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Direct Visualization of Epithelial Morphology in the Living Amphibian Urinary Bladder

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Summary. Differential interference-contrast microscopy has been applied to the study of amphibian urinary bladders, *in vitro*. It is demonstrated that well-resolved images can be obtained with little loss of tissue viability. Direct observations have been made on the structure of microvilli, the distribution of mitochondria in the mitochondria-rich cells, and the patency of lateral intercellular spaces. It is noted that the effective viscosity of cytoplasm is very high – that it is apparently a gel in which there is no Brownian movement of organelles. The frequency, shape, and pattern of distribution of granular and mitochondria-rich cells is determined for the commonly studied varieties of *Bufo marinus*. Bladders from Colombian toads contain more and larger mitochondria-rich cells than do those of the Dominican variety. There is no specific arrangement of cell-cell contacts to suggest a structural basis for cooperativeness of action. Finally, a longitudinal study of osmotically-induced changes in the structure of the "tight" or "limiting" junctions establishes the validity of previous findings by electron microscopy.

Attempts to derive quantitative correlations between structure and function in epithelia may be traced, like many active areas of research in the field of transport, to the laboratory of Hans Ussing [19]. The significance of those first observations rested on the fact that the tissue was functioning during the morphologic measurement, but the very limited resolving power of light microscopy with living tissue negated any major extension of its use. On the other hand, electron microscopy, with high resolution as its sole advantage, has been employed exhaustively and with a high degree of confidence. Structural evidence for accumulation of fluid within epithelial subcompartments has been the source of considerable theory (e.g., the "standing gradient" hypothesis of Diamond and Bossert [9]) as have permeability studies with electron-dense tracer molecules. A fairly comprehensive review of relevant contributions is found in the monograph by Berridge and Oschman [2].

Conclusions derived in this way are necessarily qualified, however. Electron microscopic (E/M) evaluations of epithelial configurations (or

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changes in the same) preclude (i) examination of living tissue, (ii) observations of an individual structure both before and after an experimental manipulation, and (iii) truly quantitative sampling. For these reasons, and because there will always be a healthy and vigorous skepticism about the preparative procedures required for E/M analysis, it seems worthwhile to seek reasonable alternatives for morphologic evaluation which might provide visual data with sufficient resolution to warrant physiological interpretation. Ideally such procedures would permit continuous observation of an epithelium in an *in vitro* situation where the physical and chemical environments can be controlled and where the techniques of electrophysiology might be simultaneously applied.

Among the many forms of light microscopy which are available, differential interference-contrast microscopy (after Nomarski) offers a number of advantages. Contrast in unstained specimens is substantial (ideally generated as constructive *vs.* destructive interference – white to black – along a gradient of refractive index within the sample); the use of large condenser aperture openings permits both optimal light microscopic resolving power (far from Airy disc limitations) and an extremely shallow depth of focus so that clear "optical" sections can be readily achieved. This report describes the results of initial attempts to apply this technique to the study of amphibian urinary bladder.

The initial findings include a quantitative description of the distribution of space among cells at the mucosal or urinary surface. Pertinent observations have also been made on the structure of microvilli, the arrangement of mitochondria, the appearance of the intercellular spaces, and the effective viscosity of epithelial cell cytoplasm. The ability to perform longitudinal studies with this nondestructive technique is detailed with a demonstration of osmotically-induced changes in the structure of limiting junctions.

Materials and Methods

Urinary bladders were obtained from doubly-pithed, female specimens of the toad, *Bufo marinus* and of the bullfrog, *Rana catesbiana*. Toads from the Dominican Republic[1] were supplied by National Reagents, Bridgeport, Conn. Those from Colombia were supplied by The Pet Farm, Miami, Fla. Bullfrogs were obtained from Connecticut Valley Biological Supply, Southampton, Mass. Toads were maintained on moist wood chips at room tempera-

1 These animals will be referred to as "Dominican" for the purpose of distinguishing them from the Colombian variety. It should be understood, nonetheless, that their origin is specifically the Dominican Republic, not the British colony, Dominica, which may well be the source of still a third varietal strain of *Bufo marinus*.

ture; bullfrogs were kept at 4 °C and moistened periodically with tap water. None of the animals were fed and all were sacrificed within 2 weeks of arrival.

The Ringer's solutions, which was used as the standard or reference medium, was of the following compositions (mM): Na^+, 117.3; K^+, 3.5; Ca^{++}, 0.9; Cl^-, 116.3; HCO_3^-, 2.4; HPO_4^{--}, 1.8; $H_2PO_4^-$, 0.3. Medium pH was adjusted to 7.8 with either HCl or NaOH; final tonicity was 225–236 mosmol/kg H_2O. Dilutions of this medium were made by addition of distilled, demineralized water except that, to reduce tonicities to less than one-half that of the reference Ringer's solution, minor components were held constant, and only appropriate amounts of NaCl were omitted. Hypertonic solutions were generated by addition of small volumes of concentrated solute (KCl, NaCl, mannitol, sucrose, urea) to the reference solution.

Epithelia were mounted for microscopy with a number of different devices with an eye to obtaining a procedure useful for manipulation of solutions. Optimal image quality was obtained by simply laying a moist strip of tissue between a standard (0.97 to 1.07 mm thick) microscope slide and a cover glass (#1) but this method was useful only for the sake of orientation with each system and allowed little control of the tissue environment and, thus, no facility for experimental manipulation. More practical for experimental purposes was the use of a Lucite ring (OD, 3.9 cm; ID, 2.9 cm; height, 0.85 cm) with the preparation mounted as a drum across one surface.

To observe urinary bladder epithelium in a maximally distended state, an individual hemibladder was suspended as a bag (mucosal surface inside) and slowly filled with Ringer's solution to a point where additional hydrostatic pressure expanded the bladder no further. It was then possible to insert the mounting ring through the open mouth of the sac and to ligate a portion of the distended epithelium in place with surgical thread. With excess tissue trimmed from the sides of the ring, the preparation was laid (serosal side down) on a $2'' \times 2''$ projection cover glass of 0.03 in. thickness (Eastman Kodak, Rochester, N.Y.). The interface between bladder and slide was moistened with several drops of aerated Ringer's solution and a meniscus of Ringer's solution was maintained outside the ring to prevent drying of the serosal surface. For water-immersion microscopy the mucosal face of the bladder was bathed with a predetermined volume (between 2.0 and 4.0 ml) of Ringer's solution; for oil immersion observations a circular cover slip (#00, Corning Glass, Pittsburgh) overlaid a moistened mucosal surface. Numerous variations of this procedure which were designed to permit better access to the serosal fluid were less satisfactory for optical examination, although we have had limited success with a modified use of the Dvorak-Stotler perfusion chamber (Nicholson Precision Instruments, Bethesda, Md.). Aeration was provided by means of a fine bubble stream in the mucosal bath which served, as well, to stir the mucosal solution. Significant warming of the specimen was readily avoided during lengthy periods of observation (up to 3 hr), since the video recording techniques employed (*see below*) required only very low levels of illumination and the mucosal solution could be stirred continuously by the aeration procedure. Ambient temperatures were 23–25 °C.

Optical components for microscopy were from Carl Zeiss (Oberkochen) with the important exception of a 50X "salt-water" immersion objective lens (Ernst Leitz, Wetzlar). The Zeiss equipment included a standard Nomarski Condenser, Wollaston prism, polarizing filters and a versatile "photo changer" for distributing the formed image to any of the several devices used. In all modes of observation it was found advantageous to use an immersion oil bridge as the interface between condenser lens and the base of the preparation slide.

