Dynamic alterations of the glomerular charge density in fixed rat kidneys suggest involvement of the endothelial cell coat

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\textbf{Short title:} Fixed kidney: Glomerular Permselectivity

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ABSTRACT

In a previous paper, we found that low ionic strength (I) reversibly reduced the glomerular charge density, suggesting increased volume of the charge-selective barrier. Since glutaraldehyde makes most structures rigid, we considered the isolated perfusion-fixed rat kidney to be an ideal model for further analysis. The fixed kidneys were perfused with albumin solutions containing FITC-Ficoll at two different ionic strengths (I = 151 and 34 mM).

At normal I, the fractional clearance, $\theta$, for albumin was 0.0049 (SEM -0.0017, +0.0027, n=6) while $\theta$ for neutral Ficoll$_{35.5\AA}$ of similar size was significantly higher 0.104 (SEM 0.010, n = 5, p<0.001). At low ionic strength $\theta$ for albumin was 0.0030 (SEM -0.0011, + 0.0018, n=6, n.s. from $\theta_{\text{albumin}}$ at normal I) and $\theta$ for Ficoll$_{35.5\AA}$ was identical to that at normal I, 0.104 (SEM 0.015, n=6, p<0.01 compared to $\theta_{\text{albumin}}$ at low I). According to a heterogeneous charged fiber model, low ionic strength reduced the fiber density from 0.056 to 0.0315 suggesting a 78 % gel volume expansion.

We conclude that (1) there is a significant glomerular charge barrier. (2) Solutions with low ionic strength increase the volume of the charge barrier even in kidneys fixed with glutaraldehyde. Our findings suggest that polysaccharide-rich structures, such as the endothelial cell coat, are key components in the glomerular barrier.

Key words: fixed kidney; glomerular permeability; charge selectivity
INTRODUCTION

In order to achieve the rapid rate of filtration required to regulate the composition and volume of body fluids, glomerular capillaries possess unique functional and structural characteristics. A striking example is an extraordinarily high hydraulic permeability. Glomerular capillaries are one to two-order of magnitude more permeable to water than are capillaries from various other microvascular beds (9). Nevertheless, the same structure normally imposes an extremely efficient barrier to the passage of plasma proteins, so that the concentrations of albumin and larger proteins are minute.

The transport of solutes across microvascular walls can be described by a two-pore theory of capillary permeability (30). Measurements of steady-state sieving coefficients (θ) of proteins from plasma to interstitium or lymph are used to predict the pore radius and distribution. In glomerular capillaries, the presences of tubular reabsorption and secretion processes that modify final urine composition are formidable obstacles to the determination of sieving coefficients for proteins, and consequently, to the study of glomerular permselectivity. Experimental in vitro systems like the isolated nephron or the isolated perfused glomerulus are free from the influence of tubular transport processes (26, 37). These systems, however, are less suitable for studies of macromolecular transport due to the small amounts of solute filtrated in minute volumes. With a normal albumin fraction of only a few tenths of a percent the measurements are less accurate than for smaller solutes.

Oliver et al (27) found that Ficolls (globular uncharged cross-linked copolymer of sucrose and epichlorohydrin that is neither secreted nor reabsorbed by the renal tubules) of various radii had a lower fractional clearance than dextrans of equal Stokes-Einstein
radius \( (a_{se}) \). This implies that Ficoll may be a reliable transport probe for the measurement of small and large pore radii.

Fractional clearance experiments of charged dextrans substantiated the hypothesis of a charge-dependent glomerular filtration of macromolecules (4). Based on fractional clearance data of dextran in the rat, in a now classic work Deen et al. (10) calculated an apparent fixed charge concentration on the glomerular capillary wall of 120-170 mEq/l. However, when using charged dextran in permselectivity studies, uptake and desulphation of dextran sulfate by the glomerular and tubular cells (6, 42, 43) and the ability of certain dextran sulfates to bind to plasma proteins (13) complicate the interpretation of the results.

We have developed a modified isolated rat kidney model, in which the tubular reabsorption processes were eliminated by glutaraldehyde fixation (5). Glutaraldehyde is a fixative that acts rapidly and offers accurate tissue preservation. There is evidence that in the isolated kidneys fixed by perfusion with glutaraldehyde no ultrastructural alteration of the glomerular capillary wall is detectable; an intact organization of the glomerular cells and an unaltered distribution of glomerular polyanions was reported (36, 37). In one of the most detailed descriptions of the fine structure of isolated kidneys after perfusion fixation with glutaraldehyde, Kriz et al. showed that the integrity of the barrier is remarkably preserved (19). No cell lysis is noticed and the structure of the glomerular capillaries is undistorted (11, 12), while the metabolic processes are eliminated. Using this model, the glomerular permeability properties can be directly studied, without interferences of the tubular apparatus and without influence of hemodynamic factors and blood constituents like hormones. The charge of the glomerular capillary wall (GCW) has been determined (5) using albumin solutions
buffered at different pHs spanning the isoelectric points of albumin and of the
glomerular basal membrane.

