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

I. The Granular Cell

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Summary. Examination of the toad urinary bladder by freeze-fracture electron-microscopy demonstrates structural specialization of the granular cell's luminal membrane compared to its basal membrane. Although both membranes appear to possess about 1,700 intramembranous particles per μm^2, those of the luminal membrane tend to be significantly larger in size. In addition, the fracturing properties of the two membranes are markedly different: the majority of particles are found on fracture face B (outer membrane face), in the case of the luminal membrane, and the majority are found on fracture face A (inner membrane face), in the case of the basal membrane. While the two fracture faces of the basal membrane possess a similar distribution of particle sizes, in the case of the luminal membrane the B face was found to possess particles generally larger than those found on the A face. It was established that the probability of luminal membrane particles adhering to face B instead of face A is closely correlated with the size of the particle. The structural specialization of the granular cell's luminal membrane may have an important relationship to the characteristic permeability properties of this membrane and the capacity of this cell type to respond physiologically to the hormone vasopressin.

Over the last twenty years the toad urinary bladder has proved invaluable as a model system for the study of fundamental transport systems and the mechanisms whereby hormones such as vasopressin and aldosterone modulate these systems. Since the epithelium of the toad urinary bladder consists of four morphologically distinct cell types, there is a strong possibility that these diverse cell types differ with respect to their transport physiology and/or with respect to their response to hormones. In addition, since the toad bladder is capable of directed mucosal-to-serosal transport of sodium, it is possible that features which distinguish the basal and lateral membranes from the luminal membrane may be important elements of such a transport system.

The granular cell is the predominant cell type of the toad urinary bladder epithelium and is estimated to represent from about 70% (Danon, Strum & Edelman, 1974) to 83% (Keller, 1963) of those cells forming the luminal surface of the bladder. Granular cells represent an even greater fraction of the surface area since they present a broad surface to the lumen compared to the thin neck of luminal membrane characteristic of the mitochondria-rich and goblet cells. Most available evidence indicates that it is the luminal membrane of the granular cell whose permeability is dramatically increased in response to the hormone vasopressin (Peachey & Rasmussen, 1961; DiBona, Civan & Leaf, 1969).

The freeze-fracture, freeze-etch technique produces fractures within the lipid interior of membranes revealing a unique view of the interior of membranes (Branton, 1966). These fracture faces have been found to possess distinctive particles which project above a smooth plane. Generally many more particles are found on the fracture face A or inner membrane face than the fracture face B or outer membrane face (Branton & Deamer, 1972). Very early in the development of the freeze-fracture technique it was appreciated that these particles might be of considerable physiological importance since metabolically active membranes such as those of chloroplasts were found to possess many particles (Branton, 1969) while those from myelin possessed few (Branton, 1967) and artificial, pure lipid membranes had none at all (Staehelin, 1968).

Considerable evidence has now accumulated which indicates that these particles represent hydrophobic protein containing moieties which are intercalated within the lipid plane of the membrane (MacLennan, Seeman, Iles & Yip, 1971; Pinto da Silva, Douglas & Branton, 1971; Tillack, Scott & Marchesi, 1972; Tourtellotte & Zupnik, 1973), although the possibility remains that in some instances particles may not represent protein (Vergara, Zambrano, Robertson & Elrod, 1974). The observations of Pinto da Silva (1973) suggest that the protein particles may represent pores which interrupt the lipid portion of the membrane and permit the transmembrane flux of hydrophilic molecules.

The present examination of the toad urinary bladder using the freeze-fracture technique was undertaken in order to characterize membrane structural specializations in an organized transporting epithelium. In particular, it was of interest to describe those structural features which might distinguish the luminal membrane of the granular cell with its special permeability properties from the lateral and basal membranes of the same cell type.

Materials and Methods

For these studies tissue was 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 the granular cells. Urinary bladders were mounted as sacs tied to tubes as previously described (Wade, Revel & DiScala, 1973). Tissue was bathed in an aerated Ringer's solution consisting of (mM): 111 NaCl, 3.5 KCl, 2.5 $NaHCO_3$ and 1 $CaCl_2$.

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 cadodylate. Tissue was frozen in either liquid Freon 22 cooled by liquid nitrogen or in undercooled liquid nitrogen. Undercooled nitrogen (liquid nitrogen cooled below its boiling point) was produced by placing a styrofoam insulated container of liquid nitrogen in a vacuum chamber and evacuating with a mechanical vacuum pump until the rapid evaporation cooled the nitrogen to its freezing point (Glover & Garvitch, 1974). The nitrogen was then removed from the vacuum chamber and the tissue frozen before the nitrogen reached its boiling point. Unfixed tissue was quickly frozen in undercooled nitrogen without exposure to cryoprotectants. The tissue was fractured in a Balzers freeze-etch unit BA 360 M or a BAF 301 (Balzers High Vacuum, Liechtenstein). 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). The platinum evaporation device was oriented with an angle of 43° with respect to the specimen table.

Replicas were examined with a Philips 200 or RCA EMU 4B electron-microscope. Particle measurements were made without prior knowledge of the membrane source from micrographs enlarged to 300,000×. Relatively flat regions of membrane without microvilli or pinocytotic vesicles were selected for measurement. The diameter of particles was estimated by measuring the width of the shadowed cap perpendicular to the direction of shadowing with a lens equipped with a micrometer grating. At least 250 particles were measured for each membrane histogram. Only particle diameter classes representing more than 1% of the total particles measured were used in the calculation of particle partition coefficients (K_p).

