Glomerular permeability to macromolecules in the *Necturus* kidney

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Glomerular permeability to macromolecules in the *Necturus* kidney. *Am J Physiol Renal Physiol* 296: F1269–F1278, 2009. First published April 1, 2009; doi:10.1152/ajprenal.00371.2007.—Many aspects of the glomerular filtration of macromolecules remain controversial, including the location of the major filtration barrier, the effects of electrical charge, and the reason the filtration barrier does not clog.

We examined these issues in anesthetized *Necturus maculosus*, using fluorescently labeled probes and a two-photon microscope. With the high resolution of this system and the extraordinary width (~3.5 μm) of the glomerular basement membrane (GBM) in this salamander, we were able to visualize fluorescent molecules in the GBM in vivo.

GBM/plasma concentration ratios for myoglobin, ovalbumin, and serum albumin did not differ from that of inulin, indicating that the GBM does not discriminate among these molecules. The GBM/plasma concentration ratios for fluoresceinated dextran 500 and 2,000 kDa were significantly below that of inulin. Glomerular sieving coefficients (GSCs) for various macromolecules decreased as molecular mass increased, and the GSCs for bovine or human serum albumin were extremely low. The effect of electrical charge on filterability of a macromolecule was also examined. The GSCs for native (anionic) and neutral human serum albumin were not significantly different, nor did GSCs for anionic and neutral dextran 40 kDa differ, indicating that charge has no detectable effect on filterability of these macromolecules. These studies indicate that the main filtration barrier to albumin is the podocyte slit diaphragm. Electron microscopic studies revealed many cell processes within the GBM. Macromolecules that penetrated the GBM were taken up by mesangial cells and endothelial cells, suggesting that these cells help to prevent clogging of the filter.


Despite extensive study, the exact anatomic location of the major barrier to transglomerular passage of macromolecules remains uncertain. The size, shape, and deformability of a macromolecule are generally believed to influence passage of a macromolecule from the blood to the urinary space, but the effects of electrical charge are controversial. It is not completely settled, moreover, why the glomerular filter does not clog (8, 10, 11, 14–16, 21, 31, 40).

In our first experiments, we determined the GSCs of a variety of macromolecules ranging in size from inulin (molecular mass ~5 kDa) to dextran 2,000 kDa. We were able to quantify probe concentrations in the GBM region of the living kidney, which enabled us to make conclusions about the site of the main filtration barrier. Second, we examined the effects of a molecule’s electrical charge on its filterability. Third, we examined the long-term fate of macromolecules that had accumulated in the GBM.

MATERIALS AND METHODS

**Animals and surgical procedures.** Thirty-seven adult *Necturus maculosus*, mostly males, weighing 84–193 g, were obtained from Carolina Biological Supply (Burlington, NC) or Nasco (Oshkosh, WI) from November to May and were housed in tanks of aerated tap water at 4°C. They were fed earthworms. Anesthesia was induced by immersion in 660 mg tricaine methanesulfonate/tap water for 10–15 min and maintained by immersion of the head and gills in a 10-fold dilution of the same anesthetic in water bubbled with 100% oxygen. The animal was placed on an animal board on its back, and the exposed skin was covered with wet paper towels. A branchial artery was cannulated with polyethylene tubing (PE-10) for injection of saline and test molecules. We injected 1–2 ml 100 mM NaCl intraruminally to maintain hydration of the animal. The kidneys were exposed by incising both sides of the lower abdominal wall, tying the medial flap of skin and muscle (with the ventral abdominal vein) between two heavy ligatures, and cutting between the ligatures. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Indiana University School of Medicine Animal Care and Use Committee.

**Two-photon microscopy.** All fluorescence images were acquired using a Zeiss LSM 510 Meta confocal/multiphoton microscope system. A coverglass was placed on the surface of one kidney, and the animal board was secured to the stage of the Zeiss Axioplan-2 upright microscope. Observations were most often made using a ×40 Apochromat/1.2-numerical aperture (NA) water-immersion objective, but sometimes a ×63 C-Apochromat/1.2-NA water-immersion objective...
or × 200/75-NA dry objective was used. Fluorescence excitation, at a wavelength of 800 nm, was provided by a titanium-sapphire laser (Spectraphysics, Mountain View, CA). The internal (descanned) photodetectors were preceded by a 545-nm long-pass beam splitter and by 500- to 550-nm or 565- to 615-nm band-pass filters, coated with infrared blocker, for the “green” and “red” fluorescence channels, respectively.

Initially, a series of background images of a single renal corpuscle was collected at laser outputs between 10 and 70% and fixed detector gains. The laser output was changed using an acoustooptic modulator. The background, at the appropriate laser output, was subtracted from all subsequent intensity measurements when the data were analyzed. To determine how variable the background was in the same animal, we determined the background level in five renal corpuscles in one Necturus at a laser output of 30%. The mean backgrounds were 6.1 (SD 0.2) intensity units in the green channel and 4.7 (SD 0.4) intensity units in the red channel.

The fluorescently labeled molecules, dissolved in 100 mM NaCl, were usually injected as a bolus over a few minutes into an afferent branchial artery. In the case of inulin, a priming dose of 1.6–3.8 mg inulin/ml 100 mM NaCl solution at 1.2–2.3 ml/h. In the case of fluoresceinated inulin was followed by a constant intra-arterial infusion of a 0.3 mg inulin/ml 100 mM NaCl solution at 1.2–2.3 ml/h.

