A MITOCHONDRIAL PUMP IN THE CELLS OF THE ANAL PAPILLAE OF MOSQUITO LARVAE

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ABSTRACT

Mosquito larvae were raised to fourth instar in distilled water in order to maximally stimulate the salt-absorbing function of the anal papillae. Two exceptional features are observed, at the fine structure level, in the epithelial lining of the papillae. At the basal (cuticular) surface of the cells, the cell membrane is thrown into deep, narrow, parallel folds. The folds not only follow a rigid pattern at the cellular level but are also arranged at right angles to the long axis of the entire organ. A complicated pattern of canaliculi connects to the distal (plasma) surface of the cells. At this surface, paired mitochondria (sometimes triplets) are clamped about the membranes of the canaliculi to form structures referred to as "mitochondrial pumps." Mitochondria are also oriented in rather precise relation to the basal folds. Glycogen granules are found throughout the cytoplasm. The endoplasmic reticulum is sparse. The Golgi zones are few and not well developed. Unidentifiable, irregular vesicles with lipid-like membranes are found associated with the basal folds. The distal (plasma) surfaces of the cells are covered by a homogeneous granular layer the composition of which is unknown.

The typical mosquito larva has a cylindrical body with a head at one end and a "y"-shaped bifurcation at the other end. One protuberance of the bifurcation contains a tracheal siphon through which the larva respires while attached to the free surface of the water. The other protuberance, which projects at a downward angle when the larvae is attached to the water surface, has an anus at the distal end. Clustered about the anus are four finger-like projections called anal "gills" or "papillae."

Until comparatively recently, the anal projections were considered to have a respiratory or gill-like function. However, Wigglesworth (16), using the methods of Fox (4), demonstrated experimentally that no significant amount of respiration occurs in the organs. He also found that the cuticle was permeable to water in the gill area but not elsewhere over the body surface, and therefore concluded that the organs had some role in water balance.

It remained for Koch (6) to clearly demonstrate, by ligature experiments, that the anal papillae are responsible for salt absorption from dilute solutions. Wigglesworth (17) arrived at the same conclusion, almost simultaneously, by more indirect lines of evidence, namely, that the papillae are larger in larvae (*Aedes aegypti* and *Culex pipiens*) raised in dilute salt solutions as compared to those raised in more concentrated solutions. He used solutions ranging from distilled water to water containing 0.9 per cent NaCl.

Later, Ramsay (8), by ingenious microsampling techniques, arrived at the conclusion that K and Na enter the papillae. The body plasma is then kept accurately in balance by a combination of secretion by the Malpighian tubules and resorption of ions by the rectum.

It is the purpose of this report to describe the fine structure of the cells to be found in the papillae of mosquito larvae which have been reared in solutions putting maximum demand on this osmoregulatory organ.

METHODS

Larvae of *Culex quinquefasciatus* (identified by E. S. Hathaway) were raised from egg raft to fourth instar in distilled water to which a small amount of tropical fish food was added as sustenance. The food plus the decomposition of a few dead larvae added a small amount of free salts to the water. Analysis at the end of the growth period revealed the water to have a chloride salinity of 5.0 parts per million or a total salinity of 0.009 parts per thousand. Thus, salts were present in only trace amounts and presumably the cellular mechanisms in the papillae would be working at a maximum to absorb the traces.

Healthy, vigorous fourth instar larvae were fixed and embedded. First attempts at fixation were frustrated by the collapse of the papillae in the process of dehydration. This was finally circumvented by cutting off the tips of the papillae with fine iridectomy scissors at the time of fixation, permitting free access of all agents to the internal epithelial lining.

A variety of fixatives was tried and the following, modified to the hyperosmotic side, proved best. Larvae were immersed in chilled glutaraldehyde (14) (one part) buffered with *s*-collidine (1) (four parts) to which 15 per cent sucrose was added in some cases and 10 per cent sucrose in others. To the final fixative 0.2 ml 1 per cent $CaCl_2$ was added per 25 ml of solution. Fixation was carried out for 30 minutes followed by 2 hours' wash in the buffer alone. The material was then postfixed for one hour in 2 per cent osmium tetroxide buffered with *s*-collidine. Dehydration was rapid, starting with 50 per cent alcohol and proceeding through propylene dioxide to embedment in a mixture of Epon and Araldite (unpublished procedure from laboratory of H. H. Mollenhauer). Sections were cut on a Porter-Blum microtome and stained with lead citrate (10). Electron micrographs were taken on an RCA-EMU-3F.

