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Structure of *Necturus* Gallbladder Epithelium during Transport at Low External Osmolarities

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Summary. Gallbladders transport isotonically over a wide range of osmolarities. This ability has been assumed to depend on the geometry of the lateral intercellular spaces. We report that this geometry in the *Necturus* gallbladder varies extensively with the external osmolarity and depends *in vitro* on the integrity of the subepithelial tissues. The structure of the living epithelium was studied by Nomarski light microscopy while ultrastructural effects were revealed by electron microscopy. The short-term effects (< 60 min) of low external osmolarities were: 1) the cells became bell-shaped with an increased cell height measured centrally, 2) lateral intercellular spaces lost their convoluted character; and 3) numerous membrane-bound cavities appeared in the cells. Furthermore, long-term exposure to the low external osmolarities caused an uneven density of epithelial cells. With subepithelial tissues intact, blistering of the epithelium cell layer was evident. Qualitative electron-microscopic data indicate that the membrane of the cavities was recruited from the basolateral cell membrane. This agrees well with light-microscopic observation that the cavities were initiated as invaginations of this cell membrane.

Key words *Necturus* gallbladder · morphology · low osmolarities · lateral intercellular spaces

Introduction

Several epithelia are exposed *in situ* to solutions both hyper- and hypo-osmotic to plasma. Futhermore, some species live in an environment which changes between seawater and freshwater. The flatworm *Procerodes ulvae* is exposed to seawater at high tide and to freshwater at low tide, and thus its ectoderm and endoderm is exposed to a wide range of osmolarities (Beadle, 1934).

The properties of leaky epithelia, as a function of external osmolarities have been studied in detail. These epithelia elaborate a secretion which is isotonic to the bathing solution over a wide range of osmolarities: rabbit gallbladder, 60–500 mOsm (Diamond, 1964); pancreas, 110–600 mOsm (Case, Harper & Scratcherd, 1968); *Rhodinius* malpighian tubule, 60–380 mOsm (Maddrell, 1969); and *Necturus* gallbladder, 1–400 mOsm (Hill & Hill, 1978) and 39–205 mOsm (Zeuthen, 1981 *a*, *b*). Furthermore, the cells maintain normal intracellular potentials and ion activities (Zeuthen, 1981 *a*, *b*).

It is a *conditio sine qua non* for any model of isotonic transport that it explains isotonic transport at low osmolarities as well as at normal osmolarities. At present, models depend not only on the parameters for water and ion permeation across the various membranes and pathways but also on the geometry of the tissue and partly that of the lateral intercellular spaces. This is particularly the case with the standing gradient model (Diamond & Bossert, 1967) and much discussion has centered around geometrical questions (Hill, 1975; *see also* recent reviews by Diamond, 1979, and Hill, 1980).

The aim of the present paper was to study the geometry of the *Necturus* gallbladder epithelium when the osmolarity of the external solution was varied in the hypotonic range of 39 to 205 mOsm. The *in vivo* condition was examined by Nomarski-optics (Allen, David & Nomarski, 1969) while further details were obtained by electron microscopy. Special attention was given to the geometry of the lateral spaces, and it was found that this depended markedly on the osmolarities of the external solutions and the time of exposure. A preliminary report has been published (Bundgaard & Zeuthen, 1981).

Materials and Methods

Gallbladders were removed from *Necturus maculosus*. The excised bladders were extended over a hole (o.d. 6 mm) in a plate of Plexiglass and placed between a water immersion lens with Nomarski attachment (Zeiss 40×) and a Nomarski-condenser (Zeiss, Phako IV 21) with a long working distance, 7 mm. The epithelial cell layer was stretched at an angle α of about 20° with the horizontal plane. This means that the basal part of the cells were in focus along one line and the top of the cells were in focus along another line. Thus the cell height could be estimated as sin α times the distance between these focal lines. The optics permitted microvilli of 1 μm to be distinguished, and these determined the position

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Table 1.

Salines[a]		Fixatives				
		Initial fixation		Rinsing solution	Osmium fixation	
mOsm	NaCl	Glutaraldehyde (%)	Na-cacodylate buffer (M)	Na-cacodylate buffer (M)	OsO_4 (%)	Na-cacodylate buffer (M)
205	105	1	0.1	0.1	2	0.1
160	78	1	0.075	0.075	2	0.1
117	52	1	0.05	0.05	2	0.05
64	26	1	0.01	0.025	2	0.01
39	13	1	0.001	0.012	2	0.001

[a] *See text* for additional ions.

Table 2. Areas of epithelial cell membranes per unit volume epithelial cytoplasm

Membrane	($\mu m^2/\mu m^3$)		
	205 mOsm	64 mOsm	
basolateral	2.18 ± 0.42	0.72 ± 0.12	$p < 0.001$
luminal	0.25 ± 0.09	0.28 ± 0.06	N.S.
cavity	0	0.83 ± 0.11	$p < 0.001$
total area investigated	2083 μm^2	5069 μm^2	
no. of samples	4	11	

Data given as mean $\pm$ SE.

of the mucosal surface. The basal termination of the cells could only be determined to accuracies of 2–3 μm.

