STRUCTURE OF THE TOAD'S URINARY BLADDER AS RELATED TO ITS PHYSIOLOGY

LEE D. PEACHEY, Ph.D., and HOWARD RASMUSSEN, M.D.

From the Department of Zoology, Columbia University, and the Laboratories of The Rockefeller Institute, New York

ABSTRACT

The structure of the urinary bladder of the toad *Bufo marinus* was studied by light and electron microscopy. The epithelium covering the mucosal surface of the bladder is 3 to 10 microns thick and consists of squamous epithelial cells, goblet cells, and a third class of cells containing many mitochondria and possibly representing goblet cells in early stages of their secretory cycle. This epithelium is supported on a lamina propria 30 to several hundred microns thick and containing collagen fibrils, bundles of smooth muscle fibers, and blood vessels. The serosal surface of the bladder is covered by an incomplete mesothelium. The cytoplasm of the squamous epithelial cells, which greatly outnumber the other types of cells, is organized in a way characteristic of epithelial secretory cells. Mitochondria, smooth and rough surfaced endoplasmic reticulum, a Golgi apparatus, "multivesicular bodies," and isolated particles and vesicles are present. Secretion granules are found immediately under the plasma membranes of the free surfaces of the epithelial cells and are seen to fuse with these membranes and release their contents to contribute to a fibrous surface coating found only on the free mucosal surfaces of the cells. Beneath the plasma membranes on these surfaces is an additional, finely granular component. Lateral and basal plasma membranes are heavily plicated and appear ordinary in fine structure. The cells of the epithelium are tightly held together by a terminal bar apparatus and sealed together, with an intervening space of only 0.02 mμ near the bladder lumen, in such a way as to prevent water leakage between the cells. It is demonstrated in *in vitro* experiments that water traversing the bladder wall passes through the cytoplasm of the epithelial cells and that a vesicle transport mechanism is not involved. *In vitro* experiments also show that the basal (serosal) surfaces of the epithelial cells are freely permeable to water, while the free (mucosal) surfaces are normally relatively impermeable but become permeable when the serosal surface of the bladder is treated with neurohypophyseal hormones. The permeability barrier found at the mucosal surface may be represented, structurally, either by the filamentous layer lying external to the plasma membrane, by the intracellular, granular component found just under the plasma membrane, or by both of these components of the mucosal surface complex. The polarity of the epithelial sheet is emphasized and related to the physiological role of the urinary bladder in amphibian water balance mechanisms.

INTRODUCTION

One of the biological membranes which has been given much attention by physiologists in the past few years is the amphibian urinary bladder. This organ is of considerable interest for a number of reasons. It is the phylogenic and functional analog of the collecting tubule of the mammalian kidney, but offers the advantages of being more readily accessible and of being simpler histologically. It is,

under resting conditions, relatively impermeable to water and urea, but becomes more permeable to these simple substances upon being treated with any of a number of neurohypophyseal hormones, including oxytocin, arginine vasopressin, lysine vasopressin, and arginine vasotocin (1–5). In addition, these hormones stimulate the net active transport of sodium ions from the mucosal to the serosal surface of the bladder (6). On the basis of their results (3, 6–9), Leaf and his collaborators have postulated that the hormones change the size of pores in some diffusion barrier at or near the mucosal surface of the bladder.

The present study was undertaken in an effort to determine the structural basis for the physiological properties of this organ and to establish, if possible, the mode of action of the neurohypophyseal hormones thereon. The latter is of particular interest since insulin, which changes the permeability of cells to small molecules such as glucose, and which, like the neurohypophyseal hormones, contains disulfide bonds possibly involved in its chemical mechanism of action, has been reported by Barrnett and Ball (10) to cause increased vesiculation of the plasma membranes of adipose cells.

This paper is a report on the first phase of this study and is concerned with the light and electron microscopic appearance of the toad's urinary bladder and some alterations in this appearance brought about by variations in ionic medium and the application of hormones to the bladder *in vitro*.

MATERIALS AND METHODS

Toads (*Bufo marinus*) were kept at a constant temperature, 20°C, well hydrated but not fed. Tissues for light or electron microscopy alone were either fixed *in situ* in pithed toads, dissected out after a few minutes, and put into bottles of cold fixative, or fixed *in vitro* shortly after removal from the animal. For the *in vitro* studies, bladder halves were removed from pithed animals and mounted according to the method of Bentley (2). Each bladder half was tied over the tip of a hollow glass tube, filled with 3 ml of Ringer's solution diluted 1 to 5 with distilled water (50 mOsM/liter), and then immersed (serosal surface outward) in 20 ml of circulating, aerated Ringer's solution (220 mOsM/liter). The rate of water movement from the mucosal to the serosal solution was measured at intervals of about 20 minutes by the decrease in weight of the bladder and the tube upon which it was mounted.

The Ringer's solution used had the following composition (mM/liter): NaCl, 110; KCl, 2.0; $CaCl_2$, 2.0; Na_2HPO_4, 1.0; NaH_2PO_4, 0.2; $NaHCO_3$, 2.0 (pH 7.4).

Specimens for paraffin embedding were fixed for 16 hours in 5 per cent trichloracetic acid and dehydrated in ethanol. Sections were cut at about 5 μ on a Spencer microtome with a steel knife.

The fixative used for phase and electron microscopic studies was 1 per cent osmium tetroxide dissolved either in Ringer's solution (sometimes with the addition of glucose, 5.5 mM/liter) or in acetate-veronal buffer at pH 7.6 (11) with $CaCl_2$ (0.001 M/liter). The bladders were fixed cold (approx. 4°C) for from 8 to 20 minutes, dehydrated through ethanol-water mixtures, and embedded in *n*-butyl methacrylate with 1 per cent 2,4-dichlorobenzoyl peroxide and 1 per cent dibutyl phthalate (Luperco CDB).

Sections of osmium-fixed tissue were cut on Porter-Blum (12) and Huxley (13) microtomes using glass knives and mounted on carbon support films. Some thin sections were treated with saturated aqueous lead subacetate, $Pb(C_2H_3O_2)_2 \cdot Pb(OH)_2$, to increase contrast, and covered with thin films of collodion. Thick sections for phase microscopy were mounted on glass slides in immersion oil of refractive index 1.460 and covered with glass coverslips.

Some observations were made on living cells obtained by scraping the mucosal surface of the bladder with a razor blade or with the edge of a glass microscope slide. Large numbers of intact epithelial cells are freed from the lamina propria by this procedure. These cells were washed, mounted in aqueous media under coverslips, and then observed by phase microscopy.

Electron micrographs were made on Eastman Contrast lantern slides at 50 kv using RCA EMU-2B and EMU-3F electron microscopes. Light micrographs were made on Eastman M plates using a coil filament lamp with a green filter. A Zeiss × 90 oil immersion phase objective, N.A. 1.25, was used for phase micrographs of methacrylate sections, and a Spencer 32 mm objective and a Zeiss 2 mm oil immersion apochromat objective, N.A. 1.3, were used for light micrographs of paraffin sections.

RESULTS

The histological arrangement of the toad's urinary bladder is illustrated in the light micrograph of a stained paraffin section shown in Fig. 1. Portions of a short loop of the bladder wall are seen sectioned transversely. This bladder was fixed while moderately distended and then emptied during subsequent treatment prior to embedding. In bladders fixed while highly stretched, all transverse dimensions of the bladder wall are proportionally reduced. A major part of the bladder wall consists of

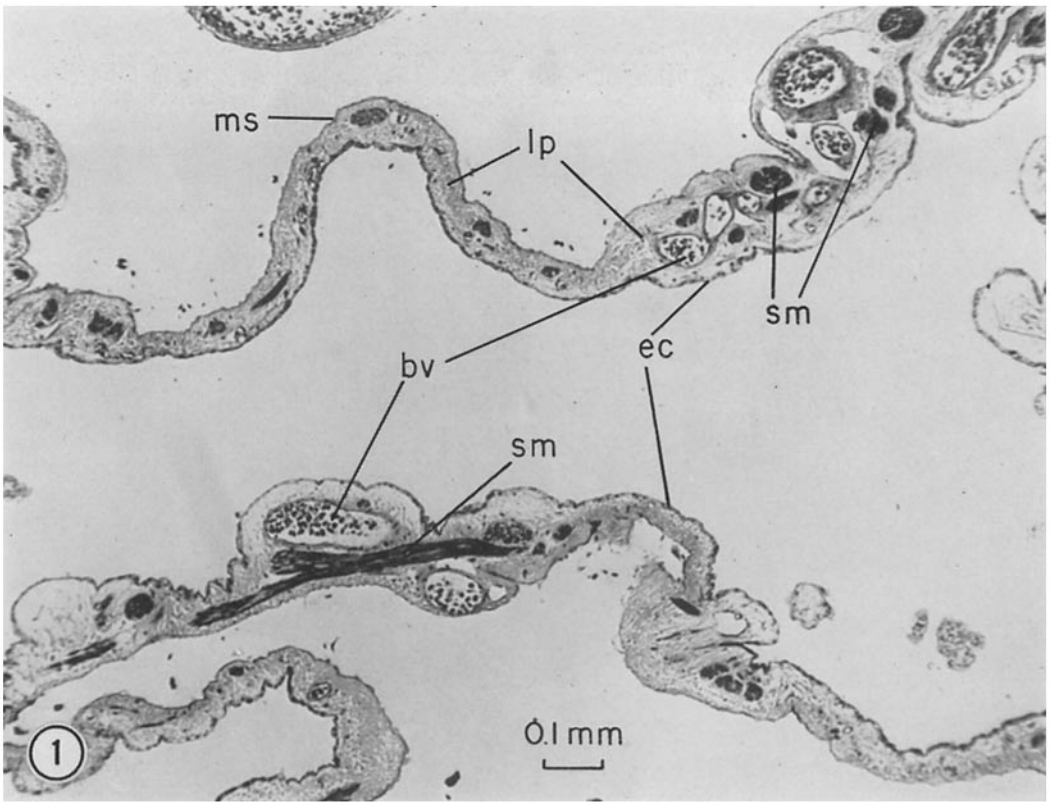


FIGURE 1

Low magnification light micrograph of a paraffin section of the toad's urinary bladder stained with iron alum–iron hematoxylin and counterstained with eosin. The epithelium (*ec*) covers the lamina propria (*lp*) on its mucosal surface. Within the lamina propria are blood vessels (*bv*) and smooth muscle fibers (*sm*). An incomplete mesothelium (*ms*) is found on the serosal side of the bladder wall. Spencer 32 mm objective. × 75.

a relatively thick connective tissue layer, or lamina propria (*lp*), made up largely of collagen fibrils, blood vessels (*bv*), and bundles of smooth muscle fibers (*sm*). The lamina propria varies considerably throughout the bladder, being rather thick where blood vessels and smooth muscle fibers are present and reaching a minimum thickness elsewhere of about 30 μ in bladders fixed while moderately distended. The mucosal surface of the bladder, bounding the lumen, is covered by an epithelium (*ec*) usually between 1 and 10 μ in thickness. This epithelium, shown more clearly in the higher magnification light micrograph in Fig. 2, appears to be one or two cells thick, although in most areas only one layer of cells is found. Even in highly distended bladders, no perforation of this cell layer is found, indicating that the cells are tightly held together at their lateral edges. The serosal surface of the bladder is covered by an incomplete squamous cell layer or mesothelium (*ms*) as seen in Fig. 1. This description is in close agreement with Lavdowsky, who apparently gave the first description of the amphibian urinary bladder (14).

Electron Microscopy

At least three morphologically distinct cell types can be identified in electron micrographs of the epithelium on the mucosal surface of the bladder. Examples of these cell types are shown in the survey electron micrograph in Fig. 3 and in later figures. The overlay of Fig. 3 indicates the boundaries of the cells and identifies the various cell types.

