A quantitative analysis of the glomerular charge barrier in the rat

JENNY SÖRENSSON,1 MARIA OHLSON,1 AND BÖRJE HARALDSSON1,2
Departments of 1Physiology and 2Nephrology, Göteborg University, SE-405 30 Gothenburg, Sweden
Received 8 August 2000; accepted in final form 5 December 2000

Sörensson, Jenny, Maria Ohlson, and Börje Haraldsson. A quantitative analysis of the glomerular charge barrier in the rat. Am J Physiol Renal Physiol 280: F646–F656, 2001.—Modifying the ionic strength (I) is a gentle way to alter charge interactions, but it cannot be done for studies of the glomerular sieving of proteins in vivo. We therefore perfused 18 isolated rat kidneys with albumin solutions of different ionic strengths at a low temperature (cIPK) to inhibit tubular uptake and protease activity. Four anionic proteins were studied, namely albumin (Alb), orosomucoid (Oro), ovalbumin (Ova), and anionic horseradish peroxidase (aHRP), together with the neutral polymer Ficoll. With normal ionic strength of the perfusate (152 mM), the fractional clearance (θ) was 0.0018 ± 0.0003 for Alb, 0.0033 ± 0.0003 for Oro, 0.090 ± 0.008 for Ova, and 0.062 ± 0.002 for aHRP. These θ values were all lower than for Ficoll of similar hydrodynamic size; e.g., θFicoll36 Å was >20 times higher than θ for albumin. Low ionic strength (34 mM) increased size selectivity as θ for anionic proteins and Ficoll fell, suggesting a reduction in small-pore radius from 44 ± 0.4 to 41 ± 0.5 Å, P < 0.01. In contrast, low I reduced the charge density of the membrane, ω, to one-quarter of the 20–50 meq/l estimated at normal I. These dynamic changes in ω seem to be due to volume alterations of the charged gel, fluid shifts that easily are accounted for by the changes in electrosmotic pressures. The finding that low ionic strength induces inverse effects on size selectivity and charge density strongly suggests that separate structures of the glomerular wall are responsible for the two properties.

capillary permeability; kidney glomerulus physiology; charge; endothelium

There is an ongoing debate as to how the restriction of molecules is carried out in the kidney. In several studies using neutral and charge-modified dextran polymers, the glomerular barrier has been shown to be both size and charge selective (2, 5). A significant charge barrier was also found for horseradish peroxidase (HRP) of different net charges (42). These classic studies suggest a charge density of 120–170 meq/l (10). However, the validity of the dextran data has been questioned, because some fractions of sulfated dextran seem to bind to glomerular cells (8) or to plasma proteins (13). Also, the data based on HRP protein clearance (42) have been criticized because of the effects of tubular degradation (39). The term “charge selectivity” refers to the phenomenon in which the fractional clearance (θ) of anionic macromolecules is less than that of otherwise uncharged solutes under similar hemodynamic conditions.

Several recent studies support the classic view of glomerular charge selectivity, albeit with a smaller charge density. Thus the θ of neutral and anionic myoglobin in rats in vivo suggests a charge density of 32 meq/l (54). We found similar results in isolated rat kidneys (IPK) perfused at low temperature (cIPK) for HRP (50), lactate dehydrogenase (28), and albumin (33).

Not all investigators share the notion of a significant glomerular charge barrier (8). Indeed, there are reports suggesting the glomerular barrier to be just as permeable as capillaries in skeletal muscle (40). However, the latter observation was performed in IPK at 37°C using lysine or NH4Cl to inhibit tubular activity, a procedure that recently has been shown to affect permeability per se (33). Another important factor for transglomerular passage is the shape of a molecule (38). In fact, elongated solutes have much higher θ than spherical molecules of similar size and charge (27, 35).

In a previous paper we studied the θ of anionic (aHRP) and neutral HRP. Neutral HRP had a θ twice as high as that of the aHRP of similar hydrodynamic size, supporting significant charge selectivity (50). In that study, we reduced the ionic strength of the perfusate without affecting osmolarity. As expected, the θ for aHRP fell during perfusion with low I, but the effects were rather small and fully reversible. The θ for neutral HRP was unaffected by a reduction in ionic strength.

In the present study, we wanted to quantify the glomerular charge density using several different anionic proteins and neutral spherical Ficolls of similar hydrodynamic sizes. The use of a broad fraction of Ficoll also allowed pore analysis, thus giving a detailed description of glomerular size selectivity. The experiments were conducted in isolated rat kidneys perfused at low temperature to reduce tubular cell and protease activity. The experimental model also allows more dramatic alterations of perfusate composition than would be possible in vivo. Thus increasing or reducing perfusate ionic strength, without affecting osmolarity, would

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be expected to markedly affect charge interactions, in line with the everyday experience of ion exchange chromatography. The two main questions at issue were the following. First, is there any evidence for charge selectivity when studied by using a variety of anionic proteins? Second, could the charge and/or size selectivity be affected by alterations of ionic strength?

**METHODS**

A total of 18 female rats of Wistar strain (Møllegaard, Stensved, Denmark) were used in the study. The rats were kept on standard food and had free access to water before the experiments. Anesthesia was induced by intraperitoneal injection of pentobarbitone (50 mg/ml, Apoteksbolaget, Umeå, Sweden). The tail artery was cannulated for recordings of arterial pressure, also serving as a route for subsequent administration of drugs. The body temperature of the rat was kept at 37°C during the preparation by means of a thermostatically controlled heating pad. The total preparation time was ~1 h. The local Ethical Committee approved the experiments.

**Kidney Perfusion Technique**

We used a modification of the isolated perfused rat kidney preparation described by Johnsson and Haraldsson in 1992 (19). Care was taken not to touch the kidneys during the preparation, and the kidneys were fully perfused with either blood or perfusate during the entire preparation. The temperature of the perfusates was kept at 8°C to inhibit tubular function as well as energy consumption and myogenic tone (6, 11) without altering capillary permeability (33, 45).