OBSERVATIONS

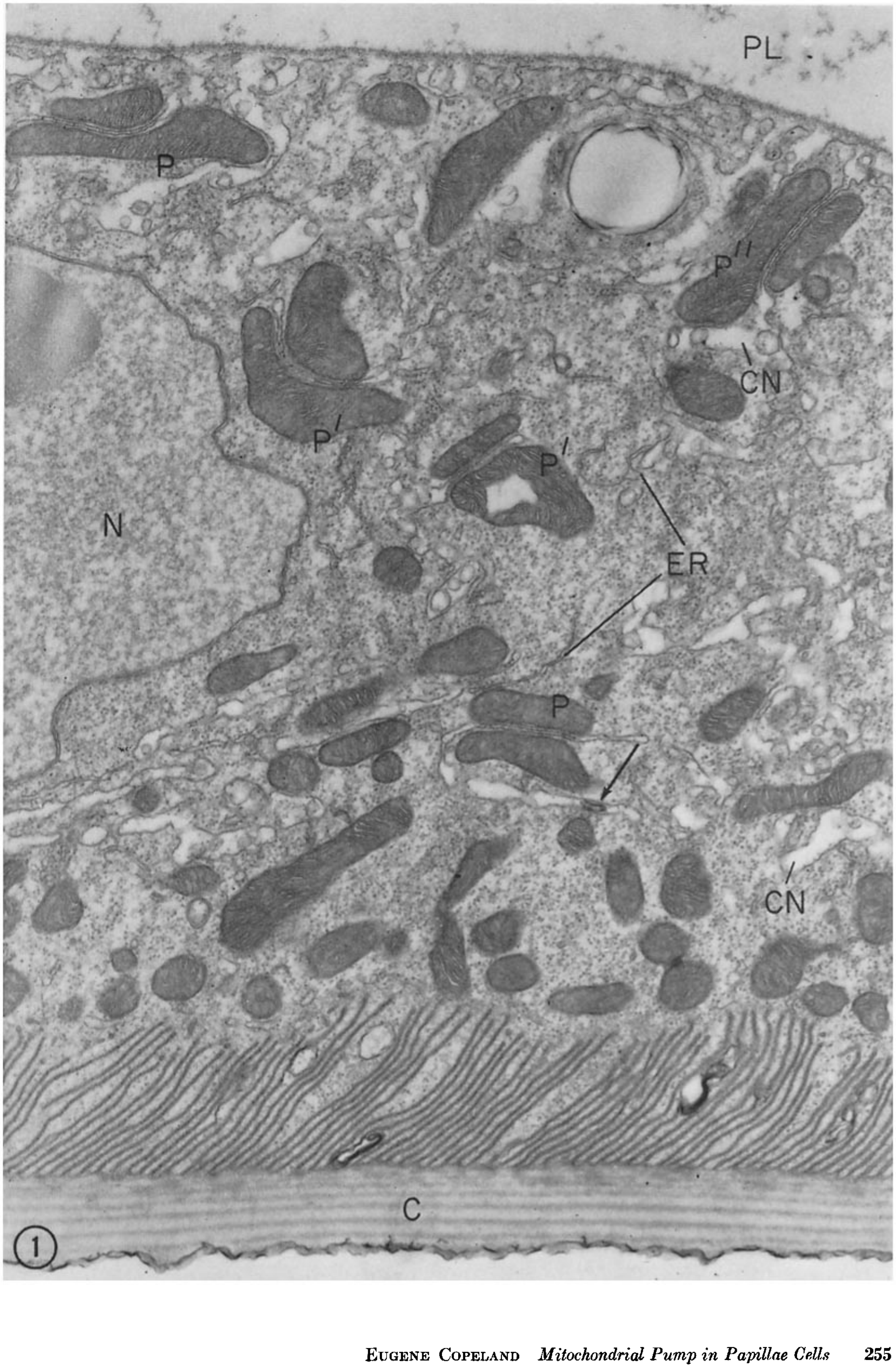
The anal papillae are composed of cuticular sacs lined with a single layer of epithelial cells possessing large nuclei.

The epithelial cell is characterized by a complex of narrow, closely packed, parallel folds of the cell membrane extending in from the external or cuticular surface of the cell (Figs. 1, 3, and 6). Sections cut at a sloping tangent to the cuticular surface show that these basal folds extend for long distances normal to the cell surface (Fig. 2). All areas of the papillae sampled to date reveal a highly unique orientation of the folds. Not only are the folds regularly spaced within the individual cells, but they are also oriented at right angles to the long axis of the entire papillary organ. The membranes of the folds are closely apposed and are evenly textured, with the exception of occasional fine, dark-staining irregularities (Fig. 6). The tips of the folds are slightly expanded and in some instances show signs of vesicle formation (Figs. 1 2, 6, and 10).

