

CODISTRIBUTION OF COLLAGEN TYPES IV AND AB₂ IN BASEMENT MEMBRANES AND MESANGIUM OF THE KIDNEY

An Immunoferritin Study of Ultrathin Frozen Sections

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ABSTRACT

Affinity-purified rabbit antibodies specific for collagen types I, III, AB₂ and for a partially characterized type IV collagen derived from a murine tumor were used to study the distribution of collagens in the normal mouse kidney. Immunofluorescence staining of conventional frozen sections demonstrated that types I and III were present in bundles around large vessels and in fibers surrounding glomeruli and tubules, whereas types IV and AB₂ were distributed in a linear fashion along basement membranes of tubules, glomeruli, and Bowman's capsule and in the mesangial stalk. The distribution of types IV and AB₂ was examined at the ultrastructural level by staining of 600- to 800-Å thick frozen sections with a three-stage procedure employing specific collagen antibodies, biotinyl sheep anti-rabbit IgG, and avidin-ferritin conjugates. Labeling by this procedure demonstrated codistribution of types AB₂ and the putative type IV in all three basement membranes. In addition, mesangial matrix was shown to contain both of these collagen types. These results support recent biochemical evidence of collagen heterogeneity in basement membranes, and also support the concept of a structural relationship between mesangial matrix and glomerular basement membranes.

The complete molecular composition of basement membranes is still unknown as is the arrangement of the constituent molecules (25). Both questions are of considerable interest because of the role of the basement membrane in such processes as filtration of urine and cell adhesion (13, 32) and because of the changes that are observed in various diseases (16).

The collagenous components of basement membranes are distinct from the classical interstitial collagens in that they have a different amino acid composition, are more highly glycosylated, and do not form striated fibrils *in vivo* (23). However,

because of the limited amounts of native, intact molecules which can be isolated and solubilized from tissues, basement membrane collagens are not yet well characterized.

Recently two basement membrane collagens have been extracted in relatively large quantities from tissues, providing a better opportunity for study of this group of collagens. Orkin et al. have described a murine tumor, the EHS sarcoma, which produces an abundant homogeneous matrix (33). They have extracted from this tumor a collagenous protein with the characteristic features of a basement membrane collagen (43). This protein

is still only partially characterized because gel electrophoresis shows several faint high molecular weight bands in addition to the α chain-sized bands (see Specificity of the Antibody Reagents). Immunofluorescence studies showed that antibodies raised against this material will stain basement membranes of kidney, lung, and skin of both human and mouse. The murine collagen isolated from this tumor has been called type IV because of its similarity to the type IV basement membrane collagen extracted from glomeruli, the lens capsule, and placenta (33).

Burgeson et al. reported the isolation of a collagen (AB₂) from placental amnionic and chorionic tissue and suggested that this may represent a second type of basement membrane collagen (6). Chung et al. have also reported the isolation of similar collagen components called A and B chains from placenta and other tissues rich in basement membranes (7). However, in both cases, the starting materials were not homogeneous basement membranes but were complex tissues.

Purified porcine glomerular basement membranes (GBMs) were found by Alexander et al. to contain small quantities of A and B chains (1). These studies suggest that AB₂ as well as type IV may be present in some basement membranes. However, questions about the composition of basement membranes are difficult to answer by chemical extraction methods alone because there may be loss or degradation of collagen during isolation and extraction, contamination of basement membranes by plasma membrane fragments or connective tissue matrix, and insolubility of many forms of collagen. Immunofluorescence studies of tissues using purified antibodies to known collagens avoid these problems, but such studies are hampered by the low resolving power of light microscopy. We have found it difficult to distinguish immunofluorescent staining of basement membranes from staining of adjacent plasma membranes or mesangial areas (29, 30). However, immunoelectron microscopy has been successfully used to localize antigen in ultrathin sections of tissue (48). This method largely avoids alteration or masking of antigenic determinants because of fixation, dehydration, or plastic embedding of conventional electron microscopy yet preserves the structural relationships of the tissue. It also provides resolution far exceeding that of fluorescence microscopy (27).

We have applied immunoelectron microscopy of ultrathin frozen sections to the problem of the

precise localization of collagen types IV and AB₂ in basement membranes of the mouse kidney and we have demonstrated codistribution of these collagens in both basement membranes and mesangium.

MATERIALS AND METHODS

All chemicals used were of analytical grade. Bovine albumin (BSA) and six times recrystallized horse spleen ferritin (Pentex) were obtained from Miles Laboratories, Inc. (Elkhart, Ind.). Glutaraldehyde was obtained from Fisher Scientific Co. (Pittsburgh, Pa.). Avidin (type III) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Amino Acid Analysis

Dry collagen samples were hydrolyzed in 6 N HCl containing 2% phenol for 24 h at 110°C in a glass chamber equipped with a Viton O-ring (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) under a nitrogen atmosphere. Analyses were done on a Durrum D500 analyzer (Durrum Instrument Corp., Sunnyvale, Calif.).

Preparation of Antibodies

The preparation of affinity-purified antibodies to rat collagen types I and III, human AB₂ collagen, and murine type IV has been described previously (30, 38).

In brief, acid-soluble type I and neutral salt-soluble type III collagens were obtained from rat skin. Human AB₂ collagen was isolated from pepsin digests of placental membranes. Murine type IV collagen was extracted with acetic acid from the EHS sarcoma maintained in Swiss Webster mice. Laminin was a kind gift of Dr. Jean-Michel Foidart (Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.). Laminin was also prepared in this laboratory by the methods of Timpl et al. (47). The isolated types I and III collagen were judged to be pure by polyacrylamide gel electrophoresis in the presence of SDS of the alpha chains and of their cyanogen bromide peptides (data not shown) (38). The basement membrane collagens used as immunogens and immunoabsorbents were also examined by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1a and b). Amino acid analysis of all these collagen preparations were in agreement with published values (6, 38, 43, 46).

Rabbit antisera to these four collagen types were raised by intradermal injection of 1–5 mg of collagen mixed with completed Freund's adjuvant. 4 wk after the first injection, three booster injections were given at 2-wk intervals. 2 wk after the last injection, the animals were exsanguinated and the sera were harvested and stored at –20°C. Each serum raised against a given type of collagen was purified by cross-absorption on affinity columns composed of all the other collagen types. Antibodies specific for the collagen type to which they were raised were absorbed on and eluted from an affinity column composed of the specific collagen type. The serological activity of the purified antibodies after concentration on Amicon filters (Amicon PM 30 Filter, Amicon Corp., Scientific Sys. Div., Lexington, Mass.) was determined by passive hemagglutination or radioimmunoassay (RIA) as previously described (4, 30, 38).

Specificity of the Antibody Reagents

In general all antibodies had a titer of 1:25,000 to 1:50,000 by

hemagglutination. The antibodies were specific for each type and no cross-reactivity could be observed with any other collagens in inhibition tests (30, 38). Furthermore, antibodies to types IV and V collagen showed no cross-reactivity with laminin or with plasma fibronectin by hemagglutination inhibition (Table I). Although the exact α chain composition of type V (AB) collagen has not been completely resolved, we have evidence for the existence of a molecule having the composition of AB₂ to which our antibodies are directed.

(a) In our hands, chemical extraction of human amnion and chorion always yields AB collagen with a α A to α B chain ratio of 1:2 (Fig. 1a, lane 2), as do extractions of human peripheral lung and mouse skin (5, 21, 29, 30, 42). Overloading the gel shows that there are no high or low molecular weight contaminants (Fig. 1a, lane 3), and both bands are susceptible to degradation by purified bacterial collagenase (Fig. 1a, lane 4) (36).

(b) Isolated α A and α B chains do not inhibit AB₂ antibodies when used as inhibitors in hemagglutination assays. AB₂ antisera or AB₂ antibodies do not exhibit any titers against α A and α B chains coupled to erythrocytes. Also, α B chains coupled to Sepharose 4B beads do not bind any antibodies in immunoadsorption experiments (reference 29 and unpublished observations).

(c) When collagens are extracted from biosynthetically labeled endothelial cell cultures, type V collagen can be specifically precipitated with our antibodies. The immunoprecipitate has an α A to α B chain ratio of 1:2 (reference 14, and Madri et al., manuscript in preparation).

These data support the concept of a collagen type V having a chain composition AB₂, though they do not exclude the possible existence in addition of α A₃ and/or α B₃ (37).

The type IV collagen we have prepared and used as an immunogen appears identical to that prepared by Timpl et al., and used by several groups studying basement membrane collagen (32, 33, 43). Gel electrophoresis shows a doublet migrating in the 140,000–160,000 mol wt region and several faint high molecular weight forms (Fig. 1b, lane 2). We cannot yet be certain that the higher molecular weight bands are not contaminating noncollagenous proteins rather than cross-linked forms or precursors of the doublet, but we would argue from the following evidence that they are not contaminants:

(a) The purification procedure includes DEAE and carboxymethyl cellulose chromatography specifically to remove any associated proteoglycans and other collagens.

(b) The amino acid analysis of this type IV collagen shows good agreement with the analysis of another basement membrane collagen, bovine lens capsule (Table II) (24). If there were a major noncollagenous contaminant in the higher molecular weight forms, one would expect a noticeable shift in hydroxyproline, glycine, and hydroxylysine residues per 1,000 residues.

