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Glomerular sieving of anionic and neutral bovine albumins in proteinuric rats

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Glomerular sieving of anionic and neutral bovine albumins in proteinuric rats. To characterize the defect in glomerular permeability leading to albuminuria in rats made nephrotic acutely by infusion of hexadimethrine (HDM) or chronically by administration of Adriamycin® (doxorubicin) (Adria), we developed and validated a tissue accumulation method for simultaneous determination of the glomerular sieving coefficients (GSC) of anionic ^{131}I-labeled bovine albumin (BSA-pI 4.9) and ^{125}I-labeled charge-modified neutral BSA (nBSA-pI 7.5 to 8.0). Total filtered marker was calculated by adding marker excreted in the urine to that filtered but reabsorbed by the tubules. The latter was determined by subtracting interstitial marker present in the left kidney, rendered non-filtering by ureteral ligation during mannitol diuresis, from the total marker accumulating within the right, filtering kidney. Experiments showed that markers circulated and were excreted intact and were neither degraded nor deiodinated during the period of the clearance studies.

In control animals the GSC of nBSA (0.026 ± 0.004) greatly exceeded that of BSA (0.0006 ± 0.0002), demonstrating the normal charge dependence of permeability. Both proteinuric groups had marked increases in the GSC of BSA (HDM: 0.021 ± 0.005; Adria: 0.025 ± 0.004), which correlated with appearance of rat albumin in their urine. HDM rats also had a twofold increase in the GSC of nBSA (0.049 ± 0.005), indicating alteration of the size dependence of permeability. The absolute increase of GSC of BSA and nBSA was similar, suggesting that albuminuria resulted from appearance of new "pores" in the glomerular filter that were not charge selective for proteins of the size of albumin. Thus, infusion of HDM, which binds to and neutralizes GBM anions, appears to produce albuminuria by inducing a structural change in the glomerular filter. Conversely, Adria rats had no significant increase in the GSC of nBSA (0.031 ± 0.005), indicating no significant change in the size dependence of permeability for proteins of the size of albumin. In these animals, the GSC of the anionic BSA approached that of the neutral nBSA, indicating that Adriamycin induces albuminuria by markedly reducing the normal charge dependence of permeability.

Filtration glomérulaire des albumines bovines anionique et neutre chez des rats protéinuriques. Afin de caractériser le défaut de perméabilité glomérulaire aboutissant à une albuminurie chez des rats rendus de faon aiguë néphrotiques par perfusion d'hexadiméthrine (HDM) ou de facon chronique par administration d'Adriamycine® (doxorubicine) (Adria), nous avons développé et validé une méthode d'accumulation tissulaire permettant la détermination simultanée des coefficients de filtration glomérulaire (GSC) de l'albumine bovine marquée à ^{131}I anionique (BSA-pI 4,9) et d'une BSA marquée par ^{125}I, à charge modifiée neutre (nBSA-pI 7,5 à 8,0). Le marqueur filtré total était calculé en ajoutant le marqueur excrété dans l'urine à celui filtré mais réabsorbé par les tubules. Ce dernier était déterminé en soustrayant le marqueur interstitiel présent dans le rein gauche, rendu non filtrant par ligature urétérale au cours d'une diurèse au mannitol, du marqueur total accumulé dans le rein droit, filtrant. Les expériences ont montré que les marqueurs circulaient et étaient excrétés intacts, et n'étaient ni dégradés, ni désiodés pendant la période de la mesure de clairance. Chez les animaux contrôles, le GSC de nBSA (0,026 ± 0,004) a largement excédé celui de la BSA (0,0006 ± 0,0002), démontrant la dépendance de la perméabilité à une charge normale. Chacun des groupes avait une augmentation marquée de GSC de BSA (HDM: 0,021 ± 0,005; Adria: 0,025 ± 0,004), qui était corrélée avec l'apparition de l'albumine de rat dans leurs urines. Les rats HDM avaient également une augmentation de deux fois de GSC de nBSA (0,049 ± 0,005), ce qui indique une altération de la dépendance de la perméabilité à la charge. L'augmentation absolue de GSC de BSA et nBSA était identique, ce qui suggère que l'albuminurie résultait de l'apparition de nouveaux "pores" dans le filtre glomérulaire, lesquels n'étaient pas sélectifs pour la charge pour les protéines de la taille de l'albumine. Ainsi, une perfusion d'HDM, qui se lie à, et neutralise les anions GBM, s'avère produire une albuminurie en induisant une modification structurelle du filtre glomérulaire. A l'opposé les rats Adria n'avaient pas d'élévation significative de GSC de nBSA (0,031 ± 0,05), ce qui indique aucune modification significative de la dépendance de la perméabilité à la taille pour les protéines de la taille de l'albumine. Chez ces animaux, le GSC de la BSA anionique était voisine de celle de la nBSA neutre, ce qui indique que l'Adriamycine induit une albuminurie en réduisant de facon marquée la dépendance normale de la perméabilité à la charge.

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The importance of proteinuria in the detection and consequences of glomerular disease has prompted many studies designed to elucidate the relationship of normal glomerular structure to the normal function of the glomerulus as a barrier to protein filtration, and the specific alteration(s) in structure responsible for the abnormal glomerular permeability to macromolecules observed in disease. Studies using urinary clearance techniques or morphologic assessment to determine the glomerular penetration of specific markers or test solutes have established that the ability of a macromolecule to penetrate the glomerular capillary wall is a function of its size, charge and shape [1–3]. In pathological conditions such as anti-GBM disease in rats [4, 5] and in human minimal change disease [6], it first appeared that proteinuria was accompanied by a loss of the charge barrier alone, with no concomitant increase in "pore" size. These observations integrated well with information from morphologic studies showing the presence of fixed negative charges in the epithelial cell glycocalyx of the glomerular capillary wall [7] and, more important, within the major protein filtration barrier, the glomerular basement membrane

[8], as well as with studies showing a reduction in fixed negative charges at both sites in proteinuric states [9, 10].

More recent studies have suggested that isolated defects in charge-dependent permeability in proteinuria are the exception rather than the rule. A sieving defect for large neutral molecules has now been demonstrated in puromycin aminonucleoside nephrosis [11], anti-GBM nephritis [12], experimental membranous nephropathy [13], Adriamycin nephrosis [14], and in numerous human nephropathies [15, 16]. Human minimal change disease remains the sole example of proteinuria not associated with a change in size dependence [6, 17]. In addition, as recently suggested by Weening and Rennke [14], it is unclear whether reductions in charge-dependent restriction contribute to proteinuria at all in some conditions, since in their studies of Adriamycin nephrosis the increased clearance of anionic horseradish peroxidase (HRP) could be accounted for by the increased clearance of neutral dextrans which were excluded from normal GBM to the same degree as the anionic HRP. As a further complication, these studies employed markers the glomerular sieving properties of which may not be identical to those of the principal protein present in proteinuric urine, albumin. In summary, the functional and structural nature of the permeability defect associated with albuminuria remains incompletely understood.

