*Kidney International, Vol. 25 (1984), pp. 527–533*

# Influence of charge on filtration across renal basement membrane films in vitro

JENNIFER BRAY and GARTH B. ROBINSON
with technical assistance from JENNIFER BYRNE

*Department of Biochemistry, Oxford University, Oxford, United Kingdom*

**Influence of charge on filtration across renal basement membrane films in vitro.** The filtration of differently charged species of myoglobin and dextran across films of isolated basement membrane in vitro showed that the filtration behavior of both polymers was influenced by charge. Rejection increased with increasing negative charge. Titration of the isolated basement membrane revealed an isoionic point of pH 5.5 and an isoelectric point of pH 5.7. The net negative charge at pH 7.4 was 0.15 mEq/g protein; this charge was attributed to carboxylate anion. Glycosaminoglycan sulphate did not contribute significantly to the net charge. Filtration of narrow range dextran fractions across films of basement membrane at the isoelectric point markedly reduced differences in filtration due to charge confirming that the differences in behavior found at pH 7.4 were due to charge interactions between the solutes and the membranes. Physical characterization of the charged and uncharged dextran fractions revealed no substantial differences in size or shape for the differently charged species.

**Influence de la charge sur la filtration à travers des films de membrane basale rénale in vitro.** La filtration d'espèces différemment chargées de myoglobine et de dextran à travers des films de membrane basale isolée in vitro a montré que le comportement de filtration des deux polymères était influencé par la charge. Le rejet s'élevait lorsque la charge négative augmentait. La titration de la membrane basale isolée a révélé un point isoionique de pH 5,5 et un point isoélectrique de pH 5,7. La charge négative nette à pH 7,4 était de 0,15 mEq/g protéines; cette charge a été attribuée à l'anion carboxylate. Le sulfate de glycosaminoglycan ne contribuait pas significativement à la charge nette. La filtration de fractions d'étendues étroites de dextran à travers des films de membrane basale au point isoélectrique réduisait de façon marquée les différences de filtration dûes à la charge, confirmant que ses différences de comportement trouvées à pH 7,4 étaient dûes à des interactions de charge entre les solutes et les membranes. La caratérisation physique des fractions de dextran chargées ou non n'a pas révélé de différence substantielle dans la taille ou la forme des espèces de charges différentes.

Studies in vivo suggest that the rejection of macromolecules by the glomerular capillary wall is influenced by the net charge on the molecules [1–5]. Briefly, rejection increases as molecular charge becomes more negative leading to the view that negative charges in the basement membrane and on adjacent cells serve to hinder the passage of negative molecules while facilitating the passage of positive molecules [6, 7]. In attempting to simplify our understanding of this problem, we examined the filtration of differently charged myoglobins and dextrans across films of isolated renal basement membrane in vitro to establish whether or not this membrane per se exhibits charge selective effects. Additionally, we determined the charge on isolated basement membrane to establish whether or not the membrane possesses a net negative charge at physiological pH as is inferred from histological studies [8].

## Methods

New Zealand white rabbits of either sex, weighing 2.5 to 3 kg, were obtained locally. Myoglobin (horse) was purchased from the Sigma Chemical Co., Poole, United Kingdom, dextrans and Sephadex from Pharmacia Ltd., Hounslow, United Kingdom; and radiochemicals from Amersham International, Amersham. Filtration cells were obtained from Amicon Ltd., High Wycombe, United Kingdom, and Millipore membranes (Type HAWP, 0.45 $\mu$m exclusion) from Millipore Ltd., Haltow, United Kingdom.

*Preparation of basement membranes.* Rabbit renal basement membranes were prepared from minced, sieved, kidney cortex after lysis of the cells with N-lauroyl sarcosine [9]. As judged by eye, 30 to 40% of the fragments recovered were derived from glomeruli, seen in preparations as clusters of loops of capillary membranes; the remaining fragments were tubular membrane.

*Filtration studies.* Coherent films of basement membrane fragments were constructed in pressure filtration cells of either 65 or 10 ml capacity (filtration areas, 13.8 and 4.9 $cm^2$, respectively) using methods described previously [10]. The cells were assembled after placing a membrane (Millipore) over a filter paper (Whatman no. 50, hardened) which in turn rested on the porous filter support of the cell. A known quantity of basement membrane, 1.5 mg for large and 0.5 mg for small cells, was suspended in buffered saline (0.15 M sodium chloride, 0.01 M tris-hydrochloric acid buffer, pH 7.4) in cells filled to two-third capacity. The cells were pressurized with oxygen-free nitrogen (200 kpa); the buffer was expelled thus forcing the fragments to form a layer on the Millipore membrane surface. The films thus formed were consolidated by continuously filtering buffered saline at the same pressure for 60 min. The films were comprised, on the average, of a depth of 20 layers of membrane; overlapping of the fragments sealed the films as judged by rejections of greater than or equal to 0.98 for $\gamma$-globulin. In filtration studies the buffered saline was replaced by

Received for publication September 1, 1982
and in revised form July 26, 1983

the solution to be filtered, 50 ml in the large and 10 ml in the small cells. Stirring was begun and pressure was applied. After 15% of the volume had been filtered, steady state conditions were reached and three sequential samples of filtrate were collected, 2.5 ml for large and 1 ml for small cells. The volume and time of each sample collected were recorded. Solute concentrations in the filtrate, in the initial, and in the final overstanding solutions were measured. Rejections ($\sigma = 1 - C_f/C_b$) were calculated. $C_f$ was the solute concentration in the filtrate; $C_b$ the concentration in the overstanding solution, was calculated for corresponding filtrates by correcting the initial concentration of the overstanding solution for losses of solute and solvent during filtration. Filtration conditions for each experiment are detailed in the text and all filtrations were at 22°C. Unless stated otherwise, filtrations were in buffered saline. Usually three filters were run in parallel using the same preparation of basement membranes.