The doses of fluorescent molecules and laser outputs were usually adjusted to achieve average plasma intensity levels ~80–150 units in both channels, if possible. We avoided saturating the detectors (intensity level of 256 or greater). Images were collected from different glomeruli (usually 3–12 in a single experiment) and most often between 10 and 90 min after injection of the probe molecules. For all of the values reported, there were no changes in GSC with time after injection of the fluorescent probes. Images were collected only from glomeruli with blood flow, as indicated by the movement of red blood cells.

In three experiments, we injected 1.6–2 mg fluorescent dextran 500 kDa in ~0.5 ml sterile 100 mM NaCl into a branchial artery in lightly anesthetized Necturus using a 28-gauge needle. The animals were allowed to recover from the anesthesia and were kept in aerated water at 4°C for 2 days.

Quantitative image analysis. Fluorescence intensities in plasma, GBM, and the urinary space of Bowman’s capsule were measured using the Metamorph image-analysis system (Universal Imaging, West Chester, PA). The GBM region was identified in the outer wall of the glomerular capillaries either 1) by virtue of its distinctly lower than plasma fluorescence intensity or 2) as the middle region in between two thin gray lines that probably represent endothelial cells and podocytes, or by using both criteria when possible. The zoom function was used to facilitate manual tracing of the region in the middle of the GBM. The width of the GBM was also measured. The calculated GSCs always used measurements on urinary space and plasma in the same image, so differences in fluorescence intensity due to the depth of focus do not affect the results.

Fluorescent probes. The following fluorescent molecules were obtained from Molecular Probes-Invitrogen (Eugene, OR): 1) dextran, rhodamine B, 10 kDa (cat. no. D1824); 2) dextran, Texas red, 40 kDa (cat. no. D1829); 3) dextran, fluorescein, 40 kDa (cat. no. D1844); 4) dextran, fluorescein, 500 kDa (cat. no. D7136); and 5) dextran, fluorescein, 2,000 kDa (cat. no. D7137). “Red” dextran 500 kDa was prepared by labeling amino dextran (cat. no. D7144) with excess Texas red to cover essentially all of the amine groups. Fluoresceinated inulin (cat. no. F3272) was obtained from Sigma (St. Louis, MO). All fluorescent molecules were exhaustively dialyzed before injection, using the following molecular mass cutoff tubing: 3.5 for inulin and dextran 10 kDa, 12–14 for dextran 40 kDa, and 50–100 for dextrans 500 and 2,000 kDa.

The numbers of moles of fluorescein per mole inulin or dextran were ~1.4 for inulin, 5 for dextran 40 kDa, 7 for dextran, 90 for dextran 500 kDa, and 96 for dextran 2,000 kDa according to the supplier. The pK values for fluorescein are such that at the pH of Necturus plasma, each fluorescein is almost completely ionized and adds two negative charges (24). Therefore, the net charges on fluoresceinated inulin and dextran 40-, 500-, and 2,000-kDa molecules are almost –3, –10, –180, and –192, respectively. Dextrans labeled with rhodamine B or Texas red dyes are essentially neutral.

Neutral [isoelectric point (pI) = 7.8] human serum albumin was prepared from native (pI = 4.9) human serum albumin (cat. no. A3782, Sigma) exactly as described before (28). These two albumin molecules have essentially the same Stokes-Einstein radius, 3.50 and 3.55 nm, respectively (28). The neutral human serum albumin was labeled with Alexa Fluor 488 in three experiments and with Alexa Fluor 568 in two experiments; the native (anionic) human serum albumin was labeled with Alexa Fluor 488 in two experiments and Alexa Fluor 568 in three experiments. The particular fluorescent label had no impact on the results.

Purified (>99%) BSA was from Sigma (cat. no. A7638) and was labeled with fluorescein isothiocyanate (20). The fluoresceinated BSA was dialyzed using 12- to 14-kDa molecular mass cutoff tubing and then passed through a Sephacryl 100-HR gel filtration column using 100 mM NaCl/2 mM sodium azide as the vehicle. Peak fractions were collected, filter-sterilized, and stored at 4°C. On the experiment day, the BSA solution was dispersed several times with 100 mM NaCl in a 30-kDa nominal molecular weight limit (NMWL) Amicon ultra-15 centrifugal filter device (Millipore, Bedford, MA) to remove the azide and any small-molecular-mass impurities.

In early experiments with BSA, reported in an abstract in 2004 (43), we had used commercial fluoresceinated BSA (cat. no. A9771, Sigma) or fraction V BSA from Serological Proteins (Kankakee, IL) labeled with Alexa fluor dyes. The fluorescently labeled BSA solutions were dialyzed using 12–14 cutoff tubing to remove free dye but were not purified using column chromatography, were not preserved with sodium azide, and were not rinsed in a 30-kDa NMWL filter device. Purity of the fluorescent BSA is an important matter. Our early measurements of glomerular sieving of BSA were likely erroneously high because of the presence of impurities and are not included in the present report.