Observations

The Luminal Membrane

With a knowledge from thin sections of those features characteristic of each membrane and cell type it is possible to use fractures which include both membrane and cytoplasm to identify the various membranes found in the toad bladder. The granular cell has a characteristic morphological appearance in thin section (*GC*, Fig. 1) which has previously been described in detail (Peachey & Rasmussen, 1961; Choi, 1963). It derives its name from the distinctive granules (*G*, Fig. 1) which line the apical cytoplasm of the cell. Most other features of the granular cell appear

to be rather typical of an epithelial cell and in sharp contrast to the mammalian urinary bladder. The toad urinary bladder shows no evidence of the luminal membrane plaques characteristic of mammalian urinary bladder (Porter, Kenyon & Badenhausen, 1967; Staehelin, Chlapowski & Bonneville, 1972). In thin section the luminal membrane appears to be a typical unit membrane with only the usual coat of glycocalyx (Fig. 2*a*) distinguishing it from the lateral (Fig. 2*b*) or basal membrane of the cell.

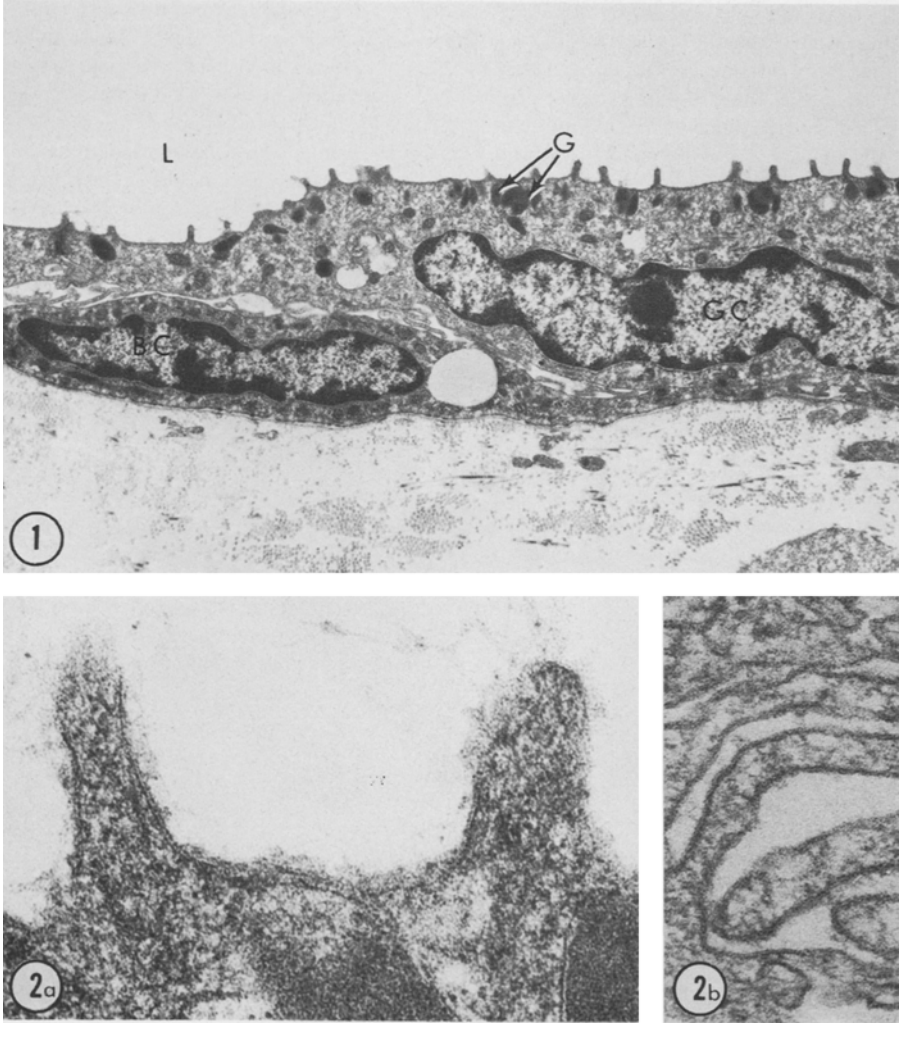


Fig. 1. The characteristic appearance of the granular cell (*GC*) in thin sections. Distinctive granules (*G*) line the apical cytoplasm. Lumen of bladder, *L*; basal cell, *BC*. (8,000×)
Fig. 2. (*a*) High magnification of the luminal membrane of the granular cell as seen in thin sections. (*b*) High magnification of the lateral membrane of the granular cell as seen in thin sections. (100,000×)

Microvilli Shape. Although in thin section the luminal surface of the granular cell would appear to have typical finger-like microvilli (Fig. 1), a surface view of extensive regions of the membrane as provided by scanning electron-microscopy (Danon *et al.*, 1974; Davis, Goodman, Martin, Matthews & Rasmussen, 1974) or favorable fractures reveals that the microvilli are frequently a series of interconnecting ridges (Fig. 3). As

