Blood Digestion in the Mosquito, *Anopheles stephensi* Liston (Diptera: Culicidae): Activity and Distribution of Trypsin, Aminopeptidase, and α-Glucosidase in the Midgut

PETER F. BILLINGSLEY[1] AND HERMANN HECKER

Swiss Tropical Institute, Basel, Switzerland

ABSTRACT   The activities of trypsin, aminopeptidase, and α-glucosidase were studied in the whole midgut, anterior and posterior midgut, and posterior midgut lumen and epithelium of the mosquito *Anopheles stephensi* Liston. Trypsin activity was restricted entirely to the posterior midgut lumen. No trypsin activity was found before the blood meal, but activity increased continuously up to 30 h after feeding, and subsequently returned to baseline levels by 60 h. Aminopeptidase was active in anterior and posterior midgut regions before and after feeding. In whole midguts, activity rose from a baseline of ≈3 enzyme units (EU) per midgut to a maximum of 12 EU at 30 h after the blood meal, subsequently falling to baseline levels by 60 h. A similar cycle of activity was observed in the posterior midgut and posterior midgut lumen, whereas aminopeptidase in the posterior midgut epithelium decreased in activity during digestion. Aminopeptidase in the anterior midgut was maintained at a constant low level, showing no significant variation with time after feeding. α-glucosidase was active in anterior and posterior midguts before and at all times after feeding. In whole midgut homogenates, α-glucosidase activity increased slowly up to 18 h after the blood meal, then rose rapidly to a maximum at 30 h after the blood meal, whereas the subsequent decline in activity was less predictable. All posterior midgut activity was restricted to the posterior midgut lumen. Depending upon the time after feeding, >25% of the total midgut activity of α-glucosidase was located in the anterior midgut. The enzyme distributions are consistent with described structural models for digestion in mosquitoes. After blood meal ingestion, proteases are active only in the posterior midgut. Trypsin is the major primary hydrolytic protease and is secreted into the posterior midgut lumen without activation in the posterior midgut epithelium. Aminopeptidase activity is also luminal in the posterior midgut, but cellular aminopeptidases are required for peptide processing in both anterior and posterior midguts. α-glucosidase activity is elevated in the posterior midgut after feeding in response to the blood meal, whereas activity in the anterior midgut is consistent with a nectar-processing role for this midgut region.

KEY WORDS   Insecta, *Anopheles stephensi*, bloodmeal digestion, midgut

INSECT-BORNE DISEASE ORGANISMS usually have an initial, often prolonged stay in the midgut of the vector, where they develop or multiply in what may become a hostile environment (Gass 1977). Knowledge of physiological events taking place in the vector midgut is important in understanding vector-parasite interactions necessary for disease transmission. Although trypsin is the major primary hydrolytic protease in the mosquito midgut (Briegel & Lea 1975), aminopeptidases in midguts of *Anopheles stephensi* Liston (Billingsley 1990b) and *Aedes aegypti* L. (Graf & Briegel 1982) also play important roles in the normal processes of blood digestion. Furthermore, differences in aminopeptidases, rather than trypsin, occurring between strains of *An. stephensi* may be partly responsible for the degree of susceptibility to *Plasmodium fal-ciparum* infection (Feldmann et al. 1990). The presence of several midgut-associated glycosidases (P.F.B. & H.H., unpublished data) in *An. stephensi* demonstrate that there are still many aspects of midgut enzymology yet to be understood.

In *Ae. aegypti*, inactive trypsin is released from small secretory vesicles within midgut epithelial cells into the posterior midgut lumen, where the enzyme is activated (Graf et al. 1986). Other studies have demonstrated the spatial distribution of enzymes across the mosquito midgut and have suggested that the peritrophic membrane may serve as a molecular filter for bloodmeal proteins, as a layer separating hydrolytic events in the lumen, and as an important layer in separating enzymes and inhibitors from one another (Borovsky 1986, Van Handel & Romoser 1987). However, the most complete and integrated data for digestion in mosquitoes is based upon ultrastructural observations complemented with morphometric analyses (Hecker 1977, Rudin & Hecker 1979, Billingsley 1990a). Such studies were used to develop a tem-

[1] Correspondence and current address: Cellular and Molecular Parasitology Research Group, Department of Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, U.K.

poral and spatial model for digestion in mosquitoes, but complementary biochemical data necessary to correlate structure and function (e.g., Billingsley 1988; Billingsley & Downe 1985, 1988) have been lacking, especially in *Anopheles* spp.