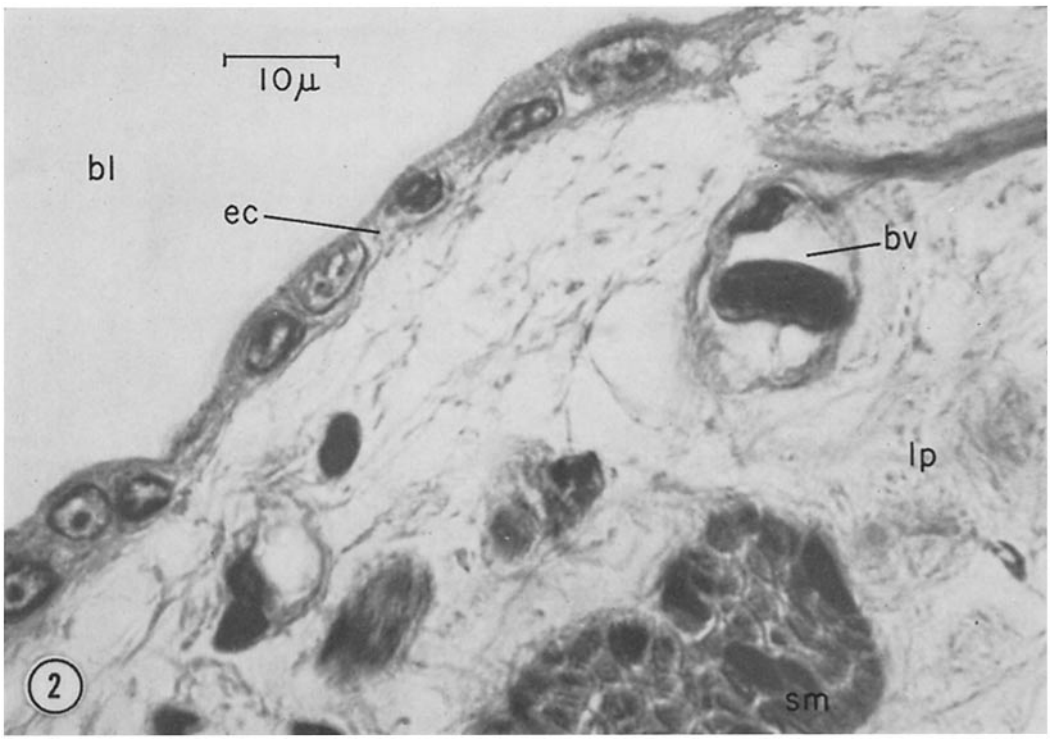


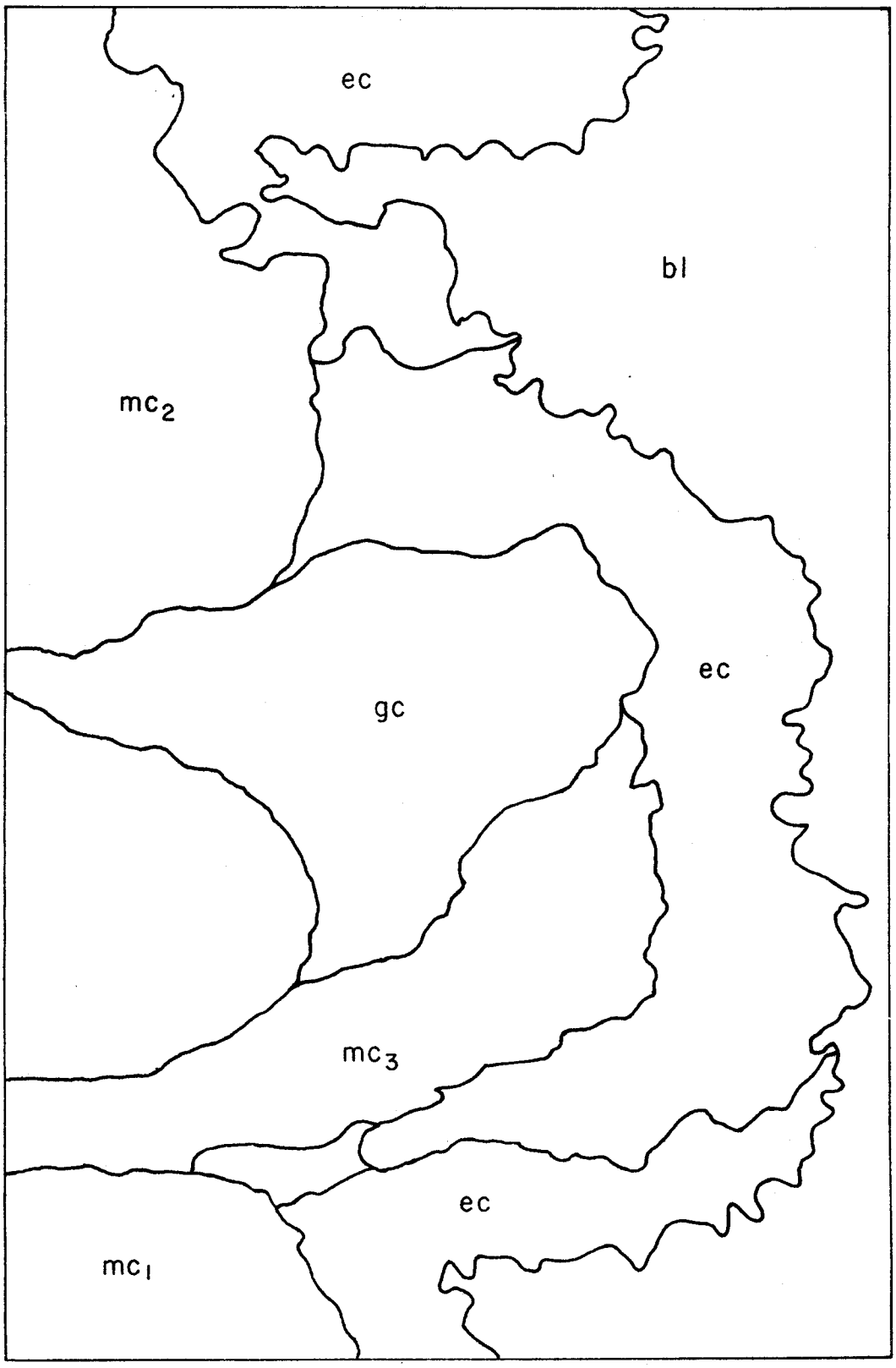
FIGURE 2

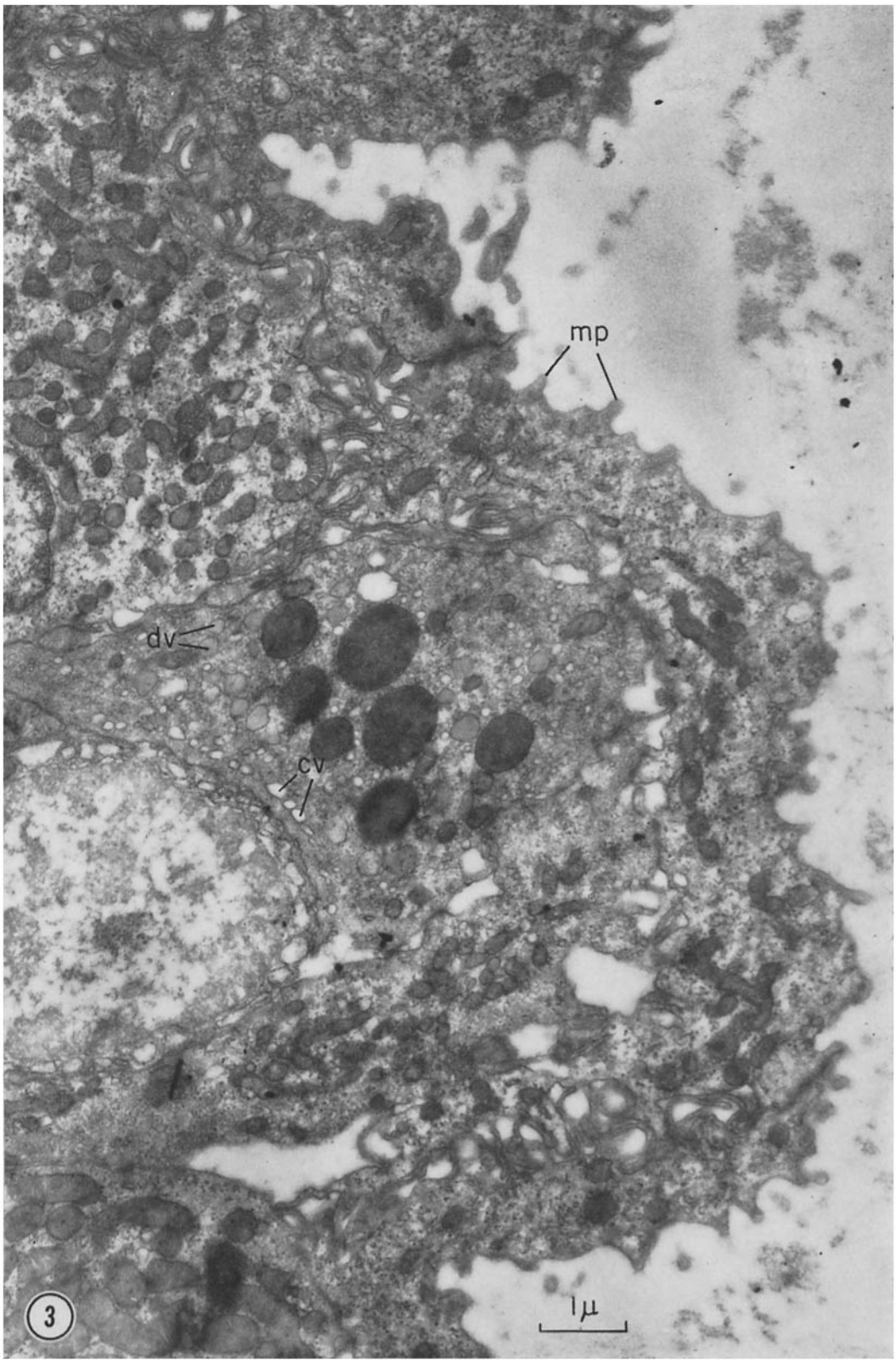
Higher magnification of the section in Fig. 1, showing the epithelial cells (*ec*) bounding the lumen of the bladder (*bl*) on the inner or mucosal surface of the bladder wall. Connective tissue fibrils of the lamina propria (*lp*), a blood capillary (*bv*), and a bundle of smooth muscle fibers (*sm*) are indicated. Zeiss 2 mm oil immersion objective, N.A. 1.3. × 1500.

The most abundant cells are the squamous epithelial cells (*ec*), which form the entire free surface of the portion of the bladder shown in Fig. 3 and, indeed, account for almost the entire area of the free mucosal surface of the bladder. Cells of the other types communicate with the bladder lumen through narrow, neck-like projections forming only a small proportion of the total free surface area (Figs. 4 and 5). For convenience, the term *epithelial cell* will be used here to refer to the more or less flat cells lying with their long axes parallel to the surface of the bladder (the squamous epithelial cells), excluding other types of cells found in the epithelial cell layer. Because of the numerical predominance of this cell type, and because another function can be attributed to at least one of the remaining cell

FIGURE 3

Survey electron micrograph of a normal bladder epithelium illustrating three types of cells found in this cell layer. The overlay indicates the boundaries of the various cells. Three squamous epithelial cells (*ec*) form the surface bounding the bladder lumen (*bl*) in this figure. Microprojections (*mp*) extend from these cells into the lumen. Also indicated are a goblet cell (*gc*) containing large, dense droplets and vesicles with either clear (*cv*) or dense (*dv*) contents. Three cells containing numerous mitochondria are also seen. Within each of these latter cells, the mitochondria are of uniform size, but there is considerable variation in size from one cell to another. The largest mitochondria are found in cell mc_1; cell mc_2 has mitochondria of an intermediate size, and cell mc_3 has the smallest mitochondria in this field. EMU-2B. × 14,000.


ec
bl
mc2
gc
ec
mc3
ec
mc1


mp
dv
cv
1μ
3

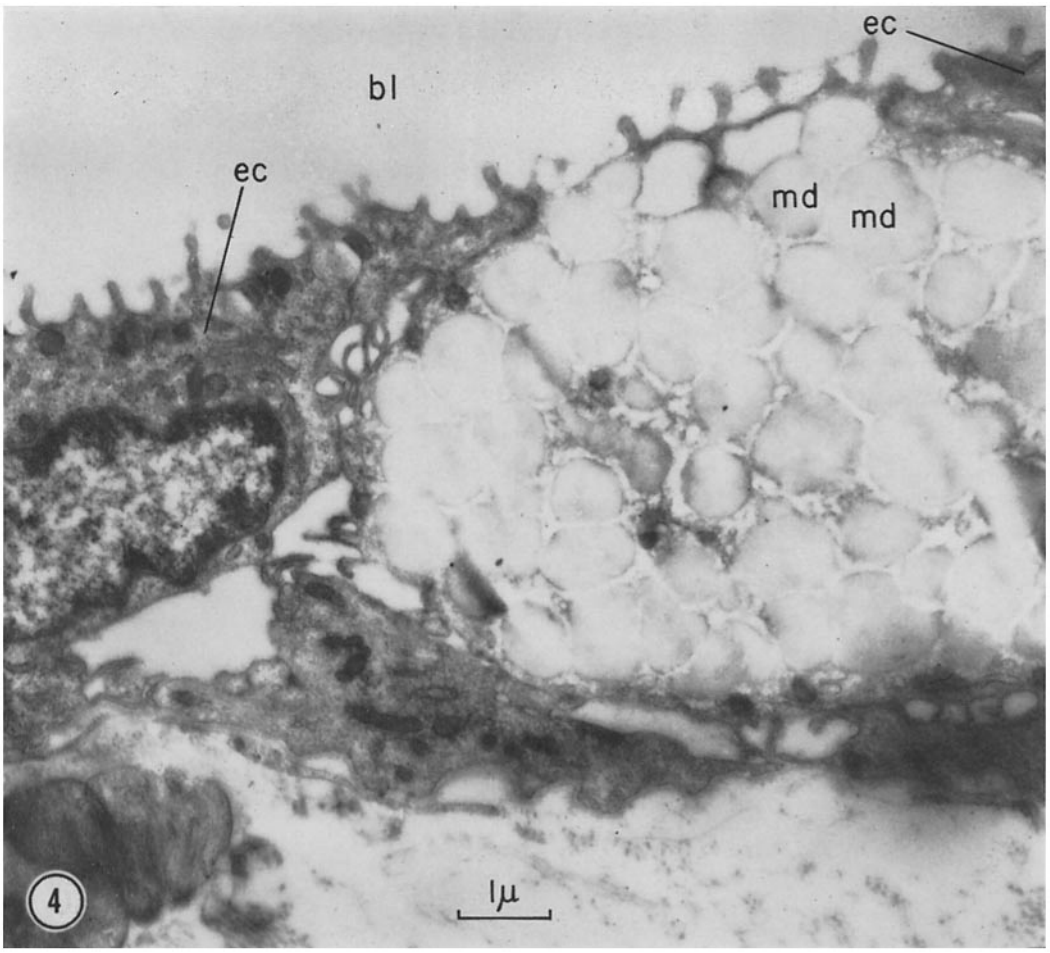


FIGURE 4

Electron micrograph showing a goblet cell packed with mucus droplets (*md*) and flanked by two epithelial cells (*ec*). This goblet cell appears as though ready to discharge its contents into the bladder lumen (*bl*). EMU-2B. × 12,000.

types, it is assumed that these cells are responsible for the unusual and interesting permeability and transport properties of the bladder.

