ACTIVE TRANSPORT BY THE CECROPIA MIDGUT

II. Fine Structure of the Midgut Epithelium

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ABSTRACT

A morphological basis for transcellular potassium transport in the midgut of the mature fifth instar larvae of *Hyalophora cecropia* has been established through studies with the light and electron microscopes. The single-layered epithelium consists of two distinct cell types, the columnar cell and the goblet cell. No regenerative cells are present. Both columnar and goblet cells rest on a well developed basement lamina. The basal portion of the columnar cell is incompletely divided into compartments by deep infoldings of the plasma membrane, whereas the apical end consists of numerous cytoplasmic projections, each of which is covered with a fine fuzzy or filamentous material. The cytoplasm of this cell contains large amounts of rough endoplasmic reticulum, microtubules, and mitochondria. In the basal region of the cell the mitochondria are oriented parallel to the long axes of the folded plasma-lemma, but in the intermediate and apical portions they are randomly scattered within the cytoplasmic matrix. Compared to the columnar cell, the goblet cell has relatively little endoplasmic reticulum. On the other hand, the plications of the plasma membrane of the goblet cell greatly exceed those of the columnar cell. One can distinguish at least four characteristic types of folding: (*a*) basal podocytelike extensions, (*b*) lateral evaginations, (*c*) apical microvilli, and (*d*) specialized cytoplasmic projections which line the goblet chamber. Apically, the projections are large and branch to form villus-like units, whereas in the major portion of the cavity each projection appears to contain an elongate mitochondrion. Junctional complexes of similar kind and position appear between neighboring columnar cells and between adjacent columnar and goblet cells as follows: a zonula adherens is found near the luminal surface and is followed by one or more zonulae occludentes. The morphological data obtained in this study and the physiological information on ion transport through the midgut epithelium have encouraged us to suggest that the goblet cell may be the principal unit of active potassium transport from the hemolymph to the lumen of the midgut. We have postulated that ion accumulation by mitochondria in close association with plicated plasma membranes may play a role in the active movement of potassium across the midgut.

Since the pioneering studies of Malpighi (47), and Swammerdam (72), the digestive system of insects has been a topic of interest and controversy among cytologists and physiologists. Although the alimentary canal consists of three basic regions, viz, foregut, midgut, and hindgut, attention has been centered on the midgut. The insect midgut is generally characterized by two cell types, columnar cells and regenerative cells. In lepidopterous and tricopterous insects a third type, the goblet cell, is present (67, 68). Regenerative cells allegedly give rise to columnar and goblet cells, although Shinoda (67) and Yung-Tai (83) reported that the latter cell types are found in newly hatched silkworm larvae in the absence of regenerative cells. Apparently, regenerative cells

are absent from the midgut of mature Cecropia larvae. Therefore, the epithelium is composed of columnar and goblet cells. Two fundamental questions regarding these cells remain unanswered: (a) Could one be a physiological phase of the other? and (b) Does one cell function principally in digestion and the other in absorption or does each cell type perform both functions?

In addition to its involvement in certain aspects of digestion and absorption (80), the midgut apparently engages in other activities such as the synthesis of blood proteins (40). Moreover, its intense endergonic role is reflected in the unusually high activity of respiratory enzymes. Indeed the important cytochrome b_5 (m) was discovered in the Cecropia midgut by Sanborn and Williams (65). It now appears that, at least in plant-eating insects, the midgut is also a primary site of ion regulation. This regulation includes the active transport of potassium from the hemolymph to the lumen of the gut. The chemical details of the process are summarized by Harvey and Nedergaard (26) and are to be described further in other communications of this series.

In our efforts to interpret and understand the chemical data on ion transport by the midgut, a detailed anatomical portrait of the cells comprising the epithelium is indispensable. Therefore, this communication explores the cytoarchitecture of the midgut cells of Cecropia with both the light and electron microscopes and attempts to relate their structure to certain aspects of ion transport.

MATERIALS AND METHODS

Mature fifth instar larvae of *Hyalophora cecropia* (L) reared on the leaves of wild cherry trees (*Prunus* spp) or willow trees (*Salix* spp) were anesthetized with frozen carbon dioxide, and the midgut was isolated by the techniques of Harvey and Nedergaard (26). After the removal of the peritrophic membrane with its enclosed leaves, the midgut was cut into small pieces. Some pieces were fixed in Bouin's or Helly's solutions and stained with Mallory's triple stain or with Heidenhain's iron hematoxylin; others were fixed in a 2% solution of phosphate-buffered (pH 7.4) osmium tetroxide and embedded in celloidin.

For electron microscope observations, small pieces of the midgut were fixed for 2 hr in a 2% solution of glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate buffer (64). After this initial fixation, the tissue was postfixed for 1 hr in a 0.1 M phosphate-buffered (pH 7.4) osmium tetroxide solution and subsequently rapidly dehydrated, infiltrated, and embedded in

Epon (45). One-micron sections of Epon-embedded material were stained according to the recommendations of Ito and Winchester (34). Thin sections were stained with uranyl acetate followed by lead citrate (78) and subsequently examined with both the RCA EMU-3E and Phillips 200 electron microscopes.

OBSERVATIONS

Bennett (11) pointed out that the term "membrane" has been used to denote many things. He divided biological membranes into the following four categories: (a) tissue membranes, (b) cellular membranes, (c) basement membranes (basement laminae), and (d) cytoplasmic membranes. The "membrane" described here consists of a single layer of epithelial cells.

A rigorous analysis of active ion transport across cellular membranes requires that the membrane be isolated so that the composition of the bathing solution, temperature, and pressure can be controlled while important parameters such as the electrical potential and the short-circuit currents are measured. The technique developed by Ussing and Zerahn (76) for studying the frog skin has been modified by Harvey and Nedergaard (26) for the study of the midgut. As illustrated in Fig. 2, the isolated midgut (M) is mounted in the gap of a section of glass tubing which forms the inner part of a perfusion chamber. Further details of techniques used in studying active ion transport across the midgut are described by Harvey et al. (27).

Light Microscopy

HISTOLOGY OF THE MIDGUT

The cylindrical midgut of a mature 15-g larva is about 3.5 cm long and 1 cm in diameter (M, Fig. 1). The inner wall of the midgut is corrugated. This corrugation is a manifestation of the major epithelial folding (EL, Fig. 3). The one-cell-thick epithelial layer consists of tall (65-μ) columnar cells (CC, Fig. 4) and shorter (35-μ) goblet cells (GCE, Fig. 4). Although the term goblet cell usually refers to a mucus-secreting cell with a definite cytology, the goblet cell in the midgut of Cecropia neither secretes mucus nor appears cytologically similar to vertebrate goblet cells. However, its superficial resemblance to the vertebrate goblet cell has prompted histologists to use this descriptive term which we will retain. The epithelium of the midgut rests on a basement lamina (BL, Fig. 4) with which are associated

tracheae, tracheoles, an inner layer of circular muscles (RM, Fig. 3) and an outer layer of longitudinal muscles (LM, Fig. 3) The longitudinal muscle layer is clearly discontinuous, and there also appear to be spaces between the fiber bundles in the circular layer. Therefore, the epithelial layer and basement lamina appear to be the only continuous diffusion barriers between the hemolymph and the lumen of the gut. In order for an ion or molecule in the hemolymph to reach the midgut lumen, it must at least traverse the basement lamina. It then may take either an extracellular pathway or pass across the basal plasma membrane, cytoplasm, and apical plasma membrane of the epithelial cell layer. Finally, it must pass through perforations in the peritrophic "membrane" (48, 84). Since the peritrophic "membrane" is removed, this structure need not be considered in discussions of the transport pathway in vitro.

Each columnar cell (CC, Fig. 4) contains a large, centrally located, sometimes oval, nucleus (NC, Fig. 4). The apical cell border is clearly striated (BB, Fig. 4) and is refractile to staining with Heidenhain's iron hemtoxylin. The apical ends of the columnar cells extend beyond the border of the goblet cells, thereby giving the epithelium a scalloped appearance. Pear-shaped buds of varied sizes are frequently seen on the luminal surfaces of the columnar cells, and in many sections spheres with a similar internal density are seen within the lumen (SB, Fig. 4; see also PB in Fig. 9). These buds are associated with healthy cells and do not seem to be signs of degenerative changes such as those described by Yung-Tai (83). Rather, they appear to be manifestations of an apocrine secretory process. The basal half of the columnar cell is densely stained with Heidenhain's iron hematoxylin and exhibits the longitudinal fibrillar appearance (BS, Fig. 4) noted by earlier cytologists.

The goblet cell is always surrounded by columnar cells, and its nucleus is consistently basally located (NG, Fig. 4). The infranuclear cytoplasmic area is devoid of the longitudinally oriented fibrillar structures that are so characteristic of this area in the columnar cell. A long oval cavity (GC, Fig. 4), which communicates freely with the lumen of the gut, dominates the architecture of the goblet cell. At light microscope levels, the border of the goblet cavity resembles the striated border of the columnar cell (GMC, Fig. 4) but is

stainable with Heidenhain's iron hematoxylin. Apparently, the lining of the goblet cavity is similar to the apical surface of the columnar cell. We shall see presently that the cavity's border is lined with numerous, rather highly specialized cytoplasmic projections.

Regenerative cells were not seen in any of the many preparations examined. However, we have observed tracheolar cells that are so closely associated with the epithelium that it is possible, at light optical levels, to interpret such cells as being an integral part of the epithelium (for example, see TC, Fig. 18). One wonders if it is not this type of cell that investigators have called regenerative cells (cf. 13).

Electron Microscopy

COLUMNAR CELLS

Fig. 5 is a low magnification image of the basal portion of a columnar cell in which the plasma membrane is thrown into an extensive array of deep parallel folds (BIF) that are perpendicular to the basement lamina. The infoldings of the plasma membrane incompletely divide the basal cytoplasm into compartments of varying sizes. In addition to containing ribosomes, cisternae of the rough endoplasmic reticulum, and microtubules, the larger compartments are characterized by long, usually slender but sometimes thick, mitochrondria with distinct cristae (MC). The mitochondria are parallel to the folds of the membrane. The smaller compartments usually contain all of the organelles previously mentioned except mitochondria.

Profiles obtained from the basal portion of the columnar cell, particularly where the plane of sectioning is somewhat oblique, give the impression that there is at least as much extracellular space as there is cytoplasm (IC, Fig. 6). Effectively, the infoldings extend about two-thirds of the way to the apical border of the cell (IC, Fig. 7).

The conspicuous brush border seen with the light microscope is resolved into a regular array of long, thin microvilli (MVC, Fig. 8; see inset Fig. 9). Scattered within the apical cytoplasm and projecting into each microvillus are fine (60- to 70-A) filaments (FMV, Fig. 8). The surface of each microvillus is covered with a fine filamentous material (MVC, inset Fig. 9). This material may be an extraneous covering; more likely, it is a "fuzzy

coat" similar to that which has been reported by other investigators on the microvilli of certain epithelial cells (33). Occasionally, evidence of micropinocytotic activity is seen in the crypts between microvilli.

Microtubules, approximately 200 to 240 A in diameter, are found throughout the cytoplasmic matrix (MT, Figs. 8 and 10). Whereas some microtubules appear randomly dispersed within the cytoplasm, the majority are parallel to the long axis of the cell, and are most numerous in the intermediate and apical regions. The microtubules were never seen projecting into the microvilli.

An abundance of both rough endoplasmic reticulum and free ribosomes is found within the cytoplasmic matrix of the columnar cell (ER, Figs. 7 and 10). Many of the ribosomes are aggregated into polysomes (PR, Fig. 8), suggesting that the cell is involved in protein synthesis (60). Also scattered within the cytoplasm are some spherical homogeneous (SP, Fig. 6) and granular bodies (GB, Fig. 17) of unknown function. Infrequently, one sees in the cytoplasm of both the columnar and goblet cells dense granular bodies such as those labeled UB in Figs. 7 and 18. The identity and function of these bodies are unknown although they may be viruses. The relatively small Golgi complexes are usually found in a supranuclear region.

The perforated nuclear envelope encompasses a granular nucleoplasm in which are suspended many masses of chromatin material (CH, Fig. 7).

GOBLET CELLS

The phenomenon of membrane-folding reaches a maximum in the goblet cell. At least four areas of extensive plications may be recognized: (a) basal, (b) lateral, (c) goblet chamber, and (d) apical. It will be helpful to describe in detail these four areas.

(a) At the base of the cell the plasma membrane is evaginated to form podocytelike extensions which appear to support the cell above the basement lamina (BOF, Figs. 11 and 18).

