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Membrane Structural Specialization of the Toad Urinary Bladder Revealed by the Freeze-Fracture Technique

II. The Mitochondria-Rich Cell

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Summary. Examination of the toad urinary bladder by freeze-fracture electron microscopy reveals that the mitochondria-rich cells of the epithelium possess distinctive and characteristic membrane structural specialization. Unique rod-shaped intramembrane particles are found in luminal and basal membranes as well as certain intracellular vesicles of this cell type. The consistent finding of two discrete patterns of luminal membrane structural organization supports the possibility that two morphological forms of mitochondria-rich cell exist within the toad bladder epithelium.

The mitochondria-rich cell has been recognized as a distinct cell type of the toad urinary bladder epithelium based on its morphology when examined with thin section electronmicroscopy (Peachey & Rasmussen, 1961; Choi, 1963; DiBona, Civan & Leaf, 1969) and scanning electronmicroscopy (Ferguson & Heap, 1970; Danon, Strum & Edelman, 1974; Davis, Goodman, Martin, Matthews & Rasmussen, 1974). This cell type has also been distinguished from other cell types of the epithelium by the localization of high levels of carbonic anhydrase within mitochondria-rich cells (Rosen, Oliver & Steinmetz, 1974; Scott, Sapirstein & Yoder, 1974). Although mitochondria-rich cells represent less than 20% of the epithelial cells in toad bladder, recent evidence indicates that this cell type may have an important role as a target cell responsive to aldosterone (Voûte, Hänni & Ammann, 1972; Sapirstein & Scott, 1975). In addition, there are reports suggesting that this cell type may also be involved in the action of vasopressin (Scott *et al.*, 1974) and urinary acidification (Rosen *et al.*, 1974).

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In the present study freeze-fracture electronmicroscopy has been used to demonstrate that the membrane structure of mitochondria-rich cells differs significantly from that previously reported for the adjacent granular cells (Wade, DiScala & Karnovsky, 1975) and that mitochondria-rich cells possess a unique membrane structural specialization, namely rod-shaped intramembrane particles. Observations are also presented which raise the possibility that different membrane structural features characterize two forms of the mitochondria-rich cell.

Materials and Methods

For these studies urinary bladders were obtained from pithed female toads (*Bufo marinus*) from both the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) and Colombia (Tarpon Zoo Co., Tarpon Springs, Florida). Toads from these two sources were found to be identical with respect to the membrane structural features of their mitochondria-rich cells although, as indicated by the work of Rosen *et al.* (1974), toads from Colombia appeared to have a higher frequency of mitochondria-rich cells. Urinary bladders were mounted as sacs on glass tubes and bathed in an aerated Ringer's solution consisting of (mM): 111 NaCl, 3.5 KCl, 2.5 $NaHCO_3$ and 1.0 $CaCl_2$.

Tissue was fixed by immersion for 15 min in 2.5% glutaraldehyde buffered by 0.1 M sodium cacodylate at pH 7.4. After fixation, tissue was washed and stored in 0.1 M cacodylate buffer. Prior to freezing, tissue was soaked in 25% glycerol in 0.1 M cacodylate. Tissue was frozen in either liquid Freon 22 cooled by liquid nitrogen or in undercooled liquid nitrogen as previously described (Wade *et al.*, 1975). Unfixed tissue was quickly frozen in undercooled nitrogen without exposure to cyroprotectants. The tissue was fractured in a Balzers freeze-etch unit BAF 301 (Balzers High Vacuum, Liechtenstein) and in most runs a Balzers mirror image replica device was used. Platinum-carbon replicas were prepared with an electron beam evaporation device (EVM 052, Balzers High Vacuum) and quartz crystal thin film monitor (QSG 201, Balzers High Vacuum). Replicas were examined with an RCA EMU 4B electronmicroscope.

Observations

Although the mitochondria-rich cell is clearly recognizable in thin sections as a single cell type morphologically different from surrounding granular cells, investigators have noted that variability exists in the ultrastructure of mitochondria-rich cells (Choi, 1963; Voûte *et al.*, 1972). Shown in Figs. 1 and 2 are two mitochondria-rich cells from the same toad bladder after identical preparative procedures and staining. Both examples illustrate the flask-like shape and large number of mitochondria characteristic of mitochondria-rich cells, but there are significant differences between them. Relative to the cell in Fig. 1, the cell in Fig. 2 appears to have more densely packed mitochondria (*M*, Fig. 2), a denser

