss due to desiccation, and the other supplied test diet for the bees.

Bees in the 4 cages were supplied with diets as follows :

1. Sucrose syrup and water only.

2. Sucrose syrup, water, and egg albumin diet (EAD; 10% egg albumin, 15% α-cellulose, and 75% saturated sucrose solution mixed to form a stiff paste; Standifer *et al.*, 1960).

3. Sucrose syrup, water, and Beltsville Bee Diet which contains lactalbumin and yeast as protein sources (BBD; Bioserv Inc., Frenchtown, NJ).

4. Sucrose syrup, water, and pollen diet (PD; equal w/v amounts of fresh, bee-collected, mixed floral source pollen stirred with saturated sucrose to form a stiff paste).

Trypsin assay

Trypsin activity was measured in bees that were five and 21 days of age. At five days after emergence when pollen consumption is elevated, workers are generally considered to be nurse bees. At 21 days, workers are in transition from hive to field bees, and pollen consumption is diminished. No further attempt was made to classify the functional caste of the experimental bees.

Twenty bees were collected from each cage, and 20 marked bees were collected from the free-flying colonies for each assay. Groups of 6 honey bees in plastic Petri dishes were chilled at 4 °C until they were immobile. The alimentary tract was extirpated by crushing the thorax with tweezers and gently pulling the terminal abdominal sclerites away from the remaining abdomen. This separated the ventriculus from the crop at the proventriculus, and the hindgut was then cut away at the anterior intestine.

Five guts per treatment were pooled and rinsed in 3 changes of ice cold *Apis* saline (AS) (Brouwers, 1982). Under a dissecting microscope, individual midguts were held submerged in a spot-well that contained 300 µl of ice cold AS, and an incision was made with fine scissors parallel to the long axis of the gut from the pyloric sphincter to the anterior end of the midgut. This released the contents of the ectoperitrophic space into AS. Ruptured samples that allowed the contents of the peritrophic membrane (endoperitrophic space) to escape were discarded. Midgut tissue was rinsed in cold AS and isolated by removing all tissue immediately posterior to the anastomoses of the Malpighian tubules. Then each of the three samples (contents of the ectoperitrophic space, the endoperitrophic space and the midgut tissue) obtained from the five pooled guts was individually diluted in 1 ml of cold AS.

All 1-ml samples were individually homogenized using a chilled glass tissue grinder with a Teflon pestle fitted into a high speed drill (4 strokes at 1 000 rpm). The homogenates were then transferred into chilled tubes and centrifuged at 10 000 rpm for 10 min at 4 °C. The supernatant fluid was collected in chilled tubes, held at 4 °C, and assayed for protein content (Bradford, 1976) and trypsin activity (Geiger and Fritz, 1984).

To run the trypsin assay on a Spec-20 spectrophotometer (Bausch and Lomb, Rochester, NY), the reaction volume was doubled. The 0.2 M triethanolamine buffer with 20 mM $CaCl_2$, pH 7.9, and the 4 mM N-α-benzoyl-dl-arginine-4-nitroanalide (Bz-Ar-NA) were brought to room temperature. Then samples were mixed with buffer and allowed to equilibrate at room temperature for 5 min prior to the addition of the Bz-Ar-NA. The reaction ran for 10 min in 10-mm tubes, and the change in absorbance was measured at 405 nm at 25 ± 1 °C against a water blank. Since the initial level of trypsin activity was stable for only one hour at 4 °C, it was measured immediately following centrifugation.

Enzyme activity is reported in units per bee and/or units per µg of protein. One unit equals one µM of substrate hydrolyzed per min at 25 °C. It is the same unit used by Dahlmann *et al.* (1978) which is numerically equal to the milliunit reported by Moritz and Crailsheim (1987). However, to compare our data to those of Grogan and Hunt (1979, 1980), a conversion factor of 10^{-3} is necessary, and the differences in substrate and methods must be recognized. Trypsin activity reported as units per bee is presented for comparative purposes, but all statistical analyses were performed using the mean values of the data reported as units per µg of protein.

A total of 15 bees from the free-flying colonies and 15 bees from each cage were assayed for trypsin activity in 3 groups of 5 bees per source, and the activity was measured five times for each group. Data were analyzed using SAS—ANOVA, Tukey's studentized range test, and Students *t*-test (SAS Institute Inc., 1985).

To determine the optimum pH range, midgut tissue and the contents of the ectoperitrophic space from a sample consisting of 15 marked 5-day-old honey bees were analyzed at pH 6.7, 7.1, 7.6, 8.1, 8.3, 8.7 and 9.7. Samples were analyzed in triethanolamine buffer with the pH adjusted using NaOH. The highest value obtained at each pH was considered 100, and lesser values were expressed as a percentage of this.

