Acta Medica Okayama

Volume 36, *Issue* 5 1982 *Article* 5

OCTOBER 1982

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KEYWORDS: glomerular basement membrance, glomerular permeability, ultrastructure, negative staining, electron microscopy

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*PMID: 6184956 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **36**, (5), 371—382 (1982)

MOLECULAR SIEVE IN RAT GLOMERULAR BASEMENT MEMBRANE AS REVEALED BY NEGATIVE STAINING

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Received April 1, 1982

Abstract. Human and bovine glomerular basement membranes (GBM) were previously shown to be a three-dimensional molecular sieve composed of pores and strands by negative staining and electron microscopy. In this study, rat GBM were isolated under several different conditions to rule out morphological changes due to isolation procedures. Rat GBM isolated under different conditions all showed the same morphological features as bovine and human GBM. The strands forming the molecular sieve were almost equal in width, measuring approximately 3.1 ± 0.8 nm. Pores were oval or polygonal. The size of pores varied a little averaging 4.4 ± 1.0 nm in the long dimension and 3.0 ± 0.6 nm in the short dimension. The average density of the pores was $16 \pm 2/1{,}000\ nm^2$. Negative staining demonstrated pores in isolated and unfixed GBM, indicating that the function of GBM is mechanical filtration of macromolecules on the basis of size.

Key words : glomerular basement membrane, glomerular permeability, ultrastructure, negative staining, electron microscopy.

Glomerular capillaries are thought to function as a mechanical filter. In normal glomerular capillaries, the glomerular basement membrane (GBM) is the only continuous layer between blood and urine, and is considered to be the main filter by many morphologists (1, 2). In 1955, Pappenheimer (3) calculated the existence of pores in the wall of glomerular capillaries with a mean radius 35 to 40 Å. Recent physiological studies (4) indicated that the glomerulus filter permits the passage of molecules with a hydrodynamic radius is no greater than 36 Å.

GBM does not show pores when conventionl methods of fixation and embedding are used. By transmission electron microscopy, the lamina densa appears as a felt-work of 30 to 40 Å fibrils which appear to be embedded in an amorphous matrix (1, 5). Infrequently, a large fibril about 100 Å wide is also found on the endothelial side (1, 6).

The failure to demonstrate pores in GBM led to several hypotheses. Menefee and Mueller (7) suggested that GBM acts as a thixotropic gel that liquefies under pressure and reforms afterwards. According to the model of Misra (8), the GBM is a gel consisting of tropocollagen molecules dispersed at random in close association with glycoproteins and lipoproteins.

However, we demonstrated pores in human and bovine GBM using negative staining (9-11). We also showed that rat tubular basement membrane (12, 13) and alveolar basement membranes (14) were made up of a molecular sieve composed of pores and strands; therefore, we proposed a molecular sieve theory of basement membranes. We isolated rat GBM under different conditions to minimize morphological changes during isolation. We describe the ultrastructure of rat GBM in more detail in this paper.

MATERIALS AND METHODS

Isolation of rat glomeruli. Approximately fifty Sprague-Dawley or Wister rats weighing 150 to 200 g were used in each experiment. Glomeruli were isolated by a modification of the method of Spiro (15). The entire procedure was performed at temperatures below 4℃. Kidneys were obtained under ether anesthesia. The capsule of each kidney was carefully removed and the medullae was dissected away from the kidneys. The cortex was forced through a 150 mesh stainless sieve by pushing it with the bottom of a beaker. Care was taken to use only moderate pressure accompanied by ample amounts of ice-cold physiologic saline. The sieved suspensions were then poured through an 80 mesh sieve and a 250 mesh sieve. Material retained on the 250 mesh sieve was then washed thoroughly with ice-cold physiologic saline. The sieving procedure was repeated until phase contrast microscope examination indicated only glomeruli free of cells, tubular fragments and Bowman's capsules. The material on the 250 mesh sieve was then transferred to a centrifuge tube and centrifuged at 120 × g for 10 min several times. The supernatant fluid was removed by suction and the glomerular pellet was re-examined for purity under a phase contrast microscope.

