

THE PERMEABILITY OF GLOMERULAR CAPILLARIES TO GRADED DEXTRANS

Identification of the Basement Membrane as the Primary Filtration Barrier

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ABSTRACT

Graded dextrans have been used as tracers to identify the primary permeability barrier(s) to macromolecules among the structural elements (endothelium, mesangium, basement membrane, epithelium) of the glomerular capillary wall. Three narrow-range fractions of specified molecular weights and Einstein-Stokes radii (ESR) were prepared by gel filtration: (a) 32,000 mol wt, ESR = 38 Å; (b) 62,000 mol wt, ESR = 55 Å; and (c) 125,000 mol wt, ESR = 78 Å. These fractions are known to be extensively filtered, filtered in only small amounts, and largely retained, respectively, by the glomerular capillaries. Tracer solutions were infused i.v. into Wistar-Furth rats, and the left kidney was fixed after 5 min to 4 h. The preparations behaved as predicted: initially, all three fractions appeared in the urinary spaces, with $32,000 > 62,000 \gg 125,000$. The smallest fraction was totally cleared from the blood and urinary spaces by 2.5 h, whereas the intermediate and largest fractions were retained in the circulation at high concentrations up to 4 h. With all fractions, when particles occurred in high concentration in the capillary lumina, they were present in similarly high concentrations in the endothelial fenestrae and inner (subendothelial) portions of the basement membrane, but there was a sharp drop in their concentration at this level—i.e., between the inner, looser portions of the basement membrane and its outer, more compact portions. With the two largest fractions, accumulation of particles occurred against the basement membrane in the mesangial regions with time. No accumulation was seen with any of the fractions in the epithelial slits or against the slit membranes. Dextran was also seen in phagosomes in mesangial cells, and in absorption droplets in the glomerular and proximal tubule epithelium. It is concluded that the basement membrane is the main glomerular permeability barrier to dextrans, and (since their behavior is known to be similar) to proteins of comparable dimensions (40,000–200,000 mol wt). The findings are discussed in relation to previous work using electron-opaque tracers to localize the glomerular permeability barrier and

in relation to models proposed for the functions of the various glomerular structural elements.

The stratified nature of the glomerular capillary wall has been known for over 15 years, but at the present time there is still controversy regarding which of its several layers serves as the main filtration barrier for plasma proteins. A number of attempts have been made to identify the filtration barrier by electron microscopy using various electron-opaque tracers (1-15). Initially, only a few such tracers were available, and these were mostly of large size (ferritin, colloidal particles of mercuric sulfide, gold, and thorotrast). Farquhar et al. (3) established that the glomerular basement membrane restricts the passage of ferritin molecules, and concluded that the basement membrane is the main filtration barrier to molecules in this size range (diam. > 100 Å [16]). Studies with other particulate tracers supported this conclusion (1, 2, 4, 5). Later a number of additional tracers became available which were not themselves electron dense, but depended for their demonstration on a histochemical reaction (involving peroxidatic activity) and deposition of an electron-dense reaction product (oxidized and polymerized diaminobenzidine or DAB). Karnovsky and his associates (7, 11) using such tracers (i.e., horseradish peroxidase, mol wt = 40,000; myeloperoxidase, mol wt = 160,000; and catalase, mol wt = 240,000) obtained evidence which in their interpretation indicated the existence of two successive filtration barriers: (a) the basement membrane which acts as a "coarse" filter to exclude very large molecules (diam. > 100 Å) and to retard smaller molecules, (b) the epithelial slits which act as a "fine" filter and represent the principal filtration barrier for molecules the size of albumin. This concept of the existence of two successive barriers has gained wide acceptance by morphologists (17) and physiologists (18) alike, in spite of the restraint exercised recently by Karnovsky and Ainsworth (13) in their interpretation of the accumulated data.

Needless to say, it is important to know if there is just one filtration barrier in the glomerulus or two in series. The problem is that most of the tracers used up to now are either too large or too small, when compared to the size of albumin, to satisfactorily bracket (with a sufficient number of probes of adequate dimensions) the size of the

restrictive glomerular passages. An additional problem with histochemically detectable tracers is that secondary erroneous localizations are possible due to diffusion and reabsorption (to other sites) of either the tracer or reaction product. Inert particulate tracers which are individually detectable would therefore be preferable. Fortunately, such tracers are now available in the case of dextrans (19, 20).

Dextrans are inert, heterodisperse polysaccharides of bacterial origin which are extremely useful as tracers because (a) they are available in a wide range of sizes from molecular weight of 10,000 to over 500,000, and (b) their filtration behavior is well known, since they have been extensively used by physiologists to study glomerular permeability (21-24). Hence, a satisfactory correlation between physiologic and morphologic data is finally possible.

In the present work we have prepared fractions of dextrans of narrow molecular weight range which bracket the size of albumin (the molecule effectively retained by the glomerulus) and have used them as tracers in an attempt to identify the filtration barrier for molecules the size of albumin (mol wt = 68,000; ESR = 35 Å) in the normal glomerulus.¹

MATERIALS AND METHODS

Materials

Crude dextrans ($T_{40} + T_{250}$) were obtained from Pharmacia, Inc., Uppsala, Sweden. 40,000 mol wt dextran was also obtained from ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio.

Animals

A total of 31 young male Wistar-Furth rats, 100-240 g body weight, were used in these experiments. Wistar-Furth strain rats were used because it has been shown that they tolerate dextran well (i.e., they do not liberate histamine in response to dextran injection) (19).

Purification of Dextrans

Commercially available dextran preparations of a specified molecular weight are well known to be

¹ A preliminary report of these findings was published previously (25).

polydisperse, with the molecular weights used in their designation representing the average of the molecules present. Hence they must be further fractionated to provide "cuts" with a molecular weight and size range narrow enough to be adequate for studies of glomerular permeability. Such narrow range fractions of selected and specified molecular weights were purified from commercial dextrans by gel filtration on a 5×100 -cm Sephadex G-150 column (bed vol = 2 liters; void volume = 631 ml) by the method of Laurent and Granath (26). Fractions of narrow range were selected, precipitated in alcohol, and air dried. The Einstein-Stokes radius (of each fraction) was determined by method of Laurent and Kilander (27), and the molecular weight was determined graphically from a calibration curve provided by Pharmacia.

Narrow-Range Fractions

Five separate fractions were purified whose characteristics are given in Table I. For preparation of the 40,000-mol wt fraction, 2.2 g of crude 40,000-mol wt dextran (Nutritional Biochemicals) dissolved in 50 ml normal saline were loaded on the column, eluted with double distilled water (at 25–30 ml/h), and collected in 10.6-ml fractions. The concentration of dextran was monitored with the anthrone reaction (30). The sample was eluted over 700 ml and the central 120 ml taken, precipitated, and dried (yielding 0.5 g). The dried precipitate thus obtained constituted the 40,000-mol wt fraction used in these experiments. For preparation of the 32,000-mol wt fraction, crude T₄₀ (Pharmacia) was purified exactly as done for the 40,000-mol wt fraction. The yields of four such runs were pooled and run again on the same column, the central 230 ml collected, precipitated, and dried (yielding 1.28 g dextran out of the 2-g load). The chromatographic profile of the final, purified preparation was much narrower than that of the crude commercial preparation (see Fig. 1). The 250,000-mol wt fraction was prepared by loading T₂₅₀ Pharmacia on the column. The void volume was collected, precipitated, and used for injection. This fractionation was done to eliminate particles of low molecular weight (<250,000). The 62,000- and 125,000-mol wt fractions were provided by Dr. Svensjö of Pharmacia, Inc., Uppsala, and were prepared on a Sepharose 4 B column. The weight average and number average molecular weights of the fractions were provided by Dr. Svensjö and were determined using light-scattering and end group analysis, respectively.

Tracer Solutions

For injection the dextrans were prepared as ~1-mM solutions by resuspension in normal saline. The 32,000- and 40,000-mol wt fractions were given as a 5% (wt/vol) solution; the 62,000-mol wt fraction as 7%; and the

TABLE I
Characteristics of Purified Dextran Fractions

Fraction	Mw	Mn	Mw/Mn	ESR
				Å
32,000*	—	—	—	38*
40,000	—	—	1.36‡	45*
62,000	62,000	53,900	1.163	55*
125,000	125,000	101,000	1.244	78*
250,000	—	—	<2.05	100*

Since it is polydisperse, the molecular weight of dextran is usually characterized as the ratio of two determinations, with the numerator consisting of the weight average molecular weight (determined by light scattering), and the denominator the number average molecular weight (determined by end-group analysis) (29). For a pure (monodisperse) solution the ratio is unity. All of the fractions used were in the range of 1.1–1.4, except the 250,000-mol wt fraction. The latter consisted essentially of the commercial preparation with the low molecular weight particles removed, whereas all the others had both higher and lower molecular weight particles removed.

* Calculated from a calibration curve supplied by Dr. William Gelb of Pharmacia.

‡ Value estimated from a similar fraction of Granath and Kvist (28).

Mw = weight average molecular weight.

Mn = number average molecular weight.

ESR = Einstein-Stokes radius.

125,000- and 250,000-mol wt fractions as 10% solutions. Before use, they were heated (or sonicated) to disperse possible aggregates.

Experimental Procedures

Rats were anesthetized with ether, and the dextran tracer solution was infused into the saphenous vein over a 5–10-min period using a Harvard infusion pump. 1 ml of tracer solution was given per 100 g of body weight. The rate varied from approximately 0.1 to 0.25 ml/min. At varying intervals (Table II) after the start of injection, the abdomen was opened, and fixation of the left kidney was initiated by injecting the fixative directly into the cortex. The renal artery, vein, and ureter were clamped simultaneously with the initiation of fixation. The kidney was allowed to fix *in situ* for 15 min. Slices of cortex were then removed, cut into small blocks, and fixation was continued for up to 3 h at 4°C. In some cases the right kidney was removed and fixed by immersion alone as follows: strips of cortex were prepared, cut into blocks in a drop of fixative, and fixed for 3 h at 4°C. The tissue blocks were routinely dehydrated in alcohol and embedded in Epon. In several cases embedding was also carried out in glycol methacrylate (31).