(b) More prominent evaginations of the plasma membrane are found along the lateral boundary of the cell and extend from the basal surface to the level of a zonula occludens (see below). These slender evaginations create a laminar pattern between the basal two-thirds of the goblet cell and adjacent columnar cells (LOF, Figs. 5, 7, and 12) and appear to communicate with each other by small cytoplasmic bridges. The extracellular space in the region of the lateral evaginations is very close to the goblet chamber and hence the midgut lumen via this chamber.

(c) Lining the entire goblet chamber (GC, Figs. 11, 12, 14, 16, and 17), but best developed on its basal (MV_1, Figs. 11 and 18) and lateral aspects

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FIGURE 1   A photograph of a dissected mature fifth instar Cecropia larva showing the large midgut (M) $\times$ 3.

FIGURE 2   A diagrammatic representation of how the midgut is mounted when measurements are made of potential differences across an isolated preparation. Midgut (M); lumen side (sparse stippling); blood side (close stippling); inlets for aerating and stirring gas (A and A'); calomel electrodes (C and C'); agar bridges (E and E'); potentiometer (mv); dry-cell battery (B); voltage divider (V); microammeter (μA); silver-silver chloride electrodes (S and S'). (After Harvey and Nedergaard, see reference 26. Reprinted, by permission, from *Proc. Nat. Acad. Sc.*, 1964, 51, 758.)

FIGURE 3   A section of a portion of the midgut fixed in a 2% solution of buffered osmium tetroxide and embedded in celloidin, depicting major folding of the epithelium (EL), an outer layer of longitudinal muscles (LM) and an inner layer of circular muscles (RM). $\times$ 100.