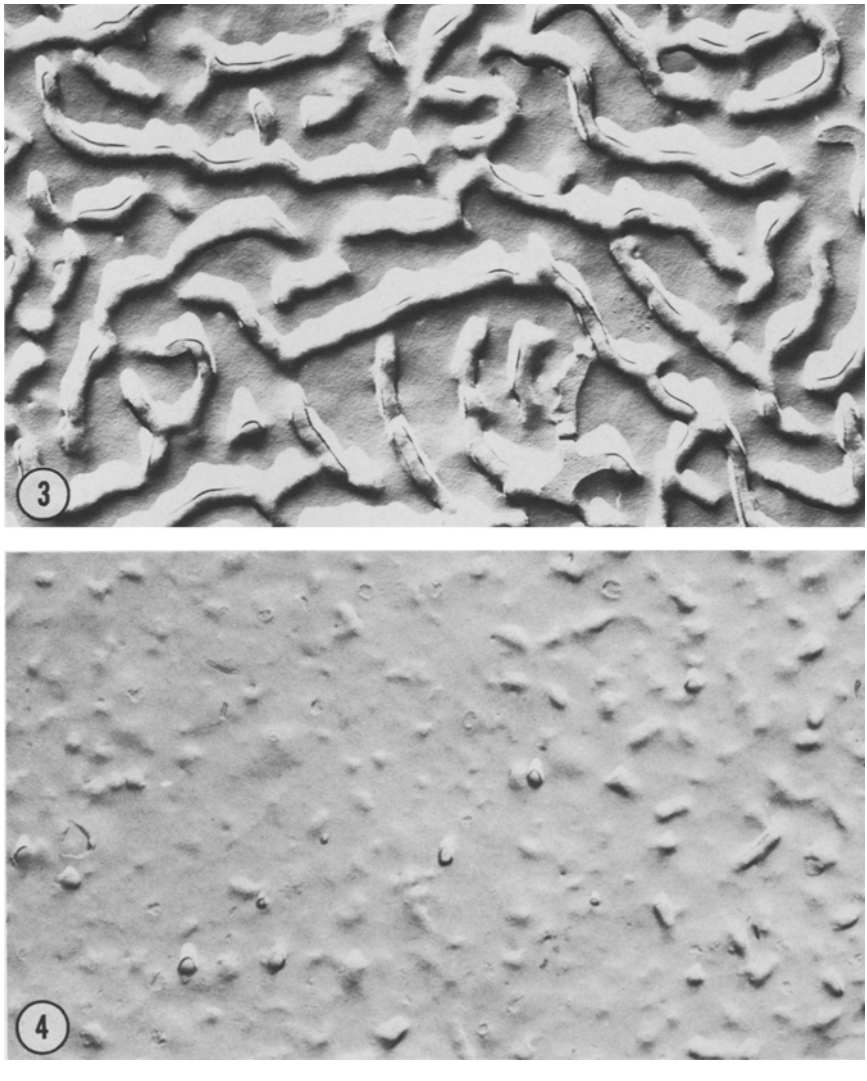


Fig. 3. Low magnification of the luminal membrane as seen in freeze-fracture (fracture face A). In this relatively unstretched bladder the microvilli are in the form of interconnecting ridges. (15,500 ×)

Fig. 4. Similar to Fig. 3 except that in this relatively stretched bladder the microvilli become shortened, finger-like projections. (12,500 ×)

has been previously reported (Gfeller & Walzer, 1971) the granular cell is very sensitive to stretch and when the bladder is stretched the microvilli may become shortened, finger-like projections (Fig. 4).

Particle Distribution and Frequency. The luminal membrane of the granular cell in contrast to most membranes previously examined using freeze-fracture possesses a high density of large particles (*P*, Fig. 5) on its fracture face B. This finding is characteristic of this membrane only and is not characteristic either of luminal membranes of the other cell types found in the toad bladder[1] or of the lateral-basal membrane of the granular cell. These particles are found in a completely random distribution but are usually less densely distributed on the tips of microvilli (*MV*, Fig. 5). The fracture face A of this membrane possesses a distribution of depressions (insert, Fig. 6) complementary to the particles of the B face with many depressions found in the flat, intervillous regions and few depressions on the tips of microvilli (*MV*, Fig. 6). In addition, there is a low density of relatively small particles (*P*, Fig. 6) on this fracture face.

Particle Distribution in Unfixed Tissue. Although unfixed tissue which has not been immersed in cryoprotectants is disrupted by the formation of ice crystals when frozen, we have been able to obtain significant regions of intact luminal membrane by freezing small pieces of tissue rapidly in undercooled liquid nitrogen (Glover & Garvitch, 1974). The fracture faces obtained from this tissue are essentially identical to those obtained from tissue which has been fixed and immersed in glycerol with the majority of particles still found on face B of the luminal membrane (Fig. 7). Thus this distinctive feature of the luminal membrane does not appear to be related to fixation or exposure to glycerol.

Intracellular Granules. In the cytoplasm adjacent to the luminal membrane are the characteristic granules from which this cell type derives its name. Freeze-fracture reveals that these granules have a disc-like shape and that their membranes are virtually bare of intramembranous particles. Both the concave fracture face A (G_A, Fig. 8) and the convex fracture face B (G_B, Fig. 8) have few particles.

Although these apical granules do not possess the large intramembranous particles characteristic of the luminal membrane, spherical vacuoles are occasionally found which do possess these particles (*P*, Fig. 8). Whether these vacuoles are involved in exocytotic or endocyto-

1 Wade, J.B. 1975. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique. II. The Mitochondria-rich cell. *(In Preparation.)*