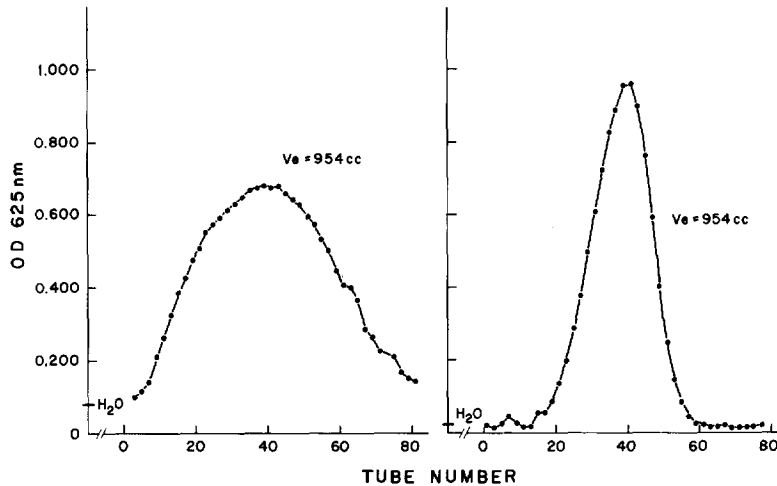


FIGURE 1 Fractionation of T_{40} dextran (Pharmacia) on Sephadex G-150 (5×90 cm). Chromatographic profiles of commercial and fractionated dextran. Left, commercial 40,000-mol wt dextran. Right, the same dextran after two purification runs. The curve on the right represents the 32,000-mol wt fraction and is much narrower in width than the commercial material. The details of the chromatography are given under Methods.

TABLE II
Experimental Animals

Tracer given	Time interval*			Total animals
	50-10 min	11-25 min	1-4 h	
<i>mol wt</i>				
32,000	2	2	2	6
40,000	2	3	2	7
62,000	1	3	4	8
125,000	1	2	3	6
250,000	1	1	2	4
				31

* Timed from the start of the tracer injection to the initiation of fixation.

Fixative

The one-step fixative introduced by Simionescu et al. (20) for demonstration of dextrans and glycogen was used throughout. This fixative contains formaldehyde (1.5%), glutaraldehyde (2.5%), OsO_4 (0.66%), and lead citrate (2-3 mg/100 ml) in 0.1 M arsenate buffer, pH 7.4. The precise mechanism by which the fixation and staining operates is unknown, but with this procedure the lead citrate (or lead- OsO_4 complex) apparently binds selectively to the dextran and enhances its contrast in comparison to the surrounding tissue. Uranyl acetate staining in block and on grid was avoided because it causes a reduction in the contrast of the dextran (20).

Sectioning

Thick sections ($0.5 \mu\text{m}$) were prepared, placed on glass slides, stained 1 min with 2% basic fuchsin at 80°C , rinsed with double distilled water and subsequently dried, sprayed with Krylon, and examined with the light microscope. This method was used because it stains plasma proteins (which appear as light pink granular material). It thereby facilitates the selection of glomeruli which are adequately fixed as judged by satisfactory retention of plasma proteins in the capillary lumina. Glomeruli were selected for subsequent electron microscopy that fulfilled the following criteria: (a) the capillary loops were distended; (b) the contents of the capillaries were retained and uniform throughout the capillary; (c) there was no obvious leakage of plasma contents into Bowman's space. Usually blocks were taken that included three to four glomeruli. Sections of 60-90 nm thickness, showing silver interference colors, were cut with Dupont diamond knives on a Porter-Blum MT-2B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.). Thin sections were stained with lead citrate (32) for 10-15 min and examined with a Siemens Elmiskop 1 or 101, or Philips 301 operating at 80 kV.

RESULTS

Morphology

The general morphology and ultrastructure of the glomerulus has been previously reported (3). The capillary wall is composed of three layers—a

fenestrated endothelium, a substantial (120–150 nm thick) basement membrane, and an elaborate epithelial layer with interdigitating foot processes which cover the outer surface of the basement membrane. In sections, these foot processes are separated (where they approach most closely) by a space of ~25 nm which is bridged by a thin (~4 nm) line, the so-called slit diaphragm or filtration slit membrane. The spaces between the foot processes up to the diaphragms are referred to as the epithelial filtration slits. A third cell type, the mesangial cell, exists in the axial regions of the glomerulus on the capillary side of the basement membrane.

The results obtained with the dextran fractions will be presented proceeding from the largest fraction to the smallest. The 125,000- and the 250,000-mol wt fractions will be considered together since their behavior is similar. The 32,000- and 40,000-mol wt fractions will also be considered together since they behave similarly.

125,000- and 250,000-Mol Wt Dextrans

At the earliest time points (~10 min) after the start of the injection, particles of dextran are seen throughout the capillary lumen. Their distribution is relatively homogeneous, but even in well fixed preparations some aggregation of particles is evident (Fig. 2). Dextran is also seen within the endothelial fenestrae and in the subendothelial areas between the endothelium and the basement membrane. Particles are not usually found in the basement membrane, but some are seen in the urinary spaces. With increasing time after injection (1–3 h), there is increasing accumulation of dextran along the luminal side of the basement membrane (Figs. 3–4) especially in the mesangial regions (Fig. 5), but no accumulation is seen in the epithelial slits or piled against the slit membranes (Fig. 3). Few particles are seen in the open urinary spaces beyond the level of the slit membranes. Even after 3.5 h the particles are still retained in the capillary lumen in a high concentration approximating that injected initially (Fig. 3), indicating that little dextran has been lost from the circulation by filtration. At all time intervals there is a sharp drop in the concentration of dextran between the inner, less compact portion of the basement membrane where particles are numerous and the dense portions of the basement membrane. At the longer time points some accumulation of dextran can also be seen within absorption droplets

in the epithelial cell cytoplasm (Figs. 8–10) and within mesangial cells (Fig. 6). The latter presumably represents uptake of dextran residues piled against the basement membrane, whereas the former represents uptake by pinocytosis occurring primarily (but not exclusively) along the base of the foot processes facing the basement membrane. Occasionally, images suggesting pinocytotic uptake of dextran particles by the epithelium are encountered (see Fig. 7).

62,000-Mol Wt Dextran

At 10 min after the start of injection the dextran is present not only in the capillary lumen, but also in the open urinary spaces beyond the level of the slits (Fig. 11). In the capillary lumina the particles are numerous and relatively evenly distributed, but in the urinary spaces they are few and aggregated into clumps. As in the case of the 125,000-mol wt fraction even at this early time, a sharp drop in the concentration of dextran is seen along the subendothelial portions of the basement membrane, but no dextran can be detected in the slits (or piled against the slit membranes) in either normal or grazing sections (Fig. 11, 12). With increasing time after injection the amount of dextran found in the open urinary spaces gradually decreases. The amount found in the capillary lumina and in the inner portions of the basement membrane remains high (Fig. 13). As with the 125,000-mol wt fraction, accumulation or piling of dextran against the basement membrane is evident in the mesangial regions, and accumulation within endocytic vacuoles (absorption droplets) is evident in both mesangial and epithelial cells. Previous observations with other tracers indicate that such vacuoles are converted in time into lysosomes (4, 33, 34).

32,000-Mol Wt and ~40,000-Mol Wt Dextrans

At the earliest time points (7.5 min) after the start of the injection, there are large quantities of the tracer both in the capillary lumina and in the open urinary spaces beyond the level of the slits. Dextran is also seen in the subendothelial space along the basement membrane but not in the subepithelial space along the epithelium (Figs. 14, 15). As with the other dextran fractions, there is a distinct drop in the concentration of the tracer along the inner surface of the basement membrane,

