THE PERMEABILITY OF RAT TRANSITIONAL EPITHELIUM

Keratinization and the Barrier to Water

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ABSTRACT

Permeability barriers must exist in transitional epithelium to prevent the free flow of water from underlying blood capillaries through the epithelium into the hypertonic urine, and such a barrier has now been demonstrated in isolated bladders. This barrier is passive in function and can be destroyed by damaging the luminal surface of the transitional epithelium with sodium hydroxide and 8 M urea solutions, by digesting it with trypsin, lecithinase C, and lecithinase D, or by treating it with lipid solvents such as Triton × 100 and saponin. From this it is concluded that the barrier depends on the integrity of lipoprotein cell membranes. The barrier function is also destroyed by sodium thioglycollate solutions, and electron microscope investigations show that sodium thioglycollate damages the thick asymmetric membrane which limits the luminal face of the superficial squamous cell. Cytochemical staining shows the epithelium to contain disulfide and thiol groups and to have a concentration of these groups at the luminal margin of the superficial cells. It thus appears that the permeability barrier also depends on the presence of disulfide bridges in the epithelium, and it is presumed that these links are located in keratin. Because of the effect of thioglycollates, both on the barrier function and on the morphology of the membrane, it is suggested that keratin may be incorporated in the thick barrier membrane. It is proposed that the cells lining the urinary bladder and ureters should be regarded as a keratinizing epitheluim.

The transitional epithelium in the mammal presents a barrier to the passage of salts and water between blood and urine (1). No dilution of the strongly hypertonic urine occurs by the passage of water across the epithelium from the intra- and extracellular fluids, and there is only a very slow movement of ions across the cells in the reverse direction, from the urine into the blood. It was evident from a study of the subcellular morphology of this epithelium that the barrier function is subdivided into an extracellular and an intracellular component (2). The extracellular barrier has been identified, by analogy with the epithelial linings of other cavitary organs, as the junctional complex formed by the lateral cell membranes of adjacent superficial squamous cells at their luminal borders, and reasons have been given for thinking that the intracellular barrier must also be at the luminal edge of the epithelium. This intracellular barrier was tentatively identified as the unusual, thick, asymmetric cell membrane which limits the free surface of the squamous cell adjacent to the urine. It was proposed that the barrier function might, in part, be due to keratin incorporated in the thick surface membrane (2). Keratin is apparently also present in the cells of this epithelium in the form of tonofilaments, which,

in other tissues, are generally believed to be keratin fibers (3, 4).

In this paper, further observations are made which confirm the effectiveness of the permeability barrier in transitional epithelium. Evidence is presented that the barrier function is destroyed by proteolytic agents, lipid solvents, and sodium thioglycollate solutions. The thioglycollate is presumed to act by breaking disulfide bridges in keratin, and it is shown that it also physically disrupts the thick cell membrane. The existence of disulfide links, both throughout the cytoplasm and in a concentrated band at the luminal edge of the epithelium, is shown cytochemically. These results support the suggestion that this is a keratinizing epithelium.

MATERIALS AND METHODS

ANIMALS: Adult male and female rats of the Wistar strain were used. They were killed by dislocation of the neck.

CHEMICALS: Triton X 100 was obtained from Rohm & Haas Co., Philadelphia; crystalline trypsin, from Armour Laboratories Ltd., Eastbourne, Sussex; sodium thioglycollate, from Difco Laboratories, Detroit; and tetrazotized diorthoanisidine (Fast Blue B salt), from George T. Gurr Ltd., London. Lecithinase D, phenyl mercuric chloride, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD reagent), saponin, and urea were supplied by British Drug Houses, Ltd., Chemicals Division, Poole, Dorset, England. A sample of *Clostridium welchii* lecithinase C was used which was originally supplied to Dr. S. J. Holt by Dr. M. G. Macfarlane, Lister Institute of Preventive Medicine, London. All other chemicals were standard laboratory reagents of the highest purity available.

Bladders in Vitro

ASSESSMENT OF PERMEABILITY: For each experiment, an excised bladder was securely tied to a small glass cannula, and the cannula and bladder were filled either with 0.05 M phosphate buffer, pH 7.4, as a control, or with an appropriate test solution as listed in Table I. The bladder was immersed in a water bath at 37°C for 1 hr, then emptied and refilled with a 50% (w/v) sucrose solution. The open end of the cannula was then attached by a rubber collar to a fine capillary tube of known diameter bore, which in turn was attached to a centimeter rule. The sucrose-containing bladder was immersed in distilled water at room temperature, and the height of the column of liquid in the capillary tube noted at 5-min intervals over the next hour. The volume of water passing through the bladder wall and entering the sucrose

TABLE I

Water Influx Across the Bladder Wall Following Chemical and Physical Damage to the Transitional Epithelium