Ultrastructure

Midguts were removed from marked five-day-old and 30-day-old honey bees from free-flying colonies. The ventriculus was extirpated as described in the trypsin assay; fixed in Karnovsky's fixative (4% formaldehyde, 5% glutaraldehyde in cacodylate buffer, pH 7.2; Karnovsky, 1965); rinsed in 0.1 M cacodylate buffer, pH 7.3; postfixed in 2% OsO_4 in sym-collidine buffer (Polysciences, Warrington, PA) or double glass distilled water; and dehydrated in a series of ethanol solutions of increasing concentration (30, 50, 70, 95, and twice in 100%). Tissues were then infiltrated overnight in L.R. White acrylic resin (London Resin Company, Ernest Fullam Inc., Latham, NY), rinsed twice in fresh resin, and polymerized at 60 °C for 24 h in gelatin capsules. Sections were cut on a Porter Blum MT-2 ultramicrotome (Ian Sorval, Inc., Norwalk, CN) using glass knives, poststained in Reynold's lead citrate and uranyl acetate (Mollenhauer, 1974), and viewed on a Hitachi H500 electron microscope at 75 kV.

All chemicals used throughout were analytical grade (Sigma Chemical Co., St. Louis, MO) unless specified.

Results

The diet of marked colony bees consisted of pollen and honey stored within the hives, and development was considered to be normal. The caged bees fed pollen received a diet that was similar to that of the marked bees, and we were able to monitor consumption. However, development of these bees cannot be considered normal because of the extended confinement. The bees fed artificial diets constituted a second level of experimental control because the diets did not contain exogenous enzymes. Thus, trypsin activity in the ectoperitrophic space could be attributed to enzymes released from the midgut tissues as opposed to exogenous enzymes that may have escaped the peritrophic membrane.

The synthetic substrate, Bz-Ar-NA, used to quantify honey bee midgut activity is highly specific for trypsin. Other trypsin-like serine proteases are capable of cleaving this substrate, but the optimum pH for these enzymes is well below neutrality. Since the optimum pH for the trypsin-like activity from the midgut tissue and ectoperitrophic space of honey bees was between 7.8 and 8.0 (Fig. 1), we are confident that the enzyme activity was due to trypsin.

Since all honey bees tested demonstrated trypsin-like activity, the enzyme appeared to originate endogenously. Table I shows that in both five-day-old hive bees and caged bees fed BBD the highest levels of activity in midgut compartments were detected within the endoperitrophic space. The activity per µg

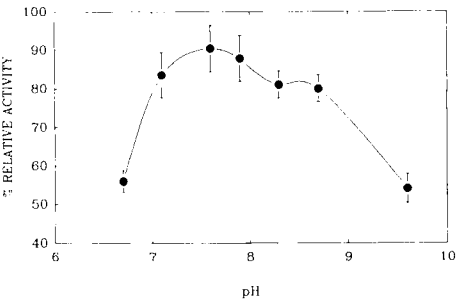

Fig. 1. pH range of trypsin-like activity from the tissue and ectoperitrophic space of the midgut from five-day-old honey bees. Data points are means and standard errors of 8 measurements each.

Table I. Trypsin activity[a] in midgut compartments and whole midguts of 5-day-old hive bees and of 5-day-old caged bees fed Beltsville Bee Diet (BBD) in February.

	Hive bees		*Caged bees fed BBD*	
Sample	*Units/bee*	*Units/μg protein*	*Units/bee*	*Units/μg protein*
Midgut tissue	12.25 ± 3.84	0.08 ± 0.02	3.40 ± 1.38	0.03 ± 0.01
Ectoperitrophic space	16.20 ± 4.16	1.41 ± 0.59	5.48 ± 2.48	0.61 ± 0.34
Endoperitrophic space	95.88 ± 22.98	1.60 ± 0.27	35.03 ± 4.22	0.90 ± 0.10
Whole midgut	138.60 ± 33.90	0.74 ± 0.07	66.20 ± 13.20	0.36 ± 0.08

[a] Values are means ± standard deviations where 1 unit = 1 μM of substrate hydrolyzed/minute at 25°C; $n = 15$.

of protein in the ectoperitrophic space was only slightly lower. These levels were up to 30 times higher than those detected in midgut tissue. The trypsin activity in all compartments as well as in whole midguts was 2—3 times higher in the hive bees than in bees fed BBD.

In tests of the effects of diet on trypsin activity, the amount of diet consumed (mg per bee per day) by the caged bees was as follows : on days 1—5 EAD = 1.00, BBD = 5.86, PD = 9.00; on days 6—21 EAD = < 0.10, BBD = 0.38, PD = 1.00. Results of enzymatic activity are in Tables II and III. In five-day-old honey bees (Table II), trypsin in the midgut tissue and ectoperitrophic space of hive bees was not significantly different from that in the bees fed PD but was significantly higher than that of bees fed BBD, EAD, or restricted to sucrose. Different levels of activity in bees of the same age reported in Tables I and II can be ascribed to seasonal variation. The data in Table I were collected in February from bees from overwintered colonies that had not yet begun population increases for the foraging season, so the trypsin activity per bee was lower than in August. These differences are consistent with seasonal variation reported in the literature (see Introduction). However, the differences were not evident when units per μg of protein were compared. This underscores the relationship of midgut protein to enzymatic activity and illustrates the importance of reporting activity per unit of protein to compare changes related to aging of honey bees when dietary intake cannot be strictly controlled.