Our impetus for performing sieving studies came from our earlier observations on acute reversible proteinuria produced by infusion of hexadimethrine (HDM) in rats [18, 19]. Despite good evidence that this polycationic compound acts initially by binding to and neutralizing GBM anionic sites [19], immunochemical studies of HDM-induced urinary proteins showed the presence of large quantities of intact IgG [18]. The urinary excretion of this large relatively neutral molecule indicated the existence of an abnormality in size-dependent permeability in HDM induced proteinuria. Because of the potential importance of this observation that charge neutralization can lead to a defect in the size dependence of permeability, we measured the glomerular filtration of radiolabeled albumin markers in animals rendered acutely proteinuric by HDM and for comparison animals with Adriamycin nephrosis. To overcome the disadvantage presented by the tubular reabsorption of protein markers, a tissue accumulation method [20, 21] was employed to account for reabsorbed marker.

Methods

Preparation and characterization of radiolabeled marker albumins

Native anionic bovine serum albumin (BSA, MW 69,000) was obtained from Reheis Chemical (Phoenix, Arizona, USA). BSA was chemically modified by the cationization method of Danon et al [22] to produce a neutral albumin marker. Dimethylpropylamine was used as the nucleophile. A chromatofocusing column, prepared and eluted in the pH range 9 to 6 using the materials and instructions supplied by the manufacturer (Pharmacia, Piscataway, New Jersey, USA), was employed to obtain a cationized BSA preparation with a narrow range of isoelectric points. For use as filtration markers, BSA and cationized neutral BSA (nBSA) were iodinated with ^{131}I and ^{125}I, respectively, by the lactoperoxidase-glucose oxidase method [23]. Specific activities of the labeled preparations ranged from 5 to 7 μCi/μg protein. The radioactivity in the preparations was 90 to 98% precipitable by hot 6% trichloroacetic acid (TCA).

Molecular weight of the markers before and after radioiodination was determined by gel permeation chromatography on 1.6 × 50 cm columns of Sephacryl S-200 (Pharmacia), eluted with 0.15M NaCl, 0.05M sodium phosphate buffer, pH 7.4 (PBS) or with human plasma obtained from outdated bank blood. Unlabeled BSA was added to samples to mark the elution position of the native parent compound. Protein concentration in column effluent fractions was determined by a Coomassie Blue dye binding assay (Bio-Rad protein assay, Bio-Rad, Richmond, California, USA), while radioactivity peaks were located by gamma counting.

Isoelectric points (pI) of the markers were determined by isoelectric focusing (IEF) in commercially prepared polyacrylamide gels (PAG plates, pH range 3.5 to 9.0, LKB, Gaithersburg, Maryland, USA) performed according to the manufacturer's instructions or by focusing in agarose gels [18].

Studies of marker properties after in vivo administration

The molecular weight of the markers following intravenous administration to rats was assessed in several ways. Two 200 g female Sprague-Dawley (SD) rats were given an intravenous bolus dose of approximately 1 μCi of ^{125}I-nBSA. Two additional rats were given 1 μCi of ^{131}I-BSA. Urine was collected for 20 min following marker bolus administration. A plasma sample was obtained at the midpoint of the urine collection period. After 20 min, the abdomen was opened and the kidneys were flushed with saline via an aortic cannula. The flushed kidneys were removed, and weighed segments of kidney were homogenized in distilled water using glass tissue homogenizers. Plasma and urine samples were applied to the same Sephacryl S-200 gel permeation columns employed for characterization of the marker preparation and eluted in the manner described above. Plasma, urine and kidney homogenate samples were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7% gel [18]. After drying, the gel slab was placed on photographic film, which was developed after an exposure time of 1 to 2 weeks. The position of labeled bands on the film was compared to the position of standard protein bands run in adjacent lanes of the same gel and identified by Coomassie Blue dye staining. IgG and BSA were used as protein standards.

Kidney tissue possesses an enzyme capable of deiodinating iodoamino acids [24]. Since post-filtration deiodination of the labeled markers might lead to underestimation of marker filtration, experiments were undertaken to determine whether significant deiodination occurred during the 20-min duration of the clearance experiments in this study. The deiodination studies were designed to evaluate the effect of monoiodotyrosine (MIT), a known competitive inhibitor of renal deiodinases [25, 26], on the proportion of TCA-insoluble radioactivity present on intact marker in urine, kidney homogenate, and plasma. Inhibition of deiodination was monitored by administering ^{125}I-MIT and quantitating the extent to which this compound was deiodinated. For these experiments, ^{125}I-MIT was prepared by radiolabeling tyrosine (Sigma Chemical, St. Louis, Missouri, USA) using a lactoperoxidase method (Enzymobeads, Bio-Rad). After labeling, ^{125}I-MIT was separated from unincorpo-

rated ^{125}I by elution of the reaction mixture from a Sephadex G25 column (PD-10, Pharmacia) with 20% ethanol in distilled water. This nonpolar eluent caused the ^{125}I to elute from the column substantially after the free ^{125}I, which eluted at the total column volume.

Two-hundred g rats were prepared as for clearance studies and allowed to equilibrate after surgery on an infusion of saline, 0.25 ml/min. Six rats were continued on saline at this rate for an additional hr, while a second group of six rats were given a 1 ml intravenous bolus of 50 mM unlabeled MIT followed by infusion of cold MIT at 10 μmoles/min in saline, 0.25 ml/min for one hr. After preloading with saline or cold MIT, each animal received a bolus and maintenance infusion of several μCi each of ^{131}I-nBSA and ^{125}I-MIT in the same volume infusion rate of saline. After administration of the labeled compounds, urine was collected for 20 min. A plasma sample was taken at 18 min. At completion of the urine collection, the abdomen was opened and the kidneys were flushed with saline via an aortic cannula prior to removal. Plasma, urine and renal homogenate samples were precipitated with 12% TCA and counted in a dual channel gamma counter to determine the relative proportion of TCA-insoluble (intact) marker to TCA-soluble ^{131}I radioactivity.

Plasma, urine and kidney homogenate samples were also analyzed to determine the extent of deiodination of ^{125}MIT. Intact ^{125}I-MIT was separated from free ^{125}I by passing samples over disposable organic absorption columns packed with octadecylsilane (C18—Sep-Pak, Waters Associates, Milford, Massachusetts, USA). These columns bound 90 to 95% of cold MIT (detected by OD_{280} measurement) or ^{125}I-MIT applied, while allowing 98% of free ^{125}I radioactivity applied to pass unbound. Rat urine, and the supernatants of plasma or kidney homogenates precipitated with TCA, did not affect binding of ^{125}MIT to the Sep-Pak column or cause binding of ^{125}I to occur. For sample analysis, the columns were pretreated with 3.0 ml of methanol followed by 5.0 ml of phosphate-buffered saline (PBS) pH 7.2. Next, samples of urine or supernatants of plasma or kidney homogenate precipitated by TCA were applied and the columns were washed with 4.0 ml of PBS. This wash was counted to determine free ^{125}I radioactivity. The column itself was then counted directly in the gamma counter, to determine intact ^{125}I-MIT bound. Intact MIT was then expressed as a percentage of total ^{125}I radioactivity in plasma, kidney, or urine.