FIGURE 4   A section of the midgut illustrating columnar (CC) and goblet (GCE) cells resting on a basement lamina (BL). Nucleus of columnar cell (NC); brush border of columnar cell (BB); basal striations of columnar cell (BS); nucleus of goblet cell (NG); cavity of goblet cell (GC) limited by a relatively dense staining region (GMC); dense spherical bodies within the lumen of the midgut (SB). Heidenhain's iron hematoxylin-stained. $\times$ 700.
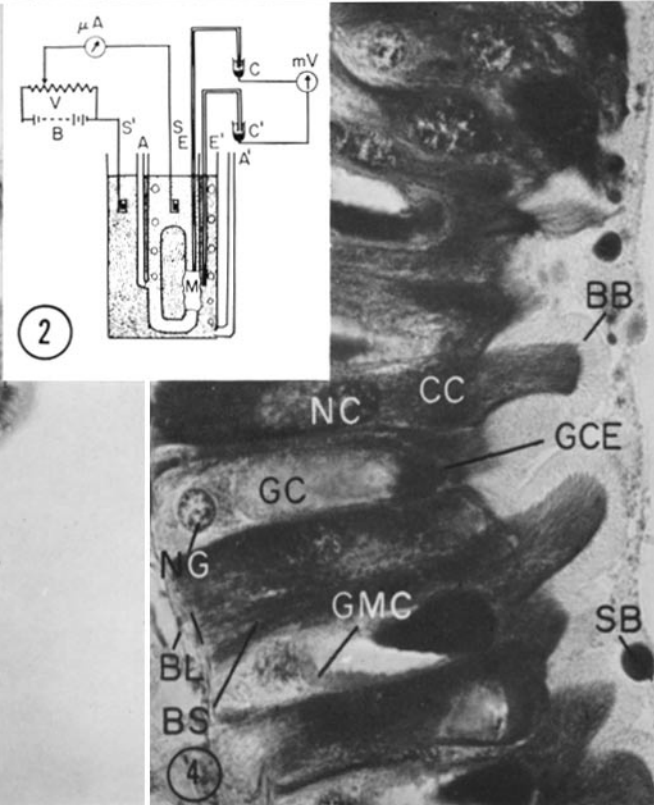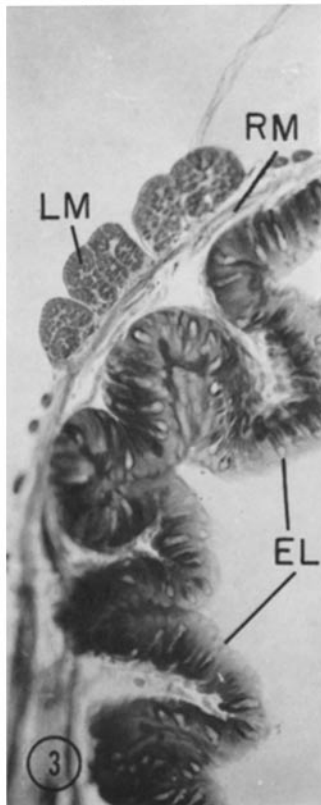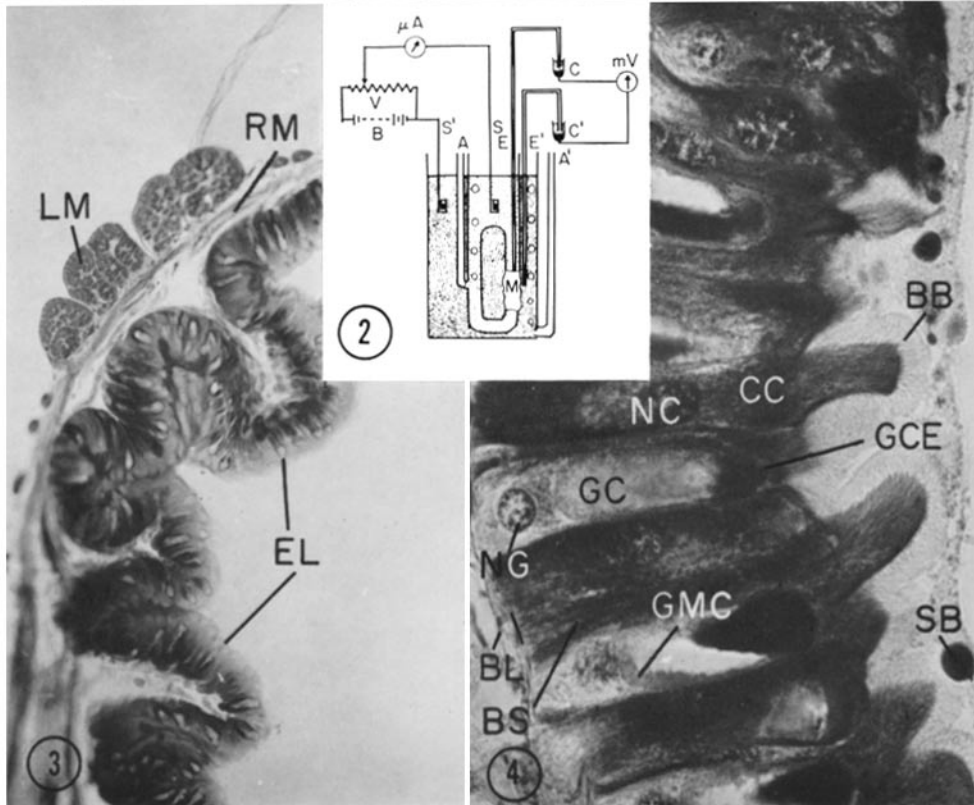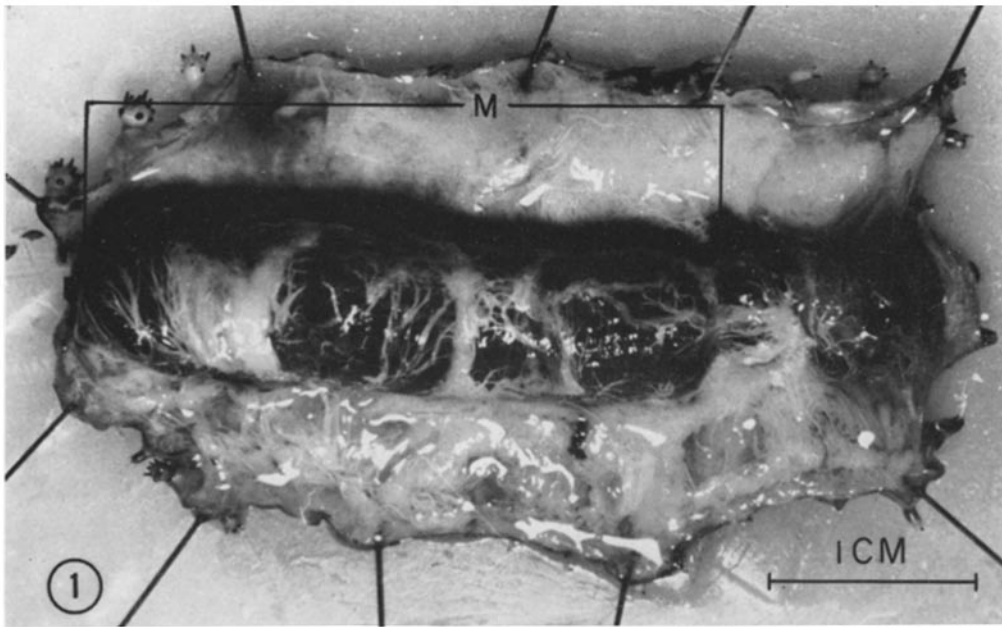
FIGURE 5 A section through the basal region of a columnar cell resting on a well defined basement lamina (*BL*). This profile also depicts the nucleus (*NC*), a mitochondrion (*MC*) and basal infoldings (*BIF*). Note the lateral outfoldings of the plasma membrane of the adjacent goblet cell (*LOF*) and the oblique section of a muscle fiber (*MS*). × 10,000.
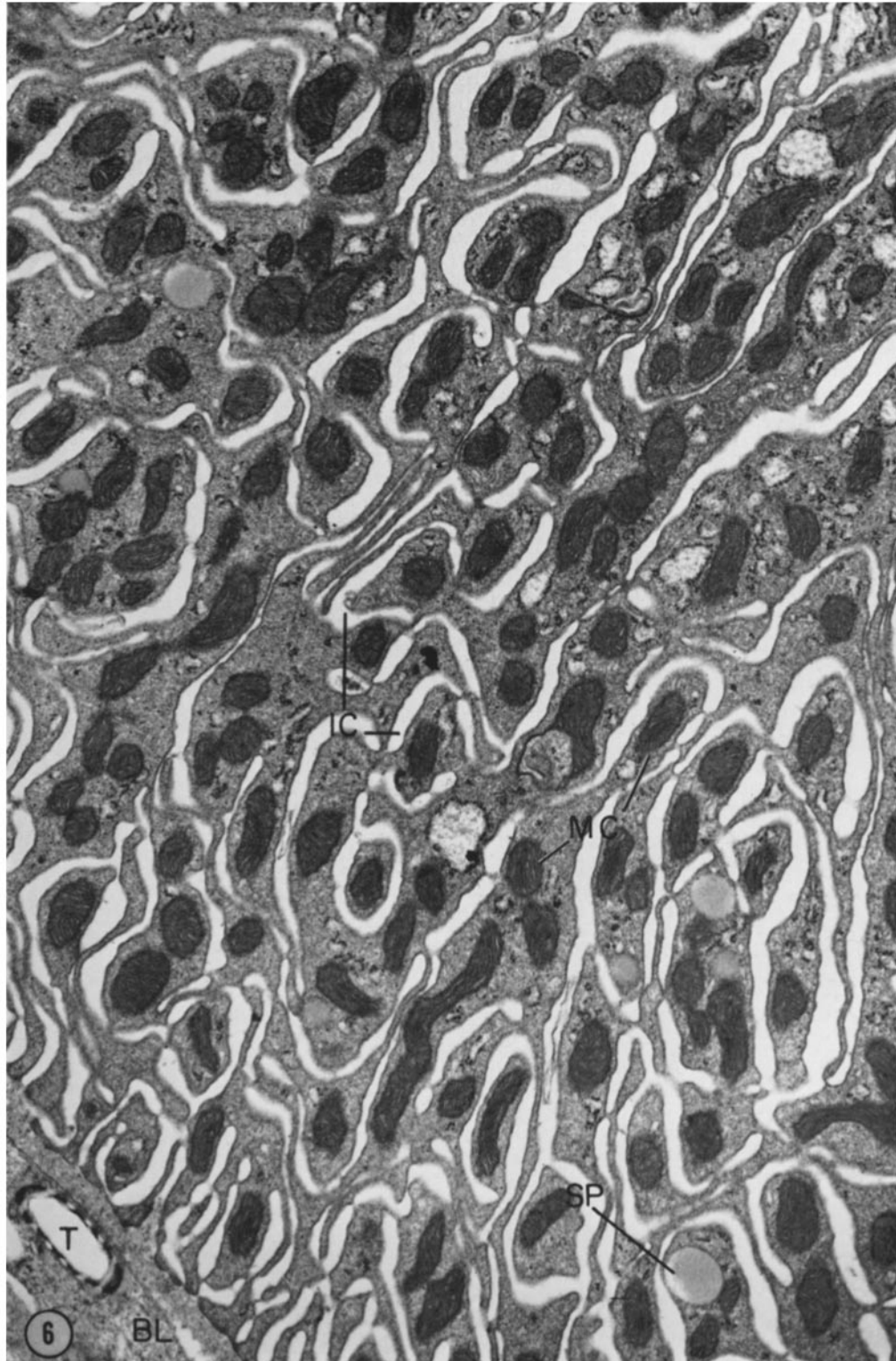
FIGURE 6   An oblique section through the basal region of a columnar cell showing the extent of the intercellular space (*IC*), mitochondria (*MC*), homogeneous spherical bodies (*SP*), basement lamina (*BL*), and tracheole (*T*). × 15,000.
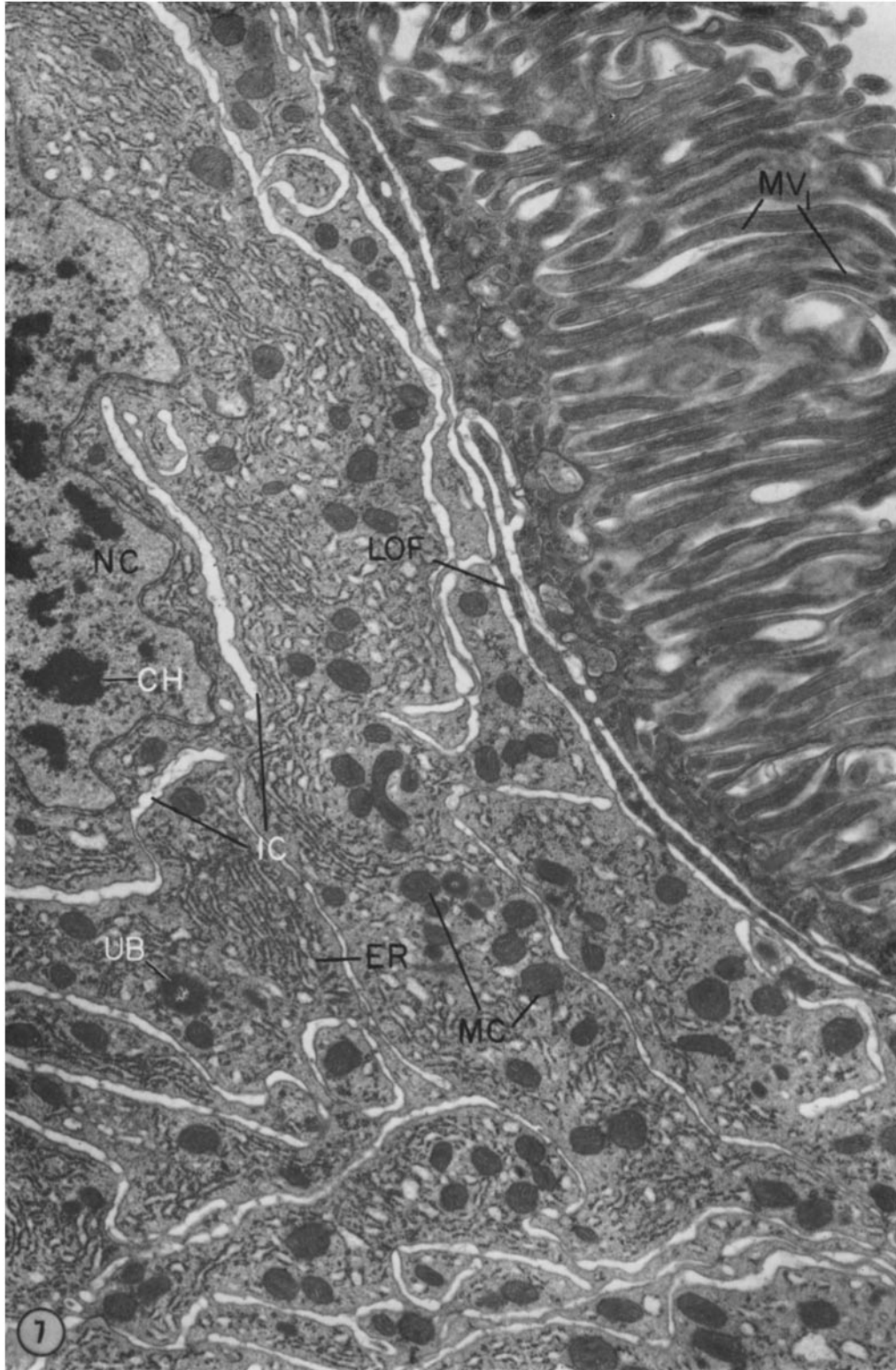
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FIGURE 7   An oblique section of a columnar cell at the level of the nucleus (*NC*), showing the intercellular spaces (*IC*), mitochondria (*MC*), endoplasmic reticulum (*ER*), and an unidentified granular body (*UB*). Note the lateral outfoldings of the adjacent goblet cell (*LOF*) and the mitochondria-filled projections (*MV₁*) that line the lateral aspects of the goblet cavity. × 12,000.
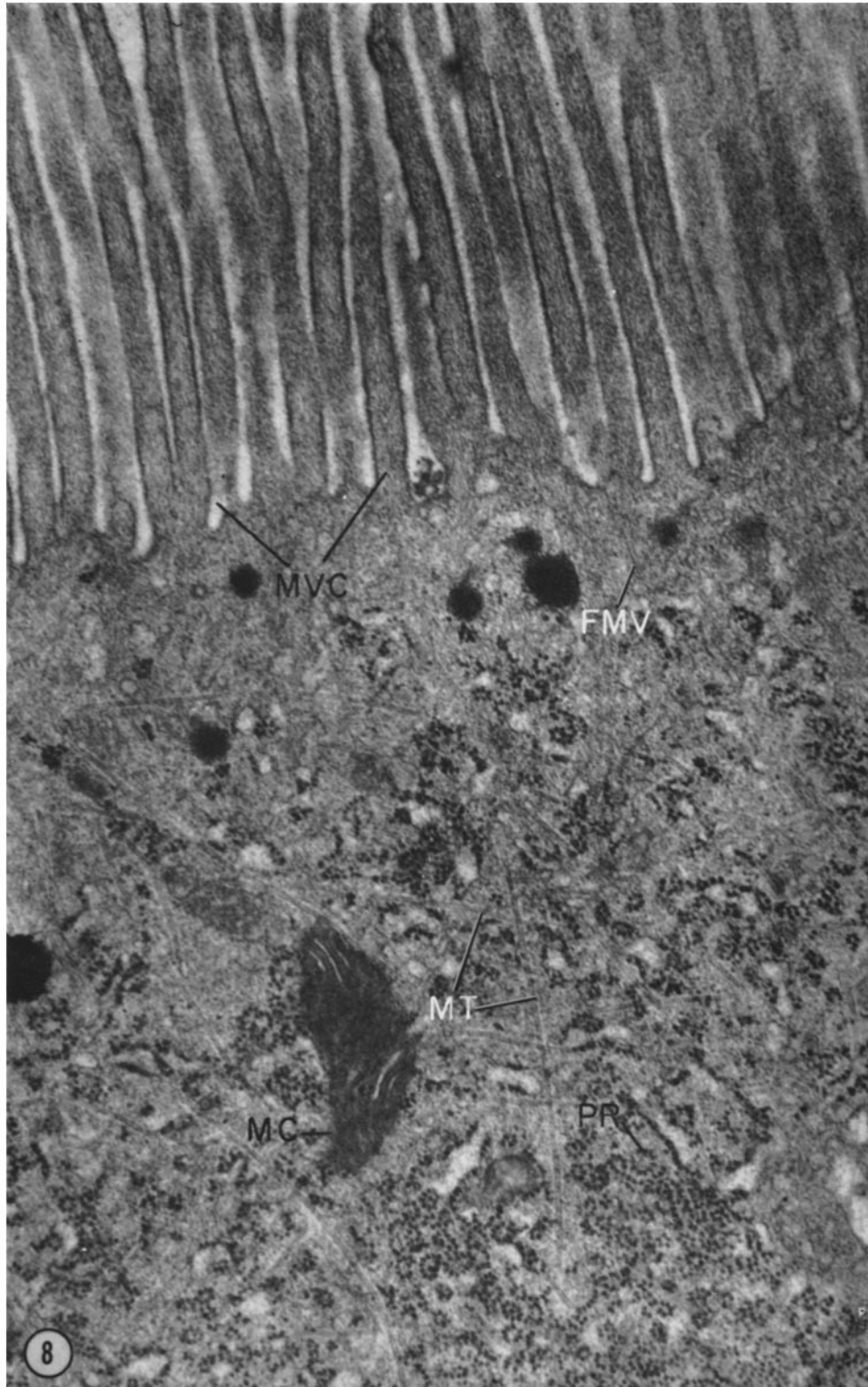
FIGURE 8 A small portion of the apical region of a columnar cell showing microvilli (*MVC*), fine filaments within the cytoplasm many of which project into the microvilli (*FMV*), microtubules (*MT*), a mitochondrion (*MC*), and polyribosomes (*PR*). × 30,000.
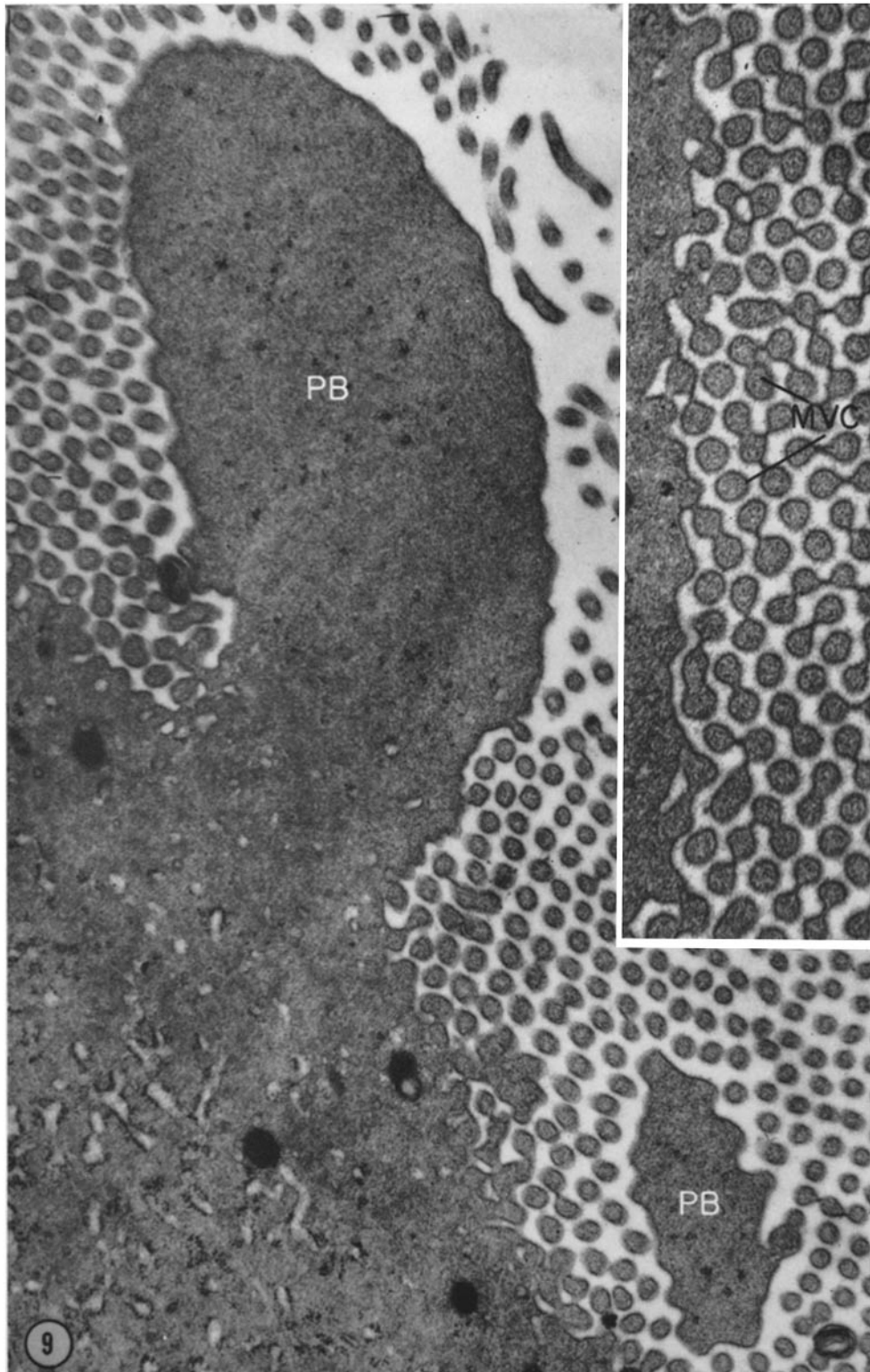
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FIGURE 9 A portion of the apical region of a columnar cell showing blebbing (*PB*). The inset illustrates cross-sections of microvilli (*MVC*) whose surfaces are adorned with a fine fuzzy material. Fig. 9, × 20,000; inset, × 30,000.
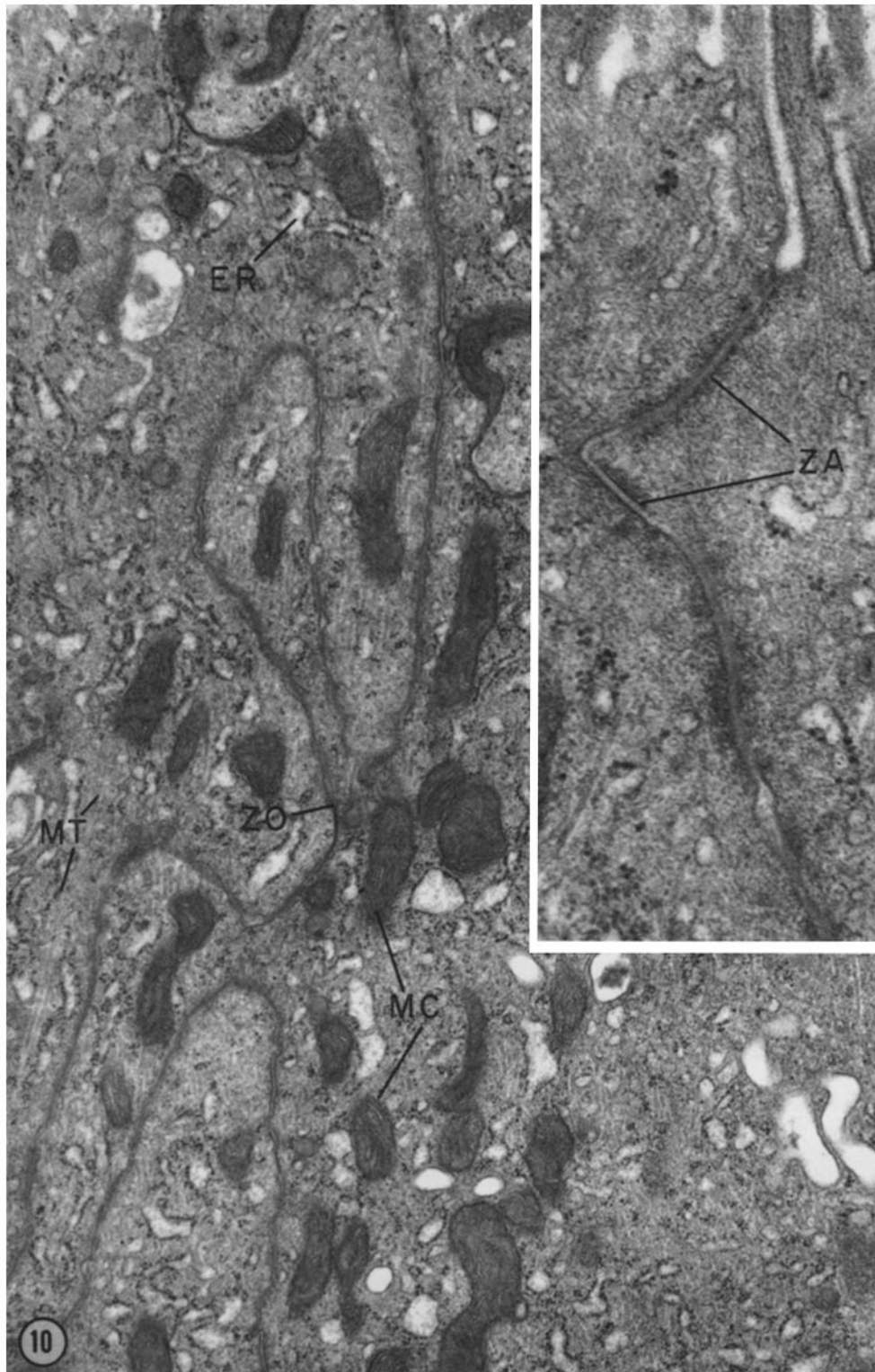
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FIGURE 10   A section through the apical region of two adjacent columnar cells depicting junctional complexes in the form of a zonula adhaerens (*ZA*, inset), and a zonula occludens (*ZO*). The cytoplasmic matrix is filled with mitochondria (*MC*), endoplasmic reticulum (*ER*), and microtubules (*MT*). Fig. 10, × 20,000; inset, × 40,000.
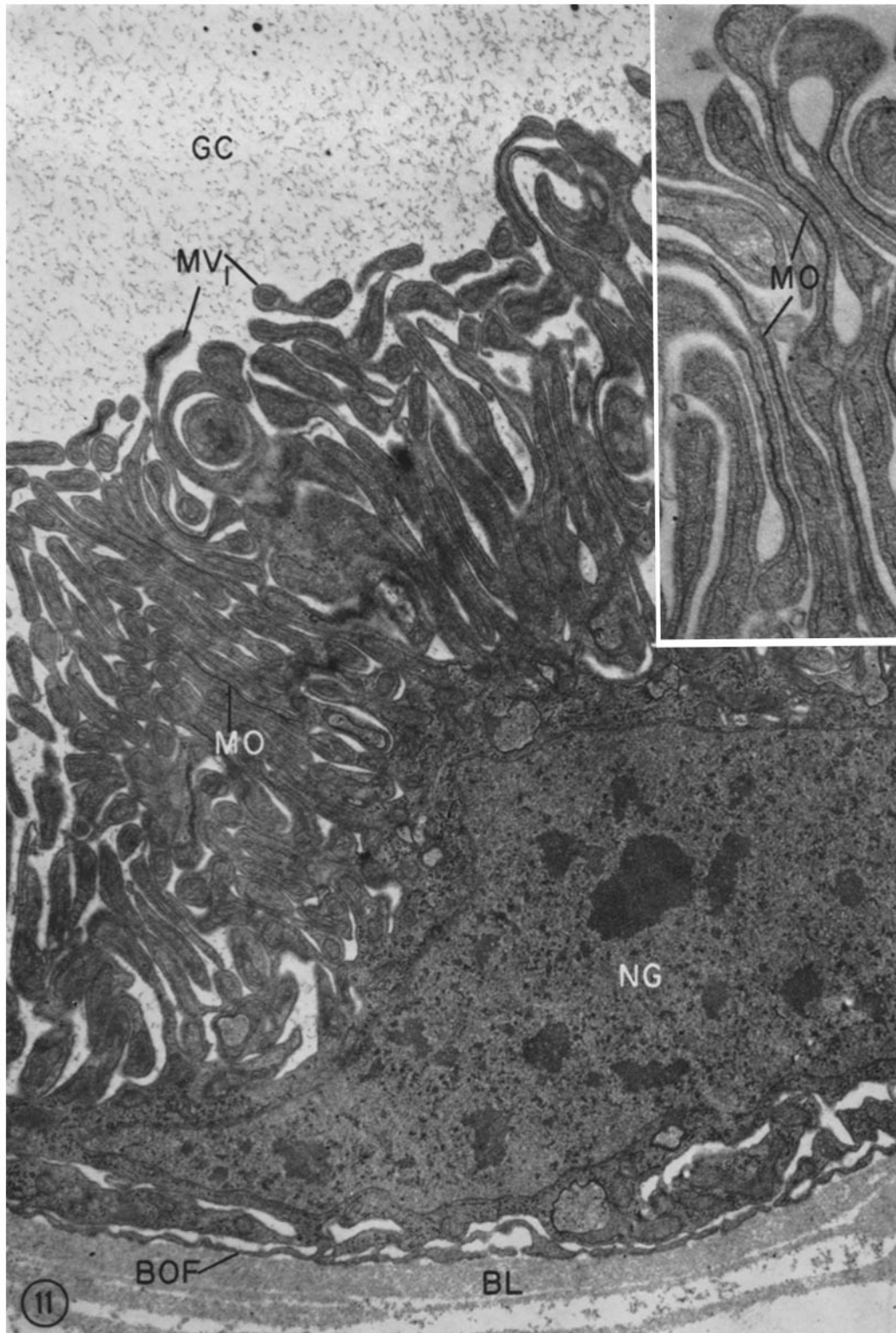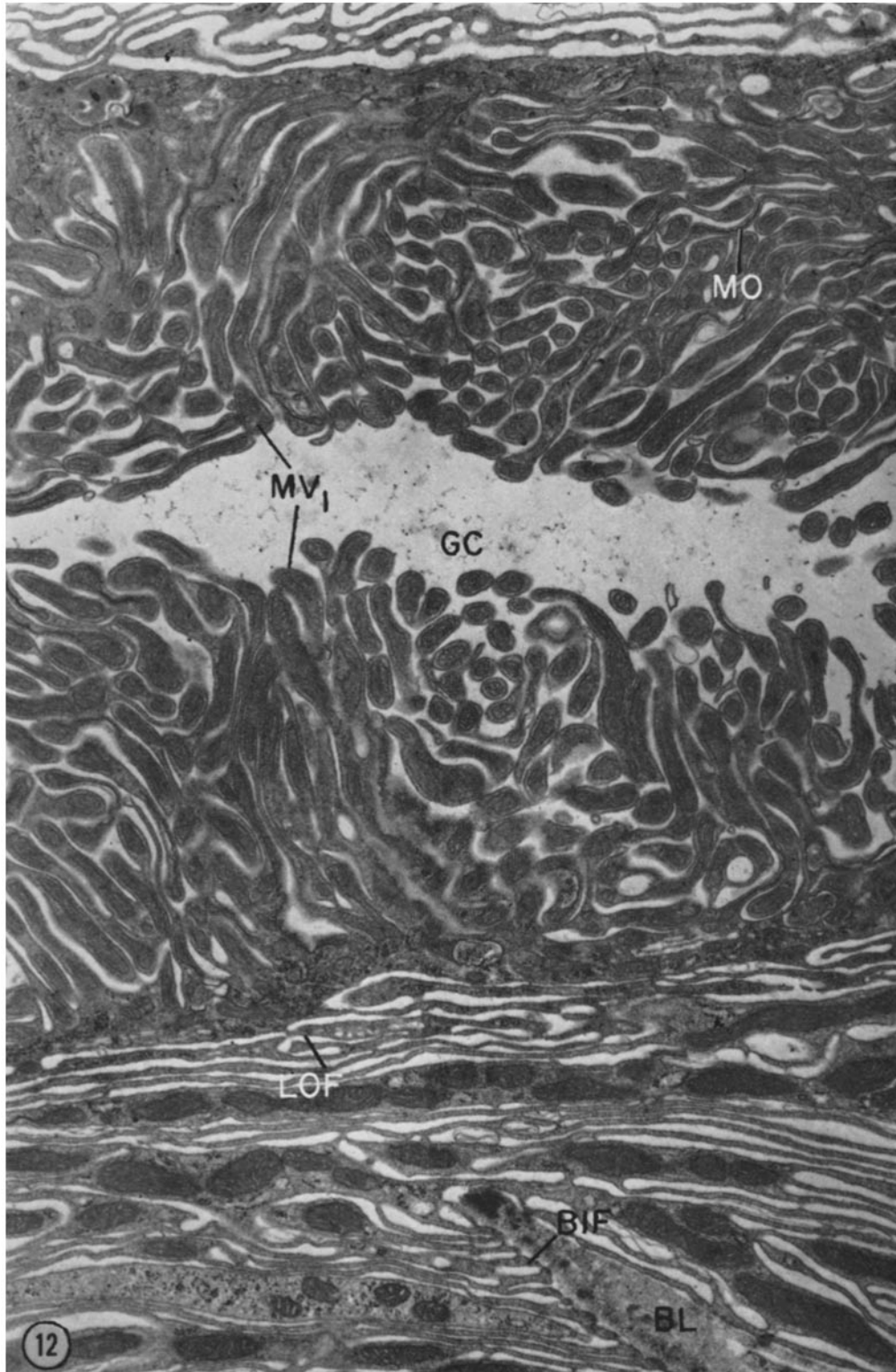
FIGURE 11 A section through the basal portion of a goblet cell showing the cavity (*GC*) containing a flocculent material, cytoplasmic projections (*MV*₁) each of which contains a mitochondrion, nucleus (*NG*), basal outfoldings (*BOF*), and basement lamina (*BL*). Note the fusion of the inner membranes of the mitochondria (*MO*; also see inset). Fig. 11, × 15,000; inset × 30,000.