The drop of solution (~15 μl) held under the objective served as mucosal solution and was renewed in less than a second by continuous flow from a peristaltic pump. The fluid, having superfused the tissue, flowed via a wick into a second chamber from where it was aspirated. The secretion could be sampled from the serosal side for analysis of osmolarity and weighing in order to determine the transport rate. For long-term adaptations the chamber was placed in a beaker containing oxygenated solutions.

In some experiments the mesothelium and some connective tissue was removed by dissection.

Solutions

Solutions were (in mM): 105 Na^+, 3 K^+, 2.7 Ca^{++}, 117 Cl^-, 2.4 HCO_3^-, osmolarity 205 mOsm. Solutions of a lower osmolarity were achieved by removing NaCl, maintaining K^+ at 3 mM, Ca^{++} at 2.7 mM and HCO_3^- at 2.4 mM. Solutions were bubbled with O_2 and had a pH of about 7.6. Osmolarities were measured in an Advance® osmometer or a Ramsay Osmometer (Ramsay & Brown, 1955).

These solutions and the corresponding fixation-solutions are shown in Table 1.

Preparation for electron microscopy

Pilot experiments showed that it was necessary to adjust the osmolarity of the fixative to the osmolarity of the bathing solutions. For example, if a gallbladder after long-term exposure at 64 mOsm was fixed in a solution of 1% formaldehyde and 1.25% glutaraldehyde in 0.1 M Na-cacodylate buffer (which is suitable for fixation at normal osmolarity ~250 mOsm) it appeared shrunken. In addition, major structural characteristics observed in the living tissue with Normarski optics, such as cavities in the epithelial cells and the irregular outline of the epithelium were not preserved. However, fixatives adjusted to the low osmolarities as mentioned below gave a reasonable preservation, characterized by the preservation of *in vivo* observations as well as by integrity of cell organelles.

Necturus gallbladders, mounted in the perspex chamber, were initially fixed by immersion in a solution of 1% glutaraldehyde in sodium cacodylate buffer. It was assumed that glutaraldehyde exerts an osmotic effect on the epithelial cells corresponding to approximately one-third of its osmolarity determined by freezing-point depression; this approximates 30 mOsm for a 1% aqueous solution of glutaraldehyde. The final osmolarity of each fixative was adjusted to the osmolarity of the medium bathing the tissue prior to fixation by varying the concentration of sodium cacodylate buffer (Table 1). After 12–16 hr in the initial fixative the tissue was removed from the perspex chamber, rinsed in sodium cacodylate buffer and post-fixed in 2% OsO_4 in sodium cacodylate buffer for 2 hr. The osmolarity of the rinsing solutions and the osmium fixatives were also adjusted to the osmolarity of the bathing media (Table 1). After osmium fixation the tissue was rinsed in distilled water, treated with 1% tannic acid (No. 1764, Mallinckrodt Inc., St. Louis, MO) in 0.05 M sodium cacodylate buffer for 45 min, dehydrated in graded series of ethanol and flat embedded in Epon resins. Thin sections cut with a diamond knife on an LKB-ultratome were poststained with lead citrate for 5 min and examined in a Zeiss 10 B electron microscope operated at 60 kV. The magnification was calibrated with a carbon replica of an optical grating (568 lines/mm).

Quantitative Measurements

Gallbladders transporting at normal osmolarity (205 mOsm) and at 64 mOsm were included in this part of the study. Sections were cut perpendicularly to the apical surface of the epithelium and were initially studied at 2500× magnification. The first segments of the epithelium which appeared to be of a quality to allow a detailed examination of the membranes were selected for the quantitative investigations. Approximately 500 μm^2 of cytoplasm were included in each sampling unit. Overlapping micrographs of these epithelial segments were taken at an electronic magnification of 12,500×. The electron micrographs which had a final magnification of 30,000× were assembled into montages on which measurements were performed. The areas of epithelial cells in montages were 2083 μm^2 (4 sample units) for gallbladders at normal osmolarity and 5069 μm^2 (11 sample units) for tissue at 64 mOsm. The unpaired students *t*-test was applied for statistics (Table 2).