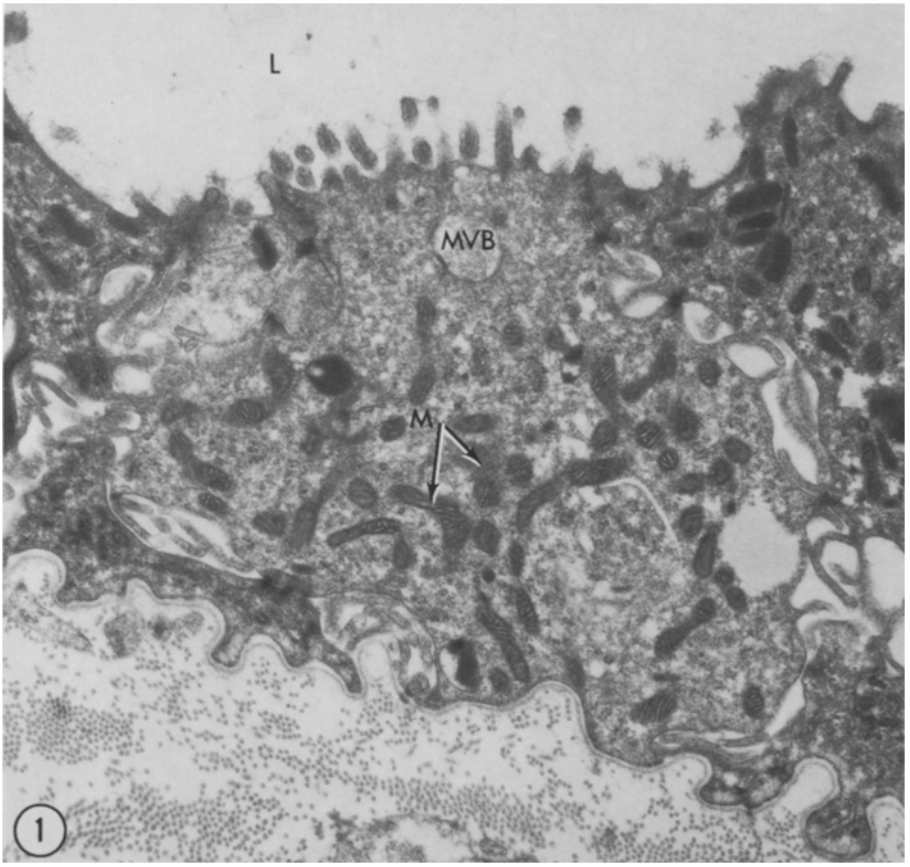

Fig. 1. A mitochondria-rich cell as viewed in thin sections. Illustrates the flask-like shape, large number of mitochondria (*M*) and the multivesicular bodies (*MVB*) characteristic of mitochondria-rich cells. Lumen of bladder, *L*. (14,000×)

cytoplasm, and more tubular vesicles (*V*, Fig. 2) in its luminal cytoplasm. In either case, their distinctive cytoplasmic features and the small area of luminal surface occupied by mitochondria-rich cells compared to adjacent granular cells makes it possible to identify the membranes of mitochondria-rich cells in freeze-fracture preparations.

The Luminal Membrane

Examination of the toad bladder epithelium with freeze-fracture demonstrates that the morphological variability of mitochondria-rich cells noted above is paralleled by a variation in membrane structural features. Two membrane structural patterns are consistently observed. The luminal surface of some mitochondria-rich cells are characterized by relatively

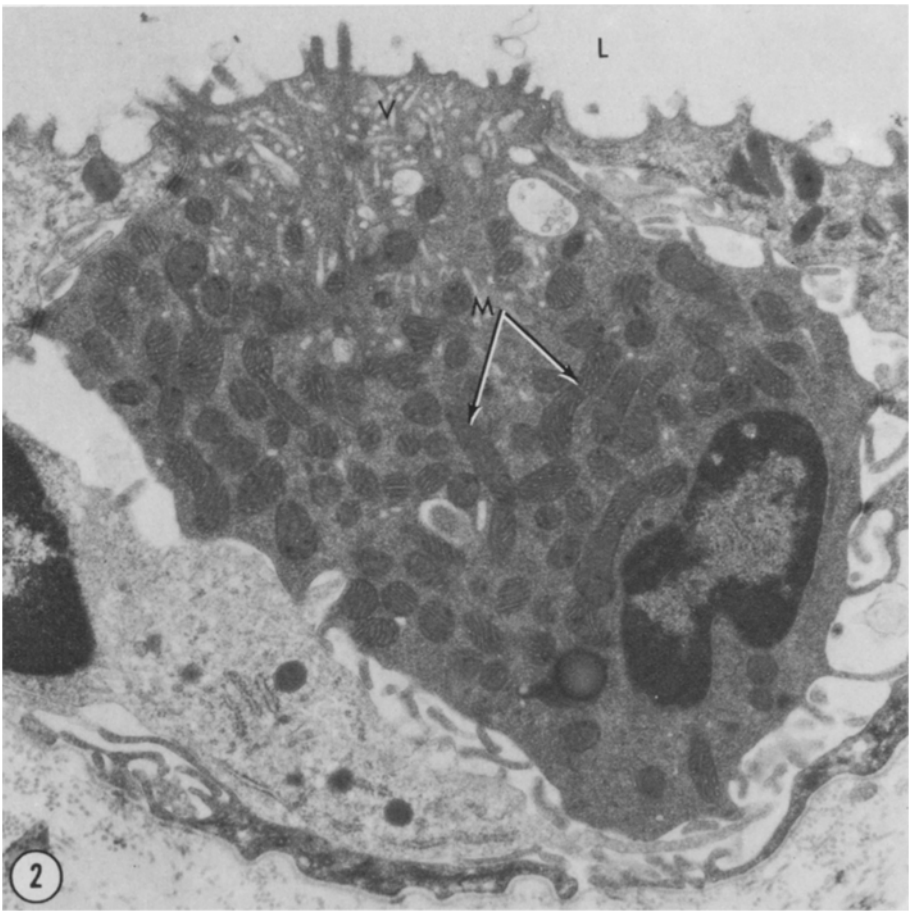

Fig. 2. Another mitochondria-rich cell as viewed in thin sections. Although from the same toad bladder and prepared by the same procedures as Fig. 1, the morphology of this mitochondria-rich cell differs significantly from that illustrated in Fig. 1. This form of the mitochondria-rich cell appears to have more densely packed mitochondria (*M*), a denser cytoplasm and more tubular vesicles (*V*) in its luminal cytoplasm than the form of mitochondria-rich cells shown in Fig. 1. Lumen of bladder, *L*. (14,000 ×)

long, finger-like microvilli. When viewed from the lumen the overall shape of these cells is usually very angular and frequently a triangular shape is observed as shown in Fig. 3. In contrast, the luminal surface of other mitochondria-rich cells is characterized by irregular, ridge-like microvilli (Fig. 4). When viewed from the lumen this form of mitochondria-rich cell is usually oval or square in shape.