Paired label marker glomerular sieving experiments

Because proteins are filtered and both reabsorbed proximally and excreted, a tissue accumulation method [20, 21] was employed to account quantitatively for filtered and reabsorbed marker. This method assumes that marker present in the saline-flushed kidney after an intravenous infusion represents the reabsorbed fraction of filtered marker. We observed in preliminary experiments that labeled marker accumulated to a significant, although reduced, degree in non-filtering kidneys. Therefore, the technique was modified to account for presumed interstitial marker by rendering one kidney of each animal non-filtering.

Two-hundred g female Sprague-Dawley rats were anesthetized with intraperitoneal pentobarbital, 50 mg/kg, and maintained on a warm pad. A femoral artery catheter (PE-50, Clay-Adams, Parsippanny, New Jersey, USA) was inserted to permit blood sampling and infusion of saline at 0.20 ml/min. A 1.0 ml priming dose of inulin 15 mg/ml dissolved in 10% mannitol was administered through a jugular vein catheter and followed by a maintenance infusion of the same solution at 0.05 ml/min. The relatively high total rate of volume infusion (0.25 ml/min) was necessary because hexadimethrine infusion is only tolerated by volume expanded rats. The total volume infused during a typical 3 hr clearance study was 45 ml. Since urine flow rate generally averaged 0.1 ml/min, rats were volume expanded by an average of 14% of body wt during this study. Through a midline abdominal incision, the bladder was catheterized (PE-90) for urine collection. Animals were then permitted to equilibrate on the infusions described until sequential 5-min urine volumes determined by weighing differed by less than 5% (approximately one hr).

Twelve control rats continued to receive saline 0.20 ml/min, via the femoral artery catheter throughout the study. Another 12 rats were made acutely proteinuric by addition of HDM, 20 μg/min, to saline infusion for approximately 45 min. HDM used in this study was obtained commercially (Sigma Chemical). The presence of heavy proteinuria was qualitatively confirmed by testing the urine with sulfosalicylic acid (SSA). A third group of 13 rats were studied after induction of chronic proteinuria by intravenous (tail vein) injection of Adriamycin® (doxorubicin, Adria Labs, Columbus, Ohio, USA), 7.5 mg/kg [27]. These rats were studied an average of 6 weeks (range 2 to 16 weeks) after Adriamycin (Adria) administration. Nine of the 13 Adria rats were placed in metabolic cages 24 hr prior to clearance study for collection of urine for albumin excretion determination.

After equilibration for all rats, and after induction of proteinuria in HDM rats, the left kidney was rendered non-filtering by ureteral ligation during the ongoing mannitol diuresis [28, 29]. Forty-five min after ureteral ligation, animals were given an intranvenous bolus of approximately 1 μCi each of ^{131}I-BSA and ^{125}I-nBSA, followed by addition to the ongoing inulin infusion of sufficient labeled markers to ensure relatively constant plasma concentration during the clearance period. The maintenance infusion supplied approximately 0.025 μCi/min and 0.05 μCi/min of ^{131}I-BSA and ^{125}I-nBSA respectively. Immediately after marker administration, a timed 20-min urine collection was begun. Plasma samples were obtained from the femoral arterial catheter at 3, 10, and 17 min after the priming marker injection. After the final arterial plasma sample was collected, a left renal vein plasma sample for determination of inulin extraction by the left kidney was obtained with a 30 gauge needle.

At the end of the clearance period, the aorta was clamped above the renal arteries with an aneurysm clamp. A large polyethylene cannula (PE-200) was rapidly inserted into the abdominal aorta. A small hole was made in the left renal vein, and the kidneys were flushed with a minimum of 100 ml of saline at a pressure of 100 mm Hg until the renal vein effluent was clear. The flushed kidneys were removed and weighed. Weighed segments of each kidney were rapidly homogenized in 10% TCA in a glass tissue homogenizer.

Determinations and calculations

Radioactivity in plasma, urine, kidney homogenate, column fractions, or gel sections was determined for both ^{125}I and ^{131}I by counting in a dual channel automatic gamma counter (Gamma 5500, Beckman Instruments, Irvine, California, USA)

with crossover correction performed by the channels ratio method. Inulin concentration in arterial and renal vein plasma was determined by the anthrone method. Urinary excretion of rat albumin in 24 hr or 20-min clearance period samples was determined by rocket immunoelectrophoresis (IEP), using goat anti-rat albumin antiserum (Cappel Labs, West Chester, Pennsylvania, USA) incorporated into 1% agarose gel at a 1:20 or 1:40 dilution. Commercially obtained rat albumin (Sigma Chemical) served as standard in this assay. The anti-rat albumin antiserum was tested by Ouchterlony immunodiffusion in agarose to determine its lower limit of detection. The antiserum produced a detectable precipitin line down to 50 μg/ml rat albumin. At the average urine flow rates observed in this study (100 μl/min), this concentration corresponds to an albumin excretion rate of 5 μg/min.

For glomerular sieving coefficient (GSC) calculations, only TCA-insoluble radioactivity was used. Right kidney filtered and reabsorbed marker was calculated as total right kidney marker minus interstitial marker present in the left (nonfiltering) kidney; that is, right kidney reabsorbed marker = right kidney wt (right kidney cpm/g − left kidney cpm/g). Total marker filtered by the right kidney for each albumin was calculated as filtered and reabsorbed marker plus excreted (urinary) marker, and divided by average plasma marker concentration to obtain a right kidney clearance for ^{131}I-BSA and ^{125}I-nBSA. The protein clearance was next divided by the inulin clearance to obtain the GSC (filtrate-to-plasma concentration ratio of the marker).

Statistics

Analysis of variance was performed using the Statistical Analysis System (SAS, Cary, North Carolina) procedure GLM (General Linear Models) to determine whether there were significant differences among means of data for the three groups. Where an overall test of significance was positive, specific differences between individual group means were tested for significance using a Duncan multiple range test. In certain cases where the variances in the three groups differed significantly, the results of the parametric analysis of variance were confirmed by non-parametric analysis of variance using the SAS procedure NPAR1WAY. Regression equations were determined and slopes tested for significance of differences by analysis of covariance, using the SAS procedure GLM. For certain comparisons (indicated in text), groups were compared using a paired or unpaired *t* test as appropriate.

Results

Characterization of radiolabeled markers in vitro and in vivo

Commercially obtained BSA produced a single band by SDS-PAGE and IEF. IEF showed that BSA and nBSA had pIs of 4.9 and 7.5 to 8.0, respectively. Gamma counting of gel sections after focusing of ^{131}I-BSA and ^{125}I-nBSA confirmed that radiolabeling did not significantly alter marker pI. Gel permeation chromatography showed that both ^{131}I-BSA and ^{125}I-nBSA co-eluted with unlabeled BSA, indicating that neither cationization nor radiolabeling significantly altered the molecular weight of BSA. In two experiments, the labeled markers were applied to a gel column and eluted with human plasma. The elution position of the markers was not altered, indicating that no binding of the markers to plasma proteins had occurred.

