

REDISTRIBUTION OF SURFACE MACROMOLECULES IN DISSOCIATED EPITHELIAL CELLS

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ABSTRACT

A number of ultrastructural and cytochemical techniques were used to study intact epithelial cells lining the frog urinary bladder: high resolution autoradiography after administration of [³H]glucosamine or [³H]fucose; ¹²⁵I iodination of external protein; concanavalin A-peroxidase, periodic acid-chromic acid silver methenamine; and colloidal thorium. Results indicate that the material (probably glycoprotein) coating the apical surface differs from that which lines the lateral and basal surfaces. After dissociation and isolation of the epithelial cells, the material previously confined to the apical surface invaded progressively the opened "tight junctions" (about 5 min), then the lateral membranes (about 40 min), and finally the basal membrane (about 80 min): at that time, the whole cell surface was entirely enveloped by the apical material.

Since, on the one hand, the reacting material was confined to the apical surface when the tight junctions were closed (in intact epithelial cells) and since, on the other hand, the apical material was sliding down the laterobasal membrane when the tight junctions were opened (in dissociated cells), it may be concluded that tight junctions contribute to maintain the cell surface specialization in epithelia.

The preparation of isolated epithelial cells from amphibian urinary bladder (5, 10, 12, 20) permits the study of various aspects of transport mechanisms across these cells.

In vivo, the behavior of these epithelial cells depends on their organization in the epithelium; the urinary bladder epithelium separates two compartments which differ from each other in their osmotic pressure and ionic content. Indeed, the apical membrane of the epithelial cells constitutes the rate-limiting barrier for water permeability (9), whereas the lateral and basal plasma membranes are the sites of a high water permeability and of an active transport of Na⁺ (4) due to the presence of Na⁺K⁺ATPase (3).

The apical and the laterobasal regions of the epithelial cell membranes may be distinguished by several ultrastructural and cytochemical characteristics. Thus, the apical plasma membrane exhibits a "fuzzy coat" (14), retains a large amount of cationic dyes (15), and displays a high concentration of surface glycoproteins (16).

Do these two types of membranes retain their different characteristics when the epithelial cells are dissociated from each other and kept isolated? To answer this question, dissociated cells were examined with light and electron microscopes after using various techniques, such as osmium tetroxide fixation, cationic staining, periodic acid-chromic acid silver methenamine staining,

concanavalin A-peroxidase complex fixation, and autoradiographic detection of various labels.

MATERIALS AND METHODS

Cell Dissociation

Epithelial cells were obtained from frog urinary bladder (*Rana esculenta*) by the method described by Scott et al. (20) with slight modification. Animals were kept in running tap water at 22°C for 8 days before the experiments. The bladders were removed from pithed frogs. To make a sac preparation, the bladders were tied to the end of a piece of polyethylene tubing with the mucosal side inwards (1). Both faces were bathed with a frog Ringer's solution (NaCl, 112 mM; KCl, 5 mM; HCO₃Na, 2.5 mM; pH 8.1, when bubbled with air) containing 2 mM of EDTA. To dissociate the epithelial cells more rapidly, the solution bathing the mucosal side was flushed in and out with a syringe for 15 min. Dissociation of the cells was completed by slowly rubbing the bladder between the thumb and the forefinger. The sac was then excised and cells were collected in a beaker. The cell suspension was sucked up and flushed back six times with a syringe and finally filtered through a nylon gauze (mesh area: 160 μm²).

The cell suspension was divided in several parts and the following experiments were carried out. Cells were (a) fixed immediately; (b) fixed after various times of incubation at 22°C; or (c) fixed after incubation in the presence of 5 μg/ml cycloheximide (Actidione, The Upjohn Company, Kalamazoo, Mich.).

Cells were fixed by adding glutaraldehyde (glutaraldehyde EM, 25% aqueous solution TAAB Laboratories, Reading, Great Britain) to a final concentration of 2%. 20 min later, the cells were sedimented by centrifugation at 100 g for 5 min. The supernate was discarded and the pellet resuspended with 2 ml of a 1% osmium tetroxide solution for 45 min at 22°C in polyethylene conical tubes. After centrifugation at 3,000 g at 4°C for 10 min, the supernate was discarded and the tube bottoms containing the pellets were cut with a razor blade. Dehydration was carried out by dipping the tube bottoms in graded ethanols. Pellets were then removed from the tubes and embedded in Epon.

Test of Viability

Cell viability was estimated by determining the fraction of the population, at various times of cell dissociation, able to exclude 0.2% trypan blue.

Colloidal Thorium

The technique described by Rambourg and Leblond (18) was used with minor modifications. Cell pellets were postfixed for 45 min in 1% osmium tetroxide. After one rinse in 3% acetic acid (pH 2.6), cells were immersed for 1 h at room temperature in a 0.5% colloidal

thorium (Testagar & Co., Detroit, Mich.) solution in 3% acetic acid (pH 2.6) and then rinsed again in 3% acetic acid. The dehydration and Epon embedding were done as above.

Periodic Acid-Chromic Acid-

Silver Methenamine

Dissociated epithelial cells fixed in 2% glutaraldehyde were embedded in Vestopal w after acetone dehydration. The sections were treated by the method indicated by Rambourg (19). They were picked up on platinum grids and floated for 20 min on a solution containing 1% periodic acid in 50% acetone; they were then rinsed by floating them twice on distilled water baths and treated for 10 min in 10% chromic acid in 50% acetone. After being kept for several hours on the surface of distilled water, the grids were transferred onto the silver methenamine solution prepared according to Rambourg and Leblond (18). Staining was allowed to proceed for 10 min at 50°C. Control sections were not treated with periodic acid and chromic acid.

Concanavalin A (Con A)-Peroxidase

Con A sites were detected with the aid of peroxidase by the technique described by Bernhard and Avrameas (2) with slight modification. The isolated cells were incubated for 15 min at 10°C in frog Ringer phosphate containing 250 μg/ml Con A (3× crystallized—Miles Laboratories, Inc., Elkhart, Ind.). After rinsing, 1 mg/ml peroxidase (grade I, lyophilized, Boehringer-Mannheim, France) was added and allowed to react for 15 min at 10°C; the temperature was maintained at 10°C to reduce the movement of Con A sites during the incubation with Con A and peroxidase. After washing out the excess peroxidase, cells were fixed with a glutaraldehyde solution either immediately or after incubation in a frog's Ringer solution at 22°C. Peroxidase was detected after the technique of Graham and Karnovsky (7). Cells were incubated for 15 min at room temperature in a solution of 0.5 mg/ml DAB (3,3'-diamino-benzidine tetrahydrochloride, Fluka A. G., Basel, Switzerland) in a 0.1 M tris buffer, pH 7.4, containing 0.1% H₂O₂. Control cells were incubated in frog Ringer phosphate without Con A. Cells were then postfixed in osmium tetroxide, dehydrated, and embedded in Epon. The unstained sections were examined at the electron microscope.