Luminal Membrane Particle Distribution and Shape

Previous freeze-fracture study of the toad bladder (Wade, *et al.*, 1975) demonstrated that the luminal membrane of granular cells has a higher

density of intramembrane particles on fracture face *E* (extracellular half-membrane formerly called fracture face "B")[1] than on fracture face *P* (protoplasmic half-membrane formerly called fracture face "A"). In both forms of mitochondria-rich cell studied the situation is reversed compared to granular cells with a lower density of particles found on fracture face *E* than found on fracture face *P*. In addition, the luminal membrane of those mitochondria-rich cells characterized by ridge-like microvilli as in Fig. 4 invariably has distinctive rod-shaped particles[2] on fracture face *P* (Fig. 5). The shape of these intramembrane particles is in striking contrast to that of the globular shaped particles of adjacent granular cells (*see GR*, Figs. 9 and 10). Shown in Fig. 6 is the complementary fracture face *E* of the region of membrane represented in Fig. 5. There are few particles but there are rod-shaped depressions corresponding to the rod-shaped particles of the *P* face. Membrane fracture faces from unfixed tissue not exposed to cyroprotectants (Fig. 7) appear similar to those obtained from glutaraldehyde-fixed tissue exposed to glycerol. Thus, neither the distinctive shape of these particles nor their tendency to adhere to fracture face *P* appears to be related to fixation or exposure to glycerol.

Another remarkable feature of this membrane is the variation observed in the frequency and distribution of rod-shaped particles (*compare* Figs. 5 and 8). Although the rod-shaped particles are occasionally found associated with the membrane of microvilli (*MV*, Fig. 8), they are more frequently associated with the membrane between microvilli. Rod-shaped particles are also sometimes found in groups (*arrows*, Fig. 8) and the particles in these groups occasionally display a tendency to be oriented parallel to each other (*double arrows*, Fig. 8).

Although the rod-shaped particles are the distinctive feature of these mitochondria-rich cell membranes, globular particles are also found (*single arrowhead;* insert, Fig. 8). In some instances rod-shaped particles

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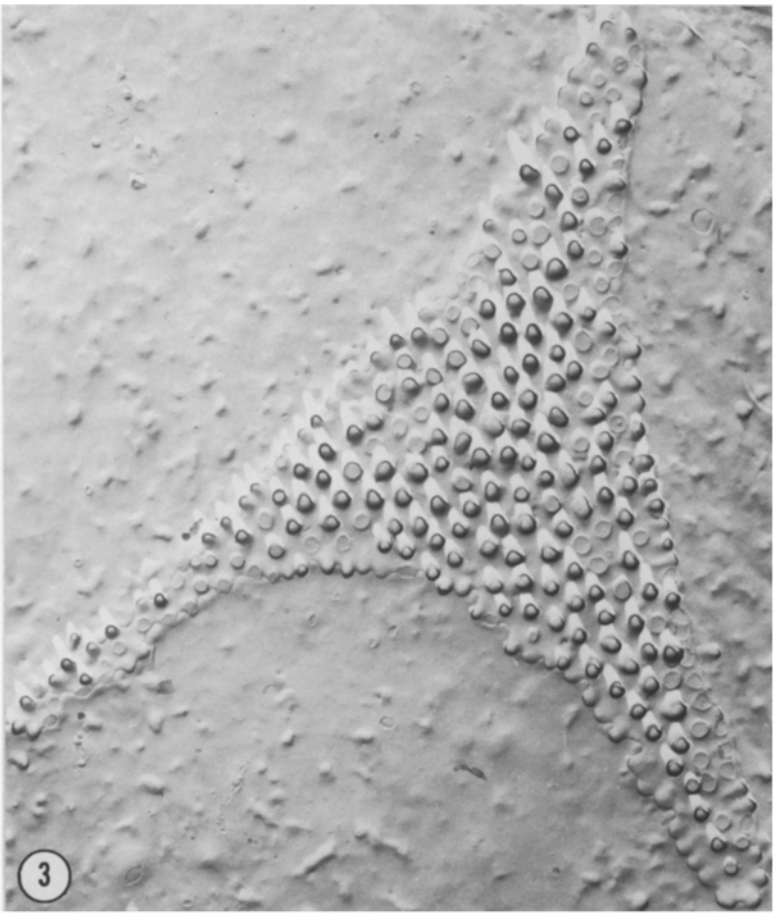
1 In keeping with the nomenclature for freeze-etching recently proposed by Branton *et al.* (1975), this paper uses *E* to refer to the half-membrane closest to the extracellular space and *P* to refer to the half-membrane closest to the protoplasm.