In the present work, the fractional clearance of FITC-Ficoll was measured to determine
the glomerular size- and charge-selectivity in the perfused-fixed isolated rat kidneys.
Previous studies suggest low ionic strength to reduce reversibly the glomerular charge
density, most likely due to volume expansion of the compartment responsible for charge
selectivity.

Therefore, it was of particular interest to estimate the charge density at different ionic
strengths of the perfusate. Our hypothesis was that lowering ionic strength would not
induce dynamic alterations of the estimated charge density in the fixed kidneys if the
glomerular charge-selectivity were to reside in the basement membrane and/or in the
podocyte slit membrane. On the other hand, marked changes in charge density could be
expected in the fixed kidneys if glomerular charge-selectivity were dependent on the
endothelial cell coat barrier, which is more resistant to glutaraldehyde (31, 35).
MATERIALS AND METHODS

Experimental animals
Experiments were performed on male Sprague-Dawley rats weighing 200-300 g. The animals had free access to food (standardized pellets, Altromin®, Altromin Gesellschaft für Tierernährung mbH Lage, Germany) and tap water until the experiment. The rats were anesthetized intraperitoneally with 100 mg/kg body wt thiopental-sodium (Trapanal®, Byk-Gulden, Konstanz, Germany).

Kidney isolation
Surgery: The rats were placed on a temperature-regulated table. The surgical procedure was a modification of that reported by Weiss et al. (40) and Nishiitsutsuji-Uwo et al. (22). The right kidney was always used for perfusion. As ureter catheters we used short (10-mm) polypropylene catheters (PP-10, Portex, Hythe, England) connected to larger polyethylene catheters (PE-50, Portex, Hythe, England), thereby preventing a buildup of ureteral backpressure. After heparin injection (Liquemin®, Hoffmann-LaRoche Grenzach-Wyhlen, Germany), the kidney was placed in a temperature-controlled metal chamber. Before starting the perfusion, the aorta was clamped distal to the right renal artery, and a double-barreled cannula was inserted into the abdominal aorta distal to the clamp. Perfusion was started in situ by opening the clamp and tying the proximal aortic ligature. Thus, zero perfusion of the experimental kidney never occurred.

Perfusion apparatus and technique: The apparatus was designed as a recirculation system with dialysis because of a higher stability. The perfusion technique and apparatus have been previously described in detail (33, 34). Experiments were performed using a substrate enriched Krebs-Henseleit bicarbonate solution containing 50 g/l bovine serum albumin (BSA, Fraction V, Sigma, Deisenhofen, Germany) (34).
Verapamil (Isoptin®, Knoll, Minden, Germany) at a dose of 4.4 µmol/l was added to the perfusion medium. The effective perfusion pressure was 100 mmHg.

Kidney fixation

After isolation, the kidney was perfusion-fixed with a 1.25% monomeric glutaraldehyde (Polyscience Inc., Warrington, U.S.A.) solution in 0.1 molar phosphate buffers (final pH 7.2). The fixation solution was made isooncotic to plasma by addition of hydroxyethyl starch (Plasmasteril®, Fresenius, Bad Homburg, Germany) to a final concentration of 60 g/l. In previous experiments it had been observed, that the perfusion resistance was increasing dramatically when the perfusate was colloid-free. For fixation, the kidney was perfused for 6 to 8 min at a pressure of 150 mmHg.

Reperfusion of the fixed kidney

Before reperfusion experiments were started, the fixed kidney was washed free from glutaraldehyde by a 60 min single-pass perfusion with 0.9% saline at a pressure of 100 mmHg. This step was necessary to avoid the formation of protein-glutaraldehyde aggregates, which can significantly reduce the perfusion flow rate in protein perfusion experiments (not shown).

Every solution used in perfusion experiments of the fixed kidney contained 100 mg/l polyfructosan. Experiments were performed at a perfusion pressure of 100 mmHg.