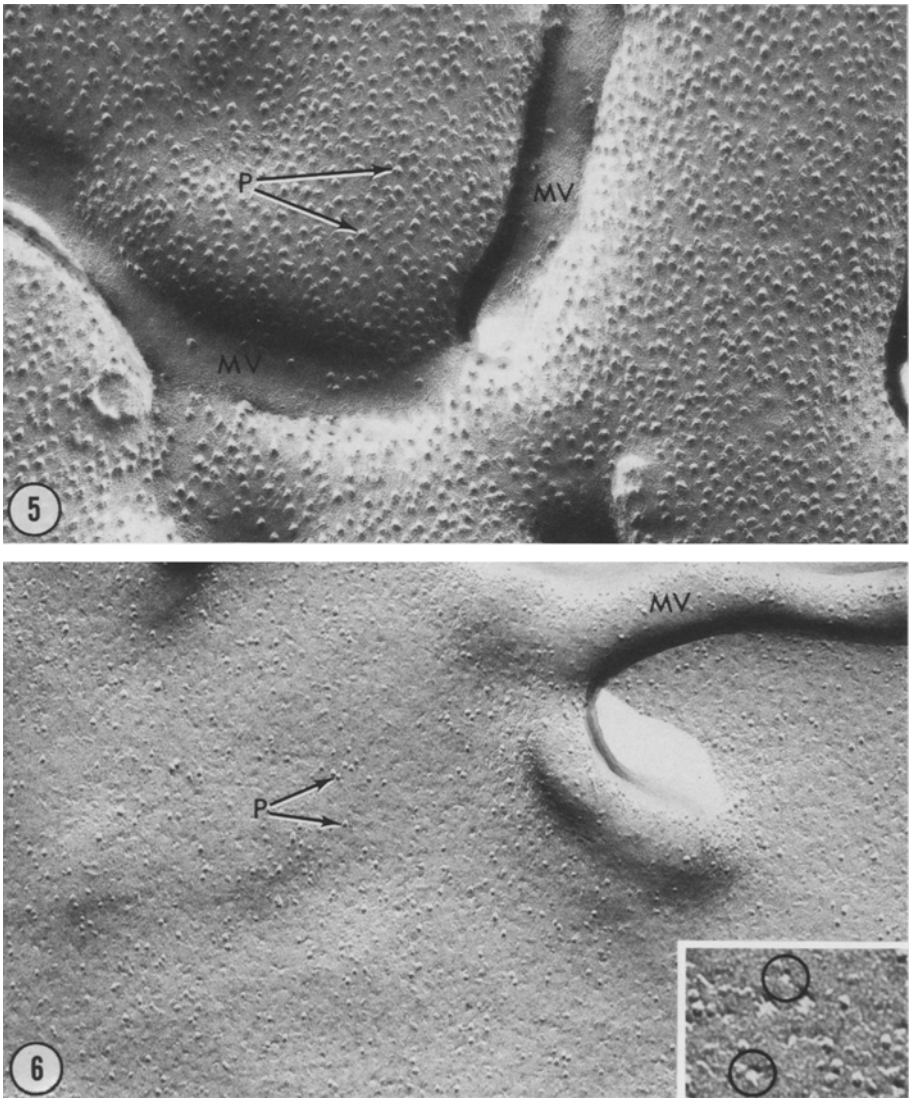
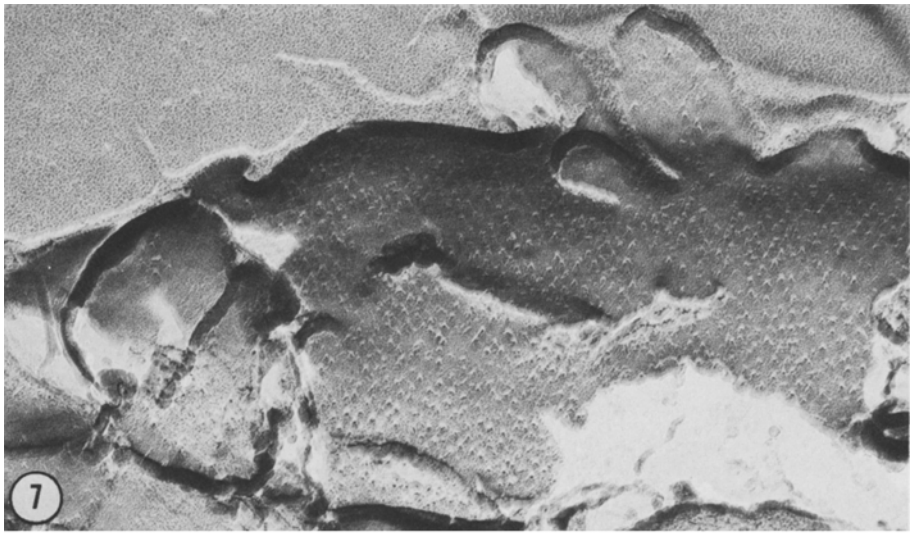


Fig. 5. Fracture face B of the luminal membrane possesses a high density of large particles (*P*). These particles are less densely distributed on the tips of microvilli (*MV*). (75,000 ×)
Fig. 6. Fracture face A of the luminal membrane possesses a relatively low density of generally smaller particles (*P*). There are also depressions which are seen most clearly in the insert (circled). Depressions are less frequently observed on the tips of microvilli (*MV*). (75,000 × ; insert, 150,000 ×)

tic events is not known, but circular pockets suggestive of membrane fusion events are found on luminal membrane fracture faces (insert, Fig. 8). We have not observed the characteristic particle arrays found in other systems to be associated with sites of exocytosis (Satir, Schooley & Satir, 1973) and endocytosis (Orci & Perrelet, 1973).