One of the other cell types found in the epithelial cell layer is the goblet or mucus-secreting cell, shown in Fig. 4. The cell labeled *gc* in Fig. 3 also has the morphological characteristics of a goblet cell. These cells contain a few small mitochondria (average width 0.2 μ) and two classes of vesicles, one with a quite clear internum and the other containing a uniform, moderately dense substance (Fig. 3). The most obvious feature of these cells is the presence of large, pale droplets, 0.5 to 1.5 μ in diameter, which can be identified with the mucus droplets seen in light microscope preparations (see *md*, Fig. 4). The fact that these cells sometimes contain these pale droplets (Fig. 4) and sometimes slightly smaller (0.4 to 1.0 μ) dense ones (Fig. 3) has been pointed out by Rhodin and Dalhamn (15) as suggesting stages in mucus formation. Fig. 4 shows a mucus cell apparently just prior to discharging its contents into the lumen of the bladder.

Cells of the third type cannot at present be identified functionally, but they clearly form a class which is morphologically distinct from the epithelial and goblet cells. Examples (*mc*) are shown in Figs. 3 and 5. They contain mitochondria in considerably greater numbers than do either epithelial

or goblet cells. The mitochondria vary considerably in size (from 0.1 to 0.4 μ in width), but those in any one cell are all of approximately the same size (Fig. 3). These cells often have a slightly less dense cytoplasmic matrix than the other cell types, and contain many small particles and isolated vesicles of endoplasmic reticulum. They communicate with the bladder lumen only through narrow necks, as seen in Fig. 5. The significance of this type of cell is not known with certainty. Early histological studies of the goblet cells of the amphibian urinary bladder showed the presence of two types of cells, in addition to the epithelial cells, with different morphological appearances when stained with various agents (16–19). For example, when stained with osmic acid one type appeared dark and coarsely granular, while the other was lighter and homogeneous or finely granular. These cells were considered to be goblet cells in different states of secretory activity, the dark, coarsely granular cells being in a "mucus-filled" state and the lighter cells with finer granulation in a "protoplasmic" state. It is quite possible that the cells with the high mitochondrial content observed in the present study represent the "protoplasmic" cells observed earlier and are goblet cells in various early stages of secretory activity. This problem will not be pursued further here.

Fine Structure of the Epithelial Cells of the Bladder

Cytoplasm: The cytoplasm of the epithelial cells is similar in fine structure to that of many other types of cells that have been described (Fig. 6). Nuclei are often proximally (basally) located, although the thinness of most of the epithelial cells does not

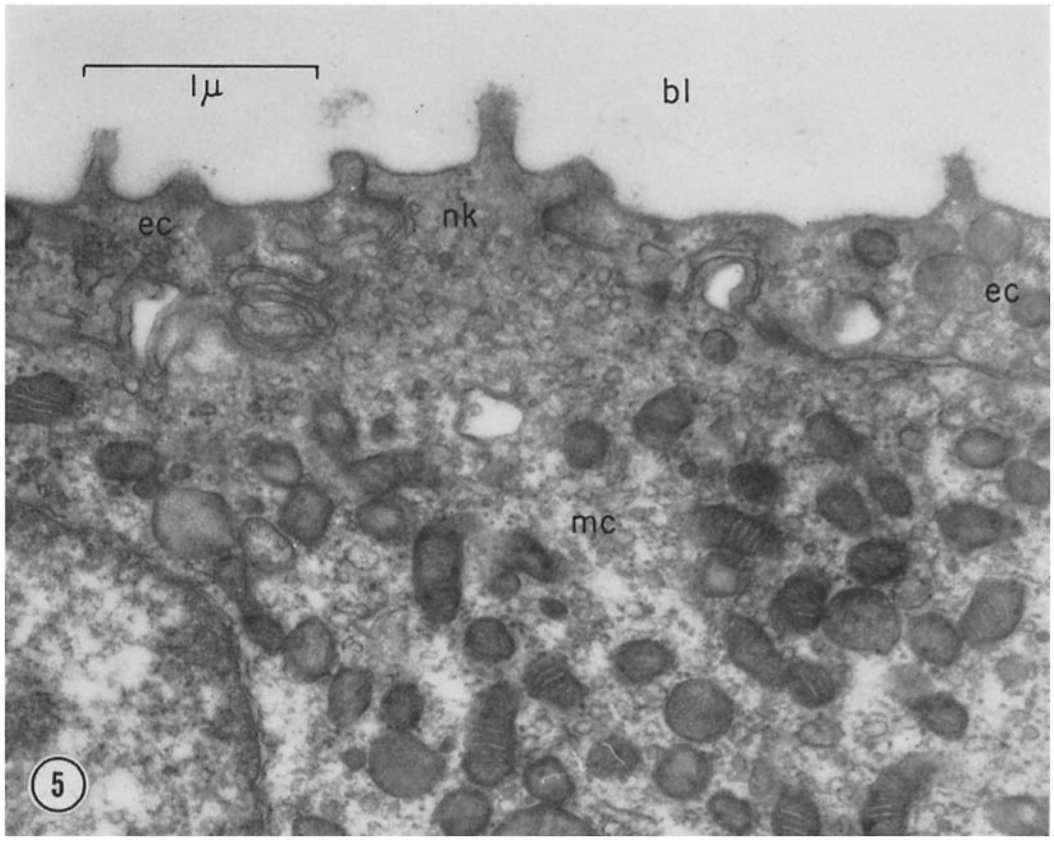


FIGURE 5

A cell of the type that contains numerous mitochondria (*mc*), showing the characteristic narrow neck (*nk*) by which these cells communicate with the bladder lumen (*bl*). Ajdacent epithelial cells are indicated (*ec*). EMU-2B. × 31,000.

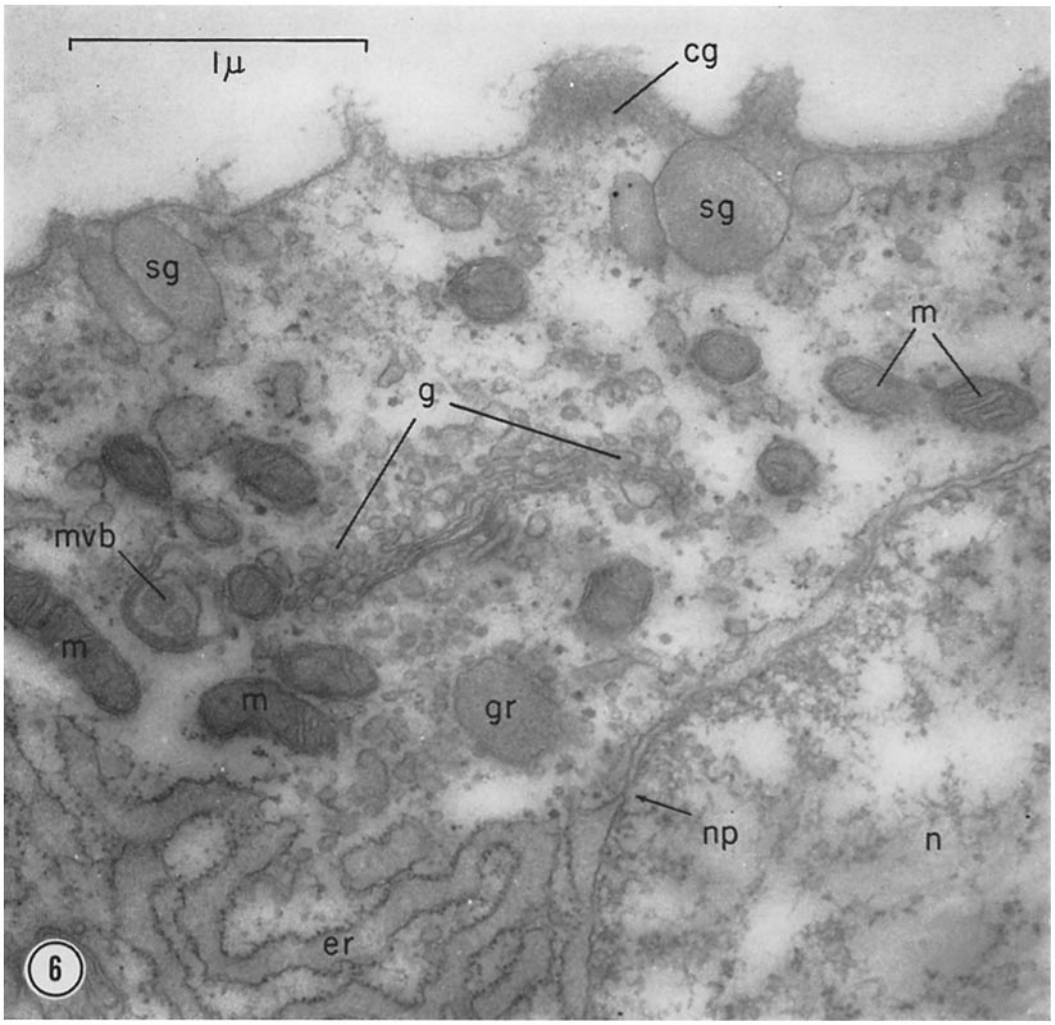


FIGURE 6

View of the cytoplasm of an epithelial cell. The Golgi apparatus (*g*) occupies the region near the center of the figure and is surrounded by mitochondria (*m*). At the lower right corner is the cell nucleus (*n*), whose double membrane shows an indication of a pore (*np*) and distention over a region covered with RNP particles and associated with an array of enlarged elements of rough surfaced endoplasmic reticulum (*er*). A multivesicular body (*mvb*) is near the left center of the figure, and several secretion granules (*sg*) are seen just under the mucosal surface plasma membrane. These granules are bounded by single membranes and contain a finely granular material similar to that found in the internum of the rough surfaced endoplasmic reticulum. A cortical layer of fine, granular material (*cg*) lies just beneath the plasma membrane of the free surface of the cell. Numerous small vesicles and dense particles are scattered throughout the cytoplasm. The granule (*gr*) seen between the Golgi apparatus and the nucleus may be a newly formed secretion granule. EMU-3F. × 39,000.

allow a sharp distinction between "proximal" and "distal." Golgi membranes are usually supranuclear, as are most of the lamellae of the rough surfaced endoplasmic reticulum and most of the mitochondria. Mitochondria and endoplasmic reticulum seem usually to be excluded from the distal half-micron of cell just under the free mucosal surface (Fig. 6).

