found in 37 of the 73 collections and at 45 of the 55 collection sites. *Cx. quinquefasciatus* were collected 151 times at 90 collection sites. At least 1 kind of pathogen was found in 137 of the 151 collections and at 81 of the 90 collection sites.

These data indicate that mosquito populations in the tropics are a lucrative source of diverse mosquito pathogens for evaluation and possible development as biological control agents. The frequency and ease with which they can be found suggests they are an important factor in natural population control.

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PERMEABILITY OF *AEDES AEGYPTI* LARVAL PERITROPHIC MEMBRANE TO PROTEOLYTIC ENZYME

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ABSTRACT. Larval mosquito peritrophic membranes and contents were removed intact, ligated at both ends, washed and each tested *in vitro* to see if proteolytic enzyme would move from the trophic to the peritrophic space. The peritrophic membrane appears to be permeable to the mosquito's own proteolytic enzyme.

The peritrophic membrane (PM) is a thin, non-cellular and commonly tubular sheet that lines the lumen of the midgut. It is composed of chitin, protein and mucopolysaccharide (Wigglesworth 1930, Day 1949, Gander 1968). Richards and Richards (1977) provided a recent review of the literature on the PM. Peters (1976) reviewed the literature dealing specifically with PM's in Diptera.

In *Aedes aegypti* larvae, the PM is secreted by the cardial cells located in the proventriculus. It lines the entire midgut and is cylindrical in shape. The PM divides the midgut lumen into two regions, the trophic space, which contains all materials ingested, and the peritrophic space, which lies between the PM and the midgut epithelium. Ultrastructurally the PM is multilaminar, consisting of fibrous

and granular layers and averages about one micrometer in thickness (Richards and Richards 1971).

Since the PM lies between the ingested food material and the midgut epithelium, passage of enzymes and digested materials must be determined to a large extent by the PM.

The significant literature on PM permeability was discussed by Peters (1976) and Richards and Richards (1977). Schildmacher (1950) reported that colloidal gold particles 2–4 nm pass through the PM of mosquito larvae, but 20 nm particles do not. Zhuzhikov (1970) determined that colloidal gold particles greater than 7nm failed to pass through the PM of *Ae. aegypti* larvae.

The purpose of this study was to determine whether or not the PM in *Ae. aegypti* larvae is permeable to the mosquito's own proteolytic enzymes.

MATERIALS AND METHODS

Ae. aegypti, Rockefeller strain, eggs were hatched in small cups of water under a vacuum. Larvae were fed a mixture of finely ground Purina® lab chow, lactalbumin hydrolysate and brewer's yeast, in equal volumes. Groups of 50 freshly hatched larvae were placed in plastic boxes (13 × 18 × 19 cm) and incubated at 27°±1°C.

Fourth instar larvae were dissected in 0.01 M sodium borate buffer (pH 8.5–9.0). PM's were removed and the ends ligated with a fiber of silk thread (Fig. 1). To wash enzyme from the surface, ligated PMs were immersed in buffer (approximately 25–30 ml of buffer for each PM) for 2 hr.

The method used to test for permeability is shown in Fig. 1. Three capillary tubes (1.0mm O.D. × 15mm) were prepared for each PM. Each tube was filled with buffer, and several crystals of azure-blue hide powder (Calbiochem) were placed in one end. Azure-blue hide powder is a dye-protein complex which is insoluble in water. When the protein is digested the water soluble dye molecules are released into solution. Thus appearance of blue color in solution indicates

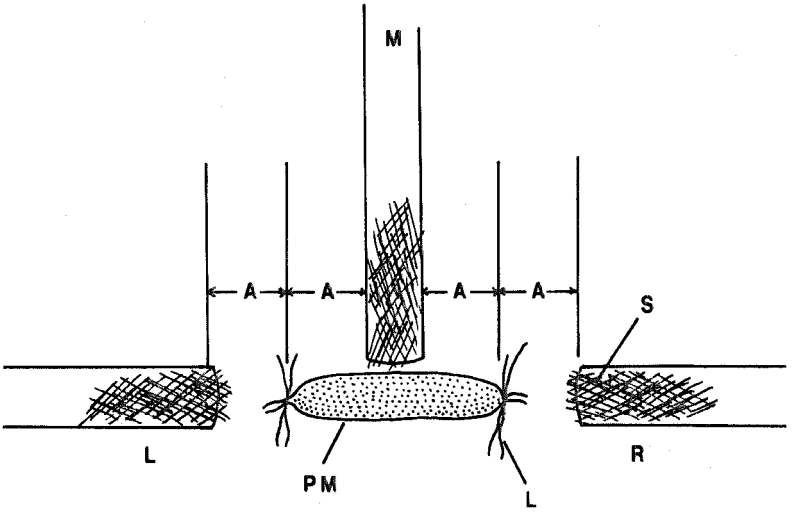

Fig. 1. Positions of capillary tubes and ligated PM (see text for explanation). A, equal distances; L. M & R, capillary tubes; Li, ligature; PM, peritrophic membrane and contents; S, azure-blue hide powder.

proteolytic enzyme activity (Rinderknecht et al. 1968.)