**Perfusates**

Modified Tyrode solutions containing human albumin (18 g/l, Immuno, Vienna, Austria), containing orosomucoid (15) were used to perfuse the kidneys. The perfusates differed in ionic strength, the standard being 152 mM, the low ionic strength 34 mM, and the high ionic strength 292 mM. All the perfusates were kept at the same high osmolality (590 mosmol/kgH2O) to enable variations in ionic strength without altering osmolality. This was obtained by adding mannitol, which has also been shown to prevent against acute renal failure (14) and act as a free radical scavenger (16). The composition of the standard perfusate was as follows: (in mM) 148 Na, 4.3 K, 2.5 Ca, 131 Cl, 0.8 Mg, 25 HCO3, 0.5 H2PO4, 5.6 glucose, and 285 mannitol. The low-ionic-strength perfusate contained (in mM) 26 Na, 4.3 K, 2.5 Ca, 8.4 Cl, 0.8 Mg, 25 HCO3, 0.5 H2PO4, 5.6 glucose, and 559 mannitol. High-ionic-strength perfusate contained (in mM) 284 Na, 4.3 K, 267 Cl, 2.5 Ca, 0.8 Mg, 25 HCO3, and 0.5 H2PO4. In addition, nitroprusside (0.27 g/l, Merck, Darmstadt, Germany) and furosemide (10 mg/l, Benzon Pharma, Stockholm, Sweden) were added to the perfusates to keep the temperature of the perfusates was kept at 8°C to inhibit tubular function as well as energy consumption and myogenic tone (6, 11) without altering capillary permeability (33, 45).

**Tracers**

The physicochemical properties of the molecules used as tracers are reviewed in Table 1.

**aHRP.** HRP is a well-characterized protein with a molecular mass of 40 kDa. Neutral HRP (303 U/mg, Genzyme Biochemicals, Maidstone, UK) was charge modified by succinylation (25, 37, 43) as previously described (50) to obtain an anionic form. The modified molecules were tested and compared with neutral HRP by agarose gel electrophoresis. There was a marked alteration of the charge, and the resulting aHRP seemed to be homogenous. The material was dialyzed and freeze-dried. The two HRP proteins had similar molecular radii as determined by gel filtration on a Superose 12 PC 3.2/30 column (SMART HPLC, Amersham Pharmacia Biotech), using 50 mM phosphate buffer with 115 mM sodium chloride and pH 7.0 as the mobile phase. The PI was 7.3 for neutral HRP and <3.5 for aHRP as determined by isoelectric focusing. The labeling of aHRP was done by using the chloramine-T method. The activity concentration was 7.8 MBq/ml for anionic 125I-labeled HRP. The net charge was ~6 as judged from the reduced electrophoretic mobility after this labeling method was used. Analyses were performed on a Superdex 200 HPLC column (Amersham Pharmacia Biotech), whereby a 0.1 M phosphate buffer (pH 7.4) was used as the mobile phase. There was no measurable change in molecular size after the iodination.

**Ovalbumin.** Ovalbumin (Sigma) has a molecular mass of 43 kDa. The ovalbumin was labeled with 131I by using N-succinimidyl-3-trimethylstannanyl) benzoate, and the activity concentration was 9.4 MBq/ml. The tracer analysis was done as for aHRP above.

**Orosomucoid.** The serum protein orosomucoid (kindly provided by Immuno) has a molecular mass of 40 kDa. The labeling with 125I was made by using iodogen, and the activity concentration was 4.6 MBq/ml. The tracer analysis was done as for aHRP above.

**Ficoll.** The neutral polymer Ficoll labeled with FITC was used in molecular radii of 14–70 Å. Ficoll is a spherical molecule (frictional ratio close to 1.0) and had no electrophoretic mobility after labeling with FITC (33).

**Table 1. Summary of the physicochemical properties of certain proteins used as tracers**

<table>
<thead>
<tr>
<th>Proportion</th>
<th>Albumin</th>
<th>Ovalbumin</th>
<th>Orosomucoid</th>
<th>aHRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kDa</td>
<td>69</td>
<td>43</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Free diffusion constant at 20°C, cm²/s × 10⁻⁷</td>
<td>6.0</td>
<td>7.8</td>
<td>5.27</td>
<td>7.05</td>
</tr>
<tr>
<td>Stokes-Einstein radius, Å</td>
<td>35.5</td>
<td>27.4</td>
<td>40.5</td>
<td>32</td>
</tr>
<tr>
<td>Sedimentation constant at 20°C, S</td>
<td>4.6</td>
<td>3.27</td>
<td>3.5</td>
<td>3.48</td>
</tr>
<tr>
<td>Axial ratio</td>
<td>3.5</td>
<td>2.7</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Fractional ratio</td>
<td>1.28</td>
<td>1.16</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.55</td>
<td>5.19</td>
<td>2.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Net charge</td>
<td>–23</td>
<td>–13</td>
<td>–24</td>
<td>–6†</td>
</tr>
</tbody>
</table>

aHRP, anionic horseradish peroxidase. †Net charge for orosomucoid was estimated from the protein chain net charge of -9 and 15 silic acid residues, i.e. -24. †After iodination of aHRP using the chloramine-T procedure, the electrophoretic mobility was reduced by half, suggesting a net charge of -6. See Refs. 4, 12, 22, 26 and 41.
**Experimental Protocol**

**Group A.** Nine rats, weighing 240 ± 4 g, were used in the first set of experiments by using $^{125}$I-aHRP and albumin as tracers. After preparation, perfusion started with the standard solution, and then low ionic strength was introduced followed by high ionic strength and finally back to standard again. The low- and high-ionic-strength values were compared with the mean value of the two standard perfusion periods. The total perfusion time was ~1 h. During each perfusion period in the first set of experiments, three urine samples were collected for determination of glomerular filtration rate (GFR) and θ for aHRP and albumin. The pressure was maintained constant. The urine and perfusate samples were subjected to gel filtration on a Sephadex PC 3.2/10 column (SMART HPLC, Amersham Pharmacia Biotech) for assessment of tracer-bound radioactivity shortly after each experiment. Radioactivity was determined by analysis of the amounts of $^{125}$I-aHRP in a gamma counter (Cobra, Auto-Gamma Counting systems, Packard Instrument, Meriden, CT). Corrections were made for background activity. Albumin concentrations were measured by radioimmunassay (PharmaciaUpJohn Diagnostics, Sverige, Uppsala, Sweden).