Study design

The fixed kidneys were perfused successively with phosphate-buffered (136.9 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 0.9 mM CaCl₂, 8.1 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.4) solutions containing 10 g/l bovine serum albumin (BSA) and 70 mg/l FITC-Ficoll (Ficoll-70®, Bioflor, Uppsala, Sweden) containing Ficoll molecules of
different size. The ionic strength, I, of the “normal” perfusate was 151 mM. The low ionic strength perfusates (I = 34 mM) contained the same concentrations of BSA and Ficoll, respectively, but otherwise had the following composition: Na 26 mM, K 4.3 mM, Ca 2.5 mM, Cl 8.4 mM, Mg 0.8 mM, HCO$_3$ 25 mM, H$_2$PO$_4$ 0.5 mM, glucose 5.6 mM and mannitol 241 mM.

Electrolytes

Na$^+$ and K$^+$ concentrations in perfusate and urine samples were determined with an ion-selective electrode analyzer (System E2A® electrolyte analyzer, Beckman, Brea, CA, USA).

Analysis of Ficoll sieving

For calculating the sieving coefficients for FITC-Ficoll, all perfusate and urine samples were subjected to gel filtration (BioSep®-SEC-S3000, Phenomenex, Torrance, CA, USA) and detection of fluorescence (RF 1002 Fluorescence HPLC Monitor, Gynkotek, Germering, Germany) using Chromeleon® (Gynkotek, Germering, Germany) software. As eluent, we used a 0.05 M phosphate buffer with 0.15 M NaCl with pH 7.0. From each sample, a volume of 5-10 µl was analyzed at an emission wavelength of 520 nm and an excitation wavelength of 492 nm; during analysis flow rate (1 ml/min), sampling frequency (1 per second), pressure (4 MPa) and temperature (8°C) were maintained constant. We estimated the error in the C$_U$/C$_P$-ratios for Ficoll to be <1% for most molecular sizes.
Other analytical methods

Total protein was determined using the Bradford method (3). Inulin was measured after acid hydrolysis by the hexokinase/glucose-6-phosphate dehydrogenase method (32) by including a phosphohexose isomerase reaction into the assay.

Calculations

Glomerular filtration rate (GFR)

The glomerular filtration rate of the isolated and of the fixed kidney was determined by measuring inulin (polyfructosan) clearance. For calculating the glomerular filtration rate (GFR), we used the following formula:

\[
\text{GFR} = \left( \frac{C_u}{C_p} \right)_{\text{inulin}} \cdot Q_u
\]

(Eq.1)

\(C_p\) is the concentration of inulin in plasma and \(C_u\) represents its concentration in urine; \(Q_u\) is urine flow rate.

Fractional clearances of albumin and Ficoll, \(\theta\)

The fractional clearance \(\theta\) for a solute X was calculated as:

\[
\theta = \frac{\left( \frac{C_u}{C_p} \right)_X \cdot Q_u}{GFR} = \frac{\left( \frac{C_u}{C_p} \right)_X}{\left( \frac{C_u}{C_p} \right)_{\text{inulin}}}
\]

(Eq.2)

Models of glomerular size- and charge-selectivity

We used two different theoretical models for analysis of glomerular size- and charge selectivity, namely the gel-membrane model (24) and a charged fiber model (16) with small discontinuities with extremely low concentrations of fibers (large pores), see below.
The gel-membrane model

The gel-membrane model (24) assumes the glomerular barrier to be composed of two separate compartments in series: one charge-selective (gel) and one size-selective (membrane). The gel contains fixed negative charges and the concentration of an anionic molecule such as albumin will be lower in the gel than in plasma. The second compartment of the barrier behaves as a membrane exerting size-discrimination but no charge-selectivity. Thus, in this model the effects of size and charge are treated in two different compartments, which greatly facilitate the calculations, but naturally represents a gross oversimplification, since the sieving coefficient for a certain solute is given by the product of $\theta$ for each individual component of a serial barrier (8). Furthermore, it can be argued that the limitations of the model affect the results leading to erroneous conclusions. However, if we consider the “gel” to be a part of the plasma compartment rather than the barrier, the model may still be valid.

Charge-selectivity is estimated from the $\theta$ for albumin and its neutral counterpart of similar size, Ficoll$_{35.5\AA}$, giving a density of fixed charges, $\omega$, see (24). The fractional clearance for Ficolls of Stokes-Einstein radii between 30-70 Å (180 data pairs) allow estimates of size-selectivity using a two-pore model, which has the following four parameters: The functional small pore radius ($r_S$), the large pore radius ($r_L$), the large pore fraction of the hydraulic conductance ($f_L$), and the unrestricted exchange area over diffusion distance ($A_0/\Delta x$). For more details please consult (24).

