Anionic charge concentration of rat kidney glomeruli and glomerular basement membrane

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Estimates of levels of glomerular and glomerular-basement-membrane anion charge should serve as useful quantitative markers for the integrity of the tissues in health and disease. We have developed a simple, rapid, technique to measure this charge through the use of ion exchange with radioisotopes $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ at low ionic strengths in phosphate buffer. When this technique is used, normal glomeruli isolated from rat have a measured net anion charge concentration of 17.4 ± 3.7 p-equiv. per glomerulus ($n = 20$). Perfused rat kidneys that lose approximately half of their glomerular heparan [^{35}S]sulphate content (owing to oxygen-radical damage) exhibited a lower anion charge, of 7.5 ± 1.6 p-equiv. per glomerulus ($n = 5$). Glomerular basement membranes prepared from rat glomeruli by a sonication-centrifugation procedure in the presence of enzyme inhibitors had a charge concentration of 6.3 ± 0.7 μ -equiv./g

wet wt. of tissue ($n = 4$), whereas membranes prepared by sonication, centrifugation, DNAase and detergent treatment had a charge concentration of 7.1 ± 1.6 μ -equiv./g wet wt. ($n = 4$). Isotope-dilution experiments with $^3\text{H}_2\text{O}$ on these detergent-prepared glomerular basement membranes demonstrated that they had a water content of approx. 93%, which would then give a net anion charge concentration of 7.6 ± 1.7 m-equiv./l ($n = 4$). These values are in good agreement with those obtained by others using titration techniques [Bray and Robinson (1984) *Kidney Int.* 25, 527–533]. The relatively low magnitude of glomerular anion charge in normal kidneys is consistent with other recent findings that glomerular anion charge is too low to affect the glomerular transport of charged molecules in a direct, passive, biophysical manner through electrostatic interactions.

INTRODUCTION

The anionic charge of the glomerulus and its two extracellular matrices, namely the glomerular basement membrane (GBM) and the mesangial matrix, is mainly contributed by the ionized univalent carboxyl and sulphate groups of heparan sulphate (Kanwar and Farquhar, 1979b; Kanwar et al., 1983). This is a class of glycosaminoglycan that is chemically heterogeneous and which has on average a charge density of 2 negative charges per disaccharide, but this may range from 1 to 4 charges per disaccharide. Chondroitin sulphate (Kanwar et al., 1983) and sialic acid-containing macromolecules (for review see Kanwar, 1984), together with collagen, may also make a contribution to the total number of anionic sites.

Evidence for the fact that the anionic charge of the GBM is responsible for glomerular charge selectivity (this is defined particularly with respect to the fractional clearance studies of differently charged dextrans; Chang et al., 1975a,b; Bohrer et al., 1978) comes from (1) qualitative electron-microscopic analysis of the distribution of ferritin molecules of widely different isoelectric points (Rennke et al., 1975; Rennke and Venkatachalam, 1977) and the binding of cationic 'stains' (Caulfield and Farquhar, 1976; Kanwar and Farquhar, 1979a), (2) the correlation between the deterioration of glomerular transport selectivity and loss of these negative charges through enzyme digestion and removal of glomerular heparan sulphate (Kanwar et al., 1980), (3) the changes associated with the binding of polycations (Seiler et al., 1975), and (4) the changes associated with the loss of anion charge that occurs with various pathologies such as nephrotoxic serum nephritis (Bennett et al., 1976) and in diabetes (Parthasarathy and Spiro, 1982).

Quantitative estimates of GBM charge concentration have been highly varied and generally indirect. A biophysical interpretation of charge selectivity in rat kidneys has been rationalized on the basis that the average GBM charge concentration is 165 m-equiv./l (Deen et al., 1980), which translates to a mass concentration of heparan sulphate of 40–45 mg/ml. NaCl distribution studies with the GBM *in vivo* give a lower value of GBM anion charge, of 25–30 m-equiv./l (Wolgast and Öjteg, 1988), and titration studies on isolated basement membranes yield a value of 0.1–0.12 m-equiv./g of GBM protein (Bray and Robinson, 1984; Kobayashi et al., 1989), which is calculated to be 12 m-equiv./l (Bray and Robinson, 1984). Chemical analysis, too, would suggest that the GBM heparan sulphate concentration is low. A value of 1% by weight for rat basement membrane was obtained by Kanwar and Farquhar (1979b) and a value of 30 μ g of heparan sulphate/ml (for GBM) was obtained by Spiro and Mohan (1987).