Test solution	Weight of water entering bladder	Effectiveness in damaging water barrier; arbitrary rating
	mg/hr	
0.05 M phosphate buffer, pH 7.4 (control)	1.0, 3.0, 3.0	
Abrasion followed by phosphate buffer	30.0	+++
0.1 N phenol	36.0	+++
0.1 N hydrochloric acid	1.0	
0.01 N hydrochloric acid	2.0	
0.1 N sodium hydroxide	55.0	++++
0.01 N sodium hydroxide	30.0	+++
8.0 M urea	30.0	+++
0.2 M sodium thioglycollate*	27.0	+++
0.1 M sodium thioglycollate*	21.0	++
0.01 M sodium thioglycollate*	7.0	+
Trypsin, 0.1 mg/ml*	17.0	++
Saponin, 1.0 mg/ml*	23.0	++
Triton X 100, 1.0 mg/ml*	15.0	++
Lecithinase C, 10 mg/ml‡	19.0	++
Lecithinase D, 1 mg/ml‡	13.0	++

* Dissolved in 0.05 M phosphate buffer and adjusted, where necessary, to pH 7.4.
‡ Dissolved in 0.05 M borate buffer, pH 7.4.

solution was approximately equal to $\pi r^2 h$, where r is the radius of the capillary tube and h the height of the column of liquid.

ENZYME DIGESTIONS OF THE EPITHELIUM: Solutions of lecithinases C and D (4 to 10 units/ml) and trypsin (0.25 mg/ml) were prepared according to Holt (5). These solutions were introduced into isolated bladders, and the bladders then incubated, as described, at 37°C for 1 hr. The enzyme solutions were then replaced by 50% sucrose and the bladder permeability determined.

Bladders and Ureters in Vivo

THIOGLYCOLLATE INJECTIONS: Animals were anesthetized with ether, and the left ureter and bladder were exposed. The bladder, if not already collapsed, was emptied by gentle pressure. A 0.1 M or 0.01 M solution of sodium thioglycollate in 0.05 M phosphate buffer, pH 7.4, was injected into the upper end of the ureter until the bladder was slightly distended. A clamp was placed over the lower end of the ureter before the needle of the syringe was withdrawn, thus trapping some of the thioglycollate solution in the ureter. After an interval of 5, 10, or 15 min, the ureter and bladder were injected in situ with osmium tetroxide, and removed for further fixation and inspection in the electron microscope.

In other animals, the common bile duct was exposed. A 0.01 M sodium thioglycollate solution in 0.05 M phosphate buffer was injected into the upper end of the bile duct and trapped in the duct by clamping the lower end. After 5 min, the duct was injected with osmium tetroxide, excised, and prepared for inspection in the electron microscope. Bile ducts were taken from other animals to act as controls.

ELECTRON MICROSCOPY: Bladders and ureters were fixed by injecting cold 4% (w/v) osmium tetroxide, buffered with phosphate (6) to pH 7.3, directly into the lumen. The organ was then removed and sectioned, under cold osmium tetroxide, into approximately 0.5 to 1.0 mm^3 cubes or cylinders. Fixation was continued for 1 hr, and the tissue was then dehydrated in ethanol and embedded in Epikote 812 (Shell Chemical Co., Ltd., London) essentially by the method of Luft (7).

Sections showing silver-to-gold interference colors were cut with glass knives on a Porter-Blum microtome, mounted on copper electromesh grids, double-stained (2) with uranyl acetate (8) and a lead salt (9), and examined in a Siemens Elmiskop 1.

LIGHT MICROSCOPY: For light microscopy, whole ureters and bladders were fixed in 4% glutaraldehyde buffered to pH 7.4 with sodium cacodylate (10) for 24 hr, then washed for 24 hr in 0.25 M sucrose buffered with 0.1 M sodium cacodylate to pH 7.4. The tissues were embedded in paraffin wax, and 5 μ sections were cut and mounted on clean glass slides. After dewaxing, the sections were stained to show —SH groups by the 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD) method of Barrnett and Seligman (11) according to the schedule given by Pearse (12). Control sections were incubated in saturated phenyl mercuric chloride for 2 days before staining. To illustrate —S—S— as well as —SH groups, the sections were reduced with a thioglycollate solution (12) before staining with the DDD reagent.

Experimental Procedure

In a first set of experiments, the permeability to water of excised bladders was assessed by measuring the rate at which water would cross the bladder wall to enter a 50% sucrose solution. The role of the transitional epithelium in controlling this was investigated by damaging the lining of the bladder, either chemically or physically, by scratching it with broken glass.