2 Although these particles are clearly rod-shaped, one of their dimensions is necessarily always obscured by the particle's white shadow. However, an estimate of rod-shaped particle dimensions can be obtained by measuring the long axis of particles which happen to be oriented with their long axis perpendicular to the direction of shadow. Similarly, the short axis of rod-shaped particles can be reasonably estimated from particles which happen to be oriented parallel to the direction of shadow. Such measurements indicate that the dimensions of rod-shaped particles are about 100×230 Å. These values actually represent an upper estimate of particle size since the replication process increases the measured size of objects (Misra & DasGupta, 1966).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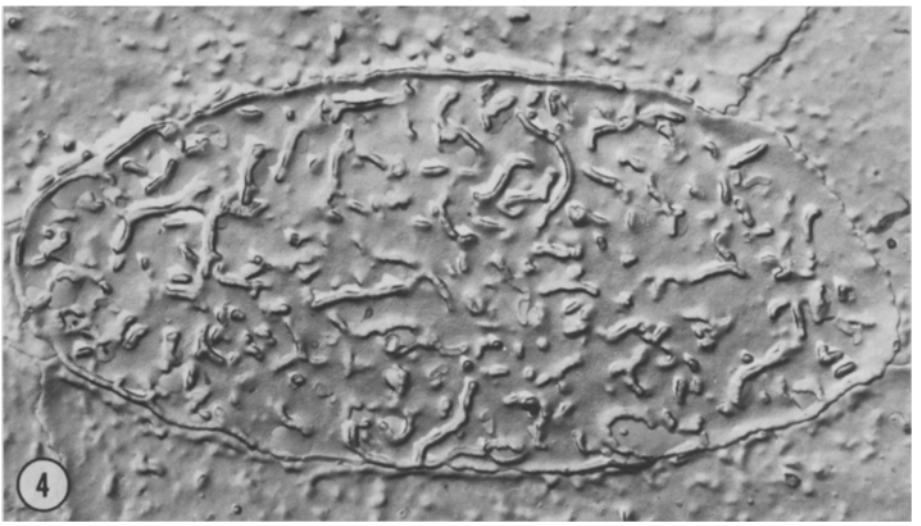
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appear to have two (*double arrowheads;* insert, Fig. 8) and, in some cases, three globular subunits (*triple arrowheads;* insert, Fig. 8). The rod-shaped particles described above are consistently observed in the luminal membrane of those mitochondria-rich cells with ridge-like microvilli but the form of mitochondria-rich cell characterized by finger-like microvilli (as in Fig. 3) has few, if any, rod-shaped particles in its luminal membrane. Instead, the luminal membrane particles of these mitochondria-rich cells appear globular in shape (*MR*, Fig. 9). As in the case of the rod-shaped particles, most of these globular particles are found on fracture face *P* (*MR*, Fig. 9) rather than fracture face *E* (*MR*, Fig. 10). Thus, this fracturing property of the luminal membrane is common to both forms of mitochondria-rich cell and contrasts with the fracturing characteristics of adjacent granular cells (*GR*, Figs. 9 and 10).

Lateral and Basal Membrane

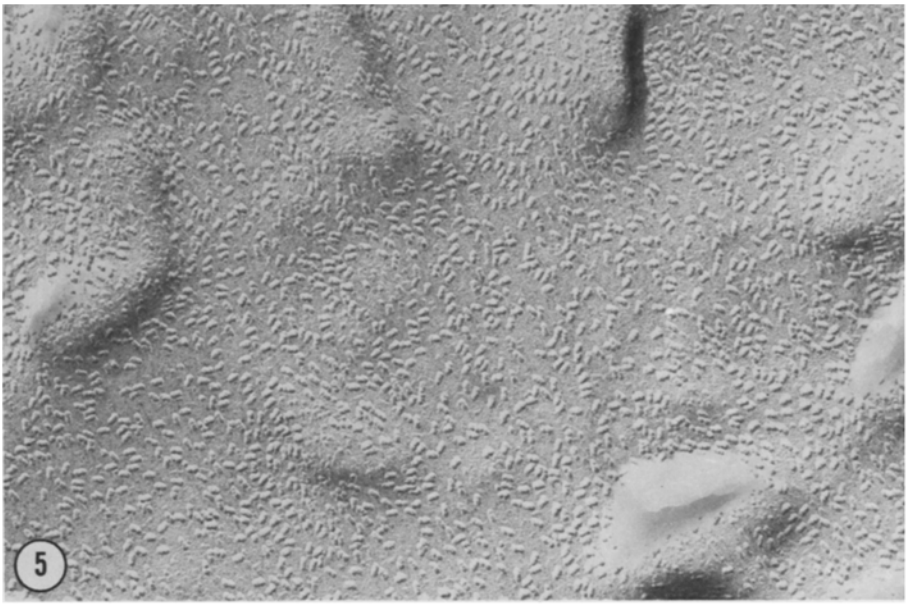
Although the luminal membranes of the two forms of mitochondria-rich cell differ very significantly, the membrane structural features of the lateral and basal membranes appear to be similar for both forms of mitochondria-rich cell. Lateral (*Lat M*, Fig. 11) and basal (Fig. 12) membranes of mitochondria-rich cells are consistently found to possess rod-shaped particles. Even when the luminal membrane (*LM*, Fig. 11) has only globular particles, the lateral membrane (*Lat M*, Fig. 11) is found to possess rod-shaped particles.

Intracellular Membranes

Rod-shaped particles are also characteristic of certain intracellular membranes of mitochondria-rich cells. Both multivesicular bodies (*MVB*, Fig. 13) and tubular vesicles (V_1, Fig. 13) are found to have rod-shaped particles associated with fracture face *P*. However, not all intracellular membranes of mitochondria-rich cells possess rod-shaped particles. Certain small vesicles (V_2, Fig. 13) as well as nuclear membranes (*N*, Fig. 14) are found to have only globular particles.