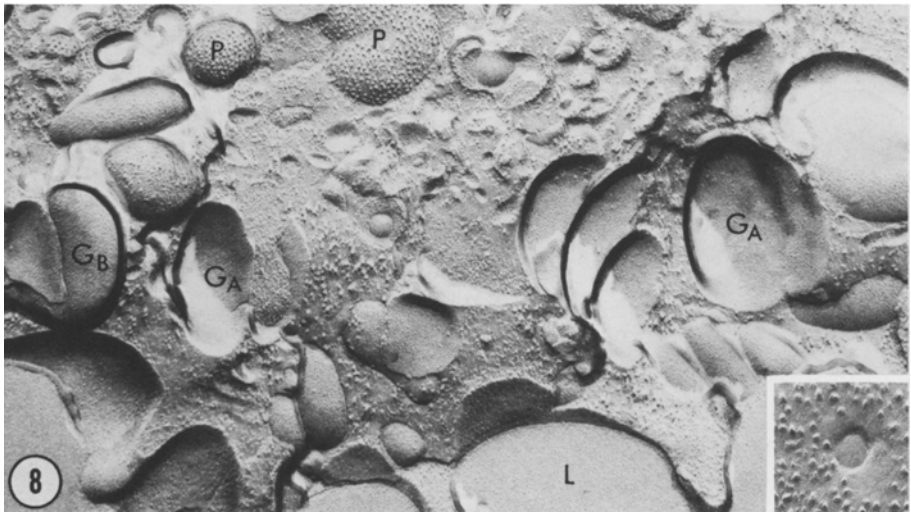


Fig. 7. Fracture face B of the luminal membrane from tissue that has not been fixed or treated with glycerol. Density and appearance of particles is similar to that observed in fixed material (compare with Fig. 5). (70,000×)

Fig. 8. In the apical cytoplasm of the granular cell are the granules characteristic of this cell type. Both the fracture face A (G_A) and fracture face B (G_B) of these granules have few particles. Occasionally spherical vacuoles are found in the cytoplasm which do possess particles (*P*) similar to those on the B face of the luminal membrane. Sometimes circular discontinuities of the luminal membrane are observed (insert) which appear to represent membrane fusion events. Lumen of bladder, *L*. (38,000×; insert, 60,000×)

The Basal and Lateral Membranes

In contrast to the luminal plasma membrane the basal and lateral membranes of granular cells possess a distribution of particles similar to that of most membranes previously described using the freeze-fracture technique. In the case of the lateral membrane there is a large number of randomly distributed particles on fracture face A (*A*, Fig. 9) and a much lower density of particles on fracture face B (*B*, Fig. 9). An identical fracture pattern is observed with the basal membrane face A (Fig. 10) and face B (Fig. 11). Occasionally the invaginations of pinocytotic vesicles are found in both basal membranes (*PV*, Fig. 11) and lateral membranes (*PV*, Fig. 9), and such invaginations possess few intramembranous particles.

Quantitation of Membrane Particles

Although the preceding observations qualitatively demonstrate that freeze-fracture reveals striking structural characteristics of the luminal membrane compared to the lateral and basal membranes, a quantitation of the freeze-fracture observations is required in order to more fully describe the differences between the membranes and establish the statistical significance of these differences. We have compared the luminal membrane quantitatively with only the basal membrane. However, qualitative and preliminary quantitative analysis of particle size and number of particles per unit area of membrane indicates that the lateral and basal membranes do not differ significantly.

Particle Size of Luminal and Basal Membranes. Histograms of the distribution of particle diameters demonstrate that, while all of the fracture faces possess a broad range of particle sizes, the particles of the luminal membrane's B face tend to be larger in diameter than those on the A face (Fig. 12). While 85% of the particles on the B face are of 100 Å or larger diameter, over 70% of the A face particles are less than 100 Å in diameter. On the other hand, an essentially identical size distribution is found on both A and B fracture faces of the basal membrane (Fig. 12). When particle size histograms for the intact membranes are constructed using the diameter data of the respective fracture faces, the luminal membrane is found to possess a significant number of particles throughout the broad range of 70 to 150 Å diameter while the basal membrane's histogram shows a relatively sharp peak at 100 Å diameter (Fig. 12).

