

LACK OF CORRELATION BETWEEN TRANSEPITHELIAL TRANSPORT CAPACITY AND PARACELLULAR PATHWAY ULTRASTRUCTURE IN ALCIAN BLUE-TREATED RABBIT GALLBLADDERS

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ABSTRACT

The effects of mucosal application of 1 mg% Alcian blue (a trivalent cationic phthalocyanine dye) on functional and ultrastructural parameters of the isolated rabbit gallbladder have been studied. Apart from minor changes in the shape of the group of central microvilli observed in thin-section electron microscopy and scanning electron microscopy, the major ultrastructural change induced by Alcian blue was an almost complete collapse of intercellular spaces in the region above the tight junctions up to the bases of the marginal microvilli as revealed by thin-section electron microscopy. Freeze-fracture electron microscopy demonstrated a complete disappearance of intramembrane particles of neighboring cell membranes corresponding to the region of interspace collapse. Transepithelial electrical resistance (R_T) increased from 44.5 to 58.7 ohm·cm² upon treatment with Alcian blue. This increase could be well accounted for by the observed structural changes in the paracellular pathway if this pathway determines the low resistance of the rabbit gallbladder epithelium. Despite the increase in R_T , net mucosa-to-serosa fluid transport and the spontaneous mucosa-positive potential difference of 3 mV were unaltered by Alcian blue treatment, supporting the hypothesis that the transepithelial transport mechanism per se is electroneutral. A calculation of the maximal paracellular mucosa-to-serosa waterflow in response to a lateral intercellular space hypertonicity of 20 mosM demonstrates that in the Alcian blue-treated gallbladder the resulting figure is about three orders of magnitude too low to keep up with the unaltered spontaneous transepithelial net fluid transport. It is therefore concluded that the tight junction pathway in rabbit gallbladders does not serve as a route for net fluid transport.

KEY WORDS rabbit gallbladder epithelium ·
transport pathways · fluid absorption ·
ultrastructure · Alcian blue

Isosmotically transporting epithelium such as that

of proximal tubule, gallbladder, and small intestine is characterized by a very low transepithelial electrical resistance compared with the resistances of apical and basolateral cell membranes (2, 12, 13). The low transepithelial resistance seems to be

caused by the presence of a high conductance paracellular shunt pathway localized in the zonulae occludentes (13, 25, 35). This shunt pathway, which probably serves as the major route for passive ion permeation, shows cation-selective properties in gallbladders, indicating the presence of an excess of free negative charges—probably in the zonulae occludentes (1). Addition of small polyvalent cations (such as Ca^{2+} or La^{3+}) causes an increase in epithelial resistance and a decrease in cation selectivity (25, 36).

It has been suggested that a low resistance of the paracellular pathway plays an important role for the function of the so-called “leaky epithelia.” Thus, it has been claimed that a substantial part of net transepithelial salt and water transport takes place between the epithelial cells, thus bypassing the transcellular transport pathway (3, 14, 20, 34). Further, the low transepithelial potential difference observed in these epithelia has been explained either by the shunting effect of the paracellular pathway (33) or as an ion-diffusion potential across the cation-selective tight junctions (24).

In this investigation we examine the role of the paracellular pathway in the process of formation of fluid absorbates in rabbit gallbladders *in vitro*. We have attempted to modify the permeability properties of the paracellular pathway by mucosal treatment of the epithelium with an appropriate concentration of a polyvalent cationic substance, 1 mg% Alcian blue, and to follow the effects on ultrastructural and functional parameters. Because of its molecular size and form the polyvalent cationic dye Alcian blue was chosen; it was expected not to penetrate the zonulae occludentes. The results presented seem to be incompatible with the hypothesis that the paracellular pathway serves as a route for net fluid movement in the process of isosmotic fluid absorption.

MATERIALS AND METHODS

Functional Experiments

White female rabbits weighing 2.5–3.0 kg were sacrificed by a blow on the neck. The gallbladder was removed, rinsed, and prepared for further use as described previously (9, 10).

FLUID TRANSPORT MEASUREMENTS: Fluid transport rates were measured gravimetrically in cannulated, nonverted sac preparations (6, 9). All experiments were carried out at 37°C with identical Ringer's solutions (see below) on both sides unless otherwise stated. Weighing periods of 10 min were used, and luminal (mucosal) content was renewed between weighing periods to ensure constant composition.

SOLUTIONS: The composition of the Ringer's solution in all experiments was (mM): 114.7 Na^+ , 7.0 K^+ , 2.0 Ca^{2+} , 1.2 Mg^{2+} ,

102.0 Cl^- , 17.5 HCO_3^- , 1.2 SO_4^{2-} , 1.2 H_2PO_4^- , 5.0 monoglucamate, and 11.0 glucose. The pH was adjusted to 7.4 by equilibration with 96% O_2 and 4% CO_2 at 37°C. All chemicals were analytical grade. Alcian blue 8GX (mol wt ~1,400) was obtained from I.C.I. United States, Inc. (Wilmington, Del.) and poly-L-lysine (mol wt = 3,500) from Sigma Chemical Co. (St. Louis, Mo.).

