Puromycin aminonucleoside damages the glomerular size barrier with minimal effects on charge density

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Hjalmarsson, Clara, Maria Ohlson, and Börje Haraldsson. Puromycin aminonucleoside damages the glomerular size barrier with minimal effects on charge density. Am J Physiol Renal Physiol 281: F503–F512, 2001.—Puromycin aminonucleoside (PAN) has been suggested to reduce glomerular charge density, to create large glomerular “leaks,” or not to affect the glomerular barrier. Therefore, we analyzed glomerular charge and size selectivity in vivo and in isolated kidneys perfused at 8°C (cIPK) in control and PAN-treated rats. The fractional clearances (u) for albumin and Ficoll of similar hydrodynamic size were 0.0017 ± 0.0004 and 0.15 ± 0.02, respectively, in control cIPKs. Two-pore analysis gave similar results in vivo and in vitro, with small- and large-pore radii of 47–52 and 85–105 Å, respectively, in controls. Puromycin increased the number of large pores 40–50 times, the total pore area over diffusion distance decreased by a factor of 25–30, and the small-pore radius increased by 33% (P < 0.001 for all comparisons of size selectivity and u). The effect of PAN was less dramatic on the estimated wall charge density, which was 73% of that of controls. We conclude that puromycin effectively destroys the glomerular size barrier with minimal effects on charge density.

proteinuria; two-pore model; capillary permeability

CONSIDERABLE EFFORTS have been made over the years to study the complex processes preventing loss of proteins in urine. Nevertheless, the underlying mechanisms of proteinuria, which is a hallmark of renal disease, are still controversial and poorly understood. Still, we lack conclusive evidence for the exact location and composition of the glomerular barrier for proteins.

Only a few human studies concerning the glomerular filtration barrier properties during nephrotic syndromes have been published, and they seem to indicate reduced size selectivity of the glomerular wall (2, 7). In particular, it has been difficult to assess size and charge selectivity in humans because of potential side effects of the required tracers.

Therefore, in an effort to understand more about proteinuria, puromycin aminonucleoside (PAN)-treated animals have been extensively studied since the late 1950s (3, 5, 9, 10, 38). PAN induces alterations in glomerular permselectivity and morphological changes as early as 24 h after a single intraperitoneal injection (32). Micropuncture experiments have clearly shown the glomerular origin of the proteinuria induced by PAN (21, 30). For a long time, PAN has been considered to be associated with a syndrome similar to the minimal change nephrosis (3, 32). Thus the charge density within the glomerular basement membrane has been reported to show early and significant reductions (1, 4, 5, 20, 24). Other groups have found less alteration or no alterations of charge density within the glomerulus (10, 16, 17). The degree of albuminuria does, however, correlate with the changes in the epithelial foot processes (27), which may be entirely lost after PAN treatment (38).

In principle, defects in the glomerular barrier may be secondary to disorders in the glomerular basement membrane, the podocytes, the endothelial cells, or the endothelial cell coat. The glomerular disialogangliosides, mainly present on the podocyte, have been shown to decrease as a direct effect of PAN (13). A recent study reported that the amount of nephrin was drastically reduced in nephrotic rats treated with PAN (18). The latter finding suggests a pivotal role of the recently cloned nephrin (19, 37), not only in hereditary diseases, but also in acquired nephrotic syndromes (23).

In sharp contrast to the studies mentioned above, it has been suggested that puromycin does not affect the glomerular barrier and that proteinuria would be due to a defective tubular uptake of intact protein (33). Glomerular size selectivity was, however, not directly estimated, and the conclusions were, to a large extent, based on the data obtained by another group under different experimental conditions (3).

Therefore, we wanted to quantitatively analyze the glomerular size and charge selectivity in rats in vivo and in vitro. Our main objective was to study the effects of PAN on the functional properties of the glomerular wall. Could the proteinuria induced by PAN be explained by glomerular or tubular mechanisms? If there is glomerular damage, does it affect the discrimination of molecular size and/or charge?

MATERIALS AND METHODS

Experimental Animals

Experiments were performed on female Sprague-Dawley rats (Møllegaards, Stensved, Denmark) weighing 250–320 g.

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The animals had free access to food (standardized pellets) and tap water. The local ethics committee approved the experiments.