Fig. 3. Low magnification view of the luminal membrane of a mitochondria-rich cell as seen in freeze-fracture. The luminal surface of this mitochondria-rich cell has finger-like microvilli. (8,200×)

Fig. 4. Another mitochondria-rich cell as seen in freeze-fracture at low magnification. In contrast to the pattern illustrated in Fig. 3, the luminal surface of this form of mitochondria-rich cell is characterized by irregular, ridge-like microvilli. (7,200×)



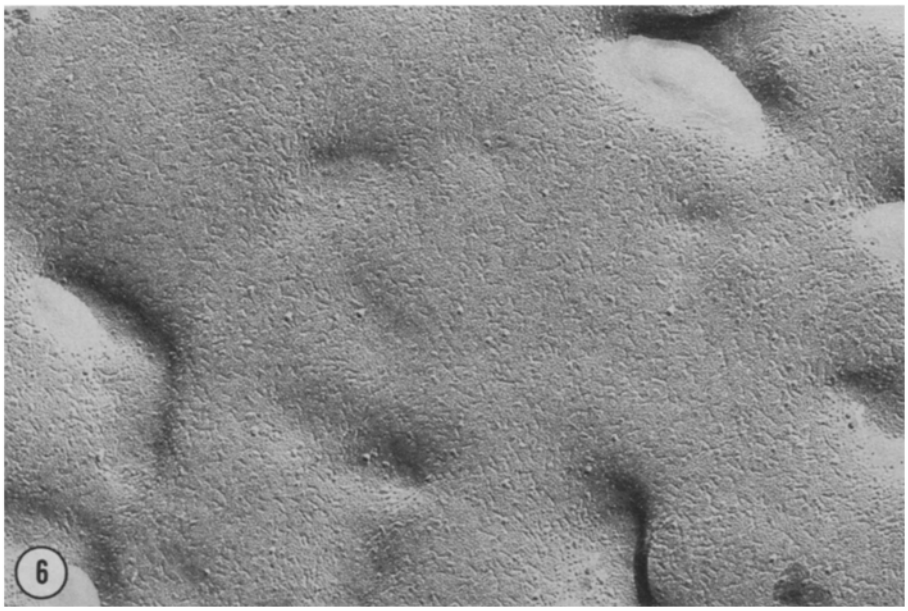


Fig. 5. Luminal membrane (fracture face *P*) of a mitochondria-rich cell which has rod-shaped intramembrane particles. (70,000 ×)

Fig. 6. The complementary fracture face *E* for the region of membrane shown in Fig. 5. This fracture face has rod-shaped depressions corresponding to the rod-shaped particles. (70,000 ×)

Fig. 7. Fracture face *P* of the luminal membrane from tissue that has not been fixed or treated with glycerol. Appearance of rod-shaped particles is similar to that observed in fixed material. (70,000 ×)