Associated with the basal folds are irregular, vesicular spaces lined with dark, usually asymmetrical membranes (Figs. 1, 2, 6, and 10). Almost all of the vesicles are continuous with the membranes of the basal folds. Although an occasional vesicle may be visualized as free of the membranes, I have not seen an identifiable one any distance from the zone of the folds. Dark-staining profiles which may be remnants of the vesicles are seen with some frequency in the spaces of the canalicular apparatus (Figs. 1 and 5). There is some indication that the vesicles may originate as enfolded proliferations of the membranes of the basal folds (Fig. 10).

Immediately above the layer of basal folds the cytoplasm is relatively homogeneous, with a sparse scattering of an irregular endoplasmic reticulum. The rest of the cytoplasm extending to the (distal

FIGURE 1 Lining epithelial cell cut in the long axis of the mosquito anal papilla. Fine, parallel folds (basal) of the plasma membrane are adjacent to the cuticle (*C*). Vesicles with asymmetrically staining walls are in the zone of the folds. The arrow points to a possible remnant of the vesicular walls within the lumen of a canaliculus. The edge of a large nucleus (*N*) is seen. Cell surface (distal) infoldings or canaliculi (*CN*) penetrate deeply. Five mitochondrial pumps are shown (at *P*, *P′*, and *P″*). At least two of the pumps (*P′*) seem clamped onto blind ends of the canalicular apparatus. One pump (*P″*) is clamped onto a continuous section of the canalicular apparatus. The endoplasmic reticulum (*ER*) is sparse. The plasma (*PL*) surface of the cell is bounded by a well defined layer of granular material. × 23,800.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plasma) surface of the cell is permeated with an irregular, branching, sinusoidal infolding of the cell surface (Fig. 3). These "canaliculi" have button-like villi protruding into their lumen (Figs. 3, 4, and 9).

All of the cytoplasm of the cell is speckled with small, dark-staining multiparticulate entities that are interpreted as glycogen particles (Fig. 9). These may or may not mask the presence of RNP granules. I am not clear on this point.

The endoplasmic reticulum is sparse and scattered throughout the cell. Frequently, in the homogeneous layer of cytoplasm just about the basal folds the endoplasmic reticulum assumes a semicircular or cusp-shaped arrangement (Figs. 2 to 4). Since granules have no marked affinity for the reticulum, the latter is assumed to be of the smooth variety.

The Golgi membranes are scattered and inconspicuous. The Golgi apparatus, when found, is small and composed of approximately three to five cisternae arranged in a semicircle, usually with well developed vesicles at the free ends (Figs. 3 to 5).

The mitochondria are numerous and have closely packed cristae (Figs. 4 and 10) that are difficult to resolve unless sectioned at right angles (Fig. 9). Although the mitochondria may be randomly scattered throughout the cell, there is always a dense population close to the tips of the folds (Figs. 1 and 3). Furthermore, the long axis of the mitochondria in that region tend to lie parallel to the long dimension of the folds (Fig. 7). Although mitochondria are rarely found between the folds, it is not uncommon for isolated folds of membrane to penetrate more deeply into the cytoplasmic body of the cell and become associated with the mitochondria (Figs. 3 and 10).

The mitochondria in the zone of the canaliculi often assume an unusual arrangement. Two or more mitochondria become closely apposed in a regular fashion and enclose constricted portions of the membrane-bounded canalicular spaces (Figs. 1, 5, 8, and 9). Although serial sections have not been attempted, survey of many of these mitochondria-canaliculi arrangements (organelles) gives the definite impression that the mitochondria are shaped like thick pancakes, thus affording a relatively large area of contact with the enclosed membranes (Fig. 4).

