

FRACTURE FACES OF OSMOTICALLY DISRUPTED ZONULAE OCCLUDENTES

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ABSTRACT

Exposing the mucosal epithelium of the toad urinary bladder to 240 mM urea in Ringer's solution is known to cause a dramatic increase in the permeability of the zonulae occludentes and the appearance of distended, bubble-like compartments within these junctions. Examination of such osmotically disrupted junctions with the freeze-fracture technique reveals that these bubbles result from a distention of the compartments existing within the meshwork of interconnecting fibrils characteristic of the zonulae occludentes in this epithelium. Frequent discontinuities in the meshwork of fibrils are also found after osmotic disruption of the junction. These observations indicate the essential role of these fibrils in maintaining the characteristic properties of the zonula occludens as a site of cell-to-cell attachment and as a permeability seal.

INTRODUCTION

Recent investigations of the zonula occludens (tight junction) indicate that this structure may in some tissues constitute part of an extracellular shunt pathway for the transepithelial movement of certain small solutes (15, 20, 26) although preventing the movement of other, larger, solutes (3, 9, 13, 16). Since the permeability of the shunt pathway appears to have considerable influence on the physiological properties of epithelia (1, 2, 11, 12), conditions which alter the permeability of the junction are of interest because they may offer some insight into the mechanisms which regulate epithelial transport.

In vitro exposure of the toad bladder or frog skin to an osmotic gradient by the addition of urea or other solutes to the mucosal bathing solution has been found to dramatically alter their permeability properties (21, 23) and this has been found to be correlated with changes in the permeability of

the zonulae occludentes of the epithelial cells (8, 22, 24). In the toad bladder such an exposure has been found to produce a deformation of the junction which results in a structure with a characteristic bubble or blister-like appearance in thin sections (6, 7, 25). It has been proposed that the osmotic gradient leads to an accumulation of solute and water within limited compartments of the junction and that the resulting hydrostatic pressure causes an increase in the permeability of the junction (7, 25).

Examination of the zonula occludens with the freeze-fracture technique has established the presence of a meshwork of intramembranous material which delineates a series of compartments within the region of the junction (4, 14, 19). Certain evidence suggests that the fibrils of this meshwork constitute the sealing component of the junction (5, 10, 13) and recent analysis indicates that the

junction fractures as might be expected if there were only a single set of fibrils which is shared by a fusion of the adjacent cell membranes at these points of attachment (18, 24).

We have examined with the freeze-fracture technique junctions from the toad bladder epithelium which have been osmotically disrupted in order to establish whether or not the distended compartments produced under these conditions were related to the compartments of the meshwork seen in normal junctions. In addition, we wished to determine whether conditions known to radically increase the permeability of the junction necessarily disrupted the continuity of the fibrils of the meshwork.

MATERIALS AND METHODS

Urinary bladders from pithed female toads *Bufo marinus* were mounted as sacs tied to tubes as previously described (25) and bathed in an aerated Ringer's solution consisting of 111 mM NaCl, 3.5 mM KCl, 2.5 mM NaHCO₃, and 1 mM CaCl₂. The tonicity of the Ringer's solution was 220 mosmol/kg H₂O and the pH was 7.6–8.2.

The epithelium was exposed to an osmotic gradient by placing a solution of 240 mM urea in Ringer's on the mucosal (urinary) side. After exposures of 1-, 10-, or 60-min duration, the tissue was fixed in 2.5% glutaraldehyde in 0.09 M cacodylate buffer at pH 7.4 for 15 min. The tissue was then washed and stored in 0.1 M cacodylate buffer.

Before freezing, tissue was soaked in 25% glycerol in 0.1 M cacodylate. Tissue was frozen in liquid Freon 22 cooled by liquid nitrogen and fractured in a Balzers freeze-etch unit BA 360M (Balzers High Vacuum, Liechtenstein) at –104°C. Replicas were examined with a Philips 200 electron microscope at 60 kV.

OBSERVATIONS

Freeze fracture of the zonula occludens in the toad urinary bladder reveals a band of five to ten interconnecting ridges (5) on the fracture face A (inner membrane face) and complementary interconnecting grooves on the fracture face B (outer membrane face). After only 1 min of exposure to a solution of 240 mM urea in Ringer's on the mucosal side, there are no detectable alterations in the appearance of the junction after freeze fracture (Fig. 1).