As part of a study on blood digestion and malaria infectivity, the current report provides an overview of activities of three major digestive enzymes in the mosquito *An. stephensi*. These enzymes have been characterized previously in this species (Berner et al. 1983, Billingsley 1990b, unpublished data), and were examined here in several defined physiological compartments of the midgut over the complete digestion period. Qualitative correlation of the results with previously published ultrastructural data is used to develop an integrated model for digestion in this important mosquito species.

Materials and Methods

Insect Rearing and Treatment. *Anopheles stephensi* Liston (all stages), originally obtained from the London School of Hygiene and Tropical Medicine in 1971, were reared in a 12:12 (L:D) photoperiod at 27 ± 2°C. Eggs were hatched in tap water containing a small amount of ascorbic acid, and larvae were fed ground hamster food. Adults were maintained at 75–80% relative humidity, provided with 10% (wt/vol) sucrose ad libitum, and were fed periodically on defibrinated pig blood (Berner et al. 1983).

For experiments, 3–4-d-old female mosquitoes were deprived of sucrose for 12–18 h and then offered a meal of warmed, defibrinated pig blood through a stretched Parafilm membrane. Fully engorged females were separated in a cold room and used for further study. At selected intervals after feeding, blood fed mosquitoes were anesthetized by cooling in a deep freeze for a few minutes and then kept on ice until dissection.

Preparation of Midgut Homogenates. Whole, anterior, and posterior midguts were dissected in cold *Aedes* Ringer's solution (Hayes 1953). All nonmidgut tissue was removed during dissection. To separate posterior midgut epithelium from the contents of the lumen, the posterior midgut was cut longitudinally and the contents were washed out onto a microscope slide. The contents were then placed into a 1.5-ml Eppendorf tube, while the intact posterior midgut epithelia were washed twice in Ringer's solution to remove any residual luminal material. Originally, we intended to separate material from each side of the peritrophic membrane, but this layer is not completely formed in *An. stephensi* until after >50% of the blood meal is digested (Berner et al. 1983). The delicacy of the peritrophic membrane during dissection prevented the preparation of uncontaminated material from each side, so the endo- and ectoperitrophic spaces were not assayed separately.

Pooled tissue was homogenized in 0.15 M NaCl in a tight fitting Teflon–Glass homogenizer, then centrifuged at 10,000 × g for 10 min at 4°C. The homogenate supernatant was used in all subsequent enzyme and protein assays, and could be stored at −80°C for several months with no significant loss of activity for the three enzymes studied. The amount of tissue per homogenate was varied according to the time after feeding and the region being assayed: unfed and from 42 h after the blood meal whole midgut, posterior midgut and posterior midgut lumen—10 mosquito equivalents per 1.0 ml saline; 2–36 h after the blood meal whole midgut, posterior midgut and posterior midgut lumen—2 mosquito equivalents per 1.0 ml saline; anterior midgut and posterior midgut epithelium—4–8 mosquito equivalents in 0.6 ml saline.

Enzyme and Protein Assays. Trypsin and aminopeptidase were assayed using the substrates benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) and leucine-*p*-nitroanilide (L*p*NA) respectively (Houseman & Downe 1986, Billingsley 1990b). Calculations of enzyme activities were made using an extinction coefficient of 8,800 nM cm^{-1} for both substrates at 410 nm (Erlanger et al. 1961). This method of tissue preparation did not detect membrane-associated aminopeptidases in the midgut (Billingsley 1990b).

α-glucosidase activity was determined by modifications to the method of Ribeiro & Perreira (1984) (Billingsley & Hecker, unpublished) using the substrate *p*-nitrophenol-α-glycoside (*p*NαG) in 0.1 M Tris·HCl buffer, pH 6.0. Absorbance was measured at 405 nm and activity was calculated from a standard curve of *p*-nitrophenol made under the same conditions.

Assays were carried out in duplicate, with a minimum of four samples (number of midguts or midgut compartments per sample as described above) for each time point after feeding unless otherwise stated. The results presented in all graphs (except Fig. 5) represent the means ± SE of 4–6 paired samples. Spontaneous breakdown of substrates and endogenous absorbance of samples were controlled by incubating paired tubes containing substrate and buffer, then adding the homogenate supernatant after the stopping agent. Absorbance readings were made on a Beckman (Fullerton, Calif.) 25 or a Cecil (Cambridge, U.K.) CE 292 spectrophotometer.