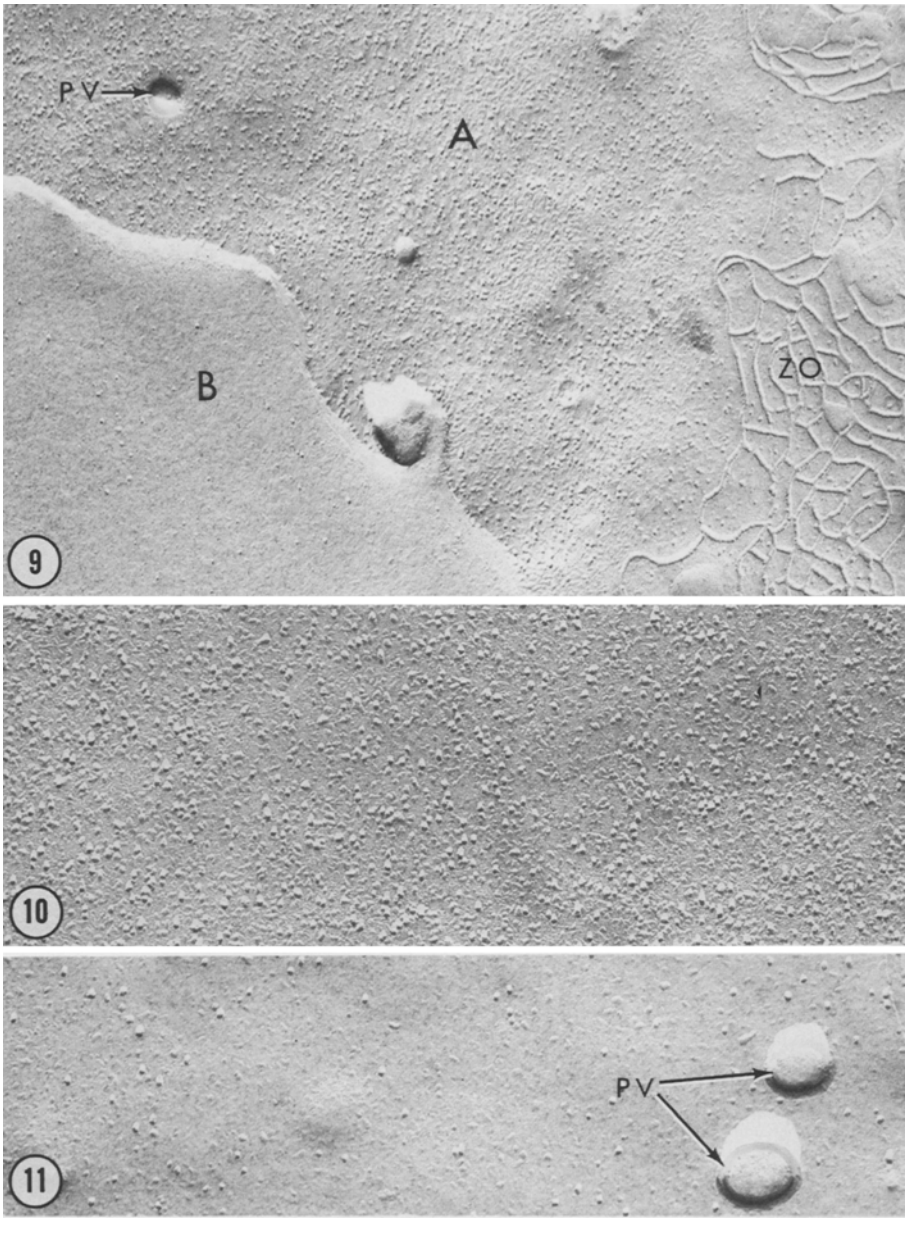


Fig. 9. The lateral membranes of the granular cell possess a large number of particles on fracture face A (*A*) and few particles on fracture face B (*B*). The invaginations of pinocytotic vesicles (*PV*) have few particles. Zonula occludens, *ZO*. (45,000 ×)

Fig. 10. Fracture face A of the basal membrane is similar to that of the lateral membrane in that it possesses a large number of particles. (75,000 ×)

Fig. 11. Fracture face B of the basal membrane possesses few intramembranous particles. The B face of pinocytotic vesicles (*PV*) is usually bare of particles. (75,000 ×)

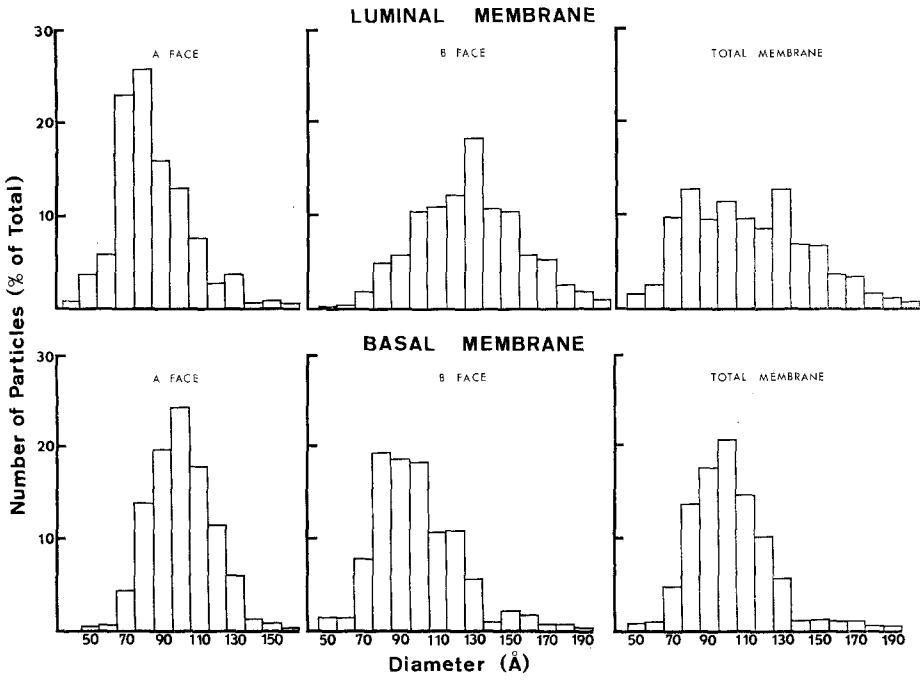


Fig. 12. Histograms demonstrating the particle size distribution of the different fracture faces of the luminal and basal membranes

The same particle diameter data summarized in Fig. 12 can also be utilized to calculate the mean particle diameter for the respective fracture faces and membranes. For the luminal membrane the mean diameter of particles on the A face differs very significantly from the mean diameter of the B face particles ($p > 0.001$), but in the case of the basal membrane there is no statistically significant difference in the mean particle diameter of the two fracture faces (Table 1). Comparison of the A and B fracture faces of the luminal membrane with the respective A and B faces of the basal membrane shows that the particles of the luminal A face are generally

Table1. Mean particle size of luminal and basal membranes

	Luminal membrane	Basal membrane	p
Fracture face A	85 ± 3 (3)	101 ± 2 (3)	< 0.025
Fracture face B	128 ± 3 (4)	99 ± 3 (4)	< 0.001
Total membrane	111 ± 1 (3)	100 ± 2 (3)	< 0.025

Grand mean of measured diameters in Å $\pm$ SEM. Number in parentheses represents the number of different toad bladders sampled. At least 250 particles were measured for each fracture face.

smaller ($p < 0.05$) and the particles of the luminal B face are larger ($p < 0.001$) in diameter than those on similar fracture faces of the basal membrane (Table 1). When the mean particle diameter is calculated for the total membrane (Table 1), there is less difference, but the mean particle diameter of the luminal membrane is significantly greater than that of the basal membrane ($p < 0.025$). Thus, although the striking difference in particle size evident when the luminal B face is compared to other fracture faces is due in part to the tendency of larger particles of the luminal membrane to remain with the B face while smaller particles adhere to the A face, it is also true that the particles of the luminal membrane are in general larger than those of the basal membrane.