MEASUREMENTS OF TRANSEPITHELIAL POTENTIAL DIFFERENCE (PD) AND RESISTANCE (R_T): These measurements were performed with gallbladders cut open and mounted between two half-chambers as described in detail previously (22). Exposed gallbladder surface area was 0.9 cm^2 ; each half-chamber contained 10.0 or 15.0 ml of Ringer's; and temperature was kept at 37°C. All PD and R_T values were corrected for values measured, without the gallbladder mounted. The polarity of streaming potentials (ΔPD 's) arising after addition of 25 mM sucrose to the mucosal solution was used to determine whether gallbladders were cation- or anion-selective, i.e., whether the high conductance pathway contained excess of free negative or positive charges, respectively (36).

STATISTICS: Data are presented as the mean of data from individual experiments or the mean of differences of paired observations in individual experiments \pm SEM. Student's *t* test was used to determine the statistical significance.

Morphological Methods

FIXATION: Sac preparations of gallbladders incubated in the standard Ringer's solution for 1 h (controls) and gallbladders treated with 1 mg% Alcian blue from the luminal side for different periods of time were fixed in 1.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). Fixation was initiated from both sides of the gallbladder wall at the same time. Bladders were cut open along the mesenteric border, and the fixation was continued for 4 h. Pieces of gallbladder wall were cut, rinsed in the above-mentioned buffer, and processed for microscopy.

MICROSCOPY: For scanning microscopy the specimens were postfixed in 2% OsO_4 buffered in 0.1 M cacodylate buffer (pH 7.3) for 2 h, rinsed in the same buffer, and dehydrated in acetone. The tissue was then critical-point dried in CO_2 , mounted on specimen stubs, and coated with carbon and gold. Specimens were observed in a Jeol JSM-U3 scanning electron microscope operated at 15 kV.

The tissue intended for thin-section electron microscopy was postfixed in the osmium tetroxide solution as described above, block-stained in 0.5% aqueous uranyl acetate for 1 h, and dehydrated in ethanol and embedded in Epon. 1- μm -thick sections stained with toluidine blue were examined in the light microscope. Silver-to-grey thin sections were cut from selected areas and, after poststaining with uranyl acetate and lead citrate, examined in a Philips EM 300 microscope.

For freeze-fracturing, small pieces of tissue were prepared after 6 h of fixation described above. After 1 h of infiltration with 25% buffered glycerol at room temperature, the specimens were mounted and oriented on gold disks so that a cleavage perpendicular to the mucosal surface could be achieved. The tissue was then frozen in the liquid phase of partially solidified Freon 22 and stored in liquid nitrogen. Freeze-fracturing followed by carbon-platinum shadowing was performed with a Balzers' apparatus (BAF 301) equipped with an electron beam gun. Specimens were fractured at a stage temperature of -115°C and replicated with platinum-carbon without etching. The thickness of the platinum carbon and carbon coats (~2 and 20 nm) was controlled by a thin film monitor. Replicas were cleaned in bleach and chromic acid.

RESULTS

Functional Observations

Control values of net mucosa-to-serosa fluid transport rates (T_{vol}), transepithelial PD, and R_T obtained ~60 min after preparation were: $T_{vol} = \sim 0.5 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg dryweight}^{-1}$; PD = 2.9 ± 0.2 mV (mucosal side positive); and $R_T = 44.5 \pm 2.6$ ohm·cm². These values are in agreement with previous observations and have been demonstrated to be nearly constant during at least 4 h of incubation (8, 9).

Fig. 1 demonstrates that addition of 1 mg% Alcian blue ($n = 4$) to the mucosal bathing solution did not cause any significant change in mucosa-to-serosa net fluid transport rate.

Fig. 2 shows that also the PD was unaltered by Alcian blue but R_T increased markedly in 12 experiments by 14.2 ± 1.0 ohm·cm² from the control value of 44.5 ± 2.6 ohm·cm² corresponding to a 32% increase; the half-time was ~4 min.

Some preliminary experiments have demonstrated that high mucosal concentrations of Alcian blue (10–100 mg%) show quite different dramatic and time-dependent destructive effects on functional (and structural) parameters. These findings will not be dealt with in the present paper. Serosal application of up to 50 mg% of Alcian blue was without effect on T_{vol} , PD, and R_T .

To test whether the effect of 1 mg% Alcian blue was because of its cationic nature or specific chemical properties, the effect of another polyvalent

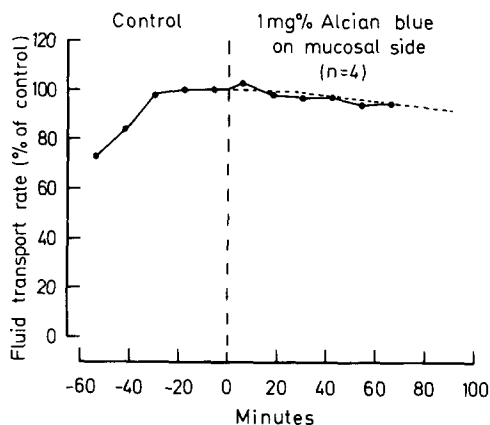


FIGURE 1 Effect of 1 mg% Alcian blue (added to the mucosal medium) on net fluid absorption rate relative to the steady-state control value; average of four experiments. The dashed line represents the spontaneous change in nontreated gallbladders ($n = 30$).