Observations were recorded by a number of means. Optimal quality images were obtained using a 35-mm camera attachment on the WL stand (or with the Nomarski equipment mounted on a Zeiss Photomicroscope) with Panatomic film (Eastman Kodak, Rochester, N.Y.). Routinely, however, video methods were employed. Continuous sequences

of events were recorded on video tape using a Panasonic recorder (Matsushita Electric Industrial Co., Ltd., Secaucus, N.J.). This procedure collects 30 images/sec, while image fidelity is somewhat limited by the 525 horizontal scanning line raster of the video display. Advantages of this technique are that contrast and brightness can be electronically modulated to enhance the image and that it is compatible with video cameras of extreme photosensitivity so that specimen illumination (and consequently, heating) can be kept to a minimum. With a Hitachi camera (HV-16s with Silicon-Vidicon tube, Hitachi Elec. Ltd., Woodside, N.Y.) it has been a routine matter to saturate the phototube (affording the camera optimal conditions for image formation) with light levels still too low to permit any visual observation and with "cool" light of short wave length as provided by any of several different narrow-pass filters. Considerably higher resolution images (1029 horizontal scanning lines/frame) were obtained with a Minicon camera (Sierra Scientific, Mountain View, Calif.). These images were collected on a video disc recorder (Data Memories, Inc., Mountain View, Calif.) which stores up to 500 individual views, each of which is composed of a pair of adjacent 1/60 sec frames. (Fixed images obtained by stopping the video tape recorder, on the other hand, are of either odd or even numbered horizontal scanning lines only, since the mode of image generation is to sample these "half-images" sequentially; consequently, such views contain but one-half of the image.) Disc recorder images were collected at various fixed intervals to permit "time-lapse" display of events. For profile plotting, such stored images were transmitted to a Princeton 801 computer graphics terminal (Princeton Electronic Products, Brunswick, N.J.) which was interfaced to a PDP 11/10 computer (Digital Equipment Corp., Maynard, Mass.) and for which software has been developed to permit extraction of geometric data by point selection through a "spark pen" (Computek Data Tablet, Computek, Cambridge, Mass.).

The quality of video images has been found to be somewhat better than one would imagine; several of the figures in this report were generated from 35-mm photographs taken directly from video images displayed on a high resolution studio monitor (Conrac QQA/15, Conrac Corp., Covina, Calif.).

Results

General Features

Using standard Zeiss/Nomarski optics, it has been occassionally possible to observe considerable epithelial detail by stretching a portion of amphibian bladder between a conventional microscope slide and a coverslip. While little experimentation is possible with this arrangement, it has proven useful for making survey observations and for understanding both the possible limitation as well as the utility of this form of optical system. The major problem with this mounting procedure is the lack of a simple means for maintaining a degree of stretch on the preparation which will minimize smooth muscle contractions. This difficulty is overcome with little or no sacrifice of potential resolving power when preparations are stretched with hydrostatic pressure and ring-mounted as described in *Materials and Methods*. Figure 1 is a view of bullfrog bladder epithelium so mounted and viewed through an oil-immersion objective.

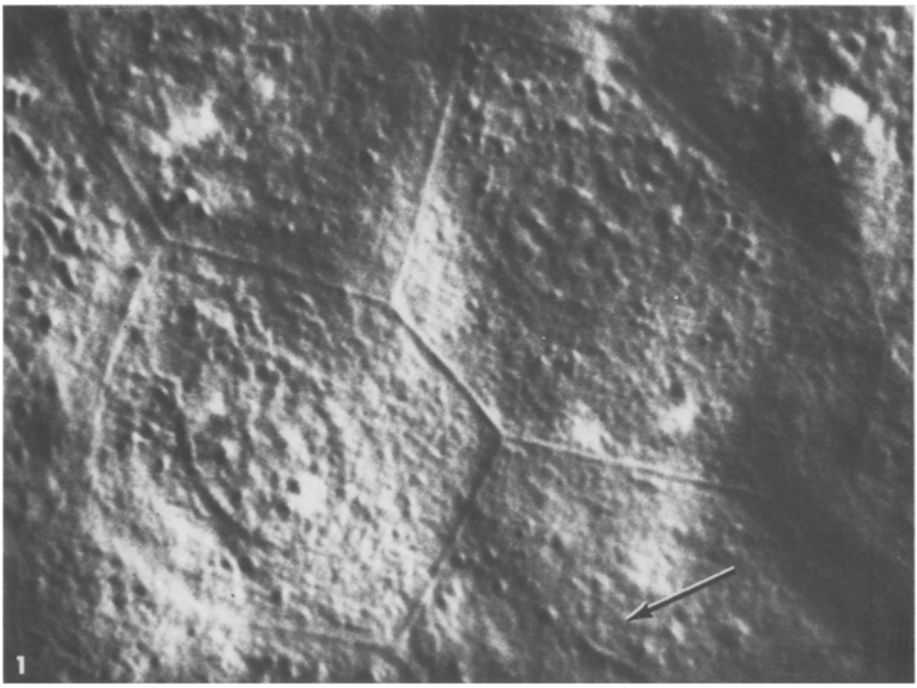


Fig. 1. Surface view of bullfrog urinary bladder by differential interference-contrast microscopy. Boundaries of granular cells are sharply resolved as elevated ridges; this high contrast image of cell-cell junction is provided by the steep gradient of refractive index in the direction of beam shearing (*see* arrow) which is directly across the axis of the boundaries in this field. Nuclei (center of profiles) and surface granularity are simultaneously focused; the preparation is very tightly stretched so that epithelial thickness is minimized. Oil-immersion lens (100/1.25) × 2,100

In a maximally distended preparation, like this one, cell nuclei, other internal organelles, and surface features are in sharp focus in a single plane. The size of the cell surfaces shown here indicates that this field contains only granular cells, appreciated as the majority species in both toad and frog bladders from electron microscopic studies [4, 7, 14].

A characteristic of differential interference-contrast microscopic images is that the sample appears to be obliquely illuminated revealing topological features as ridges or depressions. (This impression is dispelled by a 180° rotation of the image which changes apparent elevations to depressions and vice-versa.) Structural detail is actually provided by the contrast generated from the interference upon recombination of the sheared beam [1]. The rate-of-change, or gradient, of refractive index along the direction of beam shearing (arrow in Fig. 1) determines the

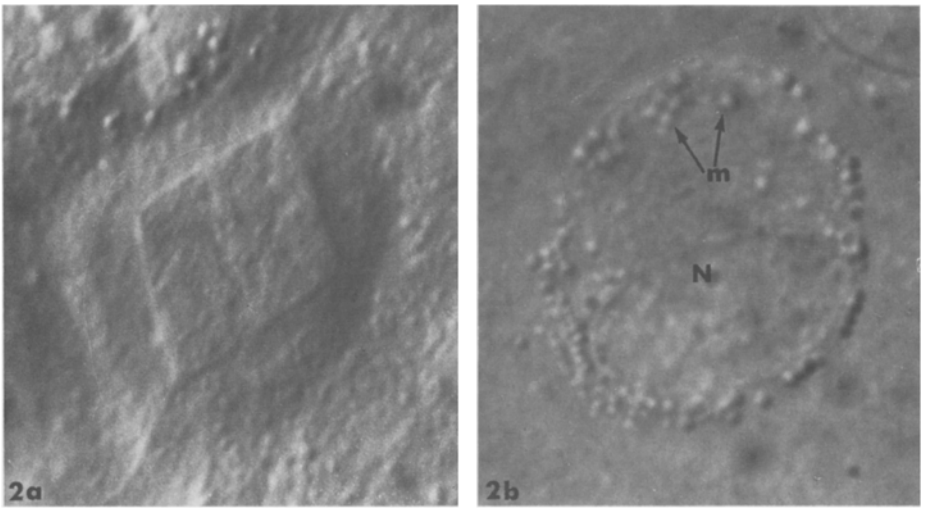


Fig. 2. Optical sections of mitochondria-rich cell from bullfrog urinary bladder. (*a*): Surface view. The rhombic mucosal face of this cell is one of the few polygonal shapes seen Quadrilaterals are most common with occasional pentagons and triangles also present. Junctions between adjacent granular cells are seen running from the upper and lower vertices of the mitochondria-rich cell profile; they are not observed as they extend from the other vertices because those junctions lie almost precisely along the direction of shearing and thus present no refractive index gradient. (*b*): Interior view. The mitochondria (*m*) of this cell type are readily seen and invariably lie in close proximity to the lateral cell membranes. This is true in toad as well as in frog bladder as shown here. Oil-immersion (100/1.25) × 2,000

extent to which contrast is generated by an object. The most striking feature of Fig. 1 is the clarity of cell boundaries so that the shape of the mucosal aspect of these cells is easily seen. A view of this type over large regions would be very informative, but it is not provided with low-power examination since the optical conditions which permit this degree of contrast also result in a very shallow depth of focus and the bladder surface is somewhat irregular even with a high degree of stretch. The impression that the "tight" [16] or "limiting" junctions [10], *per se*, are observed is probably due to two factors. A steep gradient of contrast at right angles to the cell margin is expected with a pair of closely apposed membranes positioned perpendicular to the mucosal surface. Secondly, these cells appear slightly elevated at their boundaries so that a ridge of cytoplasm protrudes to the mucosa at cell margins.