In view of the wide disparity of these estimates for such a potentially important glomerular parameter, we have performed investigations of the net negative charge in the glomerulus and the GBM by a non-perturbing NaCl equilibrium-exchange technique using radioisotopes $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$. This technique estimates the amount of $^{22}\text{Na}^+$ bound to exposed negative charges of the system as studied by $^{22}\text{Na}^+$ exchange with the resident counter-ion. The normal counter-ion to glomerular fixed charges would be Na^+ , so the exchange technique employed in this study is very much a non-perturbing one in measuring fixed charge. Anionic charges that may not have exchangeable counter-ions, such as those involved in tight electrostatic complexes with multivalent materials, would probably not be detected by this method. The exchange technique will only detect thermo-

dynamically active exchangeable counter-ions that will be associated with osmotic pressure of the system, dynamic charge selectivity and reversible binding of polyions. The technique is simple and has been used quite successfully to measure anion charge in cartilage tissue samples (Maroudas and Thomas, 1970) and anion charge concentrations of glycosaminoglycan compartments (Comper and Preston, 1974).

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (300–350 g) were obtained from the Monash University Central Animal House. [^{35}S]Sulphate (carrier-free; 10 mCi/mmol of sulphate, at 10 mCi/ml), Na^{36}Cl (0.1097 mCi/ml at 0.7 mg of Cl/ml) and $^{22}\text{Na}^+$ supplied as aqueous 20 mM NaCl (sp. radioactivity 0.37 mCi/ μg of NaCl) were from Amersham International, Amersham, Bucks., U.K. $^3\text{H}_2\text{O}$ (lot no. 26561669; 25 mCi/ml) was from DuPont (Boston, MA, U.S.A.). Ouabain (g-strophanthin) and DNAase I (bovine pancreas, grade II) were from Boehringer, Mannheim, Germany. Benzamidine hydrochloride, aminohecanoic acid, *N*-ethylmaleimide, phenylmethanesulphonyl chloride and deoxycholic acid (sodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Nembutal (60 mg/ml) was from Ceva Chemicals Australia Pty. Ltd., Hornsby, N.S.W., Australia. NaN_3 was from Ajax Chemicals, Sydney, N.S.W., Australia.

Solutions

Phosphate-buffered saline (PBS), which consisted of 0.14 M NaCl, 2.68 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4 , pH 7.5, was routinely used as a physiological buffer. Enzyme inhibitors, when used in PBS, were at the following concentrations: 0.1 mM phenylmethanesulphonyl fluoride, 0.1 mM aminohecanoic acid, 5 mM benzamidine hydrochloride and 1 mM *N*-ethylmaleimide. For Na^+ and Cl^- equilibration experiments, solutions corresponding to 1/30 dilution of PBS (1/30 PBS) and 1/15 dilution of PBS (1/15 PBS) were used.

Methods

Glomeruli preparation

Male Sprague-Dawley rats (300–350 g) were anaesthetized with an intraperitoneal injection of 1 ml of Nembutal (18 mg/ml). Both kidneys were removed, and glomeruli were isolated by the sieve method described by Spiro (1967) and suspended in 5 ml of 0.15 M NaCl on ice. After adequate mixing, a 20 μl sample of the suspension was taken up into a calibrated micro-capillary tube and the glomeruli were counted under phase-contrast microscope.

Ion-distribution studies on glomeruli

Glomeruli were centrifuged at 3300 rev./min for 5 min in a MSE Centaur 2 bench-top centrifuge and resuspended and washed in 6 ml of 1/30 PBS. The centrifugation procedure was repeated and the glomeruli were suspended in 2 ml of 1/30 PBS or other specified bathing solution. Two (1 ml) volumes, containing glomeruli, were taken and placed in separate Eppendorf tubes; to one tube was added 0.4 μCi of $^{22}\text{NaCl}$, and to the other 0.2 μCi of Na^{36}Cl . The glomeruli were kept suspended by gentle

tumbling for 24 h at 4 °C. The tubes were then centrifuged, as above, the supernatant was retrieved, and the pellet obtained was blotted gently with tissue paper to remove any excess supernatant solution and weighed. The radioactivity in the supernatant solutions and in glomeruli resuspended in 1.0 ml of 1/30 PBS was then determined. Preliminary experiments demonstrated that the distribution of $^{22}\text{Na}^+$ was constant over a period of 16–72 h. Distribution experiments were then routinely performed over a 24 h period. Glomeruli appeared normal, as determined by their appearance under the light microscope, after a 24 h equilibration period.