Autoradiographic Study with

Labeled Monosaccharides

The urinary bladders mounted in sacs were filled initially with a Ringer's solution containing 1 mM CaCl₂ and then immersed for 4 h in the same Ringer's solution containing radioactive precursors (150 μCi/ml). Two monosaccharides were used: D-[1-³H]glucosamine (sp act 16 Ci/mM, Commissariat à l'énergie Atomique (CEA),

France) and L-[6-³H]fucose (sp act 22 Ci/mM, CEA, France).

Bladders were then transferred for 14 h to a fresh Ringer's solution containing unlabeled glucosamine or fucose to concentrations 100 times higher than those used during radioactive labeling. The incubation time (18 h) was selected to allow the migration of the newly synthesized glycoprotein to the apical cell surface (16). The epithelial cells were then isolated and fixed as described above.

For light microscopy, 1- μ m thick sections were coated with K₅ emulsion. After suitable exposure, autoradiographs were developed in Kodak D₁₉ (Eastman Kodak Co., Rochester, N. Y.), and stained with 1% blue toluidine.

For electron microscopy, thin sections, stained with uranylacetate and lead citrate, were dipped into Ilford L₄ emulsion (Ilford Ltd., Essex, Great Britain). After 2 mo exposure, they were developed in Microdol X (Eastman Kodak Co.).

Enzymatic Iodination

The apical plasma membrane proteins of urinary bladder epithelium were iodinated by the technique described by Strum and Edelman (21).

Urinary bladders mounted in sacs were filled and incubated with buffer solution (NaCl, 104 mM; NaHCO₃, 12 mM; KCl, 3 mM; MgSO₄, 0.5 mM; KH₂PO₄, 0.5 mM; CaCl₂, 1 mM; glucose, 5 mM) at pH 7.2 when bubbled with a mixture of 95% O₂-5% CO₂ (8). Enzymatic iodination was carried out for 5 min by adding to the buffer solution 0.2 μ g/ml glucose oxidase (grade IV, Boehringer-Mannheim), 200 μ Ci/ml of carrier-free ¹²⁵I-Na (CEA, France), and 3 μ g/ml lactoperoxidase (Boehringer-Mannheim). Bladders were then washed with a fresh frog Ringer's solution and the epithelial cells were dissociated and fixed according to the technique described above. The labeled proteins were demonstrated by autoradiography.

RESULTS

Behavior of Epithelial Cells with Respect to Dissociation

Examination of epithelial cells after cell dissociation revealed the existence of a high percentage of structurally intact cells. This fact was borne out by the observation that trypan blue typically stained only about 5% of the cell population 20 min after the beginning of the dissociation and about 10% after 120 min, indicating that 90-95% cell viability was retained throughout the first 2 h.

When the cells were isolated, they progressively changed from trapezoidal to spherical shape. As indicated in Table I, most of the epithelial cells

TABLE I
Evolution of the Cell Shape after Dissociation

| | Dissociation time, min | | |
|------------------------------------|------------------------|--------|--------|
| | 20 | 60 | 120 |
| Percentage of epithelial cells in: | | | |
| Trapezoidal form | 87.6% | 64.85% | 14.85% |
| Spherical form | 12.4% | 35.15% | 85.15% |
| n | 900 | 400 | 500 |

The calculations were made, in the light microscope, on 1- μ m thick sections. The error introduced by counting sections perpendicular to the main axis of trapezoidal cells was not taken into account as this error occurred at all stages of dissociation.

n = number of cells calculated.

were trapezoidal after 20 min of dissociation and became spherical after 120 min. Thus, for each incubation time, we have only considered the more common cell type.