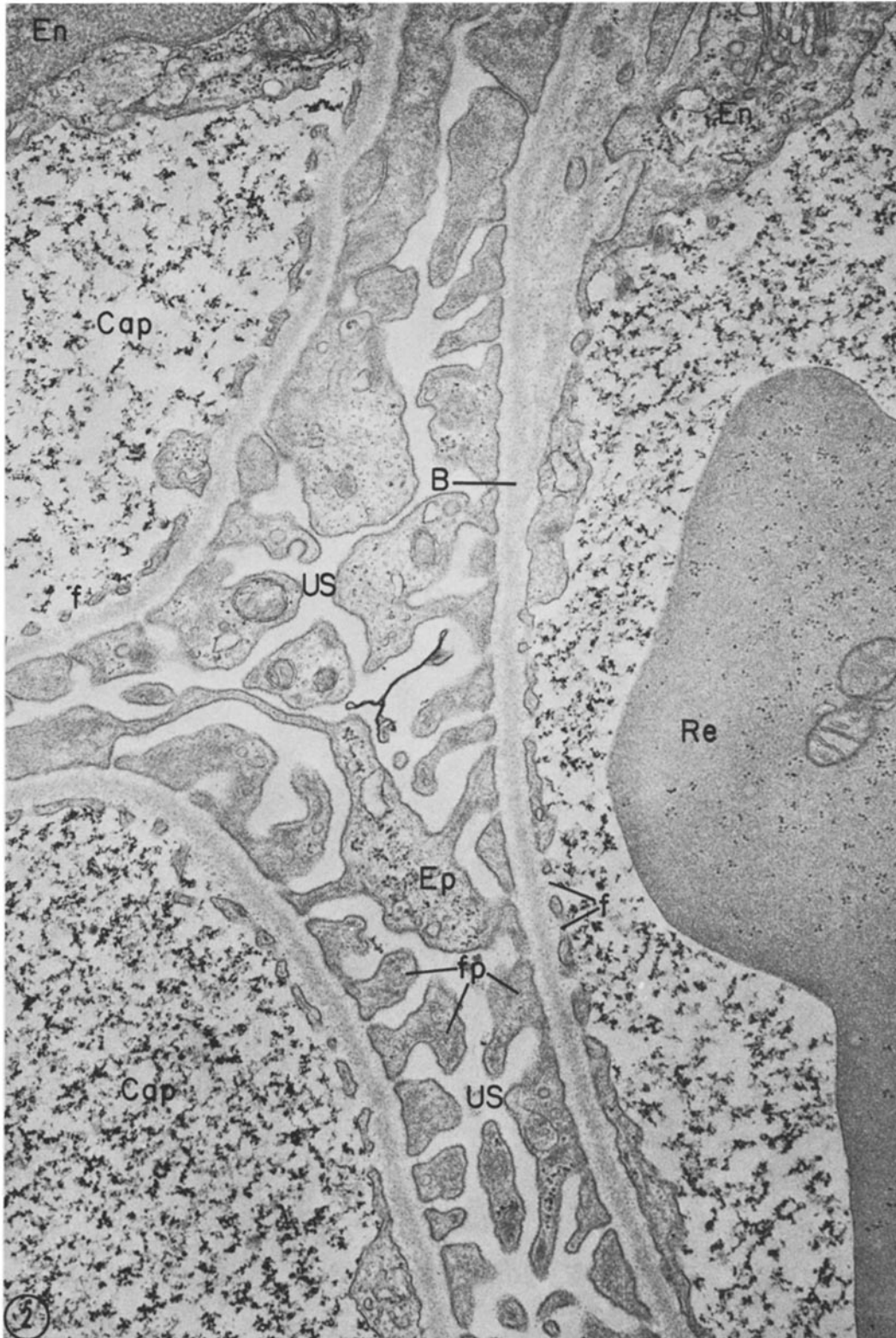
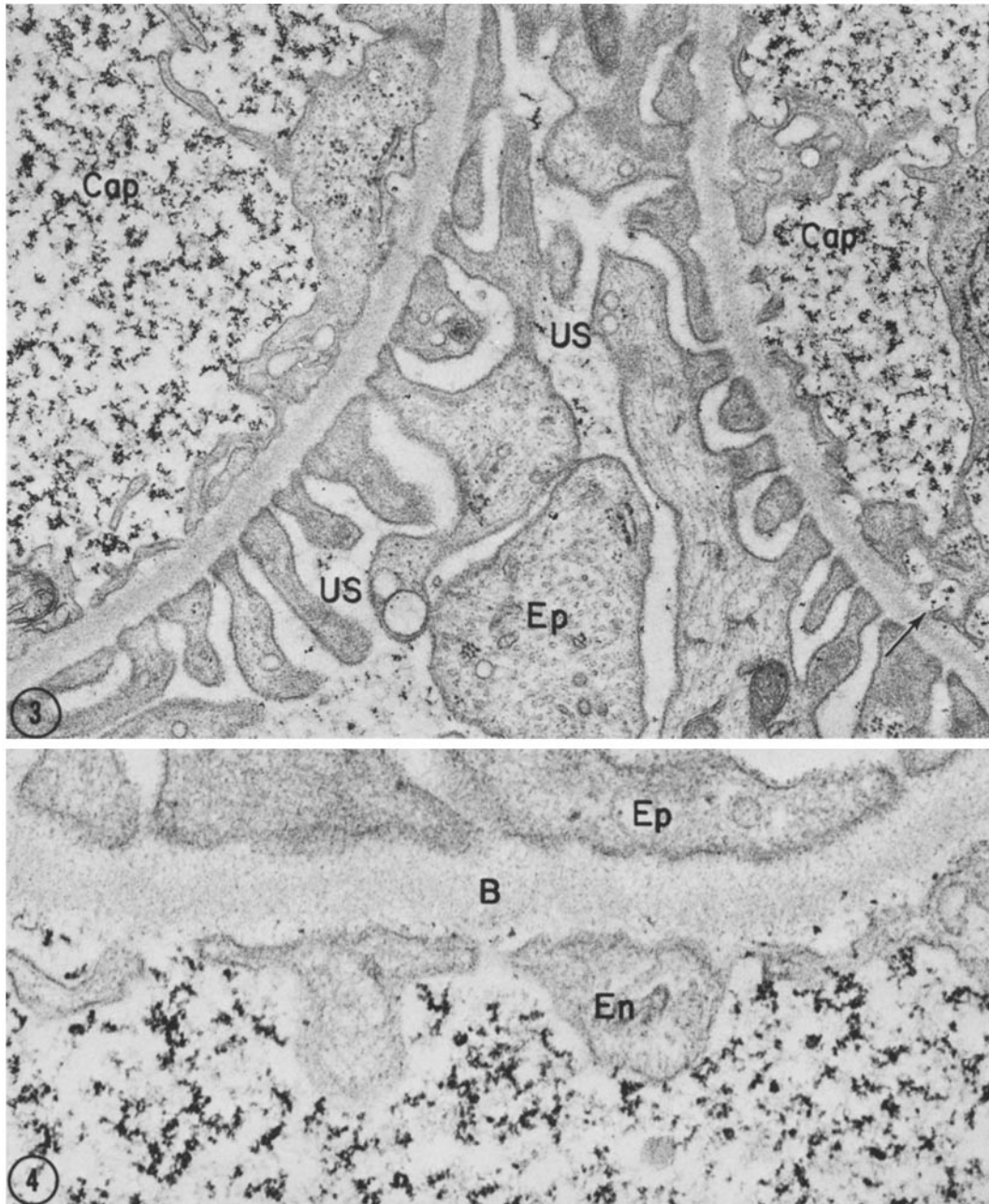
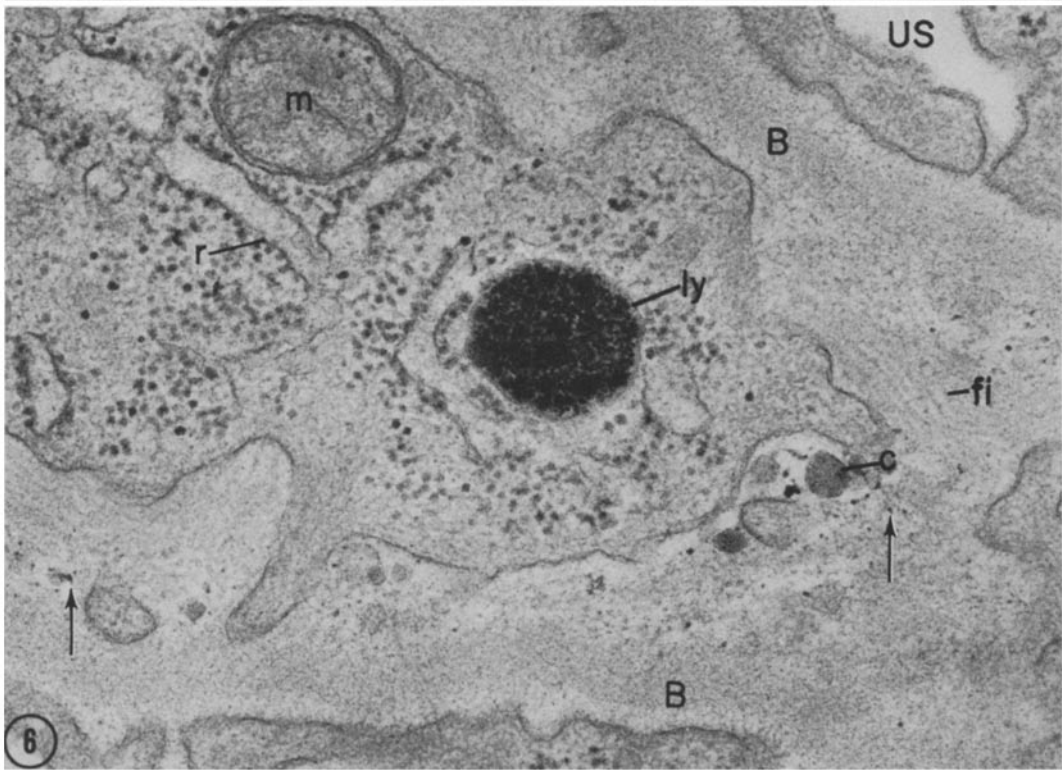
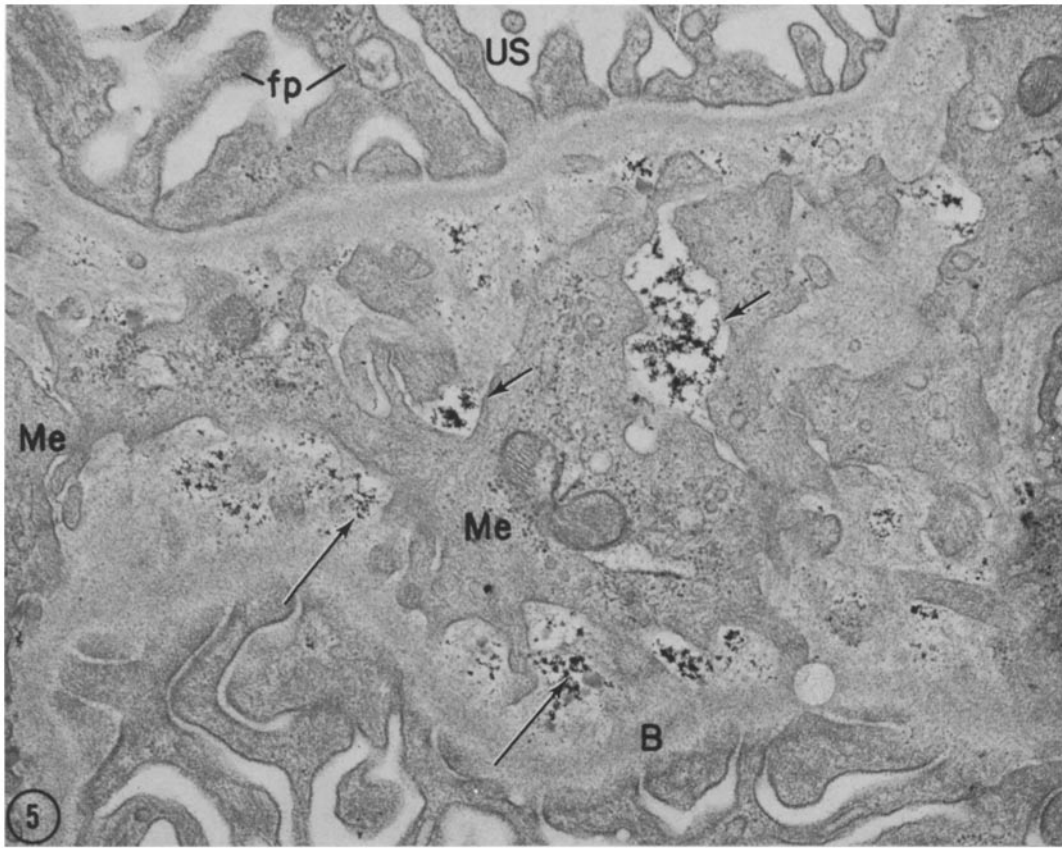


FIGURE 2 Portions of a glomerular capillary taken from an animal sacrificed 10 min after the injection of 125,000-mol wt dextran. Three capillary lumina (*Cap*) filled with particles of dextran are present in the field. The dextran appears dense and irregularly aggregated. It is seen freely penetrating the fenestrae (*f*) of the endothelium, but the basement membrane (*B*), the slits between the epithelial foot processes (*fp*), and the open urinary spaces (*US*) beyond the level of the slits are free of dextran. *Re* = reticulocyte; *Ep* = visceral glomerular epithelium. $\times 22,000$.