Isolation of rat GBM. Rat GBM was isolated from glomeruli either by sonication or by treatment with detergent. For sonic disruption, the glomerular pellets were suspended in ice cold 1 M sodium chloride. A Heat Systems sonicator (model W225R) with a 0.5-in. satinless steel probe was used. Sonic disruption was conducted at 200 watts average output power. The extent of the glomerular disruption was followed under a phase contrast microscope, and the sonic disruption was continued until complete fragmentation of the glomeruli had occurred. This required a total sonic disruption time of about 5 min. The sonically treated material was centrifuged for 20 min at 1,300 × g. The sediment was washed 5 more times in 1 M sodium chloride and 5 times in distilled water. The sediment thus obtained was pure GBM under phase contrast microscopy.

For chemical isolation of rat GBM from glomeruli, we followed the method of Carlson *et al.* (16). As modified by Langevelt *et al.* (17), lysis time in distilled water and incubation time with DNAse were prolonged to 2.5 h and treatment of with 4% sodium deoxycholate to 5 h. Between each step and before observation, the material was washed several times with distilled water to remove detergents.

Rat glomeruli or GBM were frozen for storage until observation, since it was difficult to isolate and observe GBM in one day. GBM isolated without freezing were also studied to determine the effect of ice particles on frozen tissue. In these cases, GBM was isolated by ultrasonication.

Electron microscopic procedures. Electron microscopic observation was carried out as described previously (10). After each step of isolation of GBM, the material was examined under a phase contrast microscope. The final pellet was confirmed by ultrathin sectioning

before observation. The pellets containing basement membranes were fixed with 2% glutaraldehyde and post-fixed with 1% osmium tetroxide. The blocks were dehydrated in ethanol and embedded in epoxy resin. Thin sections were cut with glass knives on a Sorvall MT2-B ultramicrotome, stained with lead citrate, and examined at 75 kV with an electron microscope (Hitachi H 500).

Specimens were negatively stained by applying a drop of suspension to formvar or collodion-coated, carbon-reinforced copper grids. After 30 seconds, excess fluid was blotted with a piece of filter paper and a drop of 1% phosphotungstic acid (PTA) (pH 7.3) was applied. The grid was blotted 20 seconds later. Preparations were examined with an electron microscope (Hitachi H 700) operated at 150 kV, and about 600 pictures were taken at the original magnification of 3,000-100,000. Occasionally, micrographs of the same part of the fragment of the GBM were taken at various magnifications or at a series of through focus to out of focus to confirm the measurement and to distinguish noise from overlapping. Stereoscopic electron micrography was also taken by inclining the plane of the grid from 8° to 10°.

Measurements of pores and strands. The long and short dimensions of pores and the width of strands were measured on both enlarged micrographs and corresponding negatives. For measurements only negatives of high magnification (30,000-100,000) were used. A long dimension was measured first, followed by a short dimension perpendicular to the long dimension.

The density of pores per 1,000 nm^2 was counted on both magnified micrographs and the corresponding negatives.