The most useful aspect of a shallow depth of focus is evident in Fig. 2 where "optical sectioning" has been employed to reveal both surface (Fig. 2*a*) and interior (Fig. 2*b*) views of a mitochondria-rich cell.

The rhombic mitochondria-rich cell surface is typical; these cells display a variety of 3-, 4-, and 5-sided surfaces. The oval profile of the nucleus and peripheral distribution of mitochondria are routinely observed as well. Such a specific arrangement of mitochondria has not been suggested by electron microscopy, but it has appeared that this arrangement is not disturbed by tissue fixation or the first few stages of dehydration in alcohol. Unfortunately, good quality optics are not achieved when the tissue is further processed.

The greater part of these efforts has been directed to the structural features of toad urinary bladder where the epithelial physiology has been more exhaustively studied than in the bullfrog. The toad bladder, however, presents additional difficulties. It possesses considerably more smooth muscle activity so that stable images are more difficult to obtain and it has a submucosa much more rich in collagen. The presence of collagen bundles, extremely birefringent in the native, fibrous state, is a major limitation of this or any direct microscopic approach to toad bladder morphology since it is so much more readily imaged than the epithelial structures of interest. With ideal differential interference-contrast optics, image focus falls off rapidly with distance from the plane of focus because of the open condenser aperture which is employed. However, highly refractile images (like those formed by collagen bundles in the submucosa) may appear as regions of bright and dark bands superimposed on a field of interest focussed at the mucosal surface as far as 15–20 μm from the submucosa. For this reason, toad bladder epithelium has been consistently more difficult to work with than bullfrog bladder where the submucosal elements are less dense, or other epithelia like gallbladder where the collagenous supporting tissue is much further removed from the mucosal surface.

To successfully image toad bladder epithelium, it has often proven advantageous to deliberately mismatch condenser and objective Wollaston prisms as has been done to obtain Figs. 3*a* and *b*. Here a 100 X oil-immersion objective (NA, 1.25) has been used with a #2 condenser which is specified for use only with 40–63 × objectives. The resulting image in this instance is among the most informative obtained in our experience to data with these methods. Here the surface features of granular cells in the Dominican toad bladder are very well resolved. The microvilli are easily seen in Fig. 3*a*; they appear as individual projections rather than forming a "brain coral" pattern as has been suggested from scanning electron microscopy [7, 8]. Junction profiles appear as ridges between cells. In Fig. 3*b*, where focus is some 4–7 μm below

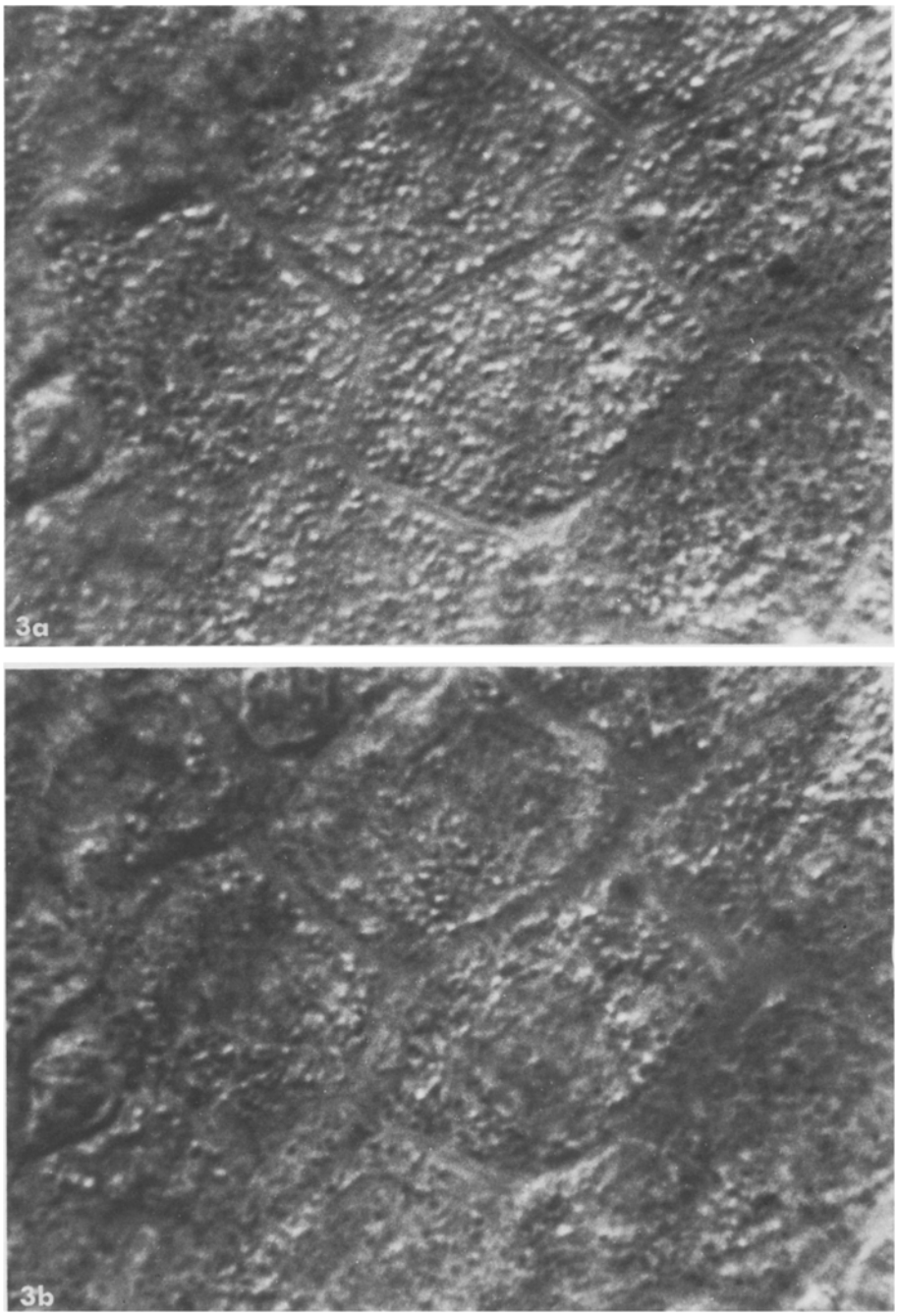


Fig. 3. Optical sections of Dominican toad bladder granular cells. (*a*): Surface view. Mucosal membrane microvilli are seen here as apparent finger-like projections. The junctions between cells appear to overlie pale, open spaces below. (*b*): Interior view. The granular content of the cytoplasm is in evidence but there seems little basis for differentiating mitochondria from granules, both of which are presumably present. Adjacent cell margins are loosely separated in this moderately stretched preparation; such "open" intercellular spaces are not seen when bladders are more tightly stretched. Oil-immersion (100/1.25) × 1,750

Fig. 3*a*, the lateral intercellular spaces are evident. This preparation was mounted with moderate tension; when bladders are more tightly stretched, the lateral spaces are not resolved; this is as expected from earlier studies with electron microscopy of this tissue [11, 12].

Cytoplasmic Viscosity

Among the organelles which might be detected by these methods, those which can be readily identified are nuclei, the mitochondria of mitochondria-rich cells, and the mucous droplets of the goblet cells. Others—granules, lysosomes, granular cell mitochondria, and lipid droplets—are obviously within recorded images but cannot be specifically identified. These intracellular organelles with radii in the range of 0.05 to 1 μm might be expected to exhibit Brownian motion as is readily observed in broken cell preparations, but they do not. A translational movement of particles within the cells can be seen when the cells are grossly swollen by dilution of the serosal bathing medium, and it might be argued that particle motion is too slight to be noticed in the viscosity of an isotonic cell interior. The sensitivity of detection by the closed-circuit video methods we have employed was tested with tape recording of the "spontaneous" motion of isolated rat liver mitochondria suspended in sucrose solutions covering a wide range of viscosities. Examining these with the identical optical system and in concentrations sufficient to mimic the crowding of particles in the cell, measurable displacements were recorded in solutions of viscosity increased by up to 50% sucrose. With some difficulty, motion was detected in 70% sucrose solutions, but it was not clear that this was always independent of the streaming effect due to drying of the suspension under the coverslip. A 50% solution of sucrose has a viscosity 15.4 times that of water; 70% sucrose is 480 times as viscous as water. It is reasonably concluded, then, that Brownian motion of mitochondria in the cells would be detectable at levels of viscosity of up to at least 13.7 cP, a value which must be less than the effective viscosity of cytoplasm at 25 °C. If, however, intense light is focused for an extended period on a single high-power field (about 6 cells), Brownian motion is eventually detected. In each case where this has been seen, its onset is abrupt and preceded by an apparent increase in cell volume. It appears that some uptake of water is essential; the consequent temperature elevation (from steady illumination) cannot be strictly responsible since the motion is often detected within a single cell in the field where others are logically at the same temperature and still provide a static view.