(c) Examination of this collagen under the electron microscope, by use of a rotary shadowing technique described by Shotton et al. (41), reveals uniform but aggregated forms of slender rodlike structures. These aggregates decrease in number but do not totally disappear upon reduction and alkylation, suggesting the possible presence of nondisulfide cross-links which would appear as higher molecular weight forms on gels (unpublished observations).

For convenience we chose to study mouse kidney. To confirm that these antibodies do cross-react with mouse collagens, the following studies were done.

Isolation of Collagens from Mouse Skin

Acetic acid-soluble types I and AB₂ and neutral salt-soluble type III collagen were isolated from skins of mice made lathyritic

by addition of 1.0 g/liter β -aminopropionitrile fumarate (Sigma Chemical Co.) to their drinking water for 8 wk. The skins were homogenized in a meat grinder and Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), and, after several washes with distilled water at 4°C, the residue was extracted with 1.0 M NaCl, 50 mM Tris-HCl, pH 7.4, and subsequently with 0.5 M acetic acid at 4°C for 24 h. The collagens were isolated from these extracts by fractional salt precipitation as described by Burgeson et al. (6), except for type AB₂ which was precipitated in the final step by dialysis against water. As shown in Fig. 1, the cross-reactivity of mouse type III (Fig. 1c) and mouse type I (Fig. 1d) is complete, or nearly complete, with the corresponding collagen type from rat skin by radioimmunoassay and passive hemagglutination-inhibition, respectively. Antibodies to human AB₂ collagen likewise cannot distinguish between human and mouse AB₂ collagen in hemagglutination-inhibition tests (Fig. 1e). Cross-reactivity of these collagens was also shown by immunofluorescence studies on human and mouse skin, which gave identical staining patterns. The staining was specific and could be abolished by addition of either mouse or human AB₂ collagen (data not shown).

Sheep anti-rabbit IgG (SAR IgG) serum was prepared as previously described (9). The antibodies were purified by immunoabsorption on rabbit IgG (Miles Laboratories, Inc.) coupled to cyanogen bromide-activated Sepharose 4B (35). After dialysis against phosphate-buffered saline (PBS), the eluted antibody solution was concentrated by ultrafiltration to 4 mgm/ml (Amicon PM 30 filter) and stored frozen at -20°C.

Tissue Preparation

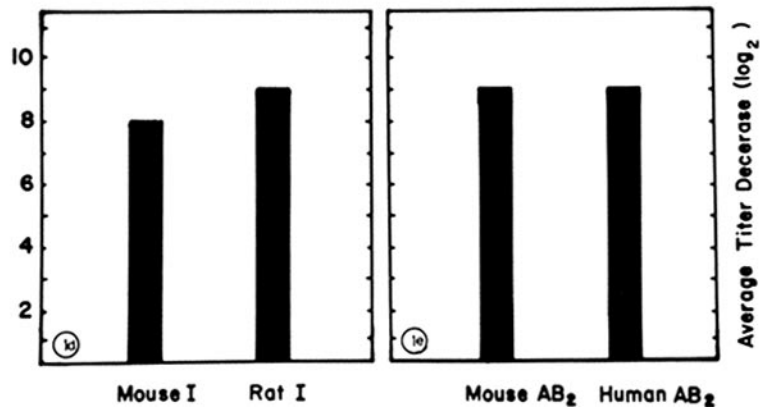
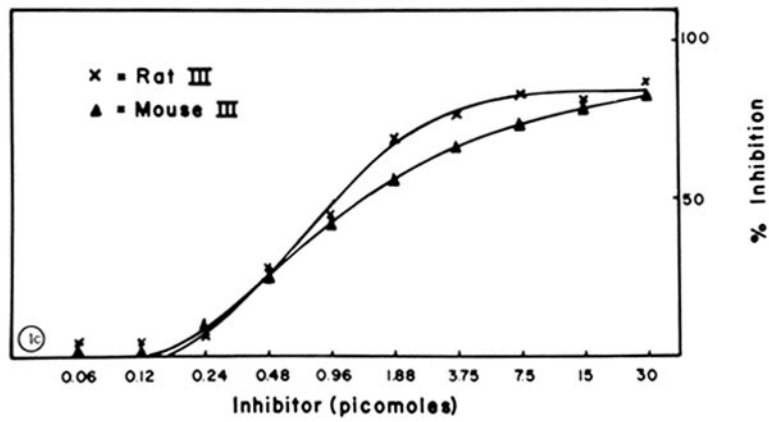
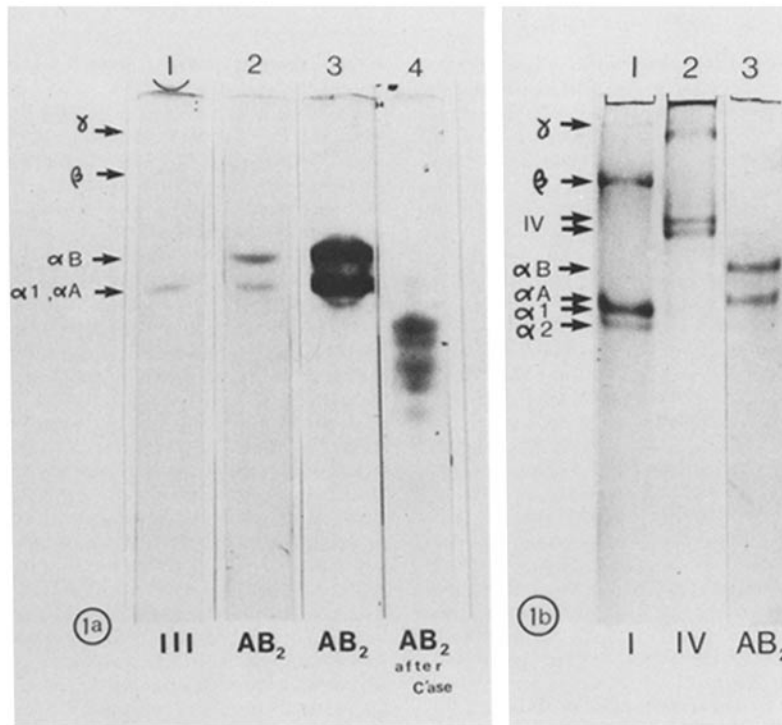
DBA and C57/B6 mice were killed by cervical dislocation. One kidney was removed, sliced longitudinally, embedded in OCT compound (LAB-TEK Products, Naperville, Ill.) and frozen immediately in liquid nitrogen. Sections 6 μ m thick were cut in a cryostat and processed for immunofluorescence. To prepare specimens for ultrathin frozen sections, the cortex was dissected from the contralateral kidney and cut into <1 mm³ pieces with a razor blade. This was done with the tissue bathed in a solution of 1% glutaraldehyde in PBS, pH 7.4. After 10 min in fixative, the small pieces of cortex were washed twice with 15 ml of PBS for 5 min, incubated in 15 ml of 0.1 M glycine, 50 mM Tris, pH 7.4 for 10 min, washed again with 15 ml of PBS for 5 min, and suspended in 2 M sucrose in PBS, pH 7.4. The tissue was stored in sucrose for at least 1 h, but often for several weeks, at 4°C before freezing for sectioning.

Immunofluorescence

Fluorescein-conjugated SAR IgG was prepared by standard techniques (17, 52). The F/P ratio was 4:1, and the labeled antibody solution was stored at -70°C at a concentration of ~0.10 mg/ml.

Tissue sections on glass slides were fixed in acetone, air dried, hydrated briefly in PBS, and overlaid with 75 μ l of a 1:50 dilution (3 μ g/100 μ l) of purified rabbit antibody to collagen. After a 1-h incubation, the slides were washed three times with PBS and overlaid with 75 μ l of a 1:40 dilution (30 μ g/100 μ l) of fluorescein isothiocyanate-conjugated SAR IgG and incubated for 40 min. The slides were washed three times with PBS and mounted under coverslips in a polyvinyl alcohol-glycerol medium (Gelvatol, Monsanto, Indian Orchard, Mass.). In controls, the antibody solution was incubated with a solution of the specific collagen before staining.

Sections were viewed with a Zeiss standard binocular 14



fluorescence microscope equipped with a mercury lamp and with a vertical illuminator using 450- to 490-nm excitation and 520 nm barrier filters. Photographs were taken with high speed Ektachrome (ASA 400) film and 4-min exposure times.

Electron Microscopy

REAGENTS: Isolated collagen antibodies were prepared as described above and diluted 1:100 to 1:200 with PBS, pH 7.4, containing 0.1% BSA.

Biotinized SAR IgG was prepared by a modification of the method of Becker and Wilchek (3). Affinity-purified SAR IgG (7 mg) was dissolved in 2 ml PBS and dialyzed into 0.1 M NaHCO₃, pH 8.0. Biotinyl-*N*-hydroxysuccinimide (9 mg) was dissolved in 1 ml *N*-*N*-dimethylformamide (Fisher Scientific Co., Pittsburgh, Pa.) and added immediately to the IgG (20). The mixture was stirred for 1 h at room temperature and dialyzed against 0.1 M NaHCO₃ at 4°C for 8 h, then against PBS with 0.02% NaN₃ at 4°C for 24 h. The biotinyl SAR IgG was passed through a Millipore filter (0.22- μ m pore size) (Millipore Corp., Bedford, Mass.) and stored at 4°C until use.