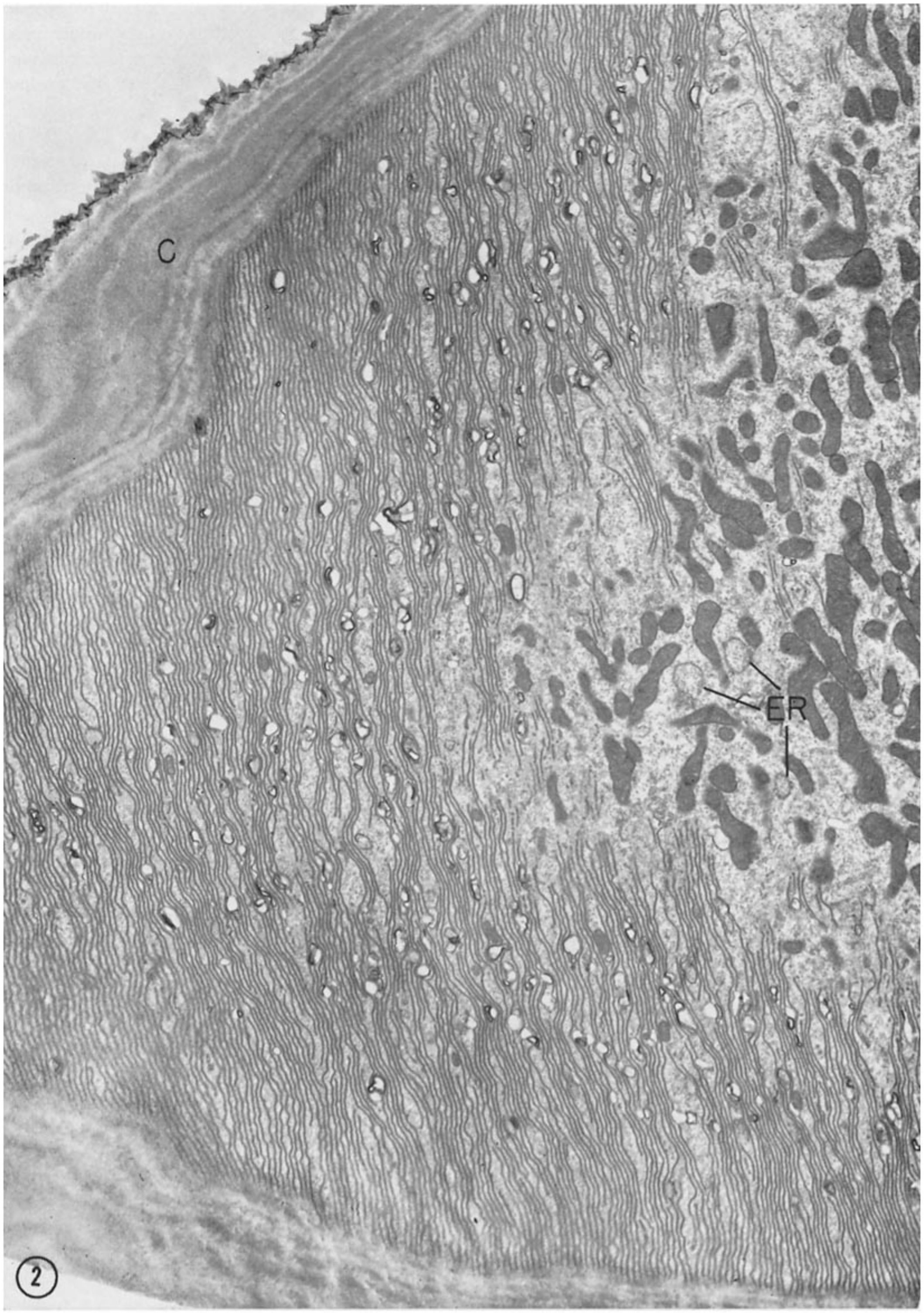
In some cases, the mitochondrial apparatus seems to surround a random section of the canaliculus (Figs. 1 and 9). In most cases, the mitochondria seem to adhere to shelf-like out-pocketings of the canaliculus with only a small fold protruding beyond the mitochondria (Figs. 1 and 9). At no time has any structure resembling the cross-section of a tubule been seen between the mitochondria. The enclosed canalicular membranes are thicker than the mitochondrial membrane or the free canalicular membrane (Fig. 8). This greater thickness is consistently observed and is not attributable to different orientations of profiles. Preferential staining, however, could be a factor.

The inner or plasma-exposed surface of the cell is interrupted frequently by the openings of the canaliculi. In fact, the cell surface is difficult to distinguish from the canalicular surface as regards both the type of membrane and its architecture in the form of blunt, button-like villi (Figs. 3 and 9). A homogeneous granular layer covers the entire inner surface of the cell (Figs. 1 and 9). The uniform thickness of this layer plus the fact that it is observed under all conditions of fixation suggests that it is not an artifact of coagulated plasma.

DISCUSSION

The description by Wigglesworth (15) of the cells in the mosquito anal papillae as seen at the light microscope level is amazingly accurate. He describes a reticulum throughout the cell and a basal striation which he surmised to be composed either of "fibres" (*sic*) or membranes. The canaliculi which I observe must, in most part, account for the reticulum, and the striations are, indeed, the folds of the cell membrane at the basal surface. Wigglesworth also describes small, dark-staining granules in the basal area which undoubtedly represent the

FIGURE 2 A tangential section sloping through the cuticle (*C*) of the cylindrical papilla toward the lumen. The long axis of the papilla runs right and left. The basal folds are quite long, evenly spaced, and lie at right angles to this axis. Note that irregular vesicles are associated with the folds. Several cusp-like cisternae of the endoplasmic reticulum (*ER*) are seen in the area of homogeneous cytoplasm together with mitochondria that tend to be oriented in the plane of the folds. × 10,000.



