Flea Ctenophthalmus: Heterogeneous Hexagonally Organized Layer in the Midgut

Abstract. *The ventriculus or midgut of adult fleas of the genus* Ctenophthalmus *(Hystricopsyllidae) is surrounded by a complex investment consisting of a "beaded layer," circumferential and longitudinal muscles, minute fibrils of the sort commonly called collagenous, and a granular layer at the boundary of the hemocoel. The beaded layer is remarkable in consisting of two types of short cylinders of greatly different diameters, each type arranged in a mixed hexagonal array.*

Many electron micrographs show the "basement membrane," "basement lamina," or "connective tissue sheath" of this or that organ in insects. Most of these basal lamina are zones that appear somewhat granular in thin sections. Commonly some or many fine "collagenous" fibrils are embedded in this granular matrix (*1–3*). But in none of the many published figures and in no other of our examinations have we found such a structure as exists around the midgut of the adult flea *Ctenophthalmus*.

For our preparations the alimentary tracts of adult fleas [*Ctenophthalmus* sp. (*4*)] were extirpated in a cold phosphate buffer and then immediately transferred to cold buffered OsO_4 (Zetterquist solution) for 2 to 3 hours. After washing in cold distilled water they were dehydrated through a series of cold ethanol solutions to propylene oxide, embedded in the epoxy resin Durcupan, sectioned, and examined with a Siemens Elmiskop. Some sections were examined without further treatment, but most were stained with uranyl acetate and lead citrate, phosphotungstic acid (PTA), or potassium permanganate. The heterogeneous hexagonal layer, which appears as a "beaded layer" in cross section, showed up with maximum clarity after treatment with $KMnO_4$ solutions; the microfibrils thought to be collagenous showed up best after treatment with PTA solutions; but the main features were revealed by each contrasting method and even by OsO_4 fixation alone.

For orientation, Fig. 1 is a diagrammatic sketch of a small portion of a section of the ventriculus (stomach or midgut). The microvillate midgut cells terminate basally at their plasma membranes; immediately beyond is the remarkable layer diagramed in Fig. 2. In the ensuing space are located (i) small muscle fibers that encircle the midgut, (ii) small longitudinal muscles that run along the gut in an anteroposterior direction, (iii) a few unidentified cells, (iv) numerous fibrils that appear to be collagenous, (v) an amorphous or slightly granular layer that demarcates the boundary of the blood cavity or hemocoel, and (vi) the nearly empty spaces separating the preceding. The total thickness of this investment ranges from 0.5 to 5 μ, mainly varying with the location of muscles.

The remarkable layer just basal to the plasma membrane of the midgut cells is some 600 to 800 Å in thickness and is composed of two types of short columns, each type in hexagonal array. The larger columns are 500 to 600 Å in breadth (most about 500 Å); the smaller ones are 170 to 180 Å in diameter. Since the interval between the larger elements is about 100 Å, the center-to-center distance of the larger columns is about 625 Å; of the smaller columns, about 375 Å. Because of undulations in the plasma membrane of the midgut cells, this layer is sometimes cut in tangential view as well as the more common cross or longitudinal section. Sometimes the beaded layer extends a greater or lesser distance between cells (up to halfway to the microvillate border) or protrudes away from the plasma membrane of the midgut cells (Fig. 3E), but usually it parallels and faithfully follows the plasma membrane at the base of the midgut cells (Fig. 3D). Numerous measurements from our many micrographs show spacings ranging from 150 to 300 Å between the plasma membrane of the midgut cells and the base of the columns that make up the beaded layer.

The two columnar units in this layer differ not only in diameter but also in stainability. After postsectioning treatment with solutions of $KMnO_4$, the smaller columns are several times more electron-dense than the larger columns (Fig. 3A); and in some regions the smaller columns appear as blunt-pointed triangles, the implication being that by pressure they are distorted by the larger columns. The material between the columnar units is scarcely detectable after treatment with $KMnO_4$ (this fact being one reason why $KMnO_4$ reveals the columnar details so clearly), but, after treatment with PTA or uranyl plus lead; intervening and surface granularity is evident. Thus it seems that there are at least three components in this layer: the large columns, the small columns, and the intervening and surface material on both sides. We do not know their chemical natures and the layer is too thin for visible-light histochemistry. The strong reaction with $KMnO_4$ suggests a

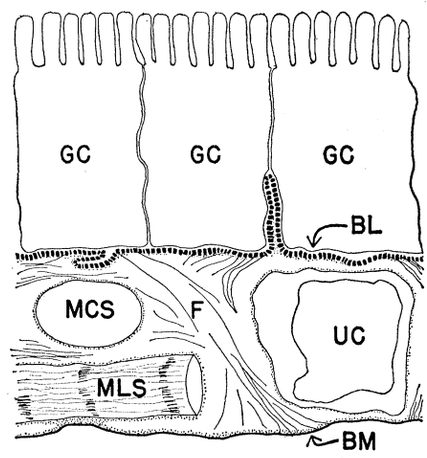

Fig. 1. Orientation diagram of a small portion of a longitudinal section of the ventriculus of an adult flea at a point where the midgut epithelial cells are short and the investment is relatively thick. Abbreviations: *BL*, beaded layer; *BM*, bounding layer at hemocoel; *F*, connective-tissue fibrils which attach to both *BL* and *BM*; *GC*, cells of the midgut epithelium; *MCS*, circumferential muscle in cross section; *MLS*, longitudinal muscle; *UC*, unidentified cell type.