Particle Density of Luminal and Basal Membranes. For both membranes the number of particles per area of membrane differs markedly between the A face and the B face (Table 2). But in the case of the luminal membrane more particles are observed on the B face than on the A face ($p < 0.005$), while in the case of the basal membrane many more particles are found on the A face than on the B face ($p < 0.005$). When the particle densities are added in order to calculate the value for the unfractured membranes, no statistically significant difference is found between the luminal and the basal membranes with respect to particle density (Table 2).

Particle Partition Coefficient and Size. Thus, although the intact luminal and basal membranes possess the same density of particles, these membranes differ radically regarding the tendency of their particles to adhere to the A face or B face when fractured. A useful index of the tendency of particles to fracture with the A face relative to their tendency to adhere to the B face has been called by Satir and Satir (1974) the "particle partition coefficient" (K_p):

$$K_p = C_A / C_B \tag{1}$$

where C_A is the density of particles on face A and C_B is the density of particles on face B. The overall K_p for the luminal membrane is 0.59

Table 2. Particle density of luminal and basal membranes

	Luminal membrane	Basal membrane	p
Fracture face A	618 ± 60 (3)	1,290 ± 169 (3)	< 0.025
Fracture face B	1,053 ± 55 (4)	319 ± 64 (4)	< 0.001
Total membrane	1,700 ± 106 (3)	1,663 ± 198 (3)	NS

Mean number of particles per $\mu m^2 \pm$ SEM. Number in parentheses represents the number of different toad bladders sampled. At least 1 μm^2 was analyzed for each fracture face.

while the K_p of the basal membrane is 4.0. While these values are strikingly different, their significance is questionable since each of the membranes clearly possesses many classes of particles and each class might well have a characteristic K_p.

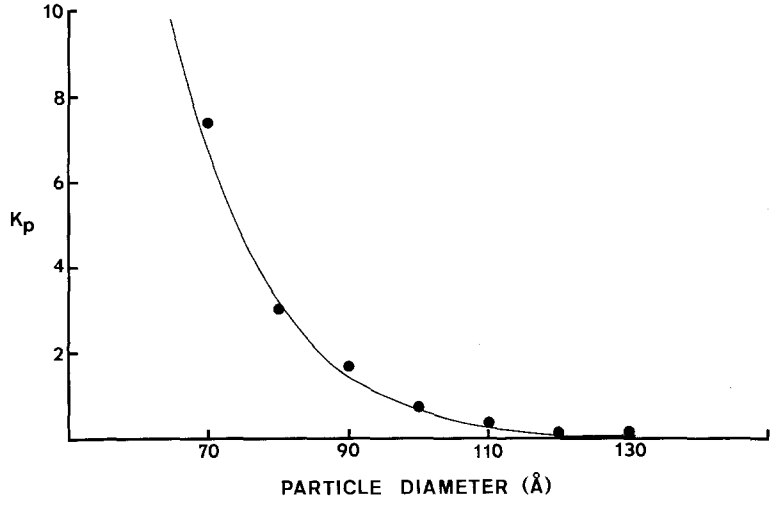


Fig. 13. Relationship between particle partition coefficient (K_p) and particle diameter for fractures of the luminal membrane. The points represent data and the solid line represents the regression Eq. (2) in the text

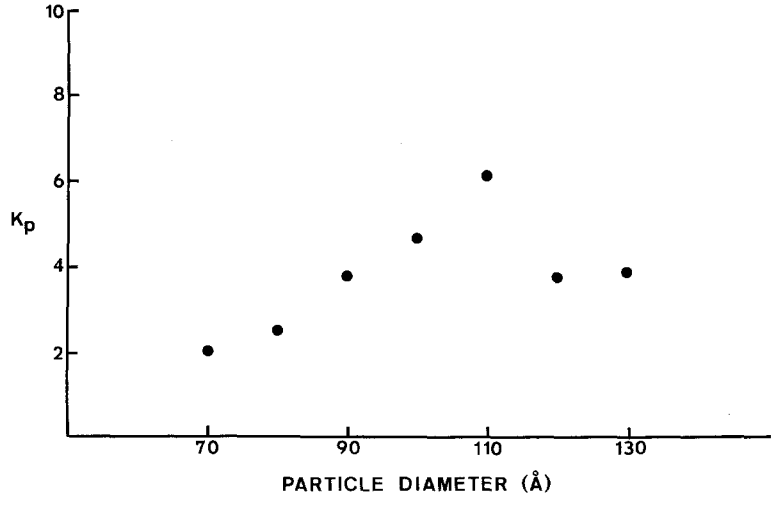


Fig.14. Relationship between particle partition coefficient (K_p) and particle diameter for fractures of the basal membrane

Indeed, this would appear to be the case at least for the luminal membrane since the two fracture faces have such different values for mean particle diameter (Table 1). The very strong correlation between K_p and particle diameter is demonstrated by Fig. 13. The decline of K_p values with increasing particle diameter is described by the equation (solid line, Fig. 13)

$$\ln K_p = 7.1 - 0.073\,D \quad (r = 0.992) \tag{2}$$

where D is the measured diameter of the particles.

A similar representation of K_p values for the basal membrane (Fig. 14) shows that in this respect the membranes are also remarkably different. Instead of the sharp drop in K_p as observed in the case of the luminal membrane there is in the case of the basal membrane a tendency for K_p to rise with increasing particle diameter. However, there is not the very close correlation between K_p and particle diameter as was observed with the luminal membrane.

