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Glomerular filtration of proteins: Clearance of anionic, neutral, and cationic horseradish peroxidase in the rat

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Glomerular filtration of proteins: Clearance of anionic, neutral, and cationic horseradish peroxidase in the rat. Glomerular permeability to horseradish peroxidase, a protein slightly smaller than rat albumin but similar in shape, was studied in Wistar-Furth rats by using a purified neutral isozyme (HRP; molecular radius, a_e=29.8 Å) as well as an anionic succinyl-derivative (sHRP a_e=31.8 Å) and a cationized enzyme (cHRP, a_e=30 Å). The clearance rate of the proteins was determined over a 20-min period using the amounts of enzyme actually filtered (i.e., protein in the urine and protein reabsorbed by tubules). Fractional clearance of cationic HRP (0.338 ± 0.019) exceeded that of neutral HRP (0.061 ± 0.005) by a factor of 5.5 and that of anionic HRP (0.007 ± 0.000) by a factor of 48. Tubular reabsorption was less than 10% of the filtered load. The experimental results indicate marked charge dependency of the filtration of proteins across the glomerulus. Fractional clearances for these proteins are significantly lower than those reported in the literature for dextrans of similar molecular radii. Other molecular properties such as shape and deformability may explain these differences.

Filtration glomérulaire des protéines: Clearance de la peroxydase de raifort anionique, neutre ou cationique chez le rat. La perméabilité glomérulaire à la peroxydase de raifort, une protéine un peu plus petite que l'albumine de rat mais semblable par sa forme, a été étudiée chez des rats Wistar-Furth au moyen d'un isoenzyme neutre purifié (HRP; rayon moléculaire, a_e = 29,8 Å), d'un dérivé succinylé anionique (sHRP, a_e = 31,8 Å) et d'un enzyme cationique (cHRP, a_e = 30 Å). La clearance des protéines a été déterminée sur une période de 20 min en utilisant les quantités de protéines réellement filtrées (c'est-à-dire les protéines de l'urine et les protéines réabsorbées par les tubes). La clearance fractionnelle de HRP cationique (0,338 ± 0,019) est supérieure à celle de HRP neutre (0,061 ± 0,059 d'un facteur 5,5 et à celle de HRP anionique (0,007 ± 0,000) d'un facteur 48. La réabsorption tubulaire est inférieure à 10% de la charge filtrée. Les résultats expérimentaux indiquent que la filtration glomérulaire des protéines dépend de façon importante de leur charge. Les clearances fractionnelles de ces protéines sont significativement inférieures à celles rapportées dans la littérature pour les dextrans de mêmes rayons moléculaires. D'autres propriétés des molécules, telles que leur forme et leur déformabilité, peuvent expliquer ces différences.

Clearance experiments have shown that glomerular transport of anionic dextran sulfate is restricted compared to that of uncharged dextran of equal size [1]. Morphologic tracer studies previously reported by us showed that penetration of the glomerular basement membrane by the large unfilterable molecule ferritin (Einstein-Stokes radius, a_e = 61 Å) varies with the isoelectric point of the protein [2, 3]. In the ferritin experiments, permeability of the entire filter could not be assessed since the tracer did not enter the urinary space regardless of charge. Even cationic ferritins were restricted distally in the capillary wall and were incorporated into glomerular epithelial cell phagosomes by mechanisms described previously by Farquhar, Wissig, and Palade [4].

Fractional clearances of proteins are considerably smaller than those of sugar polymers with equivalent hydrodynamic radii [5]. These differences may be explained: *1)* by variations in molecular charge [5, 6]; *2)* by the different tubular handling of these compounds [6] since proteins are known to be reabsorbed by the tubular epithelium, and this reabsorption would tend to lower the fractional clearances of proteins; and *3)* by differences in molecular configuration between polysaccharides and proteins [5, 6]. Molecules of sugar polymers, randomly coiled in free solution [7], may unfold during transit through the glomerulus; thus, their transport may be facilitated compared to more rigid protein molecules [5]. A similar effect of molecular conformation on the sedimentation of molecules through artificial gels has been described by Laurent et al [8–10].

The above considerations prompted a comparative study of glomerular permeability to differently charged proteins that are restricted to some extent, but nevertheless, filter into the urinary space. The present experiments, using anionic, neutral and cationic horseradish peroxidase (HRP) demonstrate *1)* that molecular charge is a major determinant of glomerular permeability to a filterable protein, and *2)* that fractional clearances of anionic, neutral, and cationic HRP corrected for tubular reabsorption are 7.1, 9, and 2 times smaller than the clearances re-

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ported for similarly sized anionic, neutral, and cationic dextran [1, 11, 12].

Methods

Purification of horseradish peroxidase (E.C.No. 1.11.1.7). Horseradish peroxidase (Type II, Sigma Chemical Co., St. Louis, Mo.) is composed of numerous isozymes and other impurities, as revealed by isoelectric focusing (Fig. 1), and has an Reinheitszahl (RZ) index of 1.0 to 1.5 (RZ = A 403 nm/A 280 nm); the main isozyme (mol wt, 40,000) with an isoelectric point (pI) of 7.3 to 7.5 was purified by gel filtration and ion exchange chromatography. For gel filtration, 500 to 800 mg of type II peroxidase was dissolved in 5 to 10 ml of 0.15 M sodium chloride solution containing 50 mM phosphate buffer (pH, 7.4) and loaded on a 50 × 2.5-cm polyacrylamide gel-agarose column (Ultrogel AcA 44, LKB Produkter AB, Sweden). Three-milliliter fractions were collected by means of a fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.). The enzymatically active components eluted as a single peak. The central portion was dialyzed against 0.02 M Tris-hydrochloric acid buffer (pH, 8.5) and concentrated by ultrafiltration through a PM-10 diaflo membrane (Amicon Corp., Lexington, Mass.). Then, 10 to 15 ml of this solution was loaded on a 50 × 2.5-cm diethylaminoethyl cellulose column (DE-52, Whatman Inc., Clifton, N.J.) equilibrated with 0.02 M Tris-hydrochloric acid buffer (pH, 8.5). The column was eluted with 1,000 ml of buffer solution containing increasing concentrations of sodium chloride (continuous gradient, 0 to 1 M sodium chloride). The central portion of the first peak so obtained contained two isozymes: a major component with isoelectric point of 7.3 to 7.5 and accounting for over 92% of peroxidatic activity and a minor component with a pI of 6.5 (Fig. 2).

