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Autor(en): **Houk, E.J. / Hardy, J.L.**

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[1] Naval Biosciences Laboratory, School of Public Health, University of California, Oakland, California 94625, USA
[2] School of Public Health, University of California, Berkeley, California 94720, USA

In vivo negative staining of the midgut continuous junction in the mosquito, *Culex tarsalis* (Diptera: Culicidae)

E. J. Houk[1], J. L. Hardy[2]

Summary

The midguts of female mosquitoes, *Culex tarsalis*, were examined electron microscopically during the digestion of a meal from either artificial sources (i.e., 100% serum or defibrinated rabbit blood) or vertebrate hosts. Intense intercellular staining was apparent when the meal was derived from the vertebrate host or defibrinated rabbit blood; less intense staining with 100% serum. The staining was attributed to the "leakage" of a component of whole blood, presumably hemoglobin, into the intercellular junctional spaces. The staining component demonstrated an affinity for the outer membrane leaflet of the plasma membrane.

This study provides evidence in support of "leaky guts" as a means of infecting an arthropod host without a midgut amplification cycle. The ramifications of this concept are pointed out with reference to vector competence and midgut barriers to infection by arboviruses.

Key words: mosquito; continuous junction; midgut; staining; ultrastructure.

Introduction

The continuous junction (*zonula continua*) is a little studied intercellular junction, primarily associated with arthropods (Satir and Gilula, 1973; Staehelin, 1974; Lane, 1978). This junction was first described from the midguts of termites and the mealworm (Noirot and Noirot-Timothée, 1967). It has since been described in the hepatic caecum of *Daphnia* (Hudspeth and Revel, 1971),

Correspondence: Doctor E. J. Houk, Naval Biosciences Laboratory, School of Public Health, University of California, Oakland, California 94625, USA

hepatopancreas of the crayfish (Gilula, 1971), insect Malpighian tubules (Dallai, 1976) and the midguts of a chelicerate (Lane and Harrison, 1978) and several insects (Oschman and Wall, 1972; Reinhardt and Hecker, 1973; Flower and Filshie, 1975; Houk, 1977).

Superficially, the continuous junction appears to be a modified septate junction (Satir and Gilula, 1973). In thin-sectioned specimens both of these junctions reveal an intermembrane distance of between 11–15 nm and septa of approximately 4–5 nm that are perpendicular to the membranes (Satir and Gilula, 1973; Staehelin, 1974). The septa of continuous junctions are often difficult to distinguish in thin-sections as the intercellular space is filled with an electron opaque granular material (Noirot and Noirot-Timothée, 1967; Gilula, 1971; Hudspeth and Revel, 1971; Oschman and Wall, 1972; Reinhardt and Hecker, 1973; Houk, 1977). This intercellular material has been demonstrated to possess the staining characteristics of glycoprotein (Dallai, 1970; Reinhardt and Hecker, 1973).

The substructure of invertebrate continuous and septate junctions has been thought to be quite distinct as demonstrated by lanthanum hydrosol studies (Hudspeth and Revel, 1971; Satir and Gilula, 1973; Staehelin, 1974; Flower and Filshie, 1975). Pleated sheet substructure, with hexagonal symmetry, has been reported for septate junctions (Locke, 1965; Gilula et al., 1970; Satir and Gilula, 1973; Staehelin, 1974; Houk, unpublished observation). Continuous junctions reveal their septa as flat, gently curving sheets (Satir and Gilula, 1973; Lane, 1978). However, Green (1978) has recently demonstrated that the continuous junction is most likely a part of a continuum that links the vertebrate tight junction with the invertebrate septate junction (Satir and Gilula, 1973; Staehelin, 1974).

The observations reported herein are the consequence of an investigation of the ultrastructural modification of midgut epithelial cells in response to blood ingestion in the mosquito, *Culex tarsalis* Coquillett. Initial observations of various temporal stages in the digestive process indicated that electron opaque material had been deposited in the intercellular spaces yielding an in vivo negative staining of the continuous junction.