FIGURE 12 A section through a portion of a goblet cell and an adjacent columnar cell with its basal infoldings (*BIF*) resting on a basement lamina (*BL*). The goblet cavity (*GC*) is lined with a host of mitochondria-filled cytoplasmic projections (*MV₁*). Many of the mitochondria show fusion of their inner limiting membranes (*MO*). Note the outfolding of the lateral plasma membrane (*LOF*) of the goblet cell. × 18,000.
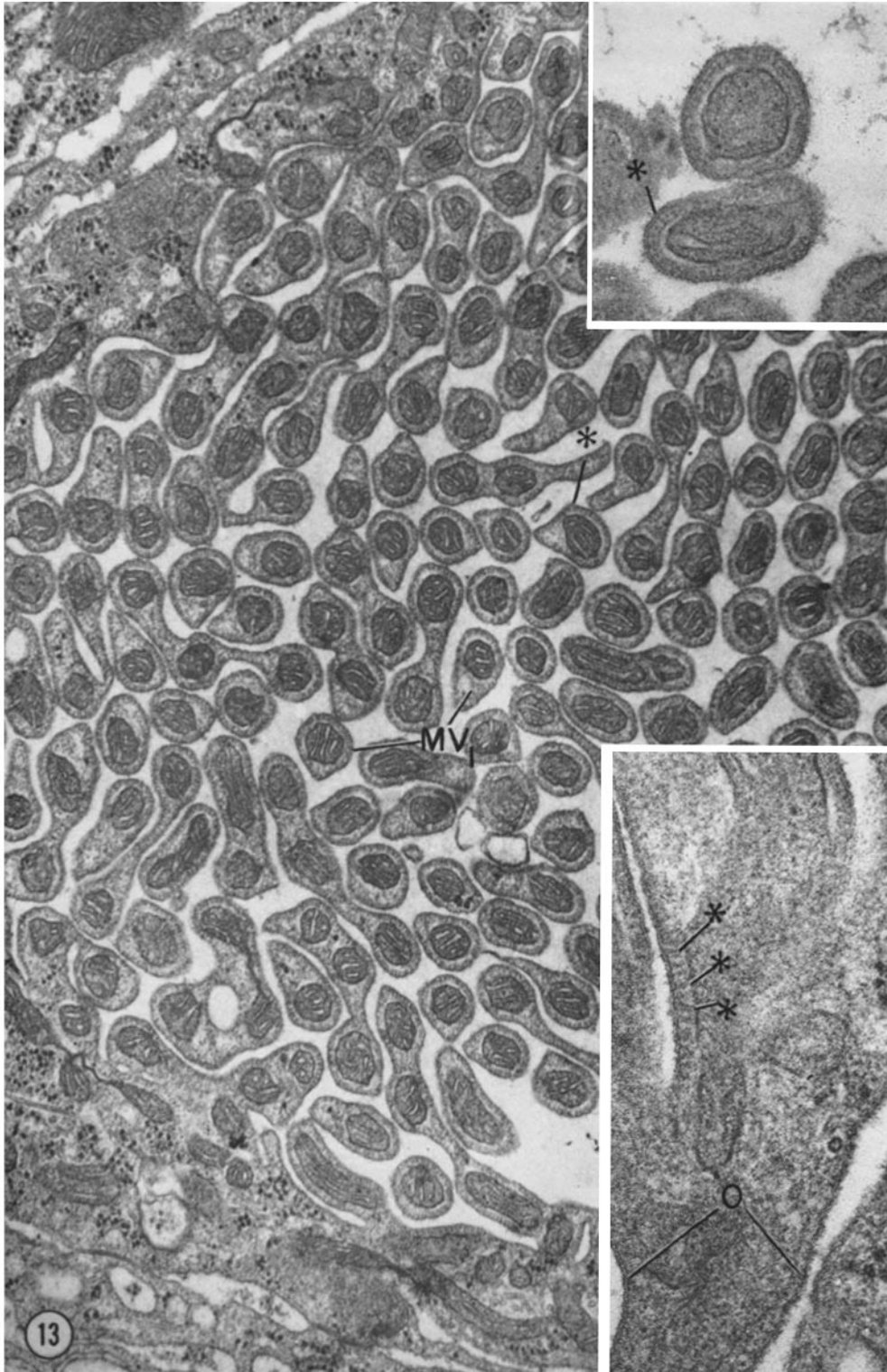
FIGURE 13   A section through cytoplasmic projections (MV_1) that line the cavity of a goblet cell. Note the spikelike units on the inner portion of the plasma membrane that form these projections (*, Fig. 13 and insets). The inset at the lower right of the figure also shows portions of the plasma membrane whose inner unit membrane is devoid of spikes (O). Fig. 13, × 30,000; upper right inset, × 60,000; lower right inset, × 70,000.