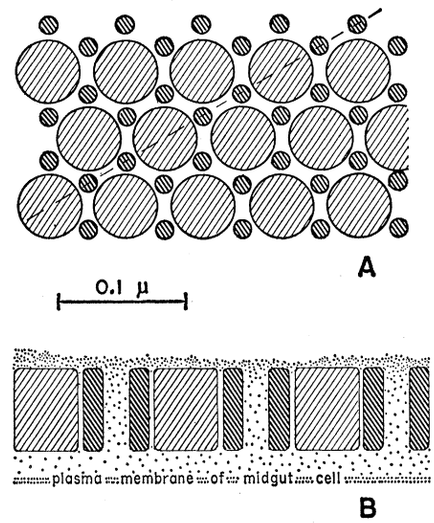

Fig. 2. Diagrammatic representation (to scale) of surface and cross-sectional views of the novel beaded layer. (A) Surface or tangential view, with all parts drawn as circles (commonly the smaller columns appear as blunt-pointed triangles; presumably they are squeezed into this shape in some areas). (B) Cross-sectional view as would be seen in a section along the dashed line in (A). Obviously appearances may vary greatly, depending on the angle of sectioning relative to the rows of columns.

polysaccharide or mucopolysaccharide, but there are no cogent data for such.

The previous paragraphs imply that there is always a hexagonal array; most surface views show such, at least over much of their area, but sometimes a row of larger columns either terminates or bifurcates. In these situations one may find either five or seven of the smaller columns surrounding a single larger column (Fig. 3A).

In the considerable space between the beaded layer and the hemocoel, one finds the small circumferential and longitudinal gut muscles. There seems to be nothing remarkable about these muscles. The few unidentified cells may conceivably be involved in formation of this investment, but too few were seen for precise characterization. In the interstices between these muscles and unidentified cells are many fibrils about 100 Å in diameter; they interlace through this space and attach to the beaded layer (Fig. 3, F and G) and to the granular layer that separates this area from the hemocoel (Fig. 3H). These fibrils are relatively faint after treatment with $KMnO_4$ but are better resolved after treatment with PTA (Fig. 3G); their attachments show most clearly in sections stained with uranyl and lead (Fig. 3, F and H). In a few favorable preparations treated with PTA, the fibrils appear faintly banded with a periodicity less than 200 Å. Presumably these correspond to the "short-spacing collagenous fibrils" described by Baccetti, Pipa, and Ashhurst [they compare favorably with fig. 19 in Ashhurst's review (*2*)]. Since the periodicity is far from the approximately 640 Å of mature vertebrate collagen we prefer the noncommittal term of "connective tissue fibril" used by Pipa and Woolever (*3*).

Peripherally there is a rather dense granular layer at the boundary of the hemocoel; it resembles the "basement lamina" of Anderson and Harvey (*5*) and the "basement membranes" in electron micrographs by various authors. To it, as we have mentioned, ends of the connective tissue fibrils attach (Fig. 3H). At some places in some sections the longitudinal muscles of the midgut (which are peripheral to the circumferential muscles) seem to touch this layer; at such points this bounding layer appears to become continuous with and indistinguishable from the sarcolemma of the muscle. Two points remain for consideration.

1) Is this beaded layer found only around the midgut? Since we were studying sections from extirpated guts, we cannot fully answer this question. But we can say that the layer is found around the midgut in its anterior, middle, and posterior parts; that it does not terminate abruptly at the midgut-foregut junction but does disappear before one reaches the proventriculus. Since the layer is not present around the proventriculus, there is no reason to assume that it occurs around organs other than the midgut. We had a few sections of the midgut of a flea larva collected in association with the adult collections. A few micrographs of these sections suggest that the larval midgut has a similar investment, but either the fixation of this single specimen was inferior or the investment is less clearly delimited (especially indistinct connective-tissue fibrils and more general granularity throughout).

2) What terms should be applied to the parts of this investment of the midgut? We have come to wonder what definition should be applied to the commonly used term basement membrane. At least our concept of basement membrane does not encompass things as heterogeneous as this. Study of connective-tissue sheaths and investments over insect organs is relatively recent, and few insects have been examined. Pending an extensive comparative study of organ investments throughout the Insecta we prefer to refer to this total ensemble noncommittally as "midgut investment."