**Group B.** In the second set of experiments with orosomucoid, ovalbumin, albumin, and Ficoll (14–70 Å) as tracers, we used nine rats weighing 263 ± 4 g. The kidneys were perfused with standard perfusate, low ionic strength, standard, high ionic strength, and finally the standard perfusate again. Low-ionic-strength values were compared with a mean value of the first and second standard period and high ionic strength to a mean value of the second and third standard perfusion. Pump flow was changed to maintain a constant perfusion pressure. Otherwise, the urine and perfusate samples were handled in the same way as in the first set of experiments. The amount of tracer-bound radioactivity was determined in a gamma counter (Cobra, Auto-Gamma Counting systems, Packard Instrument). Corrections were made for background activity, decay, and spillover from $^{131}$I to $^{125}$I.

**Analysis of Ficoll Concentrations**

For calculation of the sieving coefficients for FITC-Ficoll, perfusate and urine samples were subjected to gel filtration (BioSep-SEC-S3000, Phenomenex, Torrance, CA) and fluorescence detection (Dionex fluorimeter RF-2000, Dionex Softron, Germering, Germany) by using Chromeleon (Gynkotek, Germering, Germany) software. A 0.05 M phosphate buffer (Cobra, Auto-Gamma Counting systems, Packard Instrument) by using Chromeleon (Gynkotek, Germering, Germany) software. A 0.05 M phosphate buffer (Cobra, Auto-Gamma Counting systems, Packard Instrument) was assumed to be 1.15. We have performed numerous cIPK experiments at 8°C with Cr – EDTA, and the inhibition of tubular reabsorption is so reproducible that a constant value of 1.15 is justified (27, 15, 19).

**Fractional clearance for albumin, orosomucoid, and ovalbumin.** The renal clearance, Cl, for a molecule X is given by an equation analogous to that for calculation of GFR, that is, Cl = $(C_l/C_p)_{Cr−EDTA}Q_l$. The fractional clearance, θ, of a solute X is given by dividing its clearance with GFR giving the following simple equation

$$\theta = \frac{(C_U/C_P)_X}{(C_U/C_P)_{Cr−EDTA}}$$

(1)

**Charge Density**

We determined the charge density of the membrane during perfusion with solutions of different ionic strengths. As previously reported, this was done by using equations to describe the glomerular membrane as a gel with homogenous distribution of fixed charges (50). Such a model represents an oversimplification for several reasons, as outlined by Deen et al. (10). However, an extended and more precise analysis is presented in the APPENDIX. Briefly, the analysis is reduced to three fundamental equations with three unknown parameters, namely, the distribution of sodium (Na), chloride (Cl), and fixed charge, as well as the ion concentration of sodium (or chloride) ions in the two compartments

$$\Delta E = \frac{RT}{zF} \ln \frac{N_{aP}}{N_{agol}} = \frac{RT}{zF} \ln \frac{Cl}{Cl_{pol}}$$

(2)

where z is the valence of Na (+1) and Cl (−1), the subscript p denotes the concentrations of Na and Cl in plasma, R is the gas constant, and T is the absolute temperature. The conditions are further simplified by the assumption that sodium and chloride are the only electrolytes present, thus leaving the small contribution of other ions out of the analysis. Moreover, Na equals the sum of Cl and the negative charge due to albumin in the perfusate. The first of the fundamental equations is obtained by rearranging Eq. 2

$$N_{aP}Cl_{pol} = N_{agol}Cl_{pol}$$

(3)

The second relationship is obtained as a result of the rules of electroneutrality. Thus in a membrane with a negative charge density of ω, the positive and negative charges must be in balance. It therefore follows that

$$Cl_{pol} = N_{agol} − ω$$

(4)

Finally, the partitioning of two differently charged solutes of identical size is related to the concentrations of chloride in the perfusate and membrane. Thus the gel-to-plasma concentration ratio of a solute, X, with a net charge of $z_x$ equals

$$\frac{X_{pol}}{X_p} = \left(\frac{Cl_{pol}}{Cl_{p}}\right)^{-z_x}$$

(5)
and Nagel, and Cgel, are readily obtained by solving Eqs. 3 and 4. We have found that the following ratios between gel and those in plasma will give rise to an electroosmotic effect on aHRP (Table 3).

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### Table 2. Summary of values for recorded parameters during the 3 perfusion periods

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>Normal I</th>
<th>Low I</th>
<th>High I</th>
<th>Normal I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pump flow, ml/min</td>
<td>7.3 ± 0.6</td>
<td>7.4 ± 0.5</td>
<td>7.3 ± 0.6</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>Perfusion pressure, mmHg</td>
<td>70 ± 2.5</td>
<td>77 ± 2.4</td>
<td>75 ± 3.8</td>
<td>72 ± 2.1</td>
</tr>
<tr>
<td>Glomerular filtration rate, ml·min⁻¹·g ww⁻¹</td>
<td>0.22 ± 0.016</td>
<td>0.21 ± 0.009</td>
<td>0.19 ± 0.027</td>
<td>0.24 ± 0.014</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pump flow, ml/min</td>
<td>5.7 ± 0.5</td>
<td>5.2 ± 0.5</td>
<td>6.0 ± 0.6</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>Perfusion pressure, mmHg</td>
<td>79 ± 2.9</td>
<td>79 ± 3.21</td>
<td>78 ± 2.4</td>
<td>81 ± 2.7</td>
</tr>
<tr>
<td>Glomerular filtration rate, ml·min⁻¹·g ww⁻¹</td>
<td>0.20 ± 0.020</td>
<td>0.17 ± 0.019</td>
<td>0.21 ± 0.017</td>
<td>0.22 ± 0.018</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; I, ionic strength; ww, wet wt.