FIGURE 14 A cross-section through the upper portion of a goblet cell depicting its cavity (*GC*) and profiles of nonmitochondria-filled microvilli (*MV*₂). This goblet cell is encompassed by four columnar cells (*CC*). Note the plasma membrane (*CM*) of a columnar cell and the microtubules (*MT*) within the matrix of a columnar cell. × 15,000.

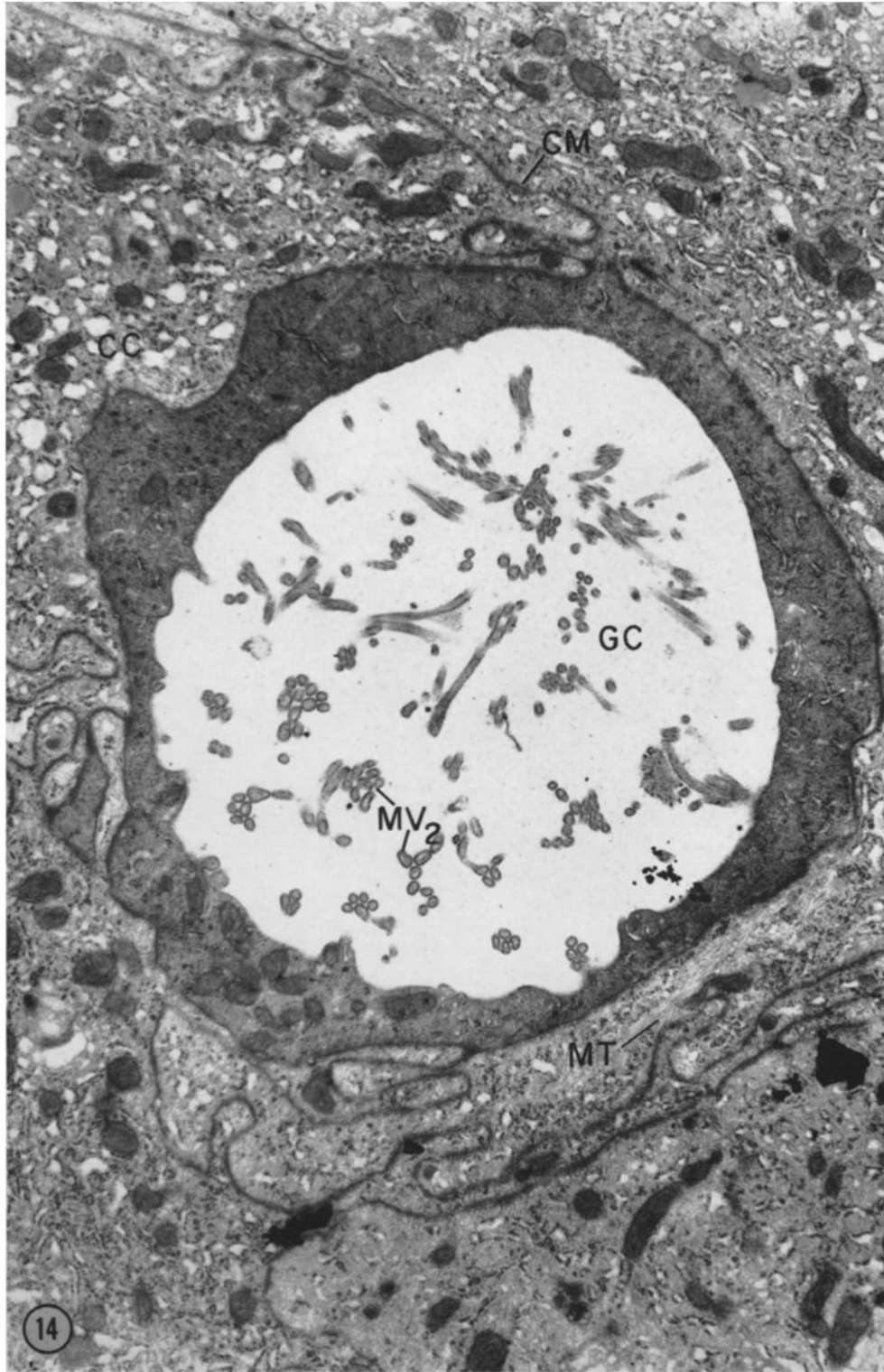(MV_1, Figs. 7, 12, and 17), are numerous cytoplasmic projections. Each projection contains a single mitochondrion. In many profiles, the mitochondria-containing projections extend into and virtually fill the chamber (MV_1, Fig. 12). Occasionally a mitochondrion forks, sending branches into two adjacent projections. Some of the mitochondria are dumb-bell shaped. The inner mitochondrial membranes are often so closely apposed that it is difficult to resolve them as separate units (MO, Fig. 11). Indeed, they appear to fuse (MO, inset Fig. 11) in much the same way that the outer leaflets of adjacent unit plasma membranes fuse when a zonula occludens is formed. Such profiles might represent stages in the duplication of mitochondria or they might equally well represent a morphological change related to a presently unknown physiological condition. Occasionally, one finds similar mitochondrial configurations in columnar cells. These mitochondria are reminiscent of the "umbo-mitochondria" in the egg of the fern, Pteridium aquilinum, as described by Bell and Mühlethaler (9). These authors interpreted such configurations as a stage in the development of this organelle.

Toward the apical end of the goblet chamber the cytoplasmic projections lining the cavity become smaller, less numerous, and contain no mitochondria (MV_2, Figs. 14, 16, and 17). The core of these projections, like the microvilli of the columnar cells, are filled with 60- to 70-A filaments.

The inner leaflet of the unit membrane of the cytoplasmic projections possesses a coat of fine spikelike units oriented perpendicular to the membrane (*, Fig. 13). Each spike is approximately 50 to 60 A long and each is separated from adjacent ones by a space of about 30 A. In some images, the spikes appear to support a globular structure 80 A in diameter (*, Fig. 13, insets). The organization of the entire unit reminds one of the construction of the "elementary particles"

found on the cristae of mitochondria (24, 51). Of particular interest is the fact that this membrane specialization is only found here and is not a common feature of any other portion of the cell's membrane (O, Fig. 13, lower inset). Moreover, we have not observed this structural feature on any portion of the plasma membrane of the columnar cell. That it is a modification of the plasma membrane which may reflect altered permeability properties is made feasible both by the electrical measurements of Loewenstein and Kanno (43) and by the chemical studies of Ashworth and Green (3) (see Discussion).

(d) In the apical region of the goblet cell the cytoplasm is drawn out into four or five large projections (PJ, Fig. 15), each of which is oriented perpendicular to the long axis of the cell. As seen in Fig. 15, these large cytoplasmic regions are further subdivided into many villuslike units whose inner cores contain a filamentous component (VU, Figs. 15 and 16). In oblique sections the individual projections are organized so that they form a lacelike pattern (VU, Fig. 16). As the villuslike projections converge toward the middle of the goblet chamber they form a small canal of variable width (CA, Fig. 15). It is through this canal that the goblet cell chamber becomes confluent with the lumen of the gut. Because the canal is rather wide in some profiles, and more or less tightly closed in others, the structural evidence suggests that it may function as a valve to control the movement of fluids between the goblet chamber and the midgut lumen.

The distribution and orientation of mitochondria within the goblet cells deserves further attention. The few mitochondria present in the cytoplasmic matrix of the main cell body are randomly oriented, whereas the mitochondria-cytoplasmic projection-associations are oriented about the circumference of the goblet cavity.