A charged fiber model with discontinuities of low fiber density

To combine size- and charge-selectivity in one model is highly complicated, but Johnson and Deen (16) extended the partitioning theory of Ogston (23) to develop a
charged fiber model to predict the concentration ratio of a solute at equilibrium in and outside a gel. The endothelial surface layer (glycocalyx) and the glomerular basement membrane are examples of such more or less charged gels. We have previously used the model in a quantitative analysis of charge selectivity (38), but the present analysis differs in two important aspects. Firstly, the present analysis takes into account that there may be heterogeneous fiber densities with regions with low fiber concentrations, i.e. large pores. Secondly, due corrections are made for the diffusivity in a gel (29).

The gel/plasma concentration ratio at equilibrium in a fiber matrix is described by the partition coefficient ($\Phi$): 

$$\Phi = \int_0^\infty g(h) \cdot dh$$  \hspace{1cm} (Eq.3)

Where $g(h)$ is the probability of finding the closest fiber at a distance, $h$, from a spherical solute in a dilute solution:

$$g(h) = \frac{2 \cdot \phi \cdot (h + r_s \cdot r_f)}{r_f^2} \cdot e^{-\phi \cdot (h + r_s \cdot r_f)^2 / r_f^2}$$  \hspace{1cm} (Eq.4)

Where $\phi$ is the volume fraction of fibers, $r_s$ the solute radius and $r_f$ the fiber radius. By integrating (Eq.4) Ogston (23) reached the following expression for $\Phi$:

$$\Phi = e^{-\phi \left[ 1 + \left( \frac{r_s}{r_f} \right)^2 \right]}$$  \hspace{1cm} (Eq.5)

Johnson and Deen (16) introduced a Boltzmann factor to describe the relative probability at different energy states in charged gels. Multiplying $g(h)$ by this factor gives:

$$\Phi = \int_0^\infty e^{-E(h)} \cdot g(h) \cdot dh$$  \hspace{1cm} (Eq.6)

Where $E(h)$ is the electrostatic free energy of the interactions between the solute and the nearest fiber divided with kT (k is Boltzmann’s constant and T is the absolute temperature). $E$ is dependent of one position variable, $h$, only. In a true system, the
solute would interact with multiple fibers (and other solutes). Johnson and Deen (16) solved (Eq.6) using a linearized Poisson-Boltzmann equation with dimensionless parameters scaled by the electrical potential RT/F (R is the gas constant, T is temperature in Kelvin, and F is Faraday’s constant).

Interactions between solute and fiber cause changes in the free electrochemical energy:

\[ \Delta G = G_{sf} - G_s - G_f \]  

(Eq.7)

Where the subscripts s, f and, sf refer to the isolated solute, isolated fiber and combined solute-fiber system. Note that in order to obtain \( \Delta G \) nested polynomial equations are required making the calculations much more complex (16).

The energy (E) needed in (Eq.6) is given by:

\[ E = \frac{(RT/F)^2 \cdot \varepsilon_s}{kT} \cdot \Delta G \]  

(Eq.8)

Where \( \varepsilon \) is the dielectric permittivity for the solvent. R, T and F were previously explained. The dielectric permittivity is the relative dielectric constant multiplied by \( \varepsilon_0 \), the constant for vacuum (\( \varepsilon_0 = 8.8542 \cdot 10^{-12} \text{ C} \cdot \text{V}^{-1} \cdot \text{m}^{-1} \)). In the case of uncharged solutes or fibers, the low dielectric constant will change the potential field of the charged solute or fiber surrounding, thus increasing the electrostatic free energy. For further details of the equations, please consult Johnson and Deen (16).

To apply the partition coefficients to experimental data, one must calculate fractional clearances (\( \theta \)). In the concept of “fiber matrix”, Curry and Michel (7) used the expression of Anderson and Malone (2) to calculate the reflection coefficient (\( \sigma \)) from the partition coefficient (\( \Phi \)):

\[ \sigma = (1 - \Phi)^2 \]  

(Eq.9)
Note that experimental observations in agarose gels give reflection coefficients that differ somewhat from those of (Eq.9) (17). There are however limited experimental studies of reflection coefficients in biological gels and to our knowledge this is the best equation available.