Table 1. Tissue viability during microscopic examination

#	(Time-min)	Initial[a] PD	Final PD	% fall/hr
1	(130)	34	21	17.7
2	(124)	22	17	11.0
3	(117)	68	50	13.6
4	(186)	47	35	8.2
5	(161)	31	27	4.8
6	(153)	51	41	7.7

[a] Transmural potential difference (mV): serosa positive to mucosa.

Tissue Viability

These techniques should offer little advantage if it were not that the epithelium was being examined in a functional state close to that obtained when using mounting chambers of the Ussing type for electrophysiologic studies. It seemed important then to verify the nondestructive nature of this optical procedure using the spontaneous transmural potential difference as an index of epithelial viability. Six preparations were secured on Lucite mounting rings as described above. The mucosal surface (interior of the ring) was bathed with 2.0 ml of isotonic Na^+-Ringer's solution and the ring was suspended, partially immersed, in a Petri dish filled as well with Na^+-Ringer's solution. In this configuration, potential differences of 22 to 68 mV, (serosa positive) were noted using matched calomel electrodes in series with 3 M KCl-agar filled bridges. Subsequently, the ring-mounted preparations were placed on lantern-slide cover glasses and examined with the optical system described for varying periods. In each case illumination of the sample was held constant at the minimal settings required for 1029 line video recording and no experimental manipulations of the media were performed. Ring-mounted preparations were then repositioned in the Petri dish where spontaneous PD was again recorded. Table 1 itemizes the findings and illustrates that over as much as 3 hr of continuous observation the decline of spontaneous potential is modest, if slightly greater than the decline usually noted when this preparation is mounted in a conventional chamber for electrophysiology.

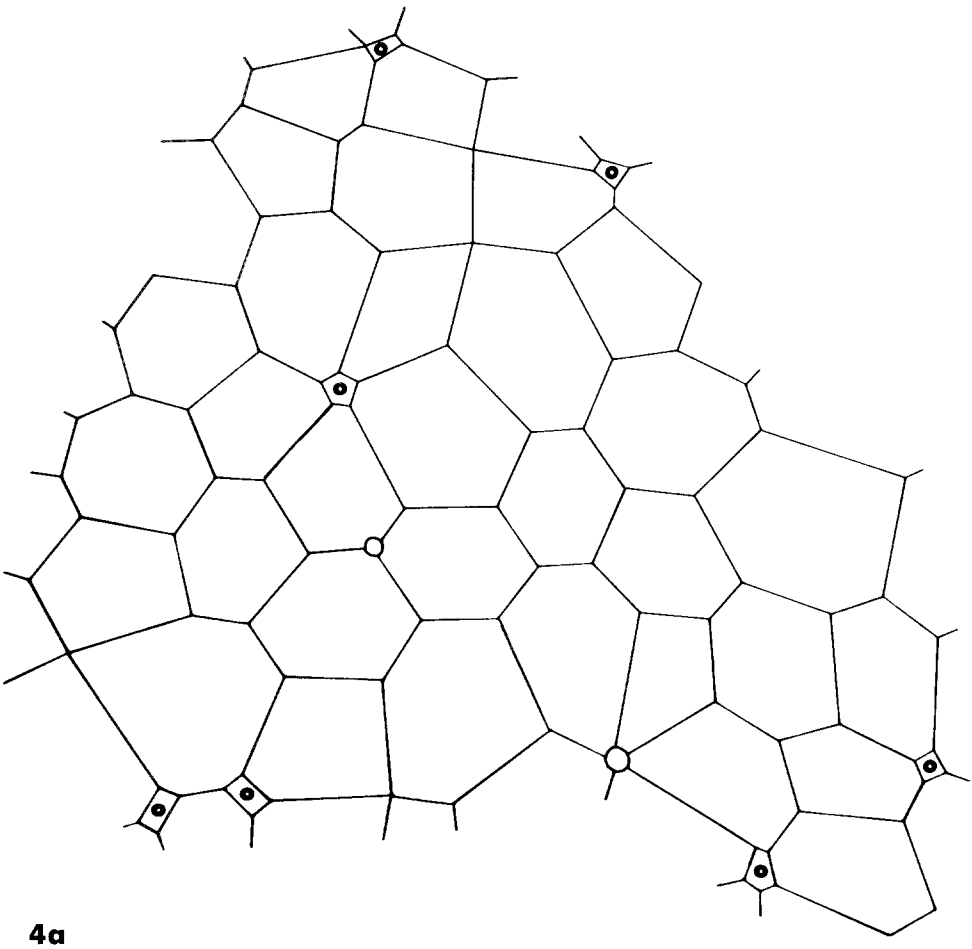
Distribution of Cells at the Mucosal Surface

An obvious advantage of these methods is that it is possible to map regions of the surface, using optical sectioning (as in Fig. 2) to verify

the cell types, and recording the dimensions of cell profiles in the living preparation. Figure 4*a* and *b* are "maps" of urinary bladders from toads of the Dominican Republic and Colombia, respectively. As comparative examination of the pictures suggests, a consistent observation has been the considerably greater frequency of mitochondria-rich cells in Colombian toads. Those with larger surface areas seem generally less refractile than their counterparts and, it would appear, are not present to any significant extent in toads from the Dominican Republic. Whether this disparity in surface appearance is indicative of a functional subdivision in this cell variety is not clear at this time. These figures show that no very obvious pattern is necessarily present in either variety and that, in each case, there are some granular cells which do not share any of their margins with mitochondria-rich cells. This fact is supported as well from studies with scanning microscopy and with freeze-fracture methods (J.W. Mills and J.B. Wade, respectively, *personal communications*).

A quantitative statement of the structure of the mucosal surfaces required more extensive sampling. Mapping of large and continuous areas is scarcely possible given the variation in image quality from region to region. In practice, the junctions in a single focal plane in a given field are traced from the video screen or computer-filed with spark pen specification of end points and vertices; focus is then progressively adjusted to reveal and permit cataloging of the junction segments not seen in the original plane of focus. A programmed correction for convolutions in and out of the dominant focal plane is then executed and the data are displayed as a two-dimensional array of lines reflecting accurate profile lengths between vertices. Only the flattest samples can be mapped this way to any extent, and any degree of correction for convolutions obliges the map to be eventually self-limiting since the two-dimensional projection cannot completely accomodate the three-dimensional information. This computer-assisted reduction of displayed images was employed to record 6–10 randomly selected fields (of 18–131 cells each) from each of six bladders of each variety. A numerical summary of these findings is provided by Table 2. An important aspect of the obtained results is the fact that, with sufficiently large sampling, it is safe to conclude that there are "average" results which describe these preparations. Individual fields do not convey this impression but suggest that a great deal of variation may occur[2]. Measurements of frog bladder surface para-

2 It may yet be true that there are seasonal variations; all of these bladders were sampled in the months of August, September, and October.