Avidin-ferritin conjugate was prepared by the glutaraldehyde conjugation method of Heitzmann and Richards (20). Biotinyl-*N*-hydroxysuccinimide and avidin-ferritin conjugate were the kind gifts of Barbara Schneider and Dr. David Papermaster (Yale University, New Haven, Conn.). Avidin-ferritin conjugate was passed through a Millipore filter (0.22- μ m pore size) and stored in 150 mM NaCl with 1.0% BSA and 0.2% NaN₃ at 4°C. The ferritin concentration of this stock preparation was 2 mg/ml. On the day of use it was diluted as described below and centrifuged at 18,000 *g* for 30 min. The supernate was used for labeling.

A 0.5% solution of uranyl magnesium acetate in 150 mM NaCl was made up by combining equal volumes of a 1% aqueous solution of uranyl magnesium acetate (ICN K & K Laboratories Inc., Plainview, N. Y.) and 300 mM NaCl. The stain was stored at 4°C and filtered through a Millipore filter (0.22- μ m pore size) before use.

ULTRATHIN FROZEN SECTIONS: The method of preparing sections of kidney for immunoferritin labeling was based on the

work of Painter et al. (34) and Tokuyasu and Singer (48).

Cubes of renal cortex, infiltrated with sucrose as described above, were placed in a small drop of sucrose on the end of a copper rod. Precooled forceps were used to pick up the rod and plunge it into just liquid (-150°C) Freon (Genetron-22, Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) for 10 s. The rod was then immediately immersed in liquid nitrogen (-196°C). The copper rod was mounted on a Sorvall MT2B microtome equipped with a Sorvall-Christensen FTS/LCT-2 frozen thin sectioner with low temperature control (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). Sections were cut at a temperature of -70 to -90°C with a dry glass knife, set at 10° clearance angle. Cutting was done manually and as rapidly as possible with the speed selector at maximum. Sections (~600-800 Å thick) were picked up from the knife with a 2-M sucrose droplet suspended from a platinum wire loop. When the sucrose melted as the loop was removed from the chamber the sections remained on the surface of the sucrose and could be retrieved by touching the droplet to a Formvar-and-carbon coated grid. Carbon coating was found to be helpful in reducing background ferritin labeling. Such sections could be kept overnight in a moist chamber at 4°C for labeling the next day.

Glomeruli were sparse in sections taken at random from renal cortex. To enrich for glomeruli, blocks of tissue containing several glomeruli were selected under a dissecting microscope with bright illumination and processed as described above.

LABELING AND STAINING OF ULTRATHIN FROZEN SECTIONS: The labeling procedure was carried out as described by Tokuyasu and Singer (48). Specifically, 50- μ l droplets of collagen antibodies (diluted 1:100 or 1:200 with PBS containing 0.1% BSA (3 or 1.5 μ g/ml) and 100- to 150- μ l droplets of buffer were placed on a parafilm strip. Grids containing sections, overlaid with a 2-M sucrose droplet, were picked up with fine forceps, inverted, and floated on the surface of the desired reagent. The sequence of labeling was as follows: Grids were washed for 1 min apiece in three successive drops of 100 mM Tris buffer, pH 7.4, to remove sucrose and then incubated for 15 min with a drop of 4% BSA to reduce nonspecific background staining. This was followed by three washes in PBS and incubation for 15 min on

FIGURE 1 (a) 5% polyacrylamide gels of type V (AB₂) collagen electrophoresed under reducing conditions and stained with Coomassie Blue. Lane 1 is a sample of type III collagen used as standard. Lane 2 is a sample of type V (AB₂) collagen noted by optical scanning to have an α A to α B chain ratio of 1:2. Lane 3 is a grossly overloaded sample of type V (AB₂) collagen demonstrating no apparent high or low molecular weight contaminants. Lane 4 is a sample of type V (AB₂) collagen after treatment with purified bacterial collagenase demonstrating collagenase sensitivity of both α A and α B chains. (b) 5% polyacrylamide gel of type IV collagen electrophoresed under reducing conditions and stained with Coomassie Blue. Lane 1 is a sample of type I collagen used as a standard. Lane 2 is a sample of type IV collagen used as immunogen and immunoabsorbent. Lane 3 is a sample of type V (AB₂) used as a standard. (c) RIA inhibition study of the cross-reactivity of rat and mouse neutral salt-soluble type III collagens. Affinity-purified rabbit anti-rat type III antibody was used at a 1:15 dilution. (d) Hemagglutination inhibition study of the cross-reactivity of mouse and rat type I collagens. Affinity-purified rabbit antibodies against rat type I collagen were serially diluted to a concentration of 1:25,000. Concentration of the mouse and rat type I acid-soluble collagens used as inhibitors was 2.5 μ g/well in all wells. Bars indicate the extent of titer decrease caused by each inhibitor. (e) Hemagglutination inhibition study of the cross-reactivity of mouse and human type AB₂ collagens. Affinity-purified rabbit antibodies against human type AB₂ collagen were serially diluted to a concentration of 1:25,000. Concentration of the mouse and human type AB₂ collagens used as inhibitors was 2.5 μ g/well in all wells. Bars indicate the extent of titer decrease caused by each inhibitor.

TABLE I
*Hemagglutination Inhibition**

Antibody	Inhibitor	Titer (-log ₂)
IV	—	8
IV	IV	1
IV	Laminin	8
IV	Plasma fibronectin	8
V (AB ₂)	—	9
V (AB ₂)	V (AB ₂)	1
V (AB ₂)	Laminin	9
V (AB ₂)	Plasma fibronectin	9

Antibodies were serially diluted from 1:100 to a final dilution of 1:12,800 (IV) or 1:25,000 (V). Inhibitor concentrations were 2.5 µg/well, in all wells. Addition of specific collagen antigen shows complete inhibition, while matrix proteins do not show any.

* Cross-reactivity studies of basement membrane collagen antibodies with noncollagenous matrix proteins.

the primary antibody solution. Grids were then washed 12 times in PBS, incubated for 15 min on biotinyl SAR IgG, and washed 12 times in PBS. They were incubated on the avidin-ferritin conjugate for 15 min and washed again 12 times in PBS. Grids were then postfixed for 8 min in 1% glutaraldehyde in PBS, washed three times in distilled water, stained with uranyl acetate for 2 min, and finally washed three times with distilled water. As a specificity control, the diluted antibody was incubated for 20 min with a freshly prepared solution of the specific collagen in 150 mM NaCl-50 mM Tris buffer, pH 7.4. The final concentration of inhibitor was 50 µg/ml. It was found to be important to ensure that the inhibiting collagen was completely solubilized. The biotinyl SAR IgG and avidin-ferritin conjugate were diluted 1:50 and 1:10, respectively, with PBS containing 0.1% BSA. All reagents were centrifuged at 20,000 g for 40 min to remove aggregates. Each experiment comprised four to six grids stained with each collagen antibody and two to four control grids labeled with inhibited antibody, with biotinyl SAR IgG and avidin-ferritin conjugate, and with avidin-ferritin conjugate only.

Grids were dried and examined with a Philips 300 electron microscope at 80 kV. After optimal conditions were found, the experiments were repeated five times to gather the data reported here.

RESULTS

Distribution of Collagens by Immunofluorescence

Type I collagen was present in the renal capsule, in the media and adventitia of blood vessels, in collecting ducts, and in reticular fibers throughout the interstitium. Thick bundles of type I around a blood vessel and a glomerulus are illustrated in Fig. 2a. Tubules and glomeruli were negative for type I, except for rare reticular fibers in the mesangial stalk.

The distribution of type III collagen was similar to that of type I except that it was more prominent in the fine reticular fibers in the renal interstitium and surrounding Bowman's capsule (see Fig. 2b).

In contrast to antibodies to the two interstitial collagens, type IV collagen antibodies intensely stained the circumference of tubules, the glomerular capillaries, and Bowman's capsule in linear patterns. All of these structures contain morphologically identifiable basement membranes (Fig. 2c). In addition, antibodies to type IV appeared to stain the mesangium and basement membranes of large vessels.

Antibodies to type AB₂ collagen stain the kidney in a pattern similar though not identical to that of type IV. The major differences are a less intense overall staining and a more diffuse staining of capillary loops (Fig. 2d).

There are differences in intensity of fluorescent staining of the various basement membranes with antibodies to type IV. They can be ranked in order of decreasing intensity of fluorescence: mesangium > Bowman's capsule > tubular basement membrane (TBM) > GBM. Staining intensity for type AB₂ shows the same order.

Control slides, incubated with normal rabbit serum or with antibodies which were preincubated

TABLE II
Amino Acid Analysis per 1,000 Residues

	IV*	IV‡	IV§
Hyp	110	104	100
Asp	53	52	55
The	34	34	29
Ser	56	60	42
Glu	102	103	93
Pro	65	66	68
Gly	281	280	275
Ala	44	45	43
Val	29	32	30
Met	13	12	8
Ile	25	25	29
Leu	52	54	58
Tyr	9	9	10
Phe	29	31	32
His	11	11	10
Hyl	37	38	35
Lys	11	13	13
Arg	32	30	43

* Data from reference 46.

‡ Our data of acid extracted type IV collagen from the EHS sarcoma.

§ Data from reference 24.