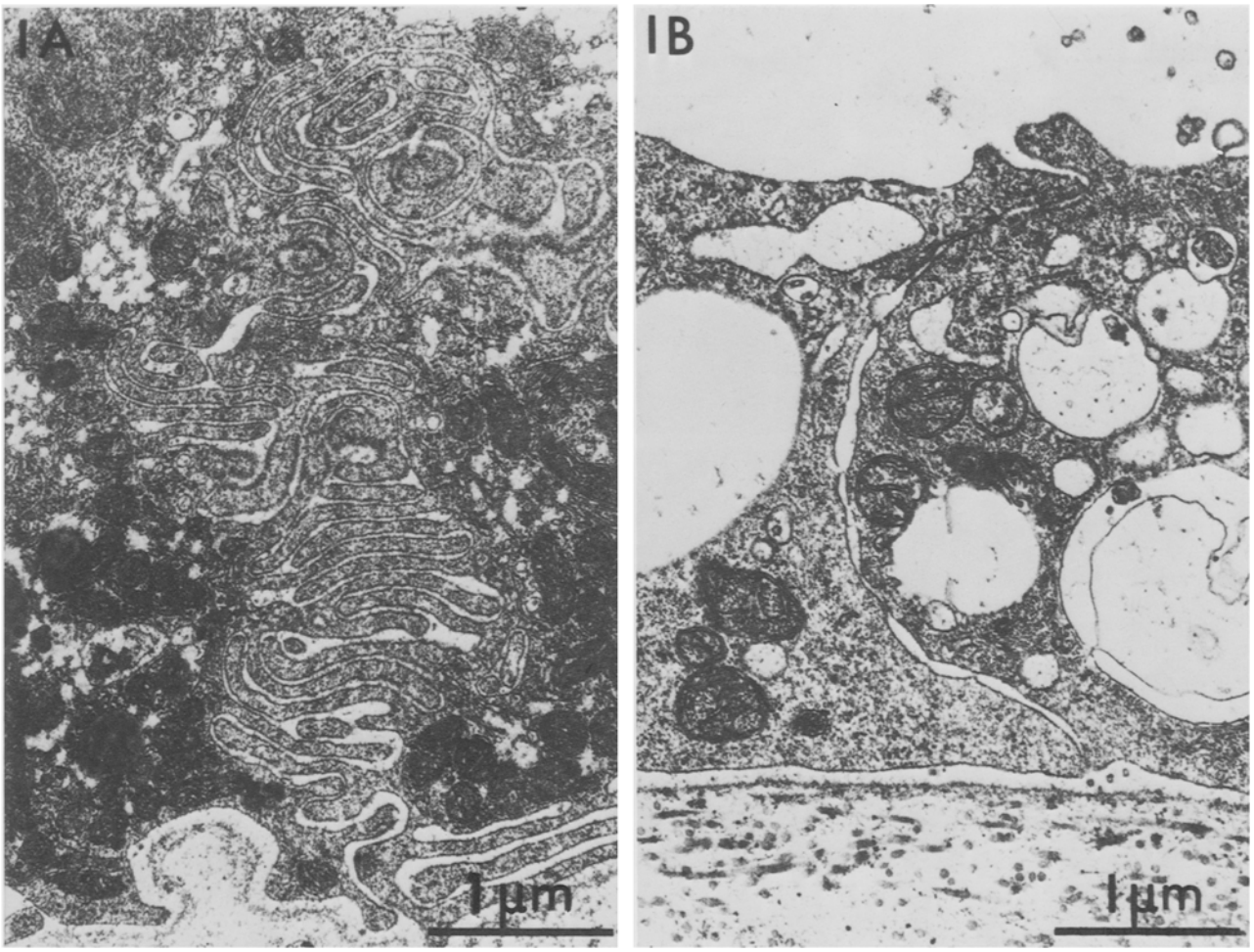

Fig. 1. *A.* Electron micrograph of the basal part of the lateral intercellular space in *Necturus* gallbladder epithelium at normal osmolarity (205 mOsm). The lateral cell membranes are extensively folded. *B.* Segment of an epithelium fixed after 3 hr incubation at 39 mOsm. The height of the cells at the junctional region has decreased from about 30 to 4 µm and the foldings of the lateral plasma membrane have disappeared

A transparent square lattice, placed at random over the montages, was used to estimate the surface densities (S_v) of the apical and basolateral membranes according to stereological principles previously described (Weibel & Bolender, 1973). The intersections of the lines of the lattice served as test points. The number of points (P) over the epithelial cells was counted. One set of lines in the lattice, served as test lines. The number of intersections (I) between test lines and apical or basolateral plasmalemma were counted and the surface densities calculated: $S_v = 2\,I/P \cdot d$, where d is the distance between test lines which equaled 5.1 cm corresponding to 1.7 µm in the tissue.

Results

Normal Osmolarities, 205 mOsm

The structure of *Necturus* gallbladder transporting at normal osmolarities is well known from the literature (Zeuthen, 1976; Hill & Hill, 1978; Schifferdecker & Frömter, 1978). Three elements form parts of the wall of the gallbladder: the epithelium, the subepithelial connective tissue and a thin layer of mesothelial cells facing the abdominal cavity. The epithelium consists of a single layer of columnar cells with a height of about 30 µm and a cell diameter of about 20 µm. These dimensions refer to bladders stretched similarly to the *in situ* condition. If the bladder is fixed unstretched the cell height is about 40 µm; if fixed while stretched maximally the height is approximately 20 µm. The cells are connected at their apical aspect by leaky tight junctions (Hill & Hill, 1978). The lateral cell membrane is extensively folded (Fig. 1*A*); and quantitative measurements performed in this study (Table 2) indicate that the folding increases the membrane area by a factor of 9.4, as compared to a hypothetical cuboidal cell with a height of 30 µm and a sidelength of 20 µm.