Ovalbumin from chicken egg (cat. no. A5503) was obtained from Sigma. Ovalbumin was mildly succinylated (anionized) as described by Qasim and Salahuddin (30) and Remke et al. (32). Ovalbumin, 100 mg, was dissolved in 40 ml, pH 7.4, PBS, and the pH was adjusted to 8.0 by adding 0.25 N NaOH dropwise. A 50-fold excess of succinic anhydride (11.2 mg) was added, and the reaction was allowed to proceed for 22 min, keeping the pH between 8.0 and 8.5. The material was then kept in the refrigerator overnight, followed by extensive dialysis against water or PBS using 12–14 cutoff tubing. Next, the material was concentrated by ultrafiltration in an Amicon ultra-15 10-kDa NMWL centrifugal filter device and passed through a Sephacryl 100-HR gel filtration column, using PBS as the vehicle. The column was calibrated with protein standards: BSA (radius = 3.55 nm) and myoglobin (radius = 1.90 nm). The peak fractions of the effluent were collected, sodium azide was added to achieve a concentration of 2 mM, and the samples were filter-sterilized (0.22-μm pore filter), and stored in a refrigerator at 4°C. Purity was checked by...
SDS-PAGE, and densitometric measurements indicated that 98–100% of the material in the fractions we injected was monomeric ovalbumin. The samples were reacted with Alexa Fluor 568. Aliquots of the fluorescent material were passed through the gel filtration column again to estimate the molecular size. The pI was measured using slab-gel isoelectric focusing. The peak fractions were rinsed several times with 100 mM NaCl and concentrated by ultrafiltration using an Amicon ultra-15 10-kDa NMWL centrifugal filter device before injection into the Necturus.

Cationic ovalbumin was prepared as described by Joh et al. (23). N-,N-1,3-dimethylpropanediamine (0.4 ml) was dissolved in 5 ml H2O, and the pH was adjusted to 6.5 or 7.5 with 6 N HCl. Ovalbumin (100 mg) was added, followed by 3.0 g 1-ethyl-3(3-dimethylaminopropyl) carbodiimide, and the pH of the mixture was kept constant for 4 h by adding 0.2 N HCl dropwise, with constant stirring. In the case of the pH 6.5 reaction, the material was then allowed to sit overnight at room temperature, but in the other case it was dialyzed immediately. The ovalbumin was then reacted with a 10-fold excess of fluorescein isothiocyanate (20). The material was subsequently dialyzed, concentrated by ultrafiltration in an ultra-15 10-kDa NMWL centrifugal filter device, and passed through a calibrated Sephacryl 100-HR gel filtration column, using 2 M NaCl as the vehicle. The peak fractions were rinsed with 100 mM NaCl and concentrated as described above.

In vitro detection of binding of cationized ovalbumin to serum albumin. We suspected that the cationized ovalbumin was bound to serum albumin in the plasma. To test this idea, we prepared a 0.1 mg/ml cationized ovalbumin solution in PBS (pH 7.44) with or without 10 mg/ml BSA. Four milliliters of both solutions (in quintuplicate) were spun in Amicon ultra-15 centrifugal filter devices with a NMWL of 50 kDa. We reasoned that if cationized ovalbumin were bound to albumin, then the combined complex would be too large to be appreciably filtered through a 50-kDa molecular mass cutoff membrane.

Structural studies. In one Necturus, we fixed the kidneys by perfusion with a 1% glutaraldehyde-Ringer solution (44). The kidneys were stored in 0.1 M cacodylate solution, pH 7.3. Part of the kidney was dehydrated through a series of graded ethanol concentrations to 100% ethanol before embedment in a 50/50 mixture of Paraplast Xtra (Fisher, Pittsburgh, PA) and Peel-away Micro-Cut (Polysciences, Warrington, PA) for light microscopic analysis. Sections were cut at 4 μm and stained with hematoxylin and eosin, periodic acid Schiff (PAS), or Jones’ silver stain for routine histological examination.

For transmission electron microscopy, kidney tissue was rinsed in phosphate buffer, dehydrated through a series of graded ethanol concentrations to 100% ethanol, passed through two changes of propylene oxide, and embedded as a single piece of tissue in Epon 812. Thick sections (~1 μm) were cut of two glomeruli and stained with toluidine blue. Subsequently, thin sections (~0.04 μm) were cut from each glomerulus, stained with uranyl acetate and lead citrate, and viewed on a FEI G2 Tecnai BioTWIN transmission electron microscope (FEI, Hillsboro, OR), equipped with an XR-60 Digital CCD system (AMT, Danvers, MA).

Statistical analyses. All data are expressed as means ± SD. Comparisons among different macromolecules were made using a one-way ANOVA with the Bonferroni correction for multiple comparisons. Comparisons between red and green fluorescent probes in the same glomerulus were made by paired t-tests. A P value <0.05 is considered significant.

RESULTS

Figures 1 and 2 show representative images from single glomeruli after injection of fluorescently labeled molecules: inulin and dextran 10 kDa (Fig. 1) and native chicken ovalbumin (Fig. 2). A remarkable finding is that the middle layer of the glomerular filtration barrier, the GBM, is discernible, and this allowed us to quantify probe concentrations in this region in vivo. The average thickness of the GBM was 3.5 μm (SD 1.5); n = 35 glomeruli.

