POROUS SUBSTRUCTURE OF THE GLOMERULAR

SLIT DIAPHRAGM IN THE RAT AND MOUSE

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ABSTRACT

The highly ordered, isoporous substructure of the glomerular slit diaphragm was re-
vealed in rat and mouse kidneys fixed by perfusion with tannic acid and glutaraldehyde.
The slit diaphragm was similar in both animal species and appeared as a continuous
junctional band, 300–450 Å wide, consistently present within all slits formed by the
epithelial foot processes. The diaphragm exhibited a zipper-like substructure with alter-
nating, periodic cross bridges extending from the podocyte plasma membranes to a
central filament which ran parallel to and equidistant from the cell membranes. The
dimensions and spacing of the cross bridges defined a uniform population of rectangular
pores approximately 40 by 140 Å in cross section and 70 Å in length. The total area of
the pores was calculated to be about 2–3% of the total surface area of the glomerular
capillaries. Physiological data indicate that the glomerular filter functions as if it were
an isoporous membrane which excludes proteins larger than serum albumin. The simi-
larity between the dimensions of the pores in the slit diaphragm and estimates for the
size and shape of serum albumin supports the conclusion from tracer experiments that
the slit diaphragm may serve as the principal filtration barrier to plasma proteins in the
kidney.

INTRODUCTION

The renal glomerulus serves as a macromolecular
sieve capable of excluding most plasma proteins
from the urine. Physiological measurements of
renal clearance rates of differently sized molecules
have indicated that the glomerular filter functions
as if it were a semipermeable, isoporous membrane
with pore dimensions compatible with the virtual
exclusion of macromolecules larger than serum
albumin (mol wt 68,000) (1, 2).

Although several hypothetical pore geometries
have been constructed from the physiological
data, the true morphological equivalent of the
glomerular filter is still a matter of debate. To gain
access to the urinary space, a molecule in the
glomerular capillary must cross sequentially three
structural layers composing the glomerular wall:
the capillary endothelium, the basement mem-
brane, and, finally, the overlying epithelial
podocytes. Tracer experiments (3–12) have shown
that the endothelium, which is perforated by
numerous, large, circular fenestrations (500–
1,000 Å diam), is freely permeable to substances
of molecular dimensions. While the passage of
large proteins such as ferritin (105–110 Å diam)
is apparently restricted by the basement mem-
brane (4), smaller proteins of a size nearer that of

serum albumin and the exclusion limit of the glomerulus appear to be restricted at the level of the slit-shaped channels formed between the interdigitating foot processes of the podocytes (5, 6, 8, 9). Previous workers (5, 9, 11, 13, 14) have suggested that molecular sieving at this level may be a property of either the fibrillar surface coats of the closely apposed membranes of the foot processes or the slit diaphragm,[1] a structure often seen bridging the epithelial slits near the basement membrane (4, 15, 16).

In the past, a fully detailed description of the slit diaphragm has not been possible because of the limited electron density of this structure after conventional fixation and staining techniques for electron microscopy. We report the use of a tannic acid fixative (17, 18) which lends considerable contrast and definition to extracellular structures in the kidney and which, in particular, clearly reveals the isoporous substructure of the slit diaphragm. The size of the pores suggests that the slit diaphragm may be the principal filtration barrier to plasma proteins in the glomerulus.

MATERIALS AND METHODS

5 adult Sprague-Dawley rats and 5 adult CD strain mice (Charles River Breeding Laboratories, Wilmington, Mass.) were used for this study. Animals were allowed free access to both food and water under standardized housing conditions for several days prior to an experiment. Kidneys were fixed by perfusion according to "method 1" of Maunsbach (19). A pressure of 140–150 cm H_2O was used for all perfusion steps. Kidneys were first rinsed free of blood with oxygenated Hanks' balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.) for 1–2 min, then fixed with a solution of TAG (18) consisting of 1% tannic acid (Fisher Scientific Co., Pittsburgh, Pa., reagent grade) and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for approximately 15 min. Pieces of tissue were then excised with razor blades and fixed for an additional 2 h by immersion in the same fixative solution at room temperature. After two 15-min rinses at 0°C in phosphate buffer containing 0.1 M sucrose, tissue was postfixed in 2% OsO_4 in phosphate buffer at 0°C, dehydrated in ethanol, and embedded in Epon

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[1] Although other authors have referred to this structure as the "filtration slit membrane" or simply "slit membrane" (4, 14, 16), we prefer to use the term "slit diaphragm" which does not presuppose a function for the structure and avoids confusion between it and the morphologically dissimilar unit membrane of the cell.