Scattered within the cytoplasm of the goblet

FIGURE 15   The inset is a photomicrograph of a Heidenhain's iron hematoxylin-stained preparation of a goblet cell, depicting its apical end (*) and cavity (GC). The cavity is bounded by a rather densely staining region (see line). To the right and left of this cell are columnar cells. The electron micrograph is a slightly oblique section made through the region marked by the asterisk in the inset. The large protoplasmic projection (PJ) is further divided into numerous villuslike units (VU). These projections all converge at the midline to form a small channel (CA). Note the microtubules (MT) within the cytoplasmic matrix of the goblet cell. The cytoplasm of a columnar cell is labeled CC. Fig. 15, × 16,000; inset, × 300.
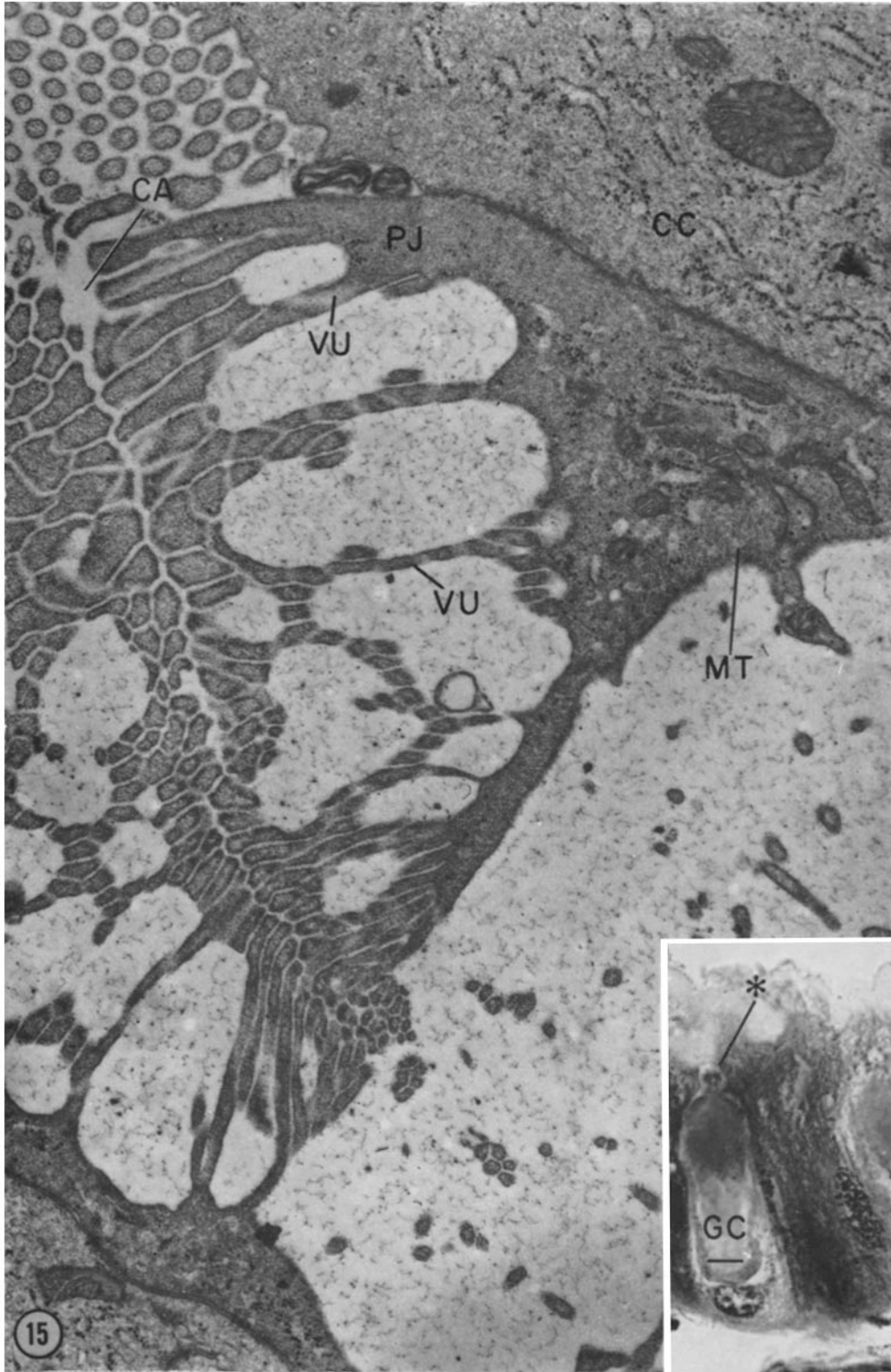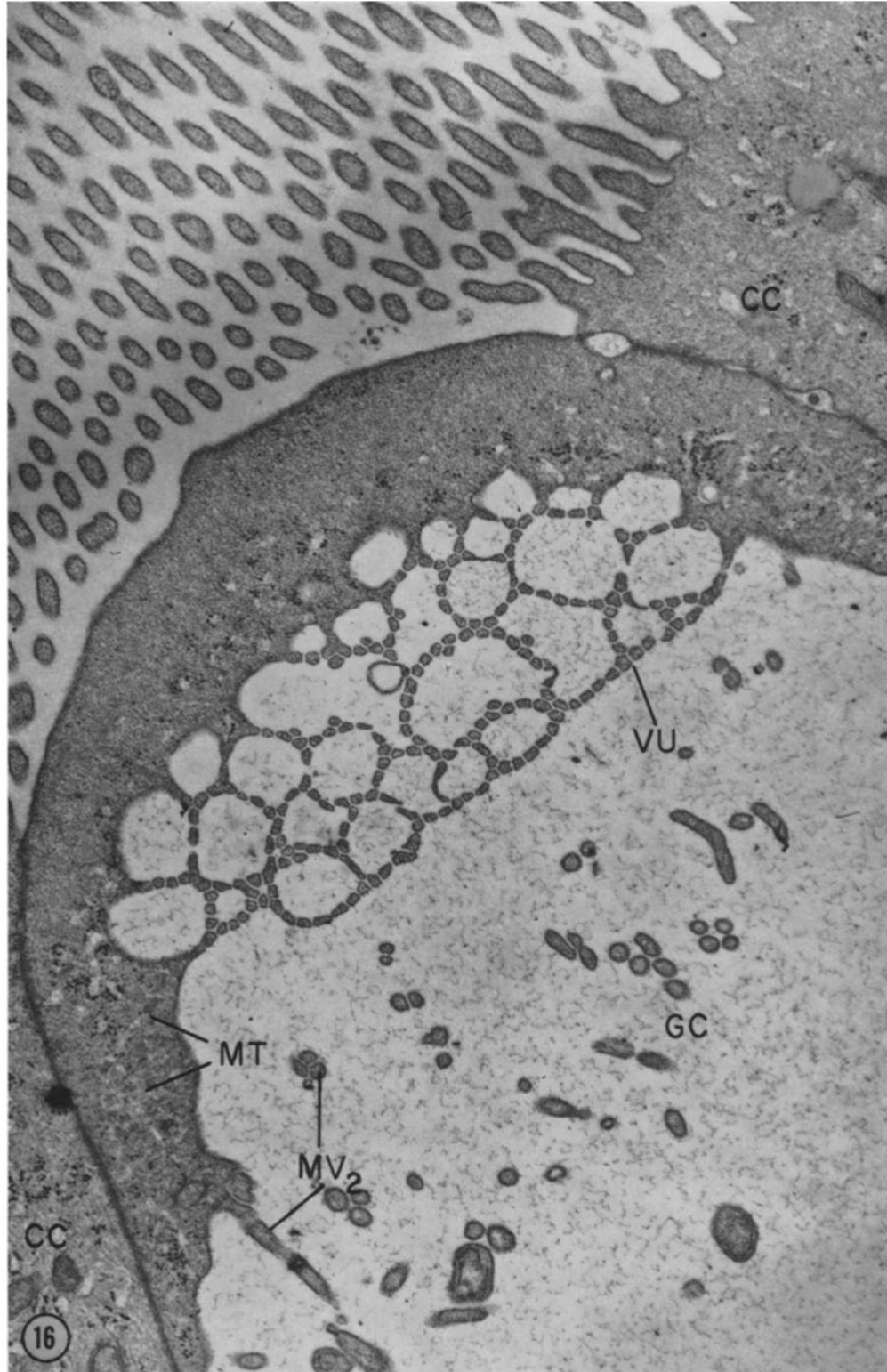
FIGURE 16   An oblique section through the apical portion of a goblet cell and two adjacent columnar cells (*CC*). Note the pattern made by the villuslike projections (*VU*) found at the apical tip of this cell, microtubules (*MT*), profiles of microvilli (*MV₂*), and cavity of goblet cell (*GC*). × 20,000.

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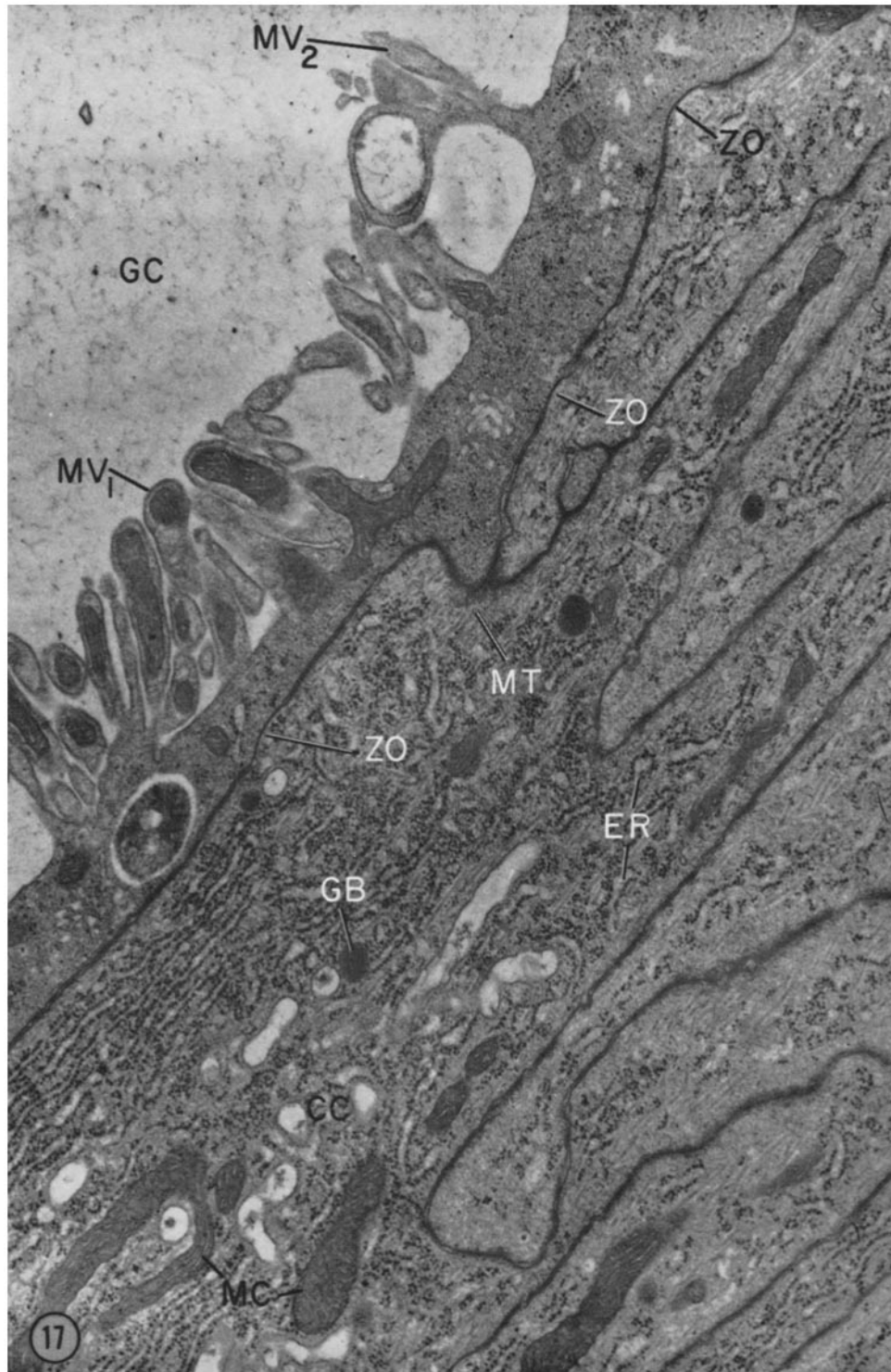
FIGURE 17   A section through a goblet cell and an adjacent columnar cell (*CC*). Note cytoplasmic projections (*MV₁*) and microvilli (*MV₂*) extending into the cavity (*GC*) of the goblet cell, mitochondria (*MC*), endoplasmic reticulum (*ER*), and microtubules (*MT*) within the cytoplasmic matrix of the columnar cell. Three zonulae occludentes are labeled *ZO*. × 18,000.

cell is a host of ribosomal units. However, the quantity of endoplasmic reticulum is scant in comparison to its abundance in the columnar cell. Microtubules are present throughout the cytoplasm, but are most numerous in the lateral and apical cytoplasm where they are randomly oriented (MT, Figs. 15 and 16). Occasionally, they are found within those cytoplasmic projections which line the apical lateral portion of the goblet chamber.