*Preparation of myoglobin derivatives.* Anionic derivatives were prepared by succinylation [11]. The reaction was allowed to proceed for 90 min; the reaction mixture was dialyzed against distilled water and then buffered saline. Cationic derivatives were prepared by conjugation with hexanediamine [12] in 1 M sodium chloride; the reaction time was 5 hr. The reaction mixture was maintained at pH 6.5 to avoid denaturation. The products were dialyzed against buffered saline.

Prior to use the myoglobin derivatives were fractionated on Sephadex G-75 to separate monomer from aggregates formed during the reactions. The anionic/succinylated derivatives were shown to have an average pI of 5.3 in isoelectric focusing. The cationic derivatives were separated on carboxymethyl-cellulose ion exchanger. The fraction used was that eluting at pH 10 using 0.1 M sodium chloride in 0.1 M glycine-sodium hydroxide buffer. The $s_{20,w}$ values were 1.91, 1.99, and 1.80 ($\times 10^{-13}$) for native, anionic, and cationic forms, respectively. In some experiments the myoglobins were labelled with $^{125}I$ using the chloramine T method [13]. The physical characteristics of the proteins remained unchanged as judged by behavior on gel-exclusion.

*Preparation of polydisperse dextrans and dextran fractions.* Dextran T-2000, dextran sulphate, and diethylaminoethyl dextran were hydrolyzed to yield polydisperse samples. For dextran and dextran sulfate, hydrolysis was continued for 60 min at 100°C in 0.1 M hydrochloric acid [14]. DEAE dextran was hydrolyzed in two batches, one for 30 min and one for 60 min; these were combined to provide a range of polydispersity similar to that obtained with the other dextrans. All hydrolyses were at a concentration of 5% (w/v). Reaction was stopped by neutralization after which the reaction mixtures were dialyzed against buffered saline. In some experiments the dextrans were labelled with $^{3}H$ by reduction with sodium borotritiide following periodate oxidation [14]. The partially hydrolyzed polymers retained their charge as judged by their adsorption to cellulose ion exchangers of opposite but not like charge. However, in the case of dextran sulfate, analyses showed that 55% of the sulfate residues had been lost during hydrolysis.

Separation of the polydisperse material was achieved by gel-exclusion chromatography on Sephadex G-100 using columns 2.5 cm × 80 cm developed at room temperature in buffered saline containing 0.02% (w/v) sodium azide. The columns were calibrated with blue dextran, bovine serum albumin, ovalbumin, myoglobin, and cytochrome c, before and after experiments.

To prepare narrow range fractions of the dextran species consecutive fractions, usually three 5-ml fractions, were pooled from different positions in the elution profile.

*Physical methods.* Viscosities were measured using a rotational viscometer (Contraves). Measurements were at 25°C, the shear rate range was 2 to 128 $sec^{-1}$, and solute concentration ranged from 0.2 to 2.5% (w/v) in buffered saline. All samples showed Newtonian behavior; values for calculation were taken at a shear rate of 94.5 $sec^{-1}$. Molecular weights were determined by equilibrium sedimentation using a three-sectioned Yphantis cell run in an analytical centrifuge (model E, Beckman Instruments, Inc., Fullerton, California). Samples were dialyzed against 0.2 M potassium chloride containing tris-hydrochloric acid buffer (0.01 M, pH 7.4). The combined optical methods were used to obtain the z-average molecular weight; runs were at 20°C and speeds were chosen to give equilibrium in 24 hr. Partial specific volumes of the parent dextrans were measured by pycnometry and were found to be 0.64, 0.50, and 0.72 $cm^3/g$ for dextran, dextran sulphate, and DEAE-dextran, respectively.

Basement membrane titrations were conducted at 25°C in a sealed stirred cell under nitrogen using a pH meter (Radiometer type 26). Analar water (BDH Ltd., Poole, United Kingdom) was used throughout, and the quantities of membrane titrated ranged from 18 to 27 mg protein in 9 ml. After measuring the isoionic point in water, solid potassium chloride was added to a final concentration of 0.1 M allowing measurement of the isoelectric point; titrations from this point were made with either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Solvent was titrated separately under the same conditions.

*Chemical methods.* Basement membrane protein was measured by the procedure of Lowry et al [15] using dried and weighed basement membrane as the standard. Myoglobin concentrations were measured by $E_{280\ nm}$ determinations. The dextran concentration was measured by the anthrone procedure [16] using the parent dextran species as standards. When radiolabelled solutes were used, radioactivity was determined by scintillation counting with internal standardization to correct for quenching.