The in vivo properties of the radioiodinated markers were examined in plasma, urine, and kidney homogenate samples from two rats given ^{125}I-nBSA and two rats given ^{131}I-BSA. Plasma and urine samples from these animals were applied to gel permeation columns. Analysis of radioactivity in the column fractions showed a major peak eluting at the same volume as unlabeled BSA and a second peak at the total column volume, representing the unbound iodide. The relationship of the size of the earlier peak to the later peak corresponded to the proportion of TCA-insoluble to TCA-soluble radioactivity. No other peaks of radioactivity were observed. When plasma, urine and kidney homogenate samples from these animals were examined by SDS-PAGE followed by autoradiography the in vivo integrity of the markers was again confirmed. For each sample, the autoradiographic film showed a single band located at a position identical to that of unlabeled, native BSA.

To assess the possibility that deiodination of labeled intact protein markers occurred during the 20-min period used in the sieving studies, rats were given ^{131}I-nBSA after pretreatment with either saline or the competitive deiodinase inhibitor MIT. The extent to which pretreatment actually influenced deiodination in vivo was determined by the simultaneous administration of ^{125}I-MIT. In six saline-treated rats, the mean (± 1 SD) percentages of intact ^{125}I-MIT were 11.6 ± 7.2, 29.0 ± 7.0, and 80.2 ± 6.9 in urine, plasma, and kidney homogenate, respectively, indicating significant deiodination of MIT in urine, plasma and to a lesser extent, kidney. In contrast, there was marked inhibition of ^{125}I-MIT deiodination in the six rats preloaded with cold MIT. In this group, mean percentages of intact MIT were 74.0 ± 6.0, 68.0 ± 11.2, and 91.0 ± 4.0 in urine, plasma, and kidney, respectively ($P < 0.01$, saline vs. MIT preload, for urine, plasma, or kidney by unpaired *t* test).

However, despite the marked inhibition of ^{125}MIT deiodination demonstrated in the MIT-preloaded rats, there was no significant increase in the proportion of TCA-insoluble intact ^{131}I-nBSA in this group. In the MIT preloaded rats the percentage of ^{131}I radioactivity precipitable by TCA was 13.0 ± 8.3, 93.5 ± 3.6 and 85.0 ± 7.6 in the urine, plasma, and kidney, respectively. In the saline-pretreated group, intact marker constituted 9.2 ± 9.7, 90.8 ± 4.5, and 80.5 ± 9.9 percent of ^{131}I cpm in urine, plasma, and kidney, respectively. None of these percentages differed significantly between the two groups ($P = 0.14$, unpaired *t* test). These studies demonstrate that deiodination of intact markers did not occur to a significant extent during the clearance studies, and that the free radioiodine present in the urine, plasma and kidney can be attributed to free iodine in the administered marker preparations.

Paired label albumin glomerular sieving experiments

The results of the glomerular sieving experiments are summarized in Table 1 and displayed graphically in Figures 1 to 5. The glomerular filtration rate of the right kidney as determined by inulin clearance (C_{IN}) during the 20-min clearance period is shown for the control and proteinuric groups in Figure 1. Although mean C_{IN} tended to be somewhat lower in the HDM group than in the controls (mean ± SEM, 0.35 ± 0.04 and 0.45 ± 0.05 ml/min/100g, HDM and control groups, respectively), this difference was not statistically significant ($P = 0.1$). This is in accordance with our previous observation that HDM infusion at the dose employed in this study produced heavy proteinuria

Table 1. Summary of albumin glomerular sieving experiments

Rat Number	C_{IN} *ml/min*	AER *μg/min*	^{131}I-BSA(pI 4.9) RK/P	LK/P	UV/P	% Reabsorbed[b]	GSC	^{125}I-nBSA(pI 7.5 to 8.0) RK/P	LK/P	UV/P	% Reabsorbed[b]	GSC
						Control (N = 12)						
1	0.54	< 5 μg/min[a]	0.075	0.072	0.002	ND	0.0004	0.45	0.19	0.004	98	0.024
2	0.68	< 5 μg/min[a]	0.038	0.039	0.003	ND	0.0002	0.68	0.21	0.028	95	0.040
3	0.59	< 5 μg/min[a]	0.035	0.041	0.003	ND	0.0003	0.39	0.26	0.073	84	0.039
4	0.80	< 5 μg/min[a]	0.039	0.039	0.005	ND	0.0003	0.92	0.20	0.045	94	0.046
5	0.78	< 5 μg/min[a]	0.037	0.033	0.004	ND	0.0003	0.58	0.16	0.017	96	0.026
6	1.54	< 5 μg/min[a]	0.043	0.025	0.032	ND	0.0010	0.37	0.11	0.060	84	0.012
7	0.94	< 5 μg/min[a]	0.020	0.008	0.001	ND	0.0007	0.23	0.08	0.020	90	0.011
8	1.37	< 5 μg/min[a]	0.020	0.034	0.003	ND	0.0001	0.82	0.38	0.025	95	0.018
9	1.26	< 5 μg/min[a]	0.046	0.017	0.032	ND	0.0013	0.91	0.26	0.022	97	0.026
10	0.47	< 5 μg/min[a]	0.054	0.042	0.003	ND	0.0019	0.53	0.18	0.009	98	0.046
11	1.81	< 5 μg/min[a]	0.027	0.036	0.003	ND	0.0001	0.67	0.28	0.041	91	0.012
12	1.78	< 5 μg/min[a]	0.061	0.032	0.009	ND	0.0011	0.65	0.26	0.063	87	0.013
Mean	1.05		0.041	0.035	0.008		0.0006	0.60	0.21	0.034	92	0.026
(± SEM)	± 0.14		± 0.005	± 0.005	± 0.003		± 0.0002	± 0.06	± 0.02	± 0.006	± 1.5	± 0.004
						HDM (N = 12)						
13	1.15	86	0.100	0.040	0.133	28	0.008	1.84	0.65	0.570	65	0.072
14	0.94	105	0.100	0.060	0.227	19	0.015	1.26	1.04	0.547	34	0.045
15	1.35	30	0.090	0.020	0.048	62	0.005	0.55	0.27	0.121	76	0.019
16	1.26	88	0.190	0.040	0.097	63	0.010	0.57	0.25	0.215	63	0.023
17	0.64	13	0.270	0.060	0.030	89	0.020	0.61	0.14	0.113	82	0.050
18	0.46	172	0.042	0.022	0.177	89	0.022	0.60	0.21	0.182	71	0.067
19	0.52	220	0.083	0.048	0.148	20	0.018	0.58	0.24	0.126	74	0.048
20	0.47	130	0.104	0.043	0.038	64	0.011	0.71	0.21	0.106	84	0.070
21	0.44	40	0.110	0.023	0.015	87	0.013	0.42	0.11	0.019	95	0.043
22	0.77	38	0.084	0.015	0.062	54	0.009	0.46	0.10	0.075	84	0.030
23	0.88	767	0.360	0.075	0.659	31	0.053	0.96	0.30	0.298	69	0.055
24	0.83	899	0.200	0.067	0.933	12	0.064	0.83	0.18	0.371	64	0.063
Mean	0.81	216	0.144	0.043	0.214	52	0.0206	0.78	0.31	0.229	72	0.049
(± SEM)	± 0.09	± 85	± 0.027	± 0.006	± 0.082	± 8	± 0.0054	± 0.12	± 0.08	± 0.052	± 4	± 0.005
						Adria (N = 13)						
25	0.57	125	0.15	0.10	0.293	17	0.031	0.47	0.22	0.269	51	0.047
26	1.20	44	0.14	0.09	0.104	34	0.007	0.36	0.27	0.081	55	0.007
27	0.44	29	0.18	0.06	0.093	59	0.025	0.33	0.20	0.093	59	0.025
28	0.63	20	0.07	0.03	0.016	67	0.004	0.16	0.12	0.011	78	0.004
29	0.44	ND	0.09	0.05	0.185	20	0.027	0.22	0.17	0.193	20	0.027
30	0.78	108	0.18	0.15	0.435	7	0.029	0.35	0.25	0.412	18	0.032
31	0.80	114	0.10	0.11	0.497	ND	0.031	0.34	0.25	0.399	27	0.034
32	1.12	43	0.14	0.06	0.057	62	0.007	0.63	0.24	0.132	77	0.028
33	0.44	100	0.18	0.18	0.205	ND	0.023	0.46	0.42	0.191	26	0.030
34	1.00	166	0.13	0.17	0.634	ND	0.032	0.53	0.39	0.599	23	0.039
35	0.76	103	0.14	0.13	0.269	6	0.018	0.46	0.28	0.152	59	0.025
36	0.60	98	0.11	0.11	0.292	3	0.025	0.52	0.23	0.228	56	0.043
37	0.86	176	0.17	0.21	1.059	ND	0.062	0.71	0.42	1.048	23	0.068
Mean	0.74	94	0.14	0.11	0.318	31	0.025	0.43	0.27	0.293	42	0.031
(± SEM)	± 0.07	± 15	± 0.01	± 0.02	± 0.079	± 7	± 0.004	± 0.04	± 0.03	± 0.077	± 7	± 0.005