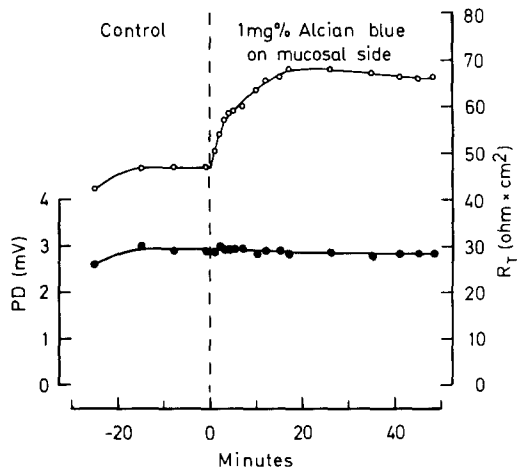


FIGURE 2 Records of an experiment showing the effects on electrical resistance, R_T (open circles), and mucosa-positive transepithelial potential difference, PD (filled circles), after addition of 1 mg% Alcian blue to the medium bathing the mucosal side.

cation, poly-L-lysine (mol wt = 3,500), on PD and R_T was studied using mucosal concentrations which would give a comparable charge concentration in the medium. The results demonstrated that mucosal application of the poly-L-lysine at concentrations between 0.25 and 1 mg% did not alter the PD but caused an increase in R_T of the same magnitude as seen with 1 mg% Alcian blue. Prolonged incubation (30–40 min) with the poly-L-lysine sometimes caused a small decrease in PD and R_T .

Streaming potentials induced by mucosal 25 mM sucrose were positive in the mucosal direction, thus confirming the cation-selective property of the gallbladder epithelium. 1 mg% Alcian blue on the mucosal side ($n = 4$) did not significantly alter streaming PDs; thus, control values and experimental values after 20-min treatment were 2.6 ± 0.1 and 2.4 ± 0.2 mV, respectively.

Morphology

There was no apparent degeneration or major alteration in the morphology of gallbladder epithelium as determined by both transmission and scanning electron microscopy during up to 30 min of incubation in 1 mg% Alcian blue (Figs. 3 and 8). However, when apical portions of gallbladder epithelium incubated in the absence of Alcian blue (Fig. 4) are compared with similar regions of Alcian blue-treated gallbladders (Figs. 5 and 6), two morphological features are different in thin-

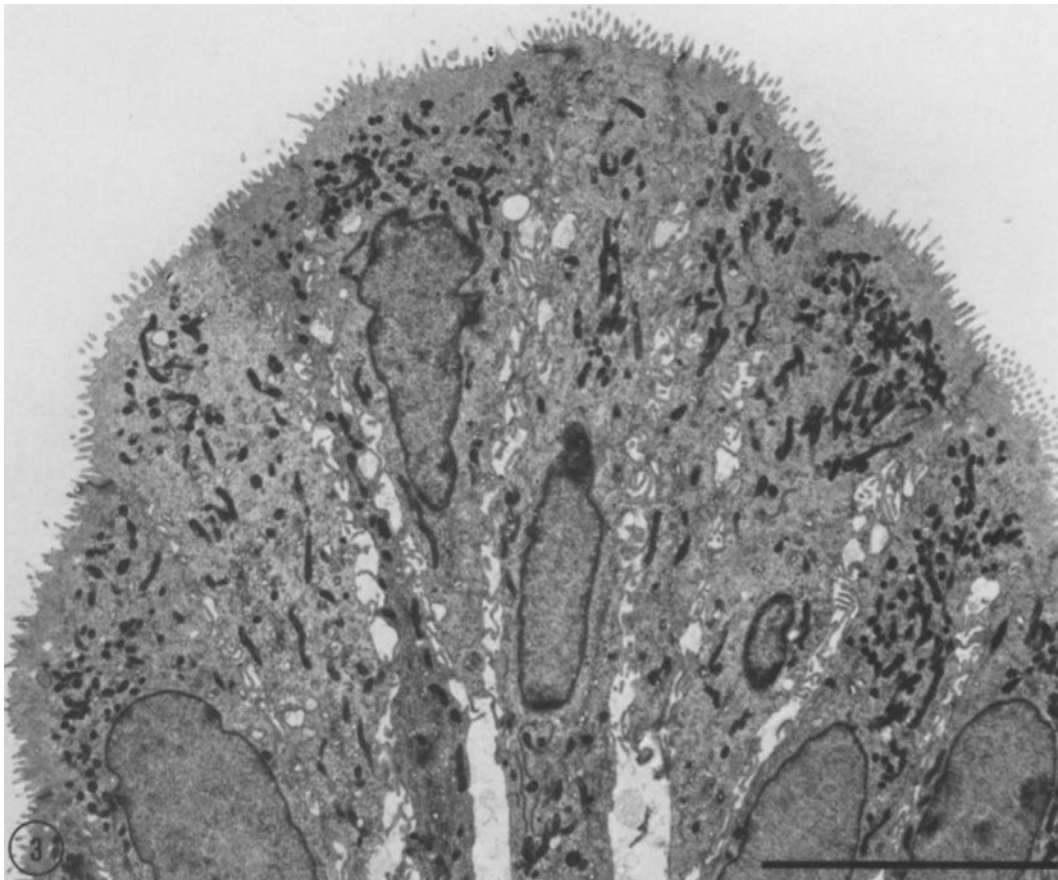


FIGURE 3 Survey picture of a rabbit gallbladder exposed to 1 mg% Alcian blue (in the mucosal solution) for 25 min after an equilibration period of ~1 h in control Ringer's solution. Apart from a slight diversity in microvillus size and shape and an apparently increased junctional depth, the overall appearance of the epithelium is remarkably normal. Bar, 10 μ m. \times 3,900.

sectioned specimens. In gallbladders not exposed to Alcian blue, the junctional region has a normal appearance and the microvilli are uniform in size and shape. In gallbladders exposed to Alcian blue, the junctional region seems to have expanded apically by an apparent fusion of neighboring cell membranes immediately apical to the tight junction zone. These Alcian blue-induced areas of close membrane apposition most often appear as pentalaminar structures ~13 nm thick, although in some cases contact areas are trilaminar, appearing as a single unit membrane shared by two adjacent epithelial cells.