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Hence, the third fundamental expression describes the θ ratio of two solutes (denoted A and B), which is given by their respective concentration ratios between gel and plasma

\[ \theta_{\text{ratio}} = \frac{\theta_A}{\theta_B} = \frac{\text{Cl}_{\text{gel}}}{\text{Cl}_{\text{pl}}} \frac{(\text{Na}_p + \text{Cl}_p)}{(\text{Na}_p + \text{Cl}_p)} \]  

The unique values of the three unknown parameters, i.e., \( \omega \), \( \text{Na}_p \), and \( \text{Cl}_p \), are readily obtained by solving Eqs. 3, 4, and 6 by using known values of \( \text{Na}_p \), \( \text{Cl}_p \), and \( \theta_{\text{ratio}} \). Every protein used in the calculations was size-matched to a neutral Ficoll. As an example, let us assume a \( \omega \) of 40 meq/l, \( \text{Na}_p = 148 \text{ mM}, \) and \( \text{Cl}_p = 142 \text{ mM} \) (6 mM of negative charges due to albumin). The chloride concentration in the gel as calculated from Eqs. 3 and 4 is 88.9%, which is close to 67%.

The imbalance between the concentrations of free ions in the gel and those in plasma will give rise to an electroosmotic pressure (\( \pi_{\text{Eo}} \)) (53) that amounts to

\[ \pi_{\text{Eo}} = R \cdot T \cdot \Delta C \approx 19.33 \cdot [\text{Na}_p + \text{Cl}_p] - (\text{Na}_p + \text{Cl}_p) \]  

where \( \pi_{\text{Eo}} \) is given in millimeters mercury and ion concentrations in millimolar. We have found that the following function describes the relationship between electroosmotic pressure (in mmHg) and charge density (in meq/l)

\[ \pi_{\text{Eo}}(\omega) = 0.0299\omega^2 + 0.119\omega \]  

### Statistics

Results are presented as means ± SE, and differences were tested by using Student’s \( t \)-test, paired design. In the case of uneven distribution, i.e., for the pore analysis parameters, the data were transformed and the geometric mean and SE values were calculated. The 95% confidence intervals for the charge densities were calculated for the fractional clearance of the anionic proteins and their size-matched neutral Ficoll control.

### RESULTS

#### General

Nine rats, weighing 240 ± 6 g, were used in group A, and nine rats, weighing 263 ± 4 g, were used in group B. Values for GFR, perfusion pressure, and pump flow are shown in Table 2. The mean wet weight of the kidneys (immediately after perfusion) was 1.07 g (n = 9). There was no evident difference between the two groups, and the results were therefore pooled for analysis.

### Albumin

During control, with normal ionic strength and high osmolarity, the θ for albumin pooled from the two series was 0.0018 ± 0.0003 (n = 18). During perfusion with the low-ionic-strength solution, the θ for albumin was 29 ± 6% lower compared with the control values adjusted for time (\( P < 0.001, n = 18 \)). High ionic strength significantly increased θ for albumin by 31 ± 11% (\( P < 0.05, n = 18 \); see Fig. 1 and Table 3).

#### aHRP

During control, with normal ionic strength and high osmolarity, the fractional clearance for aHRP was 0.062 ± 0.002 (n = 9). The θ for aHRP was lowered with 28% (\( P < 0.001, n = 9 \)) during perfusion with low ionic strength. High ionic strength had no significant effect on aHRP (Table 3).

#### Ovalbumin

During the control period, the θ for ovalbumin was 0.090 ± 0.008 (n = 9). The θ for ovalbumin decreased by 52 ± 3% (\( P < 0.001, n = 9 \)) when the kidneys were

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Fig. 1. The fractional clearances (θ) for albumin during the 5 perfusion periods. Bars, θ values for albumin (n = 18); •, data for the size-matched Ficoll364 (n = 9). Values are means ± SE. I, ionic strength.
perfused by the low-ionic-strength solution. No significant change in $u$ for ovalbumin was detected when the kidneys were perfused with high-ionic-strength solution (Table 3).

**Orosomucoid**

The $u$ for orosomucoid during the control period was 0.0033 ± 0.0003 ($n = 9$). When the low-ionic-strength perfusate was introduced, the $u$ went down by 18% ($P < 0.001, n = 9$). High ionic strength increased $u$ by 13% (not significant, $n = 9$; see Table 3).

**Ficoll**

The $u$ values were calculated for ~200 different Stokes-Einstein radii in the interval of 14–70 Å. The data were then used to compute equivalent pore sizes and numbers according to a two-pore model (see below). In Table 3, the $u$ values are given for the Ficolls with hydrodynamic radii similar to those of the anionic proteins. Figure 1 illustrates the effects of various ionic strengths on Ficoll$_{36}$. Å.

**Protein-To-Ficoll Clearance Ratios**

For orosomucoid, albumin, and ovalbumin, the $u$ was divided by that of the size-matched Ficoll in the same sample to obtain a clearance ratio. For aHRP, the analysis was based on group comparisons. All protein-to-Ficoll fractional clearance ratios are presented in Fig. 2. A ratio below unity indicates restriction of the negatively charged proteins. For albumin, the clearance ratios were between 0.05 and 0.10 irrespective of ionic strength, all being significantly lower than 1.0 ($P < 0.001, n = 18$). For ovalbumin, the ratios were close to 0.3 (less than unity, $P < 0.001, n = 9$) with no significant effects of ionic strength. The aHRP-to-Ficoll$_{32}$ Å clearance ratios were all significantly less than unity (control and high ionic strength, $P < 0.001$; final control, $P < 0.05, n = 9$) except during perfusion with low ionic-strength solutions (not significant).