Abbreviations are: C_{IN}, inulin clearance *(ml/min)*; AER, excretion of rat albumin in *μg/min* during the 20-min clearance period, determined by rocket IEP; RK/P, LK/P, ratio of right filtering (RK) or left (LK) non-filtering kidney radioactivity in *cpm/g* to plasma (P) radioactivity in *cpm/ml*; UV/P, ratio of excreted (urinary) to plasma marker; GSC, glomerular sieving coefficient; ND, not determined.

[a] All control rats excreted rat albumin at a rate below the lower limit of detection by Ouchterlony analysis (approximately 5 μg/min).

[b] Percent of total filtered marker present in reabsorbed fraction in right kidney. This value was not calculated for the anionic ^{131}I-BSA marker in control animals and in some Adria animals, in which the differences between right and left kidney marker were so small as to preclude an accurate estimate of total reabsorbed marker.

without significantly altering renal hemodynamics [19]. The mean C_{IN} of the Adria group was 0.32 ± 0.03 ml/min/100g, which was significantly less than that of the controls ($P < 0.05$). This reduction of approximately 30% in GFR is consistent with the observations of others in this model [14, 27].

To verify the non-filtering state of the ureter-ligated left kidney, the ratio of left renal vein to plasma inulin concentration was calculated for samples obtained near the end of the clearance period. In the 34 rats in which renal vein samples were successfully obtained this ratio averaged (± SEM) 1.03 ±

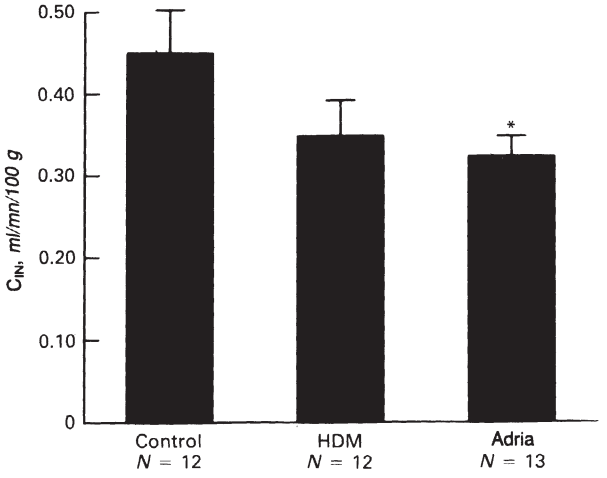

Fig. 1. *Inulin clearance (C_{IN}) in rats given saline (control), hexadimethrine (HDM), or Adriamycin® (Adria).* Bars represent mean ± SEM for each group. *$P < 0.05$, Adria vs. control.

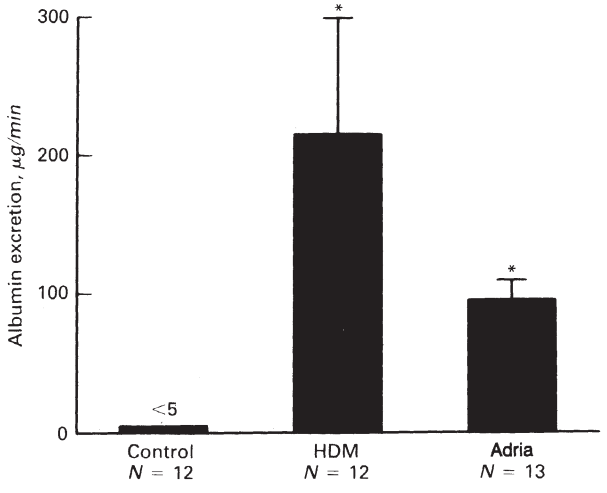

Fig. 2. *Albumin excretion rate during clearance studies in control and proteinuric rats (HDM, Adria).* All control rat urines had no detectable albumin (excretion rate, 5 μg/min). *$P < 0.001$, HDM or Adria vs. control.

