THE ISOLATION OF THE MEMBRANE OF THE TOAD BLADDER EPITHELIAL CELL

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There is increasing interest in the structure and composition of cell membranes, and in techniques for their isolation. The following report describes our method for the isolation of the membrane of the toad bladder epithelial cell, the morphology of the isolated membrane, and preliminary determinations of its phosphatase activity. Our interest in isolating the epithelial cell membrane is based on current evidence that the luminal boundary of the cell is the ultimate site of action of vasopressin (1), and that the serosal boundary is associated with active sodium transport (2, 3).

MATERIALS AND METHODS

Isolation of Membranes

Bladder halves of female toads (*Bufo marinus*) were removed and rinsed three times in distilled water which contained 1 mM sodium bicarbonate, pH 7.5. The epithelial cells were then scraped off the bladders with a glass cover slip into a Petri dish containing bicarbonate solution. From 10 to 20 bladder halves were treated in this manner, and the accumulated epithelial cells spun down for 2 min in a table model centrifuge. The cells were washed in fresh bicarbonate solution, recentrifuged, and finally resuspended in bicarbonate solution, the total volume being 4 ml for each bladder half originally used. Homogenization and isolation of the membranes was carried out by a modification of the method of Neville (4). The suspension was transferred to one or more Dounce homogenizers of 15-ml capacity equipped with loosely fitting pestles (Kontes Glass Co., Vineland, New Jersey), and the cells gently homogenized at 0° with 15 to 25 back and forth strokes of the pestle. The homogenate was examined by phase microscopy, and homogenization was continued if more than a few whole cells were seen. An aliquot of this crude homogenate was stored at 0° (crude homogenate fraction), and the remainder was freed of whole cells and nuclei by a method of filtration recently described by Warren (5). Glass beads, 50 to 80 μ in diameter (Superbrite beads, type 150-5005, Minnesota Mining and Manufacturing Co., St. Paul, Minnesota), were soaked in 0.1 M HCl for 1 hr, washed in distilled water, and placed in a Büchner funnel equipped with a coarse fritted glass disc, forming a layer approximately 0.5 cm deep on top of the disc. Filtration was carried out at 4°C; the homogenate was slowly poured through the funnel, washed through with an additional 40 to 80 ml of bicarbonate solution, collected, and centrifuged for 30 min (1500 *g* at 4°C). Phase microscopy of the pellet showed that it was free of whole cells and virtually free of intact nuclei. Abundant membrane "envelopes" were present, and retained their original appearance. One per cent of the original protein content of the crude homogenate was present in the