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vesicles as seen with the electron microscope. He says that other than the cuticle there is no visible external membrane, the cytoplasm resting directly on the cuticle. His observation is accurate in that there is no trace of the usually expected basal membrane between the cell membrane and the cuticle. The "clear cut membrane staining blue" which he describes as covering the inner surface of the cells is undoubtedly the even granular layer seen in my preparations. This layer very likely has some functional significance. It is consistently seen, and I have observed the same layer on the surface of salt cells in *Artemia* gills and Blue Crab gills (unpublished work). In the latter animal, fixation was carried out by perfusion which removed the plasma.

The fine structure of the epithelial cells of the mosquito anal papillae resembles in many respects the picture to be found in other types of transport cells and chloride cells. The basal folds might be compared to the basal folds observed by Rhodin (11–13) in kidney tubule cells of mammals and by Doyle (3) in the salt gland cells of marine birds. An increase in cell surface area results. The folds in the mosquito epithelial cells are not formed by cellular interdigitations, however, and the mitochondria do not, in most cases, lie between the folds. However, the comparison is still valid in that the folds exist and the mitochondria, in the mosquito epithelial cells, lie in close and oriented proximity to the tips of the folds.

The canaliculi observed in the mosquito epithelial cells bear some resemblance to the secretory canaliculi figured by Ito (5) in the gastric parietal cells of a variety of animals. The luminal surface of the anal papillae cells may be more irregular than and not so well equipped with microvilli as that of the acid-producing cells, but otherwise there is considerable similarity. The canaliculi might be considered analogous, if not homologous, with the crypt observed in the chloride cell of fish gills (2, 7). If it is postulated that a protected surface is essential for the secretion of salt (or acids), then the comparison is valid on the basis of functional morphology, *i.e.*, the cellular indentations take different forms but serve the same end.

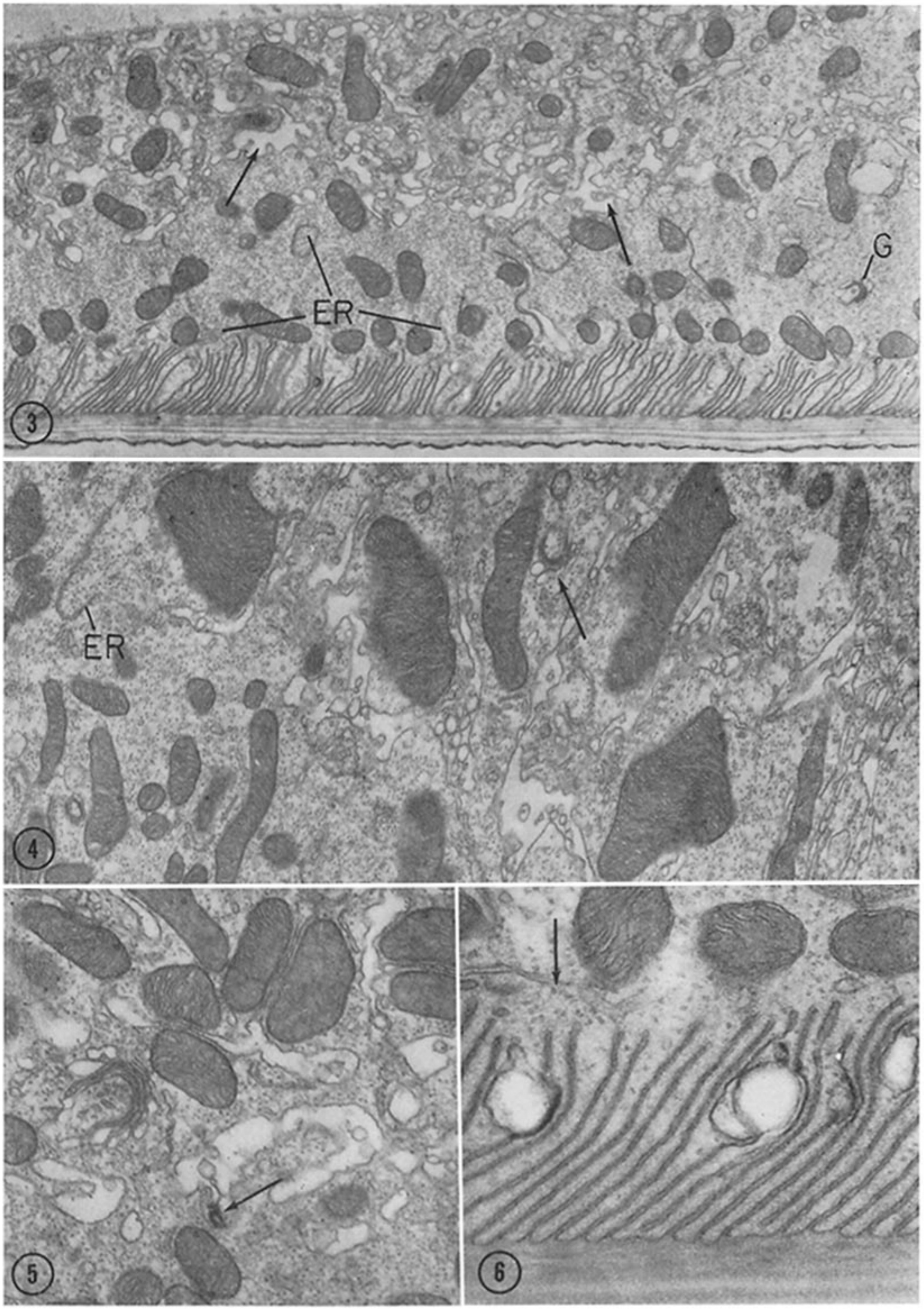
Not previously described, to my knowledge, is the association between mitochondria and canaliculi to form a distinct organelle which I would like to refer to as a "mitochondrial pump." The organelle is seen, in the cells, with a high degree of frequency and with a constancy of structure. Although it may have the same function as provided for by the mitochondria to cell membrane relationship in the cells of the salt gland of sea gull and in the distal tubule cells of the kidney, it is much more organized. The mitochondria of the organelle are flattened, and they are not trapped between cellu-