Finally, the diffusion capacity (PS) is given by: \[ PS = \frac{A_0}{\Delta x} \cdot D \cdot \Phi \cdot \frac{D}{D_0} \] (Eq.10)

Where \( A_0/\Delta x \) is the unrestricted exchange area over diffusion distance and \( D \) is the free diffusion constant. \( D/D_0 \) is the relative diffusivity in a gel as presented by Phillips (29):

\[
\frac{D}{D_0} = e^{-0.84 \cdot \Phi^{1.09}} \cdot e^{\left\{ -3.727 - 2.460 \left( \frac{r_f}{r_s} \right) + 0.822 \left( \frac{r_f}{r_s} \right)^2 \Phi \left( 0.358 + 0.366 \left( \frac{r_f}{r_s} \right) - 0.0939 \left( \frac{r_f}{r_s} \right)^2 \right) \right\}}
\] (Eq.11)

The fractional clearance (\( \theta \)) is obtained using a non linear flux equation (30):

\[
\theta = \frac{1 - \sigma}{1 - \sigma \cdot e^{\left[ \frac{GFR}{PS} \right] \cdot (1 - \sigma)}}
\] (Eq.12)

Where GFR is the glomerular filtration rate, which in this study was 0.1 ml/min/g kidney (w.w.).

In a previous study (38), we noted that the charged fiber model did not adequately describe the effects of changing ionic strength. However, introducing large pores improves the precision significantly. Moreover, introducing a heterogeneous fiber network with small regions with low fiber concentrations (1/20th of the average) further improves the agreement between theory and experimental data. Thus, the total fractional
clearance for a solute is the sum of the θ through the main gel (θ_{main gel}) and that occurring through the large pore discontinuities (θ_L). The large pores represent a small fraction (f_L) of the total hydraulic conductance and an even smaller fraction (f_L^2) of the exchange area (A_0/Δx). Hence, θ_L is calculated as for θ_{main gel} except for the fact that

\[ f_L = \frac{\theta_{main gel}}{20}, \]

which will affect σ and PS and the resulting fractional clearance can be written as:

\[
\theta_{total} = \frac{(1-\sigma_{main\_gel}) \cdot (1-f_L)}{1-\sigma_{main\_gel} \cdot e^{\left[(1-f_L) \cdot \text{GFR} \cdot (1-\sigma_{main\_gel})\right]/\sigma_{main\_gel}}} + \frac{(1-\sigma_L) \cdot f_L}{1-\sigma_L \cdot e^{\left[f_L \cdot \text{GFR} \cdot (1-\sigma_L)\right]/\text{PS}_L}}
\]

(Eq. 13)

The important parameters in the model are: the fiber radius (r_f), the relative concentration of fibers in the gel (φ), the surface charge densities of solute (q_s) and fiber (q_f), the unrestricted exchange area over diffusion distance (A_0/Δx), the large pore fraction of the hydraulic conductance (f_L) and the dilution factor for the fiber density in the large pores. Some of these parameters were constant (r_f, q_s for albumin and Ficoll, the large pore dilution factor), whereas others were modified (A_0/Δx, φ, q_f, f_L) in order to achieve a good fit between experimental and theoretical data.

*Curve-fitting procedures*

In the present study, the fractional clearances for Ficolls of different molecular sizes were modeled using Mathcad 2001i (MathSoft Engineering & Education, Inc., Cambridge, Mass., USA). Firstly, different values for A_0/Δx, φ, q_f, f_L were tested to achieve acceptable fitting between modeled and experimental data at normal ionic strength (151 mM). Secondly, the same parameter values were used to calculate the fractional clearance for Ficoll at low ionic strength, 34 mM. This resulted however in
poor fitting between experimental and modeled data particularly for the smaller solutes. Finally, the concentration of fibers were gradually reduced by low ionic strength until acceptable agreement was obtained between the experimentally determined fractional clearance for Ficoll and the values obtained by the heterogeneous charged fiber model. Details of the calculations are given in a separate PDF file available at the Journal website.

**Statistics**

Data are presented as mean values ± SEM or with 95% confidence intervals. For the two-pore model parameters and for $\theta_{\text{albumin}}$, the statistical analysis was based on the logarithmic values, due to the skewed distribution of data. Differences were tested using Student’s t-test paired design.
RESULTS

Reperfusion experiments of the fixed kidney

The ‘urine’ concentrations of sodium and potassium were equal to those in perfusate (data not shown). The inulin concentration ratio between perfusate and urine was 1.01 ± 0.02 (n=10), i.e. not significantly different from unity.

Glomerular filtration rate and renal perfusate flow

The values for the glomerular filtration rates and the renal perfusate flow (average ± SEM) are reported in Table 1.