PAN was dissolved in 1 ml of saline and then administered as a single intraperitoneal injection, and no acute toxic effects were noticed. It is important to perform the experiments before day 10 to avoid compromise by peripheral and central edema, reduced renal function, and reduced appetite.

Three groups of rats were studied: control rats (n = 9) and two groups of PAN-treated animals: 10 rats injected with PAN (Sigma-Aldrich Chemie, Steinheim, Germany) at 150 mg/kg (full-dose PAN-treated group) and studied 6 days later, and 8 rats injected with 75 mg/kg PAN (half-dose PAN-treated group) and studied 4 days after the injection.

Experiments on control rats and full-dose PAN-treated rats were done on the same day; experiments on half-dose PAN-treated rats were performed a few weeks later.

Kidney Preparation

Anesthesia was induced with pentobarbbitone sodium: 60 mg/kg ip for the control rats and 50 mg/kg ip for the PAN-treated rats. The rats were placed on a thermostatically controlled heating pad to maintain the body temperature at 37°C. The trachea was isolated, and a tracheostomy was performed with a PE-240 catheter to assist the respiration during anesthesia. The respirator (UGO Basile type 7025, Biological Research Apparatus) gave a current volume of 3 ml with a frequency of 40/min. These settings had previously been evaluated by blood gas analysis in a separate group of animals. The tail artery was cannulated by a PE-50 catheter, which was connected to a pressure transducer (PVBSchleidtechnik, Kirchensee, Germany) for recordings of arterial pressure (PA) and subsequent collection of blood. The right internal jugular vein was cannulated (PE-50) for administration of drugs, fluids, and tracers. A continuous infusion of glucose (83 mM), bicarbonate (30 mM), and saline (205 mM) was administered intravenously at a rate of 1.2 ml/h. The rats were eviscerated, and the left kidney was isolated. The abdominal aorta was then freed from surrounding tissues and prepared for cannulation at a later time.

Perfusate and Tracers

Perfusate was prepared using a modified Tyrode solution with human albumin (18 g/l; Immuno, Vienna, Austria) to which tracers were added. The solution had the following composition: 113 mM NaCl, 4.3 mM KCl, 2.5 mM CaCl₂, 0.8 mM MgCl₂, 25.5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 5.6 mM glucose, 0.9 mM nitroprusside (Merek, Darmstadt, Germany), 10 mg/l furosemide, 15 mg/l fluorescein isothiocyanate (FITC)-labeled Ficoll (Biodfr, Uppsala, Sweden), 0.16 MBq/l ⁵¹Cr-EDTA (Amersham Pharmacia Biotech, Buckinghamshire, UK), and 0.6 MBq/l ¹²⁵I-labeled human serum albumin (HSA; Isopharma, Kettel, Norway). The sieving coefficients of albumin were analyzed from the urinary output of the ¹²⁵I-HSA. The ¹²⁵I-HSA was filtered through a pre-equilibrated column (Sephadex G-25 PD-10, Amersham Pharmacia Biotech, Uppsala, Sweden) to remove the free fraction of radioactive iodine before it was added to the perfusate.

The perfusate (pH 7.4) was protected from light and gassed with 5% CO₂ in O₂.

Experimental Protocol

The rats were heparinized (2,000 IU/kg; Lövens Läke medel, Malmö, Sweden). For in vivo measurements, the tracers were injected into the jugular vein, and the vein was flushed with saline. The tracers were as follows: 0.1 ml of ⁵¹Cr-EDTA (0.4 MBq/ml), 1 ml of ¹²⁵I-HSA (0.3 MBq/ml), and 2–5 mg of FITC-Ficoll. After 10 min, 0.2 ml of blood was aspirated from the tail artery, a blood sample (500–750 µl) was collected, and the remaining blood plus 0.5 ml of saline was returned. Two other blood samples were taken 15 and 30 min later using the same protocol. Urine was collected between each sequential two of the three blood samples, with allowance for estimates of glomerular filtration rate (GFR) and fractional clearances of Ficoll and albumin.