The cells contain numerous mucous-filled vesicles with an average diameter of 0.12 µm.

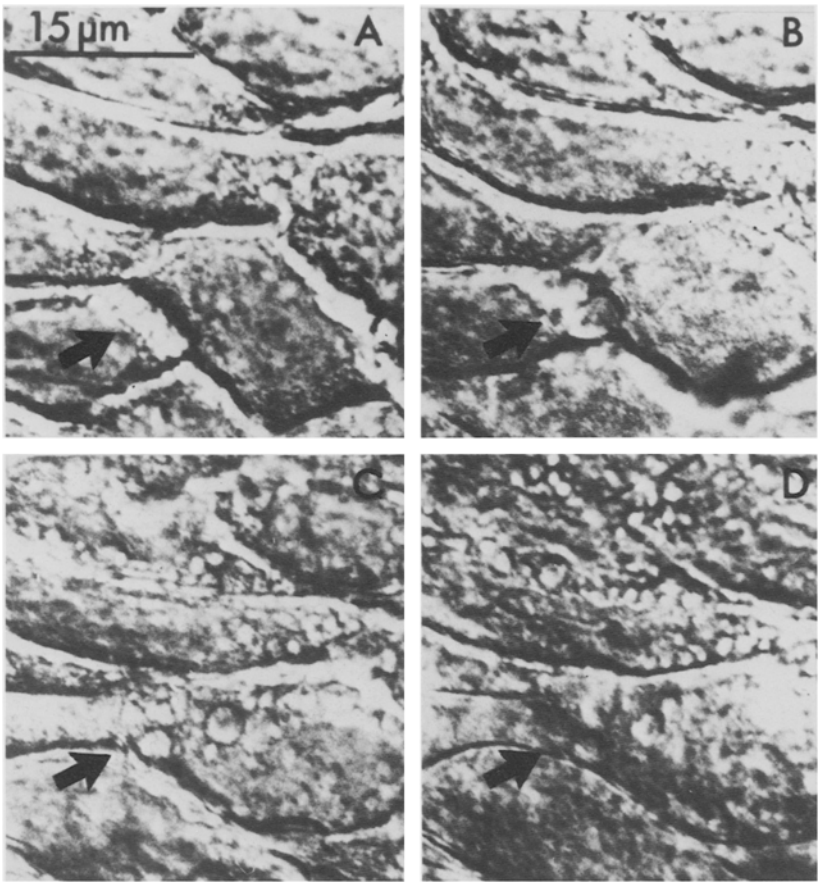

Fig. 2. Nomarski micrographs illustrating the apparent internalization of the cavities. The tissue was mounted as described on p. 97. *A.* $t=43$ min after the mucosal solution was changed from 205 to 117 mOsm. *B.* $t=72$ min. *C.* 120 min. *D.* This was taken 5 min after the mucosal solution was returned to 205 mOsm. The lateral space marked by an arrow illustrates the formation of apparently internalized cavities

Observations in the Living Tissue

The structure of the living epithelium was studied by means of Nomarski-optics. When the mucosal solution was changed from the control solution, 205 mOsm, to a lower osmolarity (Table 1) the cells began to change their shape. The cell height measured centrally increased uniformly within each field of view (250×110 µm). The effect was highly variable from tissue to tissue and was more pronounced the larger the change in osmolarity. Any strict quantitative description of the initial changes is difficult; but it can be stated that if a tissue was exposed suddenly to a solution of 117 mOsm then the increase in cell height measured centrally was no less than 10% and not higher than 60%; the change was complete within the first 5 to 10 min (8 tissues tested).

If the tissues were exposed to the low osmolarities for more than 60 min (for solutions of 117 mOsm) and 30 min (for solutions of 64 mOsm) gross morphological changes were apparent in all tissues. The number of the cells per cm^2 increased in some areas and diminished in others. Concomitantly, the distance from the junctions to the basement membrane decreased in those areas where the density of cells decreased and increased in those areas where the density increased.

The low external osmolarity had a marked effect on the shape of the lateral intercellular space. Initially, in normal osmolarities the spaces were visible as light bands which appeared 1 µm wide 10 µm below the mucosal surface. Reduced mucosal osmolarity caused a widening of the spaces. In 117 mOsm additional widening of the spaces became apparent after 15 min. This effect progressed with time and after an additional 25 min the spaces seemed to be divided into compartments. After another 30 min the compartments of the lateral spaces had become spherical and localized within the cell volume. An example is shown in Fig. 2. The rate at which it occurred was dependent on the amount of connective tissue and therefore on the amount of normal saline initially present at the serosal side. The diameter of the larger cavities increased linearly with time and their growth was not related to the initial change of cell height, as the time dependences of the two processes were different. It should be emphasized that only the behavior of the larger cavities are reported; the smaller cavities (< 2 µm) observed with the electron microscope could not be defined with certainty with the Nomarski optics.