(20). Additional tissue from nonperfused kidneys was fixed by immersion in TAG for 2–2½ h and processed in the same manner as perfusion-fixed tissue. Silver or gray thin sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), stained sequentially 5 min in a saturated aqueous solution of uranyl acetate (21) and 5 min in lead citrate (22), and examined at 60 kV in a Philips EM200 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y.). Microscope magnifications were calibrated with a diffraction grating replica, 28,800 lines per inch (Ladd Research Industries, Burlington, Vt.). Micrograph measurements with the exception of percent pore area (see text) were made from negatives with an LP-6 profile projector and micrometer stage (Ehrenreich Photo-Optical Industries, Garden City, N.Y.).

RESULTS

After TAG fixation, the kidney cortex appeared similar in most respects to tissue fixed by more conventional means without tannic acid (3–5, 11). However, a notably unique feature of the TAG-fixed tissue was the increased contrast of extracellular structures. Thus the glomerular basement membrane, various extracellular connective tissue elements such as collagen, and cell surface coats were preferentially stained and exhibited much greater electron density than these same structures after conventional fixation. In addition, plasma membranes showed increased density which clearly revealed their trilaminar substructure. In contrast, internal cell membranes as well as other intracellular structures usually exhibited staining properties similar to those of tissue fixed without tannic acid. Nevertheless, a variable number of cells, usually damaged cells near the cut edges of tissue blocks, showed general increased staining of their cytoplasm.

Slit Diaphragm

A striking feature of kidney tissue fixed with TAG was the clearly recognized slit diaphragm between epithelial foot processes. This fixation technique revealed the basic structure of the diaphragm as described by others (4) as well as several additional structural features not previously reported. The slit diaphragm appeared essentially identical in both the rat and the mouse.