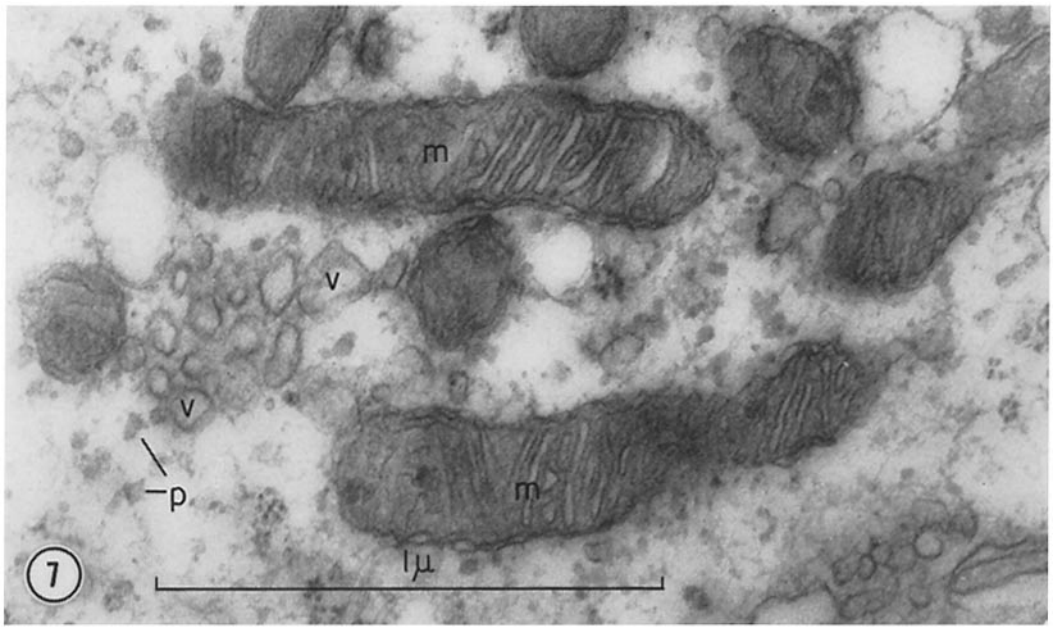


FIGURE 7

Detail of mitochondria (*m*) and cytoplasmic vesicles (*v*) and particles (*p*) in an epithelial cell. EMU-2B. × 67,000.

The mitochondria of the epithelial cells have circular to irregularly elongated profiles in section; they sometimes branch, are bounded by two outer membranes, and contain cristae (Fig. 7).

The endoplasmic reticulum of these cells consists of a smooth surfaced component, usually vesicular in form, and a rough surfaced component in the form of flat lamellae often in roughly parallel arrays (Figs. 6 and 8 *a*). The two forms of endoplasmic reticulum, the smooth and the rough, are occasionally seen in continuity with each other and also in continuity with the nuclear membrane (Fig. 8 *a* and arrow, Fig. 15).

Another membrane component of the cytoplasm is a structure which has been observed in a variety of cell types (20–22) and which has been referred to by several names, including "multivesicular body" (20). Examples of this structure are shown in Figs. 6 and 8 *b*. It is variable in form, sometimes consisting of whorls of closely packed membranes, as in Figure 8 *b*, and sometimes made up of only a few loosely packed membranes (Fig. 6). No functional significance can be attributed at present to this structure, except perhaps that it represents storage of lipid or lipoprotein molecules.

The Golgi complex of these cells is very similar in form to that which has been reported for several types of secretory cells (23). This is shown in Fig. 6. This component consists of a stack of flat lamellae, usually three to five in number, which are bounded by smooth surfaced membranes and flanked by numerous small vesicles also with smooth membranes.

Irregular dense bodies, probably lipid droplets, are occasionally observed. They often show dark, osmiophilic granules near their surfaces, as seen in Fig. 9. Granules, about 50 mμ in diameter, are also found scattered throughout the cytoplasm (Fig. 9).

The nuclei of the epithelial cells have a fine structure very similar to that commonly seen in somatic cells of vertebrates. The nuclear envelope consists of two membranes, the outer one seen occasionally to be connected with the rough surfaced component of the endoplasmic reticulum, as has already been pointed out in the electron micrographs (Fig. 15). Fenestrations or pores occur in the nuclear envelope. These are depicted in sections as areas of apparent fusion of the outer and inner nuclear membranes as shown at *np* in Fig. 6. The nucleoplasm consists of a finely granular or filamentous material disposed in patches immediately under the nuclear membrane and in clumps bordering clear areas or channels throughout the

core of the nucleus. These clear channels in the nucleoplasm are often associated with the pores of the nuclear membrane, as has been observed in pancreatic, hepatic, and other cells of the rat by Watson (24).

Secretion granules similar to the zymogen granules of the pancreas (25) are a constant finding in the epithelial cells of the bladder (*sg*, Fig. 6). They are usually located immediately beneath the free surface of the cell, in an area, as has been mentioned, from which mitochondria and endoplasmic reticulum are usually excluded. These secretion granules are bounded by single membranes and their content appears uniform or finely granular. An interesting relationship between these granules and the special structure of the free surfaces of the cells will be discussed following the description of the various surfaces of the cells.

Basal and Lateral Surfaces of the Cells: The epithelial cell layer of the urinary bladder is coated on its basal surface, adjoining the lamina propria, with an extracellular layer of amorphous material (*ms*) a little less than 0.1 μ thick, as shown in Figs. 10 and 14. This is much thinner than the usual basement membrane of histology, and will be referred to as the "subepithelial membrane," since it is not certain whether it is the basis of the staining reactions used to identify basement membranes. The basal plasma membranes covered by this subepithelial membrane are usually flat. In contrast, the lateral surfaces of all the cells and the proximal surfaces of cells not in direct contact with the subepithelial membrane are characterized by numerous loosely interlocking folds, as seen in Figs. 3 and 10.

Evidence of pinocytotic activity is often found in the basal plasma membranes adjacent to the subepithelial membrane (arrows, Fig. 10), but never in the lateral plasma membranes or on the mucosal surface of the epithelial cells.

Cellular Attachment: Specialized regions of attachment ("adhesion plates" or "desmosomes") are

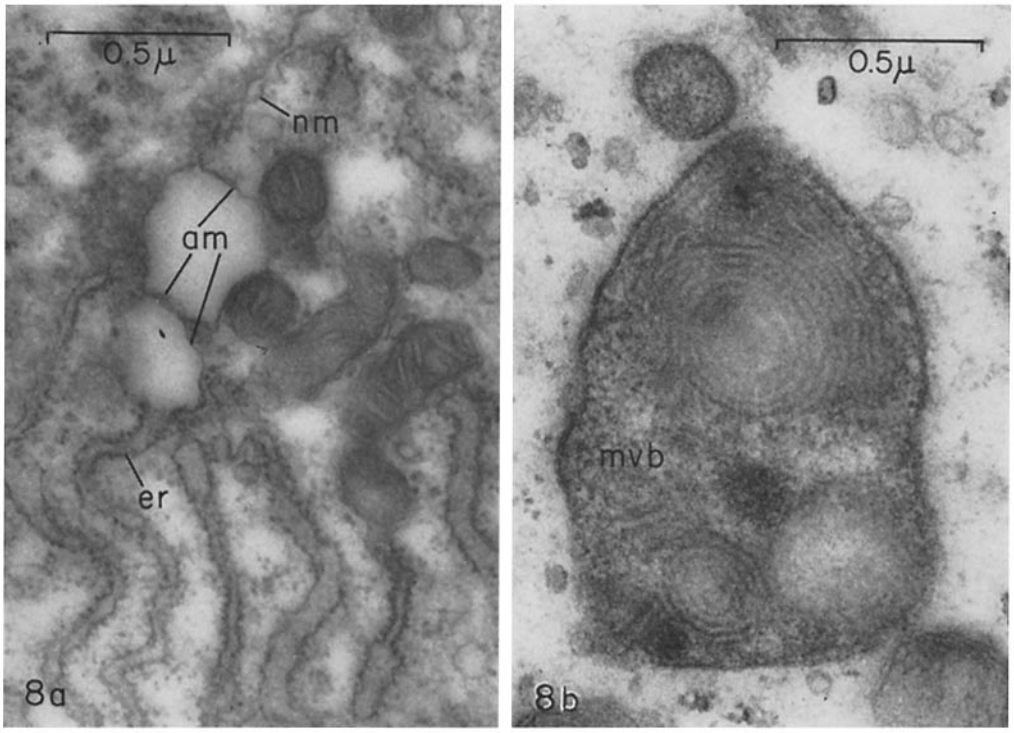


FIGURE 8

a. Showing continuity of membranes of rough surfaced (*er*) and smooth surfaced (*am*) endoplasmic reticulum with the nuclear membrane (*nm*). EMU-2B. × 48,000.

b. Detail of a complex multivesicular body (*mvb*) in an epithelial cell. EMU-2B. × 55,000.

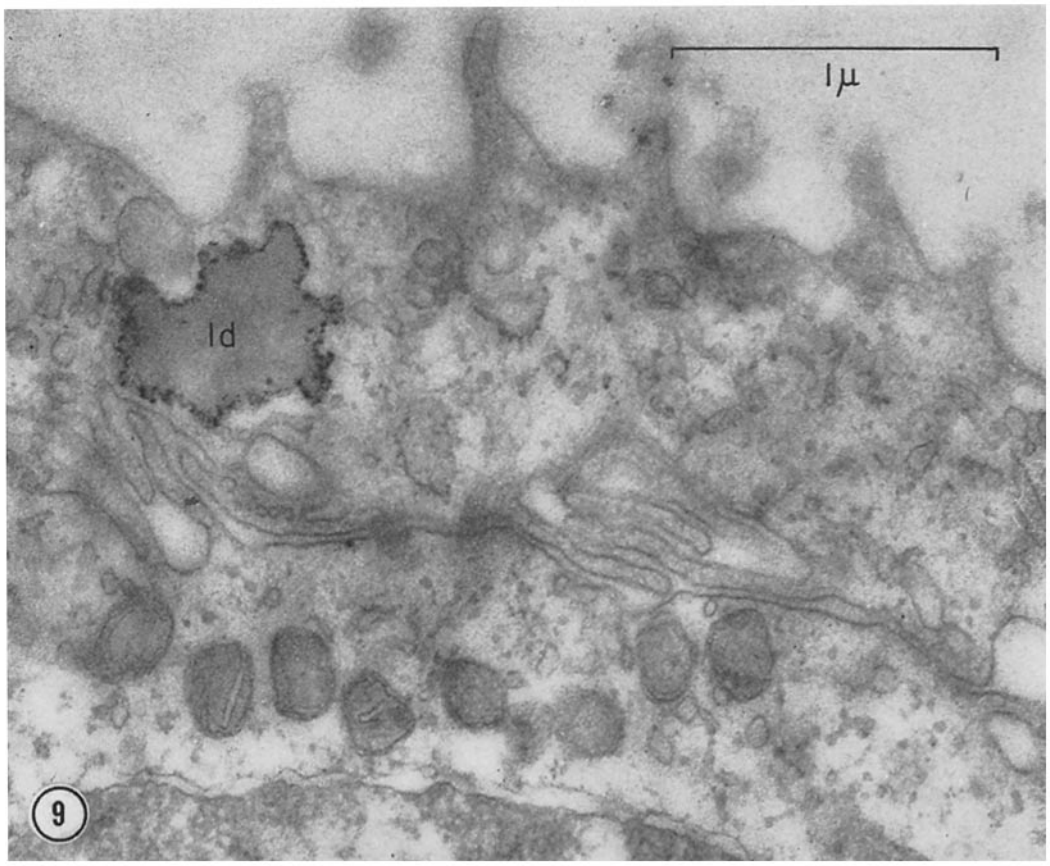


FIGURE 9

A lipid droplet (*ld*) with small, dense particles near its periphery in the cytoplasm of an epithelial cell. EMU-2B. × 43,000.

found between adjacent epithelial cells (Figs. 10 and 11). The fine structure of these attachment areas is very similar to that which has been described in many epithelia, *e.g.*, in human epidermis (26). The plasma membranes of the two attached cells run quite parallel over a short distance (about 0.4 μ) in which there is an apparent doubling of each membrane so that it shows up as two dense lines, spaced by about 0.01 μ in electron micrographs. The outer two dense lines, one from each membrane forming the desmosome, are spaced by about 0.03 μ and there is an additional faint line midway between the two. Dense areas in the cytoplasm immediately adjacent to the adhesion plates represent the "tonofibrillae," fine cytoplasmic filaments which are known to attach to the plasma membrane in these structures.

Desmosomes specially located a little less than 1 μ from the free surfaces of the cells form the so-called "terminal bar apparatus" which is common in epithelial cell layers. This region is illustrated in the electron micrograph in Fig. 11. The high frequency with which desmosomes are seen in transverse sections of the bladder epithelium at the level of the terminal bar apparatus indicates that these structures form a dense band around each cell. Since these structures remain intact under conditions where the cells are highly stretched or distended by large accumulations of fluid in the intercellular spaces, the commonly held view that the terminal bar apparatus in epithelia serves to anchor the cells together and thus maintain the integrity of the epithelial sheet seems to apply in this case.