Ion-distribution studies on GBM

A quantity of glomeruli (in the range 4000–6000) were centrifuged at 3300 rev./min for 5 min in a MSE Centaur 2 bench-top centrifuge. The glomeruli were then processed to prepare GBM by modification/combination of the techniques described previously by other investigators (Meezan et al., 1975; Kanwar and Farquhar, 1979b; Beavan et al., 1988; Bertolatus and Klinzman, 1991). The supernatant fluid was removed and the glomeruli pellet was washed with PBS containing enzyme inhibitors and resuspended in 6.0 ml of the same solution. The glomeruli were then mildly ultrasonicated for 15 s (0.3 s on and 0.75 s off cycle) with a Sonicator Ultrasonic Processor (Heat Systems, Farmingdale, NY, U.S.A.), where the output control was on 4.5. The microtip probe was used. The GBM pellet was obtained by centrifugation and washed twice with 0.85 % NaCl (Spiro, 1967). The pellet was then suspended in 40 ml of 1 M NaCl containing 4000 units of DNAase I and incubated at 4 °C with slow tumbling for 1 h. The incubation mixture was then centrifuged for 20 min at 3500 rev./min at 4 °C. The pellet was then resuspended in 40 ml of 1 % deoxycholate/0.1 % NaN_3 and incubated with gentle tumbling for 2 h. The suspension was centrifuged at 4 °C for 20 min at 3500 rev./min. The pellet was transferred into two pre-weighed Eppendorf tubes. The pellets were resuspended and washed twice with PBS. Amounts of GBM were determined by the weight of the tissue. To obtain the wet weight of tissue, the last wash/suspension was centrifuged for 5 min, the supernatant was removed and the pellet carefully blotted. The pellet was centrifuged again for 5 min, blotted again, and the Eppendorf tube with GBM pellet was then weighed. The GBM was then washed/suspended/centrifuged four times with 1 mg of BSA/ml in 1/30 PBS. The charge on the GBM was determined by a dual-label technique using $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ simultaneously. The GBM pellet was incubated with gentle tumbling at 4 °C, with 0.2 μCi of $^{22}\text{Na}^+$ and 0.4 μCi of $^{36}\text{Cl}^-$ in 1 ml of 1/30 PBS containing 1 mg of BSA/ml for 48 h. At the end of the incubation period, the GBM suspension was spun at 10000 rev./min for 5 min, the supernatant was removed, and the GBM pellet was blotted to remove excess fluid and its mass was determined. The $^{22}\text{Na}^+$ radioactivity was determined on a γ -counter (see below), and these were used to correct estimations of $^{36}\text{Cl}^-$ radioactivity with a β scintillation counter.

The effectiveness of the DNAase-detergent steps in the purification of the GBM was established through sterol analysis (Austin et al., 1988) and DNA analysis (Kim et al., 1988). Sonicated GBM preparations contained 2.28 μg of DNA/1000 glomeruli and 2.52 μg of sterol/1000 glomeruli, whereas with DNAase-detergent-treated GBM there was a 98 % and a 91.3 % decrease respectively in these two quantities. The recoveries were calculated by knowing the number of glomeruli used to prepare the GBM preparations. Electron-microscopic analysis of the GBM preparation indicated loops of GBM that were sub-

stantially free of attached cellular elements, as previously demonstrated by Kanwar and Farquhar (1979b).

Water content of the GBM

GBM in a preweighed Eppendorf tube was equilibrated with 1 ml of 1/30 PBS and 50000 c.p.m. of $^3\text{H}_2\text{O}$ for 4 days at 4 °C with gentle tumbling. The tubes were then spun in an Eppendorf centrifuge for 10 mins, and the supernatants were collected and the pellets blotted. The tubes and their pellet contents were weighed to obtain the wet weight of the GBMs. The pellets were then digested with papain. The papain-digested pellets and supernatants were then counted for ^3H radioactivity. By knowing the specific radioactivity of $^3\text{H}_2\text{O}$ in the supernatant, the water content of the weighed pellet could be determined. These experiments gave a water content of the GBM pellet of 92.9 (± 4)% ($n = 4$).