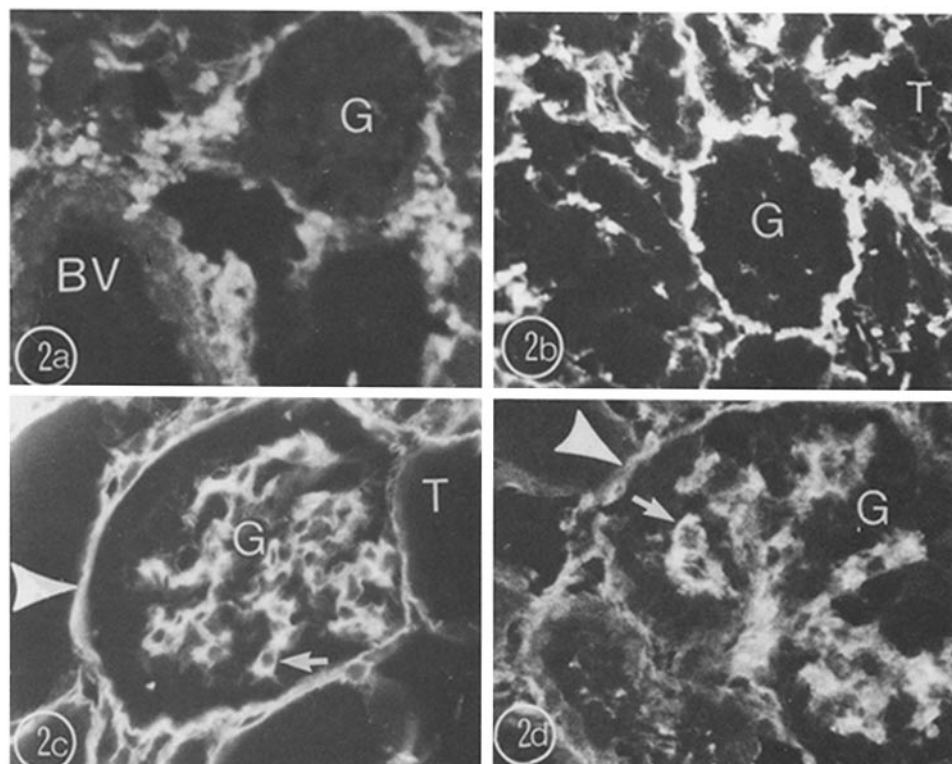


FIGURE 2 Indirect immunofluorescence patterns of mouse renal cortex stained with: (a) Anti-type I collagen, $\times 200$ original magnification. (b) Anti-type III collagen antibody, $\times 200$ original magnification. (c) Anti-type IV collagen antibody, $\times 400$ original magnification. (d) Anti-type AB₂ collagen antibody, $\times 400$ original magnification. T, tubule; G, glomerulus; BV, blood vessel; arrow, capillary loop; arrowhead, Bowman's capsule.

with the specific collagen, showed no fluorescence (data not shown).

In summary, the interstitial collagens, types I and III, were predominantly seen in bundles or reticular fibers in renal capsule, media, and adventitia of blood vessels, collecting ducts, and between adjacent tubules and glomeruli. In contrast, types IV and AB₂ were seen primarily as uninterrupted linear patterns around tubules, in Bowman's capsule, in glomerular capillary loops, and in the subendothelium of blood vessels. These results suggest the possibility that the two latter collagens are components of basement membranes. However, with immunofluorescence it is impossible to determine whether these patterns are because of staining of basement membranes, of plasma membranes, or of extracellular matrix such as mesangial matrix. Therefore we studied this localization of types IV and AB₂ collagen at an ultrastructural level.

Distribution of Collagens by Immunoelectron Microscopy

A portion of two adjacent tubular cells and of a glomerulus, respectively, are visualized on ultra-thin frozen sections stained with uranyl acetates (Fig. 3 and 4). At low magnification the ultrastructure of the kidney in these lightly fixed frozen sections is not markedly different from the ultrastructure of conventionally fixed, dehydrated, and Epon-embedded tissue. Structures such as glomerular epithelial cell foot processes, basement membranes, mesangial cells and matrix, mitochondria, and nuclei are easily identified. At higher magnification, cellular membranes seem to be less electron dense when compared to similar membranes in conventional Epon-embedded sections of approximately equal thickness. However, we were able to distinguish plasma membranes from basement membranes and to identify mesangial matrix



FIGURE 3 Ultrathin frozen section of two adjacent mouse kidney tubules fixed in 1% glutaraldehyde and stained with 0.5% uranyl acetate as described in Materials and Methods. *MV*, Tubular microvilli; *n*, nucleus; *m*, mitochondria. Arrows indicate tubular basement membrane. $\times 7,800$.

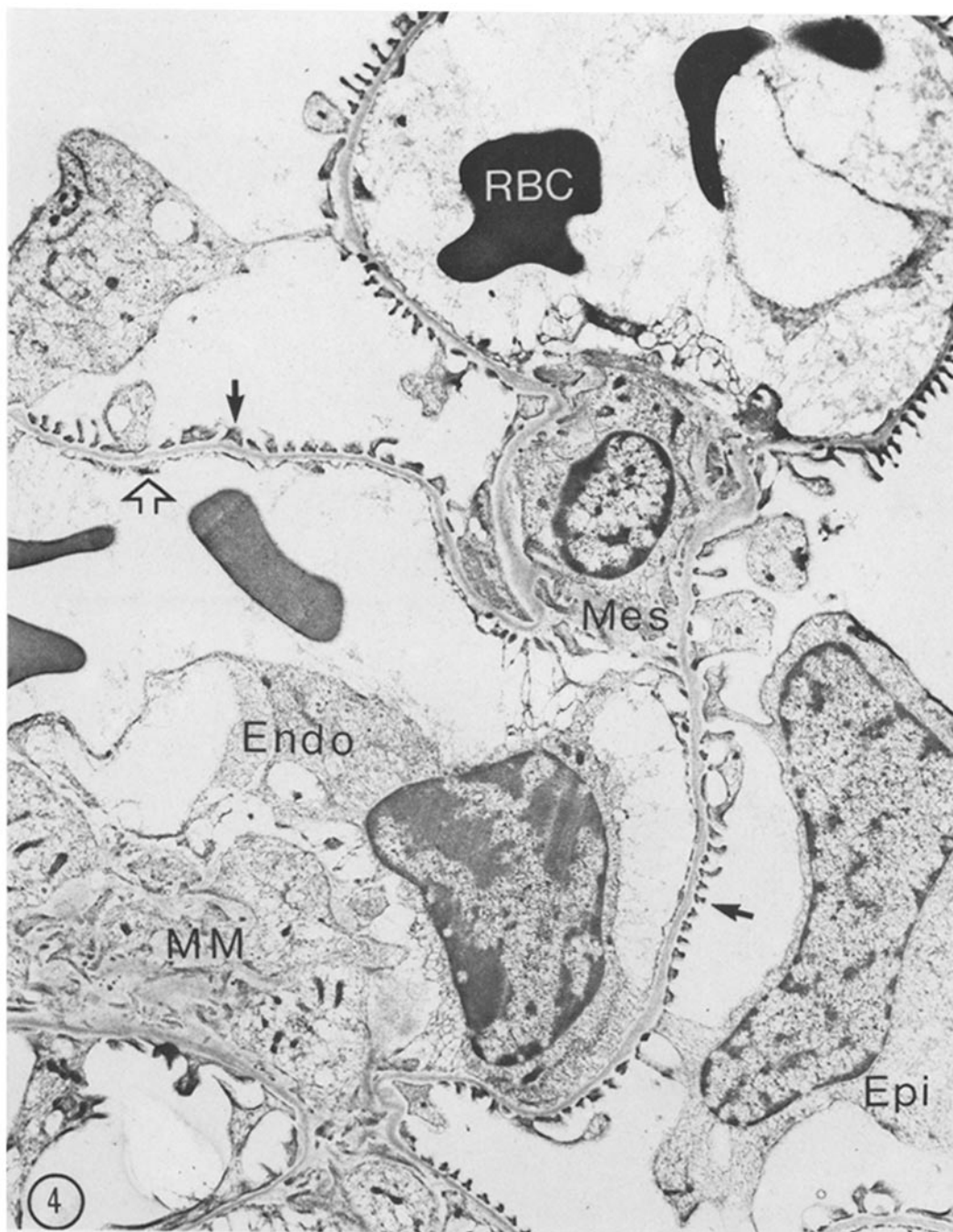


FIGURE 4 Ultrathin frozen section of a mouse glomerulus fixed in 1% glutaraldehyde and stained with 0.5% uranyl acetate as described in Materials and Methods. *RBC*, red blood cell; *Mes*, mesangial cell; *Endo*, endothelial cell; *Epi*, epithelial cell; *MM*, mesangial matrix; open arrow, endothelial cells process; solid arrows, epithelial cell foot processes. $\times 7,300$.