![Figure 2](http://ajprenal.physiology.org/)

**Table 3. Fractional clearances for tracer proteins and for Ficolls with similar hydrodynamic radii**

<table>
<thead>
<tr>
<th>Protein</th>
<th>SE Radius, Å</th>
<th>Normal I $u$</th>
<th>Low I $u$</th>
<th>Normal I $u$</th>
<th>High I $u$</th>
<th>Normal I $u$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>27.4</td>
<td>0.090 ± 0.008</td>
<td>0.037 ± 0.003</td>
<td>0.064 ± 0.003</td>
<td>0.066 ± 0.005</td>
<td>0.065 ± 0.003</td>
<td>9</td>
</tr>
<tr>
<td>aHRP</td>
<td>32</td>
<td>0.062 ± 0.002</td>
<td>0.046 ± 0.002</td>
<td>—</td>
<td>0.066 ± 0.002</td>
<td>0.068 ± 0.003</td>
<td>9</td>
</tr>
<tr>
<td>Albumin</td>
<td>35.5</td>
<td>0.0018 ± 0.0003</td>
<td>0.0013 ± 0.0003</td>
<td>0.0020 ± 0.0004</td>
<td>0.0037 ± 0.0007</td>
<td>0.0031 ± 0.0004</td>
<td>18</td>
</tr>
<tr>
<td>Orosomucoid</td>
<td>40.5</td>
<td>0.0033 ± 0.0003</td>
<td>0.0030 ± 0.0003</td>
<td>0.0039 ± 0.0003</td>
<td>0.0053 ± 0.0006</td>
<td>0.0054 ± 0.0006</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ficoll</th>
<th>SE Radius, Å</th>
<th>Normal I $u$</th>
<th>Low I $u$</th>
<th>Normal I $u$</th>
<th>High I $u$</th>
<th>Normal I $u$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll$_{27.4}$</td>
<td>27.4</td>
<td>0.25 ± 0.019</td>
<td>0.14 ± 0.015</td>
<td>0.23 ± 0.015</td>
<td>0.24 ± 0.011</td>
<td>0.22 ± 0.017</td>
<td>9</td>
</tr>
<tr>
<td>Ficoll$_{32}$</td>
<td>32</td>
<td>0.11 ± 0.01</td>
<td>0.051 ± 0.006</td>
<td>0.086 ± 0.007</td>
<td>0.108 ± 0.005</td>
<td>0.095 ± 0.008</td>
<td>9</td>
</tr>
<tr>
<td>Ficoll$_{35.5}$</td>
<td>35.5</td>
<td>0.043 ± 0.004</td>
<td>0.018 ± 0.003</td>
<td>0.038 ± 0.003</td>
<td>0.045 ± 0.002</td>
<td>0.038 ± 0.003</td>
<td>9</td>
</tr>
<tr>
<td>Ficoll$_{40.5}$</td>
<td>40.5</td>
<td>0.0089 ± 0.0008</td>
<td>0.0042 ± 0.0006</td>
<td>0.0087 ± 0.0009</td>
<td>0.0104 ± 0.0007</td>
<td>0.0091 ± 0.0006</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE. $n$, No. of rats; SE, Stokes-Einstein; $u$, fractional clearance.
Table 4. Results of the pore analysis based on the glomerular sieving of Ficoll and 95% confidence intervals

<table>
<thead>
<tr>
<th></th>
<th>Normal I (152 mM)</th>
<th>Low I (34 mM)</th>
<th>Normal I (152 mM)</th>
<th>High I (292 mM)</th>
<th>Normal I (152 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small-pore radius, Å</td>
<td>44.2 ± 0.4</td>
<td>40.6 ± 0.5</td>
<td>44.8 ± 0.4</td>
<td>44.5 ± 0.2</td>
<td>43.6 ± 0.5</td>
</tr>
<tr>
<td>Large-pore radius, Å</td>
<td>98 ± 3</td>
<td>86 ± 5</td>
<td>100 ± 8</td>
<td>91 ± 5</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>Large-pore fraction</td>
<td>0.33 ± 0.04</td>
<td>0.48 ± 0.08</td>
<td>0.46 ± 0.07, +0.21</td>
<td>0.52 ± 0.06</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>A_0 / Δx, cm</td>
<td>(5,900–27,000)</td>
<td>(8,500–20,500)</td>
<td>(5,400–34,400)</td>
<td>(9,000–34,100)</td>
<td>(10,000–31,300)</td>
</tr>
</tbody>
</table>

Values are means ± SE. LpS, total hydraulic conductance; A_0, unrestricted area; Δx, diffusion distance. Confidence intervals are in parentheses.

Results of Two-Pore Analysis

A neutral two-pore model based on the sieving data of Ficoll (14–70 Å) was carried out. The results are summarized in Table 4. At normal ionic strength (152 mM), the small-pore radius was 44 ± 0.4 Å, the large-pore radius was 98 ± 3 Å, the large-pore fraction of the hydraulic conductance was 0.33 ± 0.04%, and the total pore area-to-diffusion distance ratio (A_0 / Δx) was 14,800 (SE = 5,300 ± 3,900) cm. At low ionic strength, the small-pore radius was significantly lowered to 41 ± 0.5 Å (P < 0.001), while the other parameters were unchanged. High ionic strength did not affect the different parameters significantly. Figure 3 illustrates the small-pore radius calculated at different ionic strengths.