Scanning electron microscopy of control and Alcian blue-exposed gallbladders reveals minor differences in the configuration of the microvilli. In the control situation the densely packed micro-

villi have a uniform appearance (Fig. 7), whereas the Alcian blue treatment apparently causes some changes in the group of centrally situated microvilli which appear longer and less densely packed (Fig. 8).

In replicas of freeze-fractured gallbladder incubated for 1.5 h *in vitro* in a medium without Alcian blue, the tight junction network appears very variable with respect to both junctional depth and strand number, as described previously (5). Numerous intramembrane particles are randomly distributed at both luminal and basolateral cell membranes (Fig. 9). After incubation in 1 mg% Alcian blue, most epithelial cells exhibit a distinct particle-poor region in the P face of apical parajunctional cell membranes. The particle-poor smooth membrane region extends along the cell

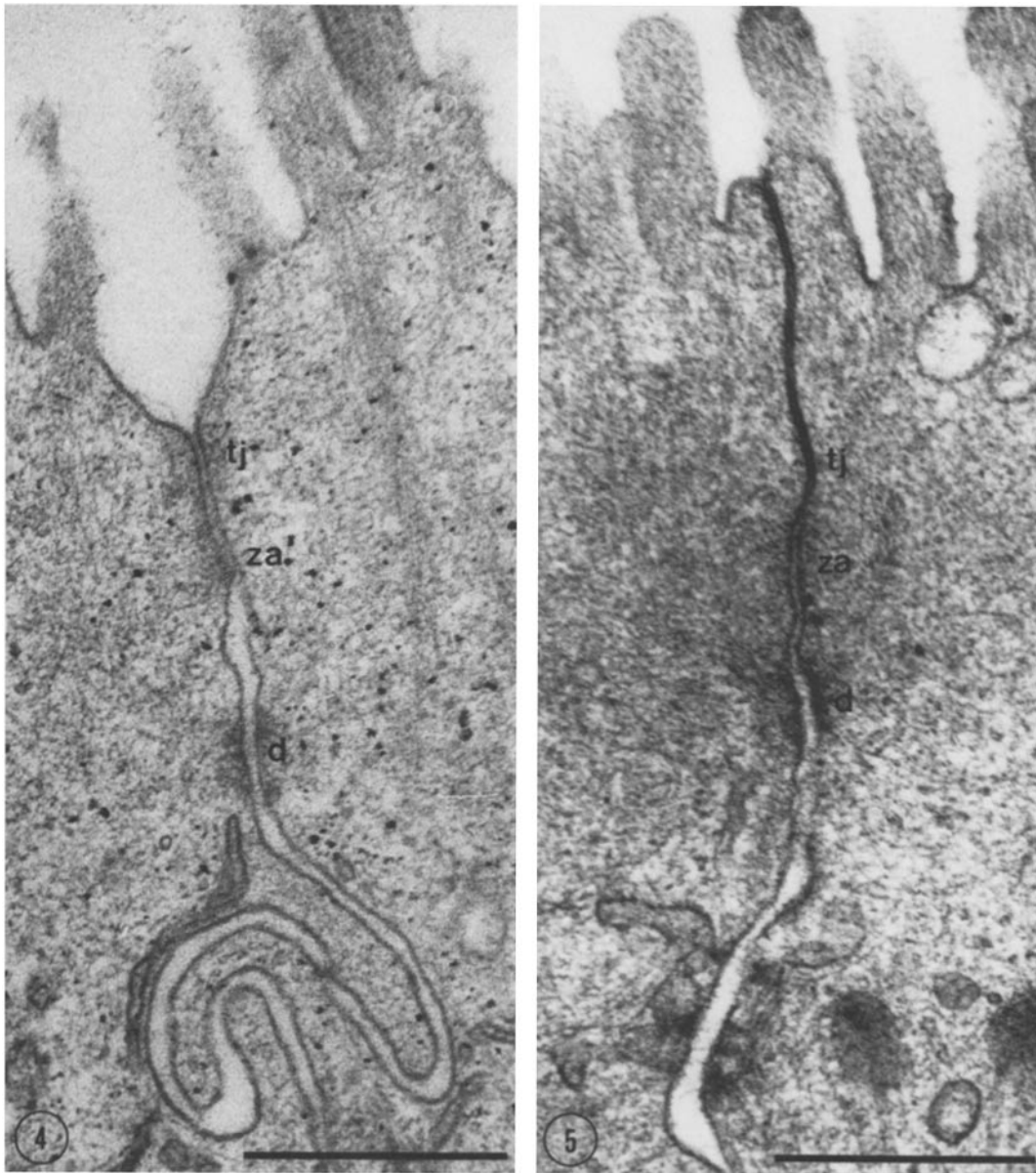


FIGURE 4 The junctional region of a gallbladder incubated in the absence of Alcian blue. Note the normal appearance of the tight junction (*tj*). *za*, Zonula adherens; *d*, desmosome. Bar, 0.5 μm . $\times 71,000$.