A 1% agar solution (prepared with sodium borate buffer) was poured into petri dishes and permitted to cool until nearly coagulated. PMs were removed from the wash, rinsed with buffer, individually placed in the agar, and the capillary tubes positioned (Fig. 1). Tubes "L" and "R" were controls and "M" was the test. "L" and "R" were the same distance (A) from the tied ends as "M." If one or both of the controls showed enzyme activity before or at the same time as the "M" tube, no conclusion could be drawn from the experiment because of the possibility of leakage at the tied ends. If the "M" tube showed activity first, then we assumed enzyme diffused through the PM.

Each preparation was covered with molten agar (approximately 43°C) and incubated at 27°±1°C. Checks for activity (i.e. release of dye into the agar) were made after 1, 2 and 3 hr of incubation. Results were judged subjectively and recorded as follows:

− = no activity
± = slight activity
+ = definite activity

To determine the effectiveness of the borate buffer wash, 4 PMs were dissected, their contents emptied and each washed as above. Each PM was then removed, rinsed and placed in a depression slide with several drops of buffer and crystals of azure-blue hide powder. In addition, the contents removed from each PM were placed in buffer along with crystals of azure-blue hide powder. All preparations were incubated at 27°±1°C for 3 hr and then examined for evidence of enzyme activity.

In order to determine if there was uniform diffusion of materials across the layers of agar, crystals of potassium permanganate and methylene blue were placed on the top layer of agar. Later, the pattern of dye diffusion was examined in sections of the agar. As a 2nd method, a slice of double-layered agar was removed and a crystal of dye placed on the interface between the 1st and 2nd layers. We assumed that uniform dye diffusion indicated uniform enzyme diffusion.

RESULTS

In 9 of 10 tests for the passage of enzyme across the PM, the test capillary tubes (M) showed activity within 3 hr (Table 1). None of the controls (L and R) for enzyme leakage via the silk ligatures showed activity before the test capillary tube and only one, following the 3rd hr, exhibited any activity at all.

None of the preparations with emptied, washed PMs showed evidence of enzyme activity even after 24 hr, and all of the preparations with gut contents showed evidence of enzyme activity within 30 min.

Table 1. Passage of enzyme across PM.[a]

Enzyme Activity	Incubation time (h)								
	1			2			3		
	L	M	R	L	M	R	L	M	R
(−)	10	4	10	10	2	10	10	1	9
(±)	0	3	0	0	2	0	0	1	0
(+)	0	3	0	0	6	0	0	8	1

[a] L, M & R same as in Fig. 1.

DISCUSSION

The larval PM appears to be permeable to proteolytic enzyme in the ectad direction.

Leakage due to tearing was a possibility, but the PMs were handled with great care and if there were tears, we would have expected much less uniformity in the results.

The heat of molten agar could have affected the results, but PMs were not exposed to more than 43°C. Room temperature was between 18° and 20°C and the 2nd layer of agar was thin and cooled rapidly. Since enzyme remained active,

we feel safe in assuming that the PM was unaffected by the brief exposure to above normal temperatures. In addition, 10 larvae placed in 43°C water which was allowed to cool to room temperature survived to the adult stage.

It is possible that the borate buffer somehow influenced PM permeability. However, the midgut lumen is hardly a uniform environment, and the PM is probably naturally exposed to a wide variety of molecules and conditions.

Permeability of the *Ae. aegypti* larval PM to proteolytic enzyme contrasts with evidence that the PM in the blow fly is impermeable to human amylase in the ectad direction (Zhuzhikov 1964).

Azure-blue hide powder is most sensitive to trypsin and chymotrypsin (Rinderknecht et al. 1968). It is not known which, or if either of these enzymes played the greater role in the results. The molecular weight and dimensions of these molecules are not known. However, Zhuzhikov (1970) found that the pore size of the PM in *Ae. aegypti* larvae was approximately 7nm. The larval mosquito proteolytic enzymes may be smaller than 7nm or Zhuzhikov may be correct in suggesting that there is a complex interaction between the enzymes and PM which allows the larger molecules to pass through.

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