After the in vivo collection of samples, the rats were prepared for the cooled isolated perfused kidney (cIPK) experiments. The abdominal aorta was cannulated distal to the renal arteries in a retrograde direction by means of PE-90 tubing. Artificial perfusion of the left isolated kidney was started without interruption of blood flow, and a ligature was placed between the renal arteries. A peristaltic pump (Labnett type MS4, Ismatec, Laboratoriumstechnik) pumped the perfusate through a cooling device placed close to the kidney, maintaining a temperature of 8°C. After a short period of equilibration, six urine samples (200–450 µl) were taken from the controls and two to six samples were obtained from the PAN-treated animals. For all animals, two different flow rates were used. First, two samples were taken at a Pₐ of ~100 mmHg (cIPK-1), and then two to four samples were taken at a Pₐ of ~125 mmHg (cIPK-2). Pₐ, urine flow, and perfusate flow were monitored using Labview computer software.

Data Analysis

The plasma and urine samples were analyzed for ¹²⁵I-HSA and ⁵¹Cr-EDTA concentrations using a gamma counter (Cobra, AutoGamma Counting Systems, Packard Instrument, Meriden, CT) to determine the sieving coefficients for albumin (θ) and the GFR, respectively. Corrections were made for the background radioactivity and the spillover of ⁵¹Cr-EDTA radiation into the iodine channels. In addition, the albumin concentrations ([Alb]) of all urine samples from the cIPKs were determined by radioimmunoassay (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden).

The sieving coefficients for FITC-Ficoll were calculated by subjecting all perfusate and urine samples to gel filtration (BioSep-SEC-S3000, Phenomenex, Torrance, CA) and detection of fluorescence (RF 1002 Fluorescence HPLC Monitor, Gynkotek, Gernering, Germany) using Chromelon (Gynkotek, Gernering, Germany) software. A 0.05 M phosphate buffer with 0.15 M NaCl, pH 7.0, was used as eluant. A volume of 5–10 µl from each sample was analyzed at emission and excitation wavelengths of 530 and 492 nm, respectively; the flow rate (1 ml/min) and the sampling frequency (1/s) were maintained constant during the analysis, as were the pressure (4 MPa) and the temperature (8°C). The errors in the urine-to-plasma (U/P) clearance ratios for Ficoll were estimated to be <1% for most molecular sizes.

Calculations

Vascular resistance. Peripheral vascular resistance in the perfused kidneys was calculated as the perfusion pressure (Pₐ – Pᵥ) over the flow rate, where Pᵥ (venous pressure) was assumed to be zero.
where GFR was determined from the U/P concentration ratio of 51Cr-EDTA times urine flow.

The free water concentration of solutes in plasma (calculated according to Waniensky et al. (44a)) was used.

Fractional clearances of albumin and Ficoll. For the in vivo experiments, the average of two sequential blood samples was used to determine the plasma concentration (P) of the solute. We performed the calculations considering a linear and a logarithmic decay of the plasma concentration of the solutes, and our results were not significantly different (<0.1 Å pore radius).

For the cIPK experiments, P represents the average of three samples collected from the perfusate and U is the concentration of the tracer in each “urine” sample collected during the perfusion period. The solute concentration increases from the arterial to the venous side of the glomerular capillary because of greater glomerular filtration of fluid than of solute (with the assumption that the solute is neither reabsorbed nor secreted). The rather small increment is dependent on the filtration fraction and on the solute U/P ratio, as described by Tencer et al. (43).

The fractional clearance (θ) of a solute X can be calculated as

$$\theta = \frac{(U/P)_x \cdot Q_U}{GFR} = \frac{(U/P)_x}{(U/P)_{CrEDTA}} \tag{1}$$

where $Q_U$ denotes the urinary flow and the other symbols have been described above.

Two-pore model. The two-pore model has four principal parameters: the small- and large-pore radii ($r_S$ and $r_L$), the large-pore fraction of the glomerular filtration rate ($f_L$), and the unrestricted total pore area over diffusion distance ($A_0/\Delta x$). The model parameters are fitted to the experimental sieving data of each experiment (228 U/P ratios vs. Stokes-Einstein radius) of spherical neutral Ficoll polymers of different molecular radii (12–72 Å) using a nonlinear regression analysis and a set of physiological equations. The net fluxes of fluid and solutes are calculated for each pore pathway separately using nonlinear flux equations (36). The effects of temperature on viscosity and diffusion were taken into account. Temperature also slightly influences the charge interactions, as evident from the equations for Debye length ($l_D$) (41), but the effect on $l_D$ is small (5%) and was not included in the analysis.