Protein contents of homogenate supernatants were determined using the trichloroacetic acid precipitation procedure in the macro-assays (2–42 h after the blood meal; whole midgut, posterior midgut, and posterior midgut lumen) or microassays (all other samples) described by Peterson (1977). Bovine serum albumin (fraction 5, Sigma Chemical Company, St. Louis, Mo.) was used as the standard.

Results were expressed as either relative activity—i.e., total activity in enzyme units (EU) per midgut equivalent or midgut compartment equivalent, or as specific activity—the enzyme activity per microgram protein per midgut equivalent determined for each sample. The latter value was used to examine the relationship between enzyme
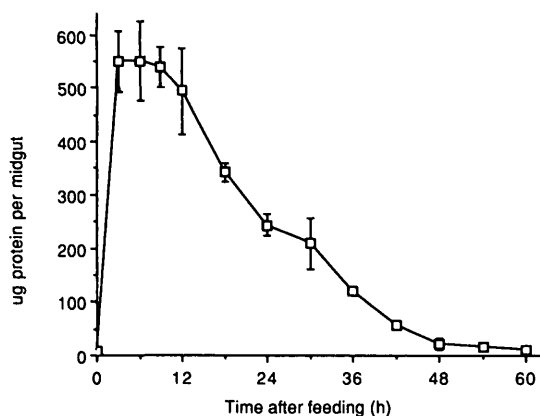
Fig. 1. Protein content of whole midgut homogenate supernatants of *Anopheles stephensi* after a blood meal.

activity and protein content of the midgut over the digestion period. One EU is described as one millimole of substrate (BApNA, LpNA, or pNαG) hydrolysed per minute.

Results

Protein Content of the Midgut. The midgut in unfed insects contained 2.5–10 μg protein per whole midgut (Fig. 1). Immediately after feeding, the whole midgut contained ≈550 μg protein, which remained constant for at least 9 h. The blood meal was digested from 12 h onwards, resulting in a rapid decline in midgut protein until 48 h after feeding. By 60 h the protein per midgut had returned to the original amounts (Fig. 1). Throughout most of the digestive period, the bulk of midgut protein was from the blood meal. The protein content of midgut epithelium did not alter significantly during the 60 h period after feeding (data not
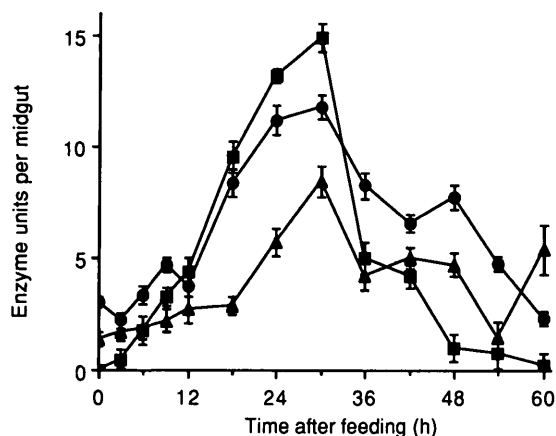

Fig. 2. Relative activities of trypsin (■), aminopeptidase (●), and α-glucosidase (▲) in whole midgut homogenate supernatants of *Anopheles stephensi*.
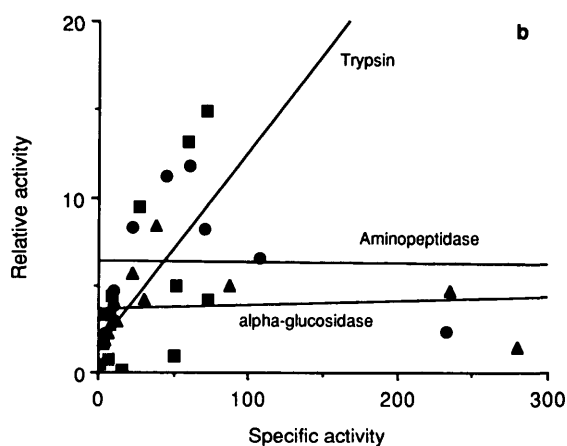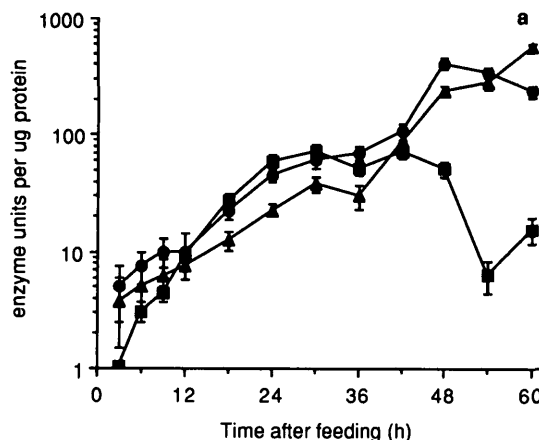