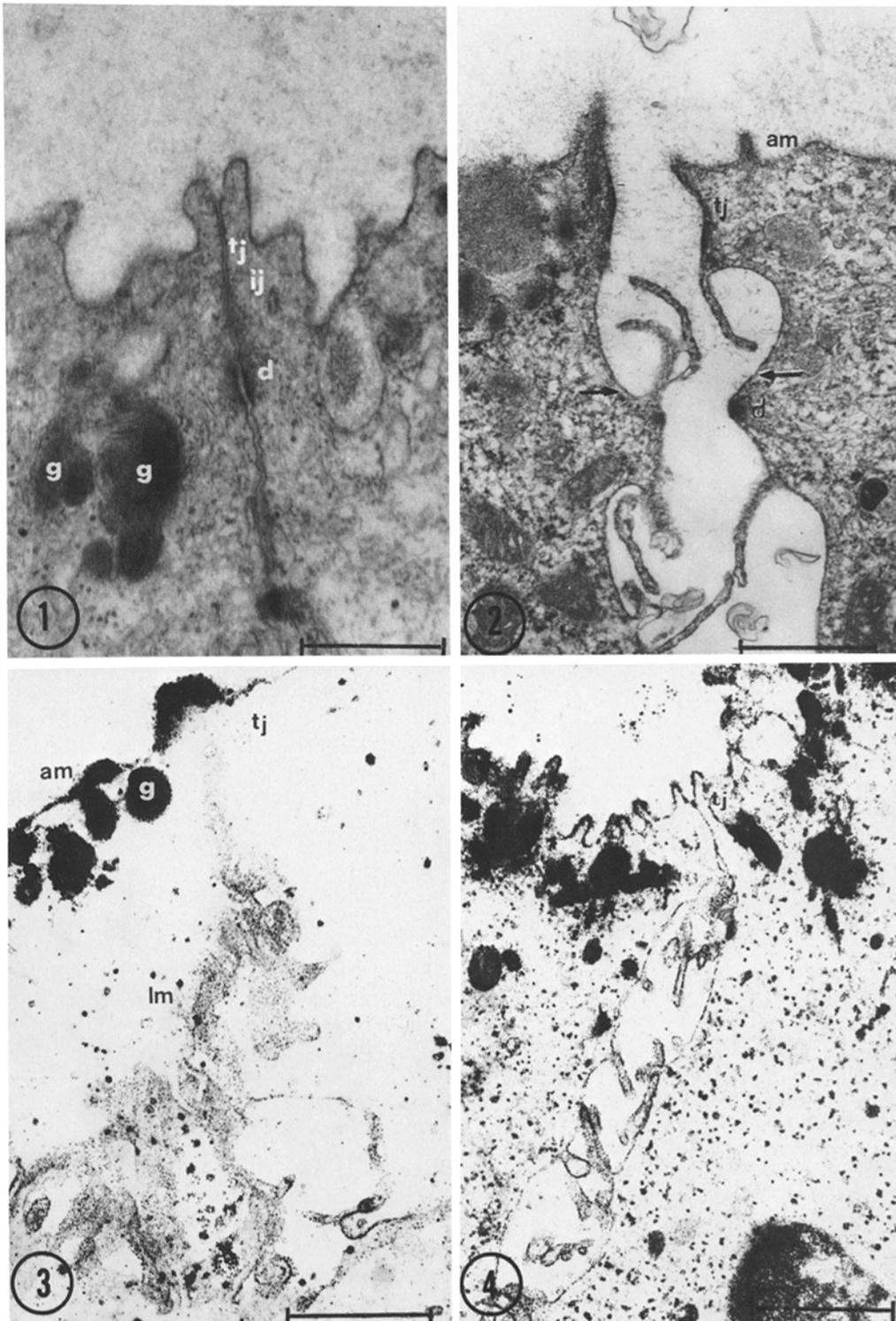
Fuzzy Coat Development

In the intact epithelium of urinary bladder fixed with OsO₄, the luminal surface of the cells was covered with a filamentous material attached perpendicularly to the plasma membrane (14). In contrast, this fuzzy coat was interrupted at the level of the tight junction and was missing in the lateral and basal parts of the cell surface (Fig. 1).

A short time after the beginning of cell dissociation (20 min), the juxtaluminal junctional complex started to open and the fuzz invaded the zone previously identified as a tight junction (Fig. 2). 30 min later, the fuzzy coat extended along the lateral membranes, whereas the shape of the cells became progressively spherical (Fig. 5). After 80 min (Fig. 6), most of the cells had become spherical and the whole plasma membrane was covered with the fuzz; at the same time, the granulations which were exclusively localized in the apical region of intact epithelial cells (Fig. 1) were uniformly distributed below the cell surface.

Detection of Polysaccharides at the Epithelial Cell Surface

Several structures (Golgi apparatus, apical granulations, and plasma membranes) could be visualized in epithelial cells after periodic acid-chromic acid-silver methenamine staining. The reaction was intense at the apical surface but weaker along the lateral and basal cell membranes (Fig. 3).



In the intact epithelium, the tight junctions were unstained (Fig. 3), however, as soon as the epithelial cells began to dissociate, the reaction extended along "the junctions" (Fig. 4). Between 1 and 2 h after dissociation, the stronger reaction observed at the apical surface gradually invaded the whole periphery of the cells.

Distribution of Surface-Negative Charges, after Staining by a Cationic Dye "Colloidal Thorium"

Within minutes after cell dissociation, the reaction with colloidal thorium was restricted to the apical membrane (Fig. 7) as seen in intact epithelial cells. After 40 min, the lateral membranes were stained (Fig. 8); finally, 80 min later, the whole plasma membrane became uniformly covered with thorium particles (Fig. 9).

Migration of Con A-Binding Sites (Carbohydrate Residues) on the Cell Membrane

In the newly dissociated cells, Con A, revealed by peroxidase was exclusively bound to the apical surface (Fig. 10). In control cell preparations, in which Con A was omitted, peroxidase was not present at the cell surface thereby indicating specific binding of peroxidase to Con A.

Later on, Con A-labeled sites migrated towards the lateral and basal cell membranes (Fig. 11) in accordance with results obtained with periodic acid-silver methenamine and colloidal thorium.

Autoradiography after Incubation with [³H]Glucosamine or [³H]Fucose

As seen in the light and electron microscopes, most of the cells isolated from bladders incubated for a long time (18 h) were well preserved. At the initial stages of dissociation (20 min) a strong autoradiographic reaction was observed over the apical membrane and apical granulations (Figs. 12, 13 and 15). After 120 min, the whole membrane became labeled without significant increase of total radioactivity, whereas the labeled granulations were then distributed uniformly below the whole cell surface (Figs. 14 and 16).

The distribution of silver grains over the plasma membrane at the two incubation periods is indicated in Table II.

Labeling of Membranous Proteins by ¹²⁵I Iodination

As in the previous experiments the silver grains were exclusively located at the apical surface of the epithelial cells 20 min after the beginning of dissociation (Figs. 17 and 18); after 120 min, the distribution of the labeling was completely modified, as the silver grains were seen over the whole plasma membrane (Fig. 19).

Cycloheximide Action

The inhibiting activity of cycloheximide was tested on the [³H]lysine incorporation in intact urinary bladder: after 2 h of incubation with cyclo-

FIGURES 1 and 2 Apical region of epithelial cells of urinary bladder fixed with osmium tetroxide, stained with uranylacetate and lead citrate. $\times 40,000$.