The clearance of a neutral solute (Cl) can be estimated using the following nonlinear flux equation (36)

$$Cl = \frac{J_x \cdot (1-\sigma)}{1-\sigma \cdot e^{-Pe}} \tag{2}$$

where $\sigma$ is the reflection coefficient of the membrane to the solute and $J_x$ is the fluid flux through each pore pathway in heteroporous models.

The Péclet number ($Pe$) describes the relative contribution of diffusion and convection

$$Pe = \frac{J_x \cdot (1-\sigma)}{PS} \tag{3}$$

where PS is the solute permeability-surface area product

$$PS = \frac{A_p}{A_0} \cdot D \cdot \frac{A_0}{\Delta x} \tag{4}$$

$A_p/A_0$ is the diffusional pore restriction factor, where $A_p$ denotes the effective pore area available for restricted diffusion, $A_0$ is the total cross-sectional area, and $D$ is the free diffusion constant for a solute (for details see Ref. 29).

For each pore pathway, the fluid flux can thus be estimated, e.g., for the small pore pathway as

$$J_x = (1-f_L) \cdot LpS \cdot (\Delta P - \sigma \cdot \Delta \pi) \tag{5}$$

where $f_L$ denotes the large-pore fraction of the hydraulic conductance, $LpS$ is the hydraulic conductance, $\Delta P$ is the hydrostatic pressure gradient across the glomerular barrier, and $\Delta \pi$ is the transvascular osmotic pressure gradient. The $LpS$ equals GFR over the filtration pressure (for details see Ref. 29). The individual pore $A_p/\Delta x$ can be calculated from $f_L$, hence, it is possible to calculate PS and Cl. Finally, by dividing the sum of clearances through the two pore pathways ($Cl_S + Cl_L$) by GFR, the sieving coefficient is obtained. The details of the two-pore model have been extensively described elsewhere (28, 29, 36).

**Charge density.** A negative molecular charge generated by some components in the glomerular wall seems to reduce the concentration of an anionic solute before it reaches the pore entrance (29, 40, 41). The main concept used for calculations is that the Na$^+$ and Cl$^-$ concentrations ([Na$^+$] and [Cl$^-$]) must be in balance to obtain electroneutrality in a membrane with a fixed charge density of $\omega$

$$[Cl^-]_m = [Na^+]_m - \omega \tag{6}$$

where [Na$^+$]$_m$ and [Cl$^-$]$_m$ are [Na$^+$] and [Cl$^-$] in the membrane. For the sake of simplicity, we assume that Na$^+$ and Cl$^-$ are the only ions present; however, we also have to consider the role of the albumin molecules in creating a negative charge

$$[Na^+]_p = [Cl^-]_p + (-z_{Alb}) \cdot [Alb]_p \tag{7}$$

where [Na$^+$]$_p$, [Cl$^-$]$_p$, and [Alb]$_p$ represent [Na$^+$], [Cl$^-$], and [Alb] in plasma and $z_{Alb}$ is the charge of albumin molecules.

If $\Delta E$ is the electrical potential between plasma and membrane and $z$ is the valence of Na$^+$ and Cl$^-$, assumed to be +1 and -1, respectively, then

$$\Delta E = \frac{RT}{z_{Na}F} \cdot \ln \frac{[Na^+]_p}{[Na^+]_m} = \frac{RT}{z_{Cl}F} \cdot \ln \frac{[Cl^-]_p}{[Cl^-]_m} \tag{8}$$

Equation 8 can be reduced to

$$[Na^+]_p \cdot [Cl^-]_p = [Na^+]_m \cdot [Cl^-]_m \tag{9}$$

Analogously, for a charged solute X with the net charge $z_X$, we can assume that the membrane-plasma partitioning is directly related to membrane-plasma partitioning of Cl$^-$, which means

$$\frac{X_m}{X_p} = \left(\frac{[Cl^-]_m}{[Cl^-]_p}\right)^{-z_X} \tag{10}$$

For example, if the [Cl$^-$] ratio is 0.9 and the charged solute has a net charge of $-23$, then the membrane-to-plasma concentration ratio is $0.9^{-23}$; i.e., the membrane concentration of the solute is 8.9% of that in plasma. Thus the fractional clearance ratio ($\theta_{ratio}$) of two solutes with similar size but different charge, e.g., albumin (Alb) and 35.5-Å Ficoll (F) is related to the membrane-plasma partitioning of Cl$^-$ as follows

$$\theta_{ratio} = \frac{\theta_{Alb}}{\theta_{F}} = \left(\frac{[Cl^-]_m}{[Cl^-]_p}\right)^{-z_{Alb}} \tag{11}$$
Table 1. Arterial pressure, pump flow rate, and vascular resistance in vivo and in clPK-1 and clPK-2