## Results

The myoglobin species, when filtered across basement membrane films with stirring, gave rejection values which reflected differences in charge with rejection increasing in the order cationic < native < anionic (Table 1); with no stirring, charge dependence was not seen. Filtering the same proteins, but labelled with $^{125}I$, in rabbit serum gave increased rejections and no charge dependence was observed (Table 1).

In the first series of dextran experiments, polydisperse samples of dextran, dextran sulfate, and DEAE-dextran were filtered across basement membrane films. The overstanding solutions and filtrates were then chromatographed on calibrated Sephadex G-100 columns to allow comparison of the profiles of molecules in each solution. In these experiments labelled dextran species were used after having first established that the chromatographic elution profiles of the original samples were identical when either radioactivity or the anthrone method was used to measure dextran concentration. These results, calculat-

**Table 1.** Rejection and flux values for charged species of myoglobin filtered across films of renal basement membrane under differing conditions[a]

| Filtration conditions | Native myoglobin | | | Anionic myoglobin | | | Cationic myoglobin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Flow $\mu l \cdot min^{-1} \cdot cm^{-2}$ | Flux $\mu g \cdot min^{-1} \cdot cm^{-2}$ | Rejection | Flow $\mu l \cdot min^{-1} \cdot cm^{-2}$ | Flux $\mu g \cdot min^{-1} \cdot cm^{-2}$ | Rejection | Flow $\mu l \cdot min^{-1} \cdot cm^{-2}$ | Flux $\mu g \cdot min^{-1} \cdot cm^{-2}$ | Rejection |
| Myoglobin *0.5 mg/ml* with stirring | 31.2 ± 1.4 | 4.0 ± 0.7 | 0.73 ± 0.04 | 29.2 ± 2.3 | 2.6 ± 0.3 | 0.8 ± 0.04 | 31.8 ± 2.1 | 6.2 ± 0.8 | 0.55 ± 0.04 |
| Myoglobin *0.5 mg/ml* no stirring | 29.3 ± 1.9 | 11.7 ± 1.2 | 0.05 ± 0.01 | 26.1 ± 2.3 | 9.5 ± 0.7 | 0.10 ± 0.01 | 29.6 ± 2.0 | 10.5 ± 0.7 | 0.05 ± 0.01 |
| Myoglobin *0.1 mg/ml* in serum | 7.9 ± 0.3 | 0.09 ± 0.01 | 0.88 ± 0.01 | 8.4 ± 0.8 | 0.1 ± 0.02 | 0.87 ± 0.01 | 8.3 ± 0.8 | 0.11 ± 0.02 | 0.85 ± 0.02 |

[a] Cationic and anionic forms of myoglobin were prepared as described in **Methods**. Filtrations were conducted at 150 kpa across films prepared from 0.5 mg of basement membrane protein in 10-ml filtration cells; the stirring rate was 1500 rpm. The myoglobins were dissolved in either buffered saline or serum after iodination with $^{125}I$. Rejections and fluxes were calculated from radioactivity measurements. Each result is the mean from two observations made on each of three separate filters ± SD.

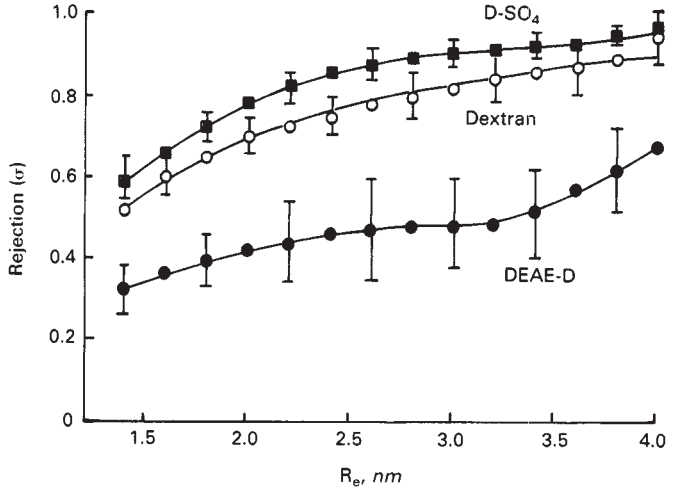


**Fig. 1.** *Filtration of polydisperse dextrans across films of basement membrane.* Polydisperse [$^3H$]-labelled dextrans, prepared as described in **Methods,** were dissolved in buffered saline (0.5 mg/ml) and filtered through films prepared from 1.5 mg of basement membrane protein in 65-ml filtration cells. Filtration was at a pressure of 150 kpa with stirring at 1000 rev/min. The overstanding solutions and the filtrates were chromatographed on Sephadex G-100 columns calibrated with proteins. Rejections were calculated by comparing the radioactivities recovered in fractions with the same partition coefficients. Average molecular radii ($R_e$) were determined from the column calibration curves. The results are the means from six separate filters. The *bars* indicate SD. Symbols are: ●, DEAE dextran; ○, dextran; ■, dextran sulfate.

ed as rejections for molecules of increasing radius (Fig. 1), showed that rejection increased with size and was influenced by charge (cationic < neutral < anionic). The experiments were then repeated using rabbit serum as the filtering medium. The results (Fig. 2) were similar except that rejections were increased; charge effects were still seen in parallel with in vivo studies [1, 2].