Where the plane of the thin sections crossed perpendicular to the basement membrane, the slit diaphragm was evident as a thin electron-dense line connecting the plasma membranes of adjacent
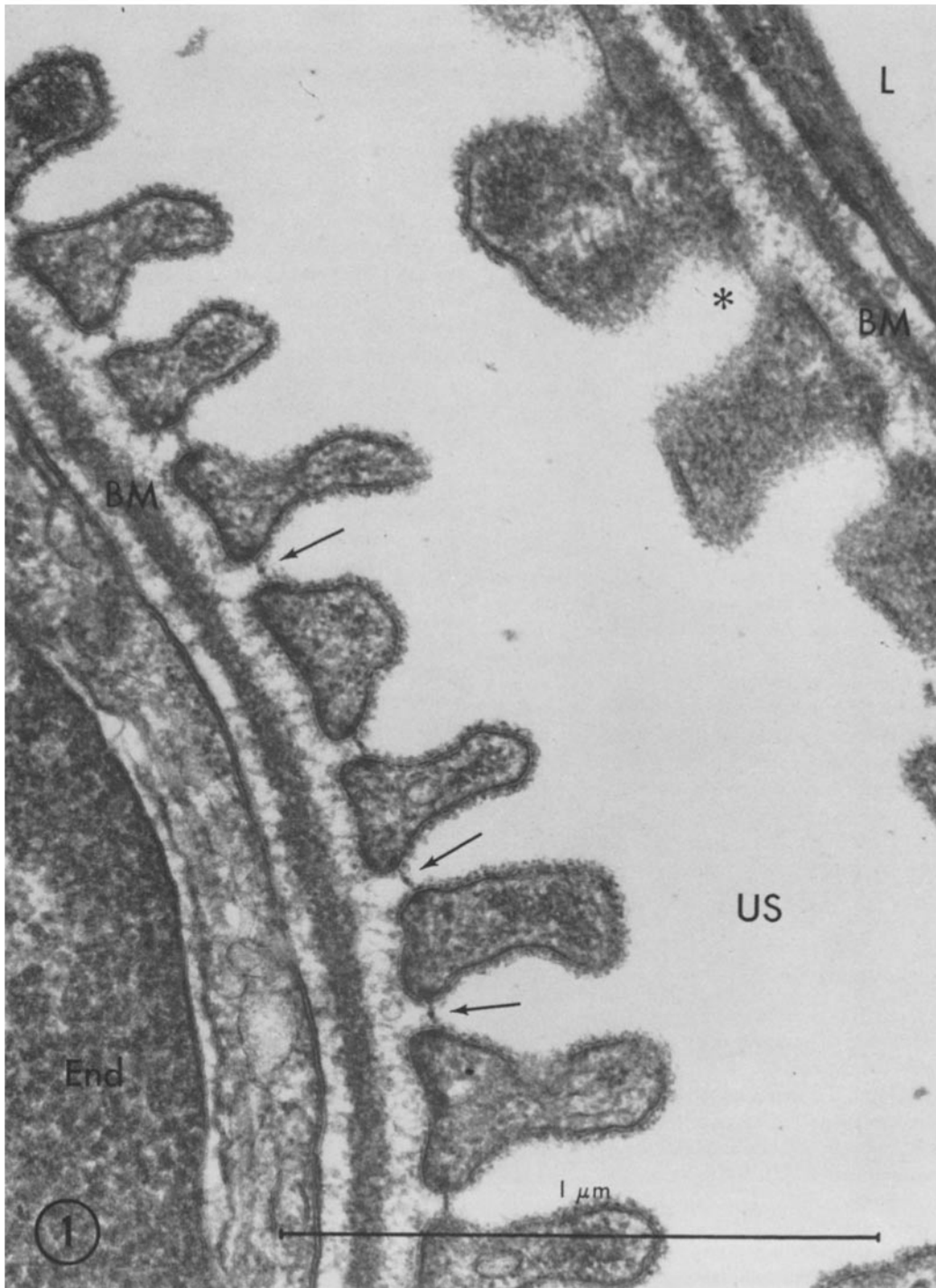
FIGURE 1   Mouse renal glomerulus. The slit diaphragm appears as an electron-dense line bridging adjacent epithelial foot processes. Where the processes appear in cross section, the central filament can often be distinguished as a dense dot in the center of the diaphragm (arrows). In these regions a clear space is usually present between the diaphragm and the basement membrane (BM). Where the foot processes have been cut obliquely, the periodicity of the cross bridges within the diaphragm can be resolved (*). L, capillary lumen; US, urinary space; End, endothelial cell body. × 88,000.

foot processes in a plane approximately 500–700 Å from the outer surface of the basement membrane (Fig. 1). Where sections cut the foot processes in cross section, a central dot (110 Å diam) could very often be distinguished in the diaphragm. In such orientation the width of the diaphragm, that is, the distance from plasma membrane to plasma membrane, varied between 300 and 450 Å with a mean value near 390 Å. Also evident was an increase in density of the cytoplasm of the foot processes directly opposite the points of attachment of the diaphragm to the plasma membranes.

Sections tangential to the basement membrane revealed the three-dimensional structure of the slit diaphragm (Figs. 2, 3). The central dot seen in cross section now appeared as a continuous filament parallel to the long axis of the epithelial slit and equidistant from the plasma membranes of the adjacent foot processes. The filament was joined to the cell membranes by regularly spaced cross bridges approximately 70 Å in diameter and 140 Å in length. The average center-to-center spacing of adjacent cross bridges was measured as 110 Å. In favorable images, the cross bridges appeared to alternate between the two sides of the central filament, thus giving the diaphragm a zipper-like appearance (Fig. 3). This highly ordered arrangement of cross bridges defined a fairly uniform population of rectangular, translucent spaces or pores between cross bridges. The cross-sectional dimensions of these pores could be calculated from the spacing and size of the cross bridges and were found to average 40 by 140 Å. The morphology and dimensions of the slit diaphragm are summarized in Fig. 4 and Table I.