FIGURE 3 Section parallel to the axis of the papilla and some distance from the nucleus of the cell, showing general topography. Arrows point to the well developed and complex canalicular spaces. A typical, small Golgi apparatus (*G*) is seen in the layer of homogeneous cytoplasm above the region of the basal folds. Random elements of the endoplasmic reticulum can be seen in association with these folds and in the form of circular or cusp-shaped cisternae. Note that the mitochondria are numerous and show a regular concentration in the area contiguous to the basal folds. Since the mitochondria lie parallel to the folds, they are seen here in cross-section. Compare Figs. 7 and 10. × 11,000.

FIGURE 4 A tangential cut sloping from the area of homogeneous cytoplasm (lower left) into the canalicular zone (upper right). Note the narrow mitochondria of the homogeneous area (associated with the basal folds) as compared to the flattened, pancake-like mitochondria of the canalicular area (associated with the mitochondrial pumps). A typical cusp of the endoplasmic reticulum (*ER*) is seen. The arrow points to a Golgi apparatus. Button-like villi are numerous in the canalicular spaces. × 17,000.

FIGURE 5 Note the small but typical Golgi element. Parts of three mitochondrial pumps are discernible. The arrow points to a dark fragment which may be a remnant of a vesicle from the region of the basal folds. × 20,000.

FIGURE 6 The basal folds rest directly on the cuticle with no visible basal membrane. The membranes of the folds are thin and closely apposed. Irregular vesicular bodies appear to be continuous with the membranes of the folds (see also Figs. 2 and 10). The tips of the folds usually expand slightly and may at times appear to vesiculate. The endoplasmic reticulum (arrow) is sometimes associated with the terminations of the folds. × 45,000.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lar folds. Quite the reverse, the mitochondria trap the cell membranes into very uniform dimensions. Furthermore, the membranes appear to be specifically thickened in the regions of intimate alignment. Since the cell is responsible for salt transport (and possibly water transport), it is reasonable to assume that this unusual structure is linked to the peculiar function of the cell. Fortunately for my morphological interpretations, there is only one known function for the anal papillae and there is only one cell type in the epithelium.

Although in this paper the emphasis has been placed on the "mitochondrial pump" organelle in the canalicular region of the cell, the possible importance of the basal fold-mitochondria relationship should not be overlooked. The next and obvious step in my investigations is to check these two types of plasma membrane-mitochondria association for the relative amounts of enzymes present, notably phosphatases.

Preliminary observations also have been made on the salt gland (epipodite segment of the gills) in the brine shrimp *Artemia* (unpublished). In forms raised to the adult stage in triple strength seawater, the mitochondrial pumps are found in highly developed form. Six or eight mitochondria may be stacked one on the next, like a pile of coins, with canalicular membranes trapped in each intervening space.