There is an additional component in the association of the plasma membranes of adjacent cells that

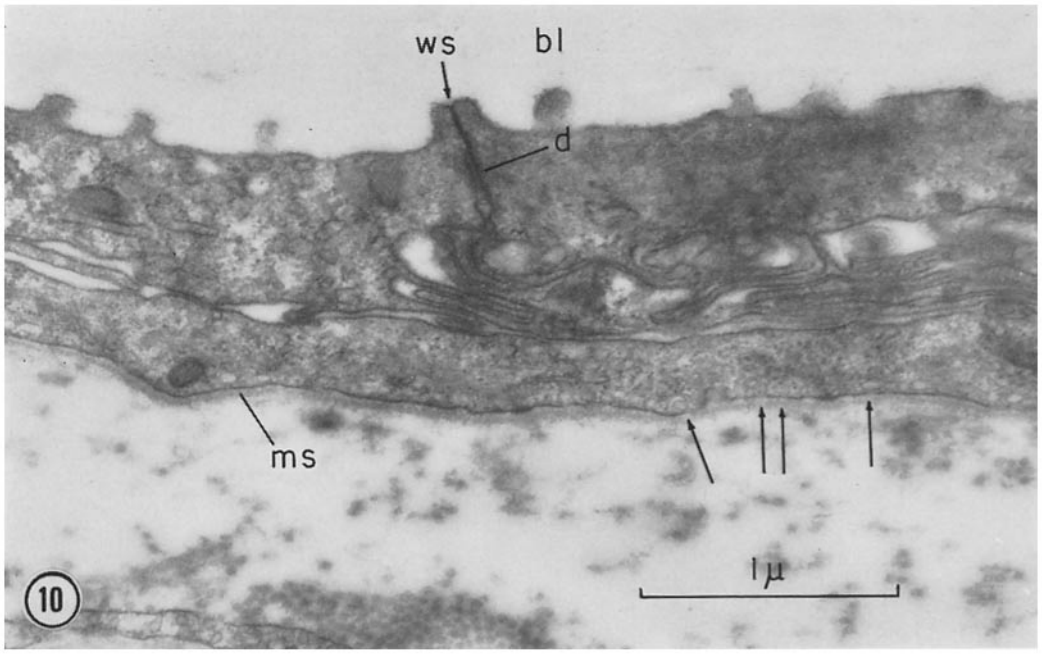


FIGURE 10

View of three epithelial cells and a portion of the lamina propria near the mucosal surface of the bladder. Close association (*ws*) is seen between the plasma membranes of the neighboring epithelial cells bounding the bladder lumen (*bl*). Below this is seen a desmosome (*d*). The proximal (lower, in this figure) plasma membranes of these two cells form deep, interlocking folds, while the plasma membrane of the lower epithelial cell, bordering on the subepithelial membrane (*ms*), is relatively flat. Evidence of pinocytotic activity in the latter plasma membrane is indicated by arrows. Bundles of collagen fibrils and a portion of a blood vessel are at the lower left. EMU-2B. × 34,000.

may serve a function in reducing the leakage path through the bladder wall between the cells. Starting just at the free surface where the two membranes of adjacent cells come together, and extending almost to the terminal bar, the space between the membranes is extremely narrow, about 0.02 μ (Fig. 11). This close apposition of cell membranes is not limited to the epithelial cells, but occurs between cells of all types in the mucosal surface of the bladder (Figs. 4 and 5).

Free Cell Surface: The free surfaces of the epithelial cells show characteristic microvilli and ridges in the plasma membranes that are most apparent in tissue that was loosely held at the time of fixation. In tissue fixed while highly stretched, these microprojections of the cell surface are reduced although not completely absent. In sections approximately parallel to the surface of the bladder, it can be seen that ridges as well as villi are present. A cortical layer of moderately dense material, finely granular in appearance, lies immediately inside the plasma membrane at the free surfaces of the cells and within the microprojections (*cf.* Figs. 6, 12, and 13).

A fine filamentous layer is found just outside the plasma membranes of the free mucosal surfaces of all the cells bordering on the urinary lumen. This filamentous material is most apparent in sections that have been heavily "stained" with lead salts (Fig. 12) and can be clearly distinguished from the plasma membrane only where the plane of the section is accurately perpendicular to the cell surface. The arrangement of this material with respect to the plasma membrane is shown at higher magnification in Fig. 13. Only cell surfaces bordering on the urinary lumen (free surfaces) have this special surface coating and the cortical granular material inside the plasma membrane. It is evident that the lower epithelial cell in Fig. 12, which is not on the inner bladder surface, does not have the fibrous coating or the granular material at its distal surface.

It is possible to find morphological evidence that this filamentous layer is derived from the epithelial cells by a secretory process (Figs. 14 and 15) which is similar to that described for the pancreas by Palade (25). Secretion granules are shown with their membranes in continuity with the plasma membrane at the mucosal surface, and a direct continuity is seen between the granule content and the fibrous coating of the cell. This suggests that the material coating these cells on their free surfaces consists of a substance synthesized and secreted by the epithelial cells. It should be mentioned here that secretion granules clearly "opening" at the mucosal surface have been seen repeatedly in bladders fixed *in vivo* or shortly after removal from the animal, but never in bladders incubated for several hours *in vitro* before fixation.

Quite often, in the epithelial cells, an extensive array of enlarged elements of the rough surfaced endoplasmic reticulum is seen (Figs. 6, 14, and 16). The thickness of these enlarged cisternae is about 0.2 to 0.3 μ, while that of the usual rough surfaced endoplasmic reticulum in these cells is only about 0.1 μ (Fig. 8 *a*). The content of the enlarged reticular elements is somewhat denser than the cytoplasmic matrix, and has roughly the same density as the content of the secretion granules, suggesting that the secretory substance or a precursor is found in the internum of the endoplasmic reticulum, as is the case in the pancreas of the guinea pig (25). Close association occurs between mitochondria and the enlarged reticulum over small areas from which RNP particles are excluded, as seen at two places in Fig. 16.

The fine structure of the epithelial cells of the toad's urinary bladder is summarized in the drawing, Fig. 17.

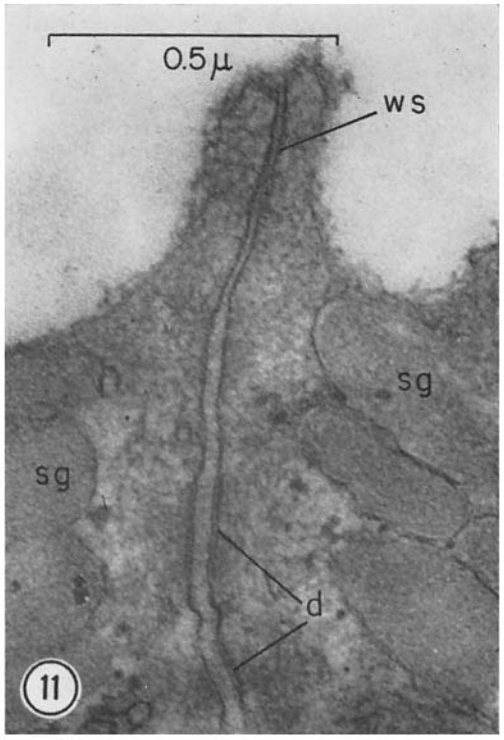


FIGURE 11

Higher magnification of the region of contact between two adjacent epithelial cells at the mucosal surface of the epithelial sheet. Desmosomes (*d*) form the terminal bar apparatus a short way from the bladder lumen, and the two plasma membranes from the adjacent cells are very closely applied to each other (*ws*) distal to the terminal bar. Secretion granules (*sg*) are seen in each epithelial cell. EMU-2B. × 76,000.

In Vitro Experiments

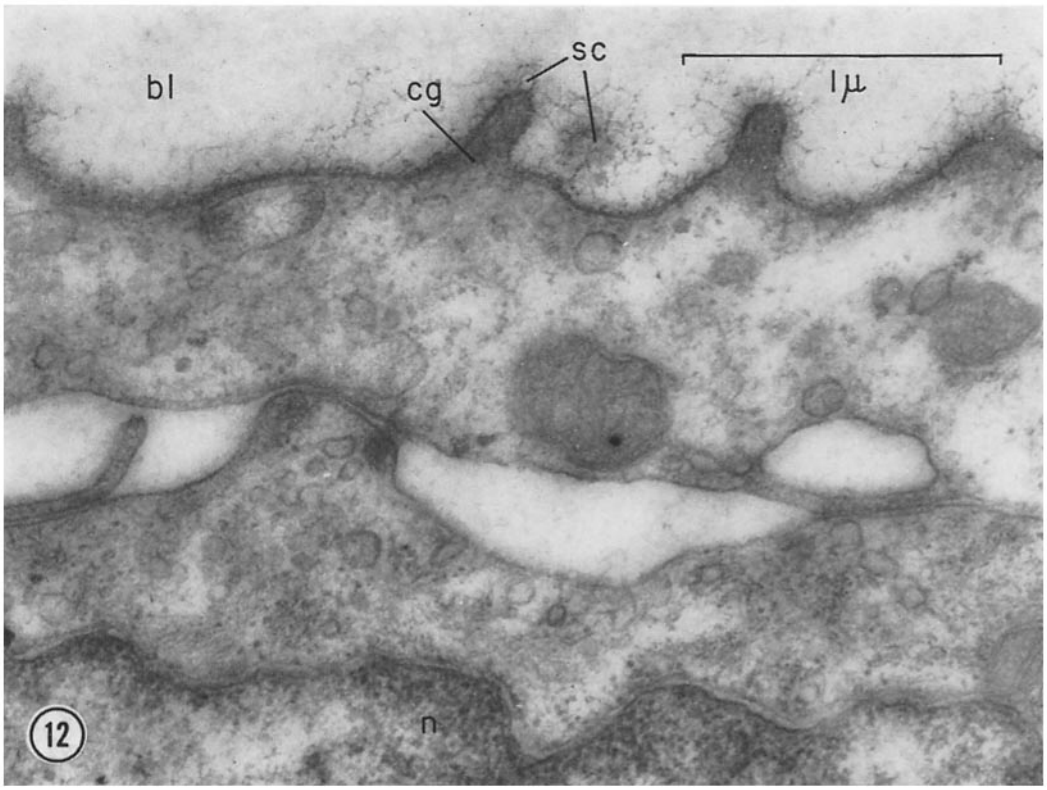
Three bladders were set up, as described under Materials and Methods with diluted Ringer's solution (50 mOsM/liter) inside (mucosal surface) and immersed (serosal surface) in Ringer's solution (220 mOsM/liter). The first of these served as a control. Weight loss of each was determined for a control period of 20 minutes and then 1.25 μg of arginine vasopressin (AVP) was added to the outside (serosal) solution of the second and third bladders. The second bladder was fixed in osmium tetroxide after its rate of weight loss had increased; the third was fixed after it had returned to a resting condition following withdrawal of the hormone. Weight loss was followed on the two experimental bladders for about an hour after they had been fixed, to make sure that the fixative was not causing extraneous effects such as reversal of the physiological states of the bladders. Weight changes were followed on the control bladder throughout the experiment and it was fixed at the end of the experiment. All three bladders were embedded in methacrylate, sectioned at 2 μ, and examined by phase microscopy. The results are shown in Figs. 18 and 19. The times at which fixative was applied to the bladders are indicated in Fig. 18. It can be seen from the graphs that the bladder which was fixed while more permeable because of the presence of hormone became slightly less permeable after fixation, and that the bladder which was fixed after hormone withdrawal

FIGURE 12

Showing the surface coating (*sc*) and the cortical granular layer (*cg*) found at the free surfaces of epithelial cells bordering on the bladder lumen (*bl*) but not found at the distal surfaces of epithelial cells not bounding the lumen, such as the lower cell in this figure. These extra layers show particularly well in sections, such as this one, that have "stained" heavily with lead salts, although they can also be seen in unstained sections. *n*, nucleus. EMU-2B. × 42,000.