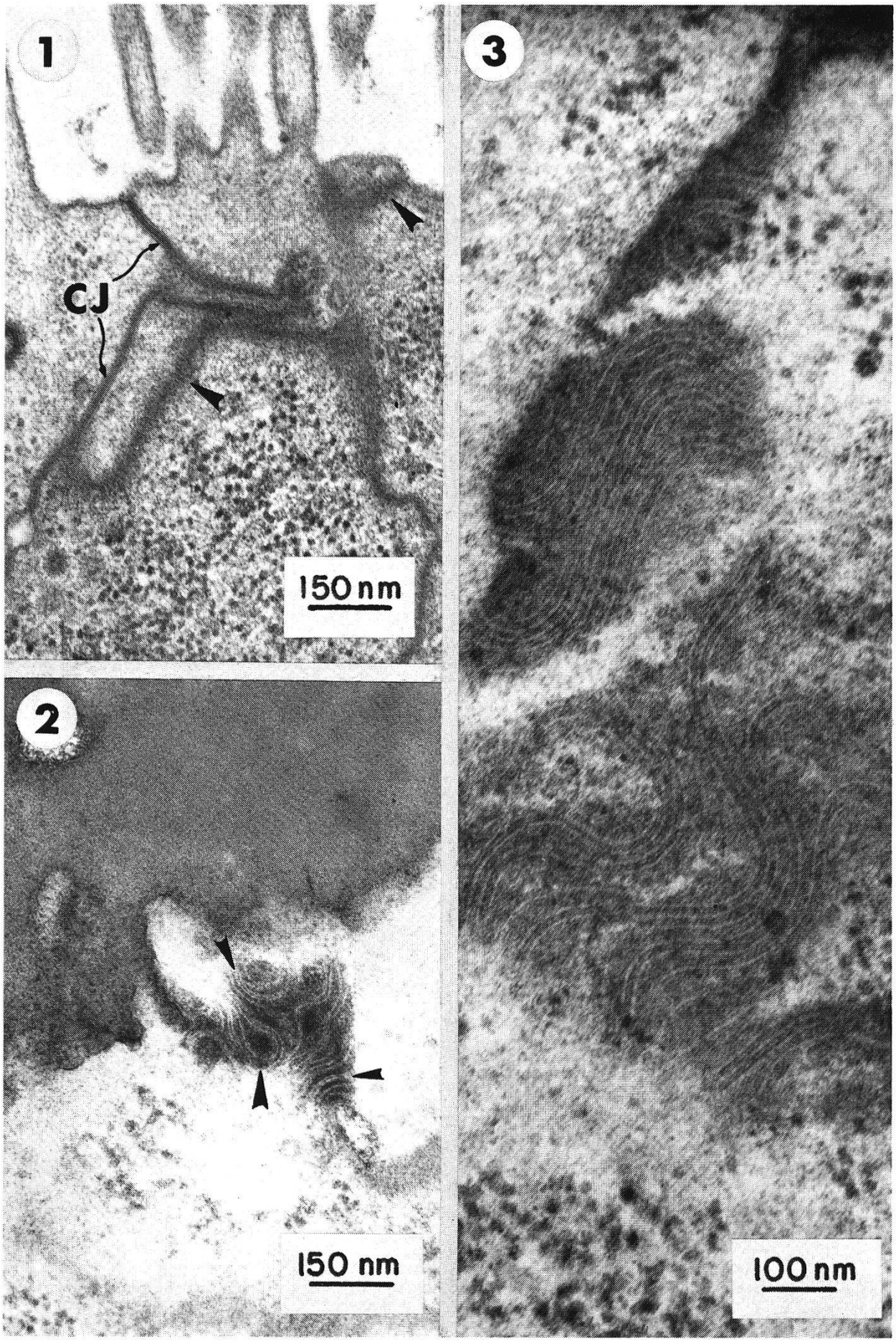
Material and methods

Mosquitoes, *C. tarsalis*, were maintained under the following laboratory conditions: a) 16L : 8D photoregime, b) 25–30° C and c) circa 80% relative humidity. At 4 days post-eclosion,

Fig. 1. Occasional indications of continuous junction (CJ) substructure (arrow heads) are observed in carbohydrate fed mosquitoes.