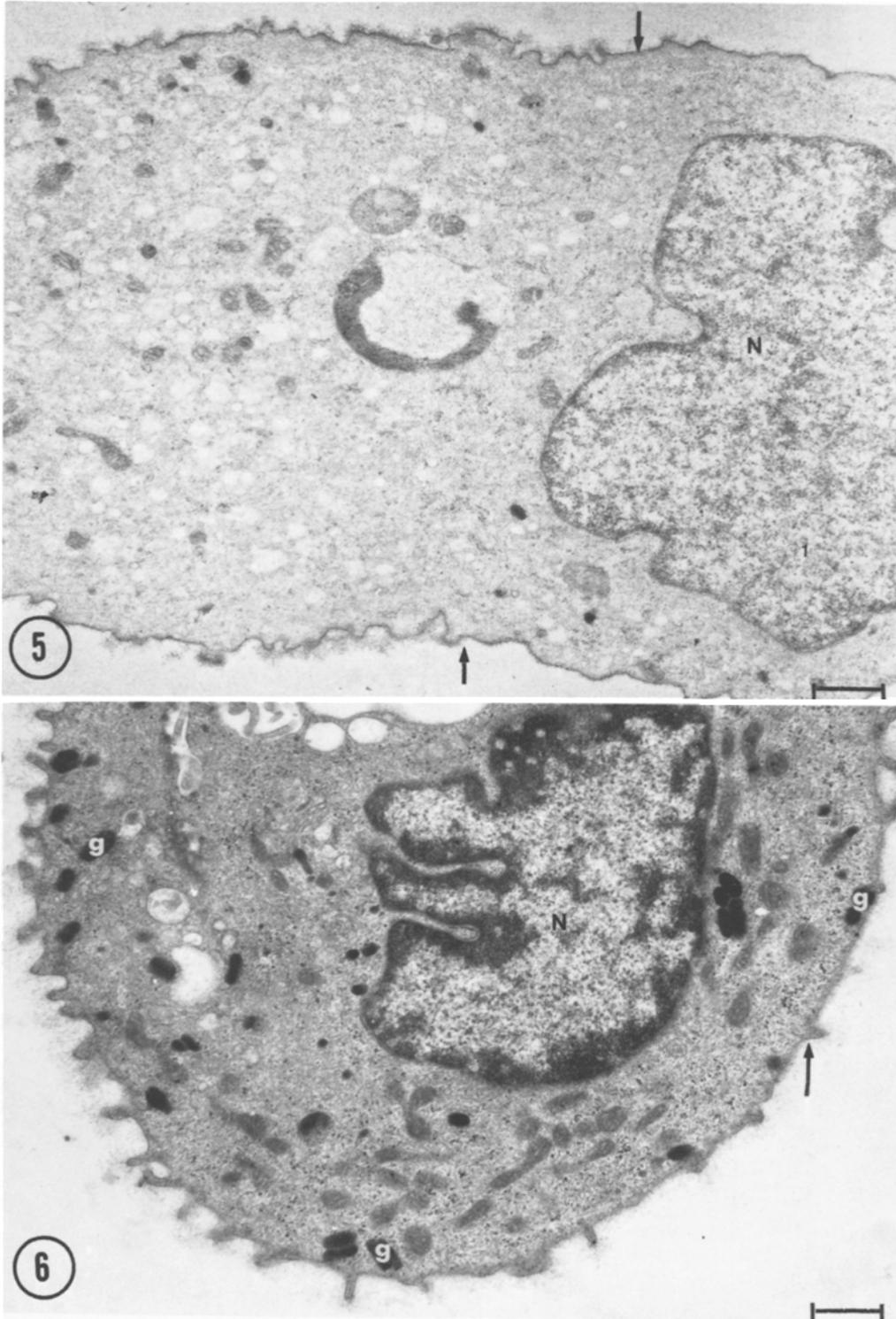
FIGURE 1 Junctional complex between two intact epithelial cells. The tight junction (*tj*) is closed. *ij* = intermediary junction; *d* = desmosome; *g* = granulation. Scale bar = 0.5 μm .

FIGURE 2 Junctional complex between two epithelial cells a few minutes after dissociation. The tight junction (*tj*) is open. Filaments similar to those of the apical membrane (*am*) now extend into the region which was formerly occupied by the tight junction and almost reach the level of the desmosome (*d*). Scale bar = 0.5 μm .

FIGURES 3 and 4 Epithelial cells of urinary bladder stained with PA-CrA- silver methenamine. $\times 20,000$.

FIGURE 3 Stained material outlines the plasma membranes of intact epithelial cells. The reaction is stronger at the apical membrane (*am*). The weaker staining observed along the lateral cell membranes (*lm*) is interrupted in the region of the tight junction (*tj*). Apical granulations (*g*). Scale bar = 1 μm .

FIGURE 4 At the beginning of cell dissociation, silver methenamine is seen in the tight junction (*tj*). Scale bar = 1 μm .



FIGURES 5 and 6 Staining with uranylacetate and lead citrate after fixation with osmium tetroxide. *N* = nucleus. $\times 10,000$.

FIGURE 5 Epithelial cell 30 min after dissociation: The fuzzy coat extends along the lateral cell membranes and reaches the lower third of the cell (arrows). *N* = nucleus. Scale bar = $1 \mu\text{m}$.

FIGURE 6 80 min later: the cell is completely spherical and the whole cell membrane (arrow) is now covered by the fuzzy coat. *g* = granulation. Scale bar = $1 \mu\text{m}$.

heximide, the lysine incorporation (TCA-precipitable material) was inhibited by 92%.

However, after 120 min of incubation with cycloheximide, the labeled molecules (with [³H]glucosamine as well as with ¹²⁵I) were distributed along the whole plasma membrane of dissociated cells, and their number was not significantly different from the one observed on the control cells 120 min after dissociation (Table II).

DISCUSSION

After cell dissociation, a stained material which was exclusively located at the apical surface in intact epithelium appeared first at the level of opened intercellular junctions; then, progressively invaded the lateral and basal cell surfaces.

The question arises as to whether this phenomenon is a true functional modification or an artefact induced by cell alteration after cell isolation. As indicated by the results after the viability test with trypan blue, 90% of the cells were still alive after 2 h of dissociation. Moreover, it was pointed out by Gatzky and Berndt (5) that the oxygen consumption of epithelial cells isolated from amphibian urinary bladder remained constant over a 2-h period. It may therefore be assumed that the majority of cell population used in this study was still alive and that the appearance of the stained material is not likely to be an artefact resulting from cell alteration.

Is the material observed on the laterobasal surface of dissociated cells identical in nature to that located at the apical surface of intact epithelial cells? And, if that is the case, what are its origin and the mechanism of its incorporation into the plasma membrane?