A sequence of images (collected at 1/s over 2 min, but compressed to 20 s) may be viewed at the following website: http://www.nephrology.iupui.edu/~gtanner/necturusglomerulus.mpg. This sequence illustrates the flow of red blood cells through glomerular capillary loops. In this experiment, fluoresceinated inulin was given by constant intra-arterial infusion and its fluorescence is equal in the blood plasma and urinary space of Bowman’s capsule.

Table 1 summarizes results for a variety of macromolecules. As expected, GSC falls as molecular size increases, suggesting that size has the major impact on filterability. The concentration of large macromolecules within the GBM is surprisingly high. This suggests that the inner layers of the glomerular filtration barrier, the endothelial cell layer and the GBM itself, only weakly impede the transglomerular passage of large molecules. Furthermore, the sharp drop-off in concentration that occurs between the GBM and filtrate in Bowman’s capsule suggests that the podocyte layer is the major barrier restricting the passage of macromolecules.

Table 2 shows data related to the role of electrical charge in filtration of differently charged macromolecules studied simul-
Succinylated ovalbumin had a significantly lower GSC than native ovalbumin. The cationic molecule had a radius of 3.26 nm, suggesting that an increased negative charge might reduce the filterability of this molecule. Mild succinylation, however, was accompanied by a significant increase in molecular radius. The size of ovalbumin is in a critical region where small differences in size might produce large changes in GSC. Therefore, the lower GSC of the more negatively charged ovalbumin might be due to a larger size.

We compared the GSC of anionic (succinylated) ovalbumin (pI = 4.4) and heavily cationic ovalbumin (pI = 9.3) in the same glomeruli (Table 2). The cationic molecule had a radius of 3.16 nm (n = 3), not significantly different from that of succinylated ovalbumin. The cationic molecule bound heavily to the entire glomerular filtration barrier (Fig. 3).

Surprisingly, the GSC of the cationic ovalbumin was significantly (P < 0.01) less than that of the anionic ovalbumin. These results do not support the idea that a positive charge on a macromolecule increases its passage through the glomerular filtration barrier (1).

The observation that cationized ovalbumin avidly binds to tissue components suggested that its low filterability might be due to binding to serum albumin (or other proteins) in Necturus plasma. To test this hypothesis, we measured the sieving coefficient (filtrate/retenate concentration ratio) of cationized ovalbumin in the presence or absence of BSA in centrifuged filter tubes with a NMWL of 50 kDa. Sieving coefficients of cationized ovalbumin averaged 0.37 (SD 0.21) and 0.08 (SD 0.02) in the absence and presence of BSA (P < 0.005), respectively. Fluid filtration averaged 24% (SD 6) and 25% (SD 4) in the absence and presence of BSA, respectively, indicating no effect of BSA on fluid filtration. These results demonstrate that cationized ovalbumin is appreciably bound by serum albumin, and this likely contributed to its unexpectedly low GSC in vivo.

The GSCs of neutral (pI = 7.8) and native (pI = 4.9) human serum albumin were very low (GSCs <0.01) and did not differ from each other (P > 0.2) (Table 2). The concentrations of neutral and native human serum albumin in the GBM averaged 0.56 (SD 0.10) and 0.52 (SD 0.10) times the plasma concentra-

### Table 2. Effects of different electrical charge on a macromolecule on its GSC

<table>
<thead>
<tr>
<th>Molecule</th>
<th>GSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native ovalbumin</td>
<td>0.68±0.15 (10)</td>
</tr>
<tr>
<td>Succinylated ovalbumin</td>
<td>0.49±0.19 (10)</td>
</tr>
<tr>
<td>Difference</td>
<td>0.19±0.17 (10)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Succinylated ovalbumin</td>
<td>0.36±0.11 (9)</td>
</tr>
<tr>
<td>Cationic ovalbumin</td>
<td>0.12±0.10 (9)</td>
</tr>
<tr>
<td>Difference</td>
<td>0.24±0.18 (9)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Neutral human serum albumin</td>
<td>0.004±0.016 (34)</td>
</tr>
<tr>
<td>Native human serum albumin</td>
<td>0.007±0.017 (34)</td>
</tr>
<tr>
<td>Difference</td>
<td>−0.003±0.015 (34)</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
</tr>
<tr>
<td>Neutral dextran 40</td>
<td>0.26±0.07 (10)</td>
</tr>
<tr>
<td>Anionic dextran 40</td>
<td>0.29±0.11 (10)</td>
</tr>
<tr>
<td>Difference</td>
<td>−0.03±0.10 (10)</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD (number of glomeruli). Succinylation increases the net negative charge on ovalbumin. NS, not statistically significant. Comparisons between differently charged forms were made in the same glomerulus, and the data were analyzed by paired t-tests.