FIGURE 13

Higher magnification view of the free surface of an epithelial cell showing that the filamentous surface coating (*sc*) lies outside the plasma membrane (*pm*), while the cortical granular layer (*cg*) lies inside. EMU-2B. × 55,000.


bl
cg
sc
1μ
n
12

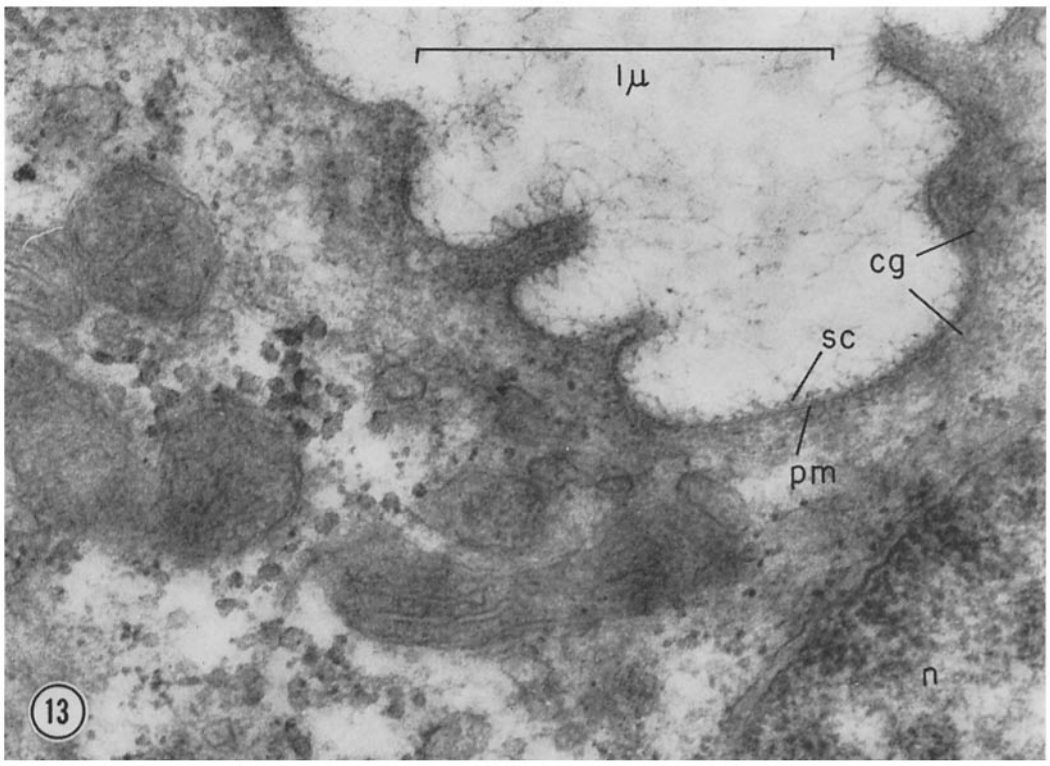
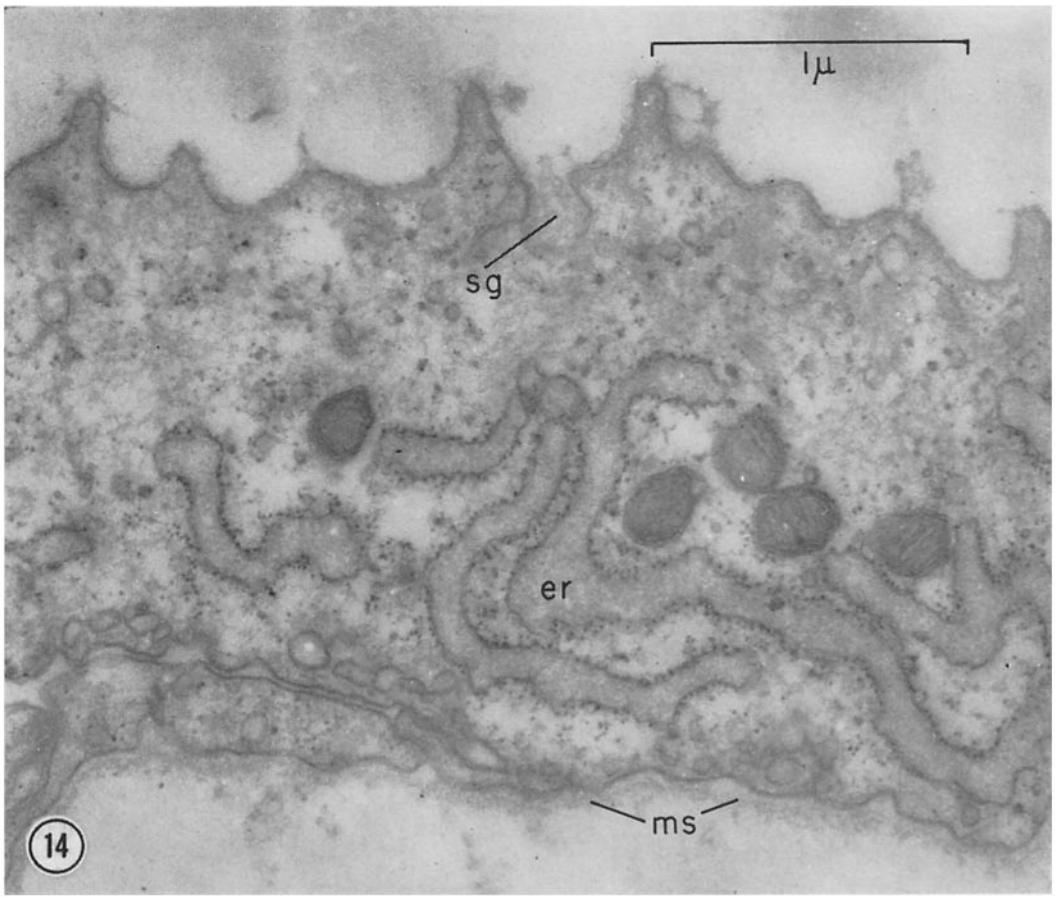

1μ
cg
sc
pm
n
13

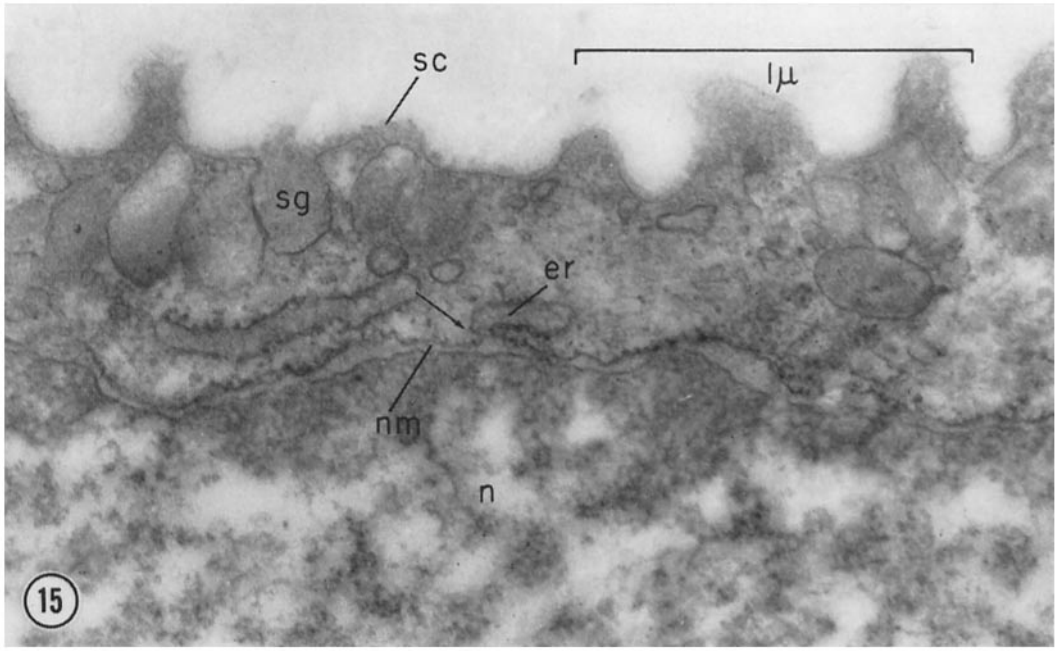
FIGURE 14

Portion of a epithelial cell showing the enlarged rough surfaced endoplasmic reticulum (*er*), and a secretion granule (*sg*) opening at the mucosal surface and discharging its contents. The subepithelial membrane is indicated (*ms*). EMU-3F. × 42,000.

FIGURE 15

View showing the content of a secretion granule (*sg*) in continuity with the surface coating (*sc*) of an epithelial cell. Also shown is continuity between the nuclear membrane (*nm*) and a portion of the rough surfaced endoplasmic reticulum (*er*) at the arrow. *n*, nucleus. EMU-2B. × 53,000.


1μ
sg
er
ms
14


sc
1μ
sg
er
nm
n
15

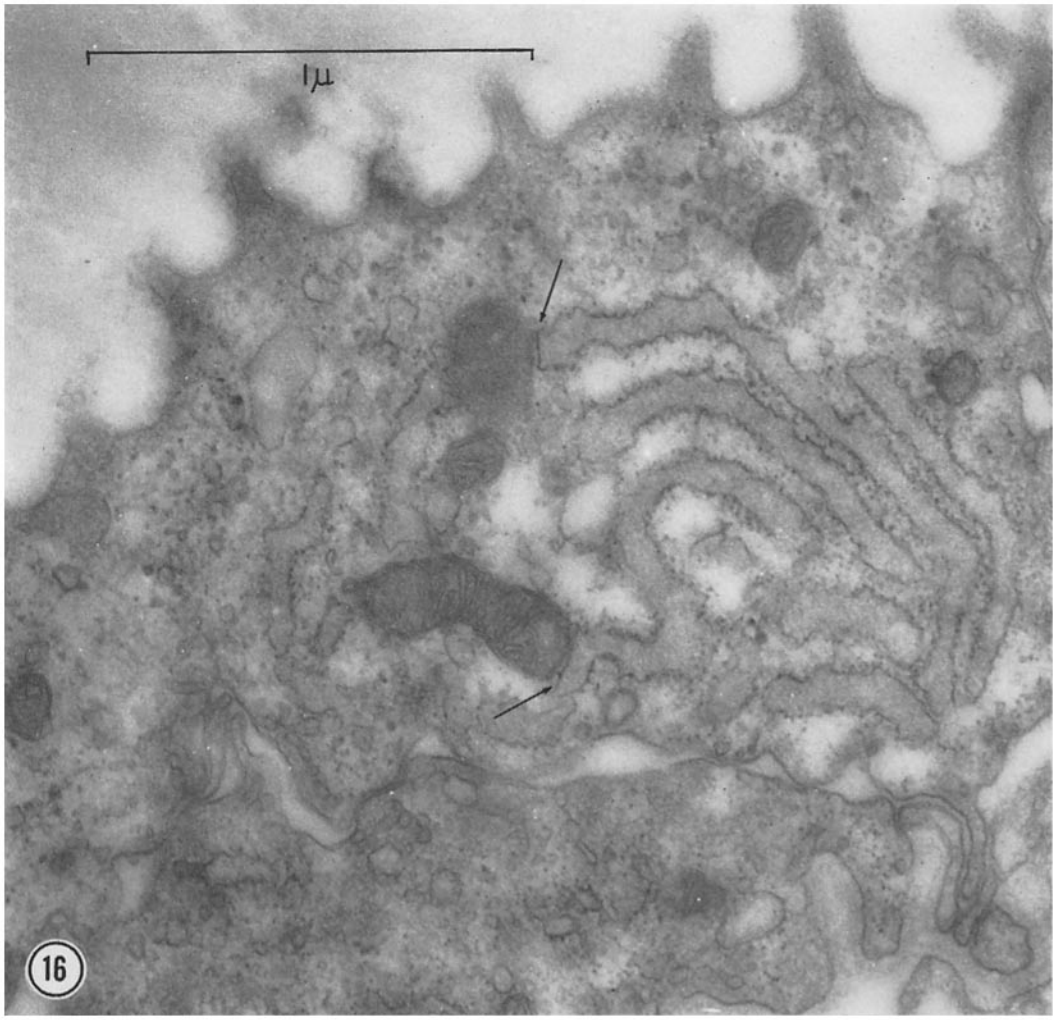


FIGURE 16

Enlarged elements of the rough surfaced endoplasmic reticulum, some of which are in close association with one or possibly two mitochondria (arrows). The upper arrow points at what is probably a mitochondrion cut so that none of its membranes appears clearly. EMU-3F. × 59,000.

became slightly more permeable following fixation, but that there was a well preserved difference in their states of permeability. As illustrated in the phase micrographs (Fig. 19), the cytological appearance of the epithelial cells varies with the physiological state of the bladders at the time of fixation. The control bladder has the same morphology as a bladder fixed *in vivo*. The epithelial cells of the bladder fixed following hormone withdrawal have the same appearance as those of the control bladder, while the cells of the bladder fixed in the permeable state show considerable swelling involving both the cytoplasm and the nuclei.

In a further experiment, three bladders were mounted as before with diluted Ringer's solution inside and were suspended in Ringer's solution. The first served as a control. After weight changes were measured for each bladder for a control period, 1.25 μg of AVP was added to the outside solution of the second bladder. The solutions were reversed on the third bladder, *i.e.*, it was filled (mucosal surface) with Ringer's solution and immersed (serosal surface) in the diluted Ringer's

solution, without hormone on either side. Weight changes were then determined after an additional period of about 20 minutes. The results, shown in Fig. 20, indicate that the bladder treated with hormone showed the expected increase in rate of water loss, while the control bladder showed no significant change, and the bladder bathed in diluted Ringer's without hormone showed a small gain in weight.

At the end of the experimental period, each bladder was fixed in osmium tetroxide, embedded, sectioned, and examined with the phase microscope (Fig. 21). The control bladder looked essentially the same as before. There was considerable swelling of the cytoplasm and nuclei of the epithelial cells of the hormone treated bladder. This swelling was even more pronounced in the epithelial cells of the third bladder.

The bladder which was fixed while in the permeable state induced by hormone (Fig. 18 *b*) was examined by electron microscopy to see whether vesiculation of the free mucosal surfaces of the epithelial cells was involved in the swelling of the cells and the transfer of water. No evidence of vesicle formation at the free surface was found, and the number of vesicles present in the cytoplasm did not appear to be significantly changed (Fig. 22). The cytoplasm appeared considerably diluted and the nuclei swollen, as had been observed in the phase micrographs.