Fractional Pore Area

The area occupied by the pores in the slit diaphragm was estimated as a percentage of the total surface area of the capillary wall. Lines were drawn on micrographs within the plane of the slit diaphragm. The portions of the lines occupied by profiles of the slit diaphragm were summed and found to be 13% of the total line length. From the measurements in Table I, the portion of the slit diaphragm surface area occupied by the rectangular pores was calculated as 26%. The product of these two percentages yielded the percentage (3–4%) of capillary surface area occupied by the pores in the regions of the epithelial slits. This percentage was further "corrected" to take into account portions of the capillary wall occupied by

endothelial or epithelial cell bodies as in Fig. 1 or otherwise lacking the slit diaphragm. In this manner it was estimated that in both animal species 2–3% of the capillary wall was occupied by the rectangular pores of the slit diaphragm.

Surface Coat and Basement Membrane

The fibrillar extracellular coat, 100–150 Å thick, covering the remaining membrane surfaces of the foot processes was prominently stained by the TAG fixation technique. However, unlike the slit diaphragm, this surface coat exhibited no discernible periodic arrangement on the cell membrane. Although the surface coat extended up to the slit diaphragm, the coat did not appear as a continuous layer across this structure (Fig. 1). On the urinary side of the slit diaphragm, the surface coats of adjacent foot processes were invariably separated from each other to form unobstructed channels up to 1,000 Å or more in width which opened into the urinary space. With respect to their size, these channels were similar to what has been observed after conventional aldehyde fixation (4, 11, 14). On the opposite side of the slit diaphragm the facing surface coats were also usually separated from each other and formed a characteristic clear zone, 400–800 Å wide, which extended from the diaphragm to the basement membrane (Fig. 1). On occasion, however, this space appeared constricted or, infrequently, completely obliterated with focal regions of contact between apposing cell membranes in or near the plane of the slit diaphragm (Fig. 2).

Certain features of the trilaminar basement membrane also appeared noteworthy in tissue fixed by TAG perfusion. Although the internal structure of the central dense layer of the membrane was difficult to resolve, fibrils of highly irregular diameter, 30–100 Å, could be recognized in the less dense subendothelial and subepithelial layers. In the subepithelial layer, these fibrils appeared to radiate from the bottom surfaces of the foot processes into the central dense zone (Fig. 1). The fibrils did not usually extend into the channel between foot processes and, in particular, did not make contact with the slit diaphragm. The slit diaphragm, therefore, appeared as a separate structure and not an extension or specialization of the basement membrane. Other features of the basement membrane were similar to descriptions previously reported (4, 11, 14).

FIGURE 2 Epithelial foot processes from mouse glomerulus. Large expanses of the slit diaphragm are readily visible within the epithelial slits in this section which has been cut parallel to the plane of the basement membrane. Focal points of contact between adjacent cell membranes are also shown (arrows). × 88,000.

FIGURE 3 Slit diaphragm from rat glomerulus. The central filament and cross bridges are readily re-
solved in this micrograph. The marks indicate a region where cross bridges appear to alternate on either
side of the central filament. Also evident is the increased density of the cytoplasm opposite the points
of attachment of the diaphragm to the epithelial cell membranes. Apparent discontinuities in the dia-
phragm, as also seen in Fig. 2, represent regions where this structure has left the plane of sec-
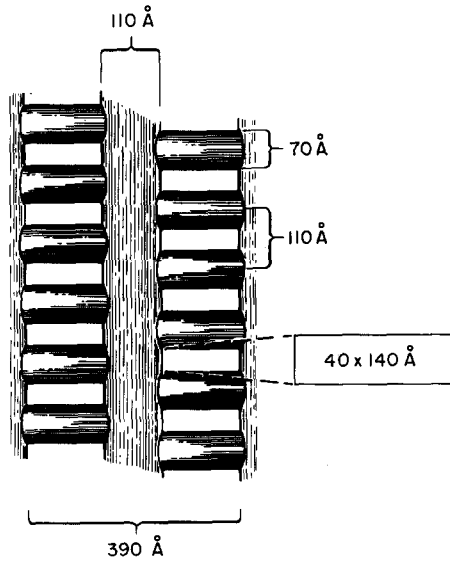tion. × 153,000.

FIGURE 4 Schematic drawing of the epithelial slit diaphragm. The average cross-section dimensions of the pores between cross bridges are indicated within the rectangle.