FIGURES 3 and 4 Portions of glomerular capillaries from an animal sacrificed 3.5 h after the injection of 125,000-mol wt dextran. The luminal concentration of tracer is still quite high. Dextran appears to penetrate the endothelial fenestrae and can be seen between the endothelium (*En*) and the basement membrane (*B*). The basement membrane proper and the epithelial slits do not contain dextran. This sharp drop in the dextran concentration which occurs in the subendothelial areas is particularly evident on the lower right in Fig. 3 (arrow) and (at higher magnification) in Fig. 4. A few molecules are seen in the urinary spaces (*US*) adjacent to the plasma membrane of epithelial cells (*Ep*). The particles present in the urinary spaces appear smaller in size than those in the capillary lumina. Fig. 3, $\times 32,000$; Fig. 4, $\times 87,000$.



no dextran is seen in the filtration slits, and no piling of the tracer is seen against the slit diaphragms. Over the course of 1–2 h after the injection, tracer gradually disappears from both the blood and urinary spaces (as it is lost by filtration), so that by 2–3 h little or no dextran remains in either location (Fig. 16). With this small molecular weight fraction there is little or no accumulation of dextran in the mesangial regions, and more limited uptake of dextran by mesangial and epithelial cells.

Appearance of Dextran

As pointed out by Simionescu et al. (20) and as seen in all figures, in sections the dextran particles appear as aggregates of considerably larger size than expected on the basis of molecular weight determinations. Data in the physiological literature (21–24) indicate convincingly that dextrans are present in molecular dispersion in the circulating plasma. Hence aggregation must occur during fixation. It appears to be inversely related to the concentration of protein in the immediate environment, since it is most pronounced in the urinary spaces, less in plasma, and least in the subendothelial portions of the basement membrane. This being the situation, the electron microscope observations indicate only the presence and relative concentration of the dextran in relevant compartments; they do not give a reliable indication as to particle size. For this parameter we rely exclusively on characterization of the fractions before injection.

Extraglomerular Location and Fate of Dextran

With all the fractions dextran is found in the lumen of all segments of the nephron. In addition, in the proximal tubule it is found in apical

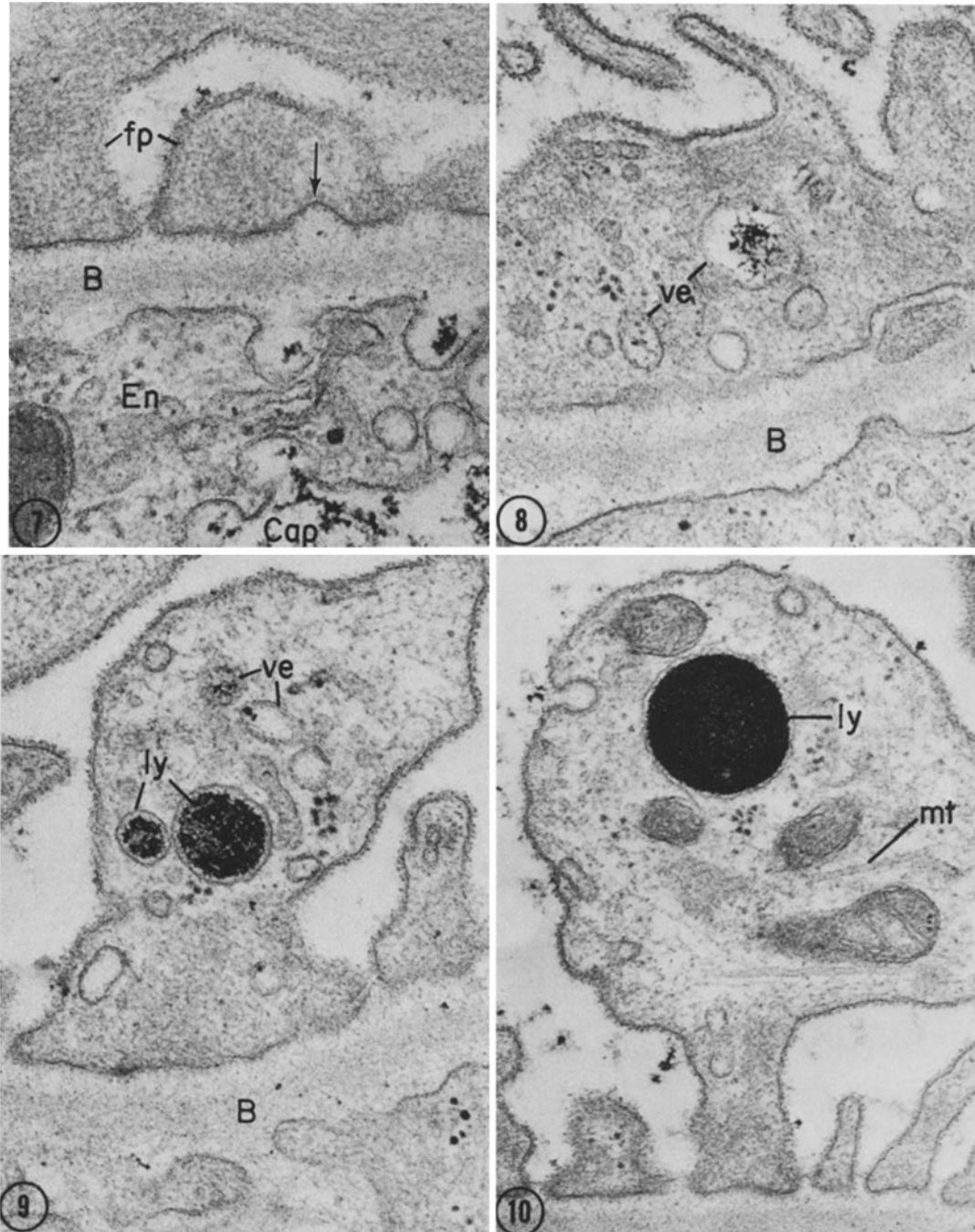
vacuoles at the base of the microvilli where it is apparently undergoing reabsorption (Figs. 17, 18). No evidence of dextran absorption is seen in distal tubules. As expected, the amount found in the proximal tubule epithelium varies inversely with the molecular weight (with $32,000 \gg 62,000 > 125,000$). All three fractions are seen in the peritubular capillaries in concentrations corresponding to that seen in the glomerular capillaries. Apparently the peritubular capillaries are quite permeable to all fractions since dextran appears in the pericapillary spaces and penetrates the intercellular spaces between cells up to the level of the tight junctions in both the proximal (Figs. 17, 19) and distal tubules as well as in the parietal epithelium of Bowman's capsule.

DISCUSSION

We have used the polysaccharide tracer dextran to identify the primary filtration barrier for macromolecules of the size of plasma proteins among the components of the glomerular capillary wall. Dextrans are known to behave like proteins as far as glomerular filtration is concerned, in that there is increasing restriction to their passage with increasing molecular weight. The only difference in their behavior is the fact that their cutoff point or their effective exclusion from filtration, occurs at a somewhat lower molecular weight than for proteins. With proteins (18, 35), restriction begins at 14,000 and increases with increasing molecular weight up to 68,000, the size of albumin (which is filtered only in small amounts [35, 36]). According to the work of Wallenius (21), Mogenson (22), and others (23, 24), effective exclusion of dextran occurs at a molecular weight of 55,000–60,000 daltons. The reasons for this are not entirely understood (18), but it is probably related to the nature of the molecule—a flexible long-chain

FIGURE 5 Mesangial area 13 min after the injection of 250,000-mol wt dextran. The dextran is seen in the intercellular spaces between the processes of adjacent mesangial (*Me*) cells (short arrows) and between these cells and the basement membrane (*B*) (long arrows). No dextran is seen in the epithelial slits or urinary spaces (*US*). $\times 20,000$.

FIGURE 6 Mesangial area 1 h after administration of 125,000-mol wt dextran, showing uptake of accumulated dextran by a mesangial cell. The dextran has been segregated into a lysosome (*ly*). A few dextran particles (arrows) and chylomicra (*c*) are seen piled in the spaces between mesangial processes and the basement membrane (*B*). Discrete (11 nM) fibrils (*fi*) of the type previously described (3) can also be seen in the field between the mesangial processes and the basement membrane. *m* = mitochondrion; *r* = rough endoplasmic reticulum. $\times 63,000$.



FIGURES 7-10 These figures show uptake of filtered dextran by glomerular epithelial cells. In Fig. 7, from an animal given 62,000-mol wt dextran 1 h before sacrifice, a single dextran particle is present facing an invagination (arrow) of the epithelial cell membrane at the base of a foot process (*fp*). In Figs. 8 and 9, from animals sacrificed 1 and 4 h, respectively, after administration of the 125,000-mol wt fraction, particles of dextran are seen in vesicles (*ve*) of variable size and in phagosomes (*ly*) within epithelial foot processes. Fig. 10, taken 4 h after injection of 125,000-mol wt dextran, shows concentration of the dextran within an epithelial phagosome (*ly*). As in the case of ferritin (3, 6) some of the dextran particles which pass through the basement membrane are picked up by the epithelium and concentrated into phagosomes which become lysosomes. *B* = basement membrane; *Cap* = capillary lumen; *mt* = microtubule. Fig. 7, \times 81,000; Fig. 8, \times 60,000; Fig. 9, \times 78,000; Fig. 10, \times 61,000.