DNA and sterol assays

The DNA content of the GBM prepared from sonicated glomeruli and sonicated/detergent-treated glomeruli was measured by a fluorimetric assay described by Kim et al. (1988). The sterol content of these preparations was measured by procedures described by Austin et al. (1988), with cholesterol as standard.

Kidney perfusion *ex situ*

The perfusion *ex situ* for 2 h with Krebs–Henseleit buffer containing 5% BSA with amino acids has been described previously (Tay et al., 1990).

Labelling *in vivo* with [^{35}S]sulphate

The labelling *in vivo* of glomerular heparan sulphate was performed by intraperitoneal injection of 1 mCi of [^{35}S]sulphate 4 h before the animal was killed. Characterization of ^{35}S -labelled material as mainly heparan sulphate has been described previously (Vassiliou et al., 1989). There was no significant loss of ^{35}S -labelled macromolecular material (as judged on Sephadex PD10 columns) from glomeruli under conditions of the ion-distribution studies.

Radioactivity counting

Sulphur radioactivity was determined by using 1.0 ml aqueous samples with 9.0 ml of a scintillant mixture as described by Fox (1968) and recorded on an LKB–Wallac 1215 Rackbeta scintillation counter. For $^{22}\text{Na}^+$, the radiation was measured in an Ekco scintillator counter type N664A (Ekco Electronics, U.K.) with a thallium-activated NaI crystal as scintillator, and also in a Wallac 1470 Wizard automatic γ -counter. For $^{36}\text{Cl}^-$ in glomeruli or GBM we used the preparation directly with the scintillant mixture. We had found that there was essentially no difference in $^{36}\text{Cl}^-$ radioactivity between the original preparation as compared with papain-digested preparations.

Papain digestion

Samples (1 ml) were incubated with 0.15 ml of papain buffer (1.0 M sodium acetate, pH 5.5, with 0.05 M EDTA) containing

10 mg/ml cysteine hydrochloride (added immediately before use) and 6.25 μl of papain suspension (16 $\mu\text{g}/\text{ml}$) at 60 °C for 24 h.

Calculations

The content of anionic charge (which will bind $^{22}\text{Na}^+$) in glomeruli or in GBM was calculated from the difference in their corresponding contents of Na^+ and Cl^- . Absolute ion concentrations can be obtained from radioactivity measurements and knowledge of specific radioactivities. All results are expressed as means $\pm$ S.D. unless stated otherwise.

RESULTS

The fixed anionic charge in rat glomeruli may exchange significant amounts of Na^+ with a bathing solution that contains $^{22}\text{Na}^+$ at relatively low ionic strengths. The results of a typical exchange experiment with 1/15 PBS and 1/30 PBS are shown in Table 1. For glomeruli in the range of 3600–6400 we obtained significant radioactivity of both ^{22}Na and ^{36}Cl in the glomerular preparation as compared with that in the bathing solution. The exchange of $^{36}\text{Cl}^-$ is assumed to be purely associated with the mobile Cl^- species associated with simple electrolytes such as NaCl. Therefore the difference in glomerular amount between Na^+ and Cl^- will give a net fixed anionic charge. The relative difference between the two ion quantities is enhanced (for a given number of glomeruli), and therefore the sensitivity of the measurement is greater when the ionic strength of the bathing solution is lowered.

In terms of expressing the units of concentration of anion charge in glomeruli, we recognize the complication that the glomerulus is an heterogeneous structure. The simple electrolyte will distribute in the extracellular matrices of the GBM and the mesangial matrix, the vascular compartment and perhaps within cells. In contrast, fixed anionic charge of the glomerulus that binds Na^+ would only be expected to be on all cell surfaces (particularly through the ubiquitous presence of heparan sulphate) and in both glomerular extracellular matrices. With the different glomerular distributions of ions in mind, we have expressed the measured glomerular fixed charge concentration in two ways (Table 2). The most direct and accurate parameter is equivalents of charge per glomerulus, which is an absolute measure of charge and makes no assumption about its distribution within the glomerular extracellular matrices. Alternatively, we have employed a charge concentration parameter of $\mu\text{-equiv.}/\text{g}$, which is obtained by measuring the weight of the glomeruli after they have been pelleted in the centrifuge (the error in this type of parameter is associated with the measurement of generally small amounts of material). The results for the charge concentrations, as measured by either parameter, were quite consistent (particularly with respect to charge/glomerulus) in spite of the different compositions of the bathing solutions; in 1/30 PBS the values were 17.7 p-equiv./glomerulus and 5.0 $\mu\text{-equiv.}/\text{g}$, and in 1/15 PBS the values were 15.9 p-equiv./glomerulus and 3.0 $\mu\text{-equiv.}/\text{g}$.