FIGURE 5 The junctional region of a gallbladder exposed to 1 mg% Alcian blue from the mucosal side for 25 min. In transmission electron microscopy the apical junctional region seems to have expanded considerably, possibly by a fusion of adjacent membranes immediately apical to the original tight junction network (*tj*). *za*, Zonula adherens; *d*, desmosome. Bar, 0.5 μm . $\times 71,000$.

perimeter in a beltlike fashion from the most apical strand of the tight junction network towards the base of the marginal microvilli, where characteristic patches or aggregates of intramembrane par-

ticle are observed (Fig. 10). Also, E-face particles are few in number in this region. In the area of contact, the fracture plane often shifts from the interior of one parajunctional apical membrane to

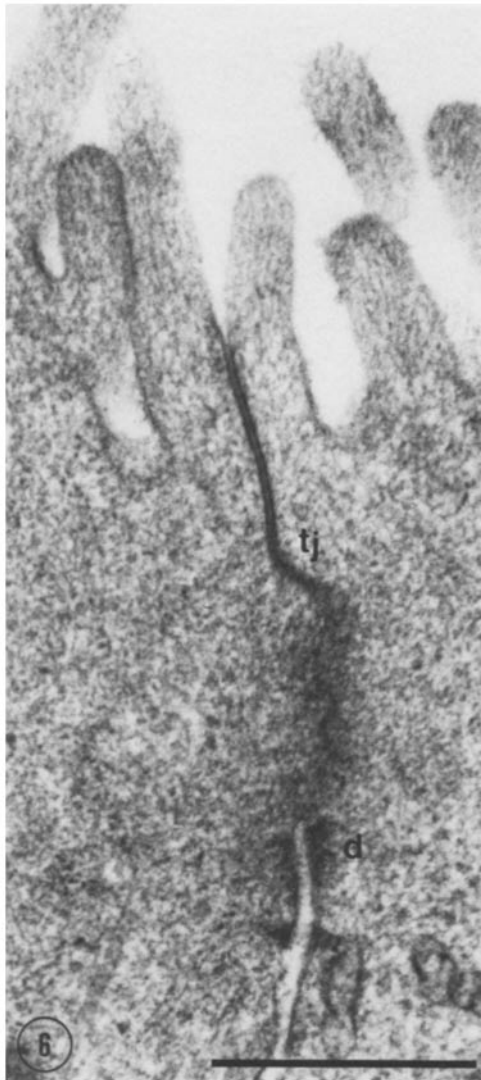


FIGURE 6 This picture is obtained from a gallbladder exposed to Alcian blue similar to the one shown in Fig. 5. Note the apparent fusion of juxtaposed apical cell membranes luminal to the original tight junction. The basal parts of adjacent microvillus membranes apparently contribute to the fusion. *d*, Desmosome; *tj*, tight junction. Bar, 0.5 μm . $\times 71,000$.

the interior of its closely apposed neighbor, exposing the P face of one membrane and the E face of the other (Fig. 10). The tight junction network of Alcian blue-treated gallbladder epithelium is similar to that of control gallbladders.