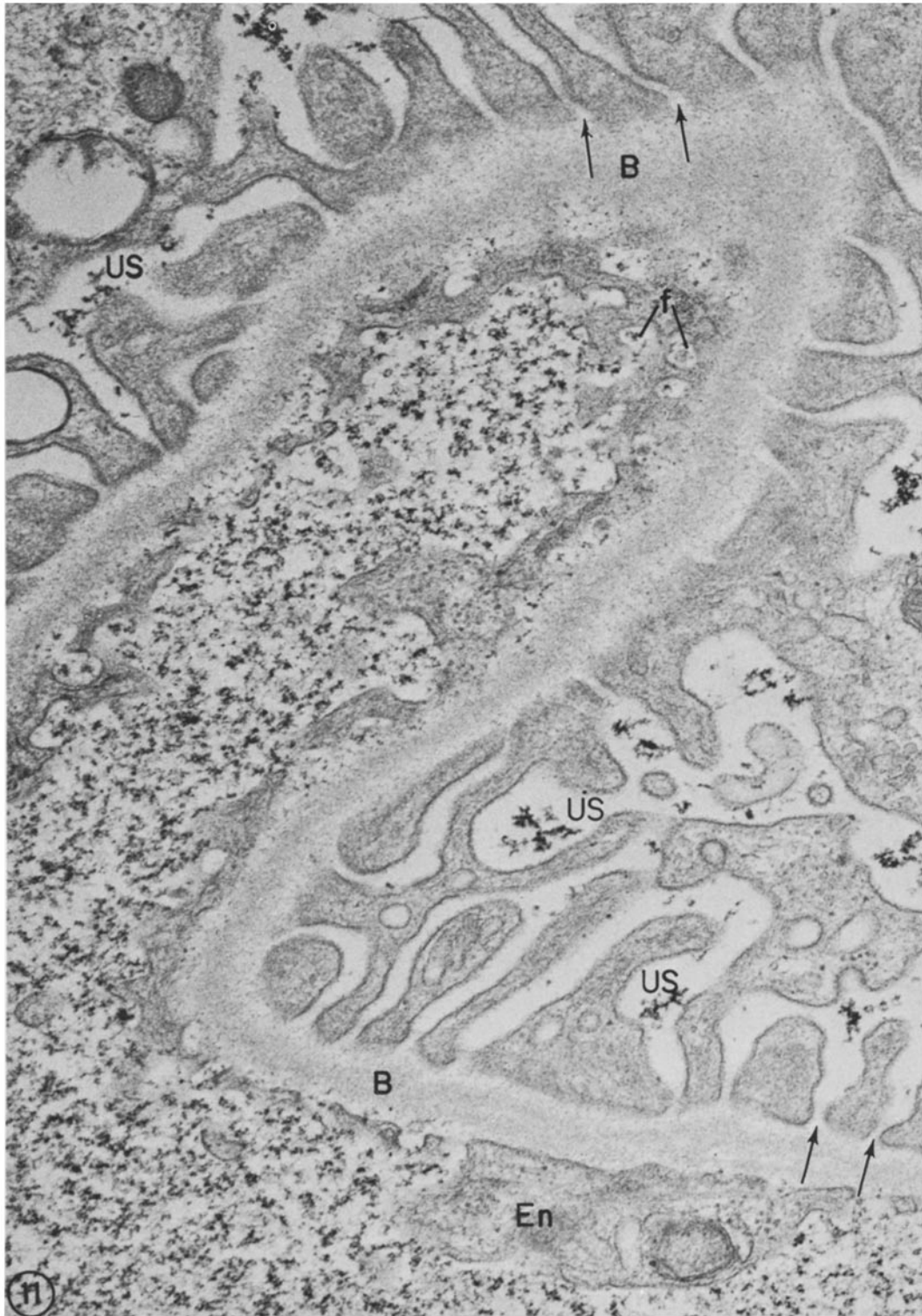


FIGURE 11 Glomerular capillary 10 min after the injection of 62,000-mol wt dextran. The tracer is present in high concentration in the capillary lumen, and a few particles, which are aggregated into coarse clumps, are present in the urinary spaces (*US*). Numerous molecules are seen between the endothelium (*En*) and the basement membrane (*B*). No dextran is present in the basement membrane proper or epithelial slits (arrows). These two main features—the sharp drop in the concentration of dextran along the subendothelial regions of the basement membrane and its absence from the epithelial slits—are particularly well shown at the top of the field where the section cuts tangentially through the capillary wall. *f* = endothelial fenestrae. $\times 36,000$.

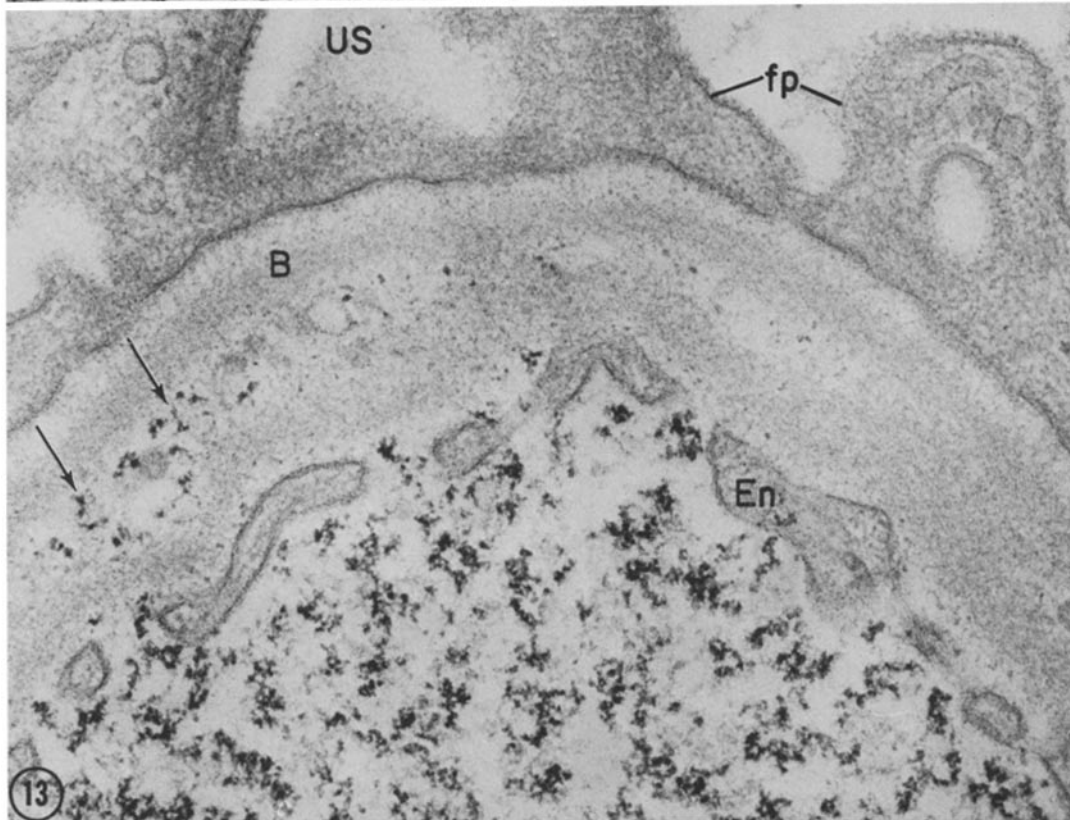
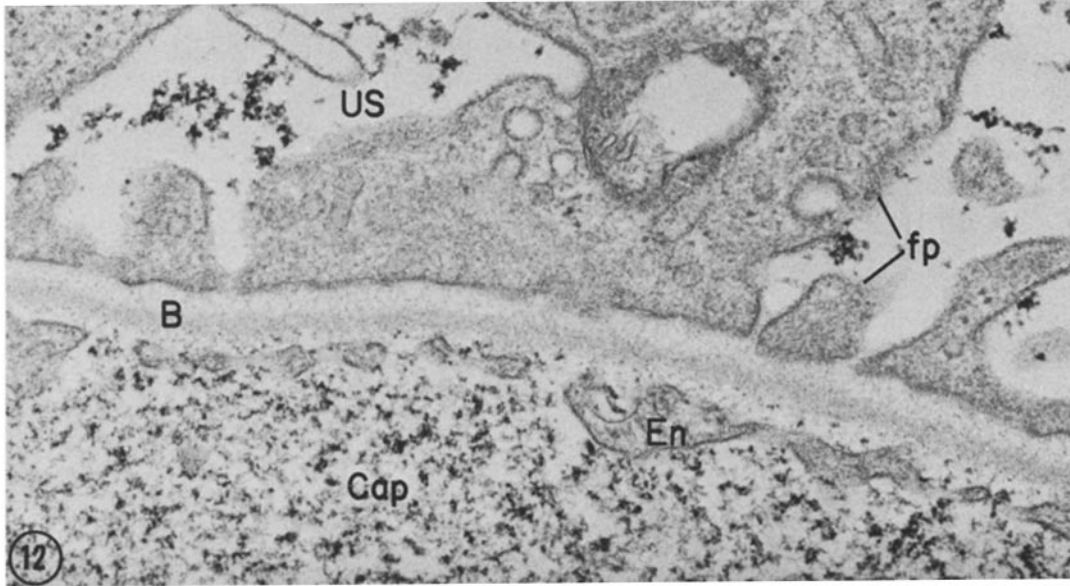


FIGURE 12 Field from the same animal as in Fig. 11, showing the sharp drop in dextran concentration that occurs between the portions of the basement membrane (*B*) adjacent to the endothelium (*En*) and the rest of the basement membrane. No dextran is seen in the slits between the epithelial foot processes (*fp*). Dextran is present in the urinary spaces (*US*) where it is aggregated into clumps larger than those present in the capillary lumen (*Cap*). $\times 40,000$.