Fig. 2. Junctional staining immediately after feeding on a vertebrate host; pairs of septae and 360° turns of septae (arrow heads).

Fig. 3. Extensive fingerprints of intercellular junctional staining 16 h after feeding on a vertebrate host.


1
CJ
150 nm
2
150 nm
3
100 nm

females were allowed to feed to repletion either on baby chicks (*Gallus domesticus* var. white leghorn) or on 5 cm squares of gauze soaked in defibrinated rabbit blood or chicken serum. Immediately upon cessation of feeding and at selected intervals thereafter mosquitoes were anesthetized with chloroform, decapitated and the digestive tract dissected in 5% glutaraldehyde (McLean and Houk, 1973). The tissue was fixed for 2 h in 5% glutaraldehyde and 4 h in 1% osmium tetroxide (Palade, 1952), stained during dehydration with uranyl acetate (Milne and deZoeten, 1967) and embedded in araldite-epon (Mollenhauer, 1964). Sections were stained for 10–12 min with 1% lead citrate (Reynolds, 1963).

Results

As noted previously (Reinhardt and Hecker, 1973; Houk, 1977) the primary apical junction of the mosquito midgut epithelium is a continuous junction (Fig. 1). This junction has an intermembrane distance of 12 nm with a total membrane width of approximately 24 nm. When apparent, septa were separated by approximately 5 nm and the septal bars themselves were about 4–5 nm in diameter (Fig. 1). Little additional information was available from obliquely sectioned regions, except for an occasional indication of intermembranous substructure (Fig. 1).

After the ingestion of a bloodmeal intense staining and substantial areas of continuous junction substructure, in obliquely sectioned areas, were apparent (Figs. 2, 3, 4). The images observed are identical to those of lanthanum treated preparations (Hudspeth and Revel, 1971; Flower and Filshie, 1975; Green, 1978; Lane, 1978). The septa were about 4–5 nm in diameter (Figs. 2, 3, 4). However, the interseptal distance was quite variable. The septa appear to take a non-parallel, meandering intermembrane pathway and occasionally were seen to make an approximate 360° turn in post-blood fed material (Fig. 2). In addition, pairs of septa were often observed (Fig. 4) with occasional indications of interseptal pegs as suggested by Flower and Filshie (1975). The depth of staining was to the basal lamina in some junctions with occasional substantial staining (Fig. 5). Significant "negative staining" persisted for about 24 h but intercellular junctional deposits have been observed up to 7 days post-bloodmeal (Houk, 1977).

Similar junctional staining was observed in those mosquitoes that engorged on defibrinated blood and serum (See Materials and methods; Figs. 6, 7). The staining was less extensive and less intense in serumfed individuals