RESULTS

The material was monitored by phase contrast microscopy throughout the

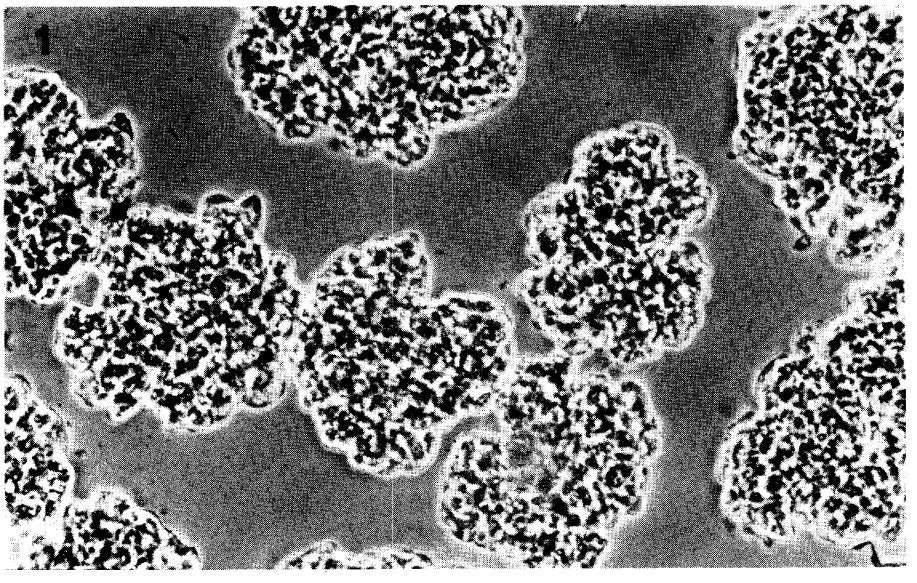


Fig. 1. Phase contrast micrograph of isolated rat glomeruli. Note the absence of recognizable cell fragments and Bowman capsules. × 350