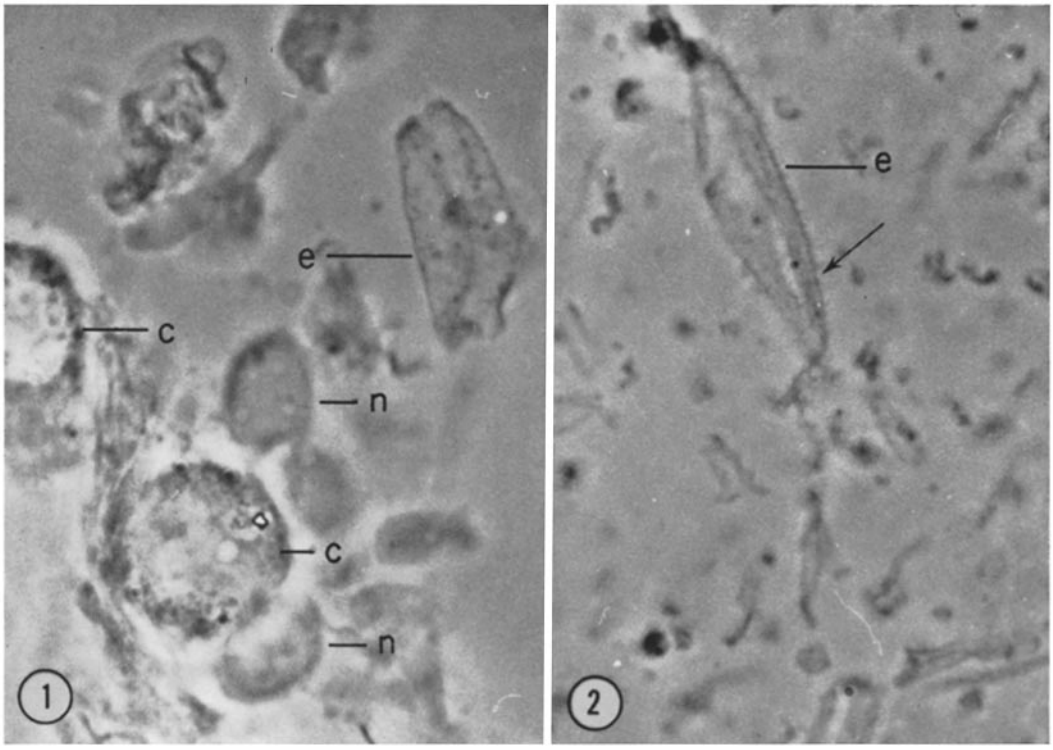


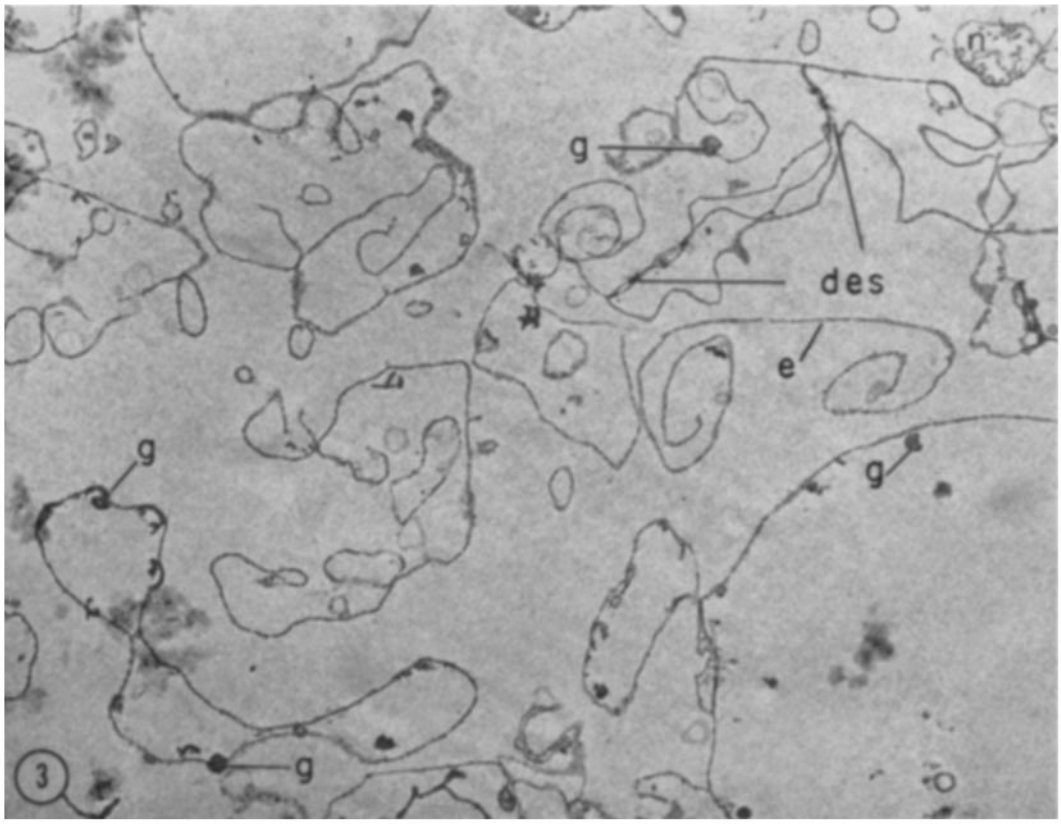
FIGURE 1 Phase photomicrograph of crude homogenate, prior to bead filtration. Intact epithelial cells (*c*), extruded nuclei (*n*), and a cell envelope (*e*) are present. × 1200.