The effect of aging on trypsin activity is evident in 21-day-old bees collected in August (Table III). Although the trypsin activity in the midgut tissue of these bees was near the lower limits of the assay, continued consumption of BBD and PD appears to have maintained slightly elevated levels of trypsin activity which were statistically intermediate between those of the hive bees and the bees fed EAD or sucrose. Activities in the ectoperitrophic space of hive and PD-fed bees were not

Table II. Trypsin activity [a] in the midgut tissue and ectoperitrophic space of 5-day-old honey bees receiving various diets in August.

	Tissue		*Ectoperitrophic space*	
Diet	*Units/bee* [b]	*Units/μg protein*	*Units/bee* [b]	*Units/μg protein*
Sucrose	7.32 ± 2.79	0.04 ± 0.02 [c]	33.15 ± 12.65	0.70 ± 0.08 [c]
Egg albumin	6.54 ± 5.73	0.03 ± 0.03 [c]	38.28 ± 16.45	0.87 ± 0.03 [c]
Beltsville bee diet	5.18 ± 4.49	0.03 ± 0.02 [c]	39.74 ± 14.80	0.89 ± 0.02 [c]
Pollen diet	21.28 ± 12.45	0.08 ± 0.04 [d]	58.08 ± 7.96	1.30 ± 0.13 [d]
Hive bees	26.20 ± 5.67	0.08 ± 0.02 [d]	63.27 ± 20.69	1.24 ± 0.13 [d]

[a] Values are means ± standard deviations; units as in Table I; $n = 15$.
[b] Means in these columns were not analyzed for variance.
[c, d] Tukey's range test; means in columns followed by the same letter are not significantly different; alpha = 0.05. Rows are not compared.

significantly different. Trypsin levels in the ectoperitrophic space of bees fed EAD, BBD, and sucrose were not significantly different but were significantly lower than those in the hive and PD-fed bees.

Data in Tables II and III support the assertion of Grogan and Hunt (1980) that young worker honey bees produce more endogenous trypsin than older bees. When the trypsin activity in the midgut tissue and ectoperitrophic space from five-day-old and 21-day-old hive bees was compared using Student's *t*-test, trypsin activities were significantly higher

Table III. Trypsin activity[a] in the midgut tissue and ectoperitrophic space of 21-day-old honey bees receiving various diets in August.

	Tissue		*Ectoperitrophic space*	
Diet	*Units/bee* [b]	*Units/μg protein*	*Units/bee* [b]	*Units/μg protein*
Sucrose	0.94 ± 1.40	0.01 ± 0.02 [c]	6.89 ± 5.62	0.23 ± 0.18 [c]
Egg albumin	1.62 ± 2.86	0.01 ± 0.02 [c]	5.18 ± 2.17	0.21 ± 0.11 [c]
Beltsville Bee Diet	4.66 ± 3.29	0.03 ± 0.02 [c, d]	7.06 ± 2.67	0.17 ± 0.08 [c]
Pollen diet	5.59 ± 5.24	0.03 ± 0.03 [c, d]	38.98 ± 14.82	0.98 ± 0.50 [d]
Hive bees	12.97 ± 6.27	0.05 ± 0.01 [d]	48.27 ± 19.79	0.79 ± 0.26 [d]

[a] Values are means ± standard deviations; units as in Table I, $n = 15$.
[b] Means in these columns were not analyzed for variance.
[c, d] Tukey's range test; means in columns followed by the same letter are not significantly different; alpha = 0.05. Rows are not compared.

(alpha=0.05) in both the tissue and the ectoperitrophic space of the five-day-old bees. Similar results were found when five-day-old and 21-day-old caged bees fed pollen diet were compared.

Ultrastructural changes in the midgut epithelial cells accompanied the reduction of protein intake associated with aging of the bee. All of the cells in the epithelium of the midgut arose from a mosaic of regenerative nidi that were distributed evenly along the basement membrane. Initial cell growth occurred in a horizontal direction until the cells of adjoining nidi abutted. Growth was then directed diagonally, and the apices of the young cells joined the gut lumen to form the crypts of the midgut. As the cells aged, their growth was directed vertically, and the apices formed protuberant pseudovilli. The apical cytoplasm of the cells in Figure 2 is representative of the columnar cells that predominated throughout the ventricular epithelium of young honey bees. The elongate tapered cells averaged 50—80 μm in length and 20—30 μm at the widest apical diameter. The nucleus, located in the central to lower third of the cell, was surrounded by extensive rough endoplasmic reticulum which extended from below the nuclear base well into the supranuclear cytoplasm. Large oval mitochondria were present in the cytoplasm near the nucleus, but those near the plasma membrane in the basal and apical portions of the cell were smaller and elongate. Mitochondrial cristae and Golgi structures were visible, but the high osmolarity of the formaldehyde—glutaraldehyde fixation or the acrylic resin caused some shrinkage and distortion. Numerous peroxisomal microbodies (Jimenez and Gilliam, 1988), lysosomes and other components of subcellular architecture were common throughout the cytoplasm.