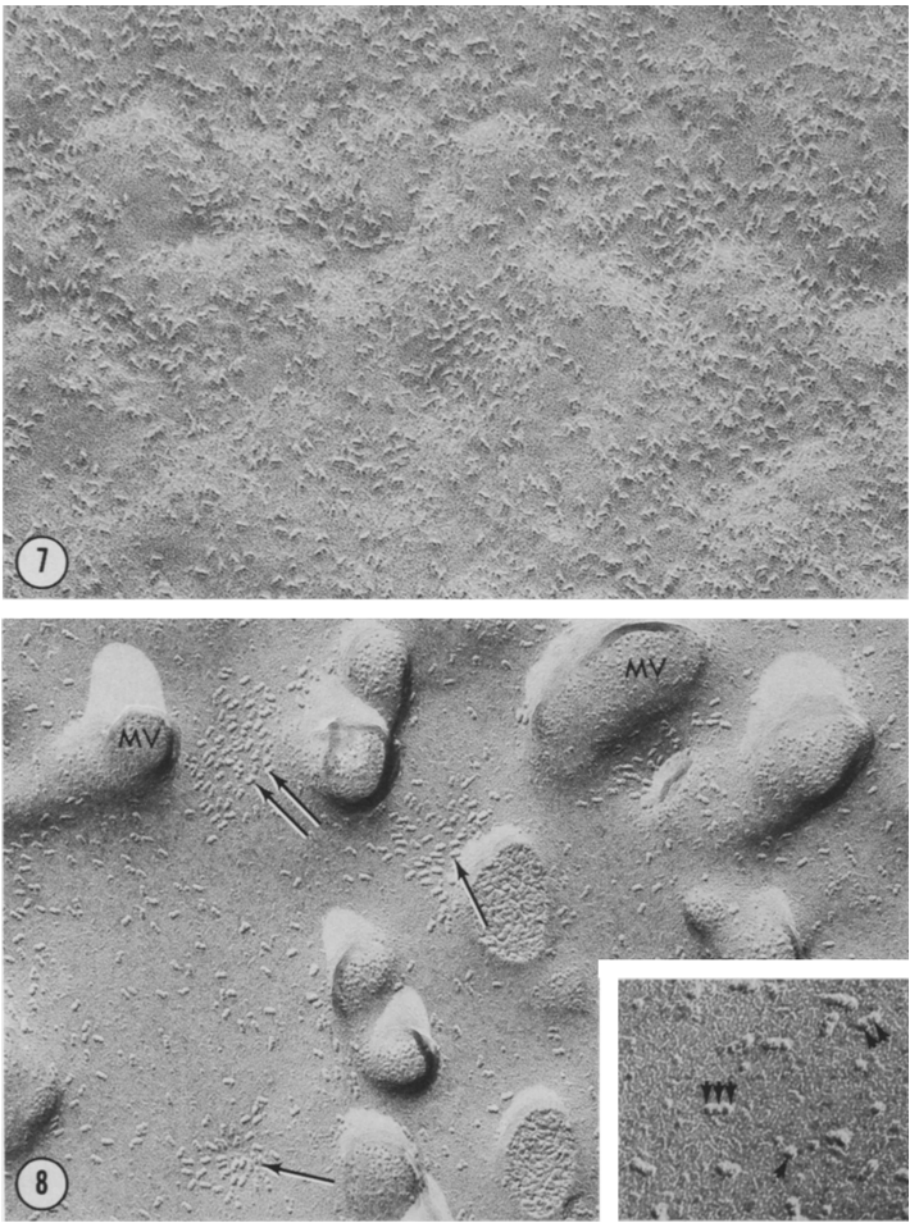


Fig. 8. Another example of fracture face *P* from the luminal membrane of a mitochondria-rich cell. Comparison with Fig.5 illustrates the variable frequency of rod-shaped particles observed. Note also that rod-shaped particles are sometimes found in groups (*arrows*) and that occasionally they display a tendency to orient parallel to each other (*double arrows*). Few rod-shaped particles are associated with the membrane of microvilli (*MV*). The insert shows a high magnification view of such a membrane illustrating that in some instances globular particles are also found (*single arrowhead*) and that in some cases rod-shaped particles appear to have two (*double arrowheads*) or three (*triple arrowheads*) globular subunits. (70,000 × ; insert, 140,000 ×)

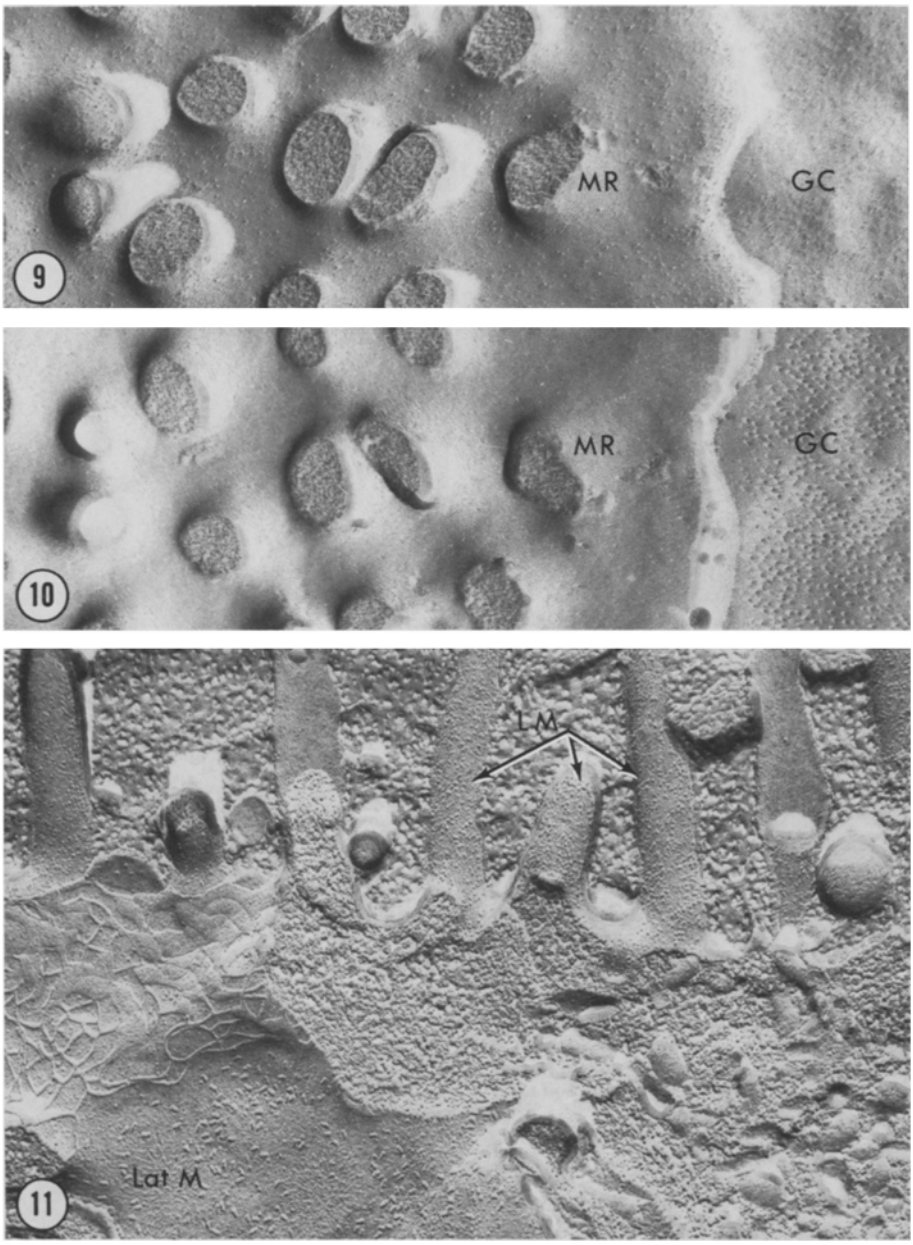
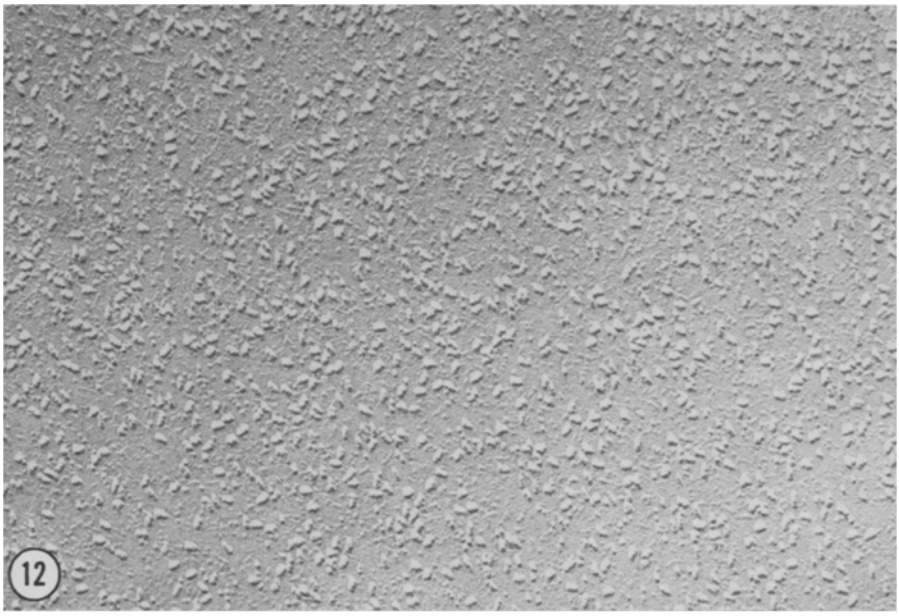