TABLE I

Dimensions of the Slit Diaphragm in the
Rat and Mouse

	Rat	Mouse
	Å	Å
Width, overall	394 ± 4*	380 ± 5
	(±29)‡	(±28)
Diameter of central filament	109 ± 2	110 ± 2
	(±10)	(±10)
Diameter of cross bridges	70 ± 2	67 ± 2
	(±6)	(±8)
Center-to-center spacing of cross bridges	112 ± 1	110 ± 1
	(±12)	(±13)

* Mean ± standard error.
‡ Standard deviation.

Immersion-Fixed Tissue

In general, the structural details of the slit diaphragm were more difficult to resolve in tissue fixed by immersion in TAG than in perfusion-fixed tissue. The diaphragm with its central filament was often recognized in cross section. However, in tangential section, both the central filament as well as the cross bridges were usually difficult to visualize as a result of a generally increased staining of surrounding material.

Immersion fixation tended to preserve flocculent material, presumably plasma proteins, within the glomerular capillaries (Fig. 5). This material ranged in size from irregularly shaped particles less than 50 Å in diameter to large aggregates in which individual particles could not be readily distinguished. Such material extended through the fenestrae of endothelial cells into the basement membrane where an increased density was readily apparent in both the subendothelial and subepithelial layers. Of particular interest was that this flocculent material often was found extending into the channels between foot processes to the level of the slit diaphragm (Fig. 5). However, this material did not extend farther into the channels past the diaphragm and was present only in small quantities within the urinary space.

DISCUSSION

Our observations on the glomerulus fixed with TAG provide a more detailed description of the morphology of the epithelial slit diaphragm than previously possible. This was due to the ability of tannic acid to provide extremely high contrast to the diaphragm as well as to other extracellular components of the tissue. Futaesaku et al. (18) have shown that tannic acid is able to form extremely strong complexes with proteins which can subsequently bind several heavy metals including osmium. Presumably similar reactions are responsible for the enhanced contrast of the slit diaphragm in our preparations. Nevertheless, because the specificity of tannic acid fixation has not been clearly established, we believe it improper to infer any chemical properties of the diaphragm from its reaction with tannic acid.

Some of the features of the slit diaphragm have been noted previously by other workers (4, 15, 16). In the most detailed study, Farquhar et al. (4) recognized the existence of the central filament and found fine strands of material sometimes bridging this central filament and the adjacent cell membranes. However, these authors were unable to resolve the number or arrangement of the cross bridges and, furthermore, could not ascertain whether the diaphragm was a continuous or discontinuous structure covering the epithelial slits. More recent electron microscope studies (5, 8, 9, 11, 12, 14) have not clarified these
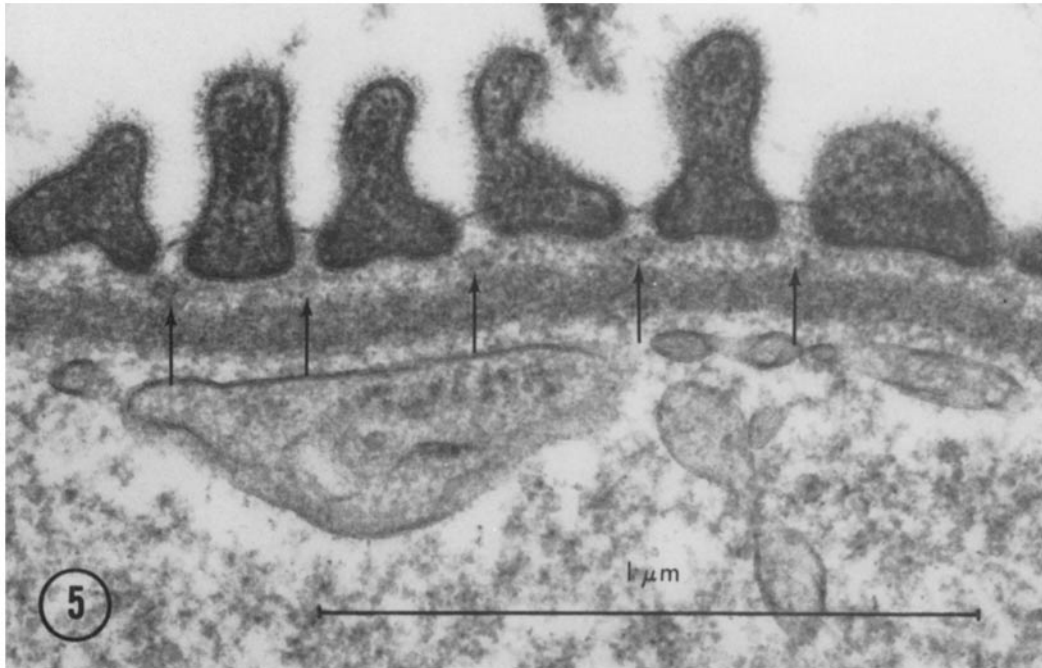
FIGURE 5 Rat renal glomerulus fixed by immersion only. A large amount of flocculent material is preserved within the capillary lumen. The material appears to penetrate into the outer layer of the basement membrane and to accumulate below the slit diaphragm (arrows). × 88,000.