The fractional clearance, $\theta$, of BSA and Ficoll\textsubscript{35.5Å}

At normal ionic strength, the sieving coefficient, $\theta$, for albumin was 0.0049 (SEM -0.0017, +0.0027, n=6), i.e. about 1/20\textsuperscript{th} of that for neutral Ficoll of similar size ($a_{\text{se}} = 35.5$ Å) 0.104 (SEM= 0.010, n=5, p<0.001). At low ionic strength perfusion, $\theta$ for albumin was 0.0030 (SEM -0.0011, +0.0018, n=6), n.s. compared to that at normal I. $\theta$ for Ficoll\textsubscript{35.5Å} was 0.104 (SEM 0.015, n=6, n.s. compared to normal I). Thus, $\theta$ for Ficoll\textsubscript{35.5Å} was significantly higher than $\theta$ for albumin at low I as well (p<0.01). For details, see Figure 1.

Figure 2 shows the sieving coefficients obtained in individual reperfusion experiments of the fixed kidney for BSA compared with that of Ficoll of $a_{\text{se}}$ 35.5 Å. All data fall to the right of the line of identity indicating restriction of the anionic albumin compared to the neutral Ficoll of similar hydrodynamic size, i.e. a significant glomerular charge barrier is evident. Figure 3 illustrates the U/P concentration ratios for Ficoll of various molecular radii.
The Gel-Membrane Model Analysis

The functional small pore radius was 36 Å (27-44 mEq/L, 95% CI) at normal ionic strength and 33 Å (23-43 mEq/L, 95% CI) at low I. The large pore radius 137 ±8 Å and 197 ±30 Å for normal and low I respectively. The large pore fraction of the total hydraulic conductance was 2 % for normal and 3 % for low ionic strength. The glomerular charge density, \( \gamma \), was estimated to be 38 mEq/L (28-71 mEq/L, 95% CI) for normal ionic strength and 13 mEq/L (11-16 mEq/L, 95% CI), during perfusion with low ionic strength perfusate, suggesting a threefold increase in volume of the “charged gel”, see Figure 4.

The heterogeneous charged fiber model

In this model, the unrestricted exchange area over diffusion distance, \( A_0/\Delta x \), was 100 000 cm. The fiber radius was 4.5 Å, the relative fiber volume, \( \phi \), was 5.6 % and the fiber surface charge density was -0.3 C m\(^{-2}\) compared to -0.022 C m\(^{-2}\) for albumin, see Table 2. The gel was heterogeneous with discontinuities with 1/20\(^{th}\) of the fiber density accounting for 8.5% of the hydraulic conductivity or 0.72% of the total area. With these parameters there was an acceptable fit between the 180 Ficoll data pairs (U/P-ratio vs. Stokes-Einstein radius) obtained at normal ionic strength and the modeled values. As the ionic strength was reduced, however, the modeled values deviated from the measured data and more so for smaller solutes, i.e. higher U/P-ratios. Figure 5 shows a Blandt-Altman plot demonstrating the deviations between measured and modeled U/P-ratios. In order to achieve a better fit at low I, a twofold (+78 %) expansion of the gel must assumed reducing the fiber density to 3.15 % see Figure 5.
In contrast to the Ficoll data, the U/P-ratios for albumin were not adequately described by the heterogeneous charged fiber model, which overestimated \( \theta \) for albumin 6-8 times. Possible explanations are presented in the Discussion.
DISCUSSION

In this study, the functional properties of the glutaraldehyde-fixed glomerular barrier were evaluated using a broad fraction of neutral spherical Ficoll molecules together with albumin using perfusate solutions of variable ionic strength. This experimental model may be considered highly artificial but it is actually ideal for the purpose of this study, as previous studies suggested marked volume changes to occur in the charge-selective compartment in response to alterations of perfusate ionic strength (38).

Our hypothesis was that if cellular structures and/or the collagen IV-rich glomerular basement membrane were responsible for charge-selectivity, then fixation would abolish dynamic changes of the charge density in response to alterations of ionic strength. On the other hand, if fixation does not affect the dynamics of charge density, then mucous structures such as the endothelial cell coat, are likely to be involved since they are more resistant to glutaraldehyde-fixation (1, 35).

Our main findings were that the fractional clearance, $\theta$, for albumin was one order of magnitude less than that for a neutral Ficoll of similar hydrodynamic size (35.5 Å).

From this charge selectivity, a charge density of 38 mEq/L could be calculated using the gel-membrane model. The value is surprisingly similar to those estimated in vivo (41) and in vitro (15, 21, 25, 38), as noted in a previous study on fixed kidneys (5). Data could also be interpreted in terms of charged fiber densities assuming a certain degree of heterogeneity. Reducing the ionic strength of the perfusate did not affect the relationship between $\theta_{\text{albumin}}$ and $\theta_{\text{Ficoll 35.5Å}}$ as much as expected based on the increased charge-charge interactions. Consequently, both theoretical models predict that the volume of the gel did increase during low ionic strength perfusion. The more accurate heterogeneous charge fiber model suggests a volume expansion of 78 %, whereas the
gel-membrane model suggests a threefold volume expansion. These dynamic alterations of the charge density in a fixed kidney suggest that the structure responsible for glomerular charge selectivity is a polysaccharide rich layer resistant to fixation such as the endothelial cell coat (or possible the glomerular basement membrane).