Preparation of polyanionic and polycationic derivatives. A polyanionic compound was prepared by succinylation [13]. For this purpose, 100 mg of succinic anhydride (Eastman Kodak Co., Rochester,

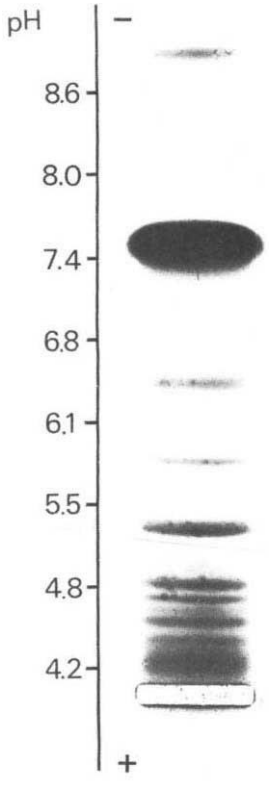

Fig. 1. *Isoelectric focusing pattern on a gel slab of horseradish peroxidase (Type II, Sigma Chemical Co.). Anode* (+) is shown on the bottom; *cathode* (−) is shown on the top of the figure. The pH gradient is indicated on the left. The main isozyme has an isoelectric point (pI) of 7.35 to 7.55.

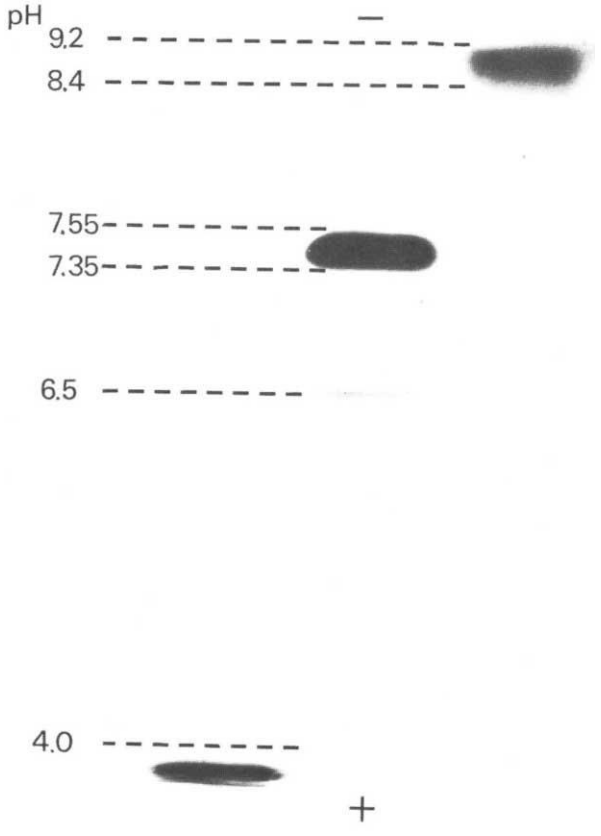

Fig. 2. *Gel electrofocusing of anionic, succinylated peroxidase on the left, native peroxidase in the central panel, and cationic peroxidase on the right.* The pH gradient was established with ampholyte in the range of 3.5 to 10. The isoelectric point (pI) of the anionic peroxidase is less than 4; the pI of the native peroxidase is 7.35 to 7.55 for the main enzyme (over 92%) and 6.5 for the minor isozyme; the pI for the cationic peroxidase is 8.4 to 9.2. *Anode* (+) is shown on the bottom (1 N phosphoric acid); *cathode* (−) on top (1 N sodium hydroxide).

N.Y.) was added in small increments to 50 ml of a solution containing 100 mg of purified tracer; the pH was maintained at 8 to 8.5 throughout the reaction (20 to 30 min). The solution was kept overnight at 4°C, dialyzed against repeated changes of distilled water, concentrated, and stored at 4°C in sterile vials after filtration through Millex Filter (0.45-μm pore size) (Millipore Corp., Bedford, Mass.). The polycationic derivative was obtained by substitution of free carboxyl groups by hexanediamine (Eastman Kodak Co.) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Story Chemical Corp., Muskegon, Mich.) as divalent reagent [14]. The pH of the solution was maintained at 6 for three hours. It was kept overnight at 4°C, dialyzed in the cold against numerous changes of distilled water, concentrated, and sterilized.

Characterization of tracer molecules: 1) Molecular charge. The isoelectric points of the tracer enzymes were determined by isoelectric focusing [15]. Slabs (9 × 20 cm) were prepared from a gel containing 4.5% acrylamide, 0.18% bis-acrylamide, 2% ampholyte (Ampholine, pH 3.5 to 10, LKB Produkter AB). A model M-150 slab electrofocusing apparatus (MRA Corp., Boston, Mass.) was used; after the proteins had focused, pH was measured on the gel slab at 5-mm intervals by means of a surface microelectrode (Ingold Electrodes Inc., Lexington, Mass.). The pH gradient so obtained was virtually linear over the range of 3.8 to 9.5. Coomassie Blue was used as a protein stain on the slab.

2) Effective molecular radius. Gel filtration of the tracer molecules was performed on a 50 × 1-cm column of an acrylamide-agarose mixture (Ultrogel AcA 44, LKB Produkter) equilibrated with 0.05 M phosphate buffer (pH, 7.4) containing 0.15 M sodium chloride and 1 mM EDTA. Samples of 0.3 to 0.8 ml were applied to the column, and 0.7-ml fractions were collected; enzyme concentrations were determined both at the absorption maximum for peroxidase (403 nm) on a Beckman DBGT spectrophotometer (Beckman Instruments Inc., Irvine, Calif.) and biochemically by the peroxidase assay [16]. The chromatographic behavior of the different tracers was studied also at different concentrations in the presence of plasma. The void volume (V_O) of the column was determined by the elution of blue dextran. Native purified horseradish perioxidase, chymotripsinogen A and serum albumin served as standards. Distribution coefficients ($Kav = V_E - V_O/V_T - V_O$, where V_E = elution volume for the test substance, V_O = void volume, V_T = total bed volume), for the molecules which served as standards were calculated and plotted against the known molecular radius (a_e) [17] on semilogarithmic graph paper (Fig. 3). The unknown molecular radii for anionic and cationic HRP were calculated from this curve.