Since charge influenced rejection of the dextrans and the myoglobins, when filtered alone, it was concluded that basement membrane exhibits a net negative charge. This inference was tested by titration of the isolated basement membrane; the titration curve is shown in Figure 3. The membrane exhibited an isoionic point at pH 5.5 and an isoelectric point at pH 5.7 in 0.1 M potassium chloride demonstrating slight binding of $Cl^-$. Analysis of the titration curve showed 1 mEq/g protein of carboxylate anion compared with 1.6 mEq/g from amino acid analysis [17]; presumably the difference is accountable by masked carboxyls, possibly amides. Titratable histidine was 0.13 mEq/g compared with 0.16 mEq/g from analysis. Total basic amino acid, equivalent to titratable carboxylate taken from the isoelectric point, was 1 mEq/g compared with 1.16 mEq/g from analysis. Thus, the titration results reasonably agree with the amino acid composition.

Having established that the isoelectric point of the basement membrane was pH 5.7, filtration studies with dextrans were conducted at this pH and at pH 7.4 for comparison. Narrow range dextran fractions were prepared as described under **Methods.** Fractions were selected so as to be sufficiently different in size to show obviously different rejections and so that rejections would fall in the 0.2 to 0.8 range; for rejections outside this range small differences in filtration behavior were less readily seen. One half of each fraction was dialyzed against phosphate-buffered saline (0.15 M sodium chloride, 0.05 M sodium phosphate buffer) at pH 5.7 and the other half against phosphate-buffered saline, pH 7.4. For filters run at pH 5.7, the basement membranes were washed centrifugally four times in phosphate-buffered saline pH 5.7; the control membranes were similarly washed in phosphate-buffered saline pH 7.4. Filtrations were then carried out in the following order: anionic, neutral, cationic. The results are shown in Figure 4. The differences in rejection between the different charge species were much reduced at pH 5.7. A second experiment using separate hydrolysates showed the same results.

The physical characteristics of narrow fraction dextrans were studied to establish whether the fractions of the different species, which had the same excluded volume on gel-exclusion, showed comparable physical behavior. These results are shown

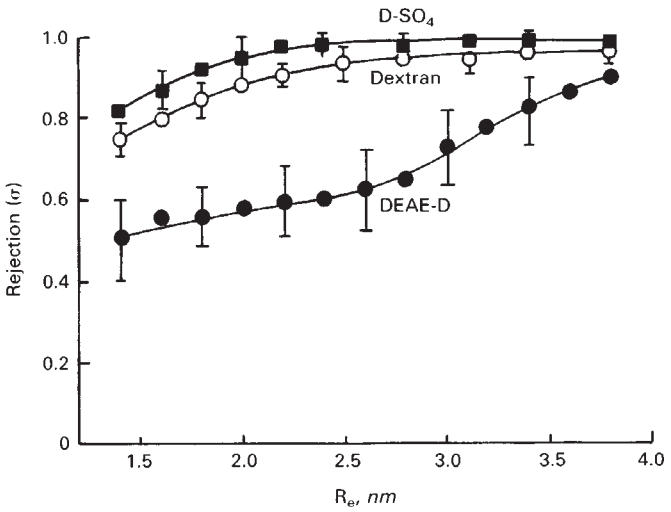


**Fig. 2.** *Filtration studies of polydisperse dextrans in serum.* The conditions were the same as those for Figure 1 except that the dextrans were dissolved in rabbit serum, 0.5 mg/ml. Symbols are: ●, DEAE dextran; ○, dextran; ■, dextran sulfate.

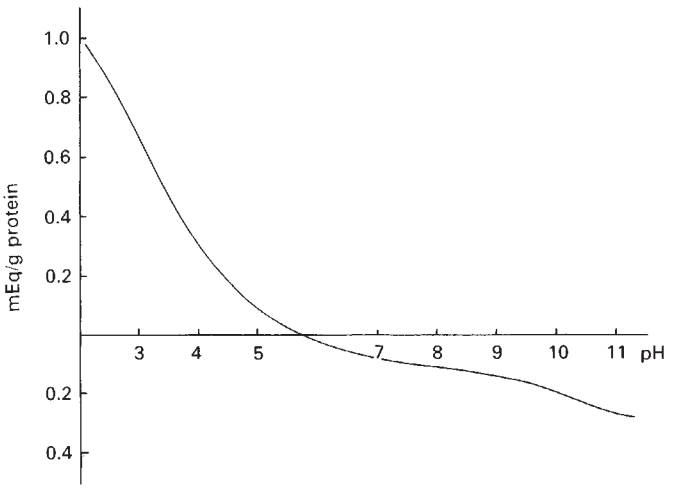


**Fig. 3.** *Titration curve for renal basement membrane.* Experimental details are described in **Methods.**

in Table 2. Generally, samples of the different species with the same average molecular radii ($R_e$) possessed similar molecular weights. Measurement of intrinsic viscosities allowed calculation of $R_e$ from the measured molecular weights; these calculated values were close to those assigned from chromatography. The intrinsic viscosities of the dextran sulfate fractions were lower than for the other two dextran species. Logarithmic plots of intrinsic viscosity against molecular weight gave exponents of 0.97, 1.1, and 0.87 for dextran, dextran sulfate, and DEAE dextran, respectively. For this limited study, these values were not significantly different from the theoretical value of 0.8 for a "self-avoiding" chain [18].