Fig. 3. The relationship between protein and trypsin (■), aminopeptidase (●), and α-glucosidase (▲) activities in the midgut of *Anopheles stephensi*. a. Specific activities plotted on a log scale demonstrate similar trends until 30–42 h. b. Linear regression analysis of mean specific activities and mean relative activities for each enzyme. Only trypsin shows a clear positive correlation. All mean data points were used (taken from Fig. 2 and 3a) for the correlation calculations, but extreme data points are not shown on the graph.

shown), therefore protein in the posterior midgut epithelium and anterior midgut was not assayed.

Post-Feeding Activity and Distribution of Trypsin. Trypsin activity increased from a baseline of no activity in unfed insects to maximum activity at 30 h after the blood meal, and then dropped to no activity by 60 h (Fig. 2). Specific activity, plotted on a log scale, showed a similar pattern with maximal activity at 30 and 42 h after the blood meal, before decreasing at 54 h (Fig. 3a). When the mean specific and relative activities of trypsin were plotted in a linear regression, there was a positive correlation between the two ($r = 0.622$; $df = 12$; $P = 0.02$) (Fig. 3b). All detectable trypsin activity was
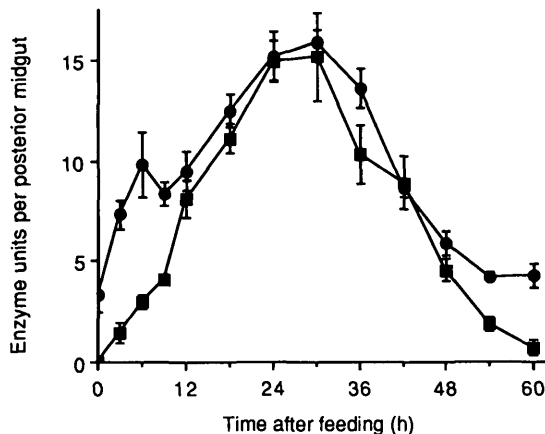
Fig. 4. Activities of trypsin (■) and aminopeptidase (●) in homogenate supernatants of the posterior midgut of *Anopheles stephensi*.
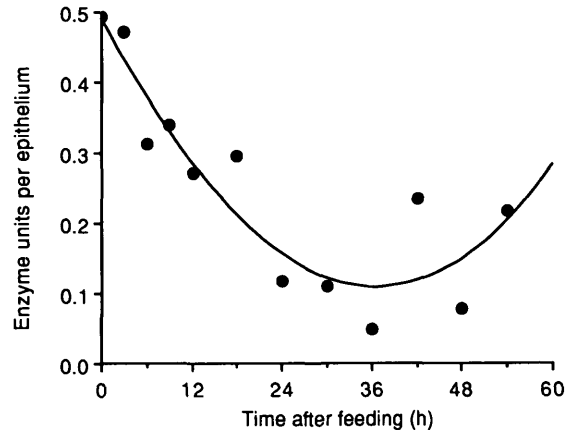

Fig. 5. Post-feeding activity of aminopeptidase in the posterior midgut epithelium homogenate supernatants of *Anopheles stephensi*. These results are the means of only two independent determinations.

restricted to the posterior midgut (Fig. 4), and all this activity was luminal. No activity could be detected in any other midgut compartment (anterior midgut or posterior midgut epithelium) at any time before or after the blood meal, even after prolonged incubation with samples containing greater amounts of tissue (up to 25 posterior midgut epithelia per 1.0 ml saline dissected at 30 h after the blood meal and incubated for over 3 h).