### Table 1. Glomerular permeability to macromolecules

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular Mass, Da</th>
<th>$r_n$, nm</th>
<th>[GBM]/[Plasma]</th>
<th>GSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>5,000</td>
<td>1.3</td>
<td>0.63±0.14 ($n = 15$)</td>
<td>1.03±0.14 ($n = 15$)</td>
</tr>
<tr>
<td>Dextran, neutral</td>
<td>10,000</td>
<td>2.7</td>
<td>0.82±0.17 ($n = 11$)</td>
<td>0.82±0.11 ($n = 12$)</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17,000</td>
<td>1.9</td>
<td>0.73±0.09 ($n = 7$)</td>
<td>0.80±0.09 ($n = 7$)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
<td>3.0</td>
<td>0.71±0.08 ($n = 5$)</td>
<td>0.65±0.14 ($n = 15$)</td>
</tr>
<tr>
<td>Serum albumin, bovine</td>
<td>66,000</td>
<td>3.6</td>
<td>0.66±0.15 ($n = 21$)</td>
<td>0.003±0.004 ($n = 23$)</td>
</tr>
<tr>
<td>Dextran, anionic</td>
<td>500,000</td>
<td>15.1</td>
<td>0.43±0.11 ($n = 14$)</td>
<td>0.048±0.018 ($n = 14$)</td>
</tr>
<tr>
<td>Dextran, anionic</td>
<td>2,000,000</td>
<td>27.7</td>
<td>0.17±0.07 ($n = 11$)</td>
<td>0.000±0.001 ($n = 11$)</td>
</tr>
</tbody>
</table>

Values are means ± SD; $n$, no. of glomeruli; $r_n$, Stokes-Einstein radius; GBM, glomerular basement membrane; GSC, glomerular sieving coefficient, i.e., concentration in urinary space of Bowman’s capsule divided by plasma concentration.
tration in 25 glomeruli. A paired \( t \)-test indicated that these values differ at the \( P = 0.06 \) level, almost statistically significant, suggesting that the negative charge on the native albumin molecule might slightly decrease its entry into the GBM. Figure 4 shows the appearance of fluorescent human serum albumin in a representative experiment.

The GSCs of neutral and anionic dextran 40 kDa were not significantly different (Table 2). We checked the molecular size of dextran 40 kDa on a calibrated Sephacryl 100-HR column, and both neutral and anionic dextrans had the same calculated Stokes-Einstein radius, 5.01 nm, which agrees exactly with the value predicted from a molecular mass of 40 kDa and published equations for converting molecular weight to radius for dextrans (29).

Figure 5 shows light and electron micrographs of the normal Necturus renal corpuscle. The renal corpuscle is unusually large and ovoid shaped (51). It possesses a vascular pole, and a urinary pole that connects to the proximal tubule via a neck segment that is lined by numerous flagella (Fig. 5A). The glomerular tuft is separated from the parietal epithelial cell layer by a wide urinary space of Bowman’s capsule. The highly attenuated parietal epithelial cells transition to the cuboidal cells of the neck segment. Serial sections of the glomerular tuft show that it is divided into several lobules with an intervening mesangium. The mesangial cells also connect capillary loops of a single lobule (Fig. 5, A and B).

The classic three layers of the filtration barrier are seen in histological sections of the Necturus glomerulus (Fig. 5). Cells of the first layer, the endothelial cells, have a rather flattened nucleus. The rest of the cell is extremely attenuated, with numerous irregularly shaped processes (Fig. 5D, double arrows) and fenestrae or openings of widely varying size.

The second layer, a very heterogeneous GBM, is positioned between the endothelial cells and podocytes and shows a lamina rara interna (subendothelial space), lamina densa, and lamina rara externa. The GBM varies greatly in both thickness and density, with the lamina rara interna showing the most variability. There are scattered cell processes of subendothelial cells (Fig. 5D, double arrowheads) and mesangial cells in this portion of the GBM, as well as filamentous structures. Near the podocytes, a dense, thin, lamina densa is easily identified. It shows a uniform thickness. Between the lamina densa and the podocytes is the lamina rara externa. It is uniformly thin, with minimal density.

Cells of the third layer, the podocytes, possess a round to ovoid cell body that branches into decreasing-sized processes or pedicels that eventually cover the entire GBM by a process of interdigitation. A filtration slit membrane is seen spanning between neighboring pedicels (Fig. 5D).

Staining of light micrograph sections of Necturus glomeruli with silver and PAS was weak, much less intense than seen with simultaneously processed rat kidney cortex tissue sections (data not shown). These results suggest a low glycosaminoglycan concentration in the GBM of the Necturus kidney.

We noted in some experiments, particularly late in the experiment (usually \( >1 \) h after fluorescent probe injection), that punctate areas of fluorescence appeared in the GBM, indicating trapping of filtered molecules. Figure 6, A and B, shows images collected \( \sim 40 \) min and \( 5 \) h after injection of fluoresceinated dextran 500 kDa into the circulation. The
appearance of punctate fluorescence in grazing sections through the filtration barrier is striking (Fig. 6B). The average distance between the centers of these fluorescent points was 3.5 μm (SD 1.1); n = 40. The fluorescent points occur most likely at the endothelial cell fenestrae, the path of least resistance for dextran to enter into the GBM. Two days after injection of Texas red-labeled dextran 500 kDa, the dye was seen in mesangial cells (Fig. 6C), which are highly branched phagocytic cells between the capillary loops, and in the glomerular capillary endothelial cells (Fig. 6D).

**DISCUSSION**

The *Necturus* kidney has several advantages for a study of glomerular permeability using in vivo microscopy. The glomeruli are unusually large and can be seen in a row right at the kidney surface (51). Individual glomerular capillaries and cells are also large, and, as we show here for the first time, the GBM can be discerned in vivo. Respiratory movements are not a problem. Experiments can be done at room temperature in this cold-blooded animal. Blood flow can be observed directly under the microscope and is typically well maintained. The two-photon system allowed us to compare simultaneously the permeability of two different fluorescent molecules in the very same glomerulus, thereby reducing experimental variability. This is especially important if the filtration rates in different glomeruli vary, because the rate of filtration of fluid affects the GSCs of macromolecules (28, 33). Numerous studies have used urinary excretion of test molecules to draw conclusions about glomerular permeability, but tubular reabsorption or metabolism of these molecules is frequently a major concern. Observations of normally functioning glomeruli provide the most direct approach to investigating glomerular permeability.