Estimated Wall Charge Density, ω

The ω derived from the equations reviewed under Calculations was ~30 meq/l for the standard perfusate. The ω of the glomerular barrier differed somewhat when estimated from each of the four anionic proteins. The albumin data indicated a ω of 48 meq/l (95% interval of confidence of 37–57 meq/l) at normal ionic strength. Ovalbumin and aHRP gave apparent ω values of 27 (95% interval of confidence of 25–33 meq/l) and 30 meq/l (95% interval of confidence of 16–42 meq/l), respectively. The elongated protein orosomucoid gave a considerably lower estimate of ω of 15 meq/l (95% interval of confidence of 11–19 meq/l; see Fig. 4).

During perfusion by the two solutions with different ionic strengths, the gel ω reversibly changed. Thus for perfusates with low ionic strength, ω fell to 22% of control, reaching values of ~7 meq/l (see Fig. 4). For high ionic strengths, the apparent ω increased almost twofold to reach values of ~52 meq/l.

Estimated Electroosmotic Pressure, Eo

The Eo of the gel was 36 mmHg using a mean gel ω of 34 meq/l for the standard perfusate. When the perfusate is shifted to one with low ionic strength, Eo would transiently be >4,000 mmHg before the ion concentration is lowered in the gel compartment. With an unchanged gel volume (and ω), the Eo would be expected to rise to 162 mmHg. This Eo elevation would be expected to cause a major fluid shift into the gel and expand its volume. Perfusion with high-ionic-strength solutions gives the opposite results. Thus Eo will initially rise to ~5,000 mmHg for fractions of a second. Once the ion concentrations have equilibrated with the new perfusate, Eo will fall to 19 mmHg. As an effect of the reduced Eo, fluid will leave the gel until a new steady state is obtained with ω above 50 meq/l.

Comparison of Experimental Data to Theoretical Models

We have analyzed the θ for albumin and Ficoll_36 Å with three different theoretical models: the gel membrane model (36), a charged fiber matrix model (17), and a charged pore model (30). The two latter models place both charge and size discrimination in the same layer, whereas the gel membrane separates charge and size selectivity into two separate components. The charged pore model is oversimplified to assess the approximate effects of charge and size. In contrast, the charged fiber matrix has a solid theoretical background and is further discussed in the Appendix. Figure 5 illustrates the results, and it is readily seen that the gel membrane model gives the best description of biological data. Please note the logarithmic scale.

DISCUSSION

This report is the hitherto most extensive analysis of glomerular charge selectivity in that four anionic proteins, and a range of neutral Ficolls, are studied. In addition, charge interactions are rapidly and revers-
ibly manipulated by altering the ionic strength in the surrounding environment. This approach, used in everyday ion-exchange chromatography, is readily performed in the IPK but is of course inaccessible for studies in vivo. Our main findings are the following. First, the glomerular barrier is highly selective and discriminates molecules on the basis of their size, charge, and shape, in qualitative agreement with the classic view. Thus all four anionic proteins studied had significantly lower $\theta$ values than their size-matched neutral Ficolls. The estimated glomerular $\omega$ was 30–50 meq/l, which is considerably less than the 120–170 meq/l predicted from the dextran data (10). In terms of $\pi_{Eo}$, the difference represents several hundred millimeters mercury (see Eq. 8). Second, altering perfusate ionic strength had far fewer effects on the $\theta$ values of negatively charged proteins than predicted for a charged gel (or membrane) with constant $\omega$. In fact, the glomerular charge selectivity was almost independent of ionic strength, because the protein-to-Ficoll clearance ratios did not change, a phenomenon that contrasts to the predictions of two different theories of charge interactions (see the APPENDIX). Third, low ionic strength caused opposite effects on glomerular size selectivity and $\omega$. The $\theta$ values for proteins and size-matched Ficolls were all reduced during perfusion with low-ionic-strength solutions. Pore analysis revealed a significant reduction of the small-pore radius without any other alterations of the size barrier. Hence, low ionic strength significantly increased glomerular size selectivity. In contrast, the $\omega$ was markedly reduced during perfusion with low ionic strength. High ionic strength increased $\omega$ without detectable alterations of the size barrier. Fourth, the inverse alteration of glomerular size selectivity and $\omega$ clearly indicates that different components of the glomerular barrier are responsible for the two selective properties. There is no theory that would allow opposite changes in size and charge discrimination in one single structure, at least not at present. Fifth, a gel membrane model of size- and charge-selective barriers in series adequately describes the transglomerular passage of proteins and Ficoll at various ionic strengths. Sixth, the effects of low ionic strength on glomerular $\omega$ can be explained by the concomitant increase of $\pi_{Eo}$ in the gel. As a consequence of the increased $\pi_{Eo}$, fluid will move into the gel and dilute the concentration of fixed charges.

The presence of significant charge selectivity is evident from the fact that the $\theta$ values for all four anionic proteins (albumin, ovalbumin, orosomucoid, and aHRP) were significantly lower than for their neutral
counterparts (size-matched Ficolls). Thus the θ for neutral Ficoll was >20 times higher than albumin despite similar hydrodynamic size. Moreover, ovalbumin had a θ that was less than one-third of that for the neutral Ficoll of similar size. Neutral Ficoll the size of HRP had a θ almost identical to the value we reported for neutral HRP in our previous study (50). However, a higher value was found for the θ for aHRP in the present work, probably reflecting the use of different labeling techniques. Thus the present chloramine-T approach was found to reduce the net charge (51) by half from −11 to approximately −6. Finally, orosomucoid had a θ that was one-third of that for the neutral Ficoll of similar size. Orosomucoid had a rather high θ considering its size (40 Å compared with 36 Å for albumin) and its negative charge (−24 compared with the albumin value of −23). However, the protein is elongated, with a frictional ratio of 1.5 (see Table 1), a factor that has been found to drastically increase the transglomerular passage of a molecule (27, 35). These biological data clearly demonstrate significant charge selectivity regardless of what kind of theoretical model is used for the analysis.