Post-Feeding Activity and Distribution of Aminopeptidase. Aminopeptidase activity was present in all midgut regions before and at all times after feeding. In whole midguts, activity rose from a baseline of ≈3 EU per midgut before feeding to a maximum of 12 EU at 30 h after the blood meal (Fig. 2). Activity then declined to baseline amounts by 60 h after the blood meal. The specific activity of aminopeptidase in the whole midgut was characterized by a delayed peak in activity at 48 h after the blood meal (Fig. 3a) compared to 30 h after the blood meal for total activity per whole midgut. The resulting correlation between mean relative activity and mean specific activity was poor ($r = -0.032$; df $= 12$; $P > 0.1$) (Fig. 3b).

In the anterior midgut, aminopeptidase activity was maintained at a very low, almost constant level that represented only 0–0.5% of total midgut aminopeptidase activity. Anterior midgut activity therefore showed no clear response to the blood meal and could not be correlated with the major aminopeptidase cycle occurring in the posterior midgut.

More than 99% of the total relative activity was always present in the posterior midgut, and activity in this region (Fig. 4) predictably demonstrated a trend similar to that in the whole midgut (Fig. 2). Aminopeptidase was distributed unevenly between the epithelium and the lumen, ranging from 12.6% at 3 h to 0.8% at 30 h after feeding. The total relative activity in the posterior midgut epithelium

decreased from 0.5 EU per posterior midgut epithelium at 3 h to 0.05 EU at 36 h after the blood meal (Fig. 5), the converse trend to luminal activity. Total activity in the epithelium then showed signs of recovery but had not returned to original amounts at 60 h after the blood meal (Fig. 5).

Post-Feeding Activity and Distribution of α-Glucosidase. α-glucosidase was active in both anterior and posterior midgut regions before and at all times after feeding. In whole midgut homogenates, α-glucosidase activity increased slowly up to 18 h after the blood meal, then rose rapidly to a maximum at 30 h after the blood meal, whereas the subsequent decline in activity was less predictable than that for the proteases (Fig. 2). The posterior midgut activity exhibited an overall increase after feeding but with no clear trend. All posterior midgut activity was restricted to the posterior midgut lumen (Table 1). The specific activity of α-glucosidase in the whole midgut showed a continuous rise from 3 h after the blood meal until 60 h (Fig. 3a), because of elevated activity late in

Table 1. Distribution of α-glucosidase in midgut compartments of *An. stephensi* during a 48-h post-feeding period

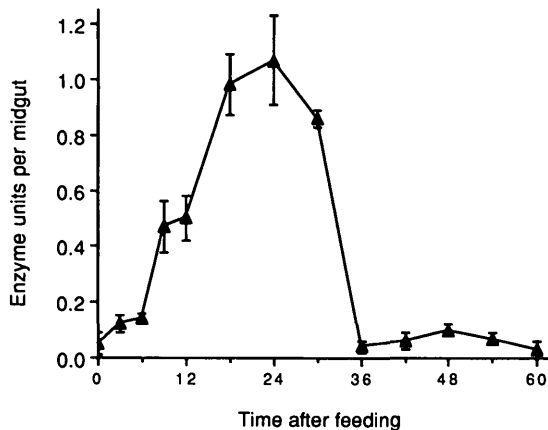| Time, h	% Total relative activity	
	Anterior midgut	Posterior midgut lumen
3	2.96	97.04
6	2.45	97.55
9	8.02	91.98
12	7.80	92.20
18	13.98	86.02
24	4.52	95.48
30	11.99	88.01
36	0.01	99.99
42	1.15	98.85
48	25.51	74.49

Fig. 6. Activity of α-glucosidase in anterior midgut homogenate supernatants of *Anopheles stephensi.*

the digestion period when very little protein was present in the whole midgut. Correlation between the mean relative activity and mean specific activity was poor ($r = 0.17$; df = 12; $P > 0.5$) (Fig. 3b).

More than 25% of the total midgut activity of α-glucosidase could be located in the anterior midgut depending upon the time after feeding (Table 1). The anterior midgut α-glucosidase activity showed a clear specific response to the presence of a blood meal, with maximum activity detectable at 24 h (Fig. 6). Activity then declined to a very low baseline and showed no correlation with other blood digestion processes in the posterior midgut.