Since one agent causing an increase in bladder permeability was found to be sodium thioglycollate, which is known to act as a keratin solvent (3), further experiments were undertaken in which the fine structure of the bladder epithelium was examined, with the electron microscope, for damage after treatment with thioglycollate solutions. The bladders used for the in vitro permeability experiments were not entirely satisfactory for electron microscope investigations. Control bladders, in which the barrier function remained intact, showed wide distension of the extracellular spaces between epithelial cells after immersion in distilled water (Fig. 1). After experimental damage to the barrier function, gross desquamation of the epithelium as well as subcellular damage was seen. Under these conditions, it was not possible to detect the *primary* site of action of sodium thioglycollate. In vivo experiments were therefore performed, in which the blood supply to the bladder was maintained so that the epithelium was subjected neither to osmotic shock nor to anoxia. The luminal side of the epithelium was then exposed to sodium thioglycollate solutions of progressively lower concentrations until a minimal amount of cell damage was detectable with the electron microscope. The damage seen could thus be attributed, with some confidence, to the thioglycollate solutions and not to the experimental conditions.

In parallel in vivo experiments, bile duct epithelium was inspected as a control before and after exposure to sodium thioglycollate.

In a final set of experiments, the epithelium was stained cytochemically to reveal the location of disulfide and sulphydryl groups and therefore, by implication, the location of keratin.

RESULTS

Bladder Permeability and Epithelial Damage

The permeability of whole excised bladders was measured by calculating the rate at which water, from distilled water in which the bladder was suspended, entered a 50% sucrose solution contained within the bladder. From the internal diameters of the bladders below their point of attachment to the cannulae, it appeared that the surface area of the epithelium in contact with the sucrose solutions was approximately 0.75 to 1.0 cm^2, but this estimate is undoubtedly low because of the folding of the epithelial surface. Moreover, those bladders in which the epithelium was damaged distended far more during the course of the experiments than did undamaged control bladders. For these reasons, no attempt has been made to relate the rate of water passage across the bladder to a unit area, and the rate figures given in Table I are not strictly comparable. They are included here to show the basis on which the arbitrary rating was made and to give an idea of the order of bladder permeability. These experiments were performed at room temperature, with no supply of oxygen or nutrients to the bladder wall, 2 to 3 hr after removing the organ from the animal.

Water entered control bladders at a relatively low rate, approximately 1 to 3 mg/hr. Damaging the epithelium, mechanically by scratching it lightly with broken glass, or by the nonspecific corrosive action of dilute phenol, damaged the barrier function of the bladder and caused a tenfold increase in the rate at which water entered the sucrose solution.

Incubation of bladders containing dilute hydrochloric acid for 1 hr at 37°C did not increase their permeability above that of control bladders incubated with phosphate buffer. Sodium hydroxide, on the other hand, increased the rate at which water entered the sucrose solution even more effectively than phenol or abrasion, and 8 M urea solutions also increased the water flow. Incubation of bladders containing trypsin, or lecithinases C or D, increased the permeability of the bladder to water, as did Triton X 100 and saponin. The effect on the bladder of three concentrations of sodium thioglycollate in phosphate buffer was investigated, and at each concentration the permeability was raised above normal.

The Effect of Thioglycollate Solutions on the Thick Cell Membrane

The membrane on the luminal surface of the squamous cells is unusually thick, measuring about 115 A across (Fig. 2). The thinner dense leaflet of the unit structure adjacent to the cytoplasm is