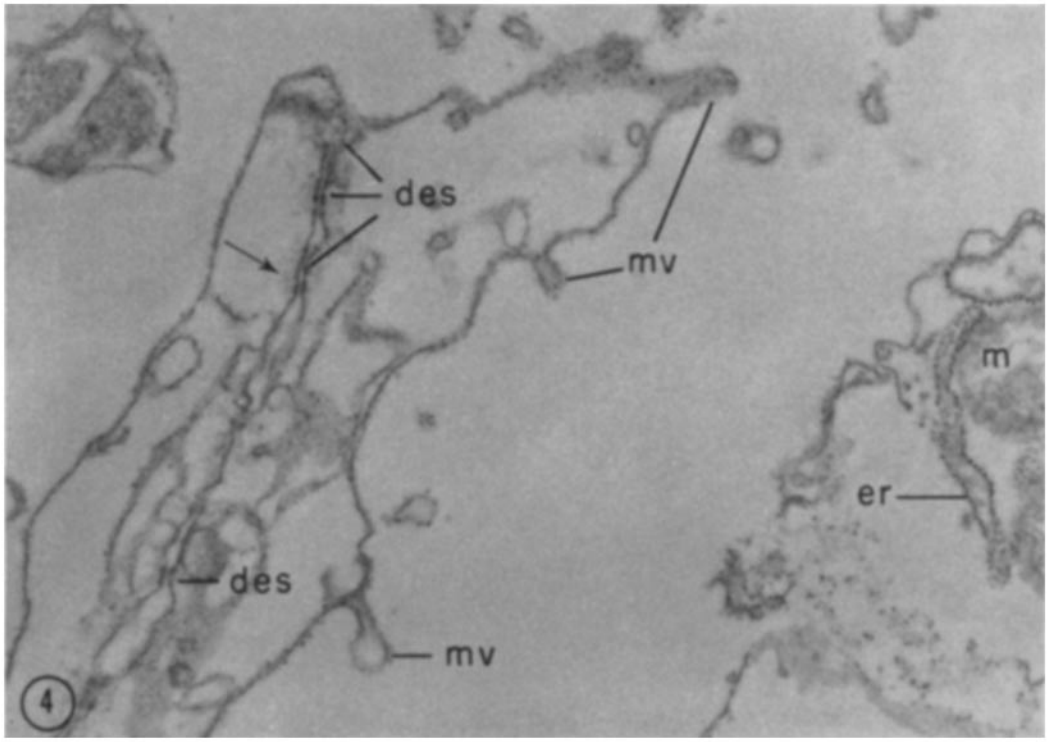
FIGURE 2 Nuclei and whole cells have been removed from the homogenate by bead filtration; cell envelopes (*e*) and fragments of envelopes are present, as shown in this phase photomicrograph. Hairlike processes (arrow) may often be seen extending from the surface of the envelopes. × 1100.

pellet. The membrane pellet was then made up to a volume of 0.3 ml with bicarbonate solution and carefully mixed with 2 ml of 69% sucrose (d, 1.34). The sucrose solution was transferred to a cellulose nitrate centrifuge tube, and overlaid with 1 ml of 55% sucrose (d, 1.26) and finally 1 ml of 36% sucrose (d, 1.16). The gradient tube was spun at 105,000 *g* for 75 min at 2° in a Spinco Model L ultracentrifuge, using an SW-39 swinging bucket rotor. Following centrifugation, a white band composed of cell membranes was observed at the d 1.16/d 1.26 interface. The membranes were collected, suspended in 4 ml

FIGURE 3 An electronmicrograph of the homogenate after bead filtration, showing long membrane segments, some of which form continuous loops (lower left). Dense granules (*g*) are seen along the inner surface of many membranes. Membranes of adjoining cells remain attached by desmosomes (*des*). A typical cell envelope form (*e*), with its edges rolled up, is shown in cross-section. A circular figure (*n*) may represent a nuclear membrane. × 6500.