Discussion

Our study provides a clear picture of the timing and sites of activity of three major enzymes, all of which have different functions in blood meal digestion, in the midgut of *An. stephensi.* Trypsin is the major primary hydrolytic protease in the mosquito midgut (Briegel & Lea 1975) and is responsible for the initial breakdown of proteins and large peptides. In *Ae. aegypti*, trypsin is synthesized de novo after blood feeding (Gooding 1973) in two cellular stages (Felix et al. 1991)—immediate translation from trypsin mRNA after feeding, followed by transcription of new trypsin mRNA several hours after the blood meal (Fuchs & Fong 1976, Hecker & Rudin 1979). Although several trypsin molecules can be detected in *Ae. aegypti* midgut (Graf & Briegel 1985, Kunz 1978), active trypsin is restricted to the posterior midgut lumen; trypsin-immunoreactive molecules that have been localized in secretory vesicles of posterior midgut epithelium are probably inactive precursors (Graf et al. 1986). Immunolocalization of trypsin clearly shows that the bulk of reactivity is restricted to the posterior midgut lumen of *Ae. aegypti* (Graf et al. 1986), where it freely crosses the peritrophic membrane to digest the blood meal. This is also the case with

An. stephensi, where all active trypsin was restricted to the posterior midgut lumen.

The quantity of trypsin produced is directly proportional to the size of the blood meal (Briegel & Lea 1975), and more specifically to soluble peptides in the blood serum (Felix et al. 1991). Consequently, trypsin and midgut protein show clear and predictable correlations (Houseman & Downe 1986). In *An. stephensi* this is reflected in the specific activity curve, which showed a clear post-feeding trend almost identical to that of total activity per whole midgut. Of the three enzymes examined, only trypsin exhibited such a correlation between relative and specific activities, supporting the theory for secretogogue control of trypsin in the mosquito midgut (Briegel & Lea 1975, Houseman & Downe 1986).

In *An. stephensi,* three aminopeptidases are responsible for the posttryptic digestion of peptides throughout the midgut (Billingsley 1990b). Low levels of activity in the anterior midgut probably represent a cytosolic soluble aminopeptidase, which would be required for routine intracellular peptide processing; the absence of any influence by the blood meal on anterior midgut aminopeptidase activity demonstrates its lack of involvement in digestion. Conversely, although posterior midgut epithelium would still require a similar cytosolic enzyme, some epithelial activity in the posterior midgut also may represent a processing or secretory step for the luminal enzyme. The decrease after feeding in the epithelium-associated aminopeptidase was a clear response to the presence of the blood meal in the lumen.

Most of the soluble aminopeptidase was secreted into the posterior midgut lumen, where its activity is probably concentrated in the ectoperitrophic space (Graf & Briegel 1982). Membrane-bound aminopeptidases constitute ≈50% of total midgut activity in *An. stephensi* (Billingsley 1990b), but there may still be a transport role for the soluble form of the enzyme. Being active close to the midgut wall, the soluble aminopeptidase is in an ideal site to drive amino acid transport from the lumen into the epithelium (and on to the hemolymph) in a fashion similar to alcohol dehydrogenase driving and directing NADH transport under experimental conditions (Vincent et al. 1988).

The specific activity of aminopeptidase peaked later compared with total relative activity or with any trypsin activity, and there was no correlation between relative and specific aminopeptidase activities. Although trypsin is responsible for primary proteolytic events in the midgut, secondary digestion of peptides is brought about by (amino- and carboxy-) peptidases, so aminopeptidase activity should correlate with peptides rather than proteins in the midgut. The peak of specific activity represents, therefore, a poor correlation with proteins, and is delayed probably to coincide with the appearance of peptides after trypsin hydrolysis of the blood meal.

The glycosidases represent a major nonproteinase group of enzymes in the mosquito midgut (unpublished data). Up to six different glycosidases are active in the midgut of *An. stephensi*, and probably occupy two major sites—the midgut lumen and the lysosomes of midgut epithelium. α-glucosidase is the major midgut glycosidase in the *An. stephensi* midgut and therefore was chosen for this study. Compared to both proteases, α-glucosidase showed a different distribution and activity after feeding. Like trypsin, α-glucosidase was active only in a soluble form and was restricted to the lumen in the posterior midgut. Like aminopeptidase, α-glucosidase was active before and after feeding, and was found in both anterior and posterior midgut regions. α-glucosidase clearly is not membrane associated and is secreted into the lumen in response to blood feeding. Because the lumen and epithelium of the anterior midgut were not examined separately, it is assumed that α-glucosidase has similar properties and distributions in both anterior and posterior midgut regions.