Discussion

It is clear from our results that, although no significant differences between the luminal and basal membranes are observed in thin sections, very striking differences are revealed by utilizing the freeze-fracture technique. Even though these differences in structure cannot at present be related to specific physiological activities of these membranes, our observations do demonstrate that physiologically diverse plasma membranes of the same cell can differ structurally in several respects and that quantitation of the freeze-fracture results can be most helpful.

While the lateral and basal membranes of the granular cell appear to be structurally similar to many other cell membranes examined with freeze-fracture, the luminal membrane is distinctive. Fracture face B of the luminal membrane is characterized by a high density of large particles while its fracture face A has fewer particles which are smaller in size. Quantitation of the freeze-fracture data demonstrates that, although the luminal and basal membranes have the same density of particles in the intact membranes, the luminal membrane's particles tend to be larger. The luminal membrane also has distinctive fracturing properties. While the tendency of a particle in the luminal membrane to adhere to the A face or B face appears to depend strongly on particle size, there is not a very close correlation with this parameter in the case of the basal membrane.

In view of the distortion of size produced by shadowing (Misra & Das Gupta, 1966), it is clear that the absolute size of particles seen in replicas does not represent the actual size of intramembranous proteins. Nevertheless, the relative diameter distributions of the intramembranous particles very likely do reflect the true variation in size. Thus, with these limitations in mind diameter measurements can be a useful tool in the examination of the relative size distribution of particles in different membranes. Yet, particle size might be expected to be a relatively crude method for characterizing the different particle classes of a membrane. Thus it may be that particles measuring 100 Å in diameter, for example, might represent many different proteins with quite different functions. If this is true, our data would imply that the fracture face to which particles adhere does not necessarily depend upon their specific characteristics but instead is a property of the membrane as a whole. In the case of the luminal membrane of the granular cell the probability of a particle adhering to the B face instead of the A face during fracturing is closely related to the diameter of the particle. A very similar correlation has also been observed in freeze-fracture studies of chloroplast membranes (G.K. Ojakian, *personal communication*). While the K_p of the basal membrane was also found to vary with particle diameter, the absence of a close relationship suggests that factors other than size may be important in the case of this membrane.

Although most regions of membrane were found to possess a randomly distributed population of particles, relatively bare regions were observed on the tips of microvilli and the invaginations of pinocytotic vesicles. Since it has been demonstrated that particles are able to move, at least under certain circumstances (Pinto da Silva, 1972; Ojakian & Satir, 1974), exclusion zones associated with microvilli and pinocytotic invaginations would suggest a significant specialization of these regions.

A high density of particles on the B fracture face has previously been observed for endothelial cell membranes (Dempsey, Bullivant & Watkins, 1973) and the luminal membrane of the rabbit urinary bladder (Staehelin *et al.*, 1972). In the case of endothelial cell membranes, the majority of particles were found on the B face in unfixed material but after glutaraldehyde fixation most particles adhered to the A face (Dempsey *et al.*, 1973). With the toad bladder most of our material has been fixed in glutaraldehyde but in unfixed material we have found a similar distribution of particles on the B face. Thus it would appear that this pattern is an intrinsic property of this membrane and not a result of fixation.

In the case of the rabbit urinary bladder, the similar finding of a high density of relatively large particles on the B face of the luminal membrane may be indicative of an evolutionary relationship to the toad urinary bladder. However, the epithelium of the mammalian urinary bladder is extremely specialized and bears little other resemblance to the epithelium of the toad bladder. The particles of the mammalian bladder are of uniform size and form hexagonal arrays (Staehelin *et al.*, 1972), while those of the toad bladder are heterogeneous in size and do not form arrays. Although it may be that the particles of the toad bladder's luminal membrane serve as anchors for cytoplasmic filaments as has been proposed for the mammalian bladder (Staehelin *et al.*, 1972), the differences in structure and physiology between mammalian and amphibian bladders are so great that it is quite possible that the B face particles found in the toad bladder have a completely different function.

The toad urinary bladder has been studied extensively by physiologists investigating its response to the hormone vasopressin. Although considerable evidence indicates that the permeability of the luminal membrane of the granular cell is dramatically increased by vasopressin (Peachey & Rasmussen, 1961; Carasso, Favard & Valérien, 1962; Civan & Frazier, 1968; DiBona *et al.*, 1969; Macknight, Leaf & Civan, 1971), the precise nature of the membrane alteration(s) remains unclear. It has been suggested that the changes may be brought about by fusion of cytoplasmic granules with the luminal membrane (Masur, Holtzman & Walter, 1972), an event possibly requiring microtubules (Taylor, Mamelak, Reaven & Maffly, 1973). Since our observations show that the membrane of the granules contains few particles, this would indicate that fusion of cytoplasmic granules is not a mechanism whereby many additional intramembranous particles could be introduced into the luminal membrane but such a fusion of granules would add radically different membrane to the luminal membrane.

Recently, Chevalier, Bourguet and Hugon (1974) have used the freeze-fracture technique to examine the frog urinary bladder. Although a comparison with our results indicates that there may be significant quantitative differences in particle density between the frog and toad urinary bladders, the frog bladder also has many more particles on the B face of the luminal membrane than on the A face (Chevalier *et al.*, 1974). Chevalier *et al.* (1974) also report that treatment with oxytocin induced a clustering of particles on the A face of the luminal membrane. A similar clustering

of particles has also been observed in the toad bladder after stimulation with vasopressin.[2]

Although the significance of these particle clusters remains to be determined, it is tempting to speculate that the structure of the granular cell's luminal membrane as revealed by freeze-fracture electron-microscopy may be closely related to its permeability properties. Since it is likely these particles represent proteins intercalated within the lipid interior of the membrane, it is quite possible that in response to vasopressin they become a system of pores or carriers for the transfer of water and/or solutes into the cell.

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