3) Enzyme activity. The enzyme activity of the three tracers was determined at different pH and various enzyme concentrations using 3,3'-diaminobenzidine as hydrogen donor [16]. Protein concentration was determined by the method of Lowry et al [18] and spectrophotometrically using a molar extinction coefficient of $E_{403\ nm} = 9.10 \times 10^4$ (l/mole × cm) [19]. The peroxidatic activity of the tracers was also measured after incubation in distilled water and rat urine, plasma, and kidney homogenate over a period of six hours. For this purpose, known quantities of the tracer enzymes in these fluids were kept in an ice-water bath. Aliquots were taken and assayed for peroxidase activity at different time intervals.

Clearance experiments. Wistar Furth rats (females; body wt, 160 to 220 g) were used for the present experiments, since this strain of rats has been shown to be resistant to mast cell degranulation after injection of horseradish peroxidase [20]. Five rats were injected with each tracer; the animals were anesthesized by i.p. injection of Inactin® (Promonta AG., Germany), 10 mg/100 g of body wt. The right jugular vein was then cannulated with two polyethylene tubes (PE-10) for infusion of solutions. The left

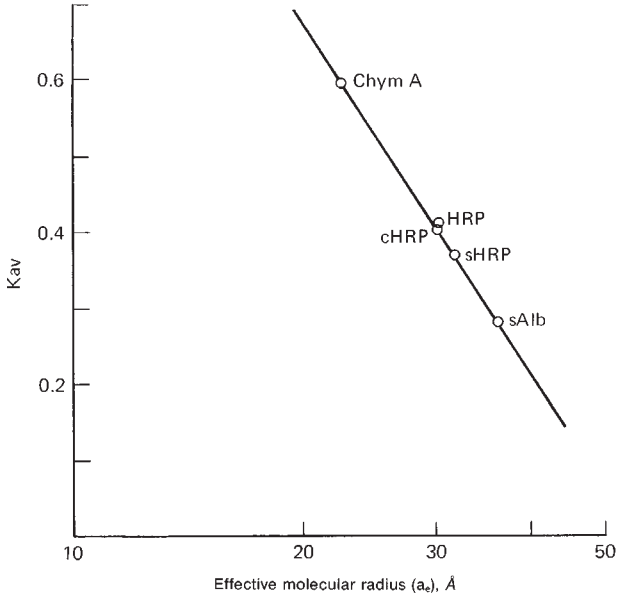

Fig. 3. *Relationship between partition coefficient Kav and effective molecular radius (log a_e = 1.7481 − 0.6668 Kav).* Chymotrypsinogen A (Chym A; a_e = 22.4 Å), native, purified horseradish peroxidase (HRP, a_e = 29.8 Å), and serum albumin (sAlb; a_e = 36.1 Å) served as controls. Calculated values for succinylated peroxidase and cationic peroxidase were 31.8 Å and 30 Å, respectively.

ureter was cannulated for urine collection. The left femoral artery was cannulated with polyethylene tubing PE-50 connected to a transducer (Statham Model P23-Dc, Statham Medical Instruments, Puerto Rico), and arterial pressures were recorded with a Grass Model 79D polygraph (Grass Instrument Co., Quincy, Mass.).

In order to achieve a mild diuresis, the animals were given a constant infusion (Harvard Apparatus Co. Inc., Millis, Mass.) of 5 g/100 ml of mannitol solution (0.036 ml/min) and 0.9 g/100 ml of sodium chloride solution (0.034 ml/min) throughout the entire length of the experiments (70 to 80 min). Whole kidney GFR's were determined using ^{14}C-inulin (New England Nuclear, Boston, Mass.) which was given as a priming dose of 20 μCi, followed by a constant infusion of 0.2 μCi/min. Urine and plasma samples were analyzed for their isotope content by standard liquid scintillation procedures (Beckman Model LS 250). The injection of the tracer substances was started 30 min after the administration of inulin. The tracer was administered i.v. as a priming dose of 0.4 to 0.5 ml of a solution containing 2 mg/ml of the enzyme dissolved in 0.15 M sodium chloride. This initial dose was given over a period of 40 to 60 seconds and was followed immediately by constant infusion of the tracer so as to minimize variations in plasma concentration. The amount necessary to maintain the initial concentration was minimal for the anionic tracer, intermediate for the neutral enzyme, and maximal for the cationic compound. Plasma concentration of peroxidase in general tended to decrease slightly over the 20-min period. The average concentration of peroxidase at 10 and 17 min was, respectively, 84 and 74% of the initial level at 3 min for the succinylated tracer (sHRP), 96 and 94% for native HRP, 85 and 81% for the cationic peroxidase (cHRP). Plasma concentrations of ^{14}C-inulin remained virtually constant during the clearance period. Urine was collected from the left ureter for a 20-min period starting at the moment of tracer injection. Blood samples (0.2 to 0.3 ml) were obtained from the left femoral artery 3, 10, and 17 min after the administration of the tracer substance. The abdominal aorta was then clamped-off above the level of the renal arteries at the end of the 20-min period. The renal vasculature was immediately perfused with 0.9 g/100 ml of sodium chloride solution for 4 to 5 min at 100 mm Hg. The "urine" obtained during this additional period was collected separately, and the amount of enzyme present in this sample was added to the amount excreted during the 20-min period, to compensate for the delay in appearance of the tracer substance in the urine due to a 1½- to 2-min transit time. The left perfused kidney was then homogenized in a grinder for the estimation of the reabsorbed tracer and maintained at 4°C to minimize possible degradation of the tracer by enzymes present in the tissue homogenate.