The pronounced increase of α-glucosidase activity in posterior midgut lumen occurred later in comparison to the proteases. The specific activity never reached a defined peak but continued to rise throughout the digestion period, in complete disassociation from other digestive events. α-glucosidase is presumably produced in response to suitable glycoside substrates in the midgut. Although some of these may be free in the serum, many more would be released by the proteolytic degradation of blood meal glycoproteins, especially those in the erythrocyte membranes (Harris & Kellermeyer 1970). There is no evidence to suggest whether α-glucosidase is active in the posterior midgut lumen in the endo- or ectoperitrophic space, but delay in onset of activity also may influence postsecretory structural modifications to the peritrophic membrane, which is highly glycosylated (Berner et al. 1983, Rudin & Hecker 1989).

By feeding radiolabelled glucose with blood to *An. stephensi*, Schneider et al. (1987) demonstrated that, even after peritrophic membrane formation is complete (18 h after feeding), sugar components of the blood meal still are absorbed in the anterior midgut. The small, but significant, increase in α-glucosidase in the anterior midgut after feeding indicates that this region may be involved in blood meal digestion, probably before the plug of peritrophic membrane at the anterior–posterior midgut junction is formed completely. The absence of any proteases in significant quantities in the anterior midgut suggests that the α-glucosidase found in this region does not originate from the posterior midgut.

Ultrastructural observations were used to develop a model for digestion in mosquitoes (Hecker 1977, 1978; Billingsley 1990a), and the current data on enzyme distribution can be used to support this model. The quantities of synthetic and secretory organelles (ribosomes, rough endoplasmic reticulum, and Golgi) in the midgut cells increase from 2 to 30 h after feeding. This clearly correlates with synthesis and secretion of all three enzymes described here, particularly in the posterior midgut. It is not possible to distinguish which organellar modifications are responsible for enzyme changes, because all enzyme activities follow a similar trend (Billingsley 1988). The absence of such organellar changes in the anterior midgut supports the model that protein digestion (at least) does not occur in the anterior midgut. The sugar digestive role also may be related to sugar feeding in mosquitoes, because the anterior midgut is thought to receive nectar passed to it from the gut diverticula (Hecker 1977, 1978; Billingsley 1990a).

References Cited

Berner, R., W. Rudin & H. Hecker. 1983. Peritrophic membranes and protease activity in the midgut of the malaria mosquito, *Anopheles stephensi* (Liston) (Insecta: Diptera) under normal and experimental conditions. J. Ultrastr. Res. 83: 195–204.

Billingsley, P. F. 1988. Morphometric analysis of *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) midgut cells during blood digestion. Tissue Cell 20: 291–301.

1990a. The midgut ultrastructure of haematophagous insects. Annu. Rev. Entomol. 35: 219–248.

1990b. Blood digestion in the mosquito, *Anopheles stephensi* Liston (Diptera: Culicidae): partial characterization and post-feeding activity of midgut aminopeptidases. Arch. Insect Biochem. Physiol. 15: 149–163.

Billingsley, P. F. & A.E.R. Downe. 1985. Cellular localisation of aminopeptidase in the midgut of *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) during blood digestion. Cell Tissue Res. 241: 421–428.

1988. Ultrastructural localisation of cathepsin B in the midgut of *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) during blood digestion. Int. J. Insect Morphol. Embryol. 17: 295–302.

Borovsky, D. 1986. Proteolytic enzymes and blood digestion in the mosquito, *Culex nigripalpus*. Arch. Insect Biochem. Physiol. 3: 147–160.

Briegel, H. & A. O. Lea. 1975. Relationship between protein and proteolytic activity in the midgut of mosquitoes. J. Insect Physiol. 21:1597–1604.

Erlanger, B. F., N. Kokowsky & W. Cohen. 1961. The preparation of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95: 271–278.