In another set of experiments described in Table 2, ouabain was used to minimize any active uptake of $^{22}\text{Na}^+$ by glomerular cells, which may then give rise to an anomalous calculation of anion charge. This was performed in case some cells were still alive after glomerular isolation and under conditions of the equilibration experiment. This experiment demonstrated that the inclusion of ouabain did not significantly affect the charge concentration.

We had previously established that significant changes in the level of prelabelled glomerular ^{35}S -labelled macromolecules (which is mainly heparan sulphate; Vassiliou et al., 1989), can be

Table 1 Quantities of glomeruli, radioactivity, Na⁺ and Cl⁻ in rat glomeruli associated with a typical equilibration experiment

External bathing solution	No. of glomeruli	Glomeruli				Bathing solution radioactivity (d.p.m./ml)	
		Radioactivity (d.p.m.)		Ion quantity (μ mol)		²² Na ⁺	³⁶ Cl ⁻
		²² Na ⁺	³⁶ Cl ⁻	Na ⁺	Cl ⁻		
1/30 PBS	6450	1668	3020	0.27	0.13	31 453	117 965
1/15 PBS	3600	1556	2130	0.24	0.175	33 060	118 310

Table 2 Total fixed anionic charge concentration in rat glomeruli and in glomeruli isolated from rat kidney perfused *ex situ*

Data presented as means $\pm$ S.D. for the number of experiments shown in parentheses: ND, not determined.

External bathing solution	Normal glomeruli		Glomeruli from perfused kidneys	
	(p-equiv./glomerulus)	(μ -equiv./g)	(p-equiv./glomerulus)	(μ -equiv./g)
1/30 PBS	17.7 $\pm$ 5.2 (7)	5.0 $\pm$ 1.3 (5)	7.5 $\pm$ 1.6 (5)	2.1 $\pm$ 0.65 (5)
1/15 PBS	15.9 $\pm$ 7.0 (6)	3.0 $\pm$ 0.8 (6)	ND	ND
1/30 PBS + 0.5 mM ouabain	18.6 $\pm$ 2.7 (7)	3.2 $\pm$ 0.8 (4)	ND	ND

Table 3 Total fixed anionic charge concentration of the GBM as measured in 1/30 PBS

DNA analysis of sonicated preparations gave values of 2280 $\pm$ 700 pg/glomerulus ($n = 3$). Using an average molecular mass per nucleotide of 330, this would be equivalent to an anion charge of approx. 6.9 p-equiv./glomerulus. For the sonicated/detergent-treated preparation a value of 0.02 μ -equiv. of DNA/g was obtained. Data are presented as means $\pm$ S.D. for the numbers of experiments shown in parentheses.

Preparation	External bathing solution	GBM charge	
		(p-equiv./glomerulus)*	(μ -equiv./g)
Sonicated	Without enzyme inhibitors	12.9 $\pm$ 3.5 (6)	2.8 $\pm$ 1.2 (4)
	With enzyme inhibitors	22.1 $\pm$ 5.4 (5)	6.3 $\pm$ 0.7 (4)
Sonicated, DNAase- and detergent-treated	With enzyme inhibitors	1.33†	7.1 $\pm$ 1.6 (4)

* Calculated knowing the number of glomeruli used to prepare the GBM.