After appropriate dilution in distilled water, two urine samples, three plasma samples, and the tissue homogenate were assayed within four hours for peroxidase actvity [16]. Clearances of inulin and of the tracer enzymes were calculated utilizing the arithmetic mean of the three plasma values obtained during the 20-min period.

Enzyme histochemistry. For enzyme histochemistry, in a separate group of rats, kidneys were perfusion-fixed for 10 min following the saline perfusion, using glutaraldehyde (1.25 g/100 ml) in 0.1 M cacodylate buffer (pH, 7.4). Chopped tissue sections (40-μm thickness) were prepared for the demonstration of peroxidase activity, following the method described by Graham and Karnovsky [21]. The tissue was then dehydrated and embedded in Epon 812. For light microscopy, 2-μm thick sections were cut with glass knives, mounted, and photographed unstained. Electron microscopy was performed on a Philips 201 electron microscope.

Results

Isoelectric points of tracer proteins. The isoelectric focusing patterns of anionic horseradish peroxidase, the native purified enzyme, and the cationic derivative are illustrated in Fig. 2. The pH range of focusing for these tracers was: less than 4 for the negatively charged succinylated horseradish peroxidase, 7.3 to 7.5 for the major component (92% of peroxidatic activity) and 6.5 for the minor component of the native enzyme, and 8.4 to 9.2 for the cationic derivative.

Effective molecular radius and interaction of the tracers with plasma proteins. The elution patterns of the three protein tracers are illustrated in Fig. 4. The elution volume of the tracer in the presence of plasma does not differ from the value obtained when the enzyme was chromatographed in the absence of plasma proteins. The elution pattern of plasma proteins is given in the three lower panels of Fig. 4 as revealed by the spectrophotometric absorption at 280 nm. These proteins eluted as three distinctive peaks in front of the peroxidase tracers, with maximal absorption at 280 nm and minor absorption at 403 nm. The same degree of absorption at 403 nm was obtained when plasma was chromatographed alone, without the addition of peroxidase tracer. Samples taken from these fractions containing plasma proteins and assayed for the enzyme failed to

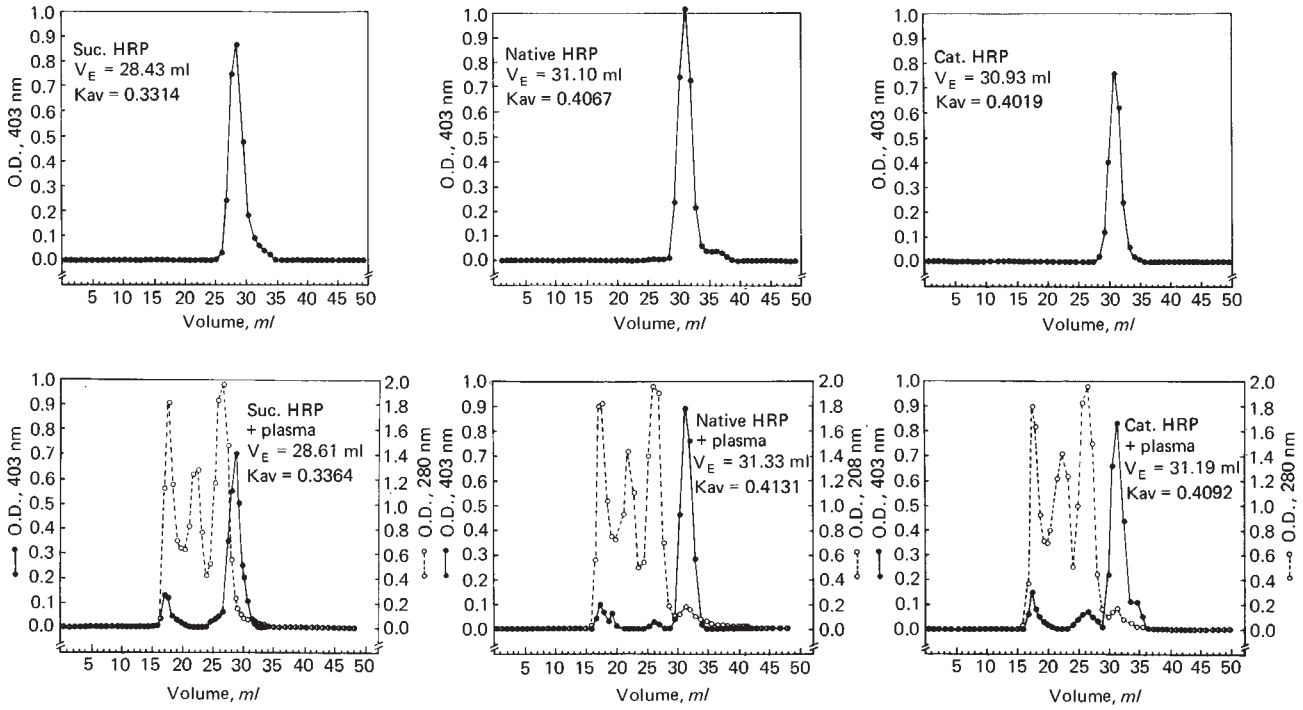

Fig. 4. *Elution patterns of anionic, succinylated horseradish peroxidase (suc. HRP), native peroxidase (native HRP) and cationic horseradish peroxidase (cat. HRP) on a 50 × 1-cm column of acrylamide-agarose mixture (Ultrogel, AcA44, LKB Produkter).* Total bed volume (V_T) = 52.1 ml, void volume (V_O) = 16.7 ml, eluent = 0.05 M phosphate buffer (pH, 7.4) + 0.15 M sodium chloride + 1 mM EDTA. *Top panels* illustrate the elution of the tracer alone; *bottom panels* show the elution of the tracer in the presence of plasma. The elution volume of the tracers is virtually unaffected by the presence of plasma proteins. No binding of peroxidase to plasma proteins could be detected when fractions containing plasma proteins (high absorbance at 280 nm) were assayed for peroxidase.

reveal peroxidatic activity, which was present only in the fractions with high absorbency at 403 nm. Binding of the tracers to plasma components and aggregation of tracer molecules thus could be ruled out. Identical results were obtained in similar gel filtration experiments, when peroxidase was added to rat plasma in smaller concentrations, similar to those obtained in the animals during the clearance experiments (100 to 150 μg/ml). Fractions were assayed for peroxidase activity; no binding of the tracers to plasma proteins was detected.