However, in tissue examined after a 10-min exposure to the osmotic gradient there are consistent and dramatic alterations in the structure of the zonulae occludentes. Most striking are concave valleys (*B*₁, *B*₂, *B*₃, *B*₄, Fig. 2) within the meshwork

of ridges on face A. These distended regions of membrane correspond in size and position to the bubble-like profiles seen in thin sections (*B*₁, *B*₂, *B*₃, *B*₄, Fig. 3) after similar exposure to osmotic gradients. The junctions of all cells in the epithelium are focally affected with some regions of the junction having an apparently normal meshwork of ridges (*R*, Fig. 2). The bubble chambers occur in a variety of sizes ranging from 0.15 up to 1.0 μm in diameter. At higher magnification it can be seen that the concave valleys seen in face A freeze fractures are always surrounded by a ridge (*R*, Fig. 4) and thus that the bubbles reflect a distention and enlargement of compartments existing between adjacent fibrils of the meshwork. It is apparent that there are now not nearly as many compartments delineated by ridges as in a normal junctional meshwork. Also, discontinuities in the ridges are now found frequently (*D*, Fig. 4).

It has not been quantitatively established whether a reduction has occurred in the total amount of material represented in the form of ridges. Many more intramembranous particles (*P*, Fig. 4) are now found within the meshwork of the junction (compare with Fig. 1). It is also observed that more particles are on the membrane face of large bubbles (*B*₁, Fig. 4) than on that of small bubbles (*B*₂, *B*₃, Fig. 4). Although some of these particles appear to be short segments of ridge (*R*_s, Fig. 4), other particles now found within the meshwork are indistinguishable in size from those particles normally observed on fracture face A of the adjacent luminal and lateral membranes of these cells.

Fracture face B of osmotically disrupted junctions is complementary to face A with the bubbles appearing as convex distentions of the membrane (*B*₁, *B*₂, *B*₃, *B*₄, Fig. 5) surrounded by grooves that are also frequently discontinuous (*D*, Fig. 5). There is also no indication that the discontinuities in the meshwork on face A have resulted from a transfer of material to face B. This evidence suggests that the discontinuities observed do indeed represent discontinuities in the junction and are not due to loss of material during fracturing or to changes in the fracturing properties of the junction. After an hour of exposure to the osmotic gradient an even more complete disruption of the junction is observed. Although some bubbles remain (*B*₁, *B*₂, Fig. 6) there are now also regions where there are only short, disconnected segments of ridge (*R*, Fig. 6). Such regions may correspond