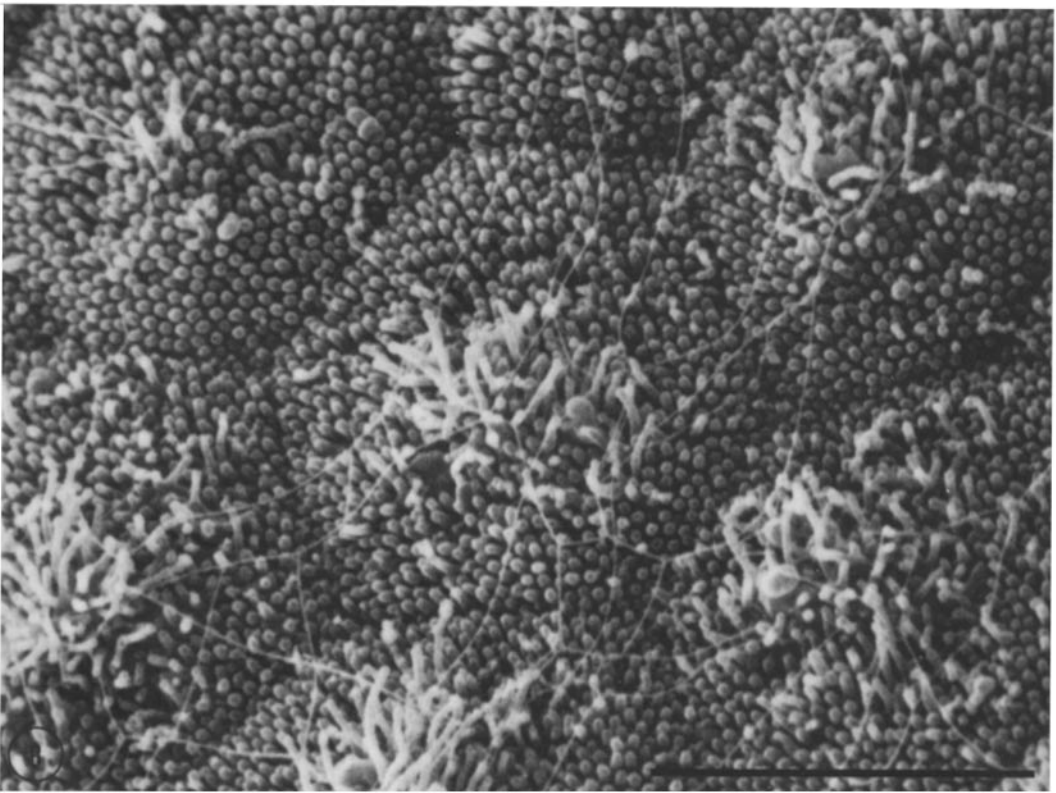
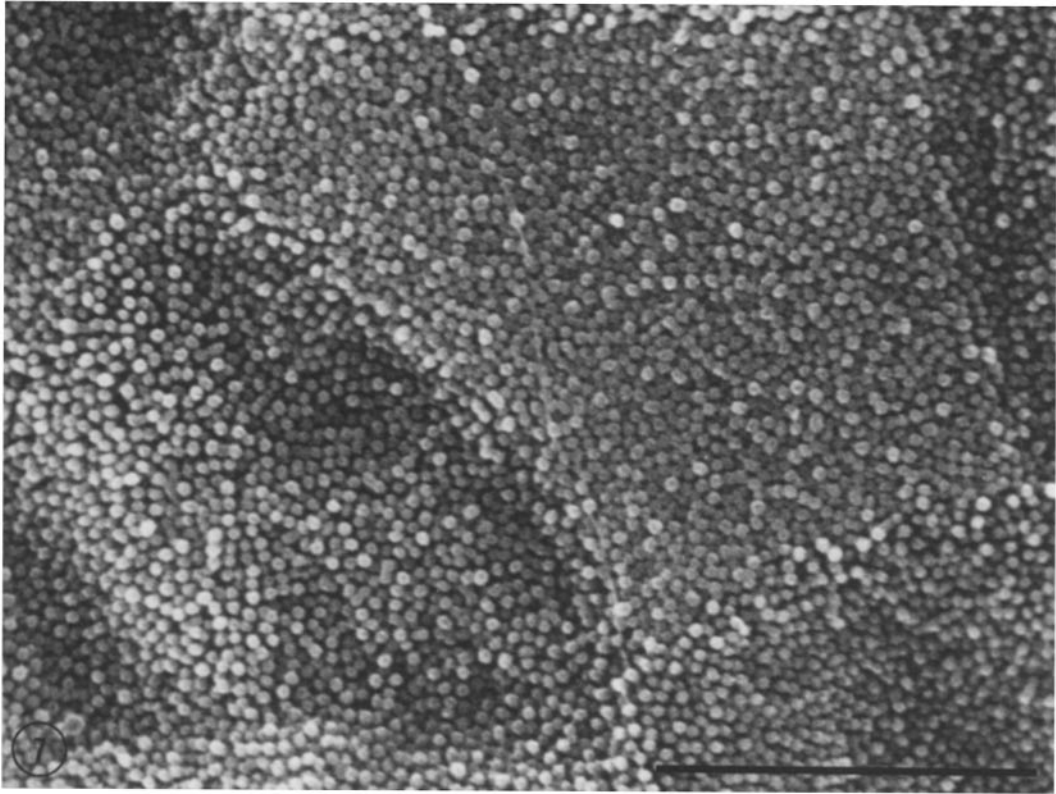
DISCUSSION

The present investigation shows that mucosal application of 1 mg% of the polyvalent cationic dye Alcian blue caused functional and structural changes in the rabbit gallbladder epithelium compatible with an increased hindrance to passive paracellular movement of ions and water. Despite these alterations, net fluid absorption did not change, indicating that the paracellular tight junction pathway does not serve as a significant pathway for vectorial net fluid transport in the process of isotonic fluid absorption.

Apart from the minor alterations in the shape of the group of central microvilli, the structural changes in the rabbit gallbladder induced by mucosal 1 mg% Alcian blue seem to be confined to that part of the paracellular pathway that is localized above the zonulae occludentes. In thin-section electron microscopy, an almost complete collapse of the intercellular space between the tight junctions and the bases of the marginal microvilli was observed (Figs. 5 and 6). Simultaneously, the closely apposed cell membranes of neighboring cells exhibited various degrees of curling and bulging. On the basis of thin-section electron microscopy, Quinton and Philpott (31) described the occurrence of "fused membranes" in poly-L-lysine-treated pieces of rabbit gallbladder. So far, these findings have been difficult to interpret on account of the fixation technique, which involved only osmium tetroxide, and the lack of uranyl block staining. The present data support the view that such a fusion actually may occur after treatment with polyvalent cations. The fact that very different cationic substances cause similar functional changes (increase in R_T without change in PD) would suggest that their mechanism of action

FIGURE 7 Scanning electron micrograph of the luminal surface of a gallbladder which was not exposed to Alcian blue, but fixed after an equilibration period of 1.5 h in control Ringer's solution. The densely packed microvilli have a uniform appearance. The borderline between adjacent cells is hardly visible. Bar, 5 μm . $\times 10,000$.

FIGURE 8 Scanning electron micrograph of the luminal surface of a gallbladder exposed to Alcian blue. Note the diversity of the microvilli. The central group seems to be elongated in comparison with the more peripherally situated ones. The fusion of adjacent cell membranes apical to the tight junction network may well be responsible for the prominent cell borders seen in this preparation. Bar, 5 μm . $\times 10,000$.