FIGURE 4 Homogenate after bead filtration, showing two cell envelopes still attached by desmosomes (*des*); filamentous strands, apparently associated with the desmosomes, radiate from the inner surface of the cell membrane (arrow). Microvilli (*mv*) contain dense amorphous material. Portions of rough endoplasmic reticulum (*er*) and a mitochondrion (*m*) are seen. × 25,000.





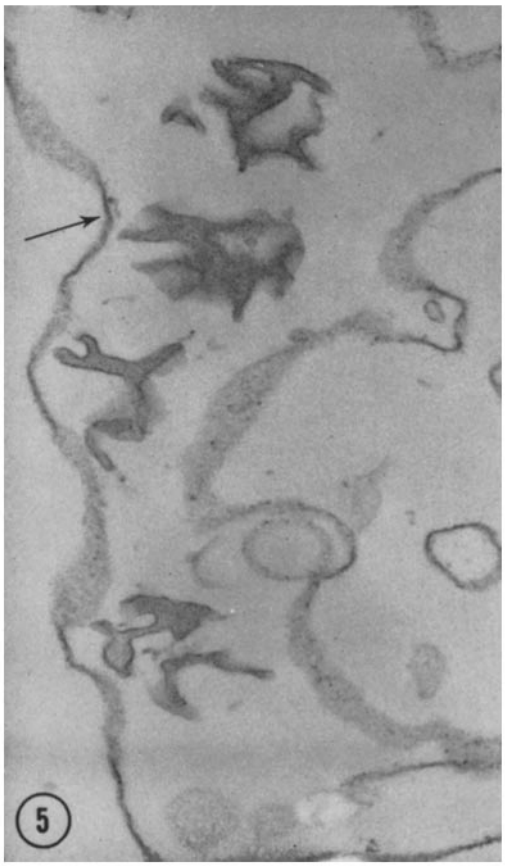


FIGURE 5 Membrane following bead filtration, demonstrating bilamellar unit membrane structure (arrow) at point of cross-section and finely granular appearance on tangential sectioning. × 37,000.

of bicarbonate solution, and centrifuged for 10 min at 105,000 *g*. The supernatant was removed, and the pellet resuspended in 0.5 to 1.0 ml of bicarbonate solution. Phase-contrast microscopy showed the suspension to consist of large cylindrical envelopes of cell membranes, fragments of cell membranes, and occasional pieces of cytoplasmic debris. Mitochondria and other cellular elements were rarely seen. The few intact nuclei that remained following bead filtration were present in a small pellet at the bottom of the sucrose gradient tube; this pellet also contained some cell membranes.

Chemical and Enzyme Determinations

Protein was measured by the method of Lowry et al. (6); inorganic phosphate (P_i) by the method of Fiske and Subbarow (7). Enzyme assays were carried out immediately after density gradient centrifugation, or following overnight storage of the membrane and crude homogenate fractions at 0°. The Mg^{++}-ATPase activity of crude homogenate and membrane fractions was determined by a modification of the method of Bonting (8); 0.05 to 0.1 ml aliquots of tissue were incubated at 37° for 30 min in the presence of 2 mM $MgCl_2$, 2 mM ATP (di Tris Form, Sigma Chemical Company), 0.1 mM EDTA, 92 mM tris(hydroxymethyl)amino methane (Sigma), pH 7.5, 58 mM NaCl, 5 mM KCl, and 0.1 mM ouabain. The total volume was 1.0 ml. Total ATPase activity was determined under the same conditions, but in the absence of ouabain. The reaction was stopped with 0.1 ml of cold 50% TCA, the suspension cleared by centrifugation, and P_i determined. The difference between the total and Mg^{++}-ATPase determinations represented Na^+-K^+-stimulated, ouabain-inhibited ATPase. ADPase activity was determined by the method of Novikoff and Heus (9).

Electron Microscopy

A drop of bicarbonate solution containing membranes was placed in a small plastic centrifuge tube, and the tube filled with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4. After standing for 5 min at 0°C, the tubes were centrifuged for 4 min (Beckman/Spinco Model 150 Microfuge). The pellet was dehydrated and embedded in Epon 812 in situ, using the method of Luft (10). Sections were made with a Sorvall MT-1 ultramicrotome, stained with 4% uranyl acetate in 50% ethanol, and examined in a Siemens Elmiskop I.