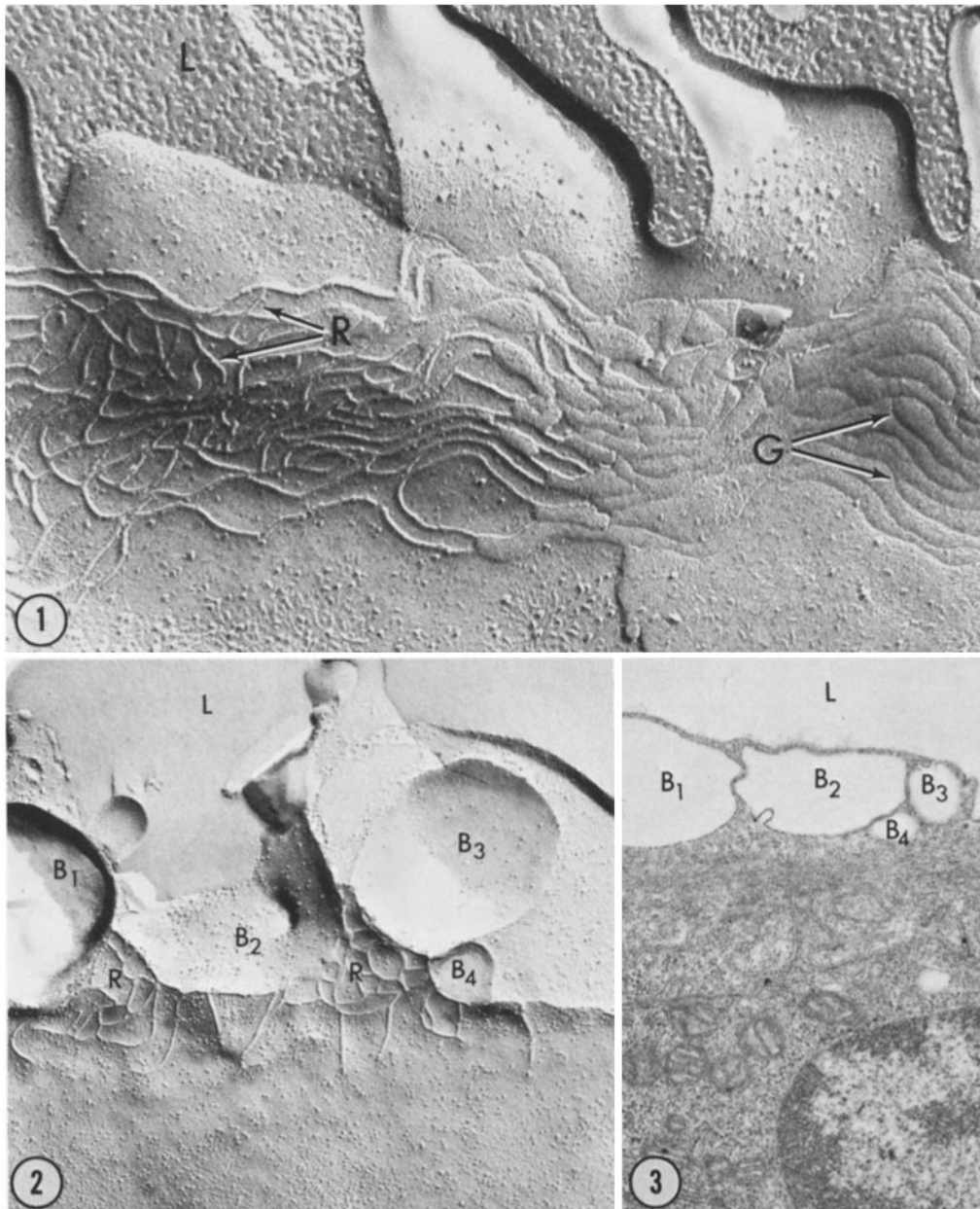


FIGURE 1 Freeze-fracture of toad urinary bladder after only 1-min exposure to 240 mM urea in Ringer's on the mucosal side. The appearance of the meshwork of ridges (*R*) on fracture face A and the complementary grooves (*G*) on fracture face B do not differ from that in control preparations. The lumen (*L*) of the epithelium (urinary side) is at the top of the figure. $\times 90,000$.

FIGURE 2 Fracture face A of the zonula occludens after 10-min exposure to 240 mM urea in Ringer's on the mucosal side. Concave valleys of various sizes (*B*₁, *B*₂, *B*₃, *B*₄) are found within the meshwork. Some regions of the junction consist of an apparently normal meshwork of ridges (*R*). The lumen (*L*) is at the top of the figure. $\times 49,000$.

FIGURE 3 Micrograph of thin-sectioned epithelium after an exposure to urea similar to that of Fig. 2. The section runs parallel to the plane of the junction and demonstrates the series of bubble-like chambers (*B*₁, *B*₂, *B*₃, *B*₄) which are found in the region of the junction under these conditions. The lumen (*L*) is at the top of the figure. $\times 22,000$.