In the mosquito epithelial cells, glycogen is found in plentiful supply throughout the cell and can be assumed to provide a basic source of energy-fuel for the two types of mitochondrial cell–membrane association (the mitochondrial pumps and the basal folds).

Although it appears that the irregular vesicles found in the base of the cell originate from the

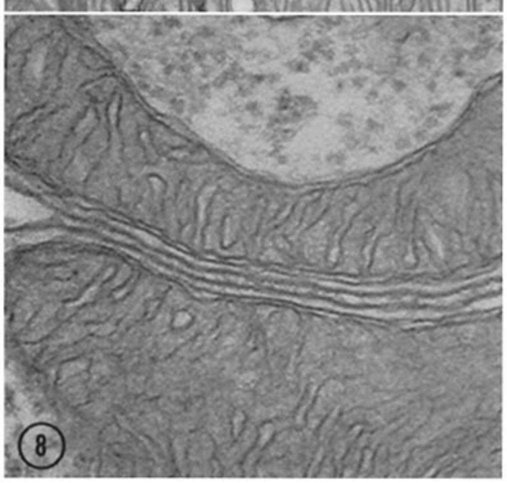

FIGURE 7 Tangential section parallel to the cuticle and sloping very gradually from the zone of the basal folds (left) into the zone of homogeneous cytoplasm and mitochondria (right). Note the length of the mitochondria and their arrangement parallel to the tips of the folds (see Fig. 3 for a cross-section of this relationship). The basal folds and the mitochondria sometimes come into association (arrow). × 17,000.

FIGURE 8 High magnification of the membranes in a mitochondrial pump (seen at lower power in Fig. 9). The two central membranes (canalicular extensions) appear to be of greater thickness than the mitochondrial membranes. × 80,000.

basal folds, their fate is not clear. The apparent coils of double membranes observed in some instances (arrows, Fig. 10) may indeed be artifacts of lipid fixation (9). Remnants of the heavy, lipid-like membranes are observed in the canalicular spaces, but a transition from one position to the other has not been clearly visualized, at least to my complete satisfaction. The vesicular structures are found consistently, and must have some bearing on cell function. The same can be said for the semicircular or cusp-shaped configurations of the endoplasmic reticulum found in greatest concentration in the relatively homogeneous cytoplasm just above the basal folds and, less often, in the canalicular area of the cell.

The close spacing of the basal infoldings might account for their parallel arrangement within any one cell. However, their consistent orientation at right angles to the long axis of the papilla itself is inexplicable.

From the above observations and the descriptions of other ion-transport cells, the following general conclusions are beginning to take shape. (*A*) Active transport is accomplished by an intimate association between an expansion of smooth membranes and mitochondria, with a plentiful supply of glycogen in the background. The membranes may take the form of the smooth endoplasmic reticulum, basal folds of cell membrane, distal infoldings of cell membrane, or cellular interdigitations. In all cases, the result is a large membranous surface area available for the exchange of materials. (*B*) As a corollary, the final stage of release of the secreted material takes place usually through an infolded or protected surface.

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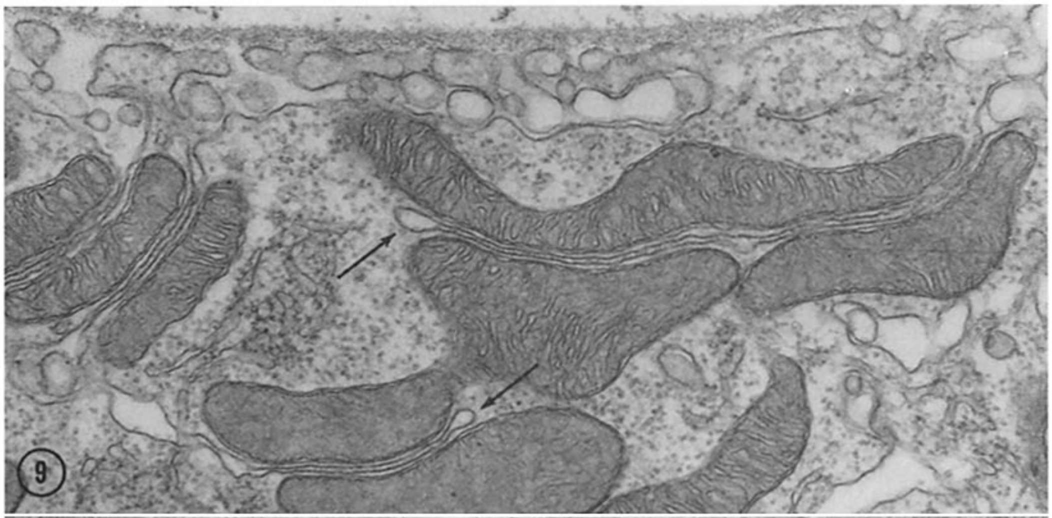
FIGURE 9 Three mitochondrial pumps are illustrated. The one to the left has three mitochondria arranged in parallel fashion to form a double pump. The central pump is more typical, and the one to the right has three mitochondria arranged in tandem fashion on one canalicular outpouching. The bulbous terminal end or flange of the canaliculus (arrows) is rather commonly seen. The particulate nature of the glycogen (?) granules can be seen best in this plate. A homogeneous layer of unknown composition covers the free surface of the cell. Note that the closely packed cristae of the mitochondria are difficult to demonstrate unless cut at right angle. × 40,000.