Fig. 3 shows the relationship between filtration coefficients of the proteins which served as standards and the respective effective molecular radii: chymotrypsinogen A, a_e = 22.4 Å (Chym A); serum albumin, a_e = 36.1 Å (sAlb); and native, purified horseradish peroxidase, a_e = 29.8 Å (HRP). The molecular radii calculated from this curve were a_e = 31.8 Å for the anionic tracer (sHRP) and a_e = 30.0 Å for the cationic enzyme (cHRP).

Peroxidase activity of tracers. The absorption spectra obtained with the modified enzymes were identical to that of the native peroxidase with a maximum at 403 nm. The optimal pH for the assay of the three tracer enzymes was 4.3 when 3,3′-diaminobenzidine was used as hydrogen donor; the activity at pH above and below 4.3 fell rapidly, as has been previously reported for the native enzyme [16]. The peroxidatic activity of the tracer enzymes was identical and linearly proportional to the amount of enzyme present over a range of 0 to 25 ng of protein (Fig. 5).

The peroxidatic activity was identical when the enzyme was added to water, urine, plasma or kidney homogenate, indicating that there was no inhibition or stimulation of the enzymatic process by either one of these fluids. The activity in all solutions maintained at 4°C remained unchanged over a time period of six hours.

Clearance experiments. Table 1 presents data from rats injected with anionic horseradish peroxidase, native enzyme, and cationic horseradish peroxidase. Mean whole kidney GFR does not differ from one group to the other. Absolute and relative clearances for the three enzyme tracers were calculated on the basis of urine excretion alone (C^U and C^U/GFR) and on the basis of urine excretion and reabsorbed peroxidase as measured in the tissue homogenate (C^{U+H} and C^{U+H}/GFR). The enzyme present in the kidney homogenate (reabsorbed

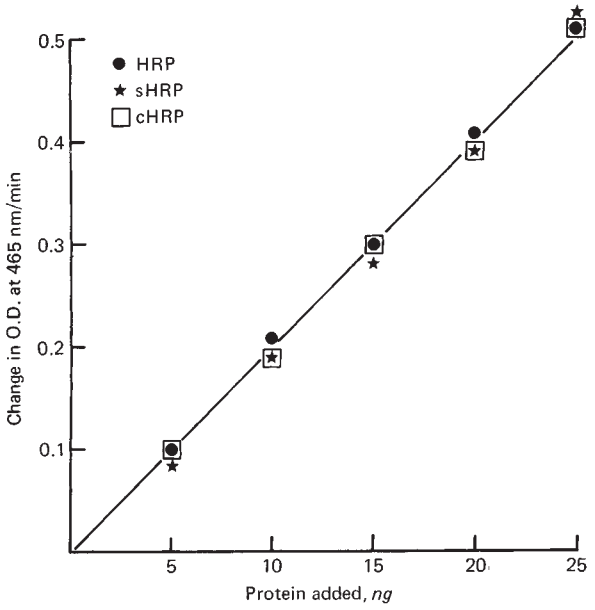

Fig. 5. *Peroxidase activity of native horseradish peroxidase (HRP), succinylated peroxidase (sHRP), and cationic peroxidase (cHRP) at different enzyme concentrations.* The incubation medium (2.95 ml) contained 0.5 mM 3,3'-diaminobenzidine, 0.1 N citrate buffer (pH, 4.3), 0.1 g/100 ml gelatine. The reaction was started by the addition of 0.05 ml of 0.18 M hydrogen peroxide. The variation in absorbance at 465 nm was recorded for 1 min [16].

tracer) when compared to the total amount filtered (excreted and reabsorbed enzyme) represents an average of 10% for anionic and neutral peroxidase and 7% for the cationic tracer. This explains the slightly smaller values of C^U and C^U/GFR calculated on the basis of urine excretion alone when compared to C^{U+H} and C^{U+H}/GFR in each group.

As can be seen from Table 1, relative clearances of native HRP and cationic HRP are 8.7 and 48 times that of the anionic tracer.

Cytochemical detection of the tracer enzymes. The renal vasculature was completely washed-out of blood elements after five minutes of saline perfusion. As can be seen from Figure 6, there is no detectable peroxidase present in the glomerular capillaries; neither is there any tracer absorbed to the cell coat or glomerular basement membrane. Transmission electron microscopy likewise did not reveal enzyme activity in the glomerular capillary wall. Figure 7 illustrates the reabsorbed tracer in proximal tubules; the uptake is minimal for anionic horseradish peroxidase, intermediate for the neutral tracer, and maximal for the cationic enzyme. The electron micrograph (Fig. 8) confirms the absence of reaction product outside reabsorption vesicles and lysosomes in a proximal tubule cell.

Discussion

Our clearance studies with horseradish peroxidase, a filterable protein with fractional coefficient and, hence, axial ratios similar to rat albumin [22, 23] indicate marked charge dependency of protein transfer across the glomerular capillary wall.

Technical considerations. The validity of our findings is based on the following considerations, which demonstrate that we were able to measure with a reasonable degree of accuracy the total protein filtered in 20 min, including the fraction reabsorbed by the tubules, and hence the rate of clearance of free plasma peroxidase through the glomeruli.