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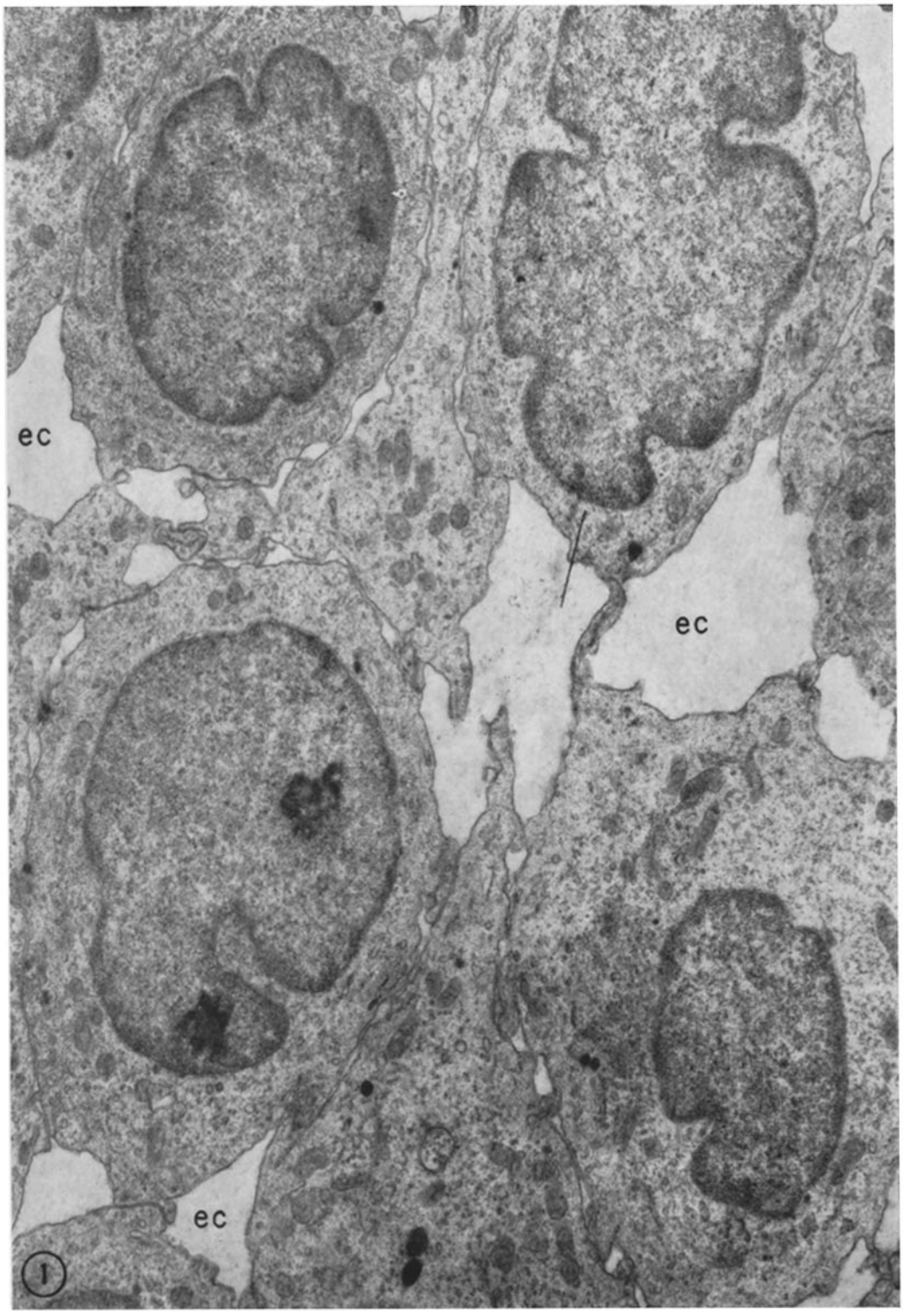
Figs. 8 to 11 are light micrographs of rat bladder and ureter, fixed in glutaraldehyde and stained cytochemically for —SH and —S—S— groups. All other figures are electron micrographs of bladder, ureter, or bile duct epithelial cells which have been fixed in osmium tetroxide, embedded in Epon, and double stained with uranyl acetate and a lead salt. The electron micrographs were taken with a Siemens Elmiskop 1.

Key to Symbols

S, squamous epithelial cell	*t*, tonofilaments
T, transitional epithelium	*cm*, cell membrane
U, lumen of ureter	*fv*, fusiform vesicle
b, projections on bile duct cell membrane	*ec*, extracellular space

FIGURE 1 The tissue illustrated in this figure is from an isolated bladder, which contains a 50% sucrose solution, and which had been suspended in distilled water for 1 hr at room temperature. The plane of section is roughly parallel to the luminal edge of the transitional epithelium and passes through the layer of intermediate cells. The extracellular spaces (*ec*) between the cells are widely distended and contain little or no electron-scattering material. The morphology of the subcellular organelles appears to be unaffected by the experimental treatment. × 12,000.



about 25 A, the central light band about 30 A, and the outer dense leaflet about 60 A thick.

The effect on the fine structure of the transitional epithelium of in vivo injections of 0.1 to 0.01 M sodium thioglycollate solutions into the lumen of the bladder or ureter was investigated with the electron microscope after 5, 10, or 15 min exposure times. The epithelium was grossly damaged by 0.1 M thioglycollate solutions and by 15 min exposure to a 0.01 M solution. Many of the superficial and intermediate cells were ruptured and others shed into the lumen. Those remaining attached to the basement membrane and subepithelial tissues showed a variable degree of cytoplasmic damage, frequently with ruptured cell membranes and large "empty" areas in the cytoplasm. After only 5 min exposure to 0.01 M thioglycollate, early signs of damage to the epithelium could be seen. The luminal surface of the epithelial cells tended to be more flattened in appearance with fewer crests than normal, and the cytoplasm immediately below the membrane became thick and dense owing to condensation of tonofilaments (Fig. 3). At intervals, breaks in the membrane were seen (Fig. 4) and the tonofilaments appeared to fray out from the cell surface into the lumen (Figs. 4 and 5). At this stage, the cytoplasmic contents only a few microns below the cell surface appeared to be substantially normal (Fig. 5), and no damage to the membranes of cytoplasmic vesicles or mitochondria, or to the cytoplasmic tonofilaments was observed, although a few spaces between the tonofilaments appeared.