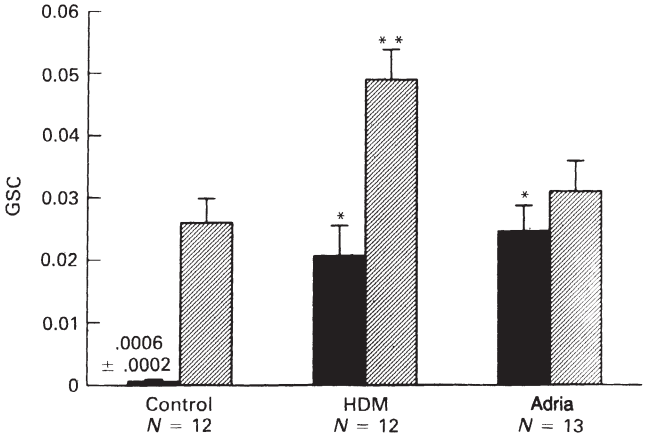

Fig. 3. *Glomerular sieving coefficients (GSC) of ^{131}I-BSA, pI 4.9 (■) and ^{125}I-nBSA, pI 7.5 to 8.0 (▨) in control and proteinuric (HDM, Adria) groups.* Bars indicate mean ± SEM. *$P < 0.001$, HDM or Adria vs. control; **$P < 0.01$, HDM vs. control.

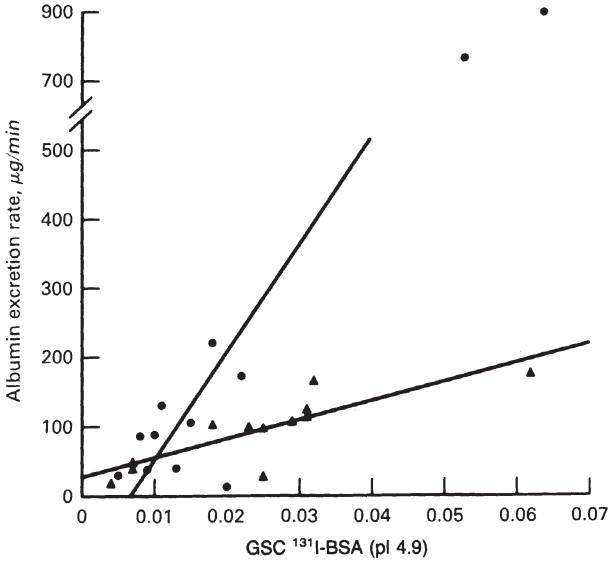

Fig. 4. *Albumin excretion rate (μg/min) plotted as a function of glomerular sieving coefficient (GSC) of ^{131}I-BSA in HDM (●) or Adriamycin® (▲) rats.* Each point represents value for a single rat. Linear regression equations (———): HDM, $y = 15{,}400 \times -102$; Adria, $y = 2{,}700 \times +28$.

0.02, which was not significantly different from 1.0. The constancy of plasma concentrations of the clearance marker was assessed by calculating the ratios of the second (10-min) and third (17-min) to the first (3-min) plasma concentrations. For ^{131}I-BSA, the second and third concentrations averaged 102 and 115% of the first concentration. For ^{125}I-nBSA, the second and third plasma concentrations averaged 99 and 117% of the initial concentration.

The mean albumin excretion rates of the three groups during the clearance period are shown in Figure 2. Albumin excretion rates of all saline-infused control animals fell below the lower limit of the assay (5 μg albumin/min). Both experimental groups were heavily albuminuric. HDM and Adria rats excreted a mean (± SEM) of 216 ± 85 and 94 ± 15 μg/min, respectively, of albumin. These values indicate a minimum 43-fold and 19-fold increase in mean albumin excretion rates for the HDM and Adria groups, respectively. In addition, 24-hr urines were collected in a subgroup of nine chronically proteinuric Adria rats 1 day prior to the clearance study. These rats excreted a mean (± SEM) of 329 ± 26 mg albumin/24 hr.

To compare renal accumulation of the two labeled marker proteins in the three groups of rats, the ratios of TCA insoluble kidney radioactivity (cpm/g) to plasma (P) radioactivity (cpm/ml) were calculated for the filtering right kidney (RK) and the non-filtering ureter-ligated left kidney (LK) for both ^{131}I-BSA and ^{125}I-nBSA (Table 1). In control animals, ^{131}I-BSA accumulated to an approximately equal extent in the right and left kidneys (RK/P = 0.041, LK/P = 0.35; not different by paired *t* test). This indicates that the marker was present in both kidneys primarily by accumulation in the interstitium, since the filtering and non-filtering kidneys had similar values. In addi-

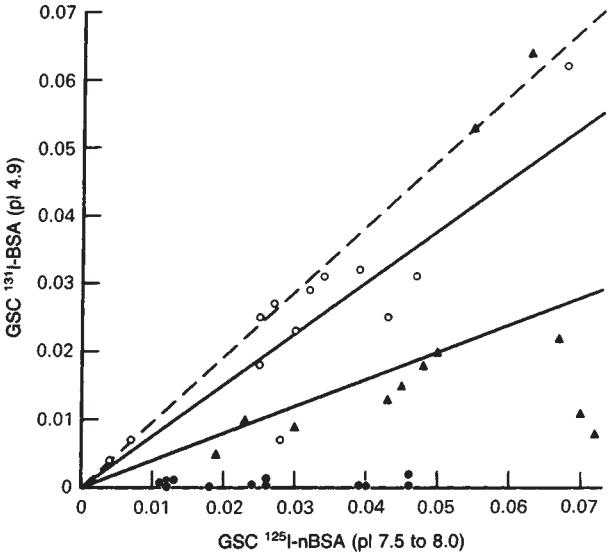

Fig. 5. *Glomerular sieving coefficients (GSC) in rats given saline (●), hexadimethrine (▲) or Adriamycin® (○). The GSC of ^{131}I-BSA is plotted on the ordinate, and the GSC of ^{125}I-nBSA on the abcissa. The ------ represents line of identity. Regression equations (———) Adria, y = .79x; HDM, y = .42x. The slope of the regression equation for control animals was not significantly different from zero.*

tion, the equal accumulation of ^{131}I-BSA in both kidneys shows that induction of the non-filtering state has not significantly altered left kidney marker accumulations. Although there undoubtedly was tubular reabsorption of some proportion of the small filtered amount of this highly restricted marker in the right filtering kidney, the renal radioactivity attributable to this reabsorbed marker was too small, in proportion to interstitial marker, to produce a consistently higher marker concentration than in the left non-filtering kidney. Since the GSC calculations for ^{131}I-BSA in the control animals reflect predominately urinary (excreted) marker, they must be regarded as minimum estimates of the GSC of this marker. A maximum estimate of ^{131}I-BSA GSC in the control group may be obtained by examining the 95% confidence limits for RK/P and LK/P (Table 1). There is less than a 5% chance that RK/P exceeded LK/P by more than 0.017. Using the average values of plasma and urinary markers in this group, one can calculate that a difference of this magnitude in kidney markers would correspond to a reabsorbed marker fraction equal to approximately 50% of the excreted urinary marker. The upper limit of the 95% confidence range for the GSC of BSA is therefore approximately 50% greater than the value calculated on the basis of urinary marker alone.

Accumulation of the neutral marker ^{125}I-nBSA was substantially greater in the right filtering kidney than in the left nonfiltering kidney. This reflects the higher glomerular filtration and subsequent tubular reabsorption of this more permeant marker. The reabsorbed fraction of ^{125}n-BSA equalled approximately 90% of the filtered load. It is not surprising that the reabsorption of nBSA exceeded that of BSA in the control group, since tubular protein reabsorption is greater for more cationic proteins. In fact, Park and Maack [30] have shown that the rate of tubular reabsorption of a nearly neutral albumin (pI 8.4) is 5 times greater than that of anionic native albumin.