JUNCTIONAL COMPLEXES OF THE MIDGUT EPITHELIUM

The same kind and sequence of junctional complexes are found both between adjacent columnar cells and between columnar and goblet cells. Fig. 10, a longitudinal section of the upper portion of two adjacent columnar cells, depicts the types of membrane specialization present. Beginning apically near the lumen of the midgut and proceeding basally, the first type of junctional complex encountered is the zonula adhaerens (ZA, inset Fig. 10). The junction is rather large and is characterized by the presence of an intercellular gap and a conspicuous dense cytoplasmic area immediately adjacent to the plasma membrane. Proceeding basally, the next type of junction encountered is the zonula occludens (ZO, Fig. 10). This junction is recognized by the fusion of the outer lamellae of the unit membrane and the obliteration of the intercellular space. Below the first zonula occludens one may find other zonulae occludentes (ZO, Fig. 17). We have observed as many as five consecutive junctions of this type, particularly between adjacent goblet and columnar cells. As indicated above, evaginations from the lateral aspect of the goblet cell extend along the basal two-thirds of the lateral boundaries between goblet and columnar cells. Junctional complexes between these evaginations have never been observed.

BASEMENT LAMINA AND MUSCLE FIBERS

A thick basement lamina subtends the cells of the midgut epithelium and appears to be composed of two components (BL, Figs. 5, 11, 12, and 18). The component most closely associated with the basal aspect of the cell is filamentous; the outer one is finely granular. The constituents of the basement lamina do not penetrate between either the infoldings of the columnar cells or the basal podocytelike extensions of the goblet cells.

The longitudinal and circular muscle fibers (MS, Fig. 5) contain the usual cytoplasmic constituents. A detailed description of them need not detain us here.

Our interpretation of the fine structure of the midgut of Cecropia is summarized in the schematic representation featured as Fig. 19.

DISCUSSION

This study has shown that the midgut and its epithelium is characterized by extensive plications. These are: (a) the midgut wall including the muscle and epithelium, (b) apical microvilli and basal infoldings of the columnar cells, (c) the deep chamber of the goblet cell, and (d) the modified (apical) mitochondria-filled cytoplasmic projections, the basal infoldings and the lateral evaginations of the goblet cell. Clearly, each order of folding represents a magnification of surface area available for the exchange of ions and molecules. This magnification of surface area is further increased by intracellular foldings such as mitochondrial cristae. In discussing the folding of the epithelium lining the gut of certain mammals, Fawcett (22) stated that, "The strategem of increasing the efficiency of organs by amplifying the areas of physiologically significant interfaces, appears to be one of nature's commonest morphological devices for 'miniaturization' of the metabolic machinery." The relationship of the greatly increased plasma membrane to its associated organelles may explain one of the most striking activities of the Cecropia midgut—its ability to transport potassium actively in massive amounts.

LOCALIZATION OF ACTIVE TRANSPORT SITE

The task of locating the cellular layer in which an active transport process occurs is a formidable one. Despite studies with the electron microscope (21), intracellular electrodes (16, 75) and classical physiological techniques, the location of the cellular site of ion transport in the frog skin and toad bladder remains controversial (cf. 32). Although the toad bladder is a simpler organ it, nevertheless, contains at least four cell types (15, 52). By contrast, insect epithelia in general, are but one-cell layer thick; herein lies a tremendous advantage for the correlation of structure and function in solute transport. Assuming that the circular muscle layer is not involved in transport, one can conclude that active transport is localized in the epithelial layer and/or its associated basal
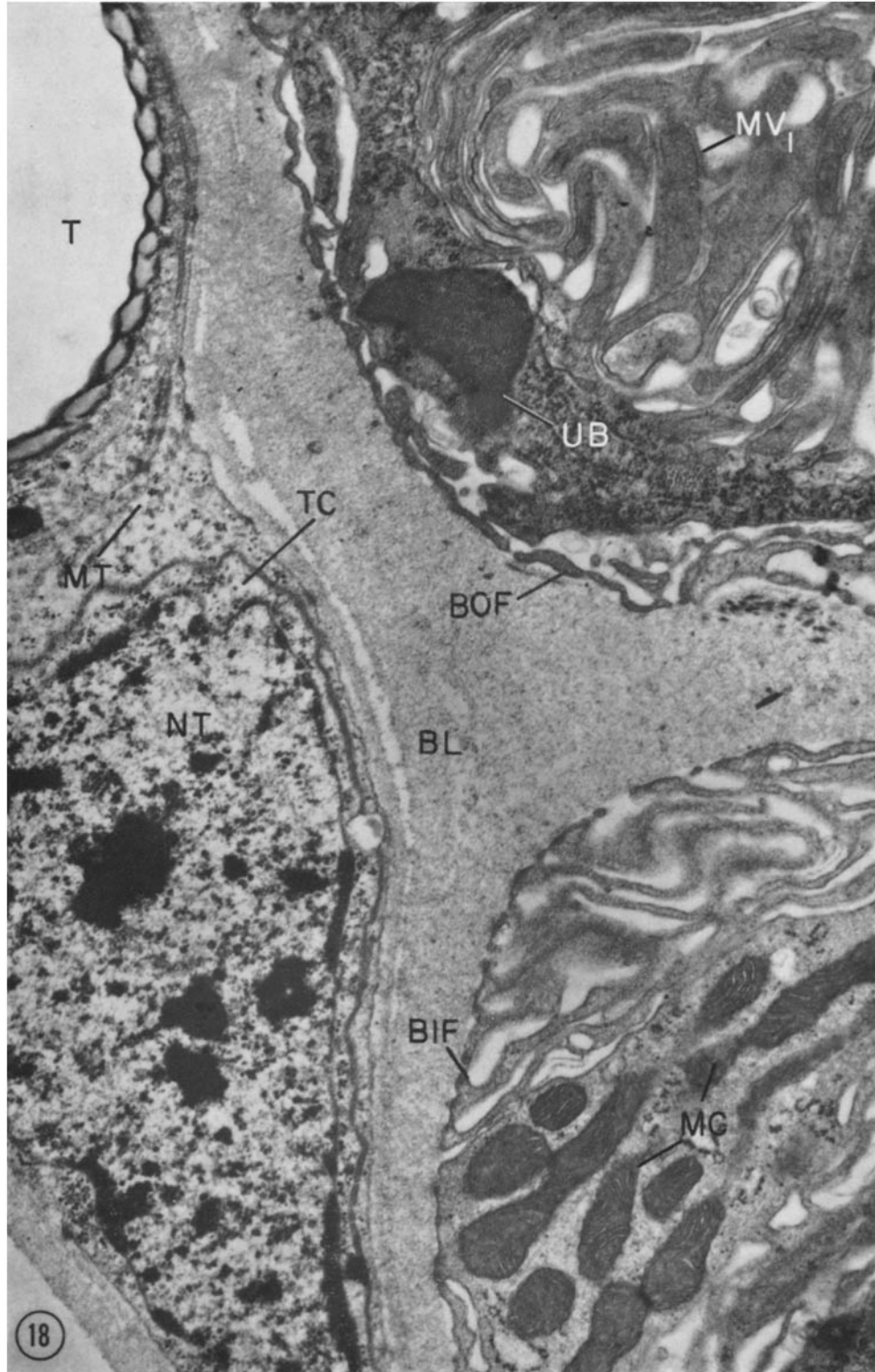
FIGURE 18 A section through the basal region of a goblet and a columnar cell resting on a basement lamina (*BL*). The goblet cell shows cytoplasmic projections (*MV₁*) that line the cavity, an unidentified granular body (*UB*), and basal outfoldings (*BOF*). The columnar cell portrays its basal infoldings (*BIF*) and mitochondria (*MC*). This micrograph also illustrates a tracheolar cell (*TC*) with its nucleus (*NT*), microtubules (*MT*), and a tracheole (*T*). × 18,000.
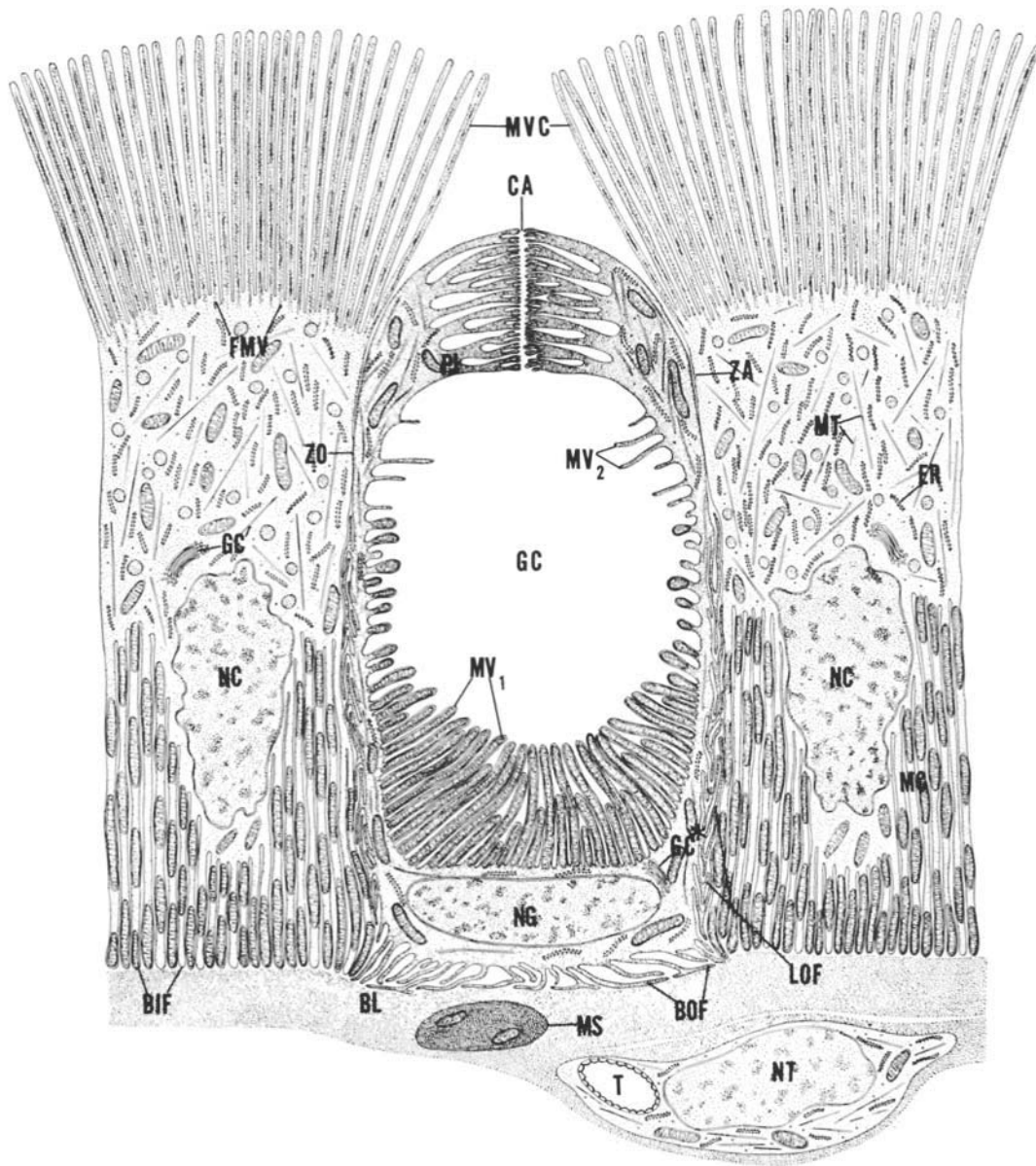
FIGURE 19   A schematic representation of the cell types comprising the epithelium of the midgut of a mature fifth instar larva of *Hyalophora cecropia. MVC*, microvilli of the columnar cells; *CA*, canal formed by the villuslike units derived from the larger protoplasmic projections (*PJ*) of the apical portion of the goblet cell; *FMV*, fine filaments within the microvilli of the columnar cell, *ZA*, zonula adhaerens; *ZO*, zonula occludens; MV_2, microvilli; *MT*, microtubules; *ER*, endoplasmic reticulum; *GC*, cavity of goblet cell; *GC'*, Golgi complex of columnar cell; *NC*, nucleus of columnar cell; MV_1, mitochondria-filled cytoplasmic projections that line the major portion of the cavity of the goblet cell; *MC*, mitochondria of columnar cell; *GC**, Golgi complex of goblet cell; *NG*, nucleus of goblet cell; *BIF*, basal infoldings of columnar cell; *BL*, basement lamina; *MS*, muscle fiber; *BOF*, basal podocytelike extensions of the goblet cell; *LOF*, lateral evaginations of the goblet cell; *NT*, nucleus of tracheolar cell; *T*, tracheole.

lamina and proceed directly to the more interesting task of locating specific transport sites within the midgut cells. Because the physiological work to date has been performed on the midgut which contains both goblet and columnar cells, the prospect that either or both cell types contribute to ion transport must be considered. Indeed the close association of mitochondria with basal infoldings in the columnar cells is very reminiscent of the modifications at the base of kidney cells thought to be involved in ion transport (see discussion below). However, the dense mitochondrial population associated with the plasma membrane of the goblet cell suggests a simple model for transcellular potassium ion transport. Therefore, principal attention in the following discussion will focus on the goblet cell.