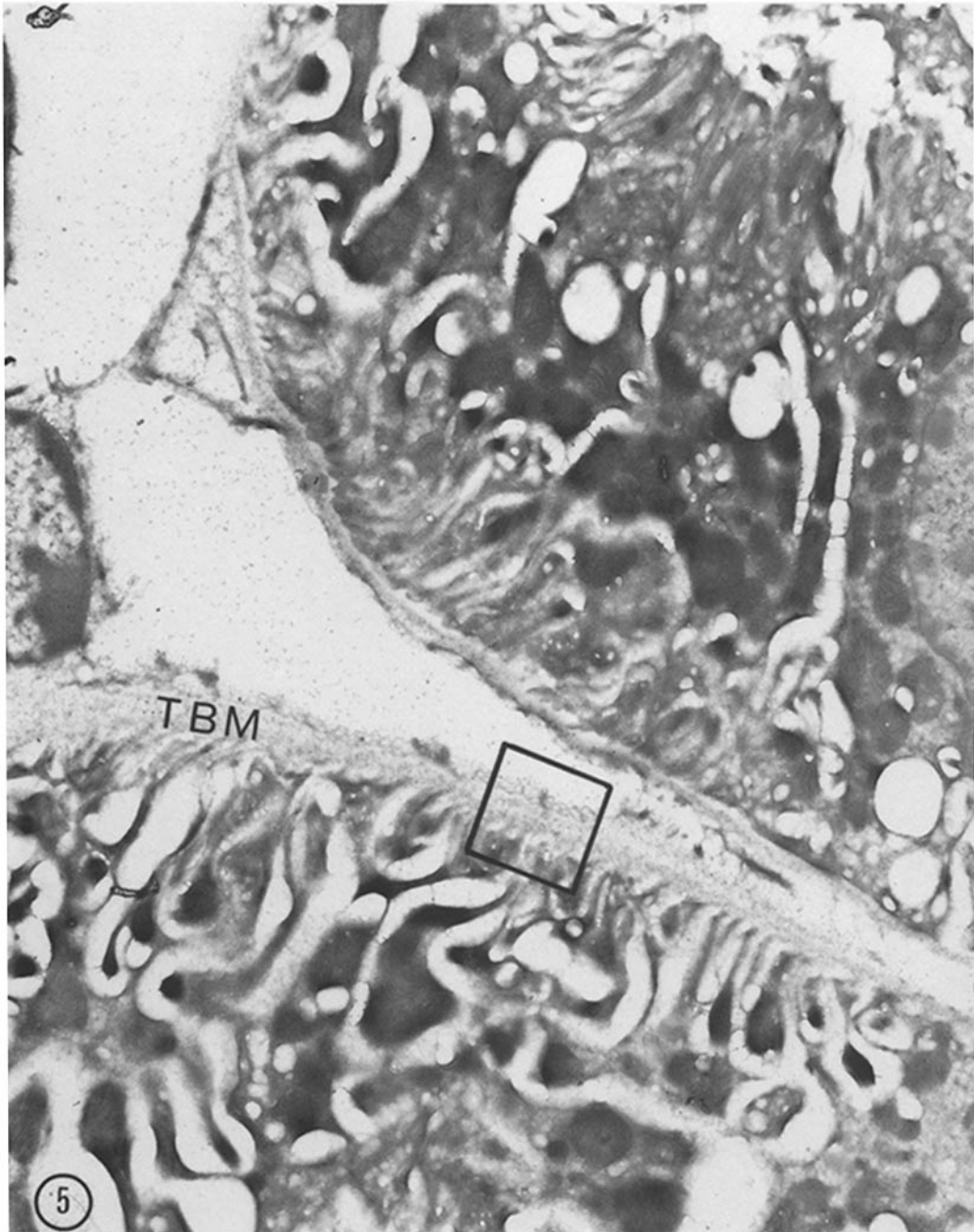


FIGURE 5 Ultrathin frozen sections of mouse tubules labeled with antibody to type IV collagen as described in Materials and Methods. *TBM*, tubular basement membrane. High magnification of the region outlined by the square is shown in Fig. 6*a*. $\times 9,900$.

and even mitochondria in these frozen sections. Therefore we did not find it necessary to try to enhance cellular membrane staining as Tokuyasu recently described (49). Occasional holes in the tissue which have been attributed to ice crystal formation during freezing were not a serious drawback to our studies.

Labeling of TBM

Fig. 5 shows a low-magnification view of two adjacent tubules labeled with antibodies to type IV collagen. The tubular epithelial cell foot processes reach out to the basement membrane. Between the two basement membranes are seen a thin layer of amorphous ground substance and a peritubular capillary in cross section. At higher magnification, the heavy ferritin labeling of the TBM can be appreciated (Fig. 6*a*). The ferritin seems to be concentrated over the electron-dense central portion of the membrane. Little labeling is seen over the epithelial cell and almost none over the empty Formvar. Labeling of the basement membrane can be prevented by incubation of the antibodies with type IV collagen before staining (Fig. 6*b*). Occasional large aggregates of ferritin were seen but, because these were considerably reduced when we centrifuged the antigen-antibody mixtures, we feel that these are probably caused by residual antigen-antibody complexes.

The question of whether the light ferritin label over the epithelial cells represents nonspecific background labeling or represents labeling of intracellular antigen cannot be answered with certainty. It is our impression that, in inhibition experiments, labeling over the cells was decreased while background labeling over the Formvar remained unchanged, indicating that the labeling over cells is specific (data not shown).

In controls in which the collagen antibody or the biotinized SAR IgG was omitted, the density of label over all the basement membranes of the kidney was as low as, or lower than, that in the inhibition studies.

Nonspecific ferritin labeling of erythrocyte cytoplasm was seen in all sections. This labeling differed from specific labeling in that it was not dependent on collagen antibody and it was not inhibited by addition of collagen to the antibody before labeling. Because omission of the biotinized SAR IgG eliminated the erythrocyte staining, we concluded that it was caused by binding of the biotinyl-SAR IgG to components of the erythro-

cyte cytoplasm. Nonspecific labeling of erythrocytes with rabbit IgG ferritin conjugates was also seen in frozen section by Ziparo et al. (56).

Fig. 6*c* shows a representative area of another tubular basement membrane labeled with type AB₂ collagen antibodies. The density of ferritin grains appears lower, but the random pattern of staining over the electron-dense portion of the membrane is the same as for type IV antibodies. Again, epithelial cell cytoplasm is lightly labeled. Pre-incubation of the antibodies with type AB₂ collagen abolished basement membrane labeling (Fig. 6*d*).

Labeling of GBM

At low magnification, the GBM in ultrathin frozen sections is easy to identify as an electron-dense ribbon between the epithelial cell foot processes and the capillary endothelial cells (Fig. 4). It appears to be separated from its flanking cells by an electron-lucent space on either side. Whether these spaces are the equivalent of the laminae rarae seen in conventional Epon-embedded sections is not known. Fig. 7*a* is a higher magnification of a portion of glomerular capillary loop labeled with type IV collagen antibody.

The basement membrane is easily identified by its electron-dense appearance and by the neighboring epithelial foot processes. We have not seen the 40-Å fibrils in the GBM that have been seen in conventionally fixed, Epon-embedded tissue (13). The ferritin labeling of type IV collagen over the electron-dense portion of the GBM is apparently random, but of lower density than over other basement membranes of the kidney. Epithelial and endothelial cells are both lightly labeled. The inhibition study (Fig. 7*b*) shows no staining of the membrane. In Fig. 7*c*, a similar area labeled for type AB₂ collagen demonstrated less dense labeling than for type IV, but label is concentrated over the GBM and is completely abolished by pre-incubation with type AB₂ collagen (Fig. 7*d*).

Labeling of Mesangium

In Fig. 8, a portion of a glomerulus, containing a mesangial cell with amorphous mesangial matrix which fills the interstices of its irregular cell borders, is labeled with antibodies to type IV collagen. A higher magnification (Fig. 9*a*) shows ferritin over the mesangial matrix. The mesangial cell itself is lightly labeled. As with basement membrane structures, labeling of the mesangium with