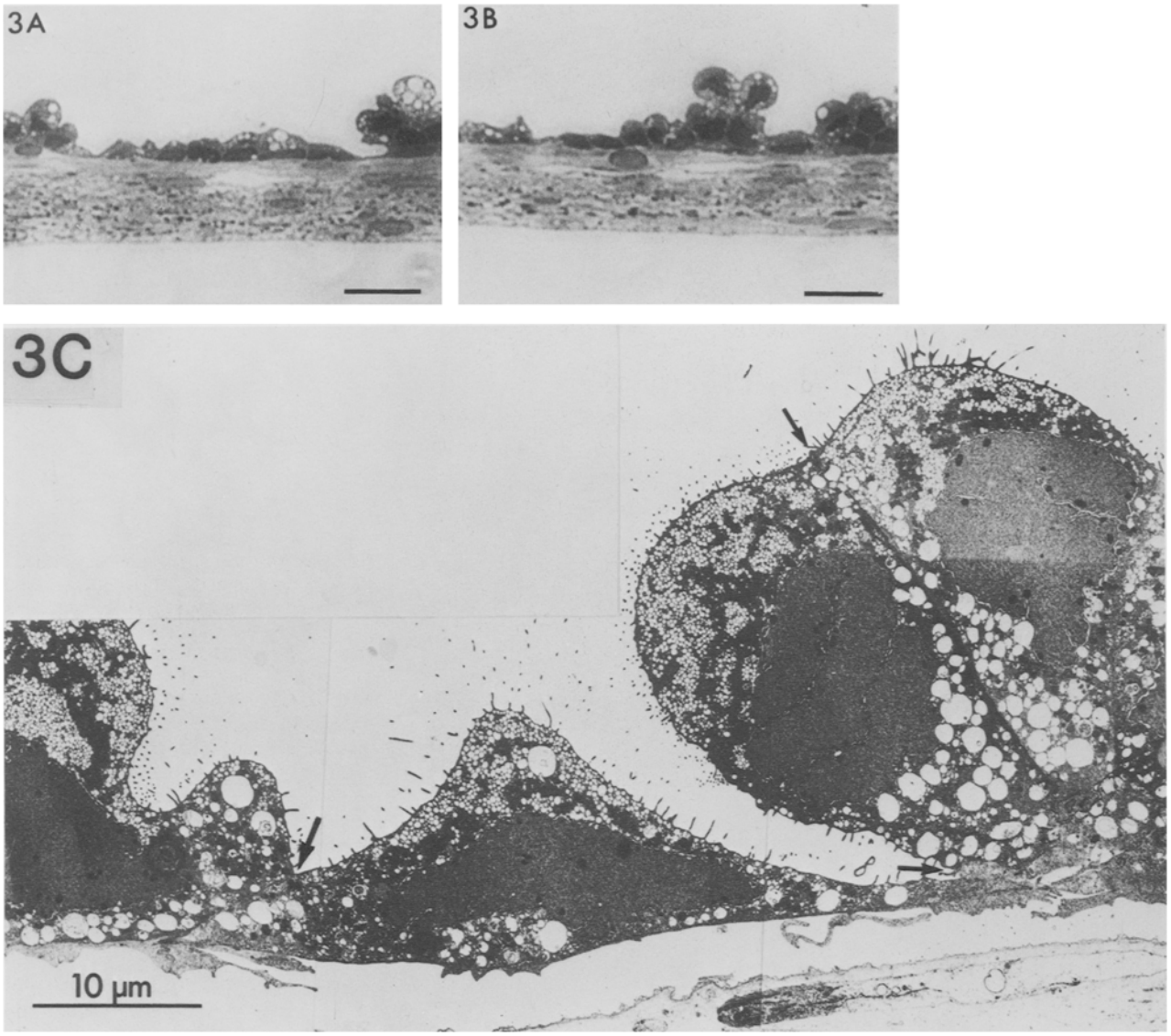

Fig. 3. Gallbladder epithelium incubated for 2 hr in 64 mOsm before fixation. *A*, *B*. Light micrographs. Notice the irregular outline of the luminal aspects of the epithelium. Bars, 50 µm. *C*. Montage of electron micrographs including 5 epithelial cells. Arrows indicate tight junctions. The length of the lateral intercellular spaces varies extensively

Observations on Gallbladders Fixed after Long-Term Exposure to Low Osmolarities

Light microscopy of one-micron sections of epon-embedded gallbladders fixed after long-term exposure at low osmolarities confirmed the major structural characteristics observed with Nomarski optics – a very irregular outline of the luminal aspect of the epithelium and appearance of cavities in the epithelial cells (Fig. 3 *A*, *B*). Electron microscopy (Fig. 3 *C*) showed that the effects of low external osmolarities were (*i*) conspicuous changes in the configuration of the lateral intercellular spaces and (*ii*) formation of numerous membrane-bound cavities located preferentially in the lateral and basal part of the cell.

Low osmolarity did not influence the morphology of the tight junctions, but substantial changes in the shape of the lateral intercellular spaces were observed. From being very tortuous in control situations (Fig. 1 *A*), they often had a much straighter outline at lower osmolarities (Fig. 1 *B*). Figure 3 *C* shows examples of lateral intercellular spaces from a bladder bathed in 64 mOsm for 120 min. It illustrates the variety of lengths the spaces can attain in the same tissue.