In another experiment, isolated cells scraped from the mucosal surface were placed in two groups. One group was washed in several changes of distilled water and the other was treated similarly in Ringer's solution. A sample from each group was then mounted under a coverslip for phase microscopy in its respective medium. The results, shown in Fig. 23, indicate that although these cells swell greatly when exposed to distilled water, most of them do not rupture.

DISCUSSION

The results of our *in vitro* experiments indicate that there is a difference in permeability to water between the mucosal and serosal surfaces of the epithelium of the isolated amphibian urinary bladder. The plasma membranes on the serosal surface of this epithelium are normally relatively permeable to water, as indicated by the gain in

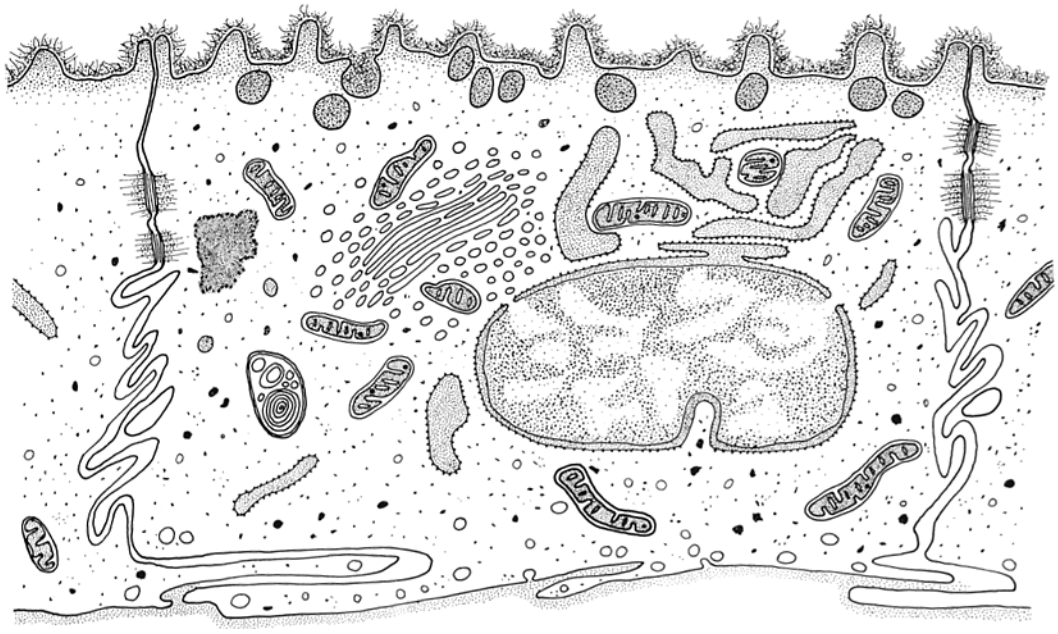


FIGURE 17

Diagram summarizing the observations on the fine structure of the epithelial cells of the toad's urinary bladder. Approx. × 20,000.

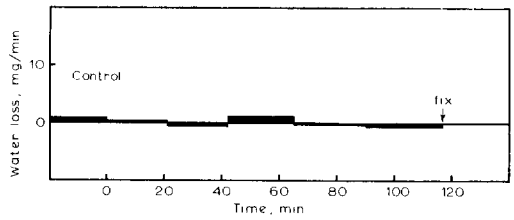


FIG. 18 *a*

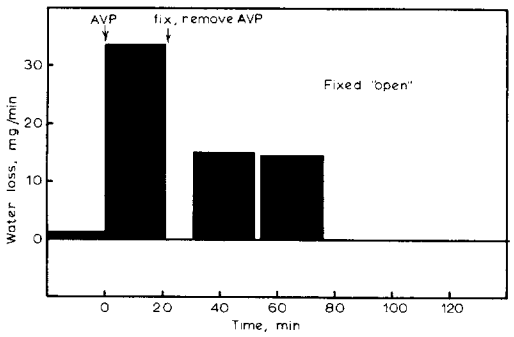


FIG. 17 *b*

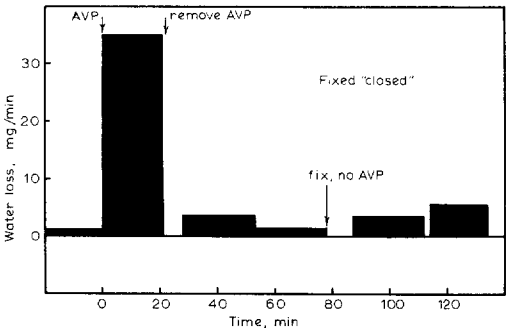


FIG. 18 *c*

FIGURE 18

Weight changes of bladders from an *in vitro* experiment in which cytological differences between bladders fixed in different physiological states were investigated. Graph *a* is from a control bladder, graph *b* is from a bladder fixed after treatment with AVP on the serosal side of the bladder, and graph *c* is from a bladder fixed after withdrawal of AVP. Each bladder in this experiment had diluted Ringer's solution on its mucosal side and Ringer's solution on its serosal side.

weight of the bladder (Fig. 20) and the marked swelling of the cells (Fig. 21, *c*) when this surface is exposed to a hypotonic medium. On the other hand, the mucosal surface of this tissue is normally relatively impermeable to water (Figs. 20 and 21, *a*), but becomes permeable following the action of neurohypophyseal hormones (Figs. 20 and 21, *b*).

These results confirm the supposition of Leaf (9) that the permeability barrier of the amphibian urinary bladder is at or near its mucosal surface. They also emphasize that one of the most striking properties of epithelial cells is their polarity, a fact generally recognized by cytologists (27) but often forgotten by workers in other disciplines.

On the basis of our cytological observations, it is possible to state unequivocally that the osmotic transfer of water from the mucosal to the serosal solution occurs through the epithelial cells and not between them. An estimate of the rate of water passage through these cells (during *in vitro* experiments) can be made by considering a typical half-bladder as a sphere 2 cm in diameter and using 30 mg/min as a representative rate of water loss after hormone administration. The calculated rate of water movement is then 24 μ^3 per μ^2 of bladder surface per minute. Since the epithelial cells are, on the average, 3 μ thick, and since, in all likelihood, only part of the volume of the cell represents diffusible water, it can be estimated roughly that a complete change in cell water takes place every 6 seconds or less.

If water were to traverse these cells at this rate in vesicles by a process named "cytopempsis" by Moore and Ruska (28) and thought by Palade (29) to be involved in the transport of ferritin and presumably other material across the endothelial cells lining blood capillaries, then one would expect to find a tremendous increase in the number or size of vesicles present in the epithelial cells of the bladder after hormone administration. It is clear from our results that no such increase occurs. This fact, together with the observation that the cytoplasmic matrix of these cells is considerably diluted in hormone treated bladders, indicates that water traversing the bladder passes through the cells.

In attempting to further correlate cytological appearance with physiological behavior, attention is immediately drawn to the difference in appearance between the serosal (permeable) and the mucosal (impermeable) surfaces of these cells. At the former surface, a typical plasma membrane is seen with the electron microscope, whereas at the mucosal surface there are two components in addition to the plasma membrane: one is the filamentous layer external to the plasma membrane, and the other is the cortical layer of moder-

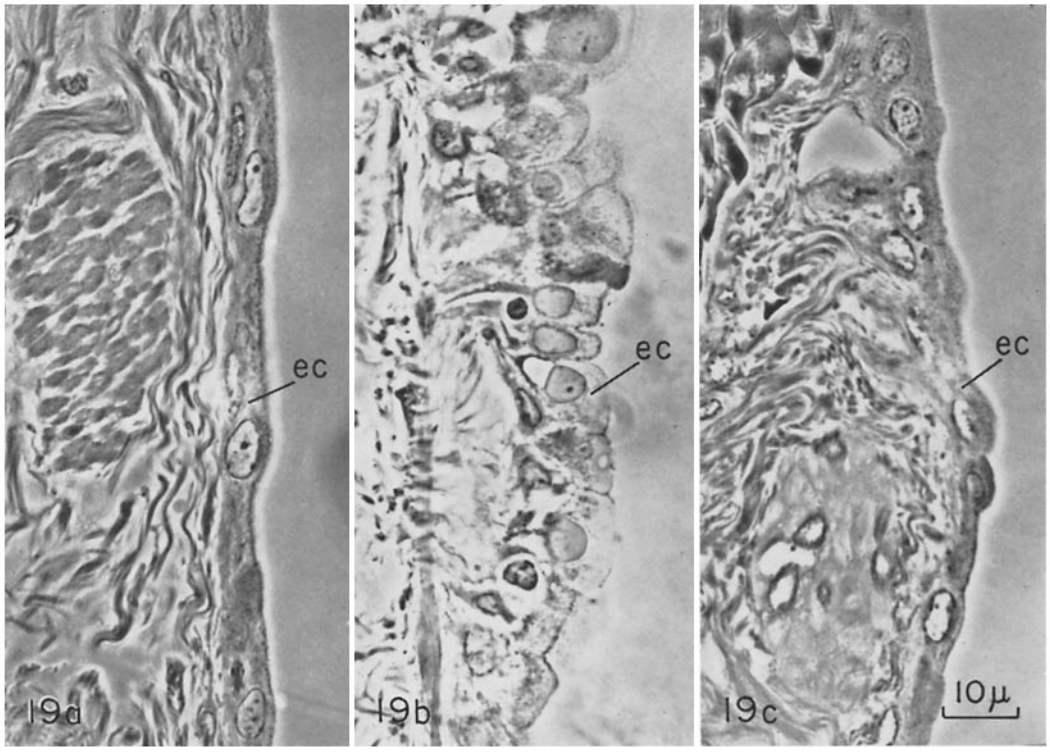


FIGURE 19

Phase micrographs of the bladders from the experiment shown in Fig. 18. The epithelial cells (*ec*) of the control bladder (*a*) show what can be called "normal" cytological structure, comparable to that of a bladder fixed *in vivo*, while those of the bladder fixed in a permeable state (*b*) are swollen. The third bladder (*c*), fixed after hormone withdrawal, looks similar to the control. Zeiss × 90 phase objective. × 900.

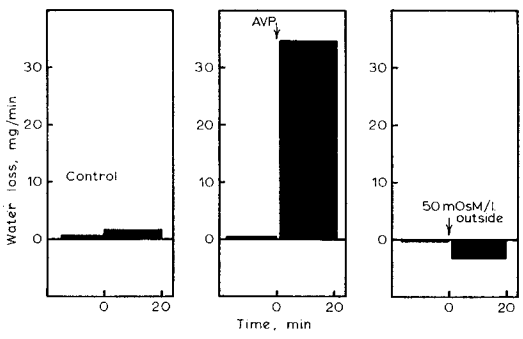


FIGURE 20

Weight changes of bladders from an *in vitro* experiment testing the effects of dilute solutions when placed on the mucosal surface without hormone present (left graph), on the mucosal surface with hormone on the serosal side (center graph), and on the serosal surface without hormone present (right graph).

ately dense, granular material lying immediately inside the plasma membrane. It has not been established whether one or the other of these layers is responsible for the unusual permeability characteristics of this cell surface.

Following application of neurohypophyseal hormones to a bladder *in vitro*, there is no apparent change in the appearance of either of these special components of the mucosal cell surface.

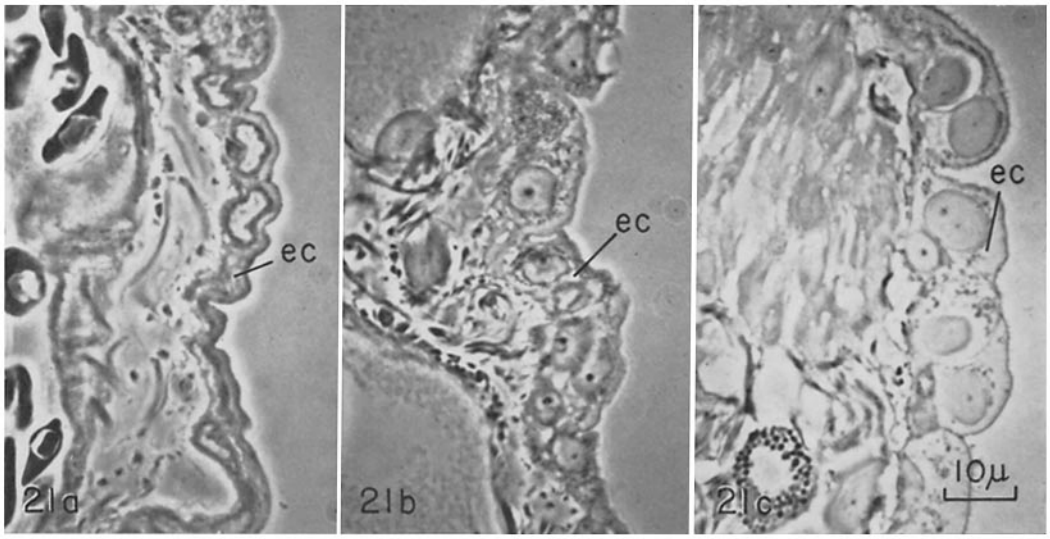
There is cytological evidence that the filamentous layer outside the plasma membrane is produced and maintained by a secretory process. However, the observation that the secretory process is halted when bladders are removed from the animal, although the bladders still respond reversibly to the application of neurohypophyseal hormones, indicates that the secretory process is not the means by which the hormones exert their characteristic effects.