Nature of the Material Redistributed along the Whole Cell Surface

The material which is observed on the whole plasma membrane 2 h after cell dissociation displays the same histochemical characteristics as the one located at the apical surface of intact epithelial cells. Its positive reaction with periodic acid-chromic acid-silver methenamine indicates the presence of glycol residues (11). Staining by colloidal thorium at low pH reveals carboxyl and sulfate groups. The specific binding of Con A is related to the presence of α -glucopyranosyl and α -mannopyranosyl residues (6), which are known to be components of glycoproteins. The autoradiographic experiments show the existence of mac-

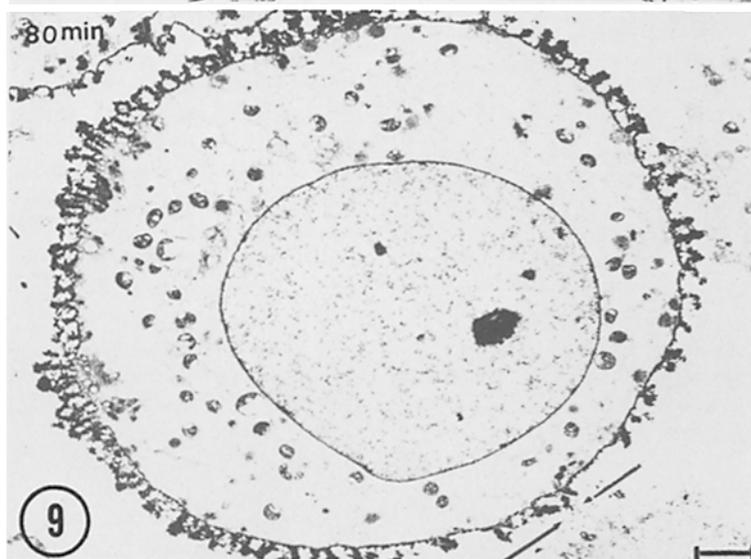
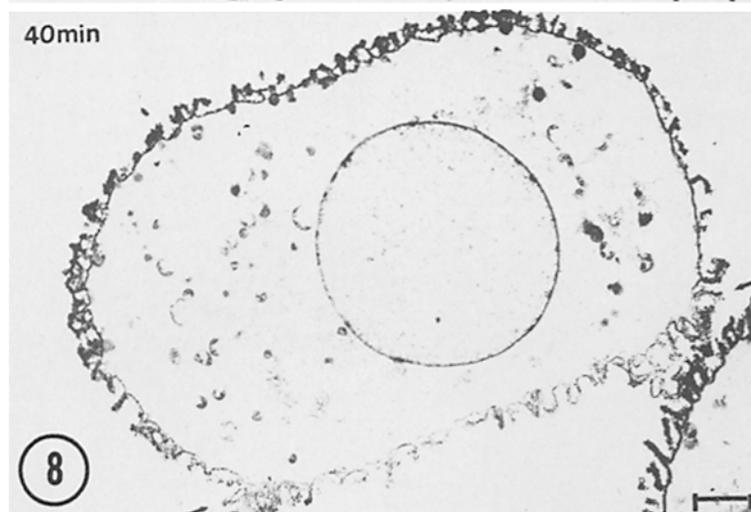
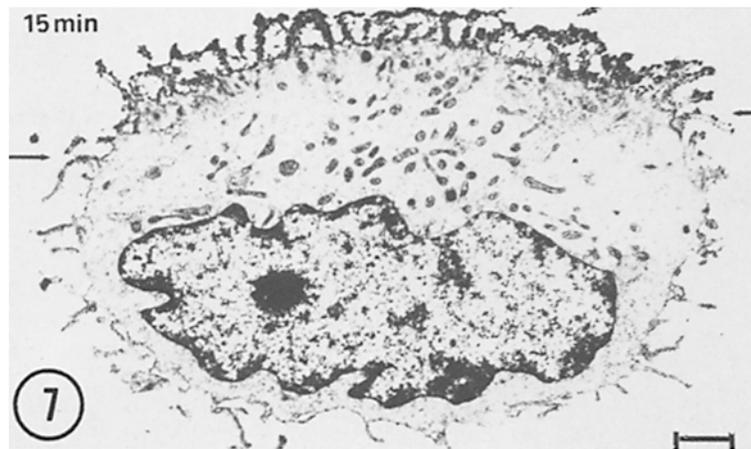
romolecules made of glucosamine and fucose. The enzymatic iodination reveals mainly tyrosyl and histidyl residues (21). Since all these results indicate the existence of polyglycans and amino acids, it may be speculated that the material present at the cell surface does contain glycoproteins although the existence of glycolipids cannot be excluded. Since, in addition, Con A and lactoperoxidase, which catalyses the iodination, are molecules too large to penetrate the cell membrane (21), it may be inferred that at least a part of this glycoproteic material is located on the external side of the plasma membrane.