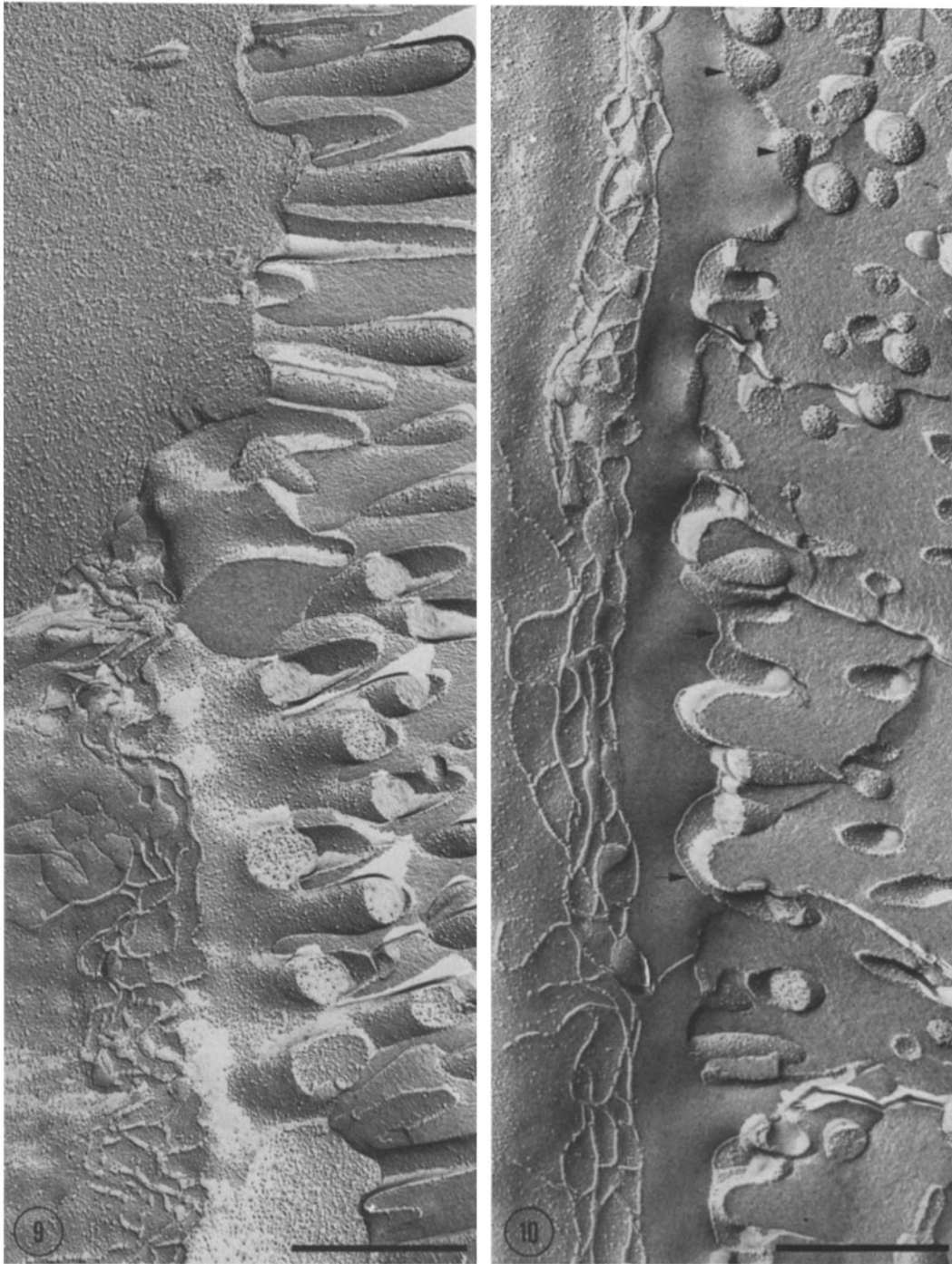


FIGURE 9 Freeze-fracture replica of a control gallbladder. In some places the tight junction is quite deep and composed of several strands. However, in other areas the junction consists of only two or three strands. Numerous intramembrane particles are randomly distributed at both luminal and basolateral membranes. Bar, 0.5 μm . \times 45,000.

FIGURE 10 The smooth particle-free zone apical to the tight junction network is exposed in this replica obtained from an Alcian blue-treated gallbladder. Note the shift of the fracture plane (arrows) which exposes P and E faces of neighboring cell membranes in intimate contact. Patches of intramembrane particles are seen at the base of some microvilli (arrowheads). Bar, 0.5 μm . \times 45,000.

is the same, namely a neutralization of cell surface negative charges at the cell margins by electrostatic interaction (28). Freeze-fracture electron microscopy of 1 mg% Alcian blue-treated gallbladders (cf. Fig. 10) showed a disappearance of intramembrane particles from the apical membrane region extending lumenally from the most apical strand of the tight junction network. Such redistribution and aggregation may stem from a neutralization of surface negative charges (29, 30). Because integral membrane protein particles are negatively charged through external attachment of acidic polysaccharides (see reviews by Glick [17] and Luft [23]), lateral movement and aggregation of particles induced by polyvalent cationic substances may be explained by a reduction in electrostatic repulsive forces between the particles (15). Similarly, the collapse of the interspaces above the zonulae occludentes observed in the present study may be caused by the lack of repulsive forces between the membranes and/or lack of surface coat material.