The Cell as the Unit of Active Ion Transport

Active transport is defined as the movement of a substance across a biological membrane which cannot be accounted for by known physical forces, i.e., transport of a substance against an electrochemical gradient or with an electrochemical gradient but at a speed greater than that predicted from a knowledge of the physical forces involved (73). The energy to oppose the physical forces is provided by the metabolism of the living cell or by isolated organelles or fragments of living cells. This definition implies *selectivity*, *energization*, and *polarization* of the solute movement. In the case of ion transport a *charge separation* is also implied.

According to Ussing (74), "Most of the current thinking about permeability problems is based on the acceptance of the membrane theory which presupposes that the main diffusion resistance in a cell is located at the surface membrane." Although this view does not necessarily presuppose that the active transport site is also located in the surface membrane, this assumption is implicit in most contemporary studies on ion transport (cf. 70). If active ion transport is to take place in the plasma membrane alone, a simple unit membrane must select, energize, and provide direction to the transported solute.

Attempts to locate the entire transport mechanism within the plasma membrane have been successful to some extent, and active ion transport in the absence of cytoplasmic constituents has been demonstrated in red cell ghosts

(29) and in extruded squid axons (4). Moreover, all of the machinery necessary for oxidative metabolism is found in the cell wall of bacteria (49). Finally, phosphatidic acid and other proposed "carrier" molecules (30) as well as "transport enzymes," such as ouabain-sensitive, sodium- and potassium-activated ATPases, are localized in the plasma membrane (31). The theory that active transport mechanisms reside in the plasma membrane has been reviewed by Skou (70).

Since the accumulation of cations by isolated mitochondria is an active process, it is clear that active transport can and does occur in the absence of the plasma membrane (14, 41, 53, 77). Large amounts of calcium and magesium and lesser amounts of potassium and sodium are taken up by isolated mitochondria through a process which is stoichiometrically linked to phosphate uptake and electron transport (61). In contrast to plasma membrane transport systems which are ouabain-sensitive (38), neither mitochondrial ion accumulation (cf. reference 41) nor potassium transport across the midgut (28) is inhibited by ouabain.

Since the pioneering studies of Sjöstrand and Rhodin (69), Rhodin (59), Pease (54), and others, it is quite clear that many cells which engage in intensive transport activities have extensively folded plasma membranes intimately associated with mitochondria. Fawcett (22) has summarized several such well known cases (see also 55). In addition to the folding demonstrated for the midgut cells of Cecropia, an augmented but incomplete list would also include: the apical canaliculi of the anal papillae of mosquito larvae (18) and cells of the branchiae of *Artemia salenis* (19), striated ducts of the parotid gland (63), Malpighian tubules of insects (2, 8), the crayfish nephron (7), the epithelial cells of the gut of *Malacosoma* and *Melanoplus* (6), tracheal end cells of the light organ of the firefly, *Photinus pyralis* (5, 71), and the labial gland of adult *Antheraea peryni* (35). In other cases, such as the chloride cells of fish gills, very abundant but randomly oriented mitochondria are associated with that portion of the plasma membrane that forms an extensive system of tubular elements (56–58). Finally, there is alignment of mitochondria in the absence of plasma membrane folding, as in the photocyte of the light organ of the firefly, *Photinus* (5, 71).

The magnitude of transport across the midgut cells is so great (short circuit currents of 2 to 4

ma for a segment of midgut 10 mm long and 5 to 10 mm in diameter are routinely measured by Harvey and associates) that it is difficult to believe that oxidative machinery sufficient to energize such massive flux could exist solely within the plasma membrane. Therefore, it is suggested that the mitochondria play an active role in this process. Their intimate membrane associations certainly shorten the diffusion distance to the plasma membrane for ATP. Equally important is the prospect that ion accumulation by mitochondria plays a central role in transcellular movement of ions (41). Either view implies that the entire cell, not just the plasma membrane, is the unit of active transport.

Ironically, neither the frog skin (21, 79) nor the toad bladder (15, 52) has cells that possess regular folds of the plasma membrane or close mitochondria-membrane associations. Mitochondria are numerous in the cells of the stratum germinativum and stratum spinosum of the complex frog skin epithelium, and the so called mitochondria-rich cells of the bladder have distinct vesicles and tubules packed in the apical region (15, 52). However, active sodium transport in the bladder and skin produces currents only about $\frac{1}{10}$ the magnitude of the potassium current of the midgut (66, 76; Harvey, Haskell, and Zerahn, unplublished data). It may be that the frog skin (cf. 73) and toad bladder utilize a plasma membrane located system because the active ion movement in each case is ouabain-sensitive, as it is in isolated plasma membrane systems (cf. 66).

MITOCHONDRIAL PARTICIPATION IN ACTIVE TRANSCELLULAR TRANSPORT

The results of an in vitro study of the potential and short-circuit current of the isolated Cecropia midgut (26–28) are far simpler to interpret by a model which invokes mitochondrial ion accumulation than by a model which interprets the transcellular potential as the sum of diffusion potentials and electrogenic pump potentials located in the plasma membrane. The presence of the goblet cavity may mean that potassium movement in vivo need not take place continuously against the entire lumen potassium concentration gradient. The cavity may function in the same way that the apical cavity in the chloride cells of fish is alleged to aid salt movement (57, 58), i.e., by providing a space of relatively low potassium con-

centration into which potassium from the cell can diffuse.

That the permeability properties of the cytoplasmic projections and associated mitochondria of the goblet cell are different from those of the free plasma membrane is suggested by the structural modification of the plasma membrane in the region of close mitochondrial association. It will be recalled that the inner leaflet of the unit membrane of the mitochondria-filled cytoplasmic projections of the goblet cell possesses recurring units, each of which appears to be composed of a stalk or spikelike portion that is capped by a globular structure. The units are not a feature of any other portion of the cell's plasma membrane, nor are they a characteristic of the plasma membrane of the columnar cells. In some respects, this coat of particles is indeed reminiscent of the fuzzy coat that is formed on the cytoplasmic side of the pits or invaginations that are formed by the plasma membrane during the initial stages of micropinocytosis. The end result of such a process is the formation of coated vesicles within the cytoplasm. The coated pits or invaginations appear to be areas of the plasma membrane specialized for the uptake of specific substances such as proteins (1, 12, 23, 62). Williamson (82) envisions that certain noncoated pits are also specialized areas of the plasma membrane; however, they are involved in the uptake of lipid.

The significance of the particles demonstrated on the inner portion of the unit membrane that forms the cytoplasmic projections of the goblet cell of Cecropia is unknown. As indicated previously, we suspect that the ability of the midgut to transport massive amounts of potassium is a function of the goblet cell. Such a structural modification as the particle-studded inner unit membrane might well play a role in the active transport of potassium in this system. A similar function has been suggested for particles on the inner leaflet of the highly folded area of the plasma membrane of cells comprising the rectal papillae of *Calliphora erythrocephala* (25). Gupta and Berridge (25) have suggested that these particles are the "seats of complex enzyme systems, generating energy for transport processes." Particles similar in structure to the "elementary particles" of mitochondria have also been observed on the outer plasma membrane of rat liver cells (10) and on the intestinal microvilli of rabbits (50).

Other plasma membrane specialization would

include the surface coat of the intestinal microvilli as described by Ito (33), as well as junctional complexes (see below). In addition to structural specialization, Berridge and Gupta (cf. 25) have shown that a magnesium and pH specific adenosine triphosphatase is localized on the leaflets of epithelial cells of the rectal papillae of *Calliphora*. Recently, Philpott (personal communication) demonstrated that horseradish peroxidase is localized on the extensive tubular system that is a modified portion of the plasma membrane of the chloride cells of *Fundulus*.

ION MOVEMENTS IN INTERCELLULAR SPACE

Farquhar and Palade (20) concluded that "the diffusion of water, ions, and small water-soluble molecules is impeded along the intercellular space of the epidermis by zonulae occludentes while it is facilitated from cell to cell within the epidermis by zonulae and maculae adhaerentes." This conclusion is based on their finding that the zonulae occludentes are continuous barriers which leave the intercellular space open toward the dermis but closed to the external medium and on evidence that hemoglobin and ferritin penetrate the epidermis only to the zonula occludens. Their interpretation also considers the work of MacRobbie and Ussing (46) on the swelling of the epidermis in hypotonic solutions. A conclusive demonstration of the impermeability of the zonulae occludentes to water is still lacking, especially since Ussing and Windhager (75) describe an extracellular shunt pathway for chloride in the isolated frog skin. At least one and as many as five zonulae occludentes are observed between columnar and goblet cells in the midgut. The finding of Harvey and Nedergaard (26) that potassium fluxes from lumen to blood-side are very small implies that these tight junctions (zonulae occludentes) or some other structure limits solute movement in the spaces between midgut cells.

In the salivary gland of *Drosophila* larvae the entire epithelium appears to be the unit of "ion environment." Microelectrode measurements of the electrical resistance of epithelial cells by Loewenstein and Kanno (43, see also 36, 37, 44, 81) show a low value for contact surfaces between adjacent cells. This work demonstrates that ions can pass freely from cell to cell but not from cell to exterior. These studies further indicate that the electrical properties of the plasma membrane of epithelial cells are not the same on all cell surfaces because the specific resistance is lower at these contact surfaces, i. e., the lowered resistance is not the result of a simple increase in membrane area. This conclusion is supported by the analyses of phospholipid and sterol in plasma membranes and intercellular membranes by Ashworth and Green (3) who found that the molar ratios of sterol to phospholipid in plasma membranes from different types of rat cells range from 0.24 to 1.32. Ashworth and Green state, "Our results do not, however, support the idea of a common repeating chemical unit in all membranes of a cell (17) since the plasma membrane seems so different from the major intracellular membranes. It appears that there is both tissue specificity and functional specificity in the composition and organization of cellular membranes."

Loewenstein and Kanno (43) postulate that the cell-to-cell ion movement occurs across septate desmosomes which abound between adjacent *Drosophila* salivary gland cells. Such septate junctions are absent in the Cecropia midgut, so that if ions move freely between midgut cells other structures such as zonulae occludentes or zonulae adhaerentes must have special permeability properties.

EVOLUTION OF TRANSPORT MECHANISMS

The morphological and physiological evidence suggests that active potassium transport by the Cecropia midgut differs radically from active sodium transport by the frog skin or toad bladder. We have suggested that the midgut employs an ouabain-insensitive mitochondrial ion accumulation device in which a close association of the plasma membrane with oriented and localized mitochondria is crucial; the skin and bladder may employ an ouabain-sensitive plasma membrane localized device. Recalling that the entire transport mechanism, as well as the machinery for oxidative metabolism, is present in the cell wall of bacteria, it is tempting to speculate that the primitive ion transport mechanism resides in the plasma membrane. When mitochondria evolved with the capacity to accumulate cations, another type of transport became possible. The ouabain-insensitive, electron transport–linked, active potassium transport of yeast (39) may provide a cellular example especially since aerobically grown yeast cells contain mitochondria (42). When epithelial cells evolved with mitochondria oriented along the basal-apical axis and in contact

with plasma membrane on either the basal or apical surface, we postulate that the conditions for mitochondria to participate in transcellular ion transport were fulfilled. Ions could either be accumulated actively from outside the cell and released into the cytoplasm or accumulated from the cytoplasm and released outside the cell. It is altogether possible that much of the controversy regarding mechanisms of active ion transport may stem from the presence of two fundamentally different transport mechanisms—a plasma membrane

mechanism and a mitochondrial mechanism—with the over-all characteristics of transport depending on the degree to which each mechanism is developed or employed in a particular tissue.

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