† Calculated knowing that the average yield of GBM/1000 glomeruli was 0.188 mg ($n = 6$).

measured when the kidney is perfused *ex situ* for 2 h. In a non-perfused kidney the incorporation of ³⁵S-labelled macromolecular material was 0.37 $\pm$ 0.09 ($n = 3$) d.p.m./glomerulus and the residual level in a perfused kidney was 0.16 $\pm$ 0.03 ($n = 3$) d.p.m./glomerulus. The release of ³⁵S-labelled macromolecules has been correlated with the production of oxygen free radicals associated with the partial ischaemic conditions of the perfusion (Tay et al., 1990). [³⁵S-labelled macromolecule release from the glomerulus can be inhibited by the use of a cocktail of oxygen free-radical scavengers in the perfusion medium (Tay et al., 1990).] When the fixed charge concentration is examined in glomeruli isolated from perfused kidneys, it is clear that there is also a substantial decrease (> 50%) in charge content within the glomerulus (expressed in terms of p-equiv./glomerulus) in accord with a similar proportional loss of ³⁵S-labelled macromolecules (Table 2).

Anion charge measurements were made of each particular

stage of the GBM preparation (Table 3). Sonicated-centrifuged GBM preparations yielded finite values of anion charge per wet weight of tissue. The yield substantially increased by the use of enzyme inhibitors during the course of the preparation. The GBM charge recovery in relation to charge originally measured in the glomeruli was over 100%. This should be corrected, however, for charged sites provided by DNA, which may contribute ~ 30% of the total anion charge for this type of preparation (Table 3). The GBM was further processed in terms of DNAase and detergent treatment, which then yielded the slightly higher anion charge of 7.1 $\pm$ 1.6 m-equiv./g. When this value is converted into a charge concentration by using the water content of the tissue (92.9%), we arrive at a value of 7.6 $\pm$ 1.7 μ -equiv./ml. Any DNA contribution to this charge was negligible (Table 3). With the knowledge that the yield of GBM from the glomerulus was 0.188 mg/1000 glomeruli ($n = 6$), we calculated that the GBM charge/glomerulus was 1.33 p-equiv./glomerulus,

which represents about 10% of the total glomerular charge (Tables 2 and 3).

DISCUSSION

The most significant finding of this study is that whole glomerular anion charge can be easily and rapidly quantified by an exchange technique using $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$. The measurements are quite reproducible when examined over a large number of samples (Table 2). This glomerular anion charge estimate also appears to correlate with levels of glomerular heparan sulphate. When this molecule is lost by the tissue, owing to oxygen-stress-related reactions, the glomerular anion charge decreases accordingly. We expect then, that this type of measurement will become useful in assessing the integrity of the glomerulus under different pathological and experimental conditions when the glomerular anion charge is altered.

In addition to the measurement of whole glomerular charge, we have made an attempt to determine the GBM charge concentration. Unfortunately, there is a considerable lack of criteria, at both the morphological and molecular level, in establishing the quality of the GBM preparation. Findings by Beavan et al. (1988) suggest that significant losses of ^{35}S -labelled macromolecules can occur at various stages of conventional GBM preparation. These losses are particularly associated with the detergent-treatment procedure after sonication or iso-osmotic lysis of the whole glomeruli. The concern is whether this loss represents a genuine purification step in the GBM preparation or a critical loss of functional GBM components (such as cell surface material that can interpenetrate the extracellular matrix of the GBM). It was for these reasons that we investigated the charge content of both sonicated GBM and sonicated/detergent/DNAase-prepared GBM. The sonication process alone is known to remove recognizable cell components (Kobayashi et al., 1989), but not to result in any significant loss of ^{35}S -labelled material (Beavan et al., 1988). The yield of ^{35}S -labelled macromolecules in such GBM (sonicated only) preparations as compared with the content in whole glomeruli was high (> 85%). The results from the $^{22}\text{Na}/^{36}\text{Cl}$ equilibration technique, shown in Table 3, demonstrate that there is substantial recovery of fixed charge in the GBM (sonicated) pellet as compared with the original charge/glomerulus, particularly when enzyme inhibitors were included in the equilibration system, although there was a significant contribution to anion charge by endogenous DNA. As it turns out, the charge content/mass of the GBM prepared by sonication was not significantly different from that prepared by the extensive sonication/detergent/DNAase treatment. When a comparison is made on the basis of charge/glomerulus, it is clear that the GBM prepared by sonication/detergent/DNAase treatment has a significantly lower anion charge than that originally present in the whole glomeruli.