The Effect of Sodium Thioglycollate on the Cell Membrane of the Common Bile Duct

The membrane on the luminal surface of the common bile duct in the rat was also found to be asymmetric (Fig. 6). The short, stubby microvilli are covered by an approximately 110 A thick membrane, with a wider than average central light band. At the tips of the microvilli, the dense leaflet on the luminal surface of the unit membrane is fimbriated and is projected into, or covered with, small dense blobs approximately 150 to 400 A in diameter (Fig. 6). It is not clear whether these blobs are a part of the membrane unit structure or a very closely applied form of extraneous coat.

This membrane was inspected after in vivo injections of sodium thioglycollate into the bile duct lumen, and no damage to the membrane or underlying tissues was observed after 5 min exposure to 0.01 M sodium thioglycollate (Fig. 7).

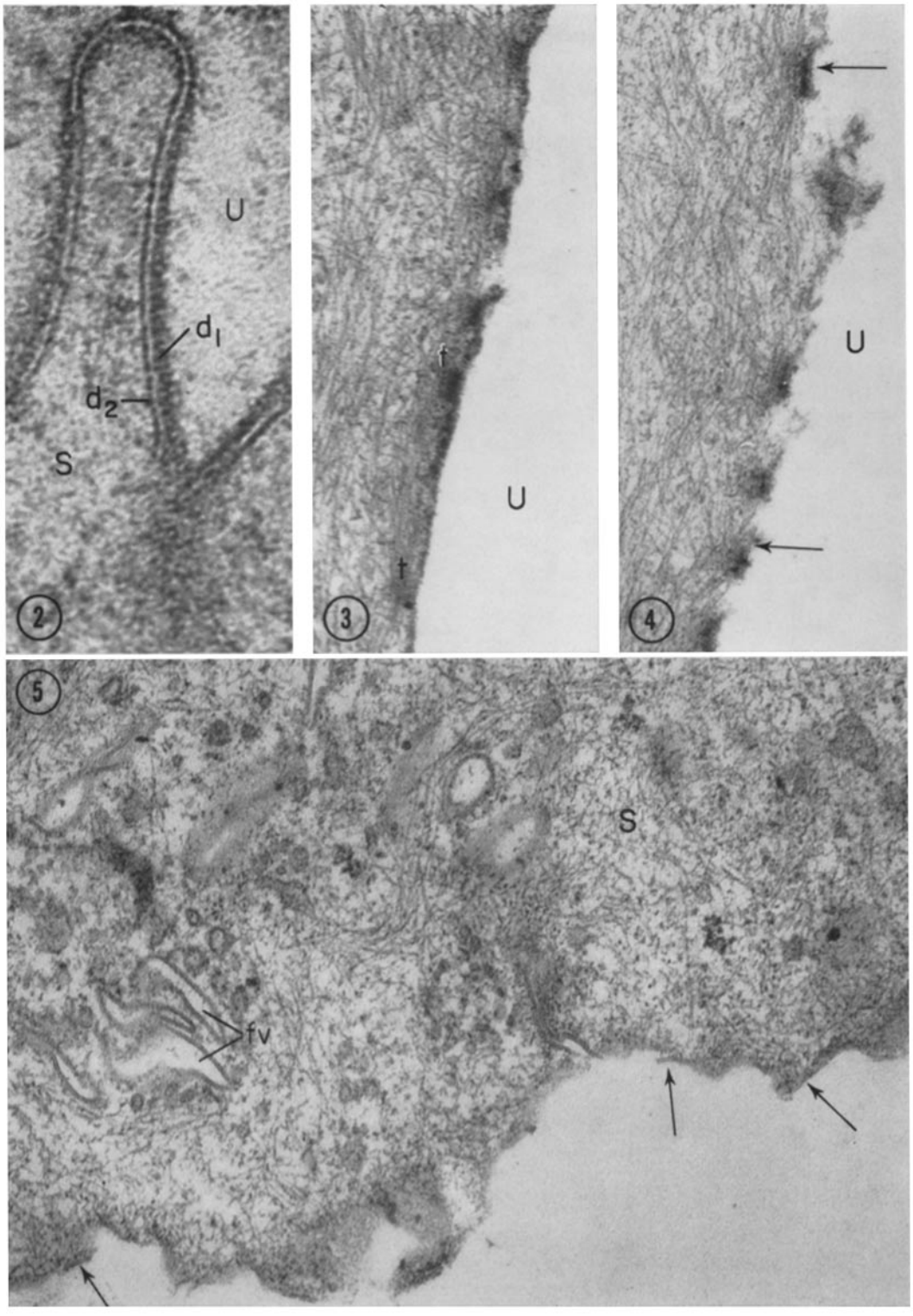
Cytochemical Staining for —SH and —S—S— groups