Fig. 9. Fracture face *P* of the luminal membrane from a mitochondria-rich cell (*MR*) with finger-like microvilli. The luminal membrane of this form of mitochondria-rich cell has globular particles and few, if any, rod-shaped particles. Luminal membrane of adjacent granular cell, *GR*. (40,000×)

Fig. 10. The complementary fracture face *E* for the region of membrane shown in Fig. 9. This fracture face of the mitochondria-rich cell (*MR*) has very few particles while the adjacent granular cell (*GR*) has many intramembrane particles. (40,000×)



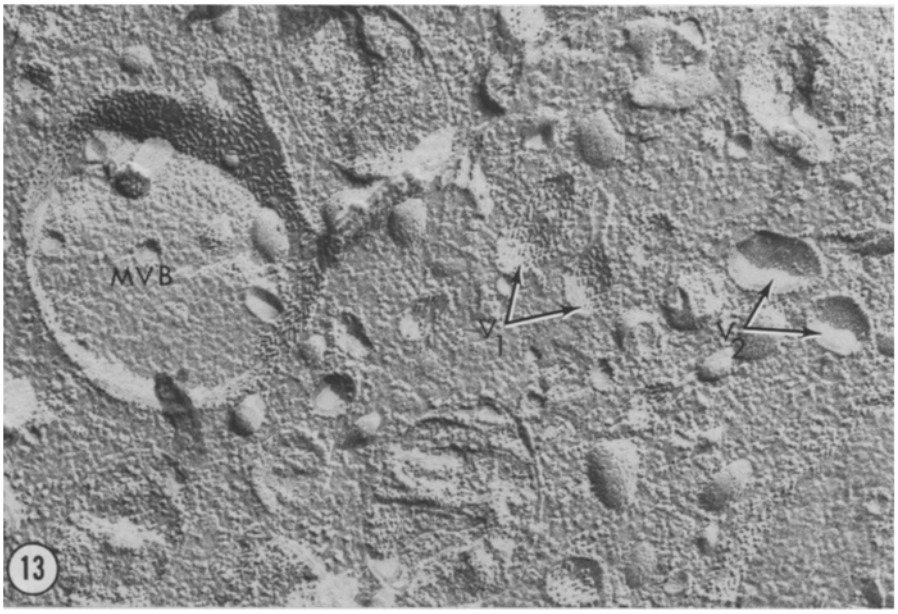


Fig. 11. The lateral membranes (*Lat M*) of mitochondria-rich cells have rod-shaped particles even when only globular particles are found in their luminal membranes (*LM*). (50,000×)

Fig. 12. Basal membranes of mitochondria-rich cells also have rod-shaped particles on their fracture face *P*. (70,000×)

Fig. 13. Rod-shaped particles are also found in the membranes of multivesicular bodies (*MVB*) and some tubular vesicles (V_1). However, some small vesicles (V_2) have only globular intramembrane particles. (50,000×)

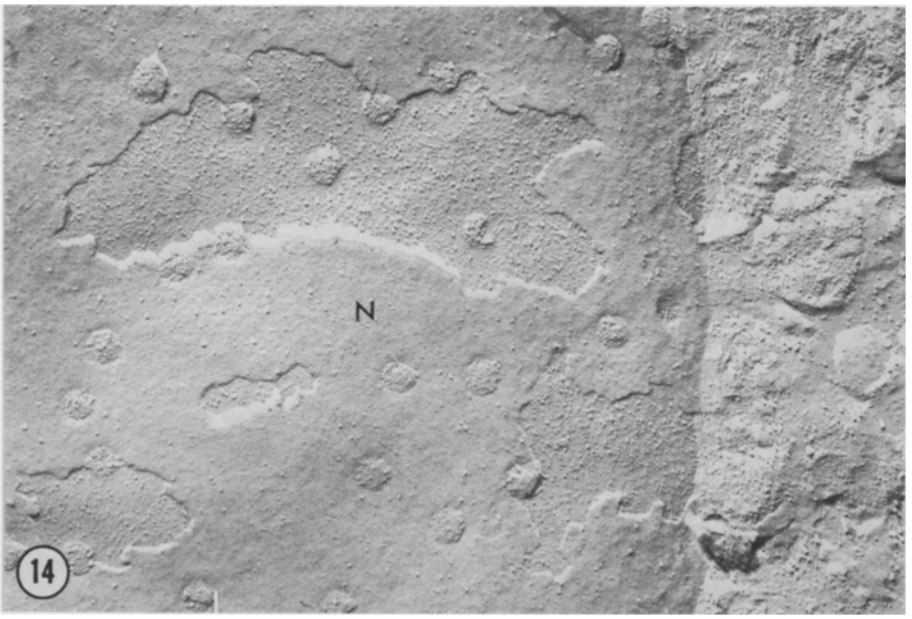


Fig. 14. The nuclear membranes (*N*) of mitochondria-rich cells have only globular intramembrane particles. (50,000 ×)