FIGURE 13 Glomerular capillary 1 h after injection of 62,000-mol wt dextran. The luminal concentration is still high, and distinct piling of dextran against the basement membrane (arrows) is seen. The particles freely penetrate the inner spongy portions of the basement membrane which appear particularly broad here due to the fact that the section grazes through the edge of a mesangial area. The urinary spaces (*US*), the epithelial slits between adjacent epithelial foot processes (*fp*) and the basement membrane (*B*) contain no tracer. $\times 76,000$.

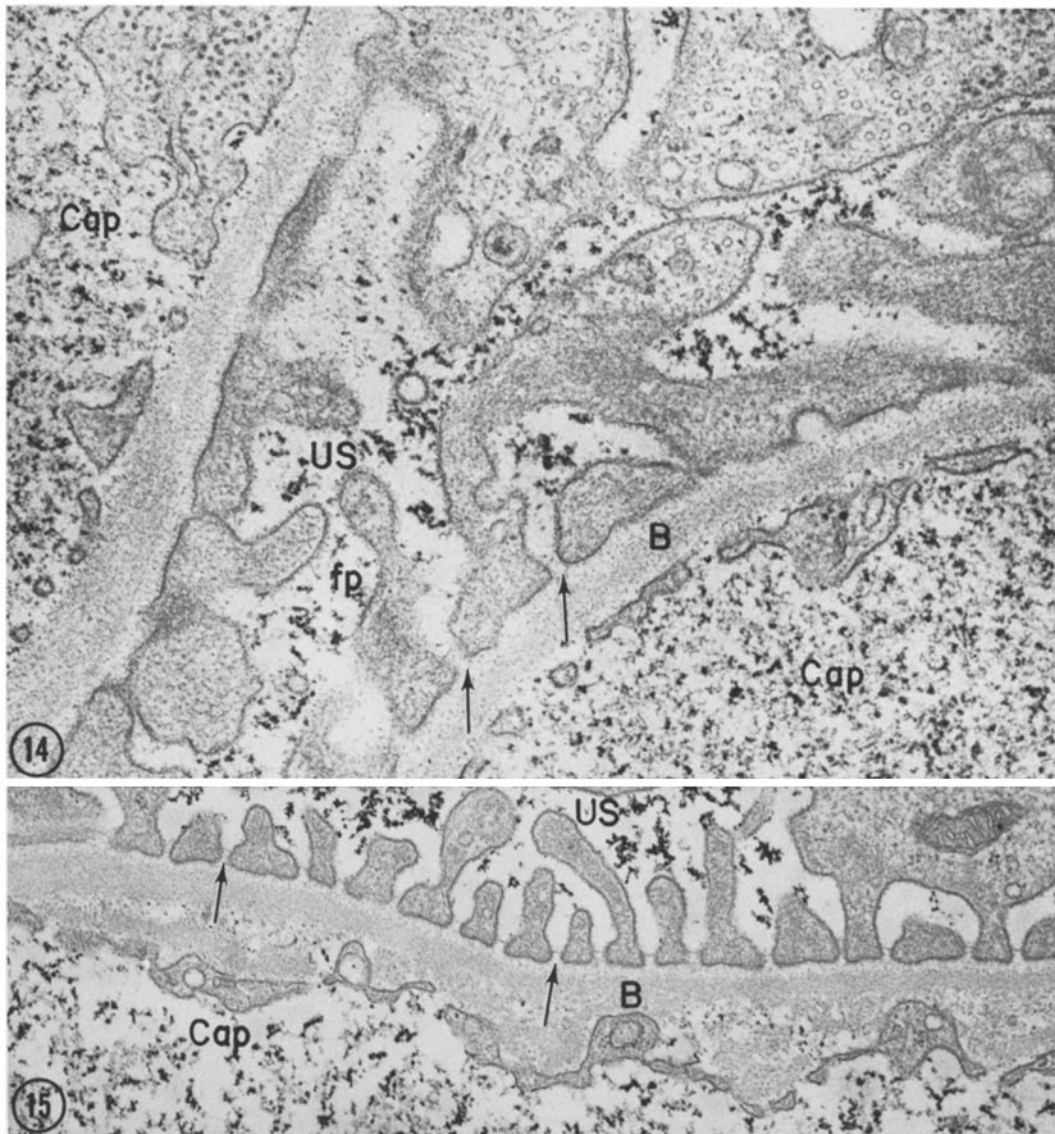


FIGURE 14 Glomerular capillary 7.5 min after the injection of 32,000-mol wt dextran. The tracer is present in large quantities in both the capillary lumina (*Cap*) and the urinary spaces (*US*). Even with this relatively small molecular weight fraction, a sharp drop in the concentration of dextran is seen along the subendothelial portions of the basement membrane (*B*) and the slits appear free of the tracer (arrows). $\times 36,000$.

FIGURE 15 Glomerular capillary 5 min after injection of 32,000-mol wt dextran. The section is from the edge of a mesangial area. As in Fig. 14, the tracer is present in both the capillary lumen (*Cap*) and the urinary spaces (*US*) in high concentration; it is also seen to be piled against the basement membrane (*B*), but it is absent from the slits (arrows). $\times 22,500$.

polymer believed to exist in solution as a random coil (37, 38).

Dextran is considered to be a nearly ideal tracer for studies of glomerular permeability or clearance because it is uncharged, inert, and does not bind to

albumin (39) or other plasma proteins. Accordingly, it is equally well suited as a tracer for the identification of the filtration barrier in the capillary wall. Most commercial preparations have the disadvantage, already mentioned, of being polydis-

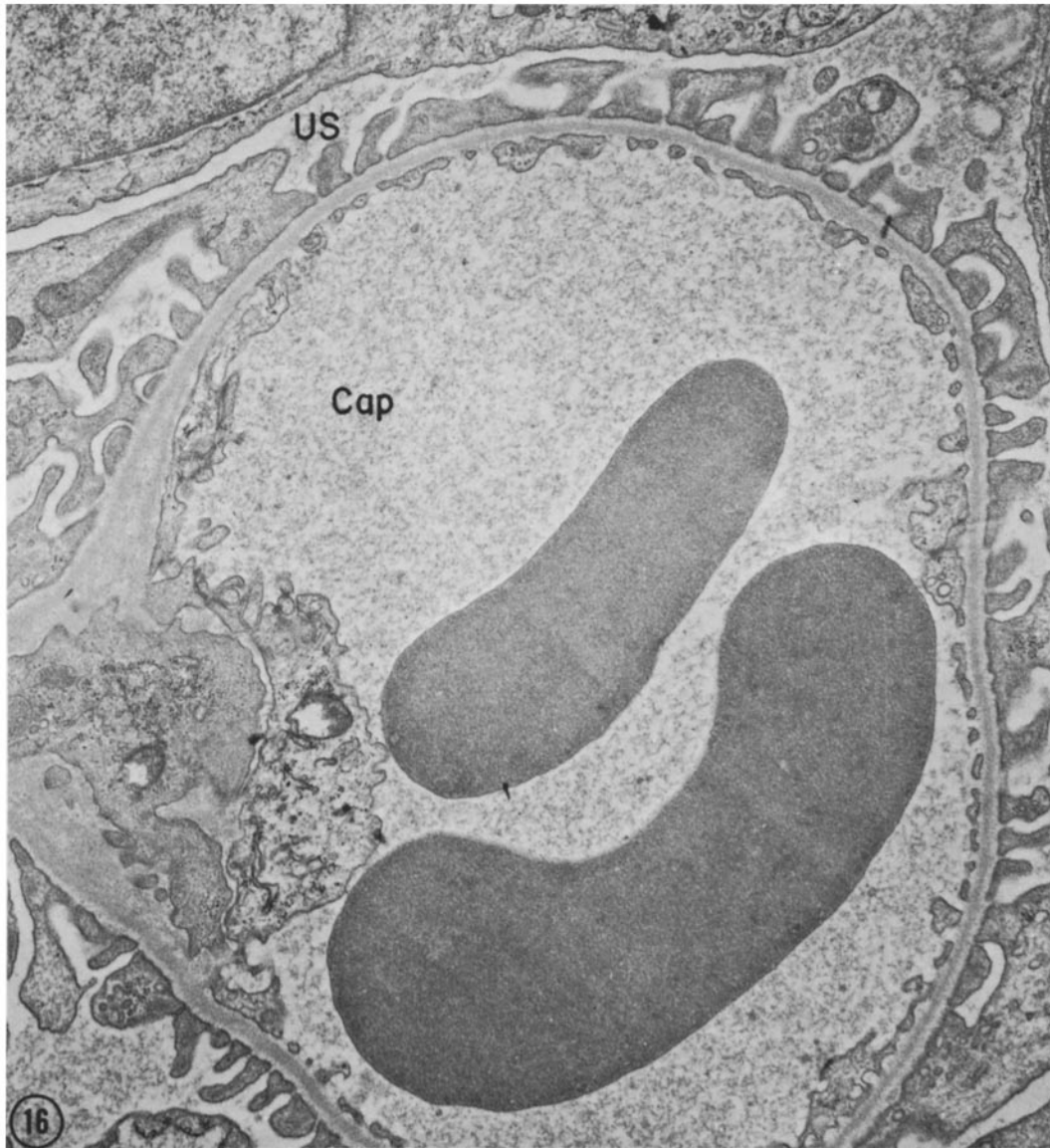


FIGURE 16 Glomerular capillary 2.5 h after the injection of 32,000-mol wt dextran. No tracer is seen in the section, i.e., in either the capillary lumen (*Cap*) or in the urinary spaces (*US*). Apparently, this small-sized dextran has been entirely lost from the circulation by filtration. $\times 13,500$.

perse, but we have considerably improved this situation by preparing fractions of narrow range in which dispersity is greatly restricted (cf. Fig. 1 and Table 1). Moreover, we have selected three fractions with molecular weights which bracket the size of albumin, the smallest protein known to be retained by the filtration barrier: (a) a relatively low (32,000) molecular weight fraction of a size

well below the filtration cutoff point which would therefore be expected to be extensively filtered (21-24); (b) an intermediate (62,000) molecular weight fraction which, like albumin, is just above the cutoff point in size and would be expected to be mostly retained by the filter; and (c) a very large (125,000) molecular weight fraction which is well above the filtration cutoff point and would be

expected to be filtered in only trace amounts. In all three cases the fractions behaved as predicted. The 32,000-mol wt fraction was gradually lost from the blood over a period of 1–2 h: initially, it was seen in high concentration in both the capillary lumina and urinary spaces, but the amount present in both locations gradually diminished with time as the dextran was cleared. The 62,000-mol wt fraction was filtered in only relatively small amounts: initially, some molecules were present in the open urinary spaces, but the amount seen in this location diminished gradually with time, whereas the luminal concentration remained high during the interval studied (up to 4 h). The 125,000-mol wt fraction was more effectively retained, since the luminal concentration remained high and relatively few molecules were seen in the urinary spaces at any time.