Feldmann, A. M., P. F. Billingsley & E. Savelkoul. 1990. Bloodmeal digestion by strains of *Anopheles stephensi* Liston (Diptera: Culicidae) of differing susceptibility to *Plasmodium falciparum*. Parasitology 101: 193–200.

Felix, C. R., B. Betschart, P. F. Billingsley & T. A. Freyvogel. 1991. Post-feeding induction of trypsin in the midgut of *Aedes aegypti* L. (Diptera: Culicidae) is separable into two cellular phases. Insect Biochem. 21: 197–203.

Fuchs, M. S. & W. F. Fong. 1976. Inhibition of blood digestion by alpha-amanitin and actinomycin D and its effect on ovarian development in *Aedes aegypti*. J. Insect Physiol. 22: 465–471.

Gass, R. F. 1977. Influences of blood digestion on the development of *Plasmodium gallinaceum* (Brumpt) in the midgut of *Aedes aegypti* (L.). Acta Tropica 34: 127–140.

Gooding, R. H. 1973. The digestive processes of haematophagous insects. IV. Secretion of trypsin by *Aedes aegypti*. (Diptera: Culicidae). Can. Ent. 105: 599–603.

Graf, R. & H. Briegel. 1982. Comparison between aminopeptidase and trypsin activity in blood-fed females. of *Aedes aegypti*. Rev. suisse Zool. 89: 845–850.

1985. Isolation of trypsin isozymes from the mosquito *Aedes aegypti*. Insect Biochem. 15: 611–618.

Graf, R., A. S. Raikhel, M. R. Brown, A. O. Lea & H. Briegel. 1986. Mosquito trypsin: immunocytochemical localization in the midgut of blood-fed *Aedes aegypti* (L.). Cell Tissue Res. 245: 19–27.

Harris, J. W. & R. W. Kellermeyer. 1970. The red cell. Production, metabolism, destruction: normal and abnormal. Harvard University Press, Cambridge.

Hayes, R. O. 1953. Determination of a physiological saline solution for *Aedes aegypti* L. J. Econ. Entomol. 46: 624–627.

Hecker, H. 1977. Structure and function of midgut epithelial cells in Culicidae mosquitoes (Insecta, Diptera). Cell Tissue Res. 184: 321–341.

1978. Intracellular distribution of ribosomes in midgut cells of the malaria mosquito, *Anopheles stephensi* (Liston) (Insecta: Diptera) in response to feeding. Int. J. Insect Morphol. Embryol. 7: 267–272.

Hecker, H. &. W. Rudin. 1979. Normal versus α-amanitin induced cellular dynamics of the midgut epithelium in female *Aedes aegypti* L. (Insecta, Diptera) in response to blood feeding. Europ. J. Cell Biol. 19: 160–167.

Houseman, J. G. & A.E.R. Downe. 1986. Methods of measuring blood meal size and proteinase activity for determining the effects of mated state on digestive processes of female *Aedes aegypti* (L.) (Diptera: Culicidae). Can. Entomol. 118: 241–248.

Kunz, P. A. 1978. Resolution and properties of the proteinases in adult *Aedes aegypti* (L.). Insect Biochem. 8: 169–175.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83: 346–356.

Ribeiro, J.M.C. & M.E.A. Perreira. 1984. Midgut glycosidases of *Rhodnius prolixus*. Insect Biochem. 14: 103–108.

Rudin, W. & H. Hecker. 1979. Functional morphology of the midgut of *Aedes aegypti* L. (Insecta, Diptera) during blood digestion. Cell Tissue Res. 200: 193–203.

1989. Lectin binding-sites in the midgut of the mosquitoes *Anopheles stephensi* Liston and *Aedes aegypti* L. (Diptera: Culicidae). Parasitol. Res. 75: 268–279.

Schneider, M., W. Rudin & H. Hecker. 1987. Absorption and transport of radioactive tracers in the midgut of the malaria mosquito, *Anopheles stephensi*. J. Ultrastr. Molec. Struct. Res. 97: 50–63.

Van Handel, E. & W. S. Romoser. 1987. Proteolytic activity in the ectoperitrophic fluid of blood-fed *Culex nigripalpus*. Med. Vet. Entomol. 1: 251–255.

Vincent, J.-C., S. Alexandre & M. Thellier. 1988. How a soluble enzyme can be forced to work as a transport system: description of an experimental design. Arch. Biochem. Biophys. 261: 405–408.