Kidney marker accumulation was also calculated for the proteinuric groups (Table 1). In the HDM group, the anionic marker ^{131}I-BSA was present in the left nonfiltering kidney to approximately the same extent as in controls (HDM group LK/P = 0.043; Control group LK/P = 0.035), indicating that HDM did not significantly alter the access of this marker into the interstitium or the relative size of this compartment. However, the HDM rats accumulated significantly more anionic marker in their filtering right kidney than did control rats (HDM group, RK/P = 0.144; control group, RK/P = 0.041), reflecting the tubular reabsorption of part of the increase in filtered load. Renal accumulation of the neutral marker was also markedly higher in the filtering kidney (RK/P = 0.78, LK/P = 0.31) in the HDM rats.

In the Adria rats, as in controls, the ^{131}I-BSA content of the right filtering and left nonfiltering kidneys was similar (RK/P = 0.14, LK/P = 0.11). These ratios are approximately three times those observed for ^{131}I-BSA in the control group, suggesting an increase in size of the interstitial compartment, or in vascular permeability, in these chronically nephrotic rats. However, these Adria rats all had marked increases in urinary excretion of ^{131}I-BSA reflecting the increased glomerular sieving of this marker. The failure of the filtering right kidney to accumulate higher quantities of this marker than the non-filtering left kidney probably reflects impairment of tubular reabsorptive processes due to the metabolic effects of Adriamycin or to the chronic proteinuria. The right kidneys of the Adria rats accumulated substantially more ^{125}I-nBSA than the nonfiltering left kidney in each case (RK/P = 0.43; LK/P = 0.27).

Reabsorbed marker was determined by subtracting interstitial marker (as estimated from the nonfiltering left kidney) from total right kidney marker and was added to excreted urinary marker to estimate total filtered marker. This value, together with values of average plasma marker and C_{IN}, was used to calculate the glomerular sieving coefficient (GSC) for ^{131}I-BSA and ^{125}I-nBSA for each rat. Mean values for the three groups are displayed in Figure 3. The estimate of the GSC of anionic native BSA obtained (0.0006 to 0.0009) agrees quite well with estimates of endogenous albumin GSC obtained by micropuncture studies in the rat [31, 32]. As expected, the GSC of neutral BSA (0.026 ± 0.004) is 29 to 43 times higher than that of anionic BSA in the control rats, demonstrating the charge-selective property of the normal glomerulus.

Changes in the GSC of ^{131}I-BSA would be expected to parallel changes in the excretion rate for native rat albumin. In both proteinuric groups, there is a dramatic and highly significant rise in the GSC of ^{131}I-BSA (Fig. 3). The correlation between albumin excretion and ^{131}I-BSA GSC values in the proteinuric animals is displayed graphically in Figure 4. There is a significant correlation between these two parameters within both the HDM and Adria groups. The slope of the relationship differs significantly between the two groups; for any given level of GSC, the Adria rats tend to have a lower albumin excretion rate, probably because of lower plasma albumin levels. Plasma albumin concentrations were not measured in these rats. However, plasma albumin levels in an equally proteinuric group of rats receiving a smaller dose of Adria were approximately one-half of those measured in controls [33]. If one assumes normal plasma albumin concentrations in the acutely proteinuric HDM rats, and uses the measured values of GSC,

C_{IN} and right kidney marker reabsorption to calculate a value for urinary albumin excretion, the value obtained corresponds quite closely with urinary excretion of rat albumin determined by rocket IEP in this group. More technically, if one assumes a mean plasma albumin concentration of 40 mg/ml in the HDM rats and uses the measured mean values for C_{IN} (0.84 ml/min), ^{131}I-BSA GSC (0.021), and percentage of filtered load reabsorbed (52%), the calculated albumin excretion rate is 339 μg/min, while the measured excretion rate in this group was 216 μg/min.

Changes in the GSC of the neutral marker, ^{125}I-nBSA, may be used as an index of changes in the size selective property of the glomerular filter. In the HDM rats, there was a statistically significant twofold rise in the mean GSC of nBSA (controls—0.026 ± 0.004; HDM—0.049 ± 0.005) (Fig. 3) demonstrating altered size dependence of permeability in this group. Although the Adria group also showed a slight rise in the mean GSC of nBSA to 0.031 ± 0.005, this difference was not statistically significant.

The difference between the GSCs of anionic BSA and neutral nBSA may be used as an index of the charge-selective property of the glomerular barrier. The relationship between the GSC of ^{131}I-BSA and ^{125}I-nBSA for the three groups of rats is shown graphically in Figure 5. In all but two cases the HDM animals retained substantial glomerular charge selectivity, with values for GSC of anionic BSA well below those for neutral nBSA. The absolute increases of GSCs for nBSA and BSA were almost equal (Fig. 3). Equivalently, the mean difference between the values of GSC for nBSA and for BSA in the HDM group was equal to that seen in controls (controls—0.025 ± 0.004; HDM—0.028 ± 0.006; $P = 0.71$). This would be the expected outcome if the increases in GSC for the anionic and neutral molecules resulted from the appearance of new "pores" in the glomerular filter that were not charge selective for protein molecules of the size of albumin. Thus, albuminuria in HDM treated animals can be satisfactorily explained as the consequence of alterations of the size selective properties of the glomerular filter.

Conversely, in the Adria rats, the increase in the GSC for anionic BSA was unaccompanied by any significant increase in the GSC for neutral nBSA. As is seen in Figure 5, in most animals of this group the GSC for BSA approached that for nBSA, indicating loss of normal charge dependent permselectivity. The mean difference between the GSC for nBSA and for BSA was 0.007 ± 0.002, significantly less than that for either the control or the HDM group ($P < 0.002$). Thus, in the Adria rats, the isolated increase in GSC for anionic BSA, and albuminuria, can be explained mainly by loss of the normal charge barrier restricting proteins the size of albumin.

Discussion

The major finding in this study was that the polycation HDM caused a significant twofold rise in the GSC of the neutral marker ^{125}I-nBSA (Fig. 3 and Table 1). The existence of a sieving defect for large neutral molecules in HDM-induced proteinuria had originally been suggested by the finding of increased urinary IgG in the HDM model [18]. While defects in the size dependence of permeability are seen in the majority of proteinuric states, we had not anticipated this finding in HDM induced proteinuria, since several lines of evidence [18, 19] indicate that the polycation HDM acts first by binding to and neutralizing GBM anionic sites, an action that might have been expected to produce an isolated defect in the charge barrier. However, both our findings [18], those of Barnes, Radnik, and Gilchrist [34] and those of Bridges et al [35] strongly suggested that infusion of a polycation leads to alterations of size dependence of permeability as well. It is clear that anionic sites within the GBM, principally the heparan sulfate proteoglycans [36, 37], play a major role in the normal charge barrier. Our present work suggests that these charged molecules also play an important structural role, since their neutralization leads to changes in the size dependence of permeability. There is precedent within relevant biological and physical systems for a charge-reduction mediated structural rearrangement. Polycation-induced neutralization of epithelial cell glycocalyx anions leads to the reversible structural change of foot process effacement [38]. In addition, Tanaka [39] has shown in charged hydrated model gels similar to GBM that charge neutralization may lead to a physical rearrangement of gel structure resulting in an increase in "average pore size" of the gel. A similar mechanism operating within the GBM may explain the association between neutralization [18, 34, 35] or reduction [10, 11] of GBM anionic sites and the appearance of abnormal size dependence of permeability typical of most proteinuric states.