From our results, we conclude that negative charge is an important determinant of glomerular capillary permeselectivity. The estimated glomerular θ was found to be 30–40 meq/l by using perfusate with normal ionic composition. Studies that confirm these magnitudes of the θ have been performed by using myoglobin in vivo (32 meq/l) (54), aHRP (34 meq/l) (50), and lactate dehydrogenase (LDH; 35 meq/l) (28) in isolated cooled perfused rat kidneys, and with albumin in fixed perfused kidneys (43 meq/l) (7).

Regarding glomerular size selectivity, the two-pore analysis showed slightly lower values for the small-pore radius compared with our previous studies (33, 36). Also, the large-pore radius was somewhat larger. These discrepancies could occur by pure chance but could also be due to the use of hyperosmolar solutions in the present study. Thus to maintain osmolarity over a wide range of ionic strengths, we used a high osmolality of 590 mosmol/kgH2O. Indeed, the changes in ionic strength on GFR, inulin clearance, and morphologic parameters. GFR was constant as ionic strength was altered, as reflected by the unchanged protein-to-Ficoll clearance ratios. This is in contrast to the marked effects predicted from the altered Debye lengths. Therefore, it seems as if the glomerular θ changed with the ionic strength. Could this reflect true changes in θ? If so, what are the underlying mechanisms? To answer these pertinent questions, it must be remembered that the concentration of fixed charges is reversibly decreased by low ionic strength, whereas the opposite occurs with high ionic strength. The reversibility can hardly be explained by loss of charges followed by renewed synthesis, because the alterations are rapid even at 8°C. We therefore conclude that the volume of the gel must change. Fluid is driven into the gel during perfusion with low ionic strength and the opposite occurs with high ionic strength. This is in total agreement with the predicted effects of ionic strength on the τEo. Thus at a normal ionic strength of 152 mM, the τEo is 36 mmHg. Reducing ionic strength to 34 mM will cause an increase in τEo to 162 mmHg, which in turn will reduce the electrochemical potential for water and cause a fluid shift into the gel. Hereby, the charge density will fall from 34 to 7 meq/l (see Results). Elevated ionic strength will give the opposite result. In addition, low ionic strength will double the Debye length, which would be expected to increase the repulsion of negatively charged groups, and hence expand the gel.

Many researchers argue that the permselective part of the glomerular barrier must lie within the basement membrane or the podocyte layer, because the endothelial cells are so highly fenestrated. The podocyte slit membrane has attracted special interest, and several new proteins have been identified such as podocalyxin (23) and nephrin (47). The latter seems to be pivotal for podocyte integrity and has been shown to be mutated in congenital nephrotic syndrome of the Finnish type (24), a condition with massive proteinuria. Regarding the glomerular basement membrane, GBM, the θ...
seems to be rather low, at least in isolated fractions in vitro (3).

As stated, the role of the endothelium has been neglected. However, there are a number of reports that recognize the presence of a cell coat or glyocalyx covering the fenestrated endothelial cells (29, 48, 52). This cell coat consists of a mesh of sialylated glycopolymers anchored in the endothelial cell surface and reinforced by proteoglycans and plasma proteins. It can be made visible by staining with a cationic dye, i.e., ruthenium red (29). Indeed, the cell coat may be a rather thick structure, in the range of 50–100 nm, covering the fenestrae and surrounding domains of the capillary wall, as demonstrated by using nonaqueous fixative (46). Such a periendothelial layer of proteoglycans may behave like the ion-exchange gel described in our model (36). The increased ion-ion interactions induced by low ionic strength may reduce charge density by simply expanding the gel volume. Figure 4 would support such an interpretation and suggests that some nephrotic syndromes may be due to endothelial dysfunction rather than kidney-specific disorders.

It may be argued that there are alternative explanations for the present findings. Could there, for example, be an uptake of negatively charged molecules by the tubular cells? This is highly unlikely. Because we perfused the kidneys at 8°C, there should be no significant cellular metabolism or uptake (31). There are, however, changes in viscosity at 8°C (~2 times higher than at 37°C); the resistance to flow and filtration is twice as high, and diffusion is reduced by 50%. Also, the Debye length is decreased by ~0.6% at 8°C. In addition, there is an increase in viscosity due to the increased concentration of mannitol in the low-ionic perfusate, to maintain the same osmolarity compared with neutral perfusate. This accounts for the slight decrease in GFR during low-ionic-strength perfusion. There is, however, no evidence that the reduced temperature affects permeability per se (33).

In summary, this study confirms that the glomerular barrier is highly charge selective. However, the magnitude of the ω is less than predicted from dextran data and amounts to 30–50 meq/l. Our results strongly suggest that there are two separate barriers in series in the glomerular wall. This is evident from the inverse changes of glomerular size selectivity and ω that occurred during perfusion with low-ionic-strength solutions. Thus there is a charge-selective gel with highly dynamic properties, the morphological counterpart of which may well be the endothelial cell coat and/or the glomerular basement membrane. This layer may act as an ion exchanger, reducing the concentration of albumin in the gel to 5% of that in plasma. A second, mainly size-discriminating component acts to further reduce the concentration of albumin in the primary urine to ~4% of that in the gel, or 0.2% of that in plasma. At present, we consider this second barrier to be situated in the podocyte slit membrane, where nephrin is a key component.

**APPENDIX**

There have been few attempts in the literature to combine size and charge selectivity because the equations derived are highly complex. In particular, the concept of a porous membrane is difficult to reconcile with the requirements of homogenous distribution of charges (10). Recently, Johnson and Deen (17) developed such a theory, which predicts the effects of electrostatic interactions on the partition coefficients of spherical macromolecules in gels with random arrays of fibers. At present, their model seems to provide the most accurate description of how macromolecules interact with a fiber matrix or gel structure.