Most cavities appeared to be internalized. However, cavities communicating with another cavity (Fig. 4 *A*) or with the lateral intercellular space (Figs. 5 and 6) were frequently encountered. In samples from an epithelium bathed in 64 mOsm for 3 hr

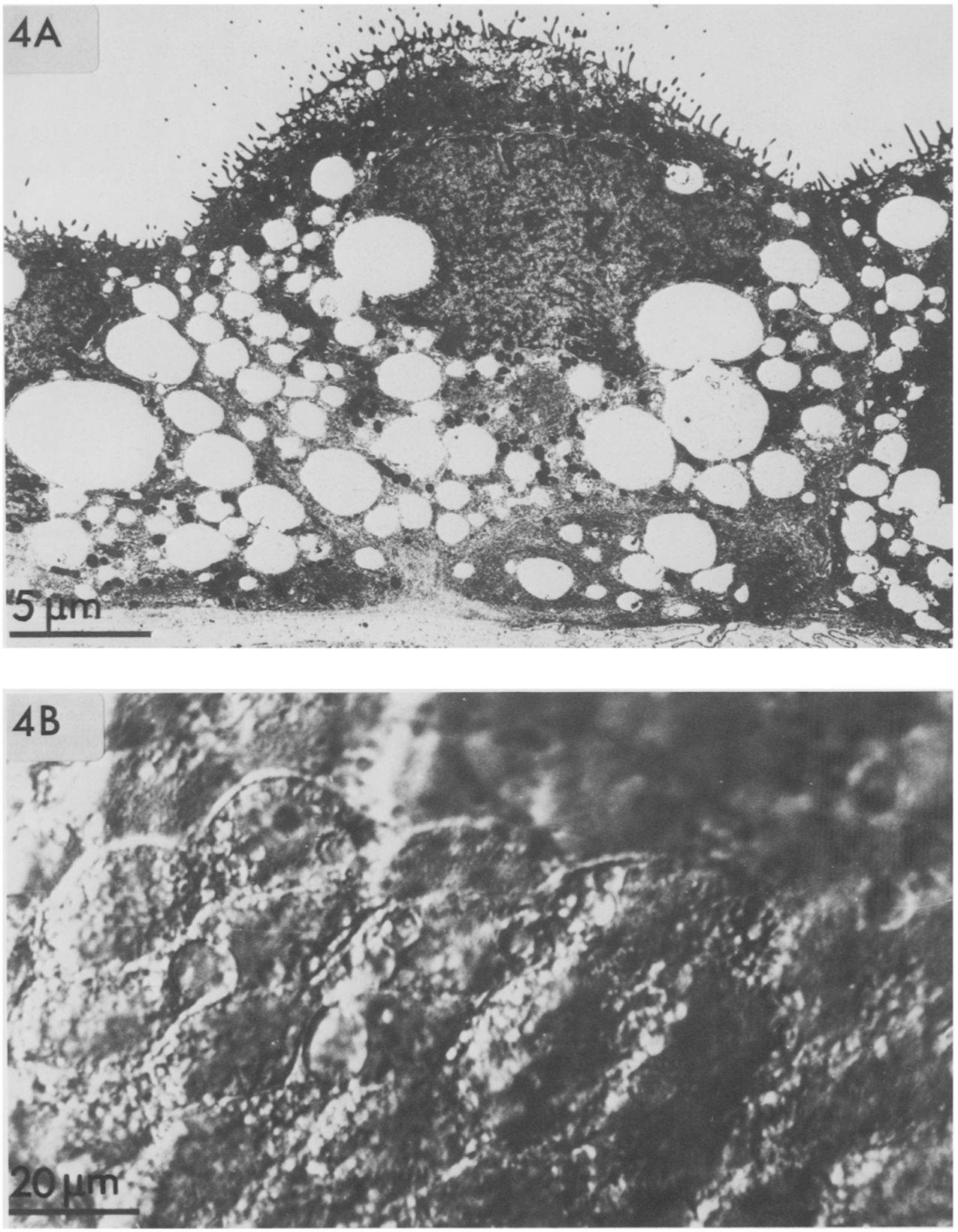

Fig. 4. Micrographs of an epithelium which has been bathed in 117 mOsm for 120 min. *A*. Electron micrograph. *B*. Nomarski micrograph. Both techniques illustrate the variation in cell heights and the heterogeneous population of membrane-bound cavities in the epithelial cytoplasm