In five-day-old pollen-feeding bees, the apical cytoplasm of the cells located in the posterior midgut was notably different from that of the cells of the anterior and middle regions of the gut. In a narrow band of cells just anterior to the anastomoses of the Malpighian tubules, the supranuclear cytoplasm was replete with unique electron-opaque vesicles that were 0.2—1.0 μm in diameter. Figure 3 shows an accumulation of the vesicles in a posterior midgut cell. Within the cell, the vesicles lacked a well defined limiting membrane, and the appearance of multilobed vesicles suggested that small vesicles may coalesce. The largest accumulations of the vesicles appeared in the

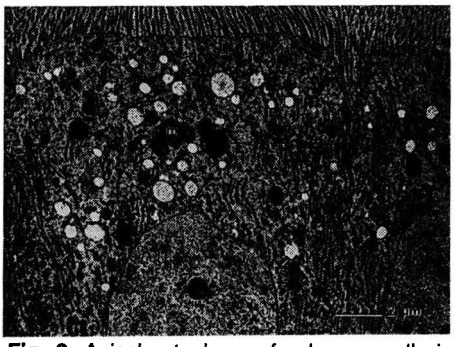

Fig. 2. Apical cytoplasm of columnar cells in the anterior midgut of a five-day-old honey bee. Mitochondria : m, nucleus = n, peroxisomal microbodies = asterisk (x 3,000).

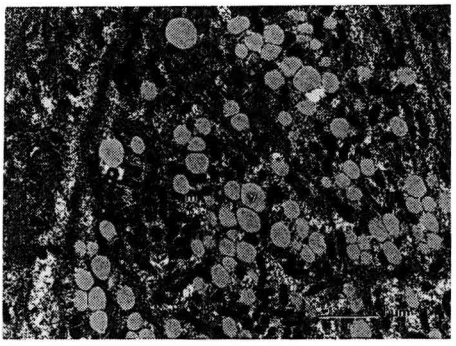

Fig. 3. Electron-opaque vesicles (v) and pleomorphic mitochondria (m) in the apical cytoplasm of a posterior midgut cell of a 5-day-old honey bee (x 9,000).

pseudovilli of the apical columnar cells. The cytoplasm of the cells was electron-dense, and the simultaneous appearance of numerous pleomorphic mitochondria indicated that the tissue was highly anabolic. Rough and smooth endoplasmic reticulum, Golgi structures, and peroxisomal microbodies were profuse.

The opaque vesicles were also observed in the gut lumen. They floated freely in the ectoperitrophic space and there exhibited an increased electron density at their perimeter as well as partial dissolution. They also appeared within large vesicles that had been released from the apices of the epithelia. Many of the large vesicles lacked remnants of the cell nucleus, but contained other organelles which suggested merocrine as opposed to holocrine release.

In addition to the unique vesicles of the distal midgut, the crypts in this region of the midgut were unusual. In the anterior and middle regions of the midgut, the microvilli (Fig. 4) of the crypts and the pseudovilli were sinuous, elongate and 11—20 μm in length. These measurements confirmed those of Vidano (1971). The microvilli of the cells situated in the crypts of the posterior midgut were pleomorphic, branching and entirely different from the elongate microvilli common throughout the remainder of the midgut. Figure 5 shows microvesiculation occurring in the pleomorphic microvilli of the posterior midgut of five-day-old bee. Electron-dense particles were visible within many of the small vesicles. Some of the particles appeared to be associated with, or protruding from, the vesicle wall.

In 30-day-old honey bees collected from free-flying colonies during the foraging season in the summer month of August, the opaque vesicles were still present within the cells of the posterior midgut, but numbers were greatly reduced. The microvesiculation in the distal crypts was also diminished, and the endoperitrophic mass was generally reduced to a small bolus of pollen grains.

Discussion

From our data, it appears that the honey bee, like many other insects, secretes proteases in response to the presence of food in the midgut (Engelmann, 1969; Briegel and Lea, 1975; Applebaum, 1985). The amounts of diet consumed

Fig. 4. Elongate microvilli in the anterior midgut of a five-day-old honey bee (x 7,000).