Therefore, the amounts of each fraction that passed through the glomerular capillary wall and appeared in the urinary spaces varied as expected (with $32,000 > 62,000 > 125,000$). The amount of 62,000 dextran seen in the urinary spaces at early time points was more than might be predicted from the physiologic data. The fact that it occurs primarily at early time points suggests that it may be due to initial clearance of smaller molecular weight components of the fraction which, although considerably purified over commercial preparations, is still polydisperse (see Table I). It should also be mentioned that the physiologic data is based on analysis of the urine and does not take into account tubular reabsorption of dextran which the results of James and Ashworth (40) as well as ours (cf. Figs. 17, 18) indicate actively takes place. Thus, like the albumin which normally leaks through the glomerulus, the dextran of corresponding size may be largely removed from the filtrate by reabsorption at the level of proximal tubule and glomerular epithelium.

The most significant finding is that in the case of all three fractions there was a sharp drop in concentration of the tracer between the luminal surface of the basement membrane and its outer, denser portions: numerous particles were seen in the inner, looser (subendothelial) portions of the basement membrane and few particles were seen beyond this level. Moreover, considerable accumulation or piling of the larger 62,000- and 125,000-mol wt fractions occurred along the luminal surface of the basement membrane in the mesangial regions. On the other hand, no accumulation or

piling was seen in the epithelial slits or against the slit membranes with any of the fractions. The results have demonstrated that the only barrier to passage of dextran particles of the sizes used ($ESR = 38\text{--}78\text{Å}$) is the basement membrane, suggesting that this layer constitutes the primary glomerular filtration barrier for plasma proteins.

Work with Other Electron-Opaque Tracers

A number of attempts have been made previously to identify the glomerular permeability barrier using electron-dense tracers (summarized in Table III). In general, two types of tracers have been used—electron-dense, particulate tracers which have the advantage that individual molecules or particles are directly visible, and mass tracers (usually proteins with peroxidatic activity) which cannot be visualized individually and whose detection and localization relies on a histochemical procedure with deposition of electron-dense reaction product. The latter have the advantage that small quantities of tracer can be more readily detected due to the amplification provided by their enzymic activity. However, they also have several potential disadvantages characteristic of histochemical reactions in general: first, diffusion of either the tracer (41) or the reaction product (42) may result in spurious localization. Secondly, quantitative differences in the distribution of tracer within the glomerulus cannot be reliably established. In addition, in the case under discussion, tracer molecules filtered in small amounts may be preferentially absorbed to the plasma membranes of the epithelial cells (see below).

Work with particulate tracers, including the dextran results reported here, has pointed to the basement membrane as the principal glomerular barrier since all these tracers [except for globin aggregates (43)] clearly accumulate with time against the basement membrane, suggesting that this is the layer that effectively restricts their glomerular passage. The results with mass tracers are less clear. They have been interpreted by Karnovsky and his associates (7, 11, 13) as indicating the existence of two successive filtration barriers—the basement membrane, acting as a coarse filter, and the epithelial slit membrane, acting as the critical barrier restricting free passage of molecules the size of plasma proteins. As far as results with individual tracers are concerned, those obtained with horseradish peroxidase (7) and cytochrome *c* (13) are not too infor-

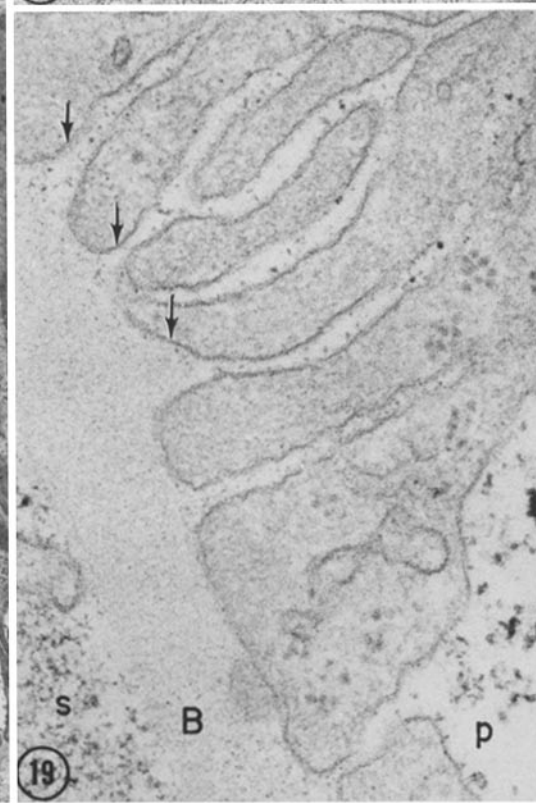
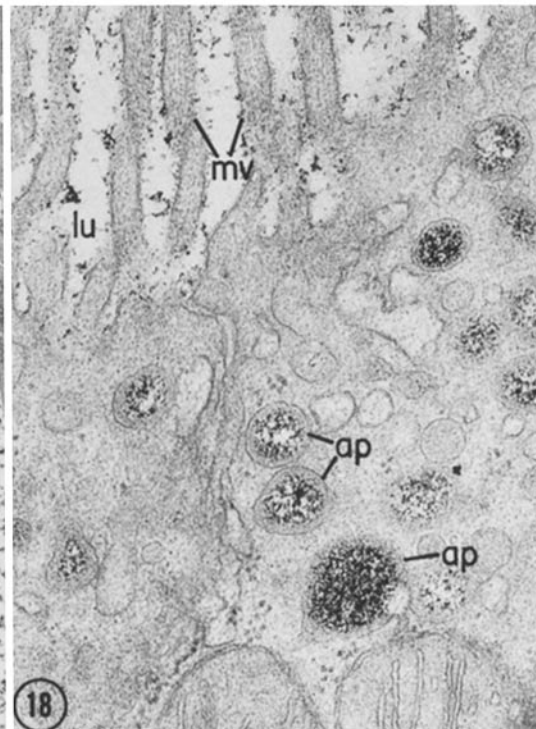
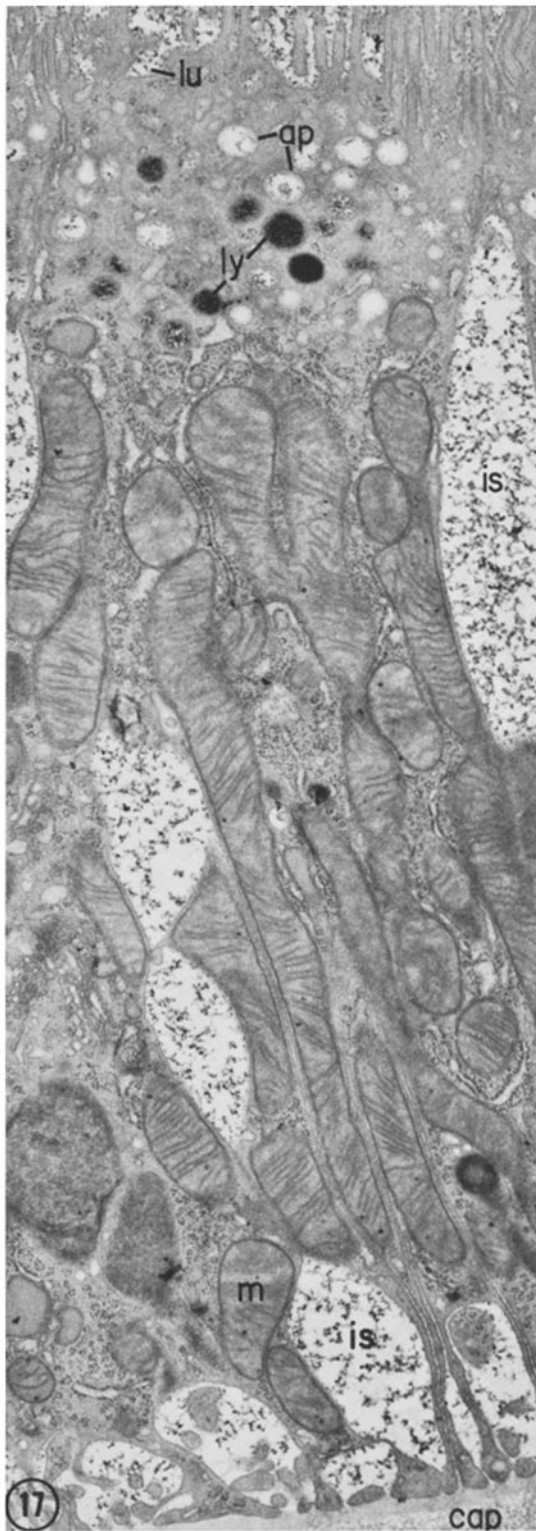


TABLE III
Ultrastructural Tracers Used to Locate the Glomerular Filtration Barrier

Tracer	Measured radius or ESR		Conclusion of author(s) regarding permeability barrier	Reference
	<i>mol wt</i>	<i>A</i>		
Particulate tracers				
1. Ferritin	480,000	61*	Basement membrane	3, 6
2. Saccharated Fe ₂ O ₃	Variable	~35*	Basement membrane	5, 15
3. Aggregated serum albumin	Variable	?	Basement membrane	8
4. Thorotrast	Variable	~100*	Basement membrane	2, 4
5. Colloidal gold	Variable	20-100*	Basement membrane	1, 3
6. Globin aggregates	Variable	250-1000*	Inconclusive	43
7. Dextran	125,000	78	Basement membrane	Present work
8. Dextran	62,000	55	Basement membrane	Present work
9. Dextran	32,000	38	Basement membrane	Present work
Mass tracers				
1. Catalase	240,000	52	Basement membrane and slits	11, 13
2. Myeloperoxidase	160,000	44	Basement membrane and slits	7, 13
3. Lactoperoxidase	82,000	~40	Basement membrane and slits	10
4. Hemoglobin	64,000	32	Inconclusive	9, 14
5. Horseradish peroxidase	40,000	30	Basement membrane and slits	7, 13
6. Tyrosinase subunits	34,000	12	Basement membrane and slits	12
7. Equine cytochrome <i>c</i>	12,000	15	No restriction	13

* Radius determined by direct measurement.
ESR = Einstein-Stokes radius.