Total glomerular filtration rate (GFR) is much lower in amphibians than in mammals. In the *Necturus*, GFR averages about 0.02 ml·min⁻¹·100 g body wt⁻¹ (31, 42), whereas GFR in the rat is about 0.8 ml·min⁻¹·100 g body wt⁻¹ (31). For this reason, small macromolecules are cleared much more slowly from the plasma in the *Necturus*, and near steady-state plasma levels were achieved soon after intravascular injection of test molecules. The *Necturus* kidney possesses far fewer nephrons...
than a rat kidney, \(750\) vs. \(30,000\), respectively (31), so the average GFR per glomerulus is the same in both species, \(13\) nl\(\times\)min\(^{-1}\)\(\times\)100 g body wt\(^{-1}\). The GFR in amphibians, however, is more variable than it is in mammals, because changes in GFR are an important pathway for altering water excretion in response to changes in hydration state in amphibians.

**Overall permeability properties.** In a classic study, Bott and Richards (3) studied glomerular permeability to a number of different proteins in frog and *Necturus* kidneys. In Ringer-perfused kidneys (chiefly of *Necturus*), they sampled, using micropuncture, fluid from the urinary space of Bowman’s capsule, and analyzed this fluid for protein using an ultramicro-method. They state that the glomerular barrier was practically completely impermeable to horse serum albumin but did not report GSCs. Permeability appeared grossly to be related in an inverse fashion to the molecular mass (size) of the molecules tested. They recognized that factors in addition to size could also influence filterability.

Our measurements of glomerular selectivity in the in vivo blood-perfused *Necturus* kidney are in good agreement with findings from the Richards’ laboratory. Inulin is freely filterable, as expected (22, 31). The GSC for inulin averaged 1.03 in our experiments (Table 1), essentially identical to average values determined by kidney micropuncture in frog and *Necturus* kidneys, 1.02 (22) and 1.04 (2), respectively. Theoretically, the GSC for inulin should be slightly higher than 1.00, because the protein concentration of *Necturus* plasma is \(2\%\) (2), and inulin is dissolved only in the plasma water. Also, the addition of negatively charged fluorescein groups to the inulin molecule (charge about \(3/molecule\)) would be expected to increase the filtrate inulin concentration above that of plasma, due to a Gibbs-Donnan effect. The GSC of native ovalbumin averaged 0.65 in our in vivo experiments, essentially identical to the average value of 0.58 (SD 0.15) reported for the Ringer-perfused *Necturus* kidney (3).

Estimates of the GSC for serum albumin in rat kidneys vary 50-fold, from 0.0006 (28, 45) to 0.03 (36), depending on the method used. Considering the magnitude of glomerular filtration rate and the high albumin concentration in the serum of mammals, such differences in GSC would have a major impact on the magnitude of tubular protein reabsorption. In the *Necturus* kidney, we found an average GSC of 0.003 (Table 1) and 0.007 (Table 2) for native bovine and human serum albumins, respectively. In earlier experiments (43), we reported a 10-fold higher GSC for BSA, but we suspect that this was mainly the result of impurities in the fluorescent albumin preparation (see MATERIALS AND METHODS). The present results indicate that the GSC for serum albumin is in fact quite low in the *Necturus* kidney. We recently found that the GSC for rat serum albumin in the rat kidney, as determined by two-photon microscopy, is also extremely low, \(0.002–0.004\) (41).
The GSCs of dextrans 40 and 500 kDa (Tables 1 and 2) are many times higher than values reported for the rat (48). Lambert (25) suggested that the salamander (or amphibian) glomerulus may be slightly more permeable to large molecules than is the mammalian glomerulus. Heterogeneity of the dextran preparations we used, however, likely also contributed to our high values. The dextran 40 kDa preparations were dialyzed using 12- to 14-kDa cutoff tubing, so the final samples contained material with a molecular mass below 40 kDa. Similarly, dextran 500 kDa was sometimes dialyzed using 50-kDa cutoff tubing, and probably contained significant quantities of dextrans with a smaller size than average. The dextrans are long, slender, and deformable molecules (48) and may replete through the glomerular filtration barrier.

Effects of electrical charge. The effect of electrical charge on the glomerular filterability of macromolecules remains controversial, with the majority of studies suggesting that net negative charge on a macromolecule reduces filterability and that net positive charge increases filterability (4, 6, 21). This effect has been attributed to the fixed negative charges associated with all three components of the filtration barrier. A few authors contend that charge plays no role (8, 9, 11, 35, 38). A possible problem with some of the studies which report that charge is important for glomerular filterability is that urinary excretion or kidney tissue uptake of proteins was measured, and the tubules reabsorb filtered proteins in a charge-dependent way (7). Positively charged proteins are more completely reabsorbed than are negatively charged proteins. Yet another problem is binding of probes to plasma proteins. Dextran sulfate, a molecule that was originally used to demonstrate charge selectivity (6), is now known to be bound to plasma proteins in a size-dependent manner (19). Finally, glomerular endothelial cells may modify the test molecules, so that molecules excreted in the urine are different from what was injected into the circulation (49). The shortcomings in some of the original studies on charge selectivity have been emphasized by Comper and colleagues (8, 9, 11).