observations and, in fact, have cast doubt on the actual existence of the slit diaphragm as a discrete structure. Karnovsky and Ainsworth (11) noted that the visualization of the diaphragm was dependent on the method of fixation and suggested that the structure might even be an artifact caused by material condensed during fixation at the surface of the basement membrane. However, our findings, in confirming and extending the results of Farquhar et al. (4), leave little doubt that the slit diaphragm is a highly ordered, real structure.

Our most important observations are, first, that the slit diaphragm is a continuous two-dimensional band which extends between all epithelial foot processes. A necessary condition for molecules in the glomerular capillary to gain access to the urinary space during filtration, therefore, is that they must be able to cross the slit diaphragm. Second, the regular, zipper-like spacing of the cross bridges within the diaphragm defines a fairly homogeneous population of rectangular pores with a mean width, 40 Å, which would be narrow enough to restrict passage of most serum proteins. We believe that these two observations lend

strong support to the notion that the slit diaphragm represents the major filtration barrier to proteins in the glomerulus.

Tracer Studies

Considerable direct evidence for the morphological site of filtration has been provided by the use of several macromolecular tracers for electron microscopy. Although studies with ferritin and thorium oxide (3, 4) have shown that the central dense layer of the basement membrane severely restricts passage of large particles (110 Å diam or greater), experiments with smaller tracers have suggested that filtration also occurs in the region of the epithelial slits as was first suggested by Hall (23). In particular, myeloperoxidase (mol wt 160,000–180,000) and lactoperoxidase (mol wt 82,000) have been shown (5, 6) to cross the basement membrane but to be restricted from entering the urinary space at the level of the diaphragm. Catalase (mol wt 240,000) also penetrates only to this level although, in addition, its passage across the basement membrane is partially retarded (8, 9). In contrast,

horseradish peroxidase (mol wt 40,000), cyto-chrome C (mol wt 12,000), and myoglobin (mol wt 16,890) have been shown to traverse both the basement membrane and epithelial slit (5, 7, 9, 10) as would be predicted from physiological evidence that molecules smaller than serum albumin (mol wt 68,000) enter the urine. Experiments with tyrosinase (mol wt 34,500) have demonstrated, in addition, a partial retention of this tracer at the slit diaphragm (12) although in other respects results were similar to those with horseradish peroxidase (5).

Similarly our observations of tissue fixed by immersion in TAG suggest that the diaphragm is important in restricting passage of normal serum proteins. In tissue fixed by this method, much serum protein appeared preserved in place as evidenced by the large amount of dense, flocculent material found within most glomerular capillaries. This material apparently penetrated the basement membrane and often filled the epithelial slit up to but not past the slit diaphragm.