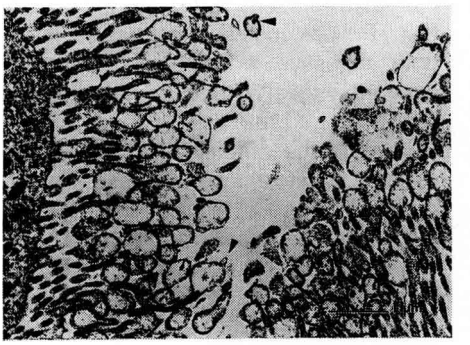

Fig. 5. Microvesiculation (asterisk) and particles (arrows) in a crypt of the posterior midgut of a five-day-old honey bee (x 10,000).

and the corresponding differences in trypsin-like activity in bees fed pollen and bees fed artificial diets suggest that enzyme synthesis is induced by a secretagogue mechanism, but the details of this regulation will require further study. Attempts were not made to correlate the midgut response to the amount of available protein nor to assess the nutritional suitability of the individual diets. The artificial diets were used to demonstrate trypsin activity in the midgut of bees consuming a diet free from exogenous enzymes. The results of the trypsin assay demonstrate that adult worker bees synthesize and secrete an endoprotease with trypsin-like activity which is active in the gut lumen. Even though the level of trypsin synthesis declined as the bees aged, substantial activity remained in free-flying bees well beyond the age of maximum pollen consumption.

The presence of trypsin activity in the midgut tissue and ectoperitrophic space of five-day-old bees restricted to sucrose solution and water implies that some synthesis and release of the enzyme occurs regardless of dietary stimulation. While on the brood comb, some of the newly-emerged bees had time to feed on stored pollen prior to being caged. This ensured survival of adequate numbers of bees restricted to sucrose through the 21-day-test. Pollen consumption would initiate both digestive enzyme synthesis and hypopharyngeal gland development (Brouwers, 1982). This might have influenced the amount of trypsin activity seen in the five-day-old bees; however, results from the 21-day-old bees suggested that even protein-malnourished bees continued to synthesize small amounts of the enzyme. The presence of luminal endoproteases would assist in the digestion and reutilization of proteins lost endogenously as the result of cell desquamations.

The small amount of trypsin-like activity in the midgut tissues compared to that in the ectoperitrophic space and the presence of specialized vesicles in the posterior midgut suggest that the honey bee might elaborate some digestive enzymes *via* a mechanism similar to the regulated secretion of the vertebrate pancreas. To protect the secretory tissues of the pancreas, a trypsin-specific inhibitor is secreted in parallel with the inactive zymogen, trypsinogen (Greene *et al.*, 1974). Whether the honey bee secretes trypsin as an inactive zymogen is not known, but a proteolytic zymogen (Felsted *et al.*, 1973) and a midgut protease inhibitor (Engelmann and Geraerts, 1980) have been reported in other insects. Graf *et al.* (1986) localized trypsin immunocytochemically in small secretory granules in the midgut of mosquitoes and suggested that it may be stored as inactive trypsinogen. It is possible that the low levels of trypsin activity in the tissue of the honey bee midgut might be the result of an endogenous inhibitor and/or intracellular storage of the enzyme in an inactive form.

Results of our ultrastructural studies suggest that some digestive enzymes produced by the midgut tissues may originate in the posterior midgut. The morphology, distribution and mechanism of release of the specialized vesicles in honey bees of various ages are similar to events correlated to digestive activity in the midguts of other insects (Lehane, 1976; Hecker, 1977; Rudin and Hecker, 1979; Houk and Hardy, 1982; Bignell *et al.*, 1982; Berner *et al.*, 1983). The small microvesicles containing electron-dense particles released from the crypts of the posterior midgut of honey bees are similar to vesicles reported in the midguts of adult flies (De Priester, 1971) and larval *Erinnysis ello* (Santos *et al.*, 1986). Recent biochemical and ultrastructural data suggest that the tips of these microvilli are a

differentiated region of the midgut epithelium which is involved in trypsin and amylase secretion in lepidopteran larvae (Santos *et al.*, 1986).

The electron-opaque vesicles are similar in size and distribution to organelles which have been documented in the midguts of termites (Bignell *et al.*, 1982) and flies (Lehane, 1976), but different methods of fixation and staining result in varied ultrastructural appearances. The absence of an obvious limiting membrane surrounding the opaque vesicle is unusual for a compartment involved in protein storage or secretion. Consequently, comparison of the vesicles is based on frequency of occurrence and relationship to enzymatic data as opposed to ultrastructural similarity. It is possible that the vesicles may contain stored lipid rather than protein and that their presence in the gut lumen was the result of lethal cell discharge. In a preliminary work, we applied Sudan black B, brominated Sudan black B, and Oil red 0 to thick cryostat sections of whole midguts from young pollen-feeding bees. Positive reactions were limited to the endoperitrophic contents, and with brominated Sudan, a slight bronze dichroism was visible in the peroxisomal microbodies. Although the small size of the opaque vesicle and the resolution of light microscopy are limiting factors in these procedures, the reduced incidence of the opaque vesicle in 30-day-old bees might be attributed to the reduced intake of pollen lipids. Localization of sites of digestive enzyme synthesis within the posterior midgut and elucidation of the mechanism of secretion require additional research.