We have analyzed our glomerular sieving data according to the theoretical model of Johnson and Deen (17). Here we will first present some of the most important equations used in the analysis and then the results of applying the theory on our data. (For a more detailed description of the model, consult Ref. 17.

The partition coefficient (Φ) of a spherical macromolecule in a random array of straight fibers was first modeled by Ogston (32)

\[
\Phi = e^{-4(1 + \alpha /r_0)^2} \quad (A1)
\]

where ϕ is the volume fraction of the fibers, α is the solute radius, and r_0 is the fiber radius. Ogston reached the elegant expression of Eq. A1 by integration of the following probability function of finding the closest fiber at a distance h from the spherical solute in a dilute solution

\[
\Phi = \int_0^\infty g(h) \cdot dh \quad (A2)
\]

where g(h) is

\[
g(h) = 2 \cdot \phi \cdot (h + \alpha + r_0) / r_0^2 \cdot e^{-4(h + \alpha + r_0)^2} \quad (A3)
\]

Johnson and Deen (17) introduced the effect of charge by including an energy term E(h), which is the electrostatic-free energy of the sphere-fiber system divided by kT (k is the Boltzmann constant and T the absolute temperature). Thus the partition coefficient in a charged fiber-matrix system when the spherical solute interacts with the nearest fiber (but not with multiple fibers) is

\[
\Phi = \int_0^\infty e^{-E(h)} \cdot g(h) \cdot dh \quad (A4)
\]

The apparent simplicity of Eq. A4 is unfortunately only an illusion, because the calculations of E(h) require quite complicated equations (see Ref. 17 for details).

Our first step was to reproduce Figs. 5–8 in the original publication (17). The second step was to adjust the model parameters to describe the partition coefficients, Φ, for albumin and the size-matched Ficoll in a normal-ionic-strength solution. Finally, we observed the theoretical effects on Φ_{albumin} and Φ_{Ficoll 36Å} of altering the ionic strength.

We assumed the fiber radius, r_f, to be 4 Å, α, to be 35.5 Å, ϕ = 0.07, and the surface charge densities (q) of fiber and albumin to be ~0.08 and ~0.022 C/m², respectively. Under these conditions, the partition coefficients for albumin were estimated to be 3.4 × 10⁻⁵ at normal ionic strengths compared with 7.6 × 10⁻⁴ for neutral Ficoll of similar size, giving a Φ ratio of 22. At low ionic strength, Φ_{albumin} was 6.3 × 10⁻⁴ and Φ_{Ficoll 36Å} was 3.6 × 10⁻⁴, giving a ratio of 5,700. High ionic
strength gave a $\Phi_{\text{albumin}}$ of 1.3 $10^{-4}$ and $\Phi_{\text{Ficoll 36Å}}$ of 8.8 $10^{-4}$, giving a $\Phi$ ratio of 7. Altering the various parameters in the model changes the absolute values for the partition coefficients, but the effects of ionic strength are qualitatively the same.

To compare these partition coefficients to our experimental data, one must calculate fractional clearances. Curry and Michel (9) introduced the concept of a “fiber matrix” (32) into the field of microvascular research. In so doing they used the expression of Anderson and Malone (1) to calculate the reflection coefficient, $\sigma$, from the partition coefficient, $\Phi$:

$$\sigma = (1 - \Phi)^2 \quad (A5)$$

Subsequent experiments have shown that Eq. A5 rather poorly predicts experimental data (20), but there is presently no better equation available. The diffusion capacity, $PS$, is given by

$$PS = A_\sigma / D \cdot \Phi \quad (A6)$$

where $A_\sigma / D$ is the unrestricted area over diffusion distance, and $D$ is the free diffusion constant. The fractional clearance, $\theta$, is obtained using a nonlinear flux

$$\theta = \frac{(1 - \sigma)}{1 - \sigma \cdot e^{-(PS \cdot (1 - \sigma))}} \quad (A7)$$

These expressions were used to model the present experimental data using the parameters given above (after Eq. 4). Under the condition of having a normal ionic strength in the perfusate, the $\theta$ values for albumin and the size-matched control were 0.0019 and 0.041, in agreement with the experimental findings. However, reducing the ionic strength of the perfusate to 34 mM would reduce $\theta_{\text{albumin}}$ to 3.5 $10^{-6}$ and $\theta_{\text{Ficoll 36Å}}$ to 0.02, implying a tremendous strengthening of the charge barrier. The model predicted the opposite for high ionic strength, giving $\theta$ values of 0.007 and 0.048 for albumin and Ficoll, respectively. Thus restricting solutes on the basis of their charge and size in the same barrier gives results that are inconsistent with our experimental findings. Therefore, the analysis supports our conclusions of two separate components in glomerular permeability. Figure 5 illustrates the effects of perfusate ionic strength on the experimentally determined fractional clearance ratios ($\theta_{\text{Ficoll 36Å}} / \theta_{\text{albumin}}$) compared with those predicted by using the present gel-membrane model, the charged fiber matrix model (17) described herein, and finally a charged pore model described by Munch et al. (30). The latter model reduces the effects of charge to a steric pore factor, which assumes the solutes to be either (negatively) charged or neutral. In the case of neutral solutes, restriction is related to the molecule-to-pore radius ratio ($a_{SE}/R$), where $SE$ is Stokes-Einstein. If the molecule is negatively charged, an adjusted radius ratio can be calculated by adding one Debye length ($l_D$) to the solute radius and subtracting one $l_D$ from the pore radius ($a_{SE} + l_D/R - l_D$).

This study was supported by Swedish Medical Research Council Grants 9898 and 2855, the Knut and Alice Wallenberg Research Foundation, the Ingabritt and Arne Lundberg Research Foundation, the National Association for Kidney Diseases, and by Sahlgrenska University Hospital LUA-B31303.

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