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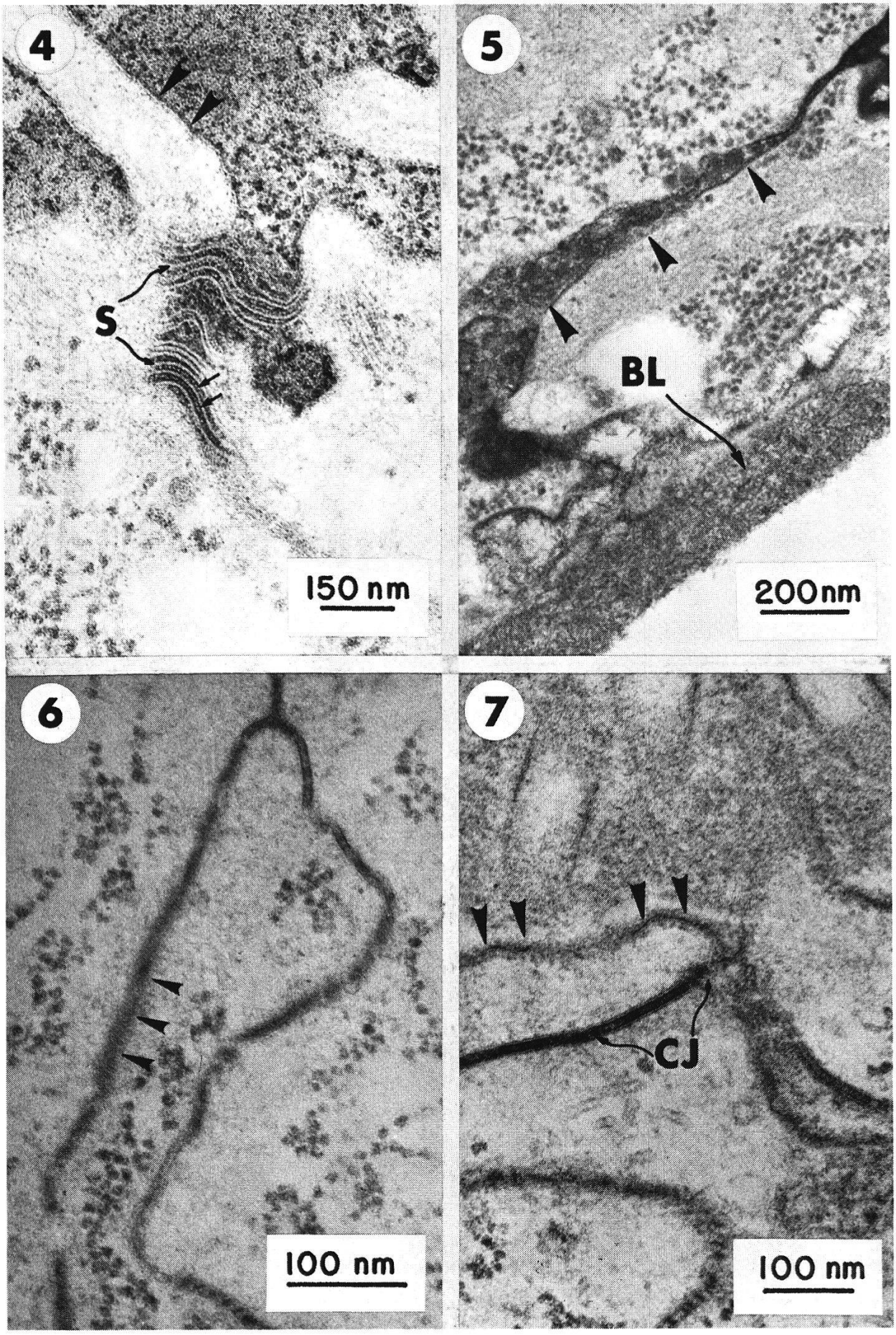
Fig. 4. Intercellular staining reveals septae (S), what may be interseptal pegs (arrows) and apparent affinity of bloodmeal component for the outer membrane leaflet (arrow heads).

Fig. 5. Extensive deposition of bloodmeal component (arrow heads) in the region of the midgut basal lamina (BL).

Fig. 6. Junctional staining of mosquito fed 100% serum demonstrates limited substructural detail (arrow heads).

Fig. 7. Defibrinated rabbit blood reveals substantial junctional staining (CJ) and affinity for the outer membrane leaflet (arrow heads).


4
S
150 nm
5
BL
200nm
6
100 nm
7
CJ
100 nm

(Fig. 6), when compared to those fed on chicks (Figs. 2, 3, 4) or defibrinated blood (Fig. 7). In the defibrinated blood fed mosquitoes the junctional staining was observed to be the consequence of a thin layer of electron opaque material immediately adjacent to the outer membrane leaflet (Fig. 7).

Unstained sections revealed no overt electron opacity, whether carbohydrate or blood fed.