To explain the results with tracers, Karnovsky and his co-workers have postulated (5, 9, 11) that the glomerulus contains two filters in series: (a) the basement membrane, a coarse filter which restricts large proteins, and (b) the epithelial slit, a fine filter which excludes smaller proteins and defines the overall permeability of the glomerulus. The results were compatible with the slit diaphragm serving as this second filter, but unfortunately the precise extent and morphology of this structure were not clearly resolved at that time. Therefore, the possibility was not excluded that some other structure near the diaphragm might at least partially contribute to filtration within the epithelial slit. Thus some authors (13, 14) have proposed that the cell surface coat, or glycocalyx, represents the main filtration barrier. Latta (14) has argued that in vivo the surface coats of adjacent foot processes are normally in close contact for the entire depth of the epithelial slits and that the clear channels which are usually found within the slits are merely shrinkage artifacts caused by preparative techniques for electron microscopy. Molecules entering the urinary space, therefore, would normally have to percolate through the surface coats within the slits. An interpretation involving shrinkage might also apply to our observations on TAG-fixed material, in which case the slit diaphragm might serve merely a supportive or other function unrelated to filtration. Nevertheless, we are inclined to believe that the finely

isoporous substructure of the slit diaphragm makes it the most likely candidate for the filter.

Pore Size

The strikingly uniform arrangement of the cross bridges within the slit diaphragm has allowed us the unique opportunity to measure with a high degree of accuracy the size of the channels between cross bridges. Their size is of particular interest since several theoretical models of the glomerular pore have been constructed from extensive physiological data. Glomerular clearance rates of several proteins and dextrans have been shown to agree well with what would be expected for an isoporous membrane perforated by cylindrical channels 70–84 Å in diameter (1, 2, 24). Landis and Pappenheimer (2), however, have emphasized that other pore geometries may be equally valid.

Although a complete theoretical analysis of rectangular pores as found in the slit diaphragm is unavailable and outside the scope of our expertise, a simple but useful comparison can be made between the size of the pores in the diaphragm and the observed exclusion limit of the glomerulus. Clearance rates show that serum albumin marks the permeability endpoint of the glomerular filter and that larger proteins do not pass through in detectable quantities (1, 2). Such data would suggest that the 40 by 140 Å pores we have measured should match the general size of albumin, values of which have been estimated by a number of physical-chemical means. Serum albumin is often represented simply as the equivalent sphere, 71 Å in diameter, which would have the same rate of diffusion in solution as the true molecule (1, 2). However, other hydrodynamic measurements indicate that albumin is closer in shape to a prolate ellipsoid with axes of approximately 150 by 38 Å (25), 168 by 34 Å (26–28), or 129 by 39 Å (28, 29). Low resolution X-ray diffraction of crystalline serum albumin has also suggested an elongated molecule with axes of 144 by 45 by 22 Å (30, 31). While such measurements must be viewed as very rough estimates for the true shape of serum albumin in solution, we are encouraged by the general similarity between these estimates and the dimensions of the channels through the slit diaphragm.

From our electron micrographs, we have also calculated that 2–3% of the total surface area of the glomerular capillaries is occupied by the pores

of the diaphragm. This percentage is compatible with the relatively large hydraulic flow across these capillaries in comparison to the much lower flow across muscle capillaries (2) where the pore area has been estimated to be 0.0033% (32) to 0.1% (2) of the total capillary surface. Using the glomerular surface area of 83 cm^2/g kidney in the rat as estimated by Renkin and Gilmore (24), we can calculate that the total pore area is approximately 2 cm^2/g kidney. This is equivalent to a total pore area per centimeter path length of about 3 $\times$ 10^6 cm/g kidney if the path length of the pore is assumed to be equal to the diameter (70 Å) of the cross bridges in the slit diaphragm. An apparent pore area per centimeter path length has also been determined independently from physiological data. Renkin and Gilmore (24), assuming a cylindrical pore model, have measured this quantity as 2.5 $\times$ 10^5 cm/g kidney in the rat and 1.5 $\times$ 10^5 cm/g kidney in the dog. Both values are similar to the earlier estimate of 2 $\times$ 10^5 cm/g kidney in the dog as determined by Pappenheimer (1, 2). Our histological estimate of the pore area per unit path length is somewhat higher than these physiological values, but a comparison is difficult since the physiological determinations are dependent on the assumed pore geometry (24). In addition, our estimate probably represents a maximum value since the functional pore area is very likely to be significantly smaller than the histological pore area as a result of intermittent or reduced blood flow in many glomeruli (33) and the formation of the bulk of ultrafiltrate over a relatively small fraction of the total capillary surface within a single glomerulus (34).

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