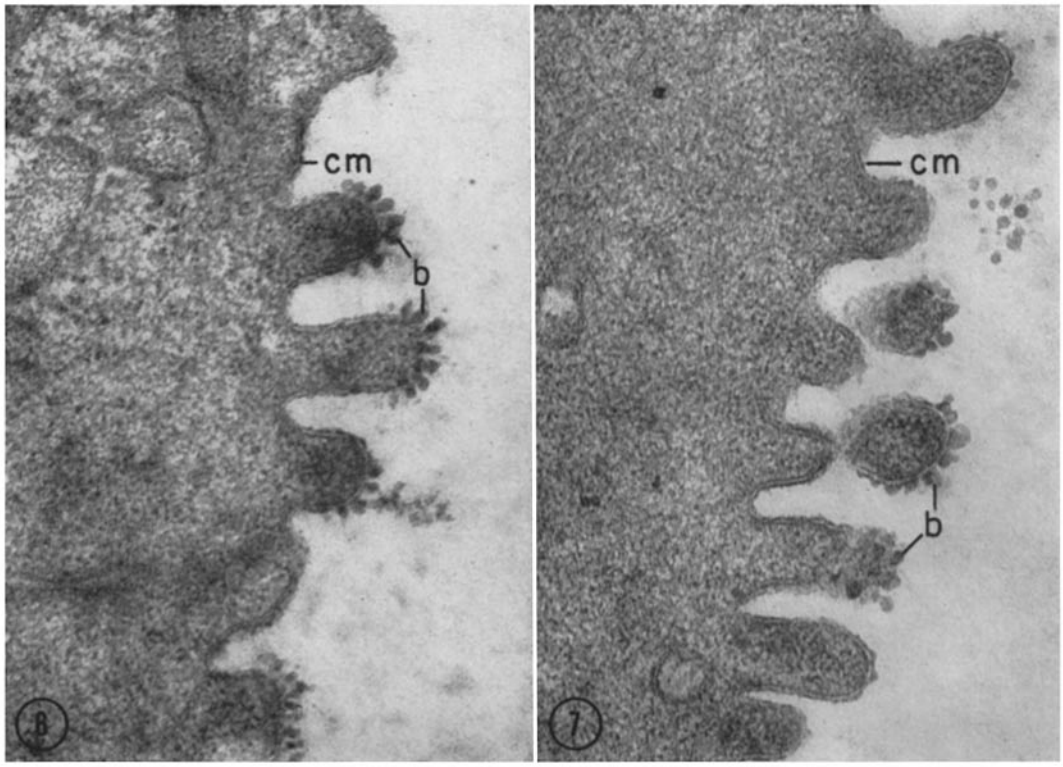
Sections of glutaraldehyde-fixed bladders were stained by the DDD method (11, 12) for sulphydryl groups. Weak positive staining of the cytoplasm throughout the epithelium was obtained, and a more intense band of stain appeared to be located at the luminal margin of the superficial cells (Fig. 8). When sections were reduced with sodium thioglycollate, before being stained, to

FIGURE 2 This figure shows part of the triple-layered cell membrane of a squamous cell (*S*) adjacent to the ureteric lumen (*U*). The asymmetric unit structure of this 115 A thick membrane can be clearly seen. The dense leaflet (d_1) adjacent to the ureteric lumen is approximately 60 A thick, while the inner leaflet (d_2) adjacent to the cytoplasm is about 25 A thick. × 300,000.

FIGURES 3 and 4 These figures are part of ureteric squamous cells and show the effect on the thick cell membrane of 5 min in vivo exposure to 0.01 M sodium thioglycollate. The unit structure of the membrane has disappeared in Fig. 3, and there is a condensation of tonofilaments (*t*) at the cell surface. In Fig. 4, remnants of the membrane can be seen (arrows), and tonofilaments appear to fray out into the ureteric lumen (*U*). × 60,000.

FIGURE 5 This field shows part of the luminal edge of a bladder epithelial cell (*S*), after in vivo exposure to 0.01 M sodium thioglycollate. Remnants of the thick surface membrane (arrows) can be seen, but elsewhere the membrane has gone, and the cell surface is ragged in appearance. The thick membranes of the cytoplasmic vesicles (*fv*), 2 to 5 μ below the surface of the cell, appear to be intact. × 60,000.