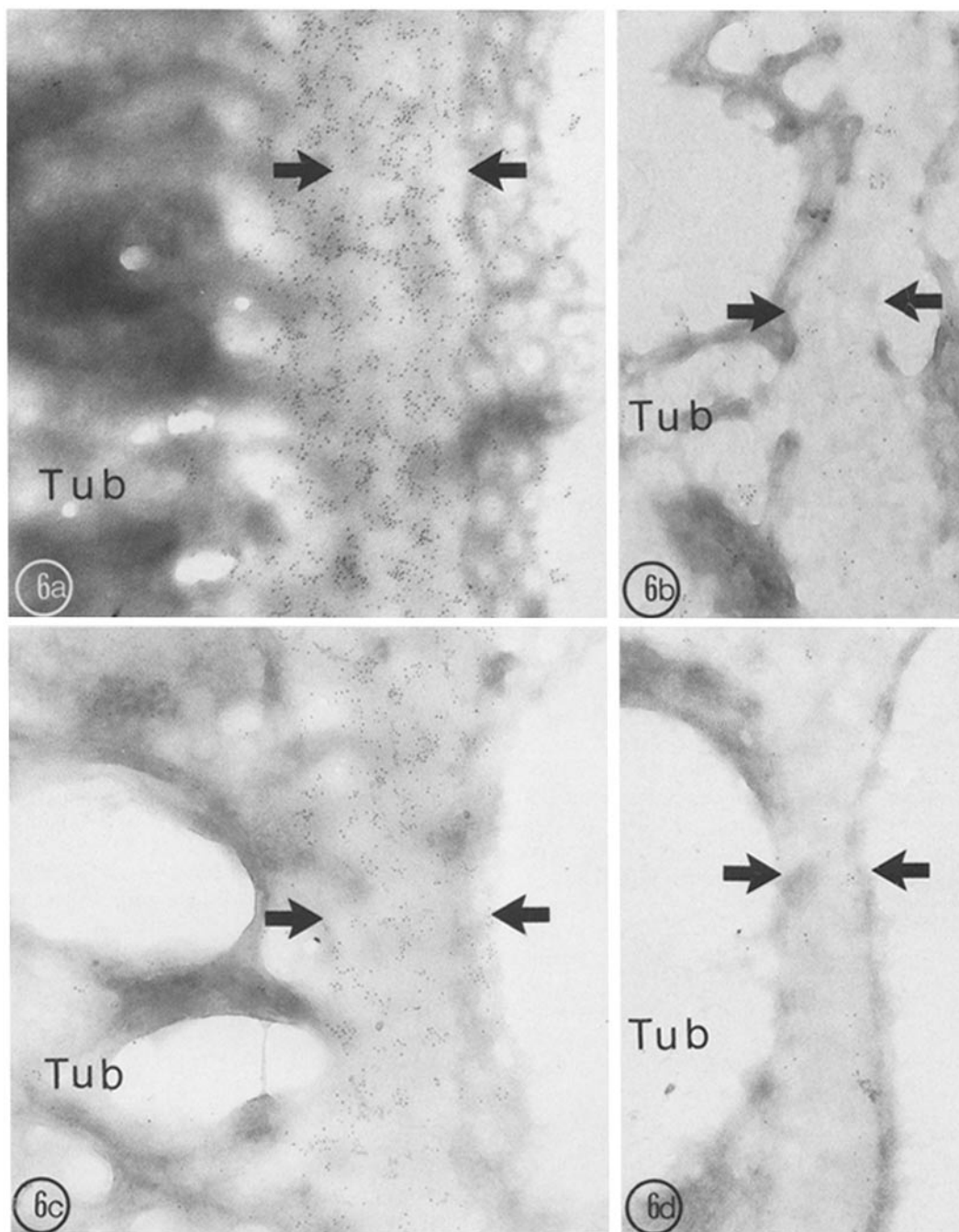


FIGURE 6 High-magnification views of tubular cell basement membranes and foot processes. (a) Basement membrane from Fig. 5 labeled with antibody to type IV collagen, biotinized SAR IgG, and avidin-ferritin. (b) Inhibition study from the same experiment as a. (c) Basement membrane from an area similar to that seen in Fig. 5, labeled with antibody to type AB₂ collagen. (d) Inhibition study from the same experiment as c. *Tub*, tubular epithelial cell cytoplasm and foot processes; arrow, TBM. $\times 60,000$.

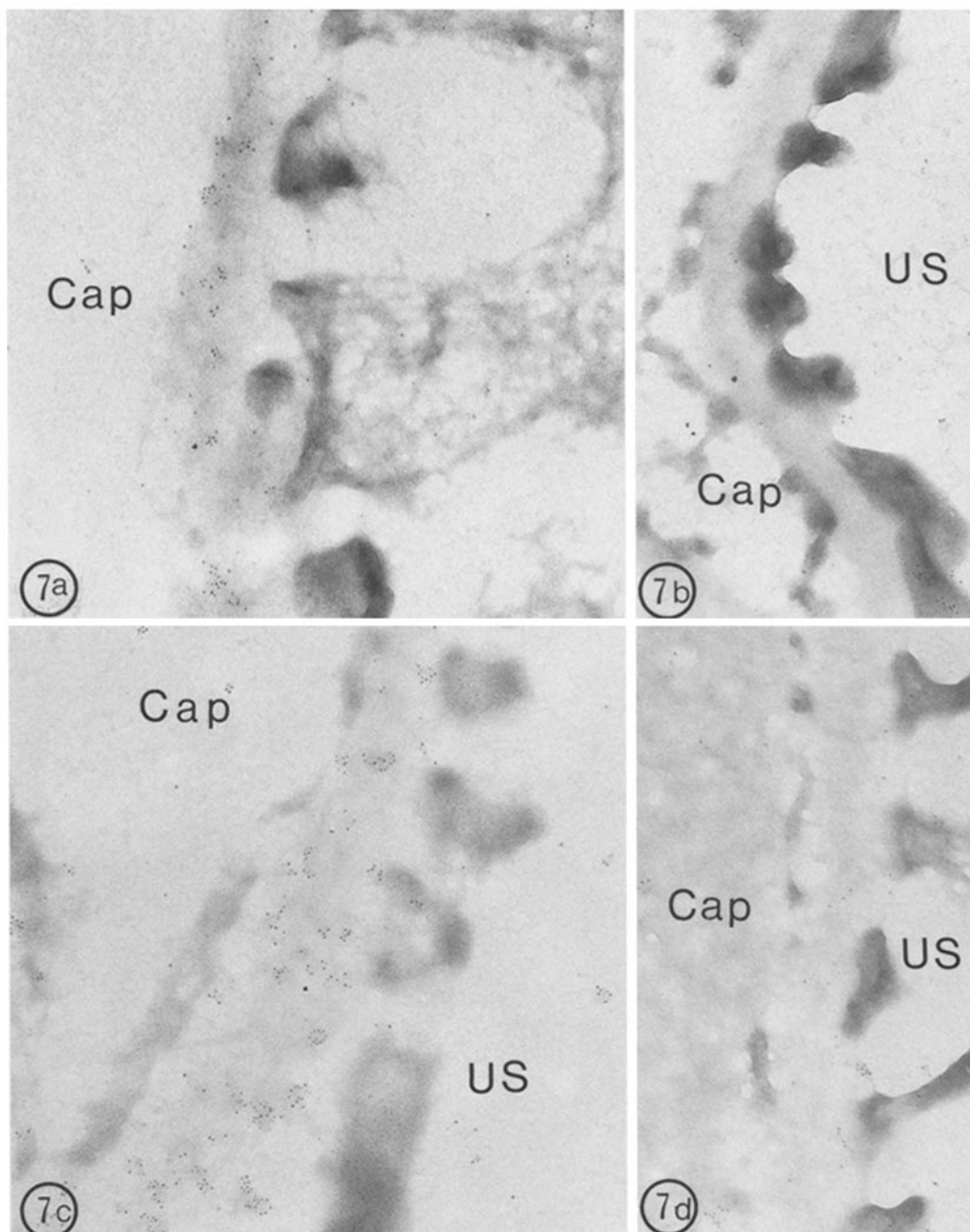


FIGURE 7 High-magnification views of GBM and adjacent cell processes. (a) GBM labeled with antibody to type IV collagen, biotinized SAR IgG, and avidin-ferritin as described in Materials and Methods. (b) Inhibition study from the same experiment as a. (c) GBM from an area similar to that seen in a, labeled with antibody to type AB₂ collagen. (d) Inhibition study from the same experiment as c. *Us*, urinary space; *Cap*, capillary. $\times 60,000$.