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>In Vivo</th>
<th>clPK-1</th>
<th>clPK-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arterial pressure, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>113 ± 4</td>
<td>81 ± 4</td>
<td>115 ± 1</td>
</tr>
<tr>
<td>PAN (7.5 mg/100 g body wt)</td>
<td>8</td>
<td>125 ± 6</td>
<td>100 ± 2</td>
<td>138 ± 4</td>
</tr>
<tr>
<td>PAN (15 mg/100 g body wt)</td>
<td>10</td>
<td>134 ± 3</td>
<td>97 ± 5</td>
<td>125 ± 6</td>
</tr>
<tr>
<td><strong>Pump flow, ml/min</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>5.2 ± 0.1</td>
<td>9.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>PAN (7.5 mg/100 g body wt)</td>
<td>8</td>
<td>5.8 ± 0.8</td>
<td>13.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>PAN (15 mg/100 g body wt)</td>
<td>10</td>
<td>8.6 ± 0.6</td>
<td>14.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td><strong>Vascular resistance, mmHg·min⁻¹·100 g wet wt⁻¹·ml⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>0.19 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>PAN (7.5 mg/100 g body wt)</td>
<td>8</td>
<td>0.17 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td></td>
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<tr>
<td>PAN (15 mg/100 g body wt)</td>
<td>10</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. clPK-1 and clPK-2, cooled isolated perfused kidneys at 100 and 125 mmHg arterial pressure, respectively; PAN, puromycin aminonucleoside.

To calculate the charge density of a membrane, we must know \([Na^+]_p, [Cl^-]_p,\) and \([Alb]_p,\) the experimentally determined fractional clearance ratio of albumin and Ficoll, and the net charge of these solutes \((z_{Alb} and z_F): z_{Alb} = -23, z_F = 0.\) Three unknown parameters remain: \([Na^+]_m, [Cl^-]_m,\) and \(\omega,\) and a unique solution can be found using Eqs. 6, 9, and 11.

A more detailed description of the calculations is provided elsewhere (28, 29, 36, 45).

**Statistics**

Values are means ± SE. Differences were tested using Student’s t-test where appropriate. The distribution of pore radii was skewed, and the values were logarithmically transformed before analysis.

**RESULTS**

**General**

General data regarding PA, pump flow, and vascular resistance (in peripheral resistance units) in vivo and in cIPKs for all three groups are presented in Table 1. The recorded PA in vivo was higher in full-dose PAN-treated rats than in control rats \((134 ± 3 vs. 113 ± 4 mmHg).\) In half-dose PAN-treated rats, PA was 125 ± 6 mmHg (Table 1).

**GFR**

GFR values in vivo and in cIPKs for the three groups of rats are shown in Table 2. As expected, GFR was lower in cIPKs than in vivo \((P < 0.01)\) in all three experimental groups. In the full-dose PAN-treated group, GFR was ~20% of control in vivo and in vitro \((P < 0.001).\) In the half-dose PAN-treated group, GFR was 60% of control in vivo and 44% in cIPKs \((P < 0.001\) for both comparisons).

**Fractional Clearance for Albumin**

The fractional clearances for albumin in the cIPK-1 experiment were 0.0017 ± 0.0004, 0.0065 ± 0.0018, and 0.022 ± 0.0009 for control, half-dose PAN-treated, and full-dose PAN-treated rats, respectively \((P < 0.001\) for all comparisons). In the cIPK-2 experiment, the sieving coefficients for albumin were 0.0022 ± 0.0007, 0.0069 ± 0.0020, and 0.0234 ± 0.0015 for control, half-dose PAN-treated, and full-dose PAN-treated rats, respectively. Thus there was a dose-dependent increase in the sieving coefficients of albumin in the nephrotic rats. Similar effects were seen in vivo, but in a lower \(\theta\) interval (Fig. 1).