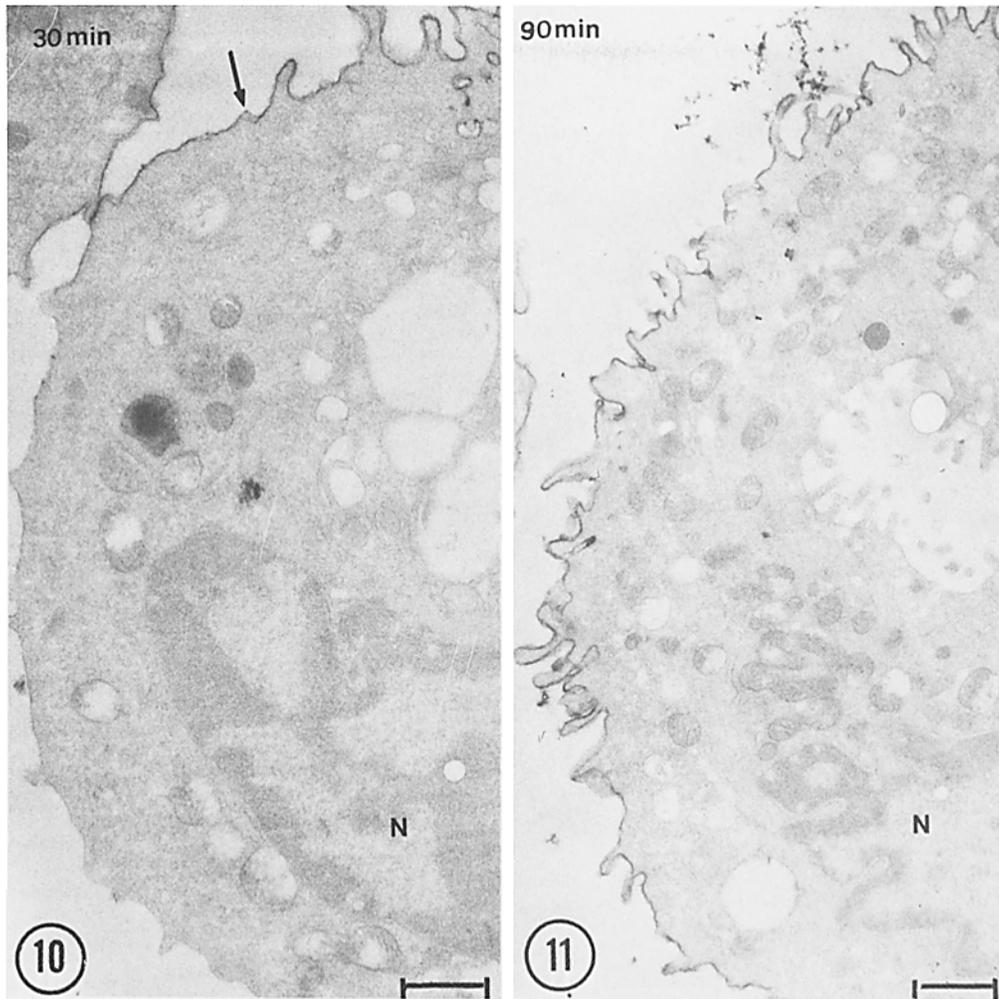
Origin of Laterobasal Coat

The progressive appearance of stained material along the laterobasal surface of dissociated cells seems to be independent of a synthesis of new proteins or glycoproteins taking place after the isolation of the cells. Indeed, it was previously shown (16) that the synthesis of glycoproteins and their migration from the Golgi apparatus to the apical surface require 12–18 h, a period of time much longer than the 1 or 2 h necessary for the appearance of the stained material along the laterobasal cell membrane. Furthermore, the spreading of surface material is, by no means, affected by a cycloheximide treatment which was found to inhibit 92% of the protein synthesis.

Other possibilities should therefore be considered for the origin of this laterobasal coat. The redistribution of markers might depend on the internalization of surface membrane; however, at no time after cell dissociation were marked vesicles, capable of transferring material from the apical to the laterobasal surface, ever observed in cells previously labeled at their apical surface by the Con A-peroxidase complex.

According to Peachey and Rasmussen (14) and Masur et al. (13), granulations could participate in the formation of the apical coat in intact epithelial cells. Indeed, autoradiographic studies have shown that a part of the newly synthesized glycoproteins first appears at the level of the apical granulations before reaching the apical membrane (16). Furthermore, since after cell dissociation the granulations, which are first confined to the apical region, migrate to the lateral and basal regions of the granular cells (Figs. 6 and 16), the glycoprotein material which appears on the laterobasal membrane could originate from the release of the material contained in these granulations. However, the results observed after iodination of cell





FIGURES 10 and 11 Con A-peroxidase staining of epithelial cells. *N* = nucleus. $\times 10,000$.

FIGURE 10 30 min at 10°C after the beginning of the dissociation. Only the apical region of the cell is stained (arrow). Scale bar = 1 μm .

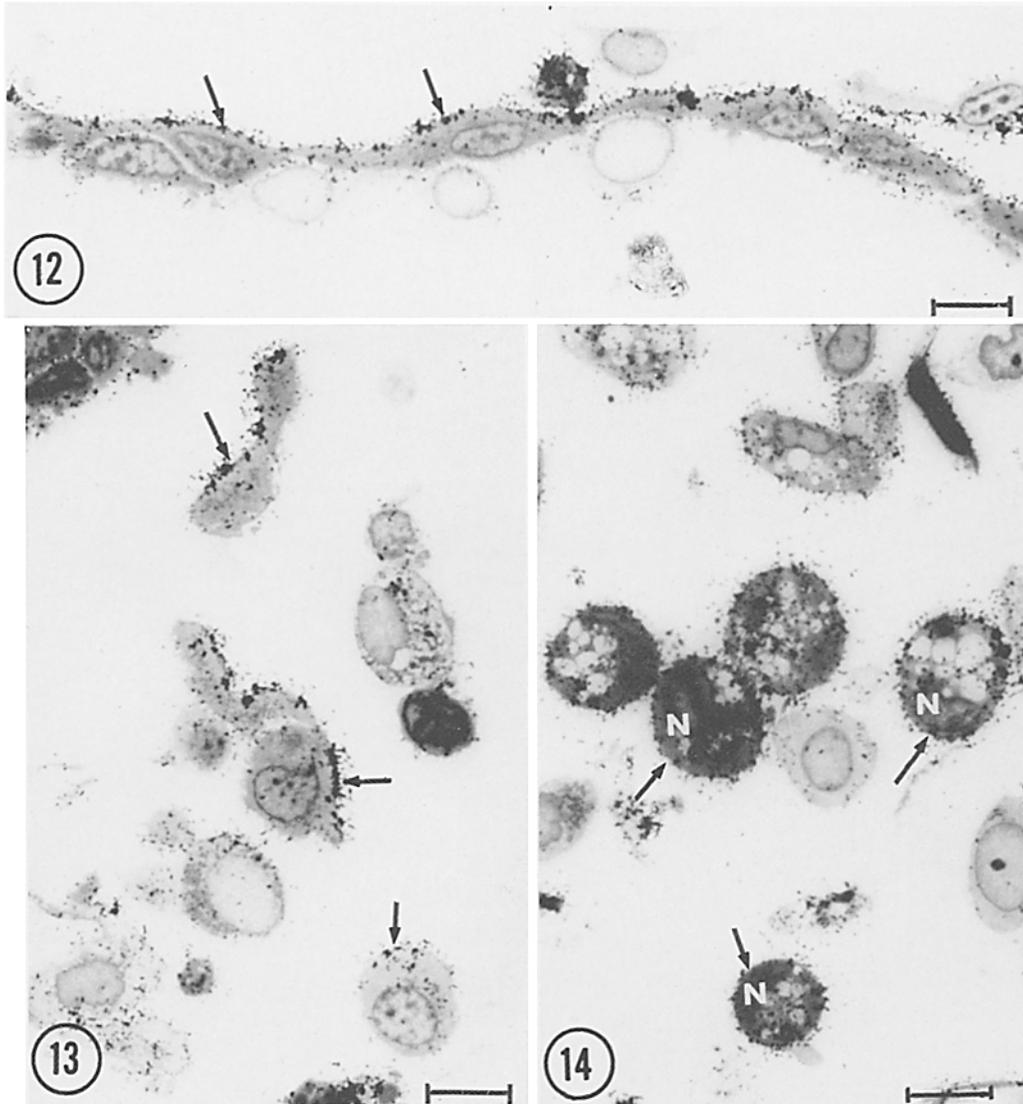
FIGURE 11 After 30 min at 10°C and 60 min at 22°C, the staining covers all the cell surface. Scale bar = 1 μm .