The markedly greater proportionate rise in the GSC of anionic native BSA compared with that of neutral nBSA (34-fold vs. twofold, respectively, Fig. 3) might suggest that a defect in charge-dependent as well as size-dependent permeability occurred in the HDM group. However, if one looks instead at the absolute increase of the GSC for BSA and nBSA over the values seen in control animals (0.20 and 0.23, respectively) it is apparent that both have increased by virtually the same absolute amount. This is exactly what one would predict if the increment in clearance of the two tracers had occurred because of the opening of a new pathway through the glomerular capillary wall which did not discriminate between charged and uncharged molecules of the size of albumin. Such a change could be conceptualized as the opening up of a few very large pores across the filtration barrier. Our findings are, therefore, compatible with the hypothesis that in HDM-induced proteinuria the increased excretion of both the exogenously administered ^{131}I-BSA and endogneous rat albumin is due to the observed defect in the size dependence of permeability. The glomerular capillary wall of HDM-treated rats retains a substantial charge barrier to a protein the size of albumin (Fig. 5).

In the Adria group, there was no significant increase in the GSC of nBSA (Fig. 3), so that the abnormality of size-dependent permeability reported previously by Weening and Rennke [14] in this model of proteinuria could not be demonstrated with the neutral albumin marker employed in this study. The discrepancy is not due to a lesser degree of glomerular injury in our Adria rats, since the drug dosage, interval between administration and the clearance study, and 24-hr urine albumin excretions were similar in the two studies. As Weening and Rennke note, the 30% increase in clearance of the anionic protein HRP (MW 40,000) that they found in Adria rats was quite small compared to increase in clearance of albumin required to account for the albuminura in these animals. They found no increase in clearance of neutral HRP. We also found no significant increase in the clearance of the larger neutral protein nBSA. Since Adria rats had increased filtration of large

neutral dextrans [14] the use of an even larger neutral protein marker would be expected to demonstrate a defect of size-dependent glomerular permeability in Adria rats. In fact, we reported the preliminary observation [40] that Adria rats do filter more neutral IgG than controls, although the increase is not as great as that seen in HDM rats. However, the current study indicates that in proteinuria due to Adria, the increased excretion of albumin (molecular wt 69,000) is principally due to a loss of the charge barrier. In the Adria group, the GSC of anionic BSA rises almost to that of nBSA (Figs. 3 and 5) indicating little remaining charge-dependent restriction for a molecule of this size.

To determine the nature of the glomerular permselectivity defect responsible for increased urinary excretion of albumin in proteinuria due to HDM or Adria we employed labeled bovine serum albumins of identical size but differing charge as test solutes in rats. The use of albumin markers avoids any assumptions about the relationship between permeability measurements performed with polysaccharide markers and the behavior of specific native proteins. The albumin markers could be easily labeled with different isotopes of iodine (^{125}I or ^{131}I) allowing the simultaneous determination of the glomerular sieving of an anionic and a neutral albumin marker in the same rat. The use of albumin markers has the disadvantage that albumin is reabsorbed so that urinary excretion of the marker underestimates the filtered load of marker. In the present study this disadvantage was overcome by employing a tissue accumulation method as described by Maack et al [20] and employed by Rennke, Patel, and Venkatachalam [21], to account for the reabsorbed fraction of the filtered load. The tissue accumulation method requires that a number of conditions must be met. First, to obtain a valid estimate of marker GSC the marker must circulate in intact form, and not bind to plasma proteins. In this study, fulfillment of these requirements was demonstrated by gel permeation chromatography of plasma samples and by elution of marker in human plasma, as well as by SDS-PAGE of plasma, kidney and urine samples. No evidence of plasma protein binding or of circulating marker fragments was found. Second, plasma marker levels must be relatively constant throughout the clearance period as was the case in these experiments. Third, the marker must not be metabolized by the kidney to an undetectable form after glomerular filtration leading to underestimation of filtered marker. Since in the present study only TCA-insoluble marker was counted and used to calculate GSC values, either deiodination of marker albumins or metabolism to small peptides or amino acids occurring after filtration could have resulted in loss of label on the filtered marker albumins to the TCA soluble pool of radioactivity. The former possibility was excluded by in vivo deiodinase inhibition studies, the results of which were consistent with observations by others that the renal deiodinase, while able to deiodinate iodinated amino acids [24], does not significantly deiodinate iodoproteins [25, 26]. The possibility that proximal tubular degradation of marker protein has occurred is unlikely since high molecular weight proteins such as albumin are hydrolyzed too slowly to have resulted in significant loss of marker in these short-term experiments [41]. Furthermore, examination of kidney homogenate radioactivity by SDS-PAGE 20 min after administration of marker disclosed no evidence of peptide fragments produced by hydrolysis.

A fourth condition is that the marker protein must accumulate in the kidney only by transport into tubular cells from the luminal side and not from the peritubular blood. There is general agreement that albumin and other large proteins are only reabsorbed from the tubular lumen and do not enter tubular cells from peritubular blood [20, 41]. However, we noted the additional complication that intrarenal accumulation of a marker may occur by direct transfer of marker from the intravascular space to the renal interstitium. Indeed, the renal interstitium is known to have a substantial albumin concentration [42], a fact that hampers micropuncture measurement of proximal tubule fluid albumin concentrations [30]. In addition, Venkatachalam and Karnovsky [43] showed that proteins even larger than albumin gain access to the renal interstitium after intravenous administration. In our experiments, this problem was overcome by performing the sieving studies in rats having one filtering and one nonfiltering kidney and using renal marker accumulation in the nonfiltering kidney as an estimate of interstitial marker. In this fashion, glomerular sieving experiments measuring the total filtered load of albumin markers could be performed.

In summary, we have demonstrated the feasibility of assessing glomerular permselectively alterations in vivo using biologically relevant protein markers as test solutes. Using this technique, we have shown that a polycation (HDM) which acts by binding to GBM anionic sites is able to produce a significant defect in size dependent glomerular permselectivity, indicating that these anionic sites are important for the maintenance of size dependent permselectivity. Conversely, in rats with Adria nephrosis, there is a reduction in the charge barrier to filtration that is of primary importance in the increased urinary excretion of albumin, the principle urinary protein.

Acknowledgments

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