Application of 10 mg% poly-L-lysines to rabbit gallbladder pieces for 30 min caused a disappearance of microvilli and an extensive swelling of the apical part of epithelial cells (31), indicating an increase in cell membrane permeability. Similarly, evidence for an increase in cell membrane permeability and cell swelling induced by high concentrations of polyvalent cationic polymers such as poly-L-lysines has been presented in other cell systems (16, 18, 21, 26, 27, 32). In rabbit gallbladders, Alcian blue at concentrations of at least 10 mg% seems to cause a similar type of permeability and structural changes (O. Frederiksen, K. Møllgård, and J. Rostgaard, unpublished observations). However, it should be emphasized that the low concentration of Alcian blue (1 mg%) used in the present study did not produce such changes (see, for example, Figs. 3 and 8).

Despite the structural changes in the region above the zonulae occludentes, no changes were observed in the tight junction network per se as revealed by thin section and freeze-fracture (cf. Figs. 9 and 10). Also, no changes were observed in the basolateral cell membranes below the tight junctions, probably indicating that Alcian blue did not penetrate into the lateral intercellular spaces.

The sustained increase in R_T upon mucosal treatment with 1 mg% Alcian blue associated with the above-described ultrastructural changes seems to support previous conclusions that the paracellular pathway contributes to the low R_T of the

epithelium (13). Although the electron micrographs seem to show complete collapse of intercellular spaces above the tight junctions, we cannot exclude the possibility of a more or less well-defined space between the neighboring cell membranes in this region. If, for example, we assume a fluid-containing space with a width of say 5 Å, an average depth of the collapsed zone of 200 nm (cf. Figs. 5, 6, and 10), a cell diameter of 10 μm, and a linear resistance of the fluid in the narrow space of 100 ohm·cm, then we arrive at a value of ~15 ohm·cm² for the increase in R_T caused by the space collapse. This value is equal to the actually observed increase in R_T of 14.2 ohm·cm². Thus, it is likely that the tight junction route is the only low resistance pathway in the rabbit gallbladder epithelium.

Addition of Ca²⁺ (36) or La³⁺ (25) in millimolar concentrations to the bathing medium of isolated gallbladder preparations causes an increase in R_T and a decrease in cation permeability. The mechanism seems to be a neutralization of free negative charges in the rate-limiting barrier of the paracellular pathway, i.e., in the tight junction. The present study demonstrates that 1 mg% Alcian blue (with about three positive charges per molecule) increases R_T without altering the ionic properties of the tight junction pathway as visualized by the lack of change in streaming potentials induced by mucosal 25 mM sucrose. The explanation could be either that Alcian blue, because of the molecular size (Stoke's radius 8 Å), does not reach the ionic groups in the tight junction pathway, or that the concentration of Alcian blue ($7 \cdot 10^{-6}$ M) is too low to neutralize the ionic groups in this pathway. In any case, the lack of changes in streaming potentials supports the conclusion that the increase in R_T induced by Alcian blue is caused by the collapse of interspaces above the tight junction level rather than by membrane charge changes.

It has been held that the high conductance of the paracellular pathway would tend to short-circuit PDs resulting from an electrogenic transepithelial ion transport mechanism (33). The present study demonstrates that 1 mg% Alcian blue increases R_T —and thus the resistance of the paracellular pathway—without affecting the mucosapositional PD of ~3 mV (cf. Fig. 2). Therefore, the transepithelial PD of the rabbit gallbladder does not seem to be determined by any shunting effect in the paracellular pathway. Rather, the results support the previous conclusion (8, 19, 24) that the transepithelial transport mechanism in this epithe-

lium is virtually neutral.

Because the tight junctions in low resistance epithelia seem to comprise the pathway through which most of the passive diffusion of many smaller solutes take place, it has been postulated that this pathway may also serve as the route for a significant part of net transepithelial water transport (3, 14, 34). Recently, it has been suggested that all transepithelial net water flow in the *Necturus* gallbladder bypasses the epithelial cells (20). The driving force for net water flow is held to be an osmotic concentration difference across the tight junction set up by active ion- or salt-transport across the lateral cell membranes from the cytoplasm into the lateral intercellular spaces (7). However, the present results seem to exclude the possibility of a paracellular contribution to net transepithelial water flow in the rabbit gallbladder. An almost complete collapse of the short interspaces luminal to the zonulae occludentes was observed between nearly all cells when 1 mg% Alcian blue was added to the luminal side. Assuming a theoretical cleft of 5 Å left between the cells, a height of the collapsed zone of 200 nm, and a cell diameter of 10 μm, it is possible to calculate (using the formula given by Civan [4]) the paracellular water flow per cm² of epithelial surface assuming a lateral space hypertonicity of 20 mosM (24). The resulting figure is $4 \cdot 10^{-3} \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}$, i.e., almost zero as compared with normal net fluid transport rates of $\sim 2 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}$. However, net fluid transport rate remained unchanged upon addition of 1 mg% Alcian blue. Thus, it seems unlikely that significant amounts of water passing between the cells contribute to the formation of isotonic fluid absorbate. Contrary to the situation in proximal tubules (14), no possibility would seem to exist in the gallbladder for a contribution from paracellular solvent drag to net salt transport. Recent results (O. Frederiksen, manuscript in preparation) have furthermore indicated that the transepithelial PD does not play any significant role as a driving force for a paracellular electrodiffusional contribution to net Na⁺ absorption. In conclusion, the present results raise serious doubt about the general validity of the concept that a paracellular water flow contributes significantly to isosmotic net fluid transfer.

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