## Discussion

It was clear from the titration results that basement membrane does possess a net negative charge at physiological pH. The charge was low, 0.1 mEq/g protein or 12 mEq/liter, assuming a void volume of 0.85 (unpublished findings) and a protein specific volume of 0.85 $cm^3/g$. This value is considerably lower than values deduced from theoretical analyses of charge effects on filtration, 120 to 170 mEq/liter [19], 60 mEq/liter [20]. The results from titration reasonably agreed with the values predicted from the amino acid composition; the isoelectric point, pH 5.7, compares with a value of pH 5.2 reported from electrophoretic analyses of rat basement membrane [21]. Glycosaminoglycan sulfate groups would not be easily measurable by titration having a pK $\simeq$ 2, but if present in significant amounts, they would contribute to the isolectric point and an appreciable disparity between titratable groups and amino acid composition would be expected. Transmission microscopy of the membranes, after allowing the binding of cationized ferritin, showed clusters of ferritin on the membrane surfaces [22]. Such binding has been judged characteristic of the presence of heparan sulfates [23]; this component of the membrane therefore does seem to have been retained during isolation as reported previously [24]. Heparan sulfate accounts for only 1% by weight of rat basement membrane [25]. If this is so in the rabbit, and assuming heparan sulfate to contain 14% by weight sulfate [25], then the negative charge contributed by sulfate would be ~20 $\mu$Eq/g. Thus, sulfate would not contribute significantly to the net charge given the large amount, relatively, of carboxylate anion.

When considering the filtration studies, it is first necessary to assess how concentration-polarization effects might influence the observations. Concentration polarization occurs when rejected protein accumulates at the filter surface and filtration behavior is then determined by this local concentration of solute rather than by the concentration in the overstanding solution. Polarization is a function of the velocity of solvent flow through the filter and, for rotary-stirred cells, the speed of stirring and the cell dimensions. The polarization parameter $\Omega$ is given by [26]:

$$\Omega = Nu/R_{ey}^{1/2}Sc^{1/3} \qquad (1)$$

where

$$Nu = 2\,U_m \cdot b/D,\ R_{ey} = \omega \cdot b^2/\nu,\ Sc = \nu/D. \qquad (2)$$

Abbreviations used are: $U_m$, solvent velocity; b, cell radius; D, solute diffusivity; $\omega$, stirrer speed (radians $sec^{-1}$); $\nu$, solvent kinematic viscosity; $R_{ey}$ Reynolds number; Sc, Schmidt number. For $R_{ey}$ greater than 32,000, that is for turbulent stirring, the exponent of the number (Eq. 1) becomes 0.75 [27]. Polarization effects become significant at $\Omega > 0.25$ [26].

The effects of polarization are evident in the results obtained with myoglobins (Table 1). When the myoglobins were filtered alone, with stirring, $\Omega \simeq 0.36$, and polarization would be expected to increase solute flux slightly (10 to 20%). When stirring was absent, polarization effects were very marked; solvent flux declined, solute flux increased, and rejection values were reduced to the point where any differences in behavior due to charge became obscured. When mixtures of proteins are filtered, polarization effects are more complex. Completely rejected proteins predominate in the rejection layer and act as a barrier to the movement of small proteins [28]. This effect was seen when myoglobins were filtered in serum. That polarization had occurred was shown by the reduction in water