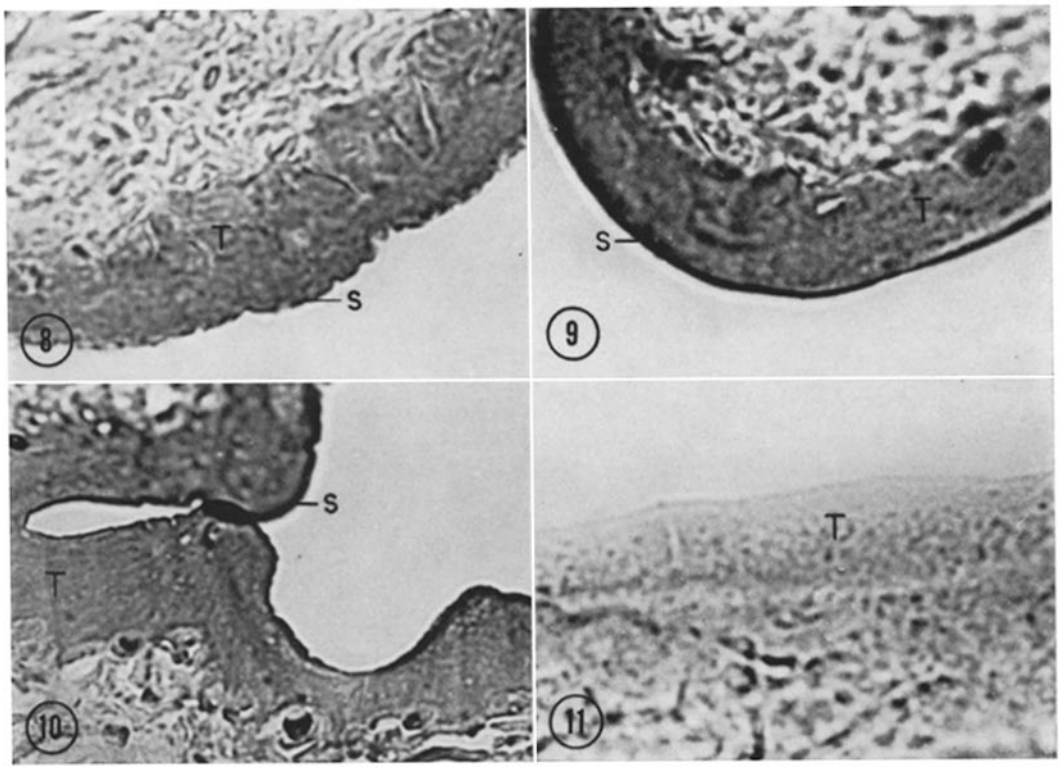
FIGURES 6 and 7 Part of the luminal edge of a bile duct epithelial cell is illustrated from a control animal in Fig. 6, and after exposure in vivo to 0.01 M sodium thioglycollate in Fig. 7. In each case, the cell surface is covered with microvilli, and the unit structure of the thick cell membrane (*cm*) can be seen. At the tips of the microvilli, the outer leaflet of this membrane is covered with, or projected into, electron-opaque blobs (*b*). No differences can be seen between the control and thioglycollate-treated membrane. × 80,000.

reveal disulfides as well as sulphydryls, the dense band of stain at the luminal edge of the epithelium was intensified, and there was a slight increase in density of the cytoplasmic stain (Fig. 9). These two results are essentially the same as those reported by Capurro and Angrist (13) for normal rat bladder. The transitional epithelium of the ureter showed a similar distribution of sulphydryl and disulfide groups (Fig. 10). No positive staining was obtained with control sections in which sulphydryl groups were blocked with phenyl mercuric chloride (Fig. 11).

DISCUSSION

The experiments reported here illustrate that the whole bladder acts as a passive barrier to water diffusion, and that this continues to function at room temperature even when the bladder is removed from supplies of nutrients and oxygen. Under these conditions, water will cross the bladder wall to enter a hypertonic solution at an approximate rate of only 1 to 3 mg/hr over an estimated epithelial area of 0.75 to 1.0 cm^2. In vivo, this barrier must depend on the transitonal epithelium, which separates blood plasma in subepithelial capillaries from the hypertonic urine which is a distance away of only 50 to 100 μ in the bladder or ureter lumen (2). The reason for using whole bladders to assess permeability rather than isolated transitional epithelium, was the difficulty encountered in stripping the thin epithelial layer, without damage, from the rest of the bladder wall. However, it was still possible to investigate the nature of the epithelial barrier by applying reagents directly to the luminal surface of epithelial cells and then by measuring permeability changes for the bladder as a whole.

The epithelial barrier appears to depend on the combined action of extracellular junctional complexes and the thick asymmetric cell membranes



FIGURES 8 to 11 These light micrographs show portions of rat bladder (Figs. 8, 9, and 11) and ureter (Fig. 10) after cytochemical staining with the DDD reagent (11, 12) for —SH groups.

Fig. 8 shows a distribution of —SH groups throughout the transitional epithelium (*T*), and a thin line of intense stain (*s*) at the luminal margin of the epithelium.

In Figs. 9 and 10, the tissue was reduced with sodium thioglycollate before staining, to show the distribution of both —S—S— and —SH groups. In ureter and bladder, the line of intense stain (*s*) at the luminal margin of the epithelium is more pronounced than it was before reduction of the tissue (cf. Fig. 8), and there is a slight increase in density of the cytoplasmic stain.