**Permeability characteristics of the fixed kidney**

The sieving coefficient for albumin obtained at neutral pH in the fixed kidney is higher than *in vivo*, but similar to that reported for the unfixed isolated perfused rat kidney(37). The glomerular permeability is, however, heterogeneous (5).

The sieving coefficient for BSA at a concentration of 50 g/l does not significantly differ from that obtained at a concentration of 10 g/l (44). Similar results have been obtained in micropuncture experiments of the isolated rat kidney (37). Therefore, perfusion experiments of the fixed kidney were performed at an albumin concentration of 10 g/l, in order to maintain the costs of the experiments low. Recent experiments have shown, however, that the albumin concentration indeed may affect the sieving of tracer macromolecules (20). This deviation from the normal physiological protein concentration, albeit disturbing, will however not affect the conclusions of the study.

**The glomerular barrier**

The finding that the sieving coefficient of albumin is much lower than that of Ficoll of equivalent size (35.5 Å) supports the classic notion of a charge barrier (4). Recently this notion has been challenged due to some limitations of the dextran used as a tracer (28). The calculation of the glomerular capillary wall charge distribution according to a simplified model of charge-charge interactions (24) gave a charge density of 38 mEq/l. Thus, glomerular charge selectivity was overestimated in the classical studies (9) due to the use of sulfated dextrans. The gel-membrane model has the virtues of being able to
describe glomerular permeability in a variety of situations and the calculations are rather straightforward. It is, however, an oversimplified view of the reality since charge- and size interactions cannot really be separated. Thus, barriers in series will contribute to the overall sieving coefficient of a tracer as products (i.e. $\theta_{\text{tot}} = \theta_1 \cdot \theta_2 \cdot \theta_3 \cdot \theta_4 \ldots \theta_n$), which suggests that there must be some degree of size restriction in the gel compartment as well (20). The charged fiber model is theoretically more correct but it suffers from being highly complex. Indeed some of the fiber matrix equations have not yet been fully developed. The equations required to estimate the partition coefficients are sophisticated but do nevertheless have certain limitations. They do for example assume random interactions between a solid sphere and one fiber. In reality, the glomerular barrier is composed of multiple fibers and plasma proteins in an orderly fashion. Moreover, the equations to estimate the reflection coefficient and the diffusion capacity in the gel are crude at present. Still, we consider the charged fiber model to be the most accurate theory for analysis of glomerular permeability.

The gel-membrane model adequately describes both albumin and Ficoll data. The heterogeneous charged fiber model grossly overestimated the fractional clearance for albumin. This probably indicates that the latter model, despite its complexity, has limitations. Alternatively, it may suggest that albumin binds to tubular structures in the fixed kidney causing underestimations of $\theta$ for albumin. There are however no indications of such binding problems in these kidneys that have been extensively pre-washed.

In the present study, we introduce heterogeneity into the charged fiber model. Hereby, the adaptation to the experimentally determined Ficoll data improved dramatically. It is important to note that similar conclusions were drawn using the two different models of
glomerular charge- and size-selectivity namely that perfusion of rat kidneys with low ionic strength seems to induce a volume expansion of the gel (by 78% or more).

*Fixation with glutaraldehyde*

The urine to perfusate ratio of one for both inulin and the electrolytes demonstrates that the urine collected represents glomerular ultrafiltrate. The tubules are therefore part of an inert system, in which the metabolic processes have been eliminated. The fixed kidney can thus be regarded as a pure "membrane". Histological studies (36) have shown that glomerular structures of isolated perfused-fixed kidneys were similar to those *in vivo*, including the distribution of anionic sites in the GBM as characterized with Ruthenium red. Moreover, in rat hindquarter preparations fixation with glutaraldehyde reduced surface area for capillary exchange, but had no effect on capillary permeability (14).