Certainly, it appears that when GBM is prepared, as in this study, by well-established procedures (Kanwar and Farquhar, 1979b), the charge concentration is low, of the order of 8 m-equiv./ml, which is in agreement with studies by Bray and Robinson (1984). This charge estimation does allow us to consider its functional role in relation to GBM charge selectivity. There is no doubt that a significant body of information has accumulated which would suggest the direct involvement of glomerular anion charge on glomerular transport charge selectivity. At this stage, criticism could be levelled at this notion, as all studies performed so far have been qualitative and there has been no direct evidence to demonstrate that glomerular anionic charge acts in an electrostatic fashion to determine the

differential trans-glomerular transport rates of the charged kinetic probes. In fact, attempts to establish this biophysical interaction through the use of model systems and theoretical considerations would suggest that it cannot account for glomerular charge selectivity.

The first serious attempt to explain glomerular charge selectivity on a biophysical basis was by Deen et al. (1980), who utilized the classical Nernst-Planck equations to describe transport of charged macromolecules across membranes with a uniform distribution of negative charge. They claimed to fit theoretically the fractional clearance data of the charged dextrans by using a membrane charge concentration of 165 m-equiv./l. This is a very high value of charge concentration as compared with the concentration of heparan sulphate, the major contributor to glomerular anion charge, found in the GBM (Kanwar and Farquhar, 1979b; Spiro and Mohan, 1987), and anion charge estimated in the present study and through titration studies (Bray and Robinson, 1984; Kobayashi et al., 1989). More recently, the assumptions and predictions of the Deen et al. (1980) biophysical approach were tested experimentally (Zamparo and Comper, 1990). It was found that systems with very high (up to 200 m-equiv./l) anionic charge (contributed by anionic polysaccharide) did not differentially affect the transport of positively and negatively charged dextran transport probes. In fact, re-analysis of the theoretical electrostatic interactions suggested that under the conditions of the fractional clearance studies differential transport would not be observed. It turns out that the major function contributing to differential transport in the biophysical model of Deen et al. (1980), namely the Donnan distribution partition function, was incorrectly used. The high anion GBM charge, proposed by Deen et al. (1980), also seems unrealistic in relation to considerations of the internal osmotic pressure of the GBM and the single nephron glomerular filtration rate (Comper et al., 1990).

In support of the arguments above, attempts to mimic transport charge selectivity in isolated basement-membrane films have resulted in very poor charge selectivity as compared with studies *in vivo* (Bray and Robinson, 1984). In fact, no significant difference could be registered between the transport of polydisperse dextran and dextran sulphate.

It is known, however, that anionic charges in the GBM and mesangial matrix are not uniformly distributed and that local areas of charge concentration along the lamina rarae interna and externa (Kanwar and Farquhar, 1979a; Stow et al., 1985) could be responsible for charge selectivity. If we are to translate the average GBM concentration of 12 m-equiv./l (Bray and Robinson, 1984; Kobayashi et al., 1989), or 8 m-equiv./l (the present study), to a local region of the lamina rarae interna (by assuming that the interna is about a third of the whole GBM), then the maximal charge concentration confined solely to this region would be in the range of 25–40 m-equiv./l. It is not likely to be higher, owing to possible microheterogeneity in distribution of charge in the region, as the interna is essentially a monomolecular layer of heparan sulphate proteoglycan (Vassiliou et al., 1989). Even then, these charge concentrations would not be sufficient to exert a charge-selective effect as studied in model systems (Zamparo and Comper, 1990). Furthermore, if this layer was able to increase its charge concentration 3-fold by compression (Robinson and Walton, 1989) (and by doing so, overcoming serious osmotic-pressure problems) its concentration would still not be enough to exert a charge-selective effect on charged dextran transport probes (see above). The likelihood that a biophysical interpretation does not explain the glomerular charge selectivity is consistent with the recent finding that the different transport for dextran and for dextran sulphate is directly

associated with glomerular cell-mediated interactions with the transport probes (Tay et al., 1991).