Spatial separation of specialized columnar cells in the insect midgut may partially confine enzyme secretion to one section of the gut and nutrient absorption to another (Berridge, 1970; Bignell *et al.*, 1982; Turunen, 1985; Espinoza-Fuentes *et al.*, 1987; Schneider *et al.*, 1987). Further segregation of digestion occurs because the peritrophic membrane partitions the gut, thus permitting sequential digestion in spatially separate compartments within the midgut lumen. Primary digestion occurs within the endoperitrophic space, followed by intermediate digestion in the ectoperitrophic space, and then terminal digestion at the brush border (Terra *et al.*, 1979, 1985). The establishment of these compartments occurs in the honey bee midgut, as it does in many insects, when the epithelial tissue secretes the peritrophic membrane. The honey bee delaminates membrane along the whole length of the midgut to surround the food bolus and form the endoperitrophic compartment (Barker and Lehner, 1972; Wigglesworth, 1972). The ectoperitrophic space forms between the epithelium and the endoperitrophic space as the result of diffusion of fluid from within the endoperitrophic space and the influx of fluid from the posterior midgut or the Malpighian tubules (Dow, 1981; Terra and Ferreira, 1981; Santos *et al.*, 1983, 1986; Terra *et al.*, 1985; Espinoza-Fuentes *et al.*, 1987). To our knowledge, there are no data supporting fluid influx through the posterior midgut of the honey bee, but since the Malpighian tubules anastomose anterior to the pyloric sphincter, any fluid influx due to diuresis enters the posterior midgut. The influx of fluid in the posterior midgut is generally balanced by an efflux from absorption in the anterior or middle portions of the gut. Recently, Crailsheim (1988a, 1988b) showed that absorption of monosaccharides and free amino acids occurs primarily in the anterior two-thirds of the adult honey bee midgut.

Fluid influx in the posterior gut balanced by efflux in the anterior gut causes the content of the ectoperitrophic space to flow posterior to anterior and thus opposite to the contents of the endoperitro-

phic space. This results in a countercurrent flux of the ectoperitrophic fluid which distributes enzymes and nutrients efficiently throughout specialized regions of the midgut. Based on current knowledge and the occurrence of specialized cells in the posterior midgut, it appears that the honey bee may utilize countercurrent flow (Fig. 6). The proventricular valve initiates the transport of consumed pollen into the midgut by selectively collecting a slurry of pollen fragments and transporting this readily digestible material in advance of the intact pollen grains (Klungness and Peng, 1984). This slurry is composed of lipids, carbohydrates, amino acids, and proteins which rapidly diffuse from pollen when it is placed in solution (Stanley and Linskens, 1974). Rapid release of small molecules through the meshwork of the peritrophic membrane may initiate a secretory cycle in the epithelium that results in the release of digestive enzymes. Enzymes released from the posterior midgut would be carried into the anterior gut by counterccurrent flow and incorporated into the endoperitrophic space along with incoming pollen grains. Enzymes capable of escaping the peritrophic membrane in the posterior midgut would be recirculated and conserved (Terra and Ferreira, 1981; Terra *et al.*, 1985). This process could account for the high concentration of digestive enzymes in the anterior midgut as observed by Zherebkin (1967) and the distribution of trypsin activity between the tissue, the ectoperitrophic space, and the endoperitrophic space in the honey bee midgut. Some of the insects in which the countercurrent flow mechanism has been proposed possess alimentary tracts in which the absorptive region of the anterior midgut is increased by gastric caeca. Honey bees