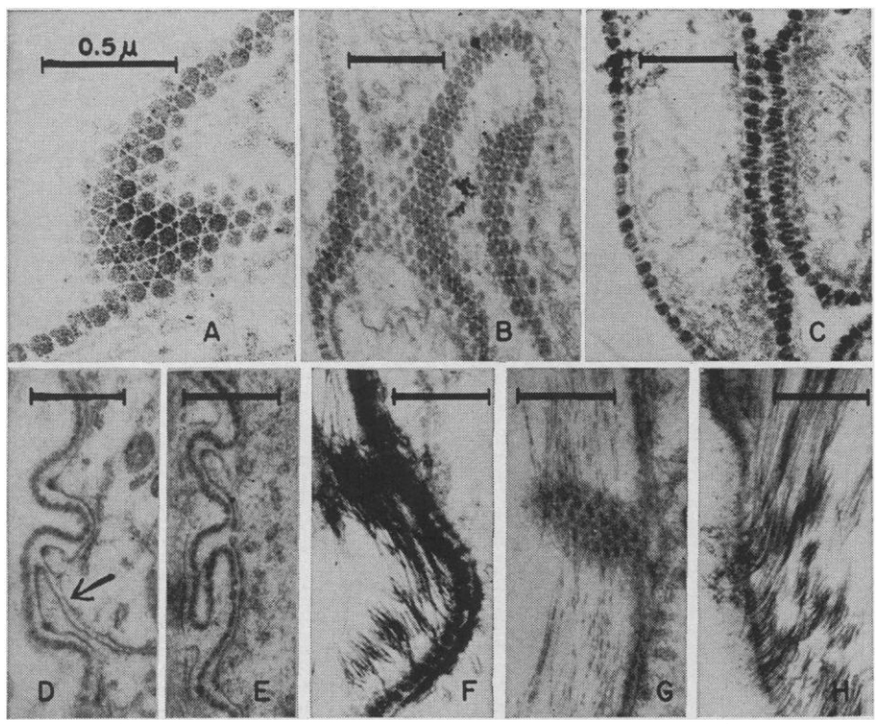

Fig. 3. Electron micrographs of representative sections from the midgut of *Ctenophthalmus* sp. The beaded layer is clearly delineated only after treatment with $KMnO_4$. Bars represent 0.5 μ. In all micrographs the hemocoel is to the left. (A) Tangential view of small area, showing a terminating row of large columns with five and seven surrounding small columns at this point (1 percent $KMnO_4$, 10 minutes). (B) Tangential view from another preparation, showing regular hexagonal array (1 percent $KMnO_4$, 10 minutes). (C) Cross section through several folds, showing (left) section through larger columns, and (right) sections through smaller columns and edges of larger columns (1 percent $KMnO_4$, 20 minutes). (D) Section of an intercellular boundary (arrow), showing positive identification of plasma membrane of the midgut cells, and beaded layer extending across this boundary (1 percent PTA, 2 minutes). (E) Loops in beaded layer, one of them free from the plasma membrane of the midgut cell (1 percent PTA, 10 minutes). (F) Connective-tissue fibrils extending into the beaded layer (2 percent uranyl acetate, 15 minutes; Reynolds lead citrate, 5 minutes). (G) Tangential view showing fibrils extending into beaded layer (1 percent PTA, 2 minutes). (H) Section through layer at periphery of investment, showing insertion of fibrils (2 percent uranyl acetate, 15 minutes; Reynolds lead citrate, 5 minutes).

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References and Notes

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4. Specimens collected from mole nests at Seewiesen über Starnberg, near Munich, Germany, in January and February 1967. Specimens saved for taxonomic identification showed that we had a mixture of *Ctenophthalmus agyrtes* Hell. and *C. bisoctodentatus* Kol. We have no way of knowing which species was used for any particular preparation for electron microscopy, but so many specimens were used that we probably had both species.
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6. Work done at the Max-Planck-Institut für Verhaltensphysiologie, Abteilung Schneider, to which thanks are due for facilities; it was aided by NSF grant GB-365. Paper 6462 of the Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul.

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X-Linked Hypoxanthine-Guanine Phosphoribosyl Transferase Deficiency: Heterozygote Has Two Clonal Populations

Abstract. *Clones of skin fibroblasts cultured from the mother of two sons with X-linked hypoxanthine-guanine phosphoribosyl transferase deficiency (Lesch-Nyhan syndrome) were assayed for activity of this enzyme by measurement of the incorporation of ^{3}H-guanine into guanylic acid as counts per minute per microgram of protein and by autoradiography. The demonstration of two populations of clones, wild-type clones with normal enzyme activity and mutant clones unable to incorporate ^{3}H-guanine, is evidence that the locus for hypoxanthine-guanine phosphoribosyl transferase on one of the X chromosomes is inactive.*

A familial syndrome consisting of hyperuricemia, mental retardation, choreoathetosis, and compulsive self-mutilation was described by Lesch and Nyhan (*1*). There is substantial evidence that the mutation is X-linked, since transmission of the disorder, which occurs only in males, is from mother to son (*2*). The disease is associated with absence of activity of an enzyme involved in purine metabolism, namely, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) (*3*).