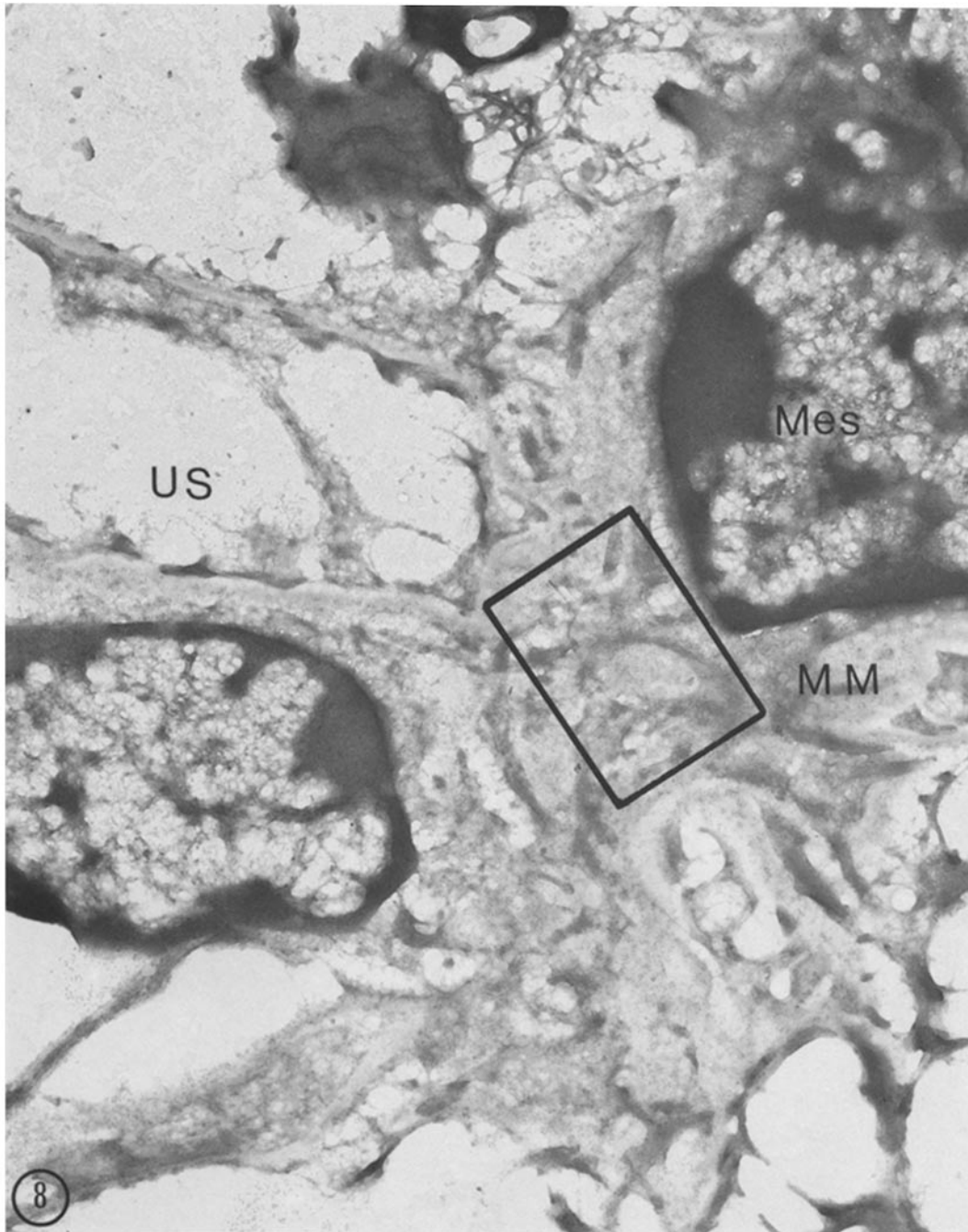


FIGURE 8 Ultrathin frozen section of mesangial area of a glomerulus labeled with antibody to type IV collagen as described in Materials and Methods. *US*, urinary space; *MES*, mesangial cell; *MM*, mesangial matrix. High magnification of the region outlined by the rectangle is shown in Fig. 9*a*. $\times 16,300$.

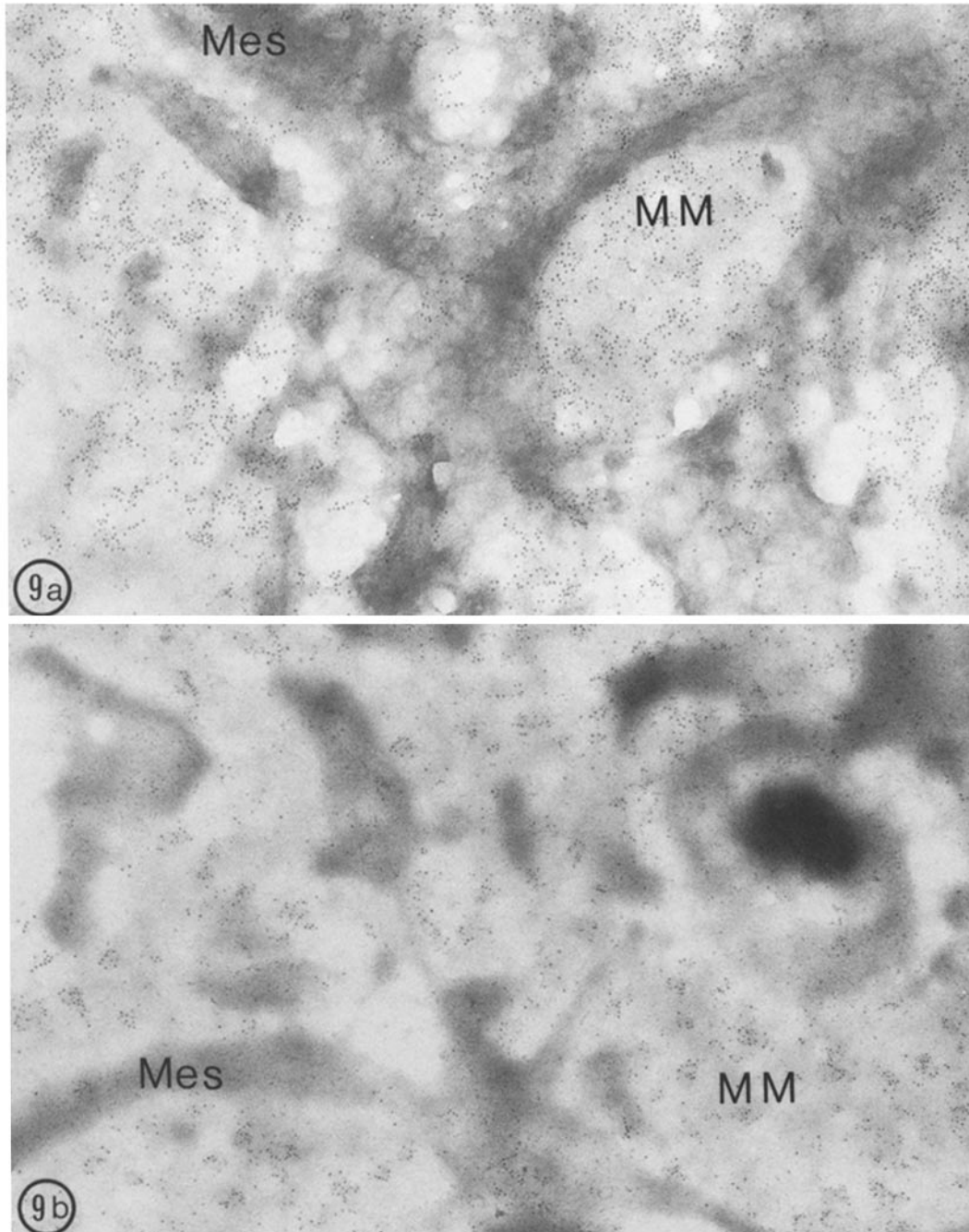


FIGURE 9 High-magnification view of mesangial area. (a) Mesangial area from Fig. 8 labeled with antibody to type IV collagen, biotinized SAR IgG and avidin-ferritin as described in Materials and Methods. (b) Similar area stained with antibody to type AB₂. Mes, mesangial cell process; MM, mesangial matrix. $\times 60,000$.

antibodies to type AB₂ collagen (Fig. 9b) was less dense than labeling with antibodies to type IV. This labeling nevertheless could also be inhibited completely (data not shown).

Labeling of Bowman's Capsule

A low-magnification view of the periphery of a glomerulus with a tubule at the outside of Bowman's capsule and a glomerular capillary loop on the inside is shown in Fig. 10. The basement membrane of Bowman's capsule is heavily labeled with antibodies to type IV collagen (Fig. 1a) and to type AB₂ collagen (Fig. 11b). Type AB₂ collagen appears to be localized in random clusters in contrast to type IV collagen which is more diffusely distributed. This patchy appearance is not caused by ferritin aggregates because the same ferritin preparation was used in both cases. Labeling could be inhibited by incubation with the corresponding collagens (data not shown). The density of ferritin grains over basement membrane of Bowman's capsule is greater than over GBM or TBM.

In summary, the ultrastructural study confirms the finding of the immunofluorescence study that both the putative type IV and type AB₂ collagen are present in all basement membranes of the mouse kidney. The density of label varied with the type of collagen and the basement membrane studied. In all instances the ferritin label was apparently randomly distributed over the electron-dense portion of the basement membrane, though AB₂ gave the appearance of being irregularly clustered in Bowman's capsule. Mesangial matrix was also shown to contain both collagen types. The relative intensity of labeling with antibodies to types IV and AB₂ collagen was similar to that observed in the immunofluorescence study.

DISCUSSION

How many types of collagen comprise the basement membranes of the kidney and whether basement membranes of the kidney are chemically similar to the mesangial matrix are not yet known (24, 26). Reports based on chemical extraction vary as to the number of collagens in glomerular basement membranes (1, 8, 11, 22, 23, 26, 50, 51). The chemical approach suffers from difficulties with dissection and sieving techniques, unknown protease sensitivity of the collagens within the tissue, incomplete solubilization and extractability of the various collagens, and the inability to determine exactly where the collagenous material orig-

inates (e.g., mesangial matrix vs. glomerular or Bowman's capsule basement membranes).

Another approach that has been taken is to study the collagen composition of basement membrane-containing structures with anticollagen antibodies (14, 43, 53-55). Here the specificity of the antibody used is critical, and this depends upon the purity of the immunogen and immunoabsorbents. This is especially pertinent to studies of type IV collagen. Recently Crouch and Bornstein (10), Sage et al. (40), Kresina and Miller (28), Glanville et al. (15), and Timpl et al. (44) have described several proteolytic fragments of type IV collagen from various tissues. Furthermore, chemical and immunochemical evidence has been adduced, supporting the existence of at least two subtypes of type IV collagen (2, 15, 28, 44, 45). These findings underscore the caution that must be exercised in making statements about type IV collagen. Much more work will have to be done before agreement can be reached. As mentioned (Materials and Methods), we have evidence that the type IV collagen we have isolated is identical to that reported by Timpl et al. (46). It has not yet been demonstrated how the higher molecular weight components seen on our gels are related to the α chain sized components; therefore, we have referred to this preparation as incompletely characterized.

The apparent codistribution of types IV and AB₂ collagen within the basement membranes and mesangium of the kidney in immunofluorescence studies prompted us to define more precisely the localization of these collagen types using immunoferritin electron microscopy.