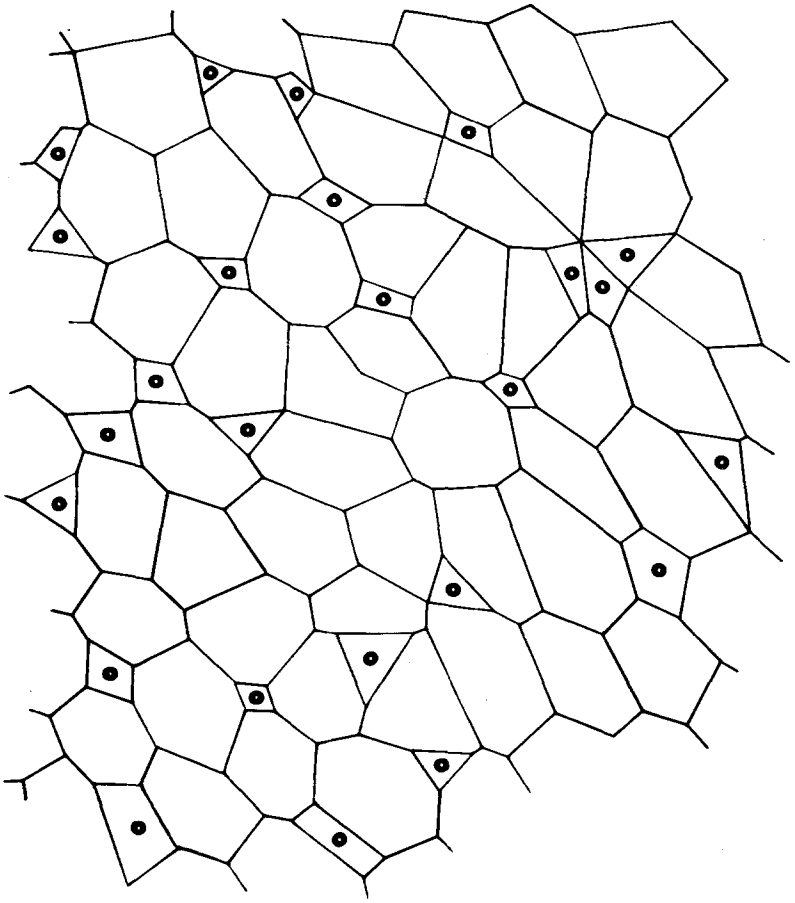


4a

Fig. 4. Schematic views of Dominican (*a*) and Colombian (*b*) toad bladder mucosal surfaces. These views from tracings of cell profiles by differential interference-contrast microscopy are not typical but serve to dramatize the fact that Colombian toad bladders contain far more mitochonria-rich cells (labeled with circles) and have somewhat smaller granular cells (unlabeled) than Dominican toad bladders. Goblet cells are the small round profiles; they are generally more abundant than in these views. Effective magnification: ×450

meters have not been made in this fashion, but examination of numerous samples confirms the observation that goblet cells are not a component of that epithelium [23]. The distribution of mitochondria-rich to granular cells there appears much like that in bladders of Dominican toads (i.e., about 1:6 as opposed to about 1:2 for Colombian toad bladders).

An important feature of the surface which is not evidenced by a numerical tabulation as in Table 2 is the packing pattern in which the cells are arranged. There is no precise regularity in either variety but a cataloging of cells on the bases of the types which share their boundaries



4b

answers a currently considered question and allows construction of a quantitatively accurate, visual picture of the mucosal surface of the epithelium in the bladder of the Dominican toad. Figure 5 is a schematic representation of such a "unit cell", the minimum description of the surface which depicts all of the pertinent characteristics. This scheme is one of very few finite solutions to the accumulated data; it contains 3 tetragonal mitochondria-rich cell faces for each triangular one; these mitochondria-rich cells oblige additional facets on the otherwise hexagonal outlines of the granular cells so that the granular cells average slightly more than 6 sides/cell; in each group of 24 granular cells, 15 are touched by a mitochondria-rich cell, 9 are not – a fairly accurate statement of the numerical finding that $34 \pm 4\%$ of the granular cells touch only other granular cells. Goblet cells are not included. Their

Table 2. Mucosal surface parameters in the urinary bladder of the toad, *Bufo marinus*[a]

Origin	Cell type	#/cm^2	Sides/cell	Perimeter (μm)	Area (μm^2)	% of cells	% of surface
Dominican Republic ($n=6$)	Granular	159,000 ±5,100	6.15±0.32	90±7	605±47	80±4	96.5±1.5
	Mitochondria-rich	26,600 ±1,150	3.75±0.14	42±3	120±31	13±1.5	3.2±0.4
	Goblet	12,300 ±1,400	round	19±2	28± 3	6±2	0.3±0.2
Colombia ($n=6$)	Granular	184,000 ±7,200	6.25±0.30	82±8	445±58	62±6	82 ±4.5
	Mitochondria-rich	105,700 ±6,350	3.77±0.22	60±3	170±44	35±5	18 ±2.5
	Goblet	9,000 ±2,300	round	19±2	28± 4	3±1	0.2±0.2

[a] Values are expressed as means±SE of the averages obtained from each animal.

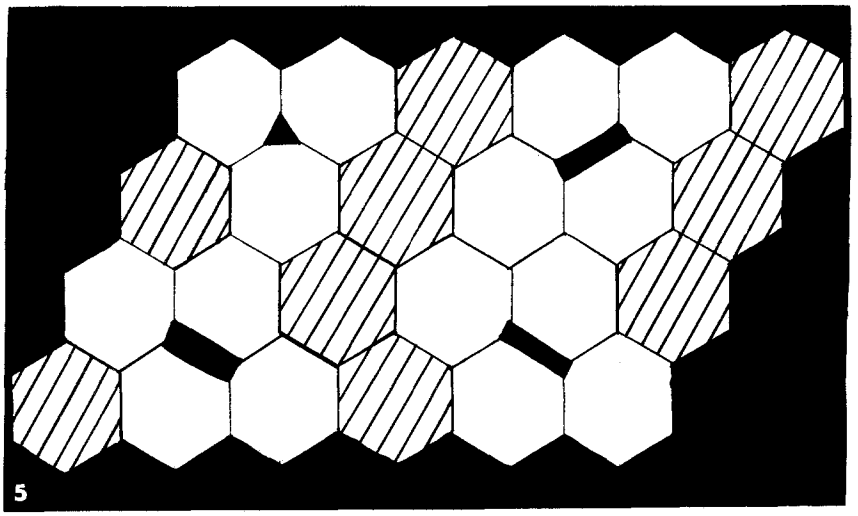


Fig. 5. Unit cell representation of Dominican toad bladder mucosal surface. This schematic drawing illustrates the features necessary to generate many of the structural characteristics of Dominican toad bladder epithelium. Portrayed are the following features: 1 mitochondria-rich cell (blackened) for every 6 granular cells; 3 rectangular to each triangular mitochondria-rich cell profile (ave. no. of sides=3.75); 3 granular cells in contact with only other granular cells (stippled) for every 5 that are touched by a mitochondria-rich cell; approximate distribution of mucosal surface area, 97:3, granular/mitochondria-rich cells. This arrangement probably never exists exactly as shown but reproduced laterally (6625 times) it would reasonably account for all of the salient features in a 1-cm^2 region of bladder mucosal surface

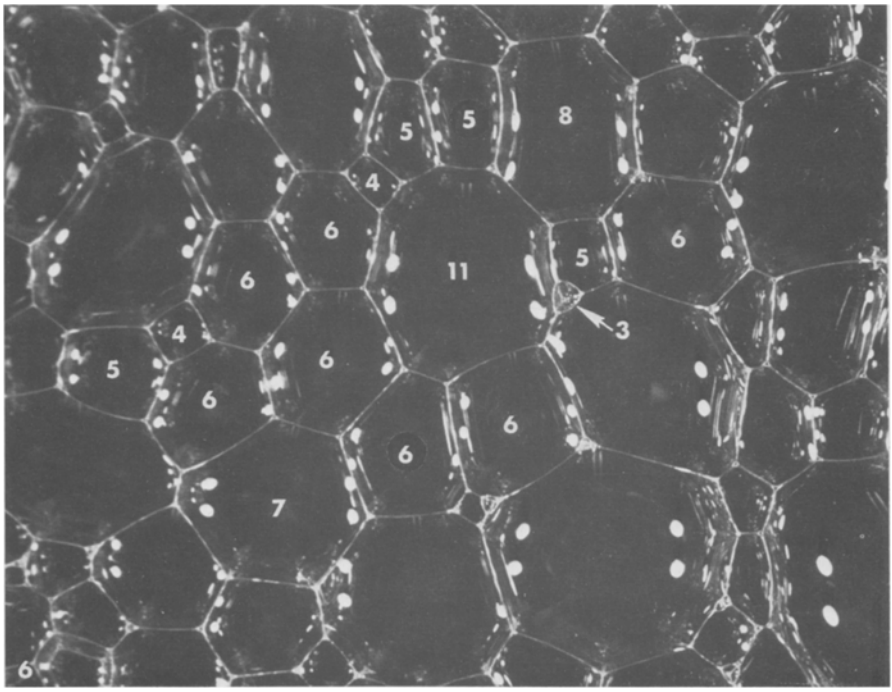


Fig. 6. Monolayer of soap bubbles (Photoflo 200, Eastman Kodak, Rochester, N.Y.). This array was generated to illustrate simply the rather complex mathematical solution to equilibration of tensions in a froth of distensible solids of identical composition (e.g., epithelial cells). It is shown here that stability of packing occurs when three edges meet to approximate three 120° angles. Where the bubbles are of unequal size, this cannot be realized; the more acute angles, then, fall to the interior of the smaller bubbles, the more obtuse to the larger, and the required distribution of tensions is satisfied by the resulting distribution of sides. The result of this is that in any local environment, the smaller bubbles will have fewer sides than the larger ones. The paired bright spots which occur repeatedly are reflections of the lights used for photography

distribution is apparently random and they must be considered of modest importance since they are completely absent in the frog urinary bladder. The Colombian toad bladder cannot be as meaningfully represented because the distribution of cells is less well understood with the data thus far collected.