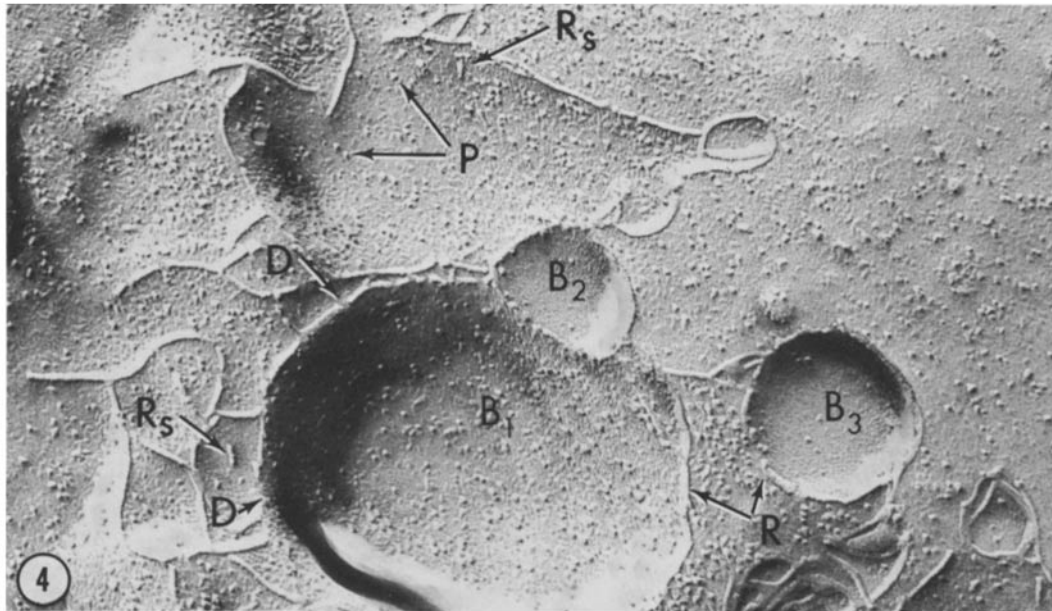


FIGURE 4 Higher magnification micrograph of fracture face A of the zonula occludens after exposure to urea as in Fig. 2. Concave valleys (B_1 , B_2 , B_3) are surrounded by ridges (R) with discontinuities (D). Intramembranous particles (P) are now much more frequent within the meshwork of the junction. Some of these particles appear to be short segments of ridge (R_s). Oriented with the most luminal side of the junction on the right-hand side of the figure. $\times 90,000$.

to portions of the junction where an altered permeability to barium and sulfate ions is found but a bubble is not evident (25).

DISCUSSION

The osmotic disruption of the zonulae occludentes of the toad bladder has been extensively studied using physiological techniques (21), thin-section electron microscopy (6, 7, 24), and ultrastructural tracers (25). The occurrence and extent of permeability changes have been found to correlate closely with the frequency with which regions of the zonulae occludentes are split into a series of widely distended chambers (7). Although we have examined these junctions with the freeze-fracture technique after 1-, 10-, and 60-min exposure to an osmotic gradient, we have not attempted to establish the presence of distinct morphological stages in the disruption of the junction. Because of the focal nature of the changes and the variability in the size of the bubble-like chambers it may prove to be difficult to establish the sequence of structural events. However, the observations presented here do demonstrate that the bubble-like chambers produced are the result of distention of the com-

partments which exist normally between the interconnecting fibrils of the zonulae occludentes. Since the cells remain adherent at those points where the fibrils are found, this would appear to be most dramatic evidence indicating that it is the fibrils rather than the regions between the fibrils which are involved in the cell-to-cell attachment function characteristic of the zonula occludens.

The disruption of the junction is not simply an effect of urea since other solutes such as NaCl, mannitol, and sucrose can produce the same results (7, 25). In addition, it has been found that an osmotic gradient is required for the effect and that both physiological and ultrastructural consequences are nearly completely reversible within 30 min after removal of the osmotic gradient (7). Because of the essential role of an osmotic gradient in the phenomenon it has been proposed (7, 25) that bubbles occur because of solute movement into the compartments of the junction and the subsequent movement of water due to the osmotic gradient. The resulting rise in hydrostatic pressure within limited compartments might reasonably cause distention of the compartments and perhaps exert sufficient force to rupture the junctional

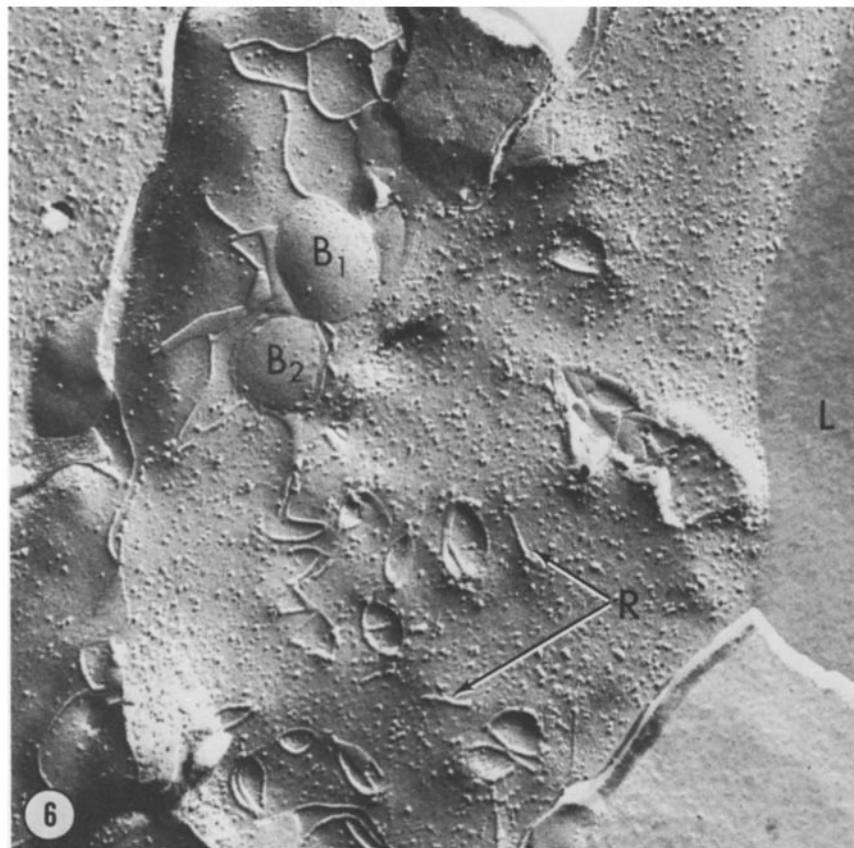
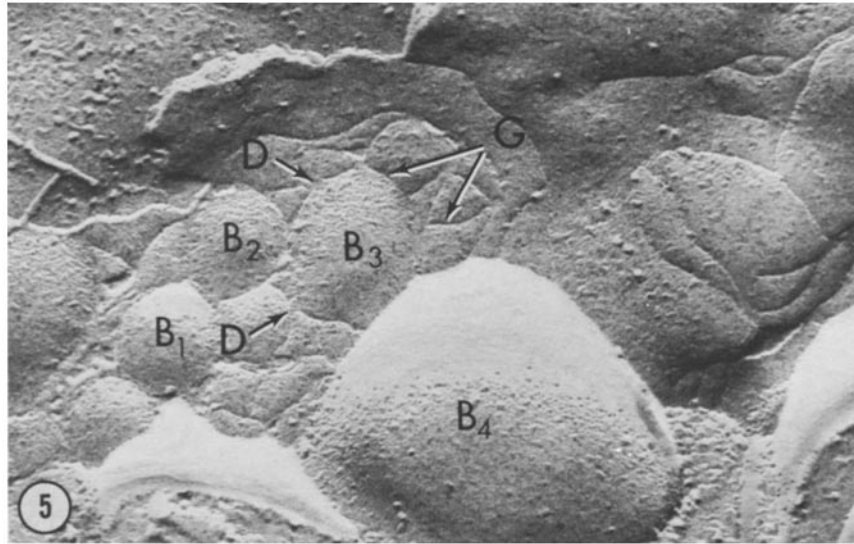