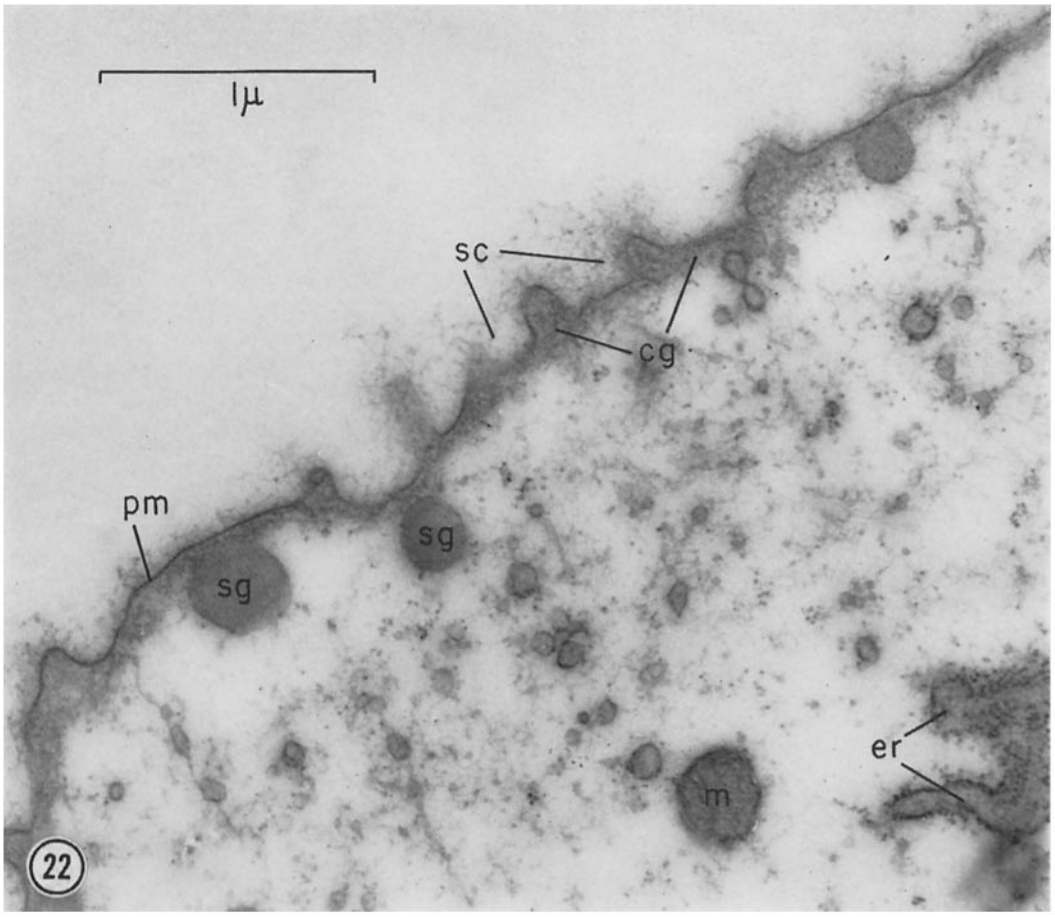
FIGURE 21

Phase micrographs of the bladders from the experiment shown in Fig. 20. Epithelial cells are indicated (*ec*). The control bladder (*a*), with a hypotonic solution on its mucosal side, shows normal appearance. The bladder with the same solutions but with hormone added to the serosal solution (*b*) shows swollen cytoplasm and nuclei. The bladder with solutions reversed, *i.e.* isotonic on the mucosal side and hypotonic on the serosal side, but with no hormone (*c*) shows even more pronounced swelling. Zeiss × 90 phase objective. × 990.

FIGURE 22

Electron micrograph of the free surface of an epithelial cell from the bladder shown in the phase micrograph in Fig. 19 *b*. The plasma membrane (*pm*), the cortical granules (*cg*), and the fibrous surface coating (*sc*) are indicated, as are secretion granules (*sg*), a mitochondrion (*m*), and some rough surfaced endoplasmic reticulum (*er*). No evidence of vesiculation on the free surface of the cell is ever observed in these cells, even when the rate of passage of water through the cell was high (approximately 24 μ^3 per μ^2 of free surface per minute in this bladder) at the time of fixation. EMU-3F. × 36,000.


ec
21a
ec
21b
ec
10μ
21c


1μ
sc
cg
pm
sg
sg
er
m
22

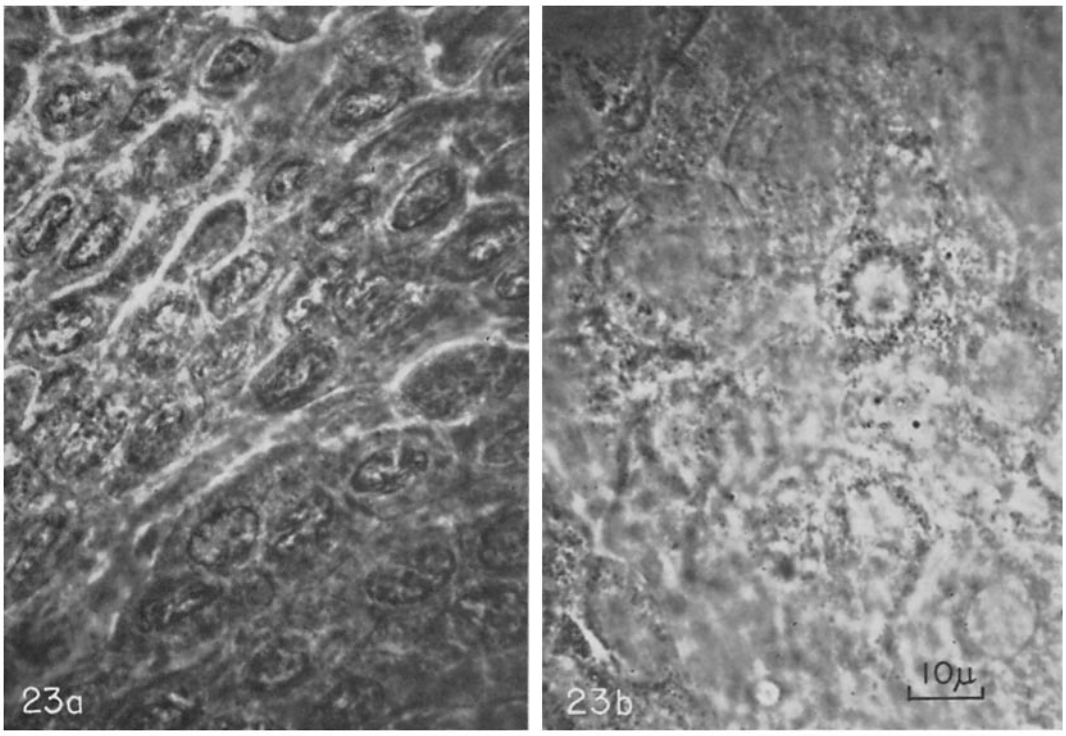


FIGURE 23

Phase micrographs of living epithelal cells isolated from the bladder by scraping. Cells washed three times and mounted in Ringer's solution, (*a*). Cells washed three times and mounted in distilled water, (*b*). Zeiss × 90 phase objective. × 990.

The fine structure of the epithelial cells of the bladder can be related to their physiological characteristics in several other ways. The tight lateral attachment of the cells near the mucosal surface assures an almost leak-proof bladder, allowing the toad to carry dilute urine for long periods of time without danger of overhydration or the necessity of refiltering the same water repeatedly through the kidneys. On the other hand, the numerous, loosely interlocking folds of the lateral membranes of the cell proximal to the terminal bars probably help to keep these cells in osmotic equilibrium with the serosal fluid, and allow a considerable increase in cell volume under conditions where the cells are subjected to low osmotic pressure, as during water resorption. In fact, our results show that these cells will not rupture even when when placed in distilled water, in spite of a considerable increase in cell volume. Odland has related a similar function to convolution of the plasma membranes of human epidermal cells (26), although this may not be the only function of such folding, as evidenced by other types of cells which exhibit folded plasma membranes but which presumably are not called on to swell under normal physiological conditions.

It is not clear how hormone applied to the serosal surface of the bladder is able to alter the permeability of a barrier located at the opposite surface of the bladder. As is well known, hormone applied directly to the mucosal surface has no effect. It is not surprising, however, that this is the case, since in the living animal the hormone normally reaches the bladder via the circulatory system, which is on the serosal side of the epithelial sheet. Furthermore, since the mucosal surface of the bladder is normally impermeable to water, it does not seem likely that molecules as large as the neurohypophyseal hormones could penetrate this barrier to express their activity. One is led to the conclusion that the hormones either enter the cell through their basal surfaces or act at these surfaces by causing the production, activation, or release

of one or more intermediates which then lead to the permeability changes observed.

One question that has not been answered by the present study is whether or not the neurohypophyseal hormones change the size of specific pores in the permeability barrier, as proposed by Leaf (9). According to our results, the surface complex at the mucosal surfaces of the epithelial cells would be the location of any pores involved in the regulation of water flow into the cells. This surface complex appears unchanged in electron micrographs of bladders treated with hormones. It must be kept in mind, however, that, in all likelihood, if the pores were less than 50 A in diameter or were of a transient nature, they would not be observable by our present techniques.

Since the completion of this work, a report has appeared by Pak Poy and Bentley, who have examined normal and Pituitrin-treated toad bladders (30). These authors, in agreement with our results, conclude that water passes through the epithelial cells, not through the intercellular spaces, and that vesiculation is not involved in the process.

This work was supported by the National Science Foundation (G-13133) and the Health Research Council of the City of New York (grant No. U-1021). Dr. Rasmussen is a New York City Investigator (I-135).

Received for publication, March 1, 1961.

BIBLIOGRAPHY

1. Steen, W. B., *Anat. Rec.*, 1929, **43,** 215.
2. Bentley, P. J., *J. Endocrinol.*, 1958, **17,** 201.
3. Maffly, R. H., Hays, R. M., Lamdin, E., and Leaf, A., *J. Clin. Inv.*, 1960, **39,** 630.
4. Rasmussen, H., Schwartz, I. L., Schoessler, M. A., and Hochster, G., *Proc. Nat. Acad. Sc.*, 1960, **46,** 1278.
5. Sawyer, W. H., *Endocrinology*, 1960, **66,** 112.
6. Leaf, A., Anderson, J., and Page, L. B, *J. Gen. Physiol.*, 1958, **41,** 657.
7. Leaf, A., Page, L. B., and Anderson, J., *J. Biol. Chem.*, 1959, **234,** 1625.
8. Leaf, A., *J. Cell. and Comp. Physiol.*, 1959, **54,** 103.
9. Leaf, A., *J. Gen. Physiol.*, 1960, **43,** No. 5, suppl., 175.
10. Barrnett, R. J., and Ball, E. G., *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 83.
11. Palade, G. E., *J. Exp. Med.*, 1952, **95,** 285.
12. Porter, K. R., and Blum, J., *Anat. Rec.*, 1953, **117,** 685.
13. Huxley, A. F., *J. Physiol.*, 1957, **135,** 17P.
14. Lavdowsky, M., *Arch. Anat., Physiol. u. wissensch. Med.*, 1872, 55.
15. Rhodin, J. and Dalhamn, R., *Z. Zellforsch.*, 1956, **44,** 345.
16. Schiefferdecker, P., *Nachr. k. ges. Wissensch. Göttingen*, 1884, 68.
17. Schiefferdecker, P., *Arch. mikr. Anat.*, 1884, **23,** 382.
18. List, J. H., *Biol. Zentr.*, 1885, **5,** 499.
19. List, J. H., *Arch. mikr. Anat.*, 1887, **29,** 147.
20. Sotelo, J. E., and Porter, K. R., *J. Biophysic. and Biochem. Cytol.*, 1959, **5,** 327.
21. Nilsson, O., *J. Ultrastruct. Research*, 1959, **2,** 331.
22. Ruska, C., *Z. Zellforsch.*, 1960, **52,** 748.
23. Palay, S. L., *in* Frontiers in Cytology, (S. L. Palay, editor), New Haven, Yale University Press, 1958, 405.
24. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1956, **6,** 149.
25. Palade, G. E., *in* Subcellular Particles, (T. Hayashi, editor), New York, Ronald Press, 1959, 64.
26. Odland, G. F., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 529.
27. Maximow, A. A., and Bloom, W., A Textbook of Histology, Philadelphia, W. B. Saunders Co., 6th edition, 1952, 31.
28. Moore, D. H., and Ruska, H., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 457.
29. Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** in press.
30. Pak Poy, R. F. K., and Bentley, P. J., *Exp. Cell Research*, 1960, **20,** 235.