The tissue in Fig. 11 was incubated in phenyl mercuric chloride to block the—SH groups before staining with the DDD reagent. Neither the cytoplasm nor the luminal edges of the epithelial cells stain after this treatment. Figs. 8 to 11, × 1400.

found only on the luminal face of the superficial cells (2). If the epithelium is damaged, by scratching it lightly with broken glass or by the nonspecific corrosive action of phenol, the barrier function is drastically reduced, and water enters the bladder (Table I). The barrier appears to be fairly acid-resistant, but is badly damaged by sodium hydroxide and 8 M urea, both of which are powerful protein solvents. It is also damaged by digestion with the proteolytic enzyme trypsin. Incubation with lecithinases, which disrupt the phospholipid lecithin, and with saponin or Triton X 100, which react with cholesterol (14, 15), also render the bladder permeable to water.

Most interesting, however, is the damage to barrier function obtained by incubation with thioglycollate solutions, which are known to break disulfide bonds by reduction to sulphydryls. These results indicate that the barrier in transitional epithelium is dependent in part upon a phospholipoprotein structure, i.e. the cell membrane, and in part upon the presence of disulfide groups. This supports the previous suggestions that the asymmetric membrane on the luminal surface of the squamous cell is the intracellular barrier and that keratin may be included in its structure (2), for keratin and keratohyalin are the only commonly found animal structural proteins rich

in sulfur, and the water-proofing properties of keratin are well known (3).

The over-all thickness of this barrier membrane in the rat is about 115 A, which agrees well with the figure of 100 to 110 A reported for the same membrane in mouse transitional epithelium (16, 17). It is damaged by sodium thioglycollate, and structural changes can be detected after only 5 min exposure to a 0.01 M solution. At this early stage of damage, tonofilaments, mitochondria, and the thick membranes of cytoplasmic vesicles below the cell surface still appear to be normal, which suggests that the thick barrier membrane is the first structure to be attacked by the thioglycollate solutions. To investigate whether this effect of thioglycollates is peculiar to the transitional epithelium or is common to all cell membranes, thioglycollate solutions were injected into the common bile duct. The asymmetric membrane of bile duct epithelial cells, unlike that of the transitional epithelium, is not damaged by sodium thioglycollate, and it may therefore be assumed that disulfide links are not an important part of its structure. These epithelial cells, in the bile duct of the rat, may be expected to function like those of the gall bladder in other species and to be active in water and cation transport (18); an impermeable keratin-containing barrier would be of no advantage in this situation.

It can be concluded from these results that the barrier function of transitional epithelium may well depend on the inclusion of some form of keratin in the thick surface membrane, unless some other sulfur-containing fibrous protein can subsequently be isolated from this tissue. The transitional epithelium undoubtedly contains some keratin or other cystine-rich protein, as the cytochemical staining results obtained with the DDD method (11, 12) for combined —S—S— and —SH groups illustrate. It is not possible to infer from these light micrographs (Figs. 8 to 11) which morphological substructures contain the reactive sulphydryl radicles. The weak cytoplasmic stain is probably due to the presence of tonofilaments throughout the cells, if tonofilaments are, in fact, a form of keratin, as is generally stated for other tissues (3, 4). The concentration of stain at the luminal edge of the squamous cells which had previously been observed by Capurro and Angrist (13) may be interpreted as due to keratin in the thick membrane of the cell surface and cytoplasmic vesicles. Alternatively, it could be due to keratin in tonofilaments, for although these fibrils are present throughout the epithelium, they are particularly plentiful toward the luminal edge of the superficial squamous cells.

The presence of tonofilaments and disulfide groups, together with the known ability of the bladder epithelium to produce keratinized plaques in response to stress (19, 20), indicates that transitional epithelium may properly be regarded as a keratinizing epithelium. This is using Kligman's definition of keratinization as "the synthesis of a peculiar fibrous protein" and "not the synonym for horny later formation" (21). This epithelium is mesodermal in derivation and is therefore an exception to the statement that "keratin-forming cells are all of ectodermal origin" (22). Transitional epithelium is thus comparable to vaginal and oral mucosae, both of which are noncornified, keratinizing epithelia. These epithelia, however, unlike transitional epithelium or epidermis, are relatively permeable to salts and water, and this has been attributed to their lack of a horny layer (23), although it is apparent that the thickness of the horny layer in epidermis is no guide to the effectiveness of the barrier (21, 23). It is equally possible that differences in permeability between oral mucosa and transitional epithelium are due to differences in the structure of their superficial cell membranes, which in oral mucosa are thickened and coated by an amorphous extracellular material, which may be polysaccharide in nature, and which originates in cytoplasmic membrane-coating granules (24). This evidently represents a form of membrane specialization which differs from that seen in transitional epithelium and which is related to a difference in function.

This work was supported by the British Empire Cancer Campaign. I am most grateful to Dr. M. A. Epstein and Dr. S. J. Holt for their advice and encouragement, and to Mr. G. Ball and Mr. T. Heather for their excellent technical assistance.

Received for publication 8 July 1965.

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