**Fractional Clearance for Ficoll**

Ficoll the size of albumin had fractional clearances of ~0.2 in the control rats in vivo and 0.15 and 0.08 in cIPK-1 and cIPK-2 experiments, respectively. Half-dose PAN did not significantly alter the sieving of Ficoll, and full-dose PAN gave at least a twofold increase of the sieving of Ficoll \((P < 0.001; Fig. 2).\) The Ficoll sieving curves for the control and the full-dose PAN-treated rats are illustrated in Figs. 3 and 4.

**Degree of Proteinuria**

The 24-h loss of albumin in urine in vivo was calculated from GFR and fractional clearance of albumin \((\theta_{Alb})\) and expressed per 100-g rat. In control rats the loss was 23 ± 11 mg; the proteinuria for half-dose and full-dose PAN-treated rats was 53 ± 6 and 407 ± 82 mg, respectively.

**Two-Pore Model Analysis**

The two-pore analysis model was used to calculate the four functional parameters in the three experimental groups of animals. The \(r_s\) was 47–52 Å in control and half-dose PAN-treated rats (not significant). In rats treated with full-dose PAN, \(r_s\) was 33% larger (Fig. 5; \(P < 0.001)\) than in...
controls. Elevating the perfusion pressure and GFR in cIPKs reduced $r_s$ by 1–3 Å in control and half-dose PAN-treated rats ($P < 0.05$ for both groups).

The $r_l$ was 85–105 Å in controls and did not differ significantly among the three groups (Fig. 6).

The $f_l$ was 0.4–0.5% during control experiments. Half-dose PAN significantly raised $f_l$ by a factor of 2–3 ($P < 0.01$). In rats treated with full-dose PAN, $f_l$ was raised by a factor of 40–50 ($P < 0.001$). Figure 7 illustrates the $f_l$ in the three experimental groups.

$A_0/\Delta x$ was 400,000 cm/g kidney (wet weight) in vivo and 250,000–300,000 cm/g kidney in cIPKs (not significant). Full-dose PAN diminished $A_0/\Delta x$ to a tiny fraction of the control values (3.6–9.1% of control, $P < 0.001$ for in vivo, cIPK-1, and cIPK-2; Fig. 8).

**Charge Density**

The $\omega$ could be adequately estimated in cIPKs from the ratio of albumin to 35.5-Å Ficoll. Thus, in controls, $\omega$ was 60 and 50 meq/l in cIPK-1 and cIPK-2 experiments, respectively, with 95% confidence intervals (CI) of 55–71 and 42–77 meq/l, respectively. In the half-dose PAN-treated group, $\omega$ was 45 meq/l during cIPK-1 (95% CI = 40–54 meq/l) and 41 meq/l during cIPK-2 (95% CI = 35–51 meq/l). In the full-dose PAN-treated group, $\omega$ was 42 meq/l (95% CI = 38–47 meq/l) during cIPK-1 and 41 meq/l (95% CI = 39–44 meq/l) during cIPK-2. There was no statistically significant difference between the half-dose and full-dose PAN-treated groups, but $\omega$ was significantly higher in controls than in nephrotic animals ($P < 0.05$; Fig. 9).

**DISCUSSION**

This is the most extensive analysis of glomerular size and charge selectivity performed in nephrotic rats, with measurements being done in vivo and in isolated kidneys perfused at 8°C (cIPK). Our main findings are as follows: 1) The normal glomerular barrier is indeed highly permselective, in qualitative agreement with the classical concept. The $r_s$ and $\omega$ are, however, of smaller magnitude than predicted from previous dextran studies. 2) Glomerular size selectivity is similar in vivo and in the isolated kidneys perfused at low temperatures. This is of particular interest, since the latter model allows for estimates of protein sieving data in the absence of tubular uptake, reabsorption, and protease activity. Furthermore, the cIPK gives us a unique opportunity to alter perfusate (e.g., ionic) composition in a manner that would be impossible in vivo. 3) As expected, PAN induced marked proteinuria (18-fold) and elevated the fractional clearances for albumin (10-fold in cIPK) and for the size-matched 35.5-Å Ficoll (2-fold). 4) Detailed heteroporous analysis revealed that PAN reduced the total pore area by a factor of 10–30, increased the number of large pores (as reflected by $f_l$) by a factor of 25–60, and increased $r_s$ by 33%. 5) The $\omega$ was lower in the PAN-treated than in the control animals, but the effects on charge selectivity were small compared with the magnitude of the size-selective alterations.