Mucous structures rich in polysaccharides, however, are more resistant to regular fixation techniques and studies on the microanatomy of such structures must employ special, non-conventional fixation regimes (1, 18, 31, 35). For this reason, it would be expected that the endothelial cell coat, a polysaccharide-rich layer creating an interface between plasma and endothelial cells, should allow volume changes even in a fixed kidney. In our study, the low ionic strength perfusion did indeed reduce the estimated charge fiber density in the isolated perfused fixed kidneys. As all fixed structures, except the endothelial cell coat, are rigid and incapable of undergoing the large volume changes required to alter the charge density observed in our experiment, this supports the hypothesis that glomerular charge selectivity is related to the cell coat covering the endothelial cells.
Finally, we have to consider some alternative interpretations of our results. Could for example the observed alteration in estimated charge density be due to something else than volume changes of the glomerular charge barrier? Indeed, the biophysical models for transport of charged solutes across charged membranes or gels are far less precise than the theories dealing with transport of neutral solutes. However, in a recent study we compared different models including the most advanced charged fiber-matrix analysis (38) and the results are more or less the same. All current theories predict that the $\theta_{\text{albumin}}$ should be reduced by more than one order of magnitude when the ionic strength is reduced from 151 to 34 mM. The experimental observations suggest a modest, but statistically significant, reduction of $\theta_{\text{albumin}}$ at low I. At present, the only plausible explanation is that the glomerular charged fiber density is reduced. The reversibility of this process demonstrated by Sörensson (39) seems to rule out other possibilities than volume changes of the charge barrier with a constant number of fixed charges. Indeed, dramatic fluid shifts are to be expected since the electro-osmotic pressure of the gel increases drastically (reaching 160 mmHg) as the ionic strength is reduced (38).

In conclusion, the glomerular barrier is size- and charge selective. Perfusion with solutions of low ionic strength reduced the estimated charged fiber density by at least 78 %, probably due to volume expansion of gel. Since almost all constituents of the glomerular barrier, except the polysaccharide-rich endothelial cell coat, are rigid in the fixed kidney, our findings support the view that the endothelial cell coat can be an important component of the glomerular barrier.
ACKNOWLEDGMENTS

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LEGENDS

**Table 1.** Mean values ± SEM for glomerular filtration rate (µl/min/g) and renal perfusate flow (ml/min) in the fixed kidneys perfused with normal and low ionic strength solutions.

**Table 2.** The parameters of the heterogeneous charged fiber model during normal conditions and during the volume expansion induced by low ionic strength kidney perfusion.

**Figure 1.** The fractional clearance (θ) ± SEM for Ficoll35.5Å (right panel) and albumin (to the left) with normal (151 mM, unfilled bars) and low (34 mM, filled bars) ionic strength perfusates. The θ for Ficoll was higher than θ for albumin at both low (p<0.01) and normal (p<0.001) ionic strengths.

**Figure 2.** The fractional clearance of Ficoll 35.5Å plotted against the fractional clearance for albumin in the perfused-fixed rat kidney. Data are shown for kidney perfusion with low (solid dots) and normal (unfilled circles) ionic strength solutions.

**Figure 3.** Urine over plasma concentration ratios for Ficoll plotted against the Stokes-Einstein radius. Data are shown for kidney perfusion with low (solid dots) and normal (unfilled circles) ionic strength solutions.

**Figure 4.** The estimated charge density using the gel-membrane model at normal and low ionic strength perfusion.

**Figure 5.** A Blandt-Altman plot describing the agreement between measured and modeled U/P concentration ratios for Ficolls of different sizes. At normal I,
there is an acceptable agreement. For low $I$, however, the fitting is poor
unless the fiber density is markedly reduced as for the diluted model.
REFERENCES


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<tr>
<td>Normal ionic strength exp. (n=6)</td>
<td>31.57 ± 3.08</td>
<td>16.36 ± 1.32</td>
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<tr>
<td>Low ionic strength exp. (n=6)</td>
<td>28.43 ± 2.23</td>
<td>16.57 ± 0.69</td>
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<tr>
<td>Parameter</td>
<td>Normal gel</td>
<td>Diluted gel</td>
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<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>Fiber volume fraction (%)</td>
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<td>Fiber radius (Å)</td>
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<td>-0.30</td>
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<td>Exchange area over diffusion distance (cm)</td>
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Figure 1

Albumin

Ficoll_35.5Å
Figure 2

Fractional clearance for Ficoll 35.5 Å

Fractional clearance for albumin

Normal I, 151 mM
Low I, 34 mM
Line of identity, 1:1
Figure 3

Stokes-Einstein radius (Å)

Ficoll U/P ratios

- Normal I, 151 mM
- Low I, 34 mM
Figure 5

-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
0.5

Measured U/P ratio for Ficoll

Difference (Measured - calculated U/P-ratio for Ficoll)

Normal Ionic strength
Low I, undiluted gel
Low I, diluted gel