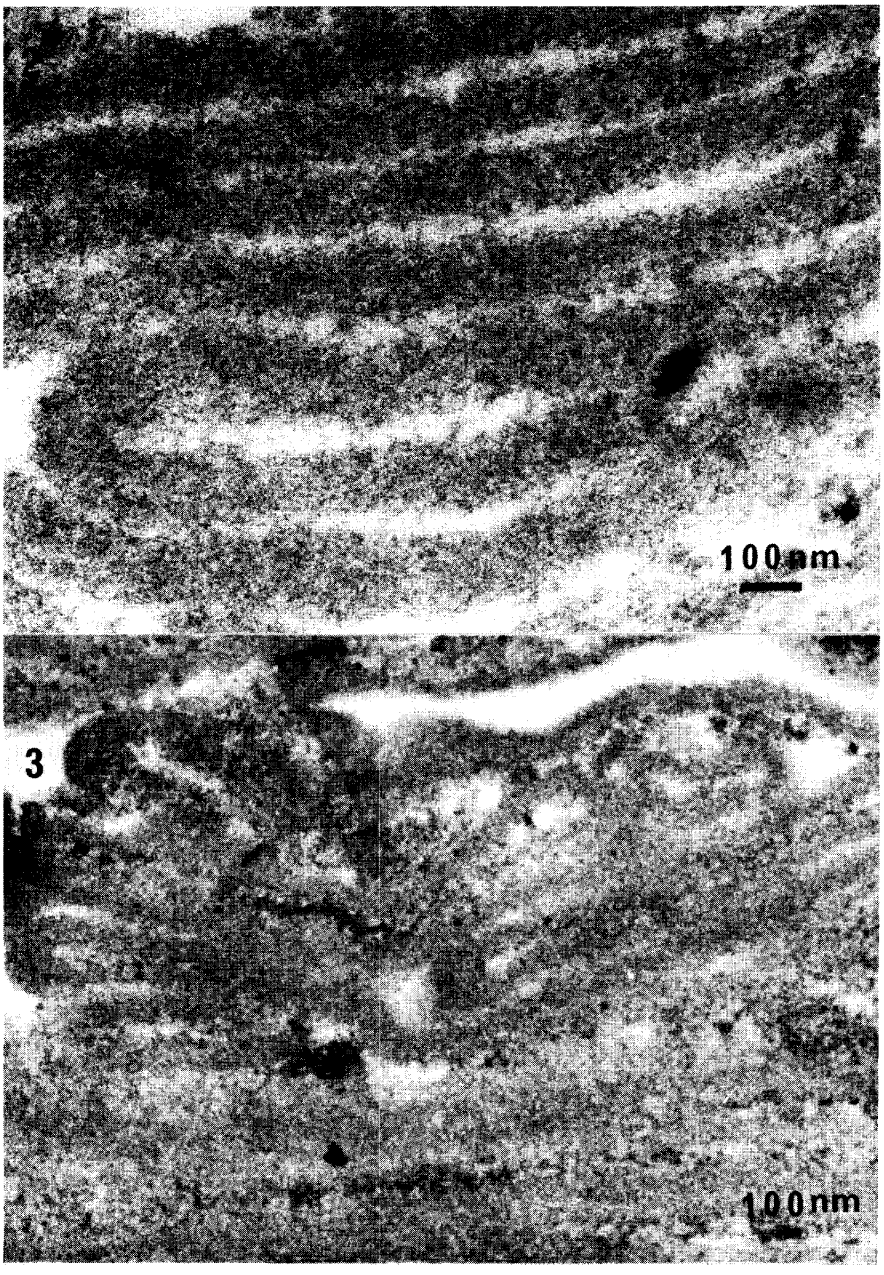
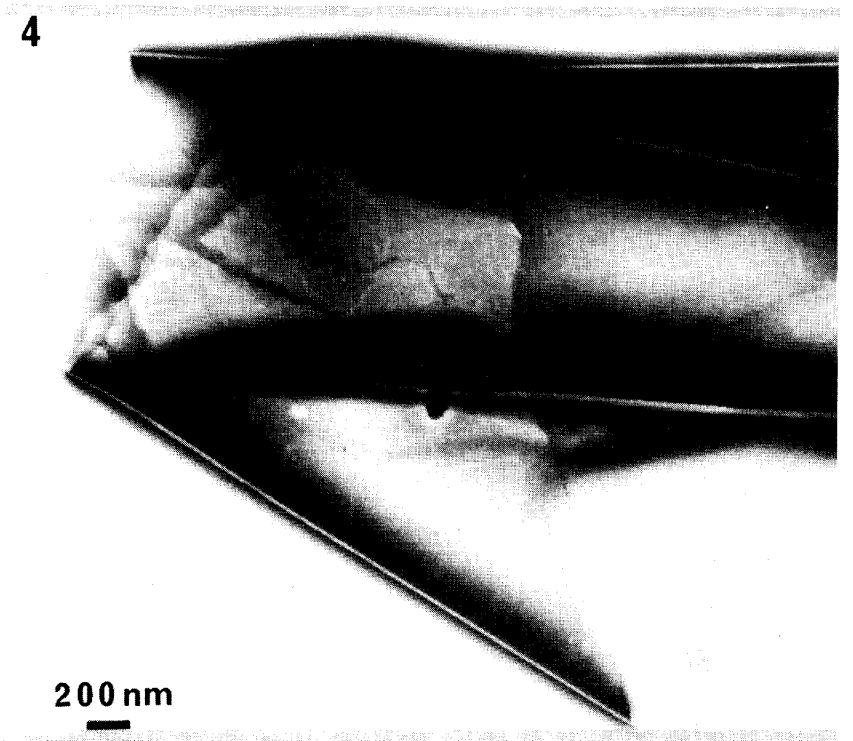


Fig. 2. Ultrathin section of rat glomerular basement membrane isolated by sonication. Note normal structure and complete absence of cell fragments. × 81,000