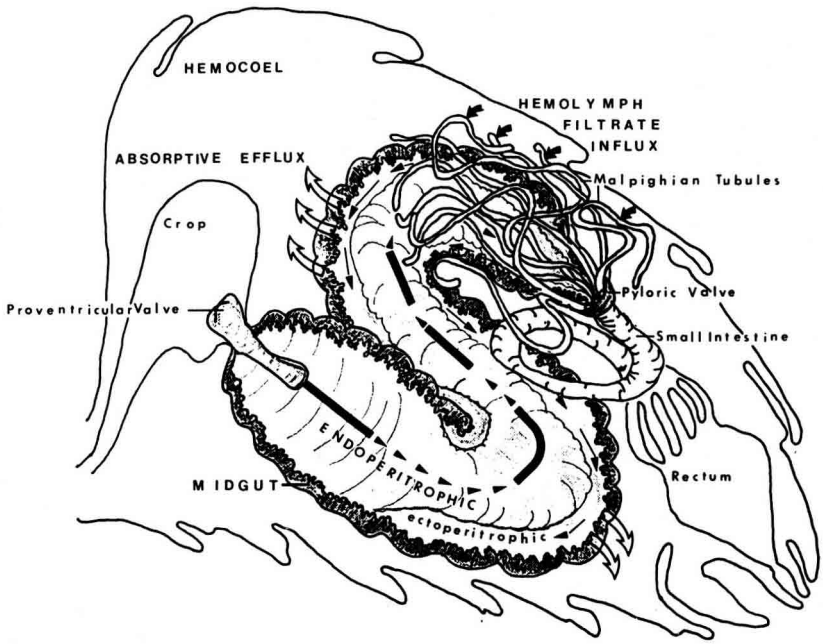

Fig. 6. Countercurrent flow model showing the fluid fluxes and the direction of flow in the endoperitrophic and ectoperitrophic spaces.

lack caeca but achieve an increase in absorptive surface area with exceptionally elongate microvilli.

Based on current data, we hypothesize that the tissues of the posterior midgut may be responsible for the synthesis and secretion of some digestive enzymes and that the adult worker honey bee may rely on countercurrent flow to distribute enzymes and nutrients efficiently throughout the midgut. Further support for this model will appear in a future publication on ultrastructure of the honey bee midgut.

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Résumé — Modifications dans l'ultrastructure de l'intestin moyen et dans l'activité de la trypsine chez l'abeille (*Apis mellifica* L.) en fonction de l'âge. On a séparé des intestins moyens d'ouvrières d'abeilles en 3 parties physiologiquement distinctes et on a analysé l'activité protéolytique. En utilisant le benzoyl-arginine-nitroanalide comme substrat artificiel, on a mesuré l'activité «trypsin-like» dans le tissu ventriculaire, dans le liquide de l'espace ectopéritrophique et dans l'espace endopéritrophique. Les niveaux d'activité les plus élevés ont été trouvés dans les espaces endopéritrophique et ectopéritrophique, les plus bas dans le tissu.

On a comparé l'activité de la trypsine dans l'intestin moyen d'abeilles d'extérieur avec celle d'abeilles encagées nourries au saccharose, avec des régimes artificiels ou au pollen. On a prélevé du couvain juste avant l'émergence des adultes; les ouvrières âgées d'un jour ont été, soit mises en cagettes grillagées, soit marquées et replacées dans des colonies d'extérieur. On a prélevé des échantillons d'abeilles pour les analyser 5 jours et 21 jours après l'éclosion. C'est chez les abeilles jeunes et d'extérieur et chez les abeilles encagées nourries au pollen que l'activité de la trypsine a été la plus forte. Les niveaux les plus bas ont été trouvés chez les abeilles encagées nourries avec un régime artificiel et chez les abeilles privées de protéines, ne recevant que du sirop de saccharose. Le niveau d'activité de la trypsine de l'intestin moyen dépend de la quantité de nourriture consommée. La consommation alimentaire et l'activité «trypsin-like» diminuent toutes deux avec l'âge.

Des abeilles marquées âgées de 5 et de 30 jours ont été prélevées dans des colonies d'extérieur. Les intestins moyens ont été fixés, montés dans de la résine acrylique et étudiés au microscope électronique. On a observé des différences dans les tissus de l'intestin moyen selon les régions. Chez les abeilles de 5 jours nourries au pollen, le cytoplasme atypique des cellules de la partie postérieure de l'intestin moyen renferme de nombreuses vésicules opaques aux électrons que l'on ne trouve pas dans les cellules des parties antérieure ou moyenne de l'intestin moyen. Dans la majeure partie de l'intestin moyen, les microvillosités de la bordure apicale en brosse et des cryptes sont sinueuses et allongées. La membrane apicale des cryptes de la partie distale de l'intestin moyen est composée de microvillosités pléomorphes, courtes, parfois ramifiées, avec de nombreuses microvési-

cules. La décharge des cellules de l'intestin moyen postérieur a provoqué la libération de vésicules opaques et de microvésicules des cryptes dans la lumière de l'intestin moyen. Chez les abeilles d'extérieur âgées de 30 jours, le nombre de vésicules opaques et la microvésiculation des cryptes de l'intestin moyen postérieur sont réduits. Ainsi, les modifications ultrastructurales dans les tissus de l'intestin moyen s'accompagnent du déclin de l'activité enzymatique liée à l'âge.

Le niveau réduit de l'activité «trypsin-like» chez les abeilles ayant reçu un régime artificiel et chez les abeilles âgées, suggère que la synthèse de l'enzyme est contrôlée par un mécanisme inducteur de sécrétion. Les divers niveaux d'activité détectés dans le tissu, l'espace endopéritrophique et l'espace ectopéritrophique de l'intestin moyen et la présence de vésicules spécialisées dans la partie postérieure de l'intestin moyen suggèrent que l'abeille peut élaborer certaines enzymes digestives au moyen d'une régulation de la sécrétion. S'appuyant sur ces observations et sur la variation régionale de l'ultrastructure cellulaire, on propose un modèle hypothétique d'un flux à contre-courant qui distribuerait les enzymes et les nutriments de façon efficace à travers l'intestin moyen de l'abeille (Fig. 6).