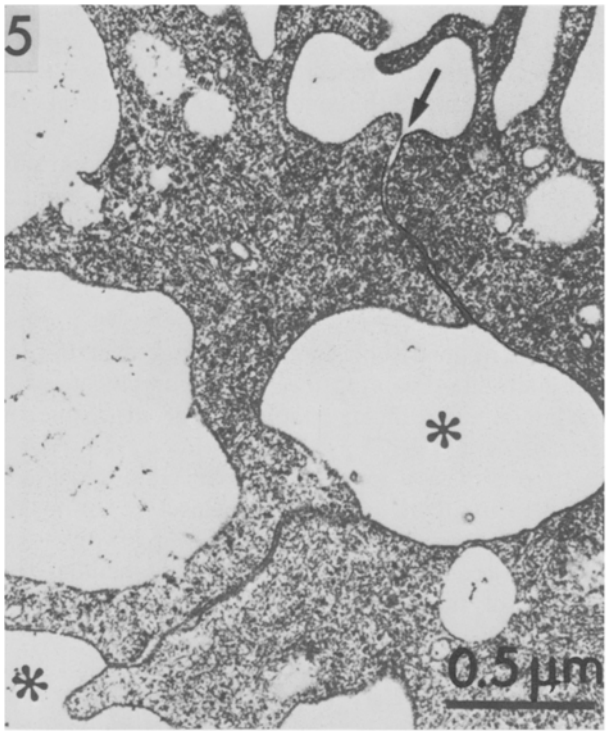

Fig. 5. Gallbladder epithelium incubated for 2 hr in 64 mOsm. Cavities in continuity with lateral intercellular space (L.I.S.) are marked by asterisks. Arrow marks the luminal opening of the intercellular space

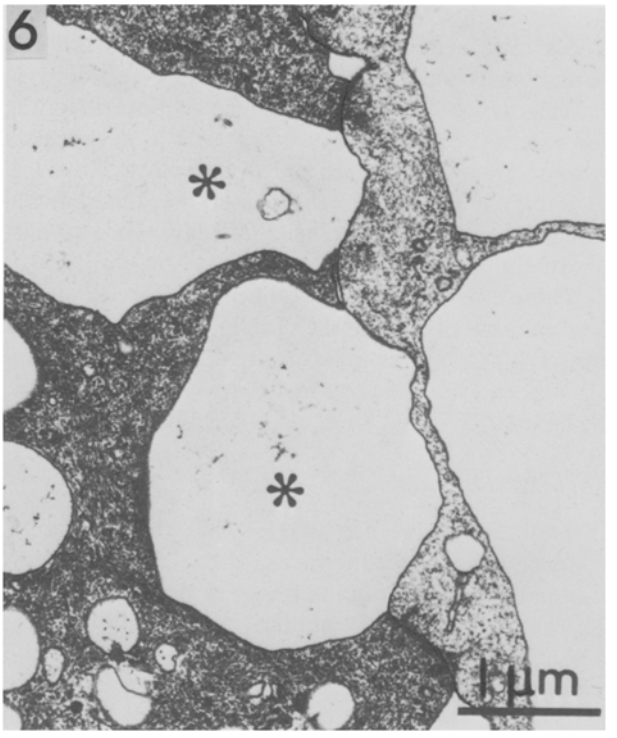

Fig. 6. Gallbladder epithelium incubated for 2 hr in 64 mOsm. Cavities in continuity with L.I.S. are marked by asterisks

we estimated the area of a) membranes of the cavities, b) the lateral cell membrane, and c) the mucosal membrane – all values expressed per unit volume epithelial cytoplasm. Standard stereological methods were employed. In Table 2 these values are compared to those obtained in an epithelium at control conditions. In the tissue incubated at low osmolarity the area of the basolateral membrane was one-third of the area found at normal osmolarities (205 mOsm). The membrane area of the cavities plus the basolateral membrane observed at low osmolarity was, however, similar to the area of the basolateral membrane determined at normal osmolarities.

Reversibility

Some observations were performed when the tissue was returned to the control solution (205 mOsm). After short exposures (< 60 min) to low osmolarities epithelial cells regained their original shape within 10 min. The diameter of the larger cavities decreased at a rate of 0.3 µm min^{-1} but remained spherical (Fig. 2*D*). After about 45–60 min a new steady state was achieved, in which the volume of the cavities was reduced to 5% of their maximal value and the lateral intercellular space had regained its convoluted character.

Effect of an Intact Mesothelium

The effects of keeping the mesothelium intact were a) to enhance the phenomenas observed, i.e. at a given external osmolarity there were at least 50% more cavities if the mesothelium was kept intact as compared to the case where the mesothelium was removed; and b) to cause blister formation. The epithelial cell layer became detached from the underlying connective tissue over circular areas of a diameter of 200–1500 µm, due to fluid accumulation between the epithelia and the connective tissue. This caused semispherical blisters to form. The blister formation was abolished if ouabain was added to the serosal side or if the mesothelium was damaged. Blisters could always be produced artificially by applying a pressure of between 8 and 10 cm H_2O from the serosal side of a tissue from which the mesothelium had been removed.