Speculation on the possible significance of cell surface shapes is forestalled by the observation that the 5-, 4-, and 3-sided profiles of mitochondria-rich cells are a logical consequence of their being small members of an otherwise nearly regular array. Figure 6 is a photograph made of a froth of soap bubbles where minimum energy considerations in a two-dimensional lattice are graphically depicted. Note that, in a general

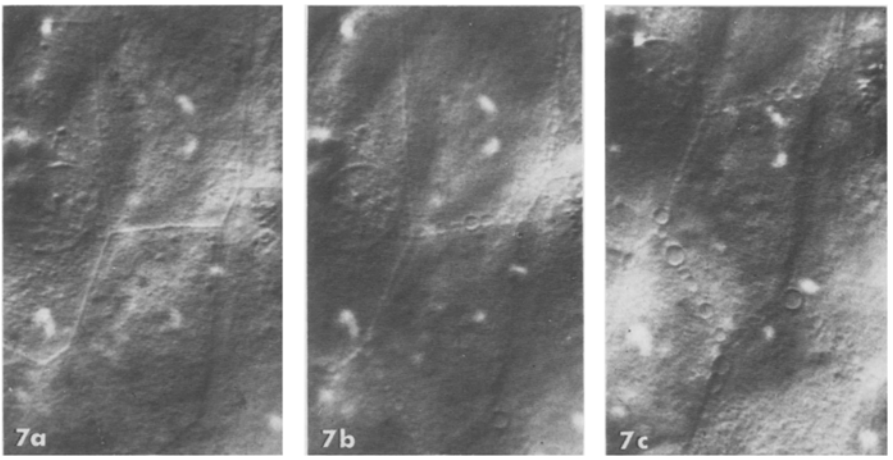


Fig. 7. Bullfrog urinary bladder surface before and during exposure to hypertonic mucosal medium. (*a*): With isotonic solutions on either side of the preparation, junctions are revealed as smooth ridges marking the cell boundaries. Immediately after this was recorded, 0.4 ml of mannitol (2.4 M in standard Ringer's solution) was added to the 2.0-ml mucosal bath so that final tonicity might reach 625 mosmol/kg H_2O. (*b*): Sixty seconds after addition of mannitol to the mucosal medium, focal spherical profiles are seen at the position of the previously smooth ridges of junction. Unfortunately, it is not possible to determine the level to which mannitol has penetrated the fluid beneath the coverslip through which the preparation is being viewed. (*c*): Five minutes after the mannitol addition, the field is shifted slightly upward to reveal additional blistering. Oil-immersion views (100/1.25) × 750

way, smaller units have less sides, a condition which optimizes the approach to equality of the contact angles at each vertex. The fact that the bladder surface approximates a "minimum energy" configuration for packed soap bubbles supports a view that the membranes of these cells are pliable, at least during growth phases of the epithelium, and that surface tension plays an important role in the eventual topology of the epithelium.

Osmotic Sensitivity of Limiting Junctions

A major objective of these efforts has been to make possible longitudinal studies of structure while function is continuously monitored. It is therefore important to establish that structural changes of interest can be observed. Osmotically-induced distensions of the "tight" or "limiting" junctions have been established by electron-microscope studies

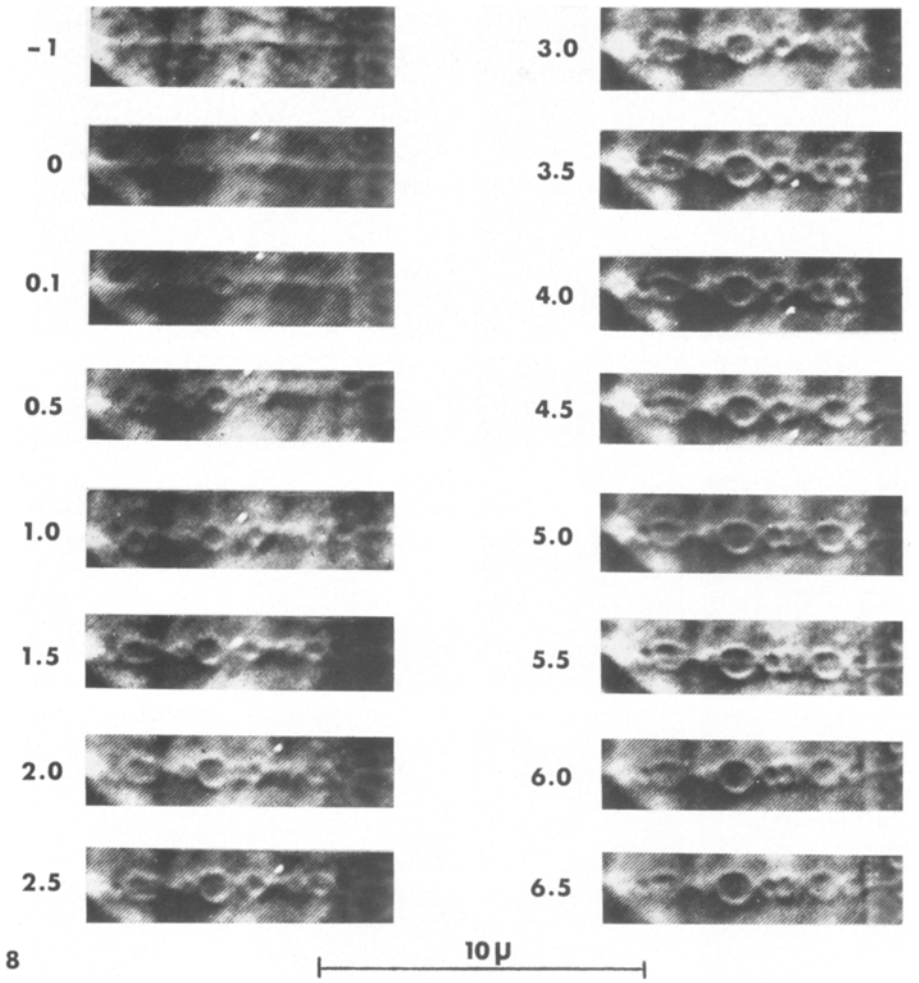


Fig. 8. Video-taped sequence of junction deformation in Dominican toad bladder after doubling of mucosal tonicity with urea. Numbers to the left of individual panels indicate the number of minutes before (−1) or after addition of urea (up to 6.5 min). In this case, with water-immersion optics, urea was added with a syringe through a fine bore needle to guarantee very rapid mixing so that attainment of a mucosal tonicity of 450 mosmol/Kg H_2O was virtually instantaneous. By 0.1 min, a small blister is evident; at least 3 are seen at 0.5 min; by 3.0 min the junction stretch (between two granular cells) is completely blistered. Fusion of blisters is apparent from views at 4.0 min to 4.5 min along the right-hand side of the junction. Water-immersion lens (50/1.00) × 4,225

[10, 13, 28] as the basis for the dramatic increases in transmural conductance which are brought about when amphibian bladder or skin is acutely exposed to hypertonic mucosal medium [24, 25]. The reliability of this phenomenon in terms of the electrical effects is sufficiently high