OBSERVATIONS

Morphology

Figs. 1 to 6 show cell membranes during the process of isolation. In the crude homogenate (Fig. 1), following a few strokes of the Dounce homogenizer, intact epithelial cells, extruded nuclei, and membrane envelopes are seen. The envelopes have a typically elongate and finely granular appearance (Fig. 2). Hairlike processes extend from their surface; whether these are microvilli, rendered visible by light-scattering, or other projections of the surface is not clear. Electron microscopy after bead filtration shows a predominance of membranes, some of which form continuous loops (Fig. 3). A moderate number of dense granules, which are characteristically seen at the luminal border of intact granular epithelial cells (11, 12), remain adherent to portions of the isolated membranes (Fig. 3); these may have been in direct contact with the luminal surface at the time of homogenization. Some microvilli persist along the borders of cell envelopes (Fig. 4);

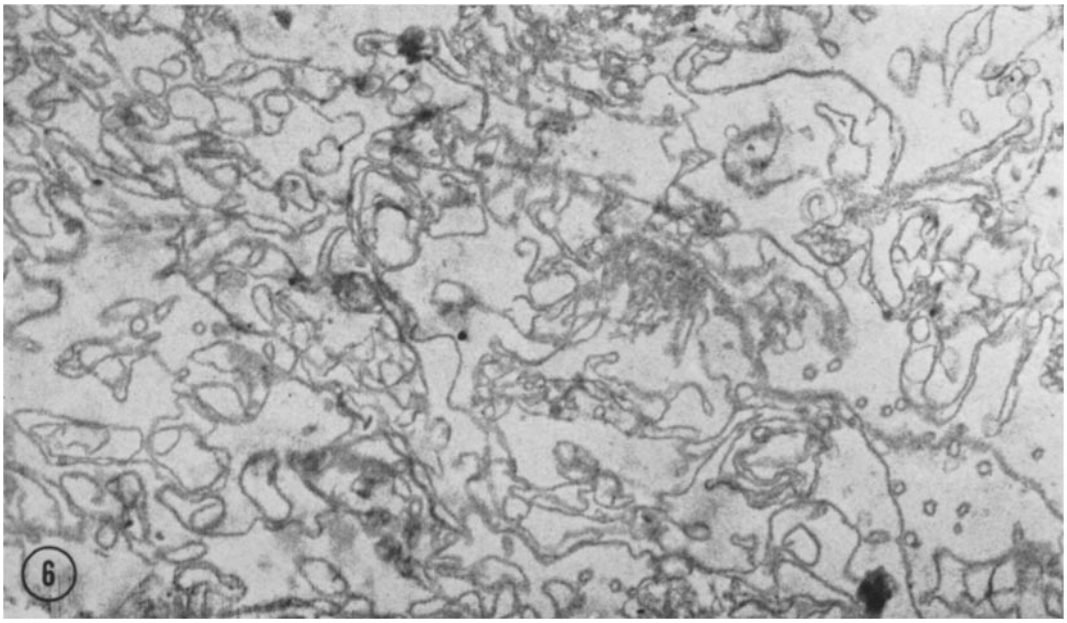


FIGURE 6 A section of the d 1.16/d 1.26 fraction after sucrose density gradient centrifugation, demonstrating the predominantly membranous composition of this fraction. × 7500.

TABLE I

Nucleoside Phosphatase Activities of Epithelial Cell Homogenates and Membranes

Enzyme	Activity (μMP_i/mg protein/30 min) Homogenate	Membrane	Enrichment $\left(\frac{P_i \text{ Membrane}}{P_i \text{ Homogenate}}\right)$
ADPase (5)*	2.8 ± 0.4 (SE)	21.5 ± 5.4	7.7
Mg^{++}-ATPase (7)	7.7 ± 0.6	47.4 ± 5.8	6.2
Na^{+}-K^{+}-ATPase (7)	1.8 ± 0.2	7.0 ± 2.4	3.9

* Numbers in parentheses refer to the number of experiments.

they often contain a dense amorphous material, suggesting that these structures are true microvilli, not simply folds created during homogenization. Desmosomes are also present (Figs. 3 and 4). Occasional mitochondria and portions of rough endoplasmic reticulum were seen in the bead filtrate (Fig. 4). At higher magnification (Fig. 5) the membranes possess a bilamellar unit membrane structure; on tangential section they appear finely granular and amorphous.