1) In animals given any one of the three enzymes followed by saline perfusion of the kidneys, peroxidase activity could be detected only in reabsorption vesicles in renal tubular cells (Fig. 7); vasculature and interstitial elements were free of reaction product as revealed by the sensitive histochemical method (Figs. 6 and 7). Saline-perfused kidneys not exposed to peroxidase had no endogenous peroxidatic activity when assayed at the dilutions used in the clearance experiments. Peroxidatic activity of plasma and urine likewise was undetectable and therefore negligible compared to the amounts of exogenous peroxidase. Incubation of horseradish peroxidase with plasma, urine, and kidney homogenate for periods up to six hours was not accompanied by decrease in enyme activity. Thus, our technique accurately measured plasma, urine, and intracytoplasmic tissue content of injected peroxidase. Furthermore, our gel filtration studies showed that there

Table 1. Comparison of anionic, neutral (native), and cationic horseradish peroxidase (HRP) in normal rats[a]

	Body wt *g*	Arterial pressure *mm Hg*	GFR *ml/min*	C^UHRP[b] *ml/min*	C^UHRP/GFR	C^{U+H} HRP[c] *ml/min*	C^{U+H}HRP/GFR
Anionic HRP	187 ± 7.23	109 ± 2.92	1.09 ± 0.11	0.007 ± 0.001	0.006 ± 0.000	0.008 ± 0.001	0.007 ± 0.000
Native HRP	194 ± 5.62	107 ± 2.55	1.13 ± 0.10	0.063 ± 0.008	0.055 ± 0.005	0.069 ± 0.008	0.061 ± 0.005
Cationic HRP	214 ± 3.69	108 ± 3.39	1.13 ± 0.09	0.349 ± 0.017	0.314 ± 0.021	0.377 ± 0.013	0.338 ± 0.019

[a] Values are means ± 1 SEM, $N = 5$ in each group.
[b] Calculated on the basis of urine excretion (C^U) alone.
[c] Calculated on the basis of urine excretion and reabsorbed tracer present in the tissue homogenate (C^{U+H}).

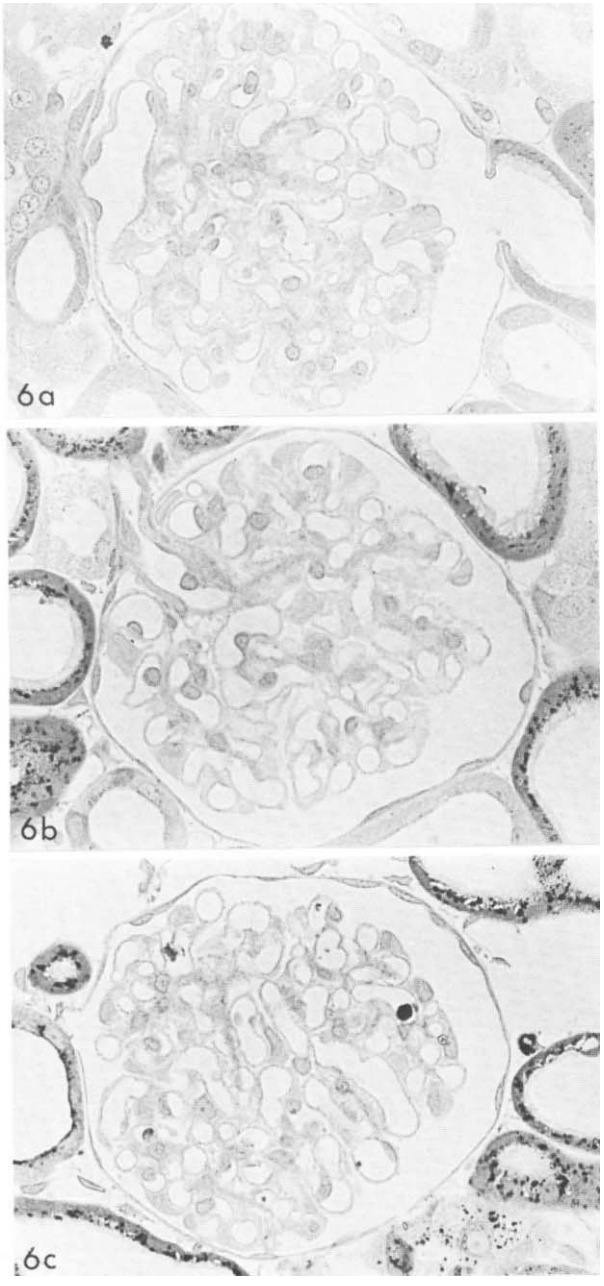

Fig. 6. *Peroxidase histochemistry of glomeruli.* Renal cortex from animals was injected with anionic *(panel a)*, neutral *(panel b)*, and cationic *(panel c)* peroxidase. The reaction product is limited to tubular cells. Glomerular capillaries do not show reaction product. (× 600.)

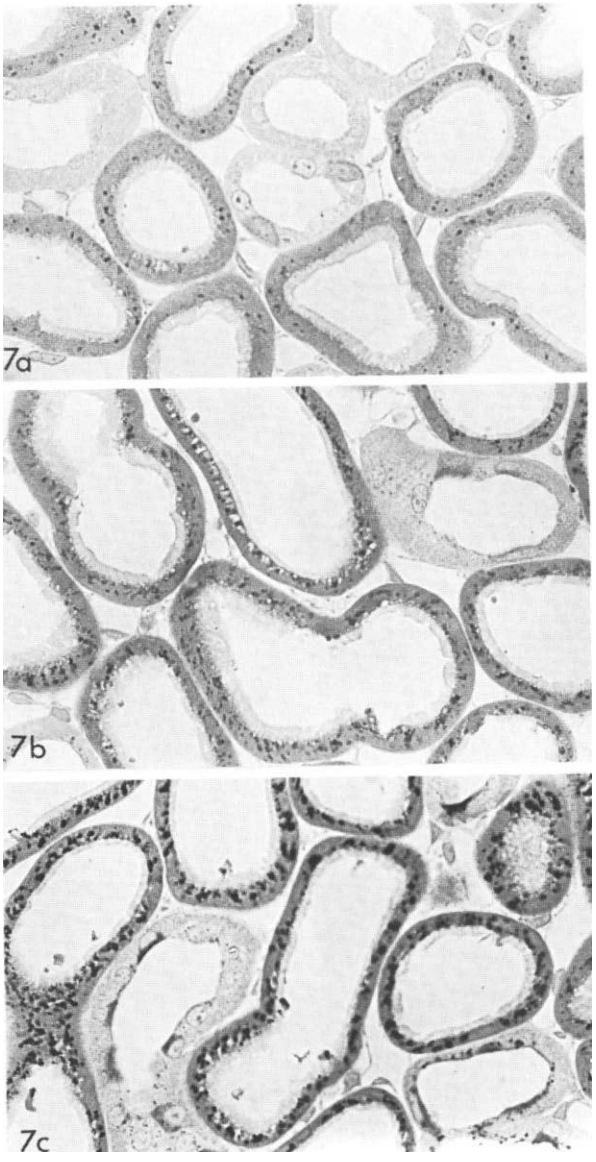

Fig. 7. *Enzyme histochemistry of cortical tubules.* Cortical tissue from animals was injected with anionic *(panel a)*, neutral *(panel b)*, and cationic *(panel c)* horseradish peroxidase. Reaction product in all three instances is limited to tubular epithelium. Peritubular capillaries and interstitium are free of reaction product. (×400.)