In a recent functional study, Tencer et al. (43) used the sieving of endogenous proteins to analyze size selectivity. The number of large pores in the glomerular wall increased 170 times in the (PAN) nephrotic rats. In addition, the $r_l$ was slightly larger (110–115 Å) and the equivalent $r_s$ was 29 Å in control and PAN-treated rats. However, Tencer et al. (11, 26) used the simplified model of charge-charge interactions, in which the restriction of anions is estimated by subtracting $l_D$ from the neutral pore radius and adding 1 $l_D$ to the hydrodynamic radius of the molecule. Therefore, for neutral molecules, the “true” $r_s$ would be closer to 45 Å, as pointed out by Tencer et al. The similarities between the two studies are remarkable, considering the huge difficulties of using proteins in vivo in the presence of intact tubular reuptake and protease activities. Careful dosing and administration of lysine (43) are probably crucial in this respect.

Lysine has been used in several studies in vivo and in the isolated perfused kidney, but it must be administered with care, since it can be nephrotoxic (25).
It has been suggested that the glomerular barrier is leaky and that PAN induces proteinuria by reducing the tubular uptake of intact albumin (33). Such interpretations are not supported by our findings. Indeed, previously published micropuncture studies have reported a marked increase in [Alb] in the initial segments of the proximal tubules (21, 30). Moreover, the tubular uptake mechanisms do not seem to be inhib-

Fig. 3. Urine-to-plasma concentration (U/P) ratios for FITC-Ficoll in cIPK-1 experiment in control rats. Values are means ± SE from all experiments in control animals (n = 9). Data from 228 U/P ratios for each sample were analyzed, but results of only 60 U/P ratios are given.

Fig. 4. U/P ratios for FITC-Ficoll in cIPK-1 experiment in full-dose PAN-treated rats. Values are means ± SE from all experiments (n = 10). Data from 228 U/P ratios for each sample were analyzed, but results of only 60 U/P ratios are given.
uted by puromycin. Instead, there is good evidence for enhanced tubular cell activities (31).

In our study, the values for fractional clearance of albumin in controls were higher in cIPKs than in vivo. Similar results were found by combined micropuncture and isolated perfused kidney studies of Stolte et al. (42). The in vivo clearance for albumin is underestimated because of potent tubular reabsorption and degradation of proteins that occur in vivo. Micropuncture measurements of [Alb] in Bowman’s space ([Alb]BS), albeit desired, have been found to be technically difficult. Tojo and Endou (44) simultaneously estimated inulin and [Alb] in different tubular structures and estimated the albumin clearance for a U/P ratio of unity, i.e., that of primary urine. They found the fractional clearance of albumin to be only slightly less than the 0.1–0.2% found in the cIPK during control. Further details of the potent tubular uptake and degradation of proteins with the involvement of cubilin and megalin complexes were recently published by Zhai et al. (47). Because of the tubular reabsorption of protein, estimates of are highly overestimated in vivo.

An advantage of the cIPK preparation is that the charge interactions can reversibly be increased by using a low ionic composition of the perfusate (41), an approach that has given us valuable insights about the glomerular barrier (29).