Fig. 3. Ultrathin section of rat GBM isolated by detergents. × 25,000



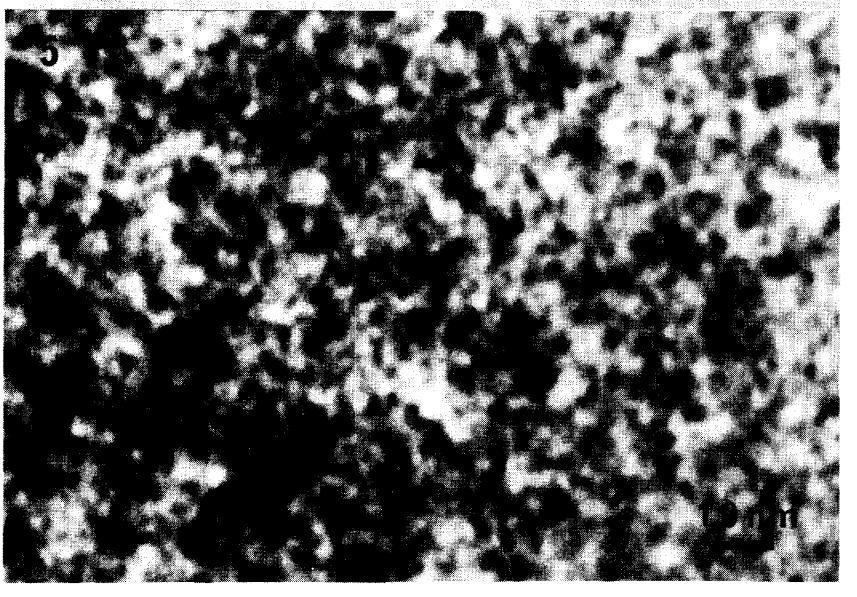


Fig. 4. Negative staining of unfixed rat GBM isolated by sonication with 1% PTA at low magnification. Note the folded broken paper-like appearance with a felt-like surface. × 28,500

Fig. 5. Negative staining of unfixed rat GBM isolated by sonication with 1% PTA at higher magnification. Note meshwork structure with numerous pores and strands. × 800,000

isolation of rat GBM. Pure glomeruli were isolated by the modified method of Spiro (15). Bowman capsules were easily separated by sieving, and pure glomeruli were obtained by repeated sieving (Fig. 1). Observation of ultrathin sections of pellets isolated by both methods confirmed the presence of pure basement membrane (Figs. 2, 3). GBM isolated by either method was characterized by long continuous sheets. Its thickness was relatively constant. The surface was homogeneous and appeared to have uniform electron density with fine fibrils embedded in an amorphorous structure. As the isolated GBM was ultrastructurally indistinguishable from its *in vivo* counterpart, the isolation procedures were considered to have caused almost no change.

GBM prepared by sonication. By negative staining, GBM was seen as fragments of various sizes. The fragments were usually triangular or square in shape. In larger fragments, GBM had a characteristic appearance of a linear contour and angular ends (Fig. 4). The surface of the membrane appeared to be felt-like at this magnification. Higher magnification of the basement membrane showed a spongy appearance. Still higher magnification (Fig. 5) revealed a meshwork composed of pores and strands. All the pictures taken at the original magnification of more than 20,000 showed the characteristic appearance of a meshwork, *i.e.*, a molecular sieve. Pores were oval or polygonal in shape and approximately equal in size in each fragment. Some of the pores were elongated and formed short channels. Strands were of similar width and formed a three-dimensional molecular sieve. The average diameter of forty strands measured was 3.1 ± 0.8 nm. The distributions of long and short dimensions measured on well-defined micrographs are shown in Fig. 6. The average long dimension of forty pores was 4.4 ± 1.0 nm and the average short dimension was 3.0 ± 0.6 nm. The average density of pores was $16 \pm 2/1000\ nm^2$. Fragments of GBM isolated without freezing also showed a molecular sieve of similar