was no interaction between peroxidase and plasma proteins, indicating that the plasma peroxidase assay measured free, unbound tracer.

2) The filtered amount of peroxidase may have been overestimated or underestimated due to transtubular transport of intact protein. This source of error is unlikely since *a)* proteins, including horseradish peroxidase, do not penetrate the tight junctions between epithelial cells in either direction in different species, including the rat [21, 24–26], and *b)* transcellular active transport of proteins likewise does not occur in either direction, as revealed by protein kinetic and ultrastructural tracer studies [21, 27–29].

3) Protein uptake into renal tubular epithelium has been studied by electron microscopic autoradiographic [28, 30, 31] and cytochemical methods [21, 32] as well as by biochemical cell fractionation tech-

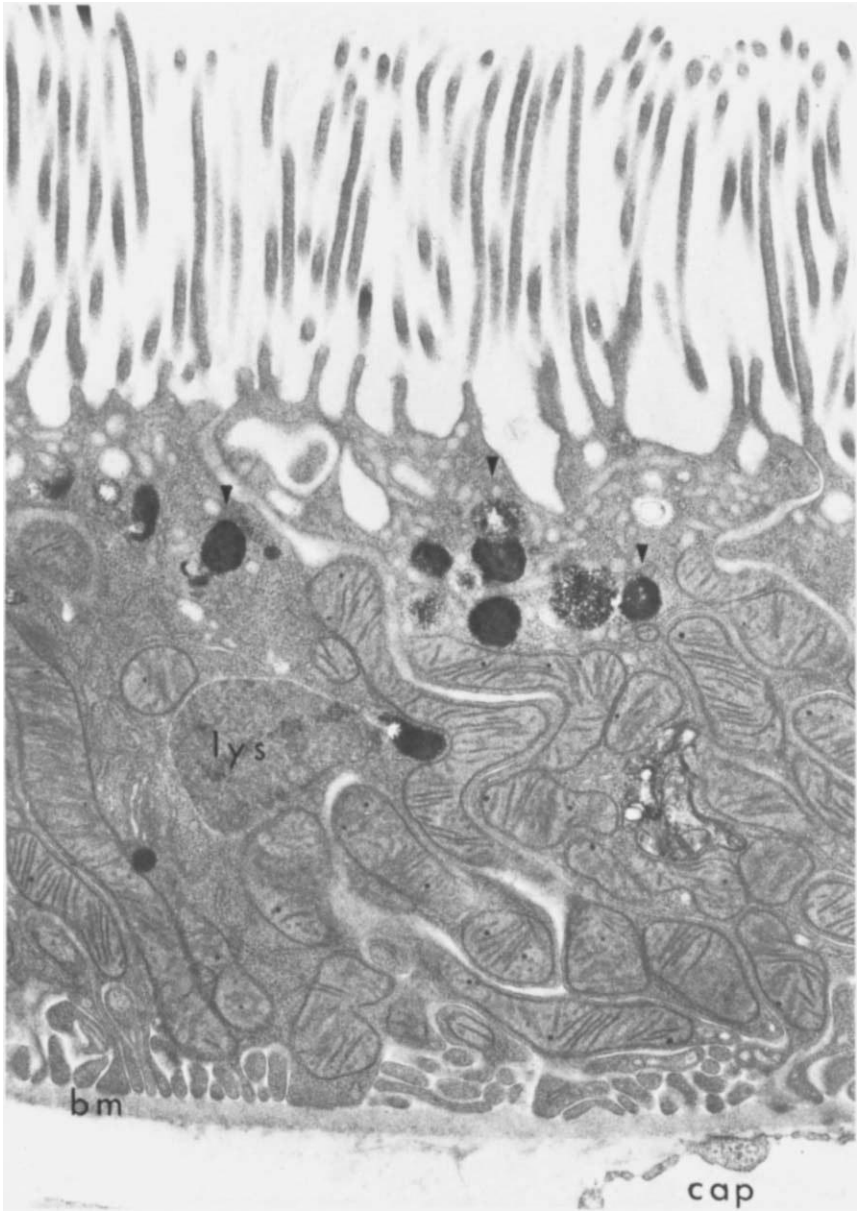

Fig. 8. *Electron micrograph of proximal tubular cell from a rat injected with native peroxidase.* Peroxidase activity *(black pigment)* is limited to reabsorption vesicles *(arrows)* and some secondary lysosomes *(lys)*. The peritubular capillary *(cap)*, the interstitium, as well as the basement membrane *(bm)* are free of reaction product. (×16,000.)