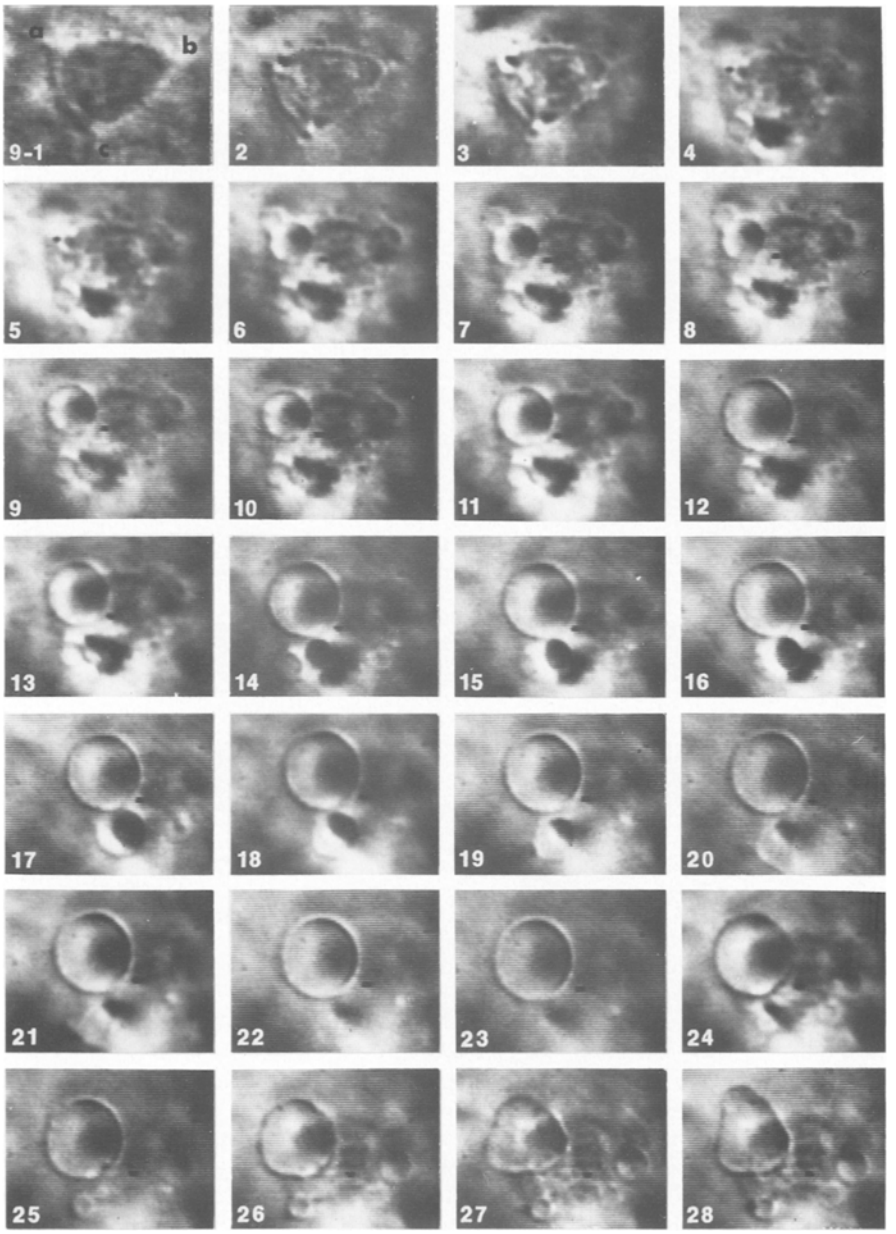
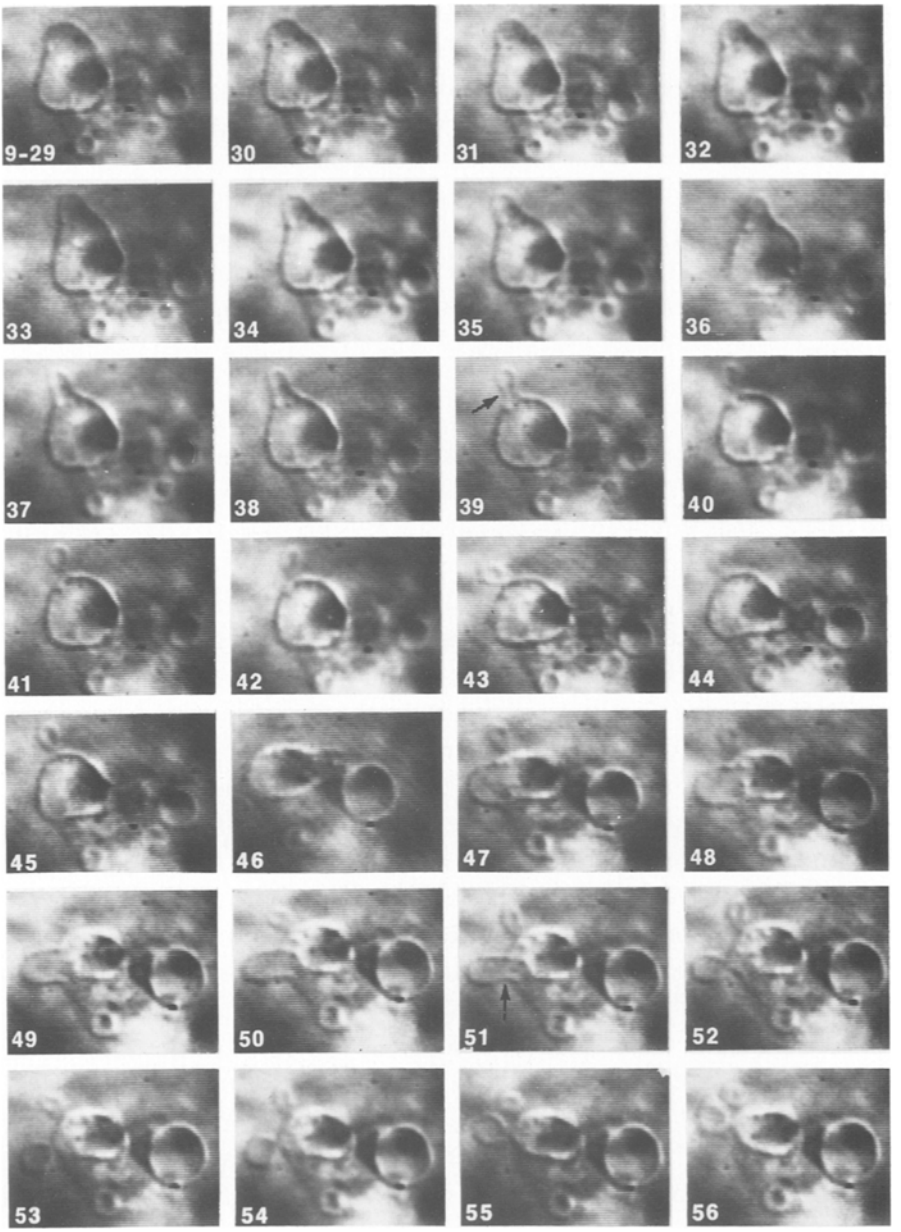


Fig. 9. Time-lapse study of osmotically-induced structural changes in a mitochondria-rich cell junction following a doubling of mucosal tonicity with KCl. Views are recorded at 12-sec intervals from 525-line video format. This sequence illustrates the development of individual blisters of the junction in considerable detail and obliges the conclusion that a considerable elaboration of membrane is required to encompass these accumulations of fluids. The breaking-off of small droplets and internalization of others are events which were implied by electron microscopy but labeled as presumed artifacts [13]. Water-immersion (50/1.00) × 2,100



that we may, in the short run, proceed confidently with a structural study apart from simultaneous electrical measurements.

Initially, with optimal resolving power as an objective, observations were made with a coverslip over a ring-mounted, drum of frog bladder through an oil-immersion objective; in this configuration the ability to

make quantitatively accurate changes in the mucosal solution tonicity is compromised. Figure 7*a* illustrates bullfrog bladder junctions with isotonic solutions bathing each epithelial surface. Figure 7*b* and *c* are taken 1 and 5 min, respectively, after a quantity of 2.4 M mannitol was added to the mucosal bath sufficient that maximum tonicity could not exceed 625 mosmol/kg H_2O. It is evident that the hypertonic mucosa resulted in a focal blistering of the junctions with no apparent change in the remainder of the epithelial features in this plane of focus.

To perform this experiment with precise knowledge of the changes in tonicity, it has been necessary to employ both a water-immersion objective to eliminate the need for a coverslip and thus provide true access to the mucosal bath, and closed-circuit video to follow changes in a continuous fashion. Figure 8 is the result of such an experiment on a Dominican toad bladder where mucosal tonicity was nearly doubled with urea (final concentration: 430 mosmol/kg H_2O). The video tape from which these photographs were made was produced with a high sensitivity silicon-vidicon camera under conditions where incident illumination was purposely reduced to a level too low for visual observation in order to keep specimen heating to a minimum. In this series of views several of the characteristics of junction deformation which had been described by electron microscopy can be observed.