FIGURES 7-9 Epithelial cells stained with colloidal thorium at various time intervals after dissociation. $\times 6,500$.

FIGURE 7 At 15 min: The apical membrane (between both arrows) is outlined by a heavy deposit of stain. A few colloidal particles are seen along the lateral and basal membranes. Scale bar = 1 μm .

FIGURE 8 At 40 min: The lateral membrane is as heavily stained as the apical membrane. The portion which probably corresponds to the basal membrane (between the arrows) remains unstained. Scale bar = 1 μm .

FIGURE 9 At 80 min: The whole surface of the spherical epithelial cell is outlined by thorium. Scale bar = 1 μm .

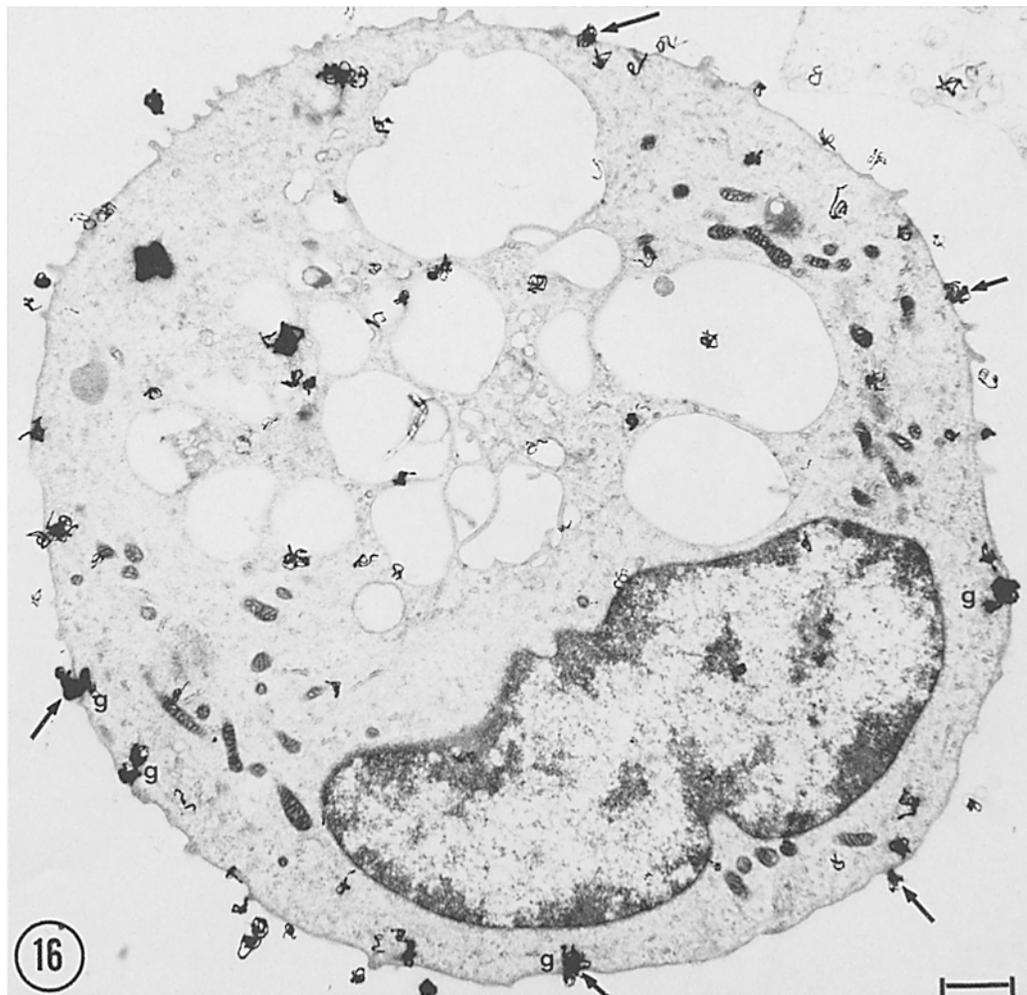
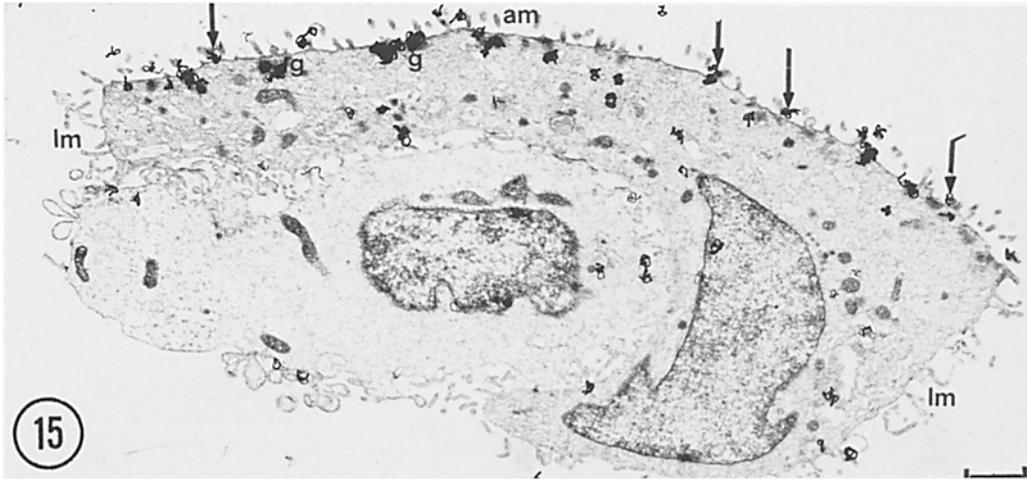


FIGURES 12-14 Light microscope autoradiographs of epithelial cells from frog bladders incubated for 4 h in a medium containing [^3H]glucosamine and subsequently incubated for 14 h in a chase medium containing unlabeled glucosamine. $\times 1,000$. Counterstained with toluidine blue.

FIGURE 12 20 min after the beginning of cell dissociation: Fragment of epithelium showing the cells still attached. Labeling is concentrated along the apical surface (arrows). Scale bar = 10 μm .

FIGURE 13 20 min after the beginning of cell dissociation: The epithelial cells are completely dissociated or in pairs. Most of the silver grains are still present at the apical pole of the cells (arrows). Scale bar = 10 μm .