FIGURE 5 Fracture face B of the zonula occludens after urea exposure as in Figs. 2 and 4. Convex distensions of the membrane (B_1 , B_2 , B_3 , B_4) are surrounded by grooves (G) with discontinuities (D). Oriented with the most luminal side of the junction at the bottom of the figure. $\times 130,000$.

FIGURE 6 Freeze-fracture of toad urinary bladder after 1-h exposure to 240 mM urea in Ringer's on the mucosal side. Regions with bubbles remain (B_1 , B_2) but there are now also regions with only short, disconnected segments of ridge (R). Oriented with the lumen (L) on the right-hand side of the figure. $\times 80,000$.

barriers. Although the present observations are completely consistent with this proposed mechanism, it is not possible to exclude the possibility that osmotic gradients alter the permeability of the junction by an unknown mechanism and that the increased movement of solute and water through the junction causes an incidental distention of the junctional compartments in the toad bladder epithelium.

Since the bubble chambers are much larger than the compartments found in the normal meshwork, the bubbles might reasonably arise from either a stretching of the preexisting compartments or breaking of the interconnections between fibrils, resulting in larger compartments. Since there are fewer compartments observed after bubbles have formed, it is likely that the latter occurs although the distended appearance of the bubbles suggests that a certain amount of stretching may also take place.

The discontinuities which are found in the meshwork might result if distention of compartments pulls the fibrils apart at new or preexisting breaks. Alternatively the material may have been removed from the freeze-fracture plane or the osmotic disruption may have altered the character of the material so that it cannot be distinguished from intramembranous particles. Although the increased number of intramembranous particles observed within disrupted areas of the junction may represent dispersed material from the fibrils, some may also be intramembranous particles from outside the junction which have moved into this region after the continuity of the fibrils was broken. Either explanation could be consistent with the observation that there are more particles found within the large bubbles than the small ones. In any case, it is difficult to account for the changes observed in the region of the junction without assuming a fluid membrane and some mobility of intramembranous material such as proposed by Singer and Nicolson (17).

The observation of discontinuities in the meshwork under conditions known to greatly increase the permeability of the junction (25) is striking evidence of the essential role of the fibrils in maintaining the characteristic properties of the zonula occludens as a permeability seal. In terms of the single fibril model (24) such discontinuities may indicate regions where fusion of the adjacent membranes is incomplete. When such discontinuities are large and frequent in number, it is apparent that solutes might detour around the remaining

regions of membrane fusion and thus cross the epithelium via a completely extracellular pathway. Thus, the size and frequency of discontinuities in the fibrils as well as the number of fibrils (5) appear to be important determinants of the permeability of the junction.

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