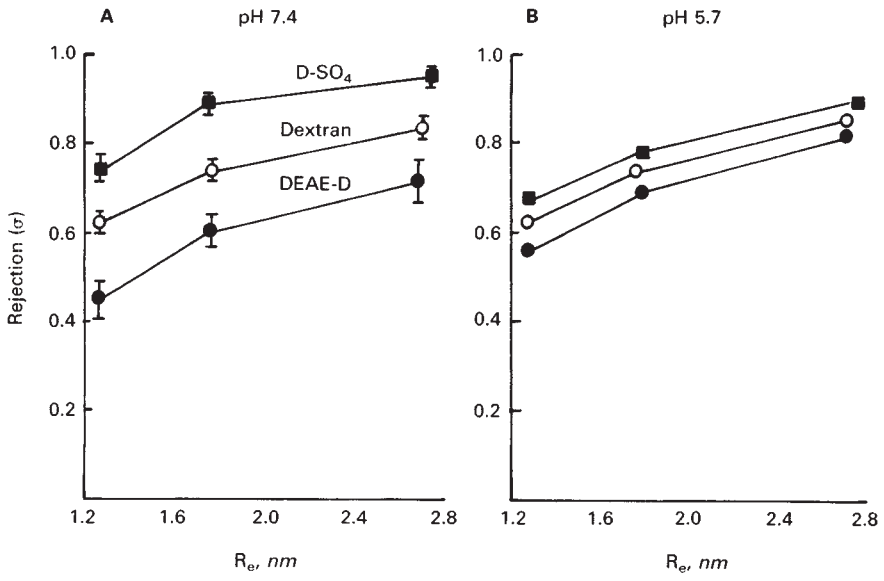


**Fig. 4.** *Filtration of narrow range dextrans across basement membrane films at pH 5.7 and 7.4.* The dextran fractions were prepared from polydisperse dextrans by chromatography on Sephadex G-100 columns as described in **Methods.** Basement membrane films were prepared from 0.5 mg of basement membrane protein in 10-ml filtration cells. Filtration was at a pressure of 150 kpa with stirring at 1500 rpm. Dextrans were dissolved in phosphate-buffered saline at the appropriate pH; the concentration was 0.5 mg/ml. The results are the means of two observations on each of three filters. The *bars* indicate SD. pH in **A** is 7.4; in **B** 5.7. Symbols are: ●, DEAE dextran; ○, dextran; ■, dextran sulfate.

**Table 2.** Physical characteristics of narrow fractions of dextran, DEAE-dextran, and dextran sulfate[a]

| Dextran species | Fraction number | $R_e$ from gel-exclusion | $M_r$ *z-average* | $[\eta]$ $dl \cdot g^{-1}$ | $R_e$ from $M_r$ (z) and $[\eta]$ |
|---|---|---|---|---|---|
| Dextran | 1 | 2.70 ± 0.2 | 18900 | 0.123 | 3.34 |
| | 2 | 2.30 ± 0.2 | 13600 | 0.096 | 2.75 |
| | 3 | 1.75 ± 0.15 | 9400 | 0.040 | 1.81 |
| | 4 | 1.45 ± 0.1 | 6500 | — | — |
| | 5 | 1.28 ± 0.1 | 5700 | 0.043 | 1.57 |
| DEAE-dextran | 1 | 2.69 ± 0.2 | 14700 | 0.144 | 3.23 |
| | 2 | 2.28 ± 0.2 | 12000 | 0.062 | 2.29 |
| | 3 | 1.77 ± 0.15 | 7300 | 0.050 | 1.80 |
| | 4 | 1.45 ± 0.1 | 5800 | 0.040 | 1.54 |
| | 5 | 1.28 ± 0.1 | 3600 | 0.035 | 1.26 |
| Dextran sulfate | 1 | 2.73 ± 0.2 | 15800 | 0.072 | 2.63 |
| | 2 | 2.30 ± 0.2 | 12900 | 0.045 | 2.10 |
| | 3 | 1.77 ± 0.15 | 8600 | 0.027 | 1.55 |
| | 4 | 1.44 ± 0.1 | 5500 | 0.022 | 1.25 |
| | 5 | 1.27 ± 0.1 | 5300 | 0.016 | 1.12 |

Abbreviations: $R_e$, average molecular radii; $M_r$, molecular weight (relative); $\eta$, dynamic viscosity.

[a] The fractions were prepared from polydisperse dextrans by gel-exclusion chromatography on Sephadex G-100 columns calibrated with proteins as described in **Methods**. Molecular weights and intrinsic viscosities were determined as described, also in **Methods**.

flow rates by ~75%; similar reductions were found in the myoglobin flux values. Thus, movement of the myoglobin has been markedly restricted presumably due to physical obstruction by the larger proteins in the polarization layer. Size rather than charge may become predominant in determining rejection in these circumstances; certainly charge selectivity had been obscured.

The polarization parameter cannot be determined accurately for the dextran filtrations since the diffusion coefficients for the solutes are unknown. Attempts to determine values by ultracentrifugation proved unsuccessful since the coefficients were found to be markedly concentration-dependent in contrast to the report by Jorgensen and Moller [29]. Diffusion coefficients, calculated from the average radii of the dextrans, gave $\Omega < 0.2$ for the largest fraction (radius 4 nm) filtered in the 65 ml cells (Fig. 1), indicated negligible polarization in these experiments. For the 10 ml cells (Fig. 4), $\Omega = 0.46$ for the largest fraction and 0.26 for the smallest so that polarization effects would have influenced these results. However since D, that is, solute diffusivity, and hence $\Omega$, would be independent of charge at the salt concentrations used, comparisons of filtration behavior of the different species possessing the same size should remain valid. Filtrations in serum were affected by polarization since both water and solute flux rates were reduced. Rejections were found to be slightly increased (Fig. 2), but charge effects could still be observed in contrast to the results obtained with myoglobin. The reason for this difference is unknown, but dextrans are more readily deformed than proteins and the polarization layer may thus be a less effective barrier to movement. There is no experimental evidence as to whether or not concentration-polarization occurs in glomerular capillaries, but judging from the present findings, charge dependence would still be observed if polarization were to occur.

The dependence of filtration behavior on charge was established using both myoglobin and dextran. In all the experiments (Table 1, Figs. 1 and 2), the rejection values of the macromolecules were found to be influenced by charge with rejections decreasing in the order: anionic > neutral > cationic. When unfractionated hydrolysates of the dextrans were filtered, there was a less marked difference in rejection between dextran and dextran sulfate than between dextran and DEAE dextran (Figs. 1 and 2). In addition the results showed a higher variance than when narrow range fractions were filtered (Fig. 4) presumably because the former experiments were more complex in design resulting in a larger margin of error.