FIGURE 10 A tangential section sloping from the basal folds (bottom) into the homogeneous cytoplasm and mitochondrial area (top). Thus, the height of the folds, including their terminal enlargements, is exaggerated. Vesicles are in various stages of formation, including two (arrows) that seem to have membranes similar to those of the basal folds. Note that the basal folds are occasionally associated with the mitochondria. × 31,000.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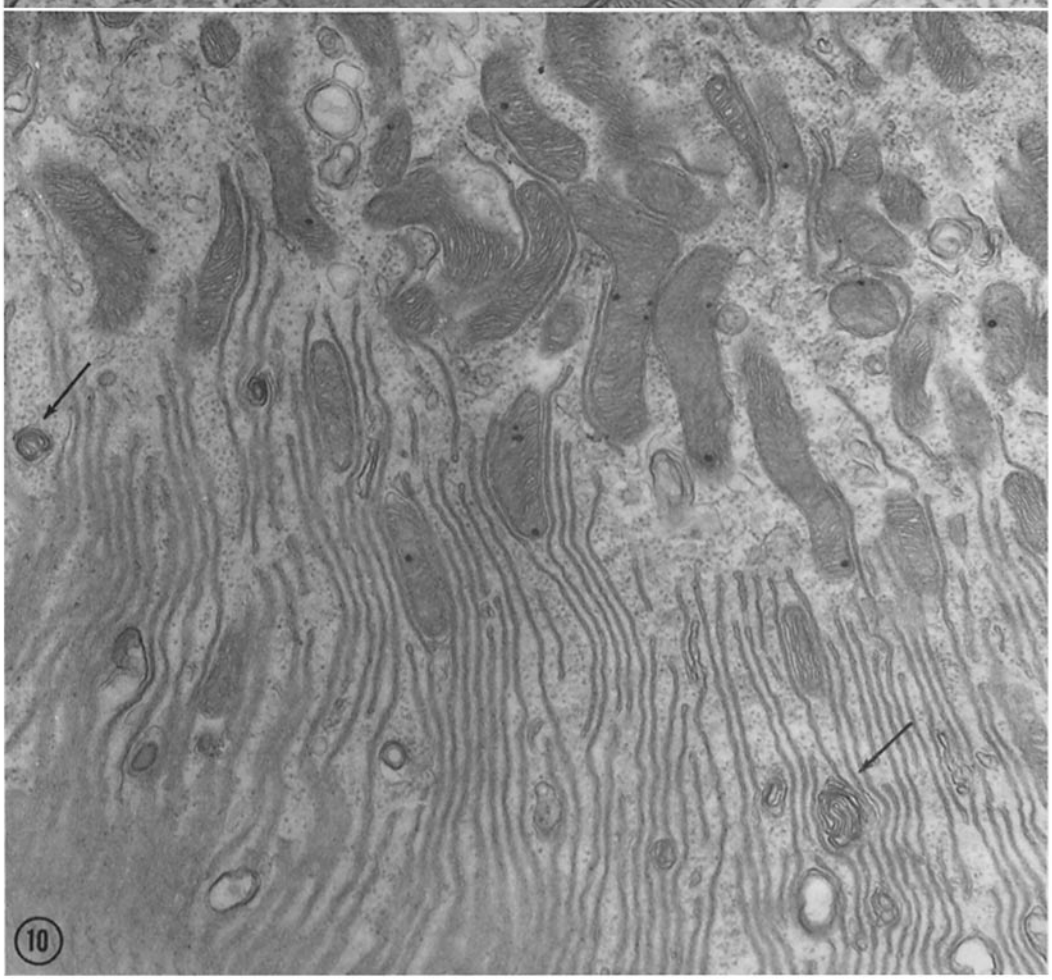
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