As determined by autoradiography, some skin fibroblasts from the mother of an affected male incorporate ^{3}H-hypoxanthine into RNA, an indication of phosphoribosyl transferase activity, whereas other cells do not (*4*). This finding is interpreted as support for the hypothesis of inactivation of one X chromosome in female somatic cells (*5*). The demonstration of a mosaic pattern in the fibroblasts grown from the mother of an affected child is compatible with inactivation of the HGPRT locus in one X chromosome; however, a critical test of X inactivation at this locus is the examination of HGPRT activity in clones cultured from a heterozygote. We have obtained clones of fibroblasts from the mother of two boys with Lesch-Nyhan syndrome (HGPRT deficiency) and have examined them quantitatively and autoradiographically for their ability to incorporate ^{3}H-guanine into guanylic acid.

Fibroblast cultures were established from skin biopsies from M.W., one of the two brothers described in the original report (*1*), and from his parents. Two biopsy specimens were obtained from the mother, one from each forearm. Cells were grown in minimum essential media enriched with 1 percent nonessential amino acids, 5 percent beef embryo extract ultrafiltrate, and 20 percent human A serum and maintained in a 5 percent CO_2 atmosphere at 37°C. Clones were obtained according to techniques of Ham and Puck (*6*). Cells in suspension after dissociation by treatment with trypsin were single when plated, and clones were plucked only if they were well isolated from neighboring clones. Each uncloned fibroblast culture and clone were assayed for HGPRT activity in two ways.

In the first method cells in logarithmic phase growth were subcultured into bottles with 15 to 20 ml of growth medium. The cell suspension was transferred into three or four Falcon plastic 60-mm petri dishes so that, for each clone or for each uncloned specimen, cultures were established at least in triplicate. After 24 to 48 hours at 37°C, the cells were incubated for 1 hour with ^{3}H-guanine (0.3 c/mmole) in a final concentration of 1 μc/ml of medium. Petri dishes were then washed twice in saline (10 minutes for second saline wash) and fixed in 95 percent ethanol. The cells were removed from petri dishes with rubber policemen, suspended in 1 ml of 0.9 percent NaCl, and disrupted by sonic vibration. The resulting cell extracts were assayed for protein concentration (*7*) and uptake of ^{3}H-guanine. The amount of radioactivity was determined on 0.6 ml of the cell extract in 10 ml of Bray's solution by means of a Packard liquid scintillation counter. The control samples (t_0) were replicate cultures to which ^{3}H-guanine was added and immediately removed by washing as previously described.

In the second method, triplicate cultures established in 35-mm Falcon plastic petri dishes were allowed to grow for 24 to 48 hours in 5 percent CO_2 before incubation with ^{3}H-guanine (9.8 c/mmole) in a final concentration of 2.5 μc/ml medium. After 6 hours of incubation at 37°C, the cells were washed in saline, fixed in ethanol, extracted with 5 percent cold trichloroacetic acid, washed in water, and dried. Petri dishes were dipped in Kodak NTB-2 liquid emulsion, and after exposure for 1 week, the autoradiographs were developed, fixed, and washed; they were examined by both phase and bright-field microscopy. Petri dishes were scored as positive when cells were labeled with ^{3}H-guanine, and negative only when all of the cells were unlabeled and visible only with phase microscopy. Each specimen was scored without knowledge of its source.

Table 1. HGPRT activity determined with ^{3}H-guanine as counts per minute per microgram of protein. Each value used to calculate the mean represents the average of the triplicate determinations. Negative clones have deficient HGPRT activity; positive clones have normal HGPRT activity.

Source of fibroblasts	Cultures examined	Clones (No.)	Mean (cpm/μg protein)	Range or standard deviation
Father	Uncloned		12.22	2.91 to 29.99
Affected son	Uncloned		.16	0.8 to .20
Mother, biopsy No. 1	Uncloned		3.43	3.42 to 3.44
Mother, biopsy No. 1	Negative clone	10	.12	± .09
Mother, biopsy No. 1	Positive clone	10	4.72	±1.75
Mother, biopsy No. 2	Uncloned		11.58	7.22 to 15.93
Mother, biopsy No. 2	Negative clone	8	.19	± .18
Mother, biopsy No. 2	Positive clone	20	10.84	±7.13