FIGURES 17-19 Portions of proximal convoluted tubule cells from animals given dextran, showing that the dextran gains access to the epithelial cell from both its apical (Figs. 17 and 18) and basal (Figs. 17 and 19) surfaces. Fig. 17, taken 12 min after injection of 32,000-mol wt dextran, shows an entire cell segment from its apical to basal surface. The tracer, which is readily filtered by the glomerulus, is present in high concentration in the tubular lumen (*lu*); between the microvilli; and in apical vacuoles (*ap*) and phagosomes or lysosomes (*ly*) in the apical cytoplasm. Fields such as this illustrate the fact that, under the conditions tested, at least part of the filtered dextran is taken up or reabsorbed and concentrated into lysosomes (absorption droplets) by the tubular epithelium. At the base of the cell, dextran particles are seen in the peritubular capillary lumen (*Cap*) and in dilated intercellular spaces (*is*). Fig. 18 is a higher magnification view of the apical cell region from the same animal as that in Fig. 17, showing dextran between the microvilli (*mv*) in the tubular lumen (*lu*) and in apical vacuoles (phagosomes). Fig. 19 shows the base of a proximal tubule cell adjoining a peritubular capillary from an animal 1 h after administration of 62,000-mol wt dextran. Enormous accumulations of dextran are seen in the perivascular connective tissue spaces (*s*) of these capillaries, which are apparently highly permeable to dextran molecules of this size. Few particles are seen in the epithelial basement membrane (*B*). Most of the particles seem to be retained by the basement membrane and to accumulate against it. However, some particles have penetrated the basement membrane and appear in the basal infoldings (arrows) and in dilated outpocketings (*p*) of the basal infoldings. Fig. 17, $\times 18,000$; Fig. 18, $\times 44,000$; Fig. 19, $\times 74,000$.

mative since both are readily filtered and are found permeating in apparent equal concentration the basement membrane as well as the epithelial slits. Hence, they do not define any restrictive barrier. The assumptions made concerning the dual role of the basement membrane and slits were made primarily on the basis of results obtained with myeloperoxidase (7) and catalase (11), both of which appeared to accumulate in the slits. Karnovsky and Ainsworth (13) have pointed out, however, that the results obtained with human myeloperoxidase can be questioned because this protein is extremely basic ($pI > 10$), and its localization in the slits could be due to absorption of this positively charged molecule onto the highly negatively charged coat (cf. 17) of the epithelial cell. It seems that the basis for the entire "two-barriers-in-series" concept rests largely on results obtained with catalase which are therefore worth examining in some detail. The results obtained with catalase are apparently dependent on the amount injected, but, according to the authors, there is often a gradient of staining intensity across the basement membrane, with a greater concentration of the enzyme present in the inner (subendothelial) layers of the membrane than in the outer (subepithelial) layers. Further, they reported that the staining trailed off in intensity toward the urinary space with "no clear-cut demarcations in most slip-pores." Therefore, it seems to us that an alternative, equally plausible, interpretation of the results obtained with catalase is consistent with the major filtration barrier being at the level of the basement membrane. The significance of reaction product in the slits is open to question in the catalase work as well as in work with all other mass tracers since in all experiments involving tracers with peroxidatic activity—catalase included—the epithelial cell membrane is stained in the urinary spaces as well as beyond their level, the staining being more extensive with tracers of small dimensions [e.g., horseradish peroxidase (cf. 7, 11, 13)]. This could be explained by a preferential absorption of the filtered tracers to the membrane of the epithelial cells or, less likely (cf. 44), by diffusion and reabsorption of reaction product. From the general standpoint, another argument against the main (size-limiting) barrier existing at the level of the slits are the findings in the glomerulus in the nephrotic syndrome. In this situation, in which glomerular permeability is greatly increased, there is no

increase in the number or width of the slits. On the contrary, as shown many years ago in humans (45) and later confirmed in experimentally induced (aminonucleoside) nephrosis in rats (cf. 46), the number of slits is sharply reduced. In addition, our preliminary work using dextrans as tracers in aminonucleoside-nephrotic rats (47) shows no piling or accumulation of dextran in the remaining slits. These changes and findings are difficult to reconcile with the postulate of the fine filtration barrier existing at the level of the slits.

Pore Theory

Based on permeability studies Pappenheimer and his associates (48, 49) postulated some time ago that the wall of glomerular capillaries—that is, the filtration barrier of the wall—behaves like a membrane provided with circular pores with an effective radius of 45 Å. More recently, Renkin and Gilmore (18) have pointed out that the glomerular filtration data could be equally well explained by the presence of circular pores with a radius of 36 Å, slits with a half-width of 36 Å, or fiber networks with fiber half-interspacing of 26 Å. Based on our observations with dextran as well as the results with other particulate tracers, the main filtration barrier—or pore-containing layer—appears to be the basement membrane. This being the case, the fiber model appears to fit in best with what is known about the morphology (cf. reference 3) and biochemistry (50) of the glomerular basement membrane. However, it should be stressed that we have seen neither pores nor particles in the basement membrane. Rodewald and Karnovsky (51) have described slitlike pores (40×140 Å) within the epithelial slit membrane or slit diaphragm in glomeruli fixed in a mixture of tannic acid and aldehydes. They have postulated that these structures represent the filtration pores; however, this postulate is based on dimensional fit rather than on retention of tracer. As already discussed, there has been no unequivocal demonstration of retention of any tracer at the level of the slits.

It is noteworthy that experiments carried out on artificial membranes (cf. 52, 53) indicate that to function effectively (i.e., to avoid formation of unstirred layers) the part of the membrane bearing the size-limiting pores should be in contact with the solution to be filtered. In the case of the glomerulus this requirement is satisfied by the

basement membrane, but it is not by the slit diaphragms which are ~200 nm removed from the plasma.

Visualization of Dextran in the Basement Membrane

We have reported that although dextran is readily visualized in the capillary lumen, the urinary spaces, and within the inner, looser subendothelial portions of basement membranes, it is not often seen within the denser portions of the basement membrane (lamina densa) where it might be expected to occur in transit. It is clear that there is some difficulty in staining dextran particles located within basement membranes. Dextran itself has only a moderate electron density (comparable to that of the basement membrane), and one relies on the method introduced by Simionescu and Palade (19) for increasing the contrast of the particles. This procedure renders individual molecules readily visible in all locations except within basement membranes (tubular as well as glomerular). The similar (carbohydrate) nature of the tracer and basement membranes or the nature and spatial arrangements of the components of the basement membrane may render staining in this location difficult. The same argument does not apply, however, to the urinary slits where likewise few particles of dextran are seen. This is an environment which, according to morphologic observations, is essentially aqueous, and the density of the content is considerably lower than that of the basement membrane. Therefore one would expect to see particles in this location, especially if they were accumulating against the slits. In fact, particles can be visualized in a structurally analogous situation in the slits between the basal infoldings of the proximal tubule epithelium (cf. Fig. 19).

Role of the Mesangium

It was previously shown (4) that the glomerular mesangium actively takes up (by endocytosis) various electron-dense tracers (ferritin, colloidal gold, and thorotrast) that accumulate against the basement membrane. The incorporated particles are segregated into phagosomes which become lysosomes (34). Based on these findings, it was suggested that the mesangium continually unclogs and reconditions the basement membrane by removing filtration residues, including plasma pro-

teins. This postulate has been supported by the findings of others, especially those of Michael and co-workers (54, 55), indicating that aggregated immunoglobulins are actively taken up by mesangial cells. The new findings with dextran reinforce this concept of the sweeping or removal function of the mesangium; they further indicate that macromolecules are rapidly and efficiently removed since no piling or accumulation of dextran particles is seen except in the mesangial regions. The mechanism of the sweeping action—whether by blood currents or endothelial activity—is still (cf. 4) unknown. It should be added that such a mechanism for reconditioning and unclogging the filter would be difficult to envisage at the level of the slits because of their complicated geometry.

Role of the Glomerular Epithelium

The present results have also reinforced previous observations indicating that the glomerular epithelium serves as a monitor to recover from the filtrate macromolecules which pass through the glomerular basement membrane. As in the case of ferritin, dextran particles are actively endocytosed by the glomerular epithelium. The latter appears to represent part of a recovery system which extends down the nephron to the proximal tubule epithelium. As in the case of ferritin (3), it is difficult to quantitate the amount of dextran which is recovered at the glomerular level vs. the amount recovered at the tubular level.

As far as the slits are concerned, it remains to be seen what precisely is their function. As has been pointed out previously (3), they have an elaborate structure which has certain features (e.g., increased density of apposed cell membranes, dense content in the intercellular spaces which is bisected by a fine intermediate line) reminiscent of shallow desmosomes. It may be that the role of the slit diaphragms is simply to provide overall stability to the filtration apparatus. In this connection it is of interest that these desmosome-like features of the slit membrane are particularly well visualized in preparations fixed in a mixture of tannic acid and aldehydes (51), since Futaesaku et al. (56) have previously demonstrated that tannic acid effectively stains the intercellular plaques of desmosomes.

In conclusion, based on results using ferritin as a tracer, Farquhar et al. (3) proposed a dynamic model of glomerular function and defined a role

for each of the components of the glomerular capillary wall in the filtration process: the basement membrane as the main filter, the endothelium as a valve which controls access to the filter, the epithelium as a monitor to reabsorb protein that leaks through the filter, and the mesangial cell as the mechanism for cleaning the filter by disposing of filtration residues. This model has been checked and put to test with graded dextrans, which represent more appropriate probe molecules than those used previously, and the model appears still to be valid.

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