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## SUBSTRUCTURE OF THE GLOMERULAR SLIT DIAPHRAGM IN FREEZE-FRACTURED NORMAL RAT KIDNEY

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In the renal glomerulus, the narrow slits between adjacent epithelial podocytes are bridged by a diaphragm (2, 8, 11). In rat and mouse kidneys fixed by perfusion with tannic acid and glutaraldehyde (TAG), it has recently been discovered that this diaphragm has a highly ordered, isoporous substructure (9). It consists of a regular array of alternating cross bridges extending from the podocyte plasma membranes to a centrally running filament. This zipperlike pattern results in two rows of rectangular pores, approximately  $40 \times 140$  Å in cross section, dimensions consistent with the proposed role of the diaphragm as an important filtration barrier to plasma proteins (6).

In the present study, we have found in freeze-cleaved and in freeze-etched normal rat glomeruli that the surface of the slit diaphragm has an appearance conforming to the pattern found in sectioned material.

### MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.), weighing 125 g, were anesthetized with intraperitoneal sodium pentobarbital (Diabotal, Diamond Laboratories, Inc., Des Moines, Iowa) 5 mg/100 g of body weight. Heparin (Liquaemin, Organon Inc., West Orange, N. J.) 100 USP units/100 g of body weight was given intravenously immediately before opening the abdomen. Renal perfusion was performed using the protocol (method 1) and apparatus described by Griffith et al. (4). Blood was rinsed from the

kidneys by perfusion (at a pressure of 140 mm of mercury) for approximately 1 min with Hanks' balanced salt solution (Microbiological Associates, Bethesda, Md.), pH 7.3, at room temperature. This was followed by perfusion for 15 min in two animals with TAG solution (3, 9) consisting of 1% tannic acid (reagent grade, Fisher Scientific Co., Pittsburgh, Pa.) and 1% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M phosphate buffer, pH 7.3, at room temperature. In another rat, perfusion was performed with formaldehyde-glutaraldehyde fixative (5) diluted 1:1 with 0.1 M cacodylate buffer, pH 7.3. A portion of the cortex from each kidney was diced with razor blades into pieces 2 mm across and then processed for freeze-fracture. To facilitate the examination of larger amounts of material by freeze-fracture, glomeruli were isolated from the remaining cortex by the method of Burlington and Cronkite (1): tissue was forced by a spatula through a stainless steel screen of no. 66 mesh (250 μm pore) (The W. S. Tyler, Co., Div. of W. S. Tyler, Inc., Mentor, Ohio) and rinsed with 0.1 M phosphate buffer through successive screens of no. 100 mesh (150 μm pore) and no. 200 mesh (75 μm pore) placed in series. Large numbers of glomeruli and some tubular fragments were found on the lowermost fine screen. After immersion in 25% glycerol in 0.1 M cacodylate buffer for 3 h, pieces of tissue and thick suspensions of the isolated glomeruli were transferred to Balzers specimen holders (Balzers AG, Liechtenstein), rapidly frozen in Freon 22 (Dupont Instruments, Wilmington, Del.) cooled by liquid nitrogen, fractured in a Balzers high vacuum freeze-etch unit BA 360 M at  $-104^{\circ}\text{C}$ , and, either immediately or after etching for up to 20 min, shadowed with platinum at an angle of  $45^{\circ}$ . Some specimens were immersed in Tris buffer 0.01 M

instead of glycerol before freezing and fracture; such samples were deep etched for up to 20 min before shadowing. The replicas were strengthened by evaporation of carbon at a 90° angle, thawed, digested from the underlying tissue with 5.25% sodium hypochlorite (Clorox Co., Oakland, Calif.), and then mounted on 200-mesh grids and examined at 60 kV with a Philips EM200 or an AEI-801 electron microscope.

## RESULTS

In preliminary experiments, it was found that when glomeruli were fixed after isolation from the kidney, freeze-cleaved podocytes showed clustering of intramembranous particles. However, if kidneys were fixed by perfusion before the isolation of glomeruli, intramembranous particles were randomly distributed (Fig. 1); such glomeruli

were indistinguishable from those found in freeze-fracture replicas of pieces of perfused cortical tissue.

Cleavage of the sides of podocytes occasionally revealed an irregular line of particles that appeared to coincide with the line of attachment of the slit diaphragm (Fig. 2), but such examples were rare and unconvincing as sites of membrane specialization. When podocytes were cleaved coplanar to the basement membrane, the complexity of interdigitation was apparent (Fig. 1); the interpodocyte space ranged from 300 to 800 Å in width, except in occasional short segments in which the space was closed (Fig. 1). In some cleaves, the space displayed segments with a regular pattern similar to the zipperlike substructure described in the slit diaphragm by Rodewald and Karnovsky (9) (Figs.



1 and 3). This was seen in both TAG-fixed and in formaldehyde-glutaraldehyde-fixed material. The width of such segments averaged 450 Å and the pattern was consistent with the presence of a series of cross bridges extending inwards from each side of the space. The cross bridges of one side showed a center-to-center spacing of approximately 100 Å and, in some areas, appeared to alternate with those of the other side like the teeth of a zipper. It was not clear whether the cross bridges interlocked centrally or whether, as suggested by Rodewald and Karnovsky (9), they terminated in a central filament. In some sites, a zigzagging shadow could be seen running centrally along the space, but the significance of this could not be determined. Deep etching of the intercellular space in dilute Tris buffer led to a somewhat distorted pattern consisting of multiple weblike strands with irregular central attachments (Fig. 4).