***Apis mellifica* — intestin moyen, ultrastructure — activité enzymatique — trypsine — âge**

Zusammenfassung — Altersabhängige Veränderungen der Ultrastruktur des Mitteldarms und der Trypsin-Aktivität bei der Honigbiene, *Apis mellifera*. Der Mitteldarm erwachsener Arbeitsbienen von *Apis mellifera* wurde in drei physiologisch verschiedene Abschnitte geteilt und auf proteolytische Aktivität analysiert. Unter Verwendung des künstlichen Substrates Benzoyl-Arginin-Nitroanalid wurde die Trypsin-ähnliche Aktivität im ventrikularem Gewebe, in der Flüssigkeit des ectoperitrophischen Raumes und in dem endoperitrophischen Raum gemessen. Die höchsten Aktivitäten wurden in den endoperitrophischen und ectoperitrophischen Räumen gemessen; in den Geweben wurden niedrigere Werte gefunden.

Es wurde die Trypsin-Aktivität bei freifliegenden Bienen mit der von gekäfigten Bienen verglichen, die mit Sucrose, künstlicher Diät oder Pollen gefüttert wurden. Eintägige Arbeiterinnen, geschlüpft aus isolierter verdeckelter Brut, wurden in kleinen Drahtkäfigen gehalten oder markiert und in freifliegende Völker eingesetzt. Am 5. und 21. Tag nach dem Schlüpfen wurden Bienenproben entnommen. Die Trypsin-Aktivität war bei freifliegenden Bienen und bei pollenernährten gekäfigten Bienen am höchsten. Niedrigere Werte wurden bei gekäfigten Bienen mit künstlicher Diät und mit Eiweiß-Mangelnahrung bei ausschließlicher Zuckerfütterung gefunden. Das Niveau der Trypsin-Aktivität im Mitteldarm war von der Menge des verzehrten Futters abhängig. Futteraufnahme wie Trypsin-ähnliche Aktivität nahmen mit dem Alter der Bienen ab.

Markierte 5 und 30 Tage alte Bienen wurden freifliegenden Völkern entnommen. Die Mitteldärme wurden fixiert, in Akryl-Plastik eingebettet und elektronenmikroskopisch untersucht. Im Mitteldarmgewebe wurden regionale Unterschiede festgestellt. Bei 5 Tage alten pollenernährten Bienen enthielt das apikale Zytoplasma der Zellen des hinteren Mitteldarms zahlreiche elektronen-opake Vesikeln, die in den Zellen des mittleren oder vorderen Mitteldarms nicht vorkamen. Die Mikrovilli des apikalen Bürstensaumes und der Krypten waren im größten Teil des Mittel-

darms geschlängelt und verlängert. Die apikale Membran in den Krypten des distalen Mitteldarms war aus kurzen, vielgestaltigen Mikrovilli zusammengesetzt, die gelegentlich Verzweigungen aufwiesen und sehr reichlich mit Mikrovesikeln durchsetzt waren. Die Entleerung aus den rückwärtigen Mitteldarmzellen führte zur Freisetzung der opaken Vesikeln und der Mikrovesikeln aus den Krypten in den Hohlraum des Mitteldarms. Bei 30 Tage alten Flugbienen war die Zahl der opaken Vesikeln und die Durchsetzung der Krypten mit Mikrovesikeln im hinteren Mitteldarm reduziert. Veränderungen der Ultrastruktur des Mitteldarmes gingen also während des Alterungsprozesses mit der Verringerung der enzymatischen Aktivität Hand in Hand.

Das verringerte Niveau der Trypsin-ähnlichen Aktivität bei Bienen, die mit künstlicher Diät gefüttert worden waren, und in älteren Bienen legt die Vermutung nahe, daß die Enzymsynthese durch einen sekretionsfördernden Mechanismus kontrolliert wird. Die unterschiedlichen Spiegel der Aktivität, die in Geweben, dem endoperitrophischen Raum und dem ectoperitrophischen Raum des Mitteldarms entdeckt wurden und das Vorhandensein von spezialisierten Vesikeln im hinteren Mitteldarm lassen vermuten, daß die Honigbiene gewisse genau dosierte Verdauungsenzyme über eine regulierte Sekretion erzeugen kann. Aufbauend auf diesen Beobachtungen und auf der regionalen Variation der Zellultrastruktur wird das hypothetische Modell eines Gegenstrom-Systems vorgeschlagen, das Enzyme und Nährstoffe wirkungsvoll durch den ganzen Mitteldarm der Honigbiene verteilen würde.

***Apis mellifera* — Mitteldarm, Ultrastruktur — enzymatische Aktivität — Trypsin — Alter**

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