Electron microscopy of the d 1.16/d 1.26 fraction after sucrose density gradient centrifugation (Fig. 6) reveals abundant membranes; intact cells and nuclei are absent.

Phosphatase Activity

Histochemical studies of the toad bladder (13–15) have demonstrated a highly active ATPase. In the studies of Novikoff et al. (13) and Keller (14), the enzyme was localized along the lateral borders of the cell; Bartoszewicz and Barrnett reported luminal localization (15). ADPase activity was also reported by Novikoff et al., and was restricted to the luminal border of the cell. The cell membrane fraction was assayed for Mg^{++}-ATPase, Na^{+}-K^{+}-stimulated ATPase and ADPase, and the activities were compared to those of the crude homogenate (Table I). The three enzymes were concentrated

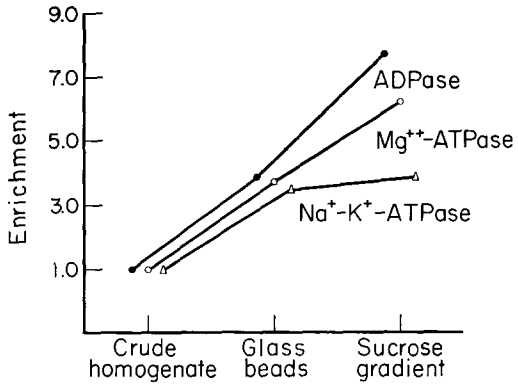


FIGURE 7 Enrichment of the three enzymes studied (P_i/mgm protein/30 min), following bead filtration (mean values for 5 experiments), and density gradient centrifugation (data from Table I).

in the membrane fraction when their activities were expressed as μM P_i hydrolyzed per mgm protein per 30 min. ADPase showed the greatest enrichment. The relatively lower enrichment of Na^+-K^+-ATPase appears to be the result of events in the gradient tube itself, since ADPase, Mg^{++}-ATPase and Na^+-K^+-ATPase exhibited the same enrichment over the crude homogenate when originally placed in the tube following bead filtration (Fig. 7). A preliminary study showed that Na^+-K^+ and Mg^{++}-ATPase activities were more widely distributed over the several phases of the gradient tube than ADPase activity, which was sharply restricted to the d 1.16/d 1.26 interface. This wider distribution suggests that ATPase activity is present in membrane fragments of differing densities. In addition, in a single experiment, total recovery from the entire gradient tube of Na^+-K^+- and Mg^{++}-ATPase activity was less than the recovery of ADPase activity, suggesting that some inactivation of ATPase may have taken place as well. Additional experiments are required to resolve these questions.

We would conclude that the present method of isolation produces a relatively high yield of outer membranes from the granular epithelial cells of the bladder. Both the luminal and basolateral portions of the membrane are concentrated in the final fraction. A number of possible sources of contamination of the preparation should be noted, including nuclear membranes, endoplasmic reticulum, and membranes from other types of cells in the bladder mucosa (mucus-secreting, mitochondrion-rich, and basal cells, which together constitute a minor proportion of the total cell population). To what extent these elements are present, and exactly to what extent the basolateral portion of the granular cells is present must await further studies.

This work was supported by grants from the National Institutes of Health, Numbers AM-03858, AM-09284, and HD-00674.

The authors gratefully acknowledge the technical assistance of Miss Gloria Duran and Miss Dorothy Popwell. They also acknowledge with thanks Dr. Leonard Warren's guidance in the design of the bead filtration method.

Dr. Hays is a Career Scientist of the Health Research Council of New York City.

Received for publication 26 May 1966.

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