In our initial studies with ovalbumin, we found that addition of negative (succinyl) groups to ovalbumin resulted in a lower GSC (Table 2). Molecular size, however, was also increased. It is often the case that when charged groups are added to a globular protein, such as ovalbumin, the effective molecular radius increases, because the molecule unravels to some degree (30). Ovalbumin is of such a size that a small change in size may profoundly affect its GSC. These experiments, therefore, did not convincingly demonstrate that addition of negative charges reduced transglomerular passage of ovalbumin.

Results with cationic ovalbumin were also problematic. The GSC of cationized ovalbumin was less than that of anionic ovalbumin, opposite of what would have been predicted by those who have suggested that a positive charge facilitates filtration (4). We demonstrated, however, that the low filterability of cationized ovalbumin was due to binding to serum albumin.

We did not detect a difference in the GSCs of native, anionic (pI = 4.9), and neutral (pI = 7.8) human serum albumin (Table 2). Because these measurements were variable, we cannot rigorously exclude an effect of net charge. Passage of neutral serum albumin into Bowman’s space was 99.6% restricted by the glomerular filtration barrier (GSC = 0.004), so net charge on albumin, even if it might be a factor affecting transglomerular passage, must play a very small role compared with other factors, such as molecular size. In the rat kidney, some investigators (28, 34) have reported that net charge on albumin influences glomerular sieving of this molecule, but others (11) have concluded that there is no charge selectivity for glomerular filtration of globular proteins. The absence of a clear charge effect in the Necturus kidney might be a consequence of a lower concentration of negatively charged proteoglycans in the Necturus glomerulus, as suggested by our PAS and silver staining studies. The extremely low filterability of serum albumin, irrespective of net charge, indicates that molecular size is clearly the major factor that limits filtration of this molecule.

We observed no difference between the GSCs of neutral or anionic dextran 40 kDa (Table 2). These results agree with recent findings by Yu et al. (52) in the rat kidney in which the same molecules were used. Venturioli and Rippe (48) reviewed glomerular sieving data for polysaccharide molecules and concluded that charge effects are often not demonstrable or are relatively small. They contend that the effects of altered charge on a molecule could be related more to changes in configuration and deformability of the molecule, and not to an electrical interaction with charges in the glomerular filtration barrier. Overall, our experiments provide no evidence for significant charge discrimination in the Necturus kidney glomerulus. We agree with the conclusion (48) that charge selectivity has been overrated in the last few decades.

Location of filtration barriers. The question, “What is the primary barrier to glomerular filtration of macromolecules?” has engendered considerable controversy. Many early studies, in rodent kidneys, identified the GBM as the main barrier (15), but more recent studies suggest that the podocytes and their slit diaphragms are most important in retaining plasma proteins in the circulation (46). Early studies that had attempted to localize the barrier to filtration of macromolecules used electron microscopy on tissue specimens collected from fixed kidneys. Electron-dense test molecules (such as ferritin) or molecules that could yield an electron-dense histochemical reaction product were used. For example, in the rat, it was observed that serum albumin was largely confined to the glomerular capillary lumen, with only small amounts detectable in the inner part of the GBM and none deeper in the GBM or in the urinary space (37). Possible criticisms of these studies include delays in fixation of the tissues (which could lead to diffusion of the probes from their original locale), binding to tissue components, or displacement or loss of probes during tissue processing. Recent studies in humans and laboratory mammals have demonstrated that a defect in the glomerular slit diaphragm protein nephrin leads to massive proteinuria, strongly supporting the conclusion that the podocytes are a major filtration barrier (46).

We report here, for the first time, in vivo measurements of the concentrations of filtered macromolecules within the GBM. These measurements were possible because of the unusually great thickness of the GBM in the Necturus kidney and the high resolution of the two-photon microscope system. GBM thickness averaged 3.5 μm in the Necturus kidney, ~10–20 times the GBM thickness in mammalian kidneys (26). All structures in the Necturus kidney (glomeruli, tubules, blood vessels, and individual cells) are unusually large for a vertebrate, and this is the major reason this animal has been a popular subject for physiological studies. Our histological studies confirm that the GBM is unusually wide in this animal. With the electron microscope, we saw that the GBM region contained many cellular processes (see, for example, Fig.
5D) that are too small to be resolved by two-photon microscopy. These processes belong to mesangial cells or subendothelial cells, a cell type also seen in the bullfrog kidney (39). Because of these cell elements in the GBM region, fluorescence intensity measurements in the matrix of the GBM are underestimated in our study.