Two approaches were adopted in an attempt to demonstrate that the differences in the behavior of the dextrans was due to charge. The first was to examine the physical properties of the dextrans to judge whether or not the charged and uncharged species behaved differently in solution. This study was restricted in the sense that it was pertinent to study only those fractions which were able to permeate the membranes. In thus restricting

the study, the small range of results obtained provided only limited data. Additionally, the small size of the molecules introduced uncertainty as to whether or not theoretical treatments of polymer folding in solution could be applied since the theories assume a large polymer chain length. The viscometric studies indicated that the charged and neutral dextrans behaved as unperturbed flexible polymers approximating in shape to spheres. Charge repulsion effects did not affect shape in an obvious manner and would not be expected to do so in 0.15 M sodium chloride [30]. The molecular weights of fractions of the different species of the same Stokes-Einstein radii from gel-exclusion were similar; these molecular weight values, combined with intrinsic viscosity values, produced calculated radii close to those measured. This indicates that polymer theory is indeed applicable to the small molecules studied. That the two measurements agree also implies that the relationships between Stokes-Einstein radius and column partition coefficients are the same for both proteins and polymers. This finding supports observations made elsewhere [31], but it has been reported that divergence of behavior is found as molecular weight increases [29]. Although fractions with the same radii possess similar molecular weights, the molecules are not identical; the monomer molecular weights of the polymers differ and hence molecules of the same weight will possess different chain lengths. This might explain why the intrinsic viscosities of dextran sulfate fractions were somewhat lower than for dextran and DEAE dextran. Attempts to determine chain lengths using end-group assay proved imprecise because of the different reactivities of the end groups of the three polymer species. No obvious physical differences between the three species were apparent from these studies supporting the view that differences in charge account for the differences in filtration behavior.

This contention was proven using the second approach of examining the filtration behavior of the dextrans at the isoelectric point of the basement membrane when it did not exhibit a net charge. These experiments (Fig. 4) showed that charge dependence was reduced or lost at the isoelectric pH. Therefore, the differences in filtration behavior seen at neutral pH must result from interactions between the charged polymers and charges in the basement membrane.

When relating these findings to glomerular filtration, one major difference to realize is that the membranes used here were derived from tubules as well as from glomerular capillaries. Clusters of glomerular capillary membrane account for 30 to 40% of the fragments seen in preparations. The clusters were larger than the fragments of tubular membrane. Hence, the weight proportion of glomerular membrane is greater than 30 to 40% and neither membrane type predominates. Comparisons of rabbit glomerular and tubular membrane have shown no major differences in gross protein or chemical composition [32]. No obvious differences were seen in the binding of cationized ferritin [22]. Thus, the two membranes appear to be chemically very similar and so might be expected to behave similarly.

The basement membrane films show charge-dependent characteristics similar to those found in vivo [1, 2]. One difference is that the films exhibit higher rejections toward dextrans than is found for capillary ultrafiltration. Here it should be noted that the films are twenty times thicker than the single basement membrane in the capillary wall and behavior of the two barriers might not be identical. Nonetheless, since the films do show charge-dependent rejection, there seems to be no reason to assume that in the capillary, the charges on cells adjacent to the basement membrane play a role in this process. It has been speculated that heparan sulfate may be important in determining charge selectivity and that loss of this material may contribute to loss of protein retention in vivo [23]. The present study shows that the charge-dependent effects are determined primarily by carboxylate anions rather than by the sulfate residues of glycosaminoglycans. Hence, the functions of the glycosaminoglycans may lie in cell basement membrane interactions rather than in sieving. Indeed, the glycosaminoglycan clusters seen on the basement membranes lie 60 nm apart leaving very large spaces through which macromolecules could penetrate the basement membrane.

## Acknowledgments

This work was supported by the National Kidney Research Fund. Dr. J. Bray was the recipient of a Medical Research Council Award for Training in Research Methods. The authors thank Drs. D. A. Hume and I. O. Walker for advice, Mr. P. Poon for assistance with viscometry, and Mrs. J. Heritage for ultracentrifugal analyses.

*Reprint requests to Dr. G. B. Robinson, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom*