It is clear that junction blistering begins within seconds after addition of solute to the mucosal bath; note that a focal distension is present at 0.1 min and at least four can be seen by the end of the first complete minute. Individual blisters may fuse with one another; the clearest example of this is in the change of image from the views at 4 min to that at 4.5 min where the paired blisters on the right of the junctions apparently fuse to form a single sphere. The focal nature of the distortions is confirmed with these images as well; in the view at 5.5 min, where the focal plane is slightly deeper, the refractile, intact ridge of junction, below the blistered profiles, is superimposed on the image.

In Fig. 9 the dynamics of junction distortion are followed on the boundaries of a mitochondria-rich cell. These views are 12 sec apart and thus cover a nearly 12-min period following a doubling of the mucosal tonicity with KCl. In this instance, it is possible to measure the volumes of some of the larger distensions making only the reasonable assumption that they are spherical. The vertices seem more susceptible than the linear portions of the margin. Following the events occurring at the upper left corner of the triangular cell profile, the development

of a large blister can be traced. The paired profiles in view #8 apparently fuse to form a single sphere with a volume of 1.77 μm^3 and a surface area of 9.42 μm^2 by view #9. There is no evidence of fusion of this sphere with another through the next 14 views (2 min 48 sec) to panel #23 where volume has increased to 8.2 μm^3 (surface area: 15.7 μm^2). Fluid accumulation here is then calculated as nearly 2.3 μm^3/min in this locus.

In the 24th view (at 3 min), there is a marked change in refractility of this profile; in subsequent views it appears progressively more flaccid with portions of the surface eventually being "pinched off" (note: views #39 and 51). It appears as well that by $9\frac{1}{2}$ min (view #48) there are small droplets within the interior of the large sphere. Neither the "pinching-off" or the "internalization" phenomena are as easily explained as junction blistering, *per se*, which may be accounted for by an imbalance of osmotic flows into and out of the compartments formed by the serial arrangement of barriers which comprise the junction [21].

Discussion

The results of this investigation establish that there are substantial advantages in the application of differential interference-contrast microscopy to the study of amphibian urinary bladder. Epithelial structures of interest are observed with a high degree of contrast and resolution under circumstances where the tissue is bathed in physiologic solutions and retains its viability. The particular advantages of this method are that no stains are required for contrast production and that the very shallow depth of focus allows an "optical sectioning" of the tissue so that features in one plane of interest may be examined free from spurious images from other levels within the sample. Coupling this form of optics with closed-circuit video methods has further increased its utility. The enhanced sensitivity of video detection methods permits registration of high quality images without intense illumination; specimen temperature is then not elevated to nonphysiologic levels.

Epithelial Fine Structure

A number of specific observations have been made which are of interest to studies of transepithelial transport in this system. Four of

these findings are related to structure at the subcellular level. The first of these is the presence of finger-like microvilli on the granular cell mucosal membrane rather than a brain-coral pattern as has been described by scanning electron microscopy [7, 8] and freeze-fracture methods [27]. The brain coral image may reflect an artifact of specimen drying or a transient surface configuration which is not resolved with the present methods.

A second point is the observed distribution of mitochondria within mitochondria-rich cells. The preferential distribution of these organelles in close proximity to the lateral cell boundaries prompts speculation of their involvement in the energetics of transmural transport. Why this distribution has not been previously detected in studies where this cell has been carefully scrutinized [4, 14, 26] may be due to the necessarily different perspective provided by electron microscopy. More likely, there is a randomization of mitochondrial positions at some point in tissue preparation; this point is suggested by the observations of Frederiksen and Rostgaard [17] who claim that there are substantial rearrangements of epithelial morphology during the latter phases of tissue preparation for electron microscopy. Their observation is in fact on the absence of dilated intercellular spaces in the frog gallbladder [17]. In toad bladders, intercellular spaces were observed as patent when the tissue is not maximally stretched, in general agreement with electron microscopic studies [11, 12]. Of course, the presumed basis for space distension in the gallbladder is isotonic fluid transport [9], a process not occurring in toad bladder, so that the present results are not necessarily incompatible with theirs.

The fourth such observation is perhaps the most provocative: that there is no detectable Brownian motion in the cytoplasm of bladder epithelial cells. The consequent conclusion that *effective* cytoplasmic viscosity is greater that 13 cP is potentially misleading. The consequences for diffusion of solutes of interest and hence for transepithelial transport depends on whether the cytoplasmic sap is a homogeneous gel of high viscosity or if it is grossly "structured" to restrict the movement of visible organelles but sufficiently fluid to permit free diffusion of solutes. The present techniques should be very useful in future pursuit of this problem.

The Mucosal Surface

The usefulness of amphibian urinary bladder as a model system for the study of salt and water transport has been predicated to a large

extent on its relative structural simplicity. However, it has long been appreciated that there are four cell types within the epithelium which must be considered [4]. The present study provides some useful information in this regard.

Goblet cells may well be completely excluded from consideration as participants in the transmural transport of salt or water. It is noted here, as well as earlier by Strum and Danon [23], that goblet cells are completely absent in the bullfrog bladder. In bladders of *Bufo marinus,* they are present in small numbers (3–6%) of the surface cells and occupy only a vanishingly small fraction of the mucosal surface area ($<0.5\%$).

Of the remaining cell types, basal cells cannot be well studied with these methods but their involvement in transepithelial transport is considered unlikely since they do not reach the mucosal surface [13].

The relative importance of granular and mitochondria-rich cells has been a concern of much recent interest. It seems irrefutable that the ADH-sensitive hydroosmotic behavior of the epithelium is exclusively a function of the granular cells [5, 15, 22]. In Dominican toads where most water-flow studies have been carried out, the mucosal surface is $>95\%$ occupied by granular cells. This can only serve to add strength to that conclusion. The examination of ADH-induced water flow by direct observation is a logical extension of these initial studies, given the demonstration that lateral intercellular spaces and the cell interior can each be well-resolved.

The role of mitochondria-rich cells is not completely resolved but here, as well, the present findings are relevant. Establishing that Colombian toad bladders contain more than twice as many mitochondria-rich cells as the Dominican variety strongly supports the contention of Rosen, Steinmetz and Oliver that this cell type is principally involved in H^+-secretion [20]; there is no published evidence that Colombian toads are more vigorous in the transport of Na^+. The frequency of "rosette" configurations of granular cells about a mitochondria-rich cell was commented upon by Danon, Strum and Edelman [7]. Recent propositions that this might be a meaningful arrangement [18] are probably unwarranted. The arrangement is far from constant in either variety of *Bufo marinus,* and fully 35% of the granular cells in bladders of toads from the Dominican Republic make no contact at all with a mitochondria-rich cell. There would also appear to be little relevance in the actual shape of mucosal surface of cells. The 3-, 4-, and 5-sided profiles of mitochondria-rich cells are likely a natural consequence of their coexistence in an array with larger cells as is shown here in examination

of packing among soap bubbles in a froth. The same considerations predict the near constancy of hexagonal profiles among the granular cells which exhibit fairly constant surface area.

Quantitative models of active transport across amphibian bladder require consideration of the extent and distribution of the cell junctions so that passive permeability (the "shunt" pathway) may be properly evaluated as an extensive parameter. In this respect, knowledge of the epithelial packing arrangement and the cell surface sizes provides the necessary data for extrapolation to the quantity of junction/unit area of the mucosal surface. In the Dominican toad bladder, there are 7.16 m of junctions/cm^2 of surface area. Roughly 85% (6.04 m) of this is junction between adjacent granular cells; 15% (1.12 m) is junction formed by apposition of a granular and a mitochondria-rich cell. Classification of the junctions in these terms may be meaningful as well; earlier results suggested a distinction where the heterocellular junctions (granular/mitochondria-rich) were specifically sensitive to the application of a mucosal bath made hypertonic with raffinose [13]. Preliminary examination of this possibility with the present techniques does not warrant amplification of this point.

Osmotic Sensitivity of Junctions

The usefulness of these methods for longitudinal studies of structure during experimental manipulations has been demonstrated here with video recording of the blistering of cell junctions in response to hypertonic mucosal medium. There has been little reason to challenge the original description of this phenomenon but its validity as the basis for the observed change in conductance has been questioned [3]. Analysis of the junction response in terms of a three-compartment model [6] has supported in detail the interdependence of the electrical and structural changes [21], and documentation of the structural events here removes any remaining contention that the results with electron microscopy were artifactual. Specifically, it is confirmed that elevation of mucosal tonicity causes a focal blistering of the junction and no apparent change in other epithelial structures or in cell volume. The apparent recruitment of membrane to contain the accumulated fluid is of potential importance to understanding junction organization and suggests that a degree of structural fluidity must be considered.

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