Discussion

Freeze-fracture electronmicroscopy has proven to be an extremely useful approach for the investigation of membrane structure. Characteristic and distinctive structural features have been described with freeze-fracture in those specialized regions of membrane involved in cell junctions (for reviews describing these examples *see* McNutt & Weinstein, 1973, and Staehelin, 1974). In addition, a growing number of nonjunctional membrane specializations have been reported including those associated with cilia (Gilula & Satir, 1972), sperm membrane (Friend & Fawcett, 1974), pinocytotic vesicles (Orci & Perrelet, 1973), mucocyst secretion (Satir, Schooley & Satir, 1973), and luminal membrane of mammalian urinary bladder (Staehelin, Chlapowski & Bonneville, 1972). Another recently described membrane structural specialization is the ordered aggregation of intramembrane particles found in neurohypophyseal hormone stimulated amphibian bladders (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & DiScala, 1975). In all of these specializations the most remarkable feature has been the arrangement of intramembrane particles. Of special interest in the present investigation is the observation

that the actual shape of individual particles rather than the arrangement of particles can represent a characteristic feature of certain mitochondria-rich cell membranes. Although in some instances these particles appear to have globular subunits, in most cases they are visualized as a single, rod-shaped unit. It therefore seems most appropriate to describe this specialization as a rod-shaped particle rather than as a linear array of particles. However, the existence of possible subunits may prove to be important in the eventual understanding of the rod-shaped particle's assembly mechanism.

Intramembrane particles have been found to be roughly globular in shape for nearly all membranes which have been examined with freeze-fracture. Elongated intramembrane particles have been reported in insect flight muscle (Smith & Aldrich, 1971), but these particles differ in size quite significantly from the rod-shaped particles of the mitochondria-rich cell. However, elongated particles which appear to be similar in size and shape to the rod-shaped particles of the toad bladder mitochondria-rich cell have recently been described in a freeze-fracture study of the rat renal collecting tubule (Humbert, Pricam, Perrelet & Orci, 1975). This observation has been independently confirmed in our own laboratory (Harmanci, Wade & DiScala, *unpublished observations*) and indicates that the amphibian bladder and mammalian renal collecting tubule may not only have in common certain functional properties but may also possess similar distinctive membrane structural specializations.

The membrane structural features of the mitochondria-rich cell are of interest not only because of their distinctively shaped intramembrane particles but because the fracturing properties of the mitochondria-rich cell's luminal membrane differs radically from that of adjacent granular cells. Whereas in the case of the granular cell luminal membrane the majority of particles are found on fracture face *E* (Wade *et al.*, 1975), in the case of the mitochondria-rich cell nearly all particles are found on fracture face *P*. Although at this time the functional significance of this observation is obscure, it is reasonable to conclude that this striking difference in membrane fracturing properties in some way reflects a cell-type specific membrane specialization as do the rod-shaped particles of the mitochondria-rich cell.

In view of the rod-shaped particle's distinctive appearance and characteristic association with only certain membranes of the mitochondria-rich cell, it is possible that rod-shaped particles represent a unique type of intramembrane protein and quite possible that they relate to a particular distinctive function of mitochondria-rich cells. Although it is premature to

speculate as to which specific activities might involve the rod-shaped particles, a possible clue to their functional significance may be the observation that the presence or absence of rod-shaped particles in the luminal membrane is correlated with the occurrence of two morphologically distinct forms of mitochondria-rich cell.

Based on the appearance of mitochondria-rich cells in thin sections Voûte *et al.* (1972) noted that they can be classified as either in a "resting" or "stimulated" state and that the frequency of stimulated cells was increased by aldosterone exposure. Stimulated mitochondria-rich cells were characterized by a denser cytoplasm containing more mitochondria and an increased number of vesicles in the apical region of the cell when compared to resting cells. Figs. 1 and 2 appear to correspond to these two states and thus support the proposal of Voûte *et al.* (1972) that two structural classes of mitochondria-rich cell are represented in the toad urinary bladder.

The membrane structural features observed in the present investigation provide additional evidence for this proposal since the surfaces of some mitochondria-rich cells were found to be characterized by finger-like microvilli while others had irregular, ridge-like microvilli. These two strikingly different patterns can also be recognized in published scanning electron micrographs of the toad bladder (*see* Fig. 9 of Danon *et al.*, 1974). An examination of luminal membrane structure with freeze-fracture also reveals that the cells with ridge-like microvilli invariably have many rod-shaped intramembrane particles while cells with finger-like microvilli have few, if any, rod-shaped particles. Since examination of the same cell with both thin sections and freeze-fracture is not technically feasible, it is not possible to state unequivocably that the two classes of mitochondria-rich cells characterized here by their membrane structural features correspond precisely to the two states of mitochondria-rich cell described by Voûte *et al.* (1972). This is, however, an attractive hypothesis. Based on a comparison of thin section and freeze-fracture images, it is likely that the cells possessing ridge-like microvilli and rod-shaped particles in their luminal membrane correspond to those with dense cytoplasm which were identified as "stimulated" by Voûte *et al.* (1972) while the cells with finger-like microvilli correspond to the "resting" state.

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