niques [27]. These experiments have revealed that the tubular epithelium incorporates protein, including peroxidase, uniquely through a vesicular reabsorptive mechanism located at its luminal surface [21, 25, 28–31]. Thus, intracytoplasmic foreign protein in the renal tubules may be traced to an origin in the glomerular filtrate rather than the peritubular interstitium. Consequently, the only possible source of error in the estimation of filtered protein appears to be inactivation of reabsorbed enzyme by the kidney epithelial cells. Straus has shown by a double labeling technique that the fusion of reabsorption droplets containing horseradish peroxidase with proximal tubular lysosomes containing hydrolytic enzymes does not commence till 15 to 20 min following i.v. injection of the enzyme in the rat [33]. Lysosomal degradation and inactivation of horseradish peroxidase is an extremely slow process not only *in vitro* [34, 35] but also *in vivo*. In this regard, the rate of breakdown of reabsorbed peroxidase in the renal cortex was shown by Straus [27, 35] to be only 6 to 8% per hour. This value is consistent with the half

life of 7 to 9 hr for horseradish peroxidase within the lysosomal apparatus of murine macrophages reported by Steinman and Cohn [36]. For ^{125}I-cytochrome C, a faster rate of degradation *in vitro* by rat proximal tubules has been reported by Christensen [31]. Thus, 20% of the tracer incorporated into the kidney cortex is digested within 20 min, as revealed by liberation of non-protein-bound ^{125}I from tissue slices. Even if this higher figure (not necessarily indicative of degradation of enzymatic activity) is accepted for peroxidase in our experiments, the maximum error in estimation of the filtered amount would not exceed 2%, since the tissue content of the enzyme was not more than 10%, on the average, of the total amount present in the urine and tissue homogenate.

Charge-dependent filtration of proteins. Previous studies have demonstrated that molecular size is an important determinant of glomerular permeability to macromolecules [5, 11, 37–42]. Since our experiments were performed with protein molecules within a very narrow range of size (31.8 Å, 29.8 Å, 30.0 Å), the influence of this factor on glomerular permeability to horseradish peroxidase could not be assessed. For a minor increment in molecular radius of only 1.8 Å, however, the clearance of anionic horseradish peroxidase was 8.7 and 48 times smaller than that of the neutral and cationic tracer, respectively. For a difference in relative clearance of 48 times in the case of dextrans, the required variation in molecular radius would have to be 10 Å [1, 11], as inferred from previous studies using dextrans and dextran-sulfates. Even though they are similarly sized molecules, the relative clearances of cationic horseradish peroxidase and that of the neutral molecule differed by a factor of 5.5. These facts together suggest that molecular charge was the factor of overriding importance in determining the observed marked variations in clearance of the differently charged peroxidases.

The glomerular capillary wall is a negatively charged structure, rich in polyanionic glycoproteins [43–46]. The results of the present experiments, our previous tracer studies with ferritin [2, 3], and the differences in clearance between dextran and dextran sulfate [1] suggest that macromolecules may interact electrophysically with these charged components of the glomerular capillary wall during filtration. Thus, in addition to restriction due to steric factors, negatively-charged macromolecules may be expected to be repelled and, therefore, to be excluded preferentially from the matrix of the glomerular capillary wall and from the glomerular filtrate. The opposite is true for cationic macromolecules; in this case, filtration is facilitated due to attractive forces. For neutral molecules, these electrophysical forces are of minor importance only.

Permeability of proteins and sugar polymers: Possible effect of molecular shape and deformability. There are significant differences between the clearance values of peroxidase in our experiments and dextrans of equivalent hydrodynamic radii reported in the literature [1, 11, 12] (Table 2). The fractional clearances of 32 Å dextran sulfate (0.05 ± 0.01), 30 Å uncharged dextran (0.55 ± 0.01), and 30 Å cationic DEAE dextran (between 0.75 ± 0.09 and 0.60 ± 0.11) are, respectively, 7.1, 9, and about 2 times greater than those of similarly sized anionic, neutral and cationic peroxidases used in our experiments.

The clearance technique used by us for peroxidases differs from that used for the dextrans. Unlike the method used in the dextran studies [1, 11], the clearance period in our experiments commenced simultaneously with the administration of peroxidase, i.e., without a prior period of equilibration. This was necessary to estimate the amount of peroxidase reabsorbed by the kidneys during the clearance period. Accurate determination of this reabsorbed peroxidase was essential to calculate the total amount filtered, a value crucial to the validation of fractional clearances in terms of *glomerular permeability*. Such considerations do not arise in the case of dextran and dextran sulfate, which are reported not to be reabsorbed in any significant amounts [1, 11]. Since we have also estimated the fraction of reabsorbed

Table 2. Comparison of fractional clearances (C/GFR) of dextrans (D) and horseradish peroxidase (HRP)

	Anionic HRP	Dextran sulfate[a]	Native HRP	Dextran[b]	Cationic HRP	DEAE-dextran[c]	
Size	31.8Å	32Å	29.8Å	30Å	30Å	28Å	32Å
C/GFR	0.007 ± 0.000	0.05 ± 0.01	0.061 ± 0.005	0.55 ± 0.01	0.338 ± 0.019	0.75 ± 0.09	0.60 ± 0.11
$\frac{C_D/GFR}{C_{HRP}/GFR}$	7.1		9.0		≈2		

[a] Ref. [1].
[b] Ref. [11].
[c] Ref. [12].

peroxidase with a reasonable degree of accuracy, it is unlikely that tubular reabsorption underestimated the clearances of the peroxidases.

Differences in methodology alone are unlikely to explain the large variations in the clearances of peroxidases and dextrans. Dextrans are linear flexible polymers, and form loose, random coils of hydrated spheres in free solution [7]. Such polymers are, however, vulnerable to deforming forces, and may unfold and assume an elongated configuration [47]. These forces are minimal under conditions of free diffusion, as in gel filtration [48]. During convectional transport across the glomerulus, however, dextran molecules may unfold as they encounter obstacles in the capillary wall [5] and behave as if they were of smaller dimensions. This type of anomalous behavior of linear polymers has been shown to occur during sedimentation through hyaluronate solutions. Forced migration of linear, rod-like or randomly coiled flexible molecules through solutions of chain polymers [8] is much greater than that of globular molecules of equivalent hydrodynamic radii [9, 10]. Rigid interpeptide cross-links, particularly disulfide bonds, are present in proteins, including horseradish peroxidase [49]. Thus, molecular deformation is less likely to occur in proteins.

The differences in clearance values between dextrans and peroxidases, considered in the context of the above theoretical considerations, suggest that molecular shape and flexibility may also be a factor that determines glomerular permeability to macromolecules, in addition to size and charge. Experimental verification of this possibility under controlled conditions appears warranted.

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