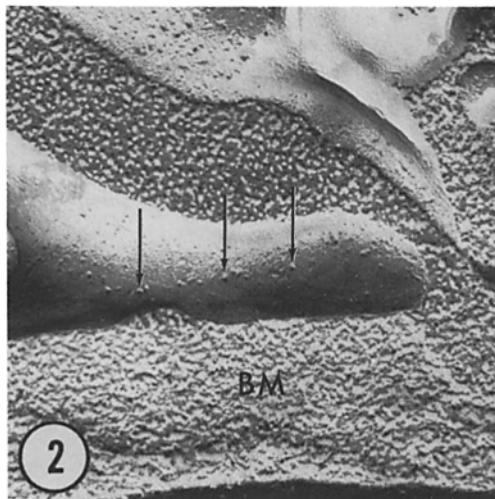


FIGURE 2 Freeze-fracture of normal rat glomerulus. Cleavage has occurred through the membrane of a podocyte, approximately perpendicular to the basement membrane (BM). An irregular line of particles (arrows) is seen in a position that may coincide with the line of attachment of the slit diaphragm. TAG fixation.  $\times 89,000$ .

## DISCUSSION

These findings provide further evidence that the glomerular slit diaphragm has a complex periodic substructure consisting of multiple, regularly spaced cross bridges; the spacing of the cross bridges in the replicas approximated that measured in sections (9). The demonstration of this substructure in freeze-cleaved glomeruli fixed with formaldehyde-glutaraldehyde indicates that the pattern seen in sectioned material after TAG fixation (9) is not simply an artifact caused by exposure to tannic acid. The diaphragm, then, appears to constitute a unique and peculiarly specialized type of intercellular junction, although it is not consistently associated with areas of intramembranous specialization like those found in gap junctions, desmosomes, or zonulae occludentes (7).

The demonstration that the slit diaphragm is a real entity with a highly ordered isoporous substructure supports the view (6) that it may be a significant filtration barrier in the glomerulus. The dimensions of the rectangular pores within the diaphragm match the general size of the elongated albumin molecule (10). This could result in severe, although incomplete, restriction to the passage of albumin molecules that had succeeded in traversing the basement membrane. However, the filtration function of the diaphragm has not, as yet, been directly proven. It could be argued that it simply plays an architectural role in maintaining the normal interpodocyte space and podocyte interdigitation.

## SUMMARY

In freeze-cleaved and in deep freeze-etched preparations of normal rat glomeruli, segments of the interpodocyte space displayed a regular pattern consistent with the zipperlike substructure proposed for the slit diaphragm.

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FIGURE 1 Freeze-fracture of normal rat glomerulus. Cleavage has occurred through epithelial podocyte membranes, coplanar to the basement membrane. The podocytes show complex interdigitation and are separated from one another by a space, except in occasional short segments in which the space is closed. In some segments (bracket), the space displays a regular substructure; a higher power view of similar segments is shown in Fig. 3. Such segments presumably reflect the cleavage pattern at the level of the slit diaphragm. In wider zones of the interpodocyte space (I), cleavage has occurred at some distance from the diaphragm and no pattern is seen. In this and the subsequent micrographs, shadowing has been performed from bottom to top. Formaldehyde-glutaraldehyde fixation.  $\times 49,000$ .

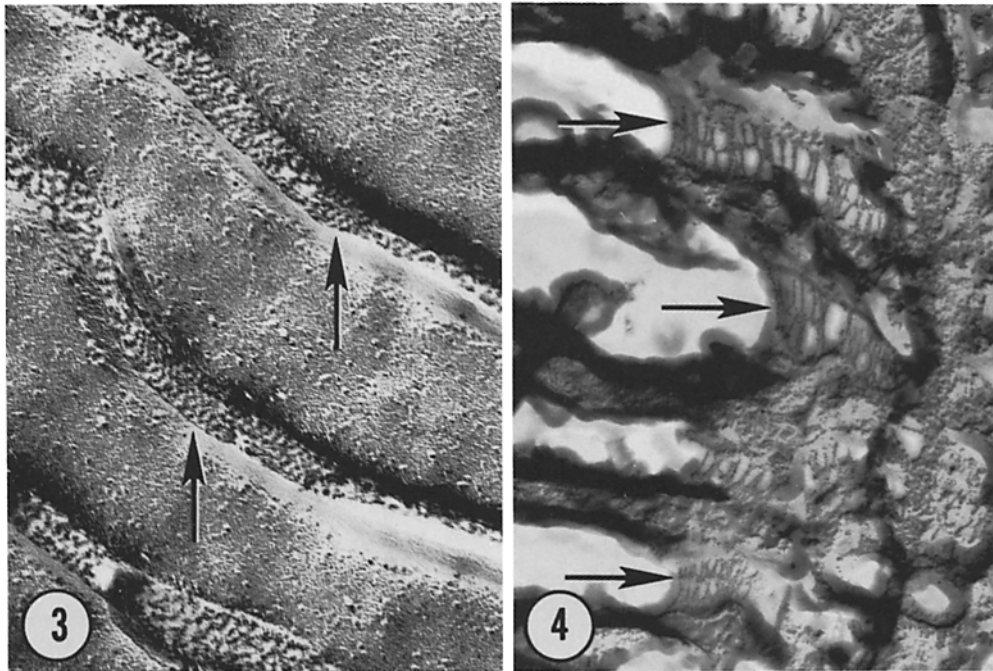


FIGURE 3 Freeze-fracture of normal rat glomerulus showing detail of interpodocyte spaces. Note segments displaying regular pattern (arrows). TAG fixation.  $\times 104,000$ .

FIGURE 4 Deeply etched freeze-fracture of normal rat glomerulus showing detail of interpodocyte spaces. Note areas (arrows) showing a pattern consisting of multiple weblike strands with irregular central attachments. TAG fixation.  $\times 53,000$ .

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