Discussion

This paper describes the effects of low osmolarities on the morphology of *Necturus* gallbladder epithelium. The changes can be summarized as follows: 1) the cells became bell-shaped, with increased distances from the central part of the mucosal membrane to

the basement membrane; 2) lateral intercellular spaces lost their convoluted character (Fig. 1 *A*, *B*); 3) numerous membrane-bound cavities appeared in the cells (Figs. 3, 4); 4) long-term exposure to the low external osmolarities caused cells to accumulate in some areas and to flatten in other areas (Fig. 3 *A*, *B*, *C*); 5) if the mesothelium and the subepithelial tissue were intact, the epithelial cell-layer formed blisters or domes[1].

These extensive changes did not, however, impair the function of the epithelium in terms of rate of transport and intracellular electrical and chemical potentials as was ascertained previously (Zeuthen, 1981 *a*, *b*).

Origin of the Cavities

Three lines of evidence suggest that the membrane of the cavities is recruited from the serosal cell membrane: 1) cavities originated at the periphery of the cell (Fig. 2); 2) the membranes of the cavities were in some cases found to be continuous with the lateral cell membrane (Figs. 5 and 6); and 3) the stereological analysis showed that the decrease in area of the lateral membrane of the cells was similar to the membrane area of the cavities (Table 2). In other words, the total lateral and basal area of cell membrane measured at normal osmolarities is close to the sum of the membrane area of the cavities and the basolateral membrane measured at low osmolarities. The remaining difference of 25% (Table 2) might be due to the fact that the membrane areas are expressed as area per unit cell volume and that this parameter is not constant when the external osmolarity and Na^+ concentration are varied. Furthermore, cell shrinkage which occurs during preparation for electron microscopy might vary with the osmolarity of the applied solutions. The total effect of these changes in volume, however, cannot be too different at normal and low osmolarities since the areas of the mucosal membrane per unit cell volume were calculated to be the same in the two cases (Table 2). Stereological analysis has been performed on the rabbit gallbladder by Blom and Helander (1977) and the area of the serosal membrane was estimated as 2.9 to 4.1 $\mu m^2/\mu m^3$. These values are slightly larger than the present results for the *Necturus* gallbladder. A direct comparison is difficult because the cells from rabbit gallbladder are comparatively long and thin and because their lateral cell membrane is less folded.

In terms of epithelial transport it is important to know whether the interior of the membrane-bound cavities communicates with the serosal solutions. It was clearly shown that some cavities are part of or open to the lateral intercellular spaces (Figs. 5 and 6). A majority of the cavity profiles, however, appear to be free in the cytoplasm and they may either represent internalized compartments or communicate with the lateral intercellular spaces out of the plane of section.

In preliminary experiments with ruthenium red added to the fixatives the inner aspect of the membrane of some apparently internalized cavities was labeled. Because of low and variable ruthenium red labeling of the basolateral cell membranes a rigid quantification of labeled versus unlabeled cavities was not possible. Some experimental facts suggest that the membranes of the cavities are continuous with the cell membrane; when an apparently internalized cavity grows or shrinks during recovery (Fig. 2), then it retains its spherical shape. Thus membrane material is supplied or taken away during growth or shrinkage. Unless mechanisms of membrane synthesis and catabolism are involved it appears that the membrane of the cavity is continuous with the lateral membrane. Finally, the tissues retain their capacity for salt transport (Zeuthen, 1981 *a*, *b*). If lateral cell membranes were internalized this capacity would decrease.

The finding that the geometry of the lateral intercellular spaces changes markedly when the osmolarity of the external solution changes, should be taken into account when mathematical models of isotonically transporting epithelia are considered. In the standing gradient model proposed by Diamond and Bossert (1967) Lr^{-2}, where L is the length of the lateral intercellular space and r is the width of the space, is an important parameter for the isotonicity of the secretion; obviously this parameter must now be considered a (complicated) function of the osmolarity. The paracellular route can no longer be modeled by a tight junction in series with a lateral space. At low osmolarities, at least, the paracellular route must be modeled by a parallel combination of tight junctions each in series with lateral space, the lengths of which are distributed.

Spring and Hope (1978) reported that the hydrostatic pressure in the lateral intercellular spaces of the gallbladder of *Necturus maculosus* was 3.2 cm H_2O and that this pressure was the final driving force for the secretion. The rate of volume transport at

[1] Induced changes in the geometry of the lateral intercellular spaces have previously been observed. Spring and Hope (1978) observed alterations in the geometry of the spaces when the hydrostatic pressure of the serosal solution was elevated. Maunsbach and Boulpaep (1980) described a marked widening of the lateral intercellular spaces in proximal tubules when these were fixed with a peritubular pressure gradient. Beadle (1934) observed in the light microscope the formation of vacuoles in the intestinal epithelium of *Procerodes ulvae* (formerly named *Gunda ulvae*) after transition of the animal from sea to freshwater. This could be the same phenomenon as the one observed in this study.

reduced external osmolarities is increased proportionally to the degree of dilution (Zeuthen, 1981*b*). At an external osmolarity of 64 mOsm the rate was about three times larger than that observed at normal osmolarities. This suggests that the pressure in the interspaces under these circumstances may be as high as 10 cm H_2O and we suggest that it is this pressure which is responsible for the deformation of the lateral spaces observed at low external osmolarities.

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