It is well known that the isolated perfused kidney has reduced filtration fractions and low GFRs. 1) Erythrocyte-free perfusates have approximately half the viscosity of blood in vivo. As a consequence, the flow rate must be doubled (at 37°C) to maintain a given PA, hence reducing the filtration fraction by a factor of 2. At 8°C, the increased viscosity due to the low temperature will compensate for the loss of erythrocytes. However, low temperature will also reduce the hydraulic conductivity by a factor of 2 as a consequence of Poiseuille’s law. Moreover, the increased tubular fluid viscosity at 8°C will raise the intratubular pressures, hence reducing the filtration gradient across the glomerular capillary wall (14). 3) There are fewer functionally active nephrons in the isolated perfused kidney with marked heterogeneity (22). All these effects reduce the magnitude of GFR and the absolute values of renal clearance, but the fractional clearance will not be affected. Indeed, no difference has been found in fractional albumin clearance between homogeneously and heterogeneously perfused kidneys (22). There are three alternative descriptions of the functional properties of the glomerular barrier. One is the classical concept of a highly selective glomerular barrier with a charge density of 120–170 meq/l, according to which [Alb]BS is 10^{-6} of that in plasma ([Alb]P). The daily albumin losses in humans would be ~9 mg (GFR × [Alb]P × 10^{-6} = 180 l/day × 50 g/l × 10^{-6} = 9 mg/day). Another is the recently launched “albumin retrieval hypothesis,” which suggests that the glomerular capillaries are as leaky as those in skeletal muscle and that [Alb]BS is close to 8% of [Alb]P. Because there are almost no proteins in the final urine, the authors have suggested an “albumin retrieval” mechanism whereby albumin is taken up passively in intact form.

**Fig. 5.** Small pore radius ($r_S$) in vivo and in cIPK-1 and cIPK-2 experiments in control (filled bars), half-dose PAN-treated (hatched bars), and full-dose PAN-treated rats (open bars). Values are means ± SE. The $r_S$ was significantly larger (+33%) after full-dose PAN. ***P < 0.001 vs. control.

**Fig. 6.** Large pore radius ($r_L$) in vivo and in cIPK-1 and cIPK-2 experiments in control (filled bars), half-dose PAN-treated (hatched bars), and full-dose PAN-treated rats (open bars). Values are means ± SE. No significant difference was observed among the groups.

**Fig. 7.** Large pore fraction of the glomerular filtrate ($f_L$) in vivo and in cIPK-1 and cIPK-2 experiments in control (filled bars), half-dose PAN-treated (hatched bars), and full-dose PAN-treated rats (open bars). The huge $f_L$ increase in the full-dose PAN-treated rats. Values are means ± SE. ***P < 0.001 vs. control.
by the tubular cells and returned to blood (34). If the rat data were applicable to humans, ~700 g/day of albumin would be filtered and reabsorbed in intact form (GFR $\times$ [Alb]$_{P}$ $\times$ 0.08 = 720 g/day). Finally, we have proposed a modified charge- and size-selective model for glomerular permeability (29) in qualitative agreement with the classical concept. It differs, however, in being more size discriminating and having less charge density (30–60 meq/l) than the classical dextran studies suggested (150 meq/l) (8). It is interesting to mention that the charge density is very similar to that obtained in the perfused-fixed rat kidney model (6). Thus recent data from the literature, including some of our studies, suggest that [Alb]$_{BS}$ is close to 0.1% of [Alb]$_{P}$. In humans, this would mean albumin losses close to 9 g/day, which would suggest mainly renal elimination of albumin or (more likely) that the experimental conditions have raised the albumin losses by a factor of 2.

Interestingly, the values for fractional clearance of albumin in full-dose PAN-treated animals were higher in vivo than in the cIPK. What could be the rationale for this difference between controls and nephrotic animals? One explanation could be found in terms of inflammatory responses. Thus PAN is known to stimulate the interstitial infiltration of mononuclear leukocytes and CC chemokines, a subgroup of chemokines characterized by adjacent cysteines in the molecule, in the renal tissue, contributing to the kidney impairment (35). Furthermore, it has been suggested that inflammation has a role in the progression of PAN nephrosis in the chronic and acute forms (12, 15, 39, 46). Low temperature is normally an effective way of reducing inflammatory responses. We may therefore speculate that exposing the nephrotic kidneys to low temperature (8°C) inhibited the inflammatory cell activity and, consequently, reduced the degree of proteinuria.

We conclude that the normal glomerular capillaries indeed are highly permselective with significant charge selectivity. PAN markedly reduces glomerular size selectivity with minimal effects on charge density. Thus PAN substantially reduced the total pore area, $A_0/D_x$, and even increased the $r_s$ somewhat. Our findings are in agreement with previously published data on protein sieving in vivo and with the observation of reduced nephrin content in the slit diaphragm of glomerular epithelial cells during nephrotic syndromes (18, 19, 37). Different structures seem to be responsible for glomerular charge and size selectivity (29, 40). The glomerular size barrier may be situated at the podocyte.
slit diaphragms, while the endothelial cell coat may exert the charge interactions.

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