Discussion

The continuous junction (*zonula continua*), since its original description (Noirot and Noirot-Timothée, 1967), has defied functional categorization. Satir and Gilula (1973) observed that this junction possessed characteristics of both tight and septate junctions. Both junctions are known to be occlusive and perhaps adhesive (Satir and Gilula, 1973; Staehelin, 1974). Thus, one would expect that if the continuous junction is truly a hybrid junction its limit of occlusion would lie somewhere between the 350–40,000 daltons of tight and septate junctions respectively (ref. cit.; Satir and Gilula, 1973). Recent studies by Green (1978) would place the continuous junction in a junctional continuum; between the invertebrate pleated sheet septate junction and the vertebrate tight junction. In addition, cellular adhesion and transport functions have been ascribed to continuous junctions (Satir, and Gilula, 1973; Lane, 1978).

In those insects examined to date, the continuous junction is the predominent midgut epithelial junction (Noirot and Noirot-Timothée, 1967; Dallai, 1970; Oschman and Wall, 1972; Reinhardt and Hecker, 1973; Flower and Filshie, 1975; Houk, 1977). Since the midgut is lacking in tight junctions and apical desmosomes (Reinhardt and Hecker, 1973; Houk, 1977), the continuous junction probably has both adhesive and occlusive properties. However, some hematophagous insects (arthropods) represent an unusual situation in that they periodically engorge on massive quantities of blood (Wigglesworth, 1943; Clements, 1967; Gooding, 1972). The midgut distension or over-distension may place excessive strain on the adhesive properties of the continuous junction. To emphasize this point, Wigglesworth (1943) reported that *Rhodnius* had often been observed to continue feeding until the midgut ruptured. A similar situation, wherein the hemocoel was filled with blood after engorgement, has been observed several times in *C. tarsalis* (Houk, unpublished observation).

The data presented herein indicate that after *C. tarsalis* mosquitoes feed to repletion on a bloodmeal the midgut intercellular junctions stain excessively, when compared to carbohydrate and serum fed individuals. This staining situation has been observed in all mosquitoes examined under varying feeding-fixation schemes. Although the extensiveness of individual junctional staining varies from nearly zero to the extreme situation seen in Fig. 5, within the same mosquito midgut. That the staining is related to midgut distension and bloodmeal composition was demonstrated by the feeding experiments comparing

100% serum and defibrinated blood. The serum fed individuals revealed reduced intercellular staining (Fig. 6). Whereas, defibrinated blood fed mosquito continuous junctions stained quite densely (Fig. 7). Additional support for the concept of leaky cellular junctions has been presented by Simionescu et al. (1978a, b). The investigators report that up to 30% of the junctions in the pericytic venules of the mouse diaphragm are open to a gap of 3–6 nm.

It appears from examination of serum and defibrinated blood fed mosquitoes that the component responsible for intercellular staining is present in whole blood (Figs. 2, 3, 4, 6, 7). The stained component revealed an intimate association with the outer membrane leaflet and most likely the glycosaminoglycans both at the luminal cellular surface and in the intercellular junctional space (Fig. 7). As conjecture, one might suspect that hemoglobin arising from spontaneous hemolysis would be the most likely candidate for the whole blood component responsible for the intercellular staining. Hemoglobin has a net positive charge at or slightly below neutrality (Dodge et al., 1963) and would be bound strongly by anionic glycoproteins in the midgut epithelium membranes. Subsequent glutaraldehyde fixation would allow detection of hemoglobin bound in this manner. In fact, hemoglobin has been implicated in a similar membrane phenomenon within red blood cells (Baker, 1967).

The major implication of this study is perhaps not in the area of continuous junction occlusion limits at the molecular level. Instead, the importance lies in the finding of bloodmeal materials within the area of the basal lamina (Fig. 5). Two studies of arbovirus acquisition by mosquitoes (Boorman, 1960; Miles et al., 1973) have indicated the presence of infectious virus in the hemocoel as rapidly as 30 min after engorgement. The speed with which the virus appeared within the hemolymph led to the suggestion that some mosquito midguts may be "leaky". The leaky midgut speculation has, we feel, been visually documented herein.

Our studies on this problem are continuing along two lines. First, we are attempting to identify the intercellular electron opaque material. Second, penetration of midgut continuous junctions by polystyrene spheres and western equine encephalomyelitis virus are being correlated to verify that "leaky guts" do indeed exist.

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