FIGURE 14 After 120 min: The silver grains are now evenly distributed along the whole cell surface (arrows). *N* = nucleus. Scale bar = 10 μm .



FIGURES 15 and 16 Electron microscope autoradiographs of epithelial cells from frog bladders incubated in vitro for 4 h in medium containing [^3H]glucosamine followed by incubation for 14 h in chase medium containing unlabeled glucosamine.

FIGURE 15 20 min after the beginning of the cell dissociation. $\times 7,000$. The label is concentrated along the apical membrane (*am*) (arrows); the lateral membranes (*lm*) show very few silver grains. Some apical granulations (*g*) are labeled. Scale bar = 1 μm .

FIGURE 16 After 120 min: The whole cellular membrane has become labeled (arrows). Granulations (*g*) are distributed along the whole cell periphery. $\times 8,000$. Scale bar = 1 μm .

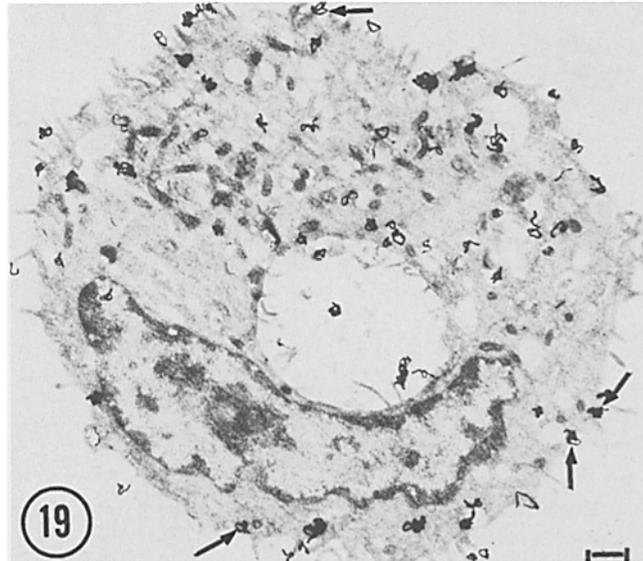
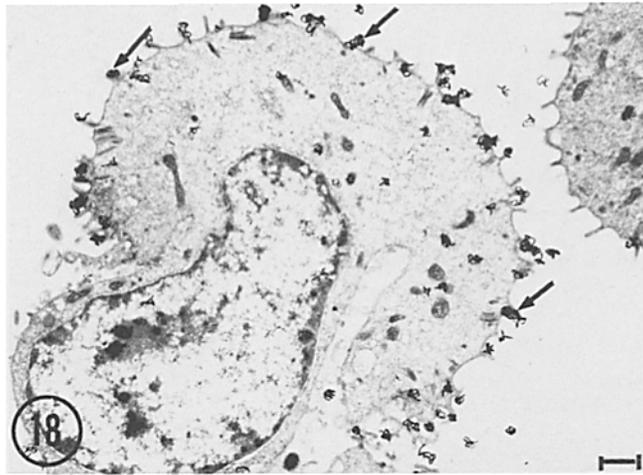
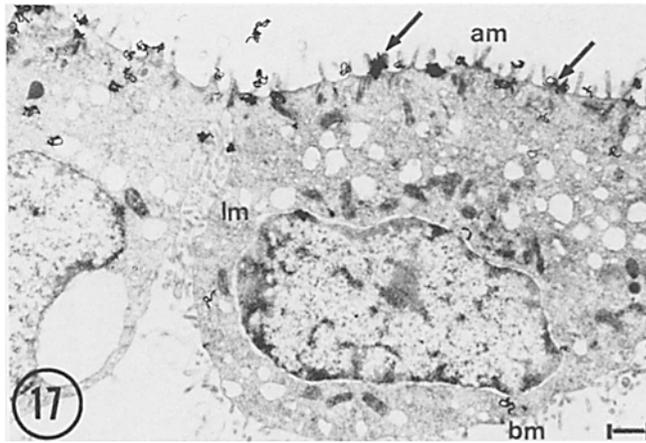


TABLE II
Silver Grain Distribution over the Plasma Membrane

| | Time after dissociation | | P |
|--|-------------------------|--------------------|------------------------|
| | Period I, 20 min | Period II, 120 min | |
| Apical membrane | 5.58* ± 0.25 | | (A - B) <0.001 |
| Laterobasal membrane | 2.49 ± 0.58 | | |
| Whole plasma membrane | 3.90 ± 0.40 | 4.73 ± 0.34 | (I - II) NS >0.5 |
| Whole plasma membrane After cycloheximide treatment | | 4.25 ± 0.38 | CII-DII NS >0.2 |
| <i>n</i> | 9 | 19 | 23 |

At 120 min after dissociation, only the whole plasma membrane was considered, as at this time it was impossible to distinguish clearly the apical membrane from the laterobasal membrane.

* Number of silver grains/10 μm of membranous profile ± SE.

n = number of experiments.

surface proteins indicated that this hypothesis does not fully account for the phenomenon. Indeed, it was shown, in this experiment made on the intact bladder, that the labeling only concerned protein material located on the external side of the plasma membrane and did not penetrate the granulations. Thus, if the material found at the laterobasal surface, after cell isolation without further labeling, had exclusively originated from these granulations, it would have remained unlabeled, in contrast to what was observed.

The last and most probable hypothesis would be the sliding down of the apical material along the laterobasal membrane. Indeed, with all the techniques used in this study, the stained material was found to invade progressively and continuously first the region of the tight junction, then the lateral surface and finally the basal surface of cells. Furthermore, 120 min after dissociation, the whole membrane became labeled without significant increase of radioactivity, as compared with the one observed at 20 min after dissociation (Table II). Hence, the glycoprotein material

which characterizes the apical surface of cells making up the intact epithelium would slide along the whole cell membrane when cells are dissociated. Under these conditions, the apical junctions would play a leading part in the maintenance of a cell surface heterogeneity in the normal bladder epithelium by preventing the apical glycoproteins from migrating basally.

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FIGURES 17-19 Electron microscope autoradiographs of epithelial cells after iodination of their apical membranous proteins. × 4,000.

FIGURES 17 and 18 20 min after the beginning of cell dissociation, the silver grains are localized along the apical membrane (*am*) (arrows). *lm* = lateral membrane; *bm* = basal membrane. Scale bar = 1 μm.

FIGURE 19 After 120 min: The autoradiographic reaction is seen all over the plasma membrane (arrows). Scale bar = 1 μm.

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