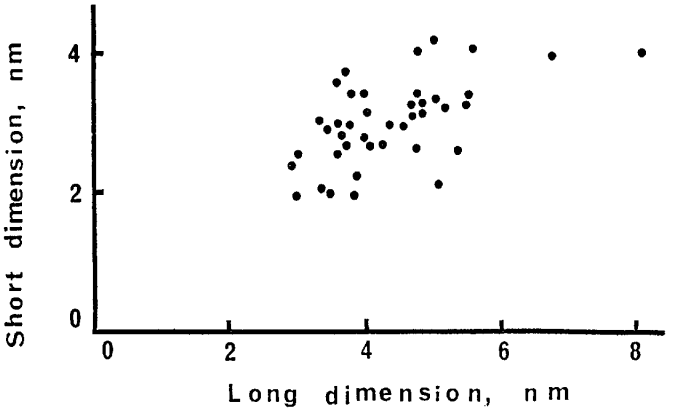


Fig. 6. Distribution of long and short dimensions of forty pores in rat GBM.

size. Stereoscopic observation clearly demohstrated the three-dimensional molecular sieve of GBM.

GBM prepared by detergents. These showed essentially the same morphological features as those isolated by sonication. Some of the fragments showed a rough or loosened appearance (Fig. 7). The molecular sieve may be damaged

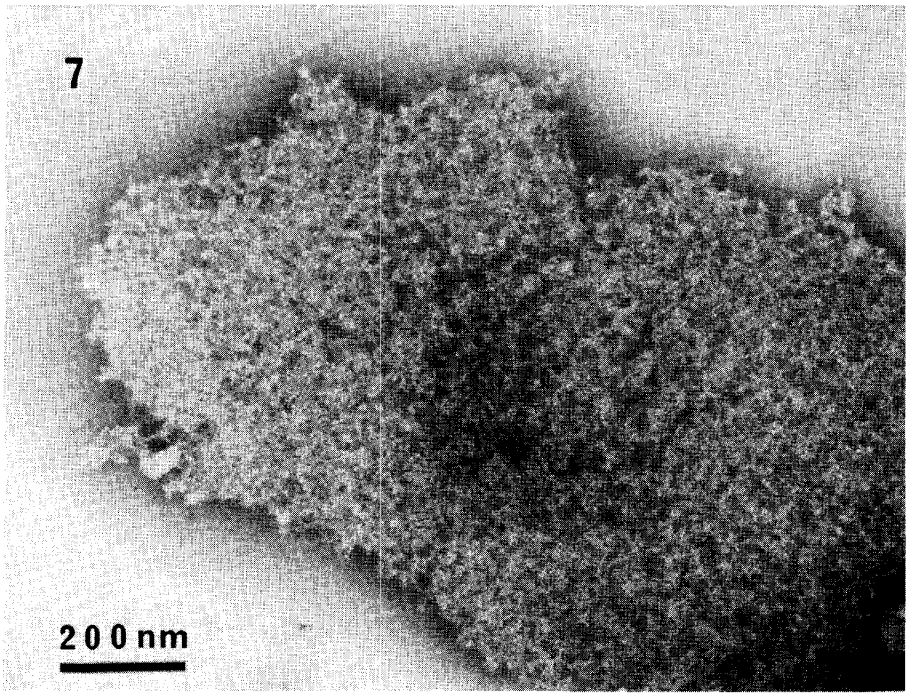


Fig. 7. Negative staining of unfixed rat GBM isolated by detergents with 1% PTA. Note rough and loosened surface. × 84,000

a little by the action of detergents. At higher magnifications a molecular sieve of equal size was observed.

Under different conditions of isolation the same structure was observed, so the ultrastructure of GBM is a molecular sieve composed of pores and strands.

DISCUSSION

Morphologists (1, 5, 6, 18-21) have failed to demonstrate pores in GBM because they observed GBM mostly by ultrathin sectioning. The resolving power of ultrathin sectioning is at most 10 angström owing to the influence of heavy metal staining or embedding materials. On the other hand, negative staining devised by Horne and Brenner (22) is capable of resolving particles separated only a few angström apart. As negative staining does not require

fixation, dehydration or embedding, specimens have less chance to degenerate.