## References

1. Chang RLS, Deen WM, Robertson CR, Brenner BM: Permselectivity of the glomerular capillary wall. III. Restricted transport of polyanions. *Kidney Int* 8:212–218, 1975
2. Bohrer MP, Baylis C, Humes HD, Glassock RJ, Robertson CR, Brenner BM: Permselectivity of the glomerular capillary wall. Facilitated filtration of circulating polycations. *J Clin Invest* 61:72–78, 1978
3. Rennke HG, Cotran RS, Venkatachalam MA: Role of molecular charge in glomerular permeability: tracer studies with cationised ferritins. *J Cell Biol* 67:638–646, 1975
4. Rennke HG, Patel Y, Venkatachalam MA: Glomerular filtration of proteins: clearance of anionic neutral and cationic horseradish peroxidase in the rat. *Kidney Int* 13:278–288, 1978
5. Purtell JN, Pesce AJ, Clyne DH, Miller WC, Pollack VE: Isoelectric point of albumin: effect on renal handling of albumin. *Kidney Int* 16:366–376, 1979
6. Brenner BM, Hostetter TH: Mechanisms of glomerular barrier function. *Contrib Nephrol* 26:9–16, 1981
7. Venkatachalam MA, Rennke HG: Glomerular filtration of macromolecules: structural, molecular and functional determinants, in *Renal Pathophysiology—Recent Advances,* edited by Leaf A, Giebisch G, Bolis L, Gorini S, New York, Raven Press, 1980, pp 43–56
8. Farquhar MG, Kanwar YS: Characterisation of anionic sites in the glomerular basement membrane of normal and nephrotic rats, in *Renal Pathophysiology Recent Advances,* edited by Leaf A, Giebisch G, Bolis L, Gorini S, New York, Raven Press, 1980, pp 57–74
9. Ligler FS, Robinson GB: A new method for the isolation of renal basement membranes. *Biochim Biophys Acta* 468:327–340, 1977
10. Cotter TG, Robinson GB: Effects of concentration — polarisation on the filtration of proteins through filters constructed from isolated renal basement membrane. *Clin Sci Mol Med* 55:113–119, 1978
11. Habeeb AFSA, Cassidy HG, Singer SJ: Molecular structural effects produced in proteins by reaction with succinic anhydride. *Biochim Biophys Acta* 29:587–593, 1958
12. Carraway KL, Koshland DE Jr: Carbodiimide modification of proteins, in *Methods in Enzymology,* edited by Grossman L, Moldave K, New York, Academic Press, 1972, vol 25, pp 616–623
13. Greenwood FC, Hunter WM, Glover JS: The preparation of

$^{131}$I-labelled human growth hormone of high specific radioactivity. *Biochem J* 89:114–123, 1963
14. CHANG RLS, UEKI IF, TROY JL, DEEN WM, ROBERTSON CR, BRENNER BM: Permselectivity of the glomerular capillary wall to macromolecules. II. Experimental studies in rats using neutral dextran. *Biophys J* 15:887–906, 1975
15. LOWRY FS, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
16. ROE JH: The determination of sugar in blood and spinal fluid with anthrone reagent. *J Biol Chem* 212:335–343, 1955
17. ROBINSON GB, LIGLER FS: The effects of protein extraction on the structure and filtration properties of renal basement membranes. *Eur J Biochem* 111:485–490, 1980
18. MORRIS ER, ROSS-MURPHY SB: Chain flexibility of polysaccharides and glycoproteins from viscosity measurements. *Tech Life Sci* B3:B310/1–B310/46, 1980
19. DEEN WM, SATVAT B, JAMIESON JM: Theoretical model for the glomerular filtration of charged solutes. *Am J Physiol* 238:F126–F139, 1980
20. DEEN WM, BRIDGES CR: Correction: Molecular charge of horseradish peroxidase. *Am J Physiol* 242:F750, 1982
21. KALANT N, MISRA RP, MANLEY RSTJ, WILSON J: Glomerular basement membrane in experimental nephrosis: X-ray diffraction and electrophoretic studies. *Nephron* 3:167–174, 1966
22. BRAY J: Studies on renal basement membranes. PhD Thesis. Oxford, United Kingdom, University of Oxford, 1983
23. KANWAR YS, FARQUHAR MG: Presence of heparan sulphate in the glomerular basement membrane. *Proc Natl Acad Sci USA* 76:1303–1307, 1979
24. KANWAR YS, FARQUHAR MG: Anionic sites in glomerular basement membrane. *J Cell Biol* 81:137–153, 1979
25. KANWAR YS, FARQUHAR MG: Isolation of glycosaminoglycans from glomerular basement membrane. *Proc Natl Acad Sci USA* 76:4439–4497, 1979
26. MUNCH WD, ZESTAR LP, ANDERSON JL: Rejection of polyelectrolytes from microporous membranes. *J Membr Sci* 5:77–102, 1979
27. BLATT WF, DRAVID A, MICHAELS AS, NELSON R: Solute polarisation and cake formation in membrane ultrafiltration, in *Membrane Science and Technology*, edited by FLYNN JE, New York, Plenum Press, 1970, pp 47–97
28. ROBINSON GB, COTTER TG: Studies on the filtration properties of isolated renal basement membrane. *Biochim Biophys Acta* 551:85–94, 1979
29. JORGENSEN KE, MOLLER JV: Use of flexible polymers as probes of glomerular pore size. *Am J Physiol* 236:F103–F111, 1979
30. LIBERTI PA, STIVALA SS: Physicochemical studies of fractionated bovine heparin. *Arch Biochem Biophys* 119:510–518, 1967
31. LAURENT TC, GRANATH KA: Fractionation of dextran and Ficoll by chromatography on Sephadex G-200. *Biochim Biophys Acta* 136:191–198, 1967
32. LIGLER FS: The isolation and structure of renal basement membranes. PhD Thesis, Oxford, United Kingdom, University of Oxford, 1977