We observed that even though inulin was freely filtered across the glomerular filtration barrier, its concentration in the GBM was 37% lower than in plasma or Bowman’s space; i.e., the GBM/plasma concentration ratio averaged 0.63 (Table 1). This lower ratio most likely reflects the presence of gel, fibrous, and cellular elements in the GBM region which exclude macromolecules. The GBM/plasma concentration ratios for proteins from myoglobin to BSA averaged −0.7 (Table 1), and were not significantly different from that of inulin. These findings indicate that the inner layers of the filtration barrier, i.e., the endothelial cell layer and the GBM, do not discriminate among these molecules. On the other hand, dextran 10 kDa appeared to have a significantly higher GBM/plasma concentration ratio than that for inulin, 0.82 (P < 0.01), and large, highly anionic molecules, fluoresceinated dextran 500 and 2,000 kDa, had significantly lower ratios, 0.43 and 0.17, respectively (P < 0.001) (Table 1). It is possible that the higher ratio for dextran 10 kDa could be due to the fact that this molecule is neutral, whereas fluoresceinated inulin is anionic. The low ratios for dextran 500 and 2,000 kDa indicate restricted entry into the GBM, probably because of their large size and highly anionic nature. For serum albumin, there is a steep fall in concentration between the GBM and urinary space of Bowman’s capsule (Table 1), suggesting that the podocyte slit diaphragms are the main barrier to filtration of this molecule.

Our findings strongly support earlier conclusions from electron microscopic studies by Schaffner and Rodewald (39) in the bullfrog kidney. They reported that the lamina rara interna in this amphibian kidney was often greatly enlarged and was infiltrated by cellular elements. The large anionic molecule ferritin (molecular mass 480 kDa) readily penetrated the GBM; it was found throughout the GBM and was often found in clusters adjacent to slit diaphragms. These authors concluded that the slit diaphragms are the principal filtration barrier to ferritin and smaller plasma proteins in the bullfrog kidney. Our results in the Necturus, using different probes and methods, are consistent with the findings in the frog.

It should be noted that the GBM is usually depicted as consisting of three layers: lamina rara interna, lamina densa, and lamina rara externa (26) (Fig. 5). This appearance, however, may be an artifact that results from dehydration of the tissue with organic solvents (such as ethanol) during conventional processing for electron microscopy. If the tissue is gently dehydrated by freeze-substitution, then the GBM appears as a single layer and this probably corresponds to the living state (5). Thus it is unlikely that there was a barrier within the GBM (a lamina densa), which we could not resolve by fluorescence microscopy, that might have restricted filtration of macromolecules.

Deen et al. (12–13, 21) have championed the view that all components of the filtration barrier can influence to some extent the glomerular filtration of macromolecules and the three components act in concert. The relative importance of different components might differ in amphibian (mesonephric) and mammalian (metanephric) kidneys. In the Necturus kidney, the GBM is much wider, but less compact and uniform, and it probably has a lower negative charge density than in mammalian kidneys. In the amphibian kidney, the endothelial cell layer and GBM do not appreciably restrict filtration of serum albumin, and, therefore, the podocyte cell layer must constitute the major barrier for this molecule. In the mammalian kidney, the GBM consists of a tighter, stronger meshwork of fibers, which may be an adaptation to much higher glomerular capillary hydrostatic pressures. Consequently, the GBM in mammals may assume a significant role in restricting the transglomerular passage of serum albumin, even though the podocytes can function as a major filtration barrier.

**Accumulation of filtered molecules in the GBM.** The kidney glomerulus filters large quantities of fluid, yet does not appear to clog. Results in the present study indicate that large molecules such as dextran 500 kDa may visibly accumulate in the GBM after circulating in the plasma for more than an hour or so. The punctate appearance of fluorescence in the GBM (Fig. 6) strongly suggests that these molecules accumulate beneath the endothelial fenestrae, since this would be the path of least resistance for fluid filtration. Smithies (40) recently suggested that the GBM is the major barrier to filtration and, because of its gel-like properties, helps to prevent clogging of the filter. According to his model, however, the fraction of the gel space available to serum albumin is only 0.02; this value is far below the serum albumin concentration we observed in the Necturus GBM. Furthermore, Smithies’ model assumes that the podocyte slit diaphragms do not act as a molecular sieve.

Cellular components of the glomerulus have the ability to take up large molecules, and may keep the filter clean. Vyas et al. (49) suggested that glomerular endothelial cells take up and process anionic dextrans in the rat kidney. In the mouse, at least under pathological conditions, glomerular endothelial cells have been shown to phagocytose immunoglobulins trapped in the GBM (47). In the Necturus kidney, we observed accumulation of dextran 500 kDa around the nucleus (i.e., in the cytoplasm) of endothelial cells 2 days after administration of this molecule (Fig. 6D). Farquhar and Palade (17) and Lewis et al. (27) found that podocytes phagocytosed proteins or protein complexes that had deposited in the GBM in the rat kidney. It has also been suggested that mesangial cells keep the glomerular filter clean by ingesting trapped macromolecules (18). Indeed, we saw accumulation of dextran 500 kDa in these cells in the Necturus kidney (Fig. 6C). Thus glomerular cells may clean the filtration barrier and keep it from clogging with filtration residues. In addition, simple diffusion of accumulated macromolecules out of the GBM would reduce clogging (14).

**Conclusions**

The major barrier to glomerular filtration of macromolecules such as serum albumin is at the level of the podocytes and their slit diaphragms in the amphibian (Necturus) kidney. We found no compelling evidence that a negative electrical charge reduces the filterability of macromolecules. Cellular elements in the glomerular filtration barrier appear to take up macromolecules that have accumulated in the GBM and in this way may help to prevent clogging of the filter.

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