In our immunoelectron microscope study antibodies to type AB₂ and this type IV collagen react with the mesangial matrix and all basement membranes in the kidney: glomerular and tubular basement membranes and Bowman's capsule. Labeling is more intense with anti-type IV than with anti-type AB₂ for any given basement membrane, and the various membranes label with different intensities. The intensity of the ferritin labeling is qualitatively in accord with our immunofluorescence findings. It is not clear whether these differences are a reflection of varying amounts of these collagens within the membranes, or whether they are because of differences in the structural organization of these components. It is likely that basement membranes contain other macromolecules, and inaccessibility of antigenic determinants could be caused by variations in composition of these non-

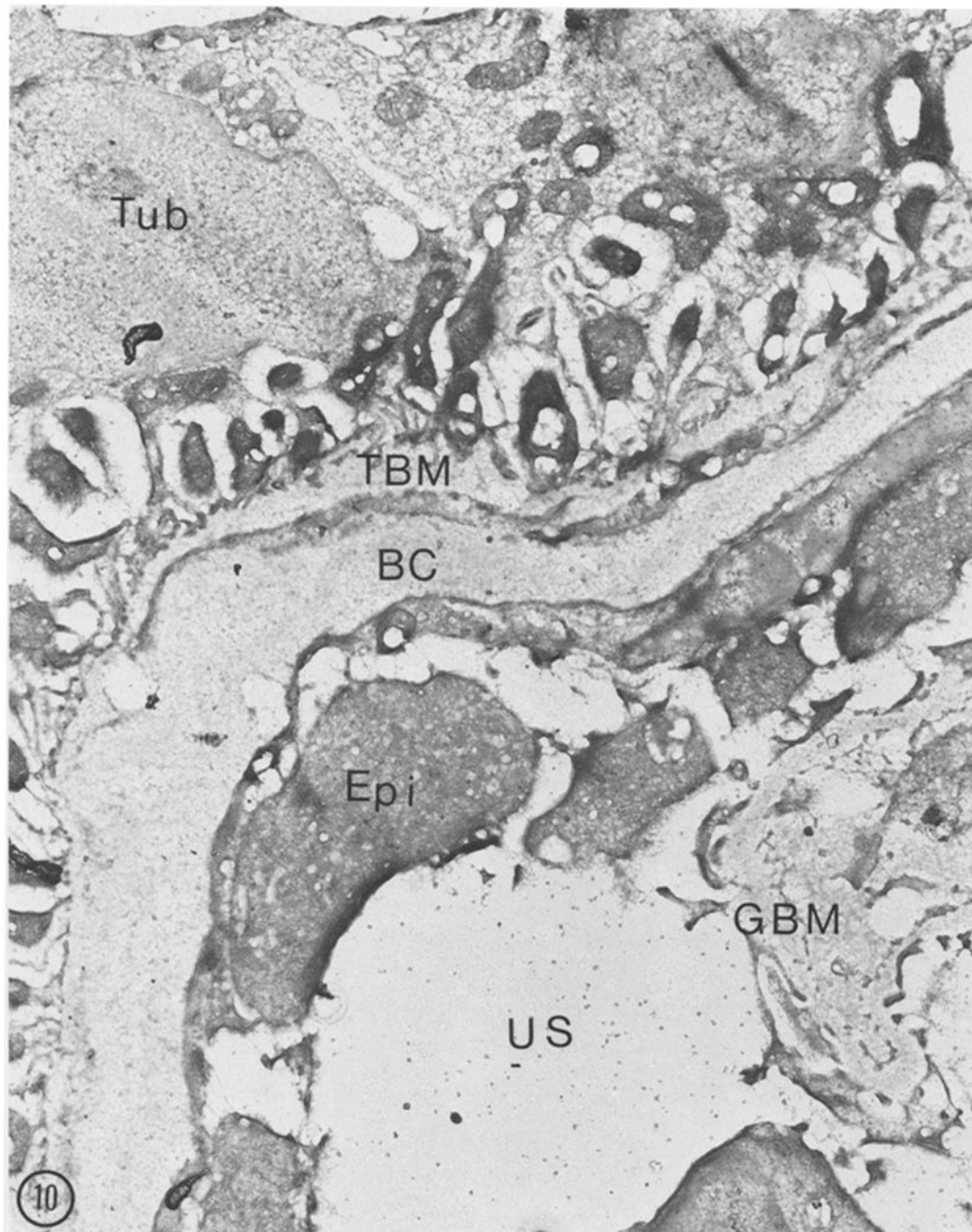


FIGURE 10 Ultrathin frozen section of Bowman's capsule flanked by a tubule above and glomerular structures below. Micrograph taken from an inhibition study with type AB₂ antibody. High magnification not shown. *Tub*, tubular cells; *TBM*, tubular basement membrane; *BC*, Bowman's capsule; *Epi*, epithelial cell; *US*, urinary space; *GBM*, glomerular basement membrane. $\times 11,700$.

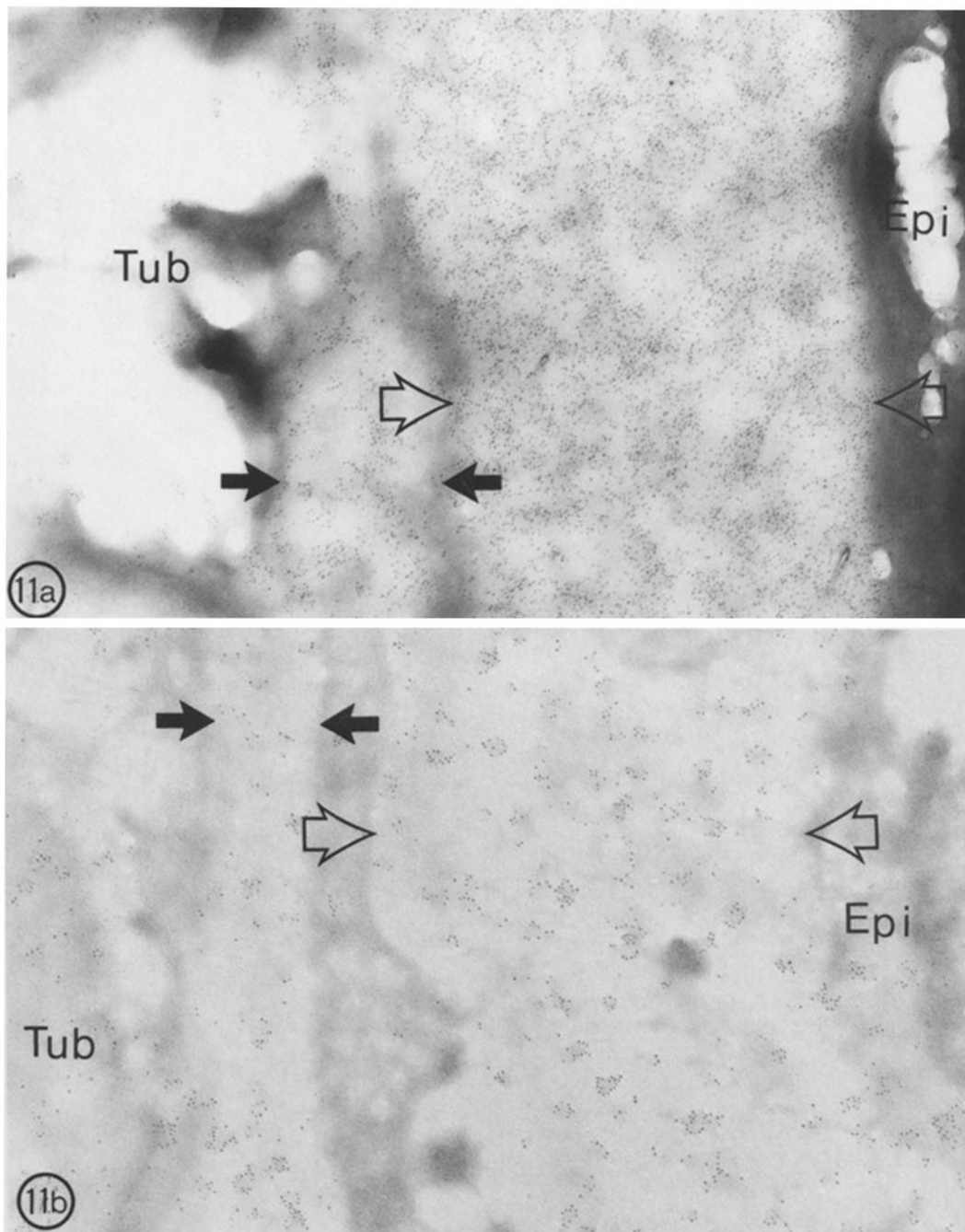


FIGURE 11 High-magnification view of Bowman's capsule and adjacent structures. (a) Bowman's capsule labeled with antibody to type IV collagen, biotinized SAR IgG, and avidin-ferritin. (b) Similar area labeled with antibody to type AB₂ collagen. *Epi*, parietal epithelial cell; *Tub*, tubular cell; closed arrows, TBM; open arrows, Bowman's capsule basement membrane. $\times 60,000$.

collagenous moieties. Similarly, the apparent clustering of type AB₂ in Bowman's capsule in contrast to the diffuse distribution of type IV is difficult to interpret. At present, we do not feel that the different patterns suggest necessarily a different arrangement of the two collagen types within the basement membrane.

The finding of "basement membrane collagens" in the mesangial matrix is intriguing. Previous morphological studies have been interpreted as showing that the mesangial matrix is distinct from the lamina densa (the principal part of the basement membranes) (31). Farquhar and Palade felt that there is a relationship between the matrix and the basement membrane (12). Because there is no known method of obtaining pure mesangial matrix