Freeze-etching is also known to be useful for studying the ultrastructure of GBM. As previously reported (23), GBM as seen by freeze-etching consisted of fine granules 40 to 200 Å in diameter. As the water frozen within the tissue appeared as round particles in our experiment, freezing was not suitable for ultrastructural study. So, at present, negative staining is preferred for observing the molecular sieve of GBM, although GBM has to be isolated *in vitro*.

As it is hard to distinguish GBM from tubular basement membrane and Bowman's capsule, pure glomeruli had to be obtained. Pure glomeruli were isolated by a modification of the Spiro. As rat GBM isolated by both sonication and detergents showed the same morphological features as that observed *in vivo* by ultrathin sectioning, it is clear that pure GBM was obtained by both methods.

GBM are usually prepared from glomeruli using ultrasound (15, 24-26). Recently GBM were also isolated by the use of detergents (16, 27, 28). This time we also used the detergent method of Carlson *et al.* (16) in order to rule out changes in membrane structure during the isolation procedure of harsh sonication. With these two methods for isolating GBM from glomeruli, the same meshwork structure was observed. Langevelt *et al.* (17), comparing sonication and the detergent method for the isolation of bovine GBM favoured the detergent method on the grounds of morphological and chemical studies. However, from our experiments, sonication was better for the observation of ultrastructure of GBM, as the ultrastructure of the GBM was occasionally altered by detergents.

Rat kidneys and GBM were stored frozen. However, in another experiment to rule out the influence of freezing and thawing, GBM were isolated and observed with electron microscopy immediately after rats were sacrificed and without freezing the tissue. Whether frozen or not, the same meshwork was observed. These results prove that the ultrastructure of GBM is indeed a meshwork composed of fine strands with pores.

Exactly which structure in the capillary wall represents the primary filter retaining plasma protein in the circulation has been the source of controversy (29). After the failure to detect pores by direct morphological observation, attempts were made to localize the permeability barrier using tracer macromolecules of different size and electric charge. Farquhar *et al.* (1, 30) proposed that the basement membrane is the critical barrier. On the other hand, Karnovsky *et al.* (31, 32) suggested that the basement membrane represents only a crude filter, with the critical barrier being the slit pores. Latta *et al.* (33) suggested that the lamina rara interna is the restrictive barrier for serum albumin during normal flow. Recently Farquhar also agreed that the main barrier lies along the inner surface of GBM (34, 35). Slit diaphragms may play some role in filtration, but they do not seem rigid enough to be the main filter.

Though molecular electric charge has been stressed recently, molecular size is another main factor in the glomerular restriction of macromolecules. In normal glomerular filtrate, proteins the size of albumin or larger can not be demonstrated. According to hydrodynamic studies, bovine albumin is a prolate ellipsoid with major and minor axes of 14.0 and 4.0 nm (36). As the molecular weight of rat albumin is close to that of bovine, the size of the rat albumin molecule must be close to that of bovine. The sizes of the pores in this study are similar to the minor axis of the albumin molecule. This value supports the contention that GBM acts as a filtration barrier. Macromolecules may change or be deformed during filtration (37). In addition to molecular size, molecular charge (38-43) and hemodynamic factors (44) influence the transglomerular passage of macromolecules (45).

Filtration experiments have been made using isolated GBM (46-49). Gekle *et al.* (50) estimated a mean pore equivalent radius of 2.9 ± 1.0 nm. This value does not differ much from the values obtained in our experiment.

By the use of negative staining, we demonstrated pores in isolated and unfixed GBM which, so far, had only been postulated in theory. We demonstrated that GBM functions as a size barrier in the mechanical filtration of macromolecules.

Acknowledgments. This study was supported in part by a Research Grant for Glomerular Lesions from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, Japan.

The author acknowledges the photographic assistance of Mr. Takashi Nakamura, Mrs. Mutsuko Imajo and Mrs. Sumiko Kikuta. The author is deeply grateful to Tadashi Ofuji, president of Okayama University and to Prof. Zensuke Ota for their suggestions.

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