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REFERENCES

- Austin, L., Arthur, H., Iannello, R. and Jeffrey, P. L. (1988) in *Molecular Pathology of Nerve and Muscle* (Kidman, A. D., Tomkins, J. K., Morris, C. A. and Cooper, N. A., eds.), pp. 385–390. Humana Press, Clifton, NJ
- Beavan, L. A., Davies, M. and Mason, R. M. (1988) *Biochem. J.* **251**, 411–418
- Bennet, C. M., Glascock, R. J., Chang, R. L. S., Deen, W. M., Robertson, C. R. and Brenner, B. M. (1976) *J. Clin. Invest.* **57**, 1287–1294
- Bertolatus, J. A. and Klinzman, D. (1991) *Microvasc. Res.* **41**, 311–327
- Bohrer, M. P., Baylis, C., Humes, H. D., Glascock, R. J., Robertson, C. R. and Brenner, B. M. (1978) *J. Clin. Invest.* **61**, 72–78
- Bray, J. and Robinson, G. B. (1984) *Kidney Int.* **25**, 527–533
- Caulfield, J. P. and Farquhar, M. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1646–1650
- Chang, R. L. S., Ueki, I. F., Troy, J. L., Deen, W. M., Robertson, C. R. and Brenner, B. M. (1975a) *Biophys. J.* **15**, 887–906
- Chang, R. L. S., Deen, W. M., Robertson, C. R. and Brenner, B. M. (1975b) *Kidney Int.* **8**, 212–218
- Comper, W. D. and Preston, B. N. (1974) *Biochem. J.* **143**, 1–9
- Comper, W. D., Williams, R. P. W. and Zamparo, O. (1990) *Connect. Tissue Res.* **25**, 3–16
- Deen, W. M., Satvat, B. and Jamieson, J. M. (1980) *Am. J. Physiol.* **238**, F126–F139
- Fox, B. W. (1968) *Int. J. Appl. Radiat. Isot.* **19**, 717–730
- Kanwar, Y. S. (1984) *Lab. Invest.* **51**, 7–21
- Kanwar, Y. S. and Farquhar, M. G. (1979a) *J. Cell. Biol.* **81**, 137–153
- Kanwar, Y. S. and Farquhar, M. G. (1979b) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4493–4497
- Kanwar, Y. S., Linker, A. and Farquhar, M. G. (1980) *J. Cell. Biol.* **86**, 688–693
- Kanwar, Y. S., Jakubowski, L. M. and Rosenzweig, L. J. (1983) *Eur. J. Cell. Biol.* **31**, 290–295
- Kim, Y.-J., Sah, R. L. Y., Doong, J.-Y. H. and Grodzinsky, A. (1988) *Anal. Biochem.* **174**, 168–176
- Kobayashi, S., Nagase, M., Honda, N., Adachi, K., Ichinose, N. and Hishida, A. (1989) *Kidney Int.* **35**, 1405–1408
- Maroudas, A. and Thomas, H. (1970) *Biochim. Biophys. Acta* **215**, 214–218
- Meezan, E., Hjelle, J. T. and Brendel, K. (1975) *Life Sci.* **17**, 1721–1732
- Parthasarathy, N. and Spiro, R. G. (1982) *Diabetes* **31**, 738–741
- Rennke, H. G. and Venkatachalam, M. A. (1977) *Kidney Int.* **11**, 44–53
- Rennke, H. G., Cotran, R. S. and Venkatachalam, M. A. (1975) *J. Cell Biol.* **67**, 638–646
- Robinson, G. B. and Walton, H. A. (1989) *Microvasc. Res.* **38**, 36–48
- Seiler, M. W., Venkatachalam, M. A. and Cotran, R. S. (1975) *Science* **189**, 390–393
- Spiro, R. G. (1967) *J. Biol. Chem.* **242**, 1915–1922
- Spiro, R. G. and Mohan, P. S. (1987) in *Renal Basement Membranes in Health and Disease* (Price, R. G. and Hudson, B. G., eds.), pp. 11–23, Academic Press, London
- Stow, J. L., Sawada, H. and Farquhar, M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3296–3300
- Tay, M., Comper, W. D., Vassiliou, P., Glasgow, E. F., Baker, M. S. and Pratt, L. (1990) *Biochem. Int.* **20**, 767–778
- Tay, M., Comper, W. D. and Singh, A. K. (1991) *Am. J. Physiol.* **260**, F549–F554
- Vassiliou, P., Tay, M. and Comper, W. D. (1989) *Biochem. Int.* **19**, 1241–1251
- Wolgast, M. and Öjteg, G. (1988) *Am. J. Physiol.* **254**, F364–F373
- Zamparo, O. and Comper, W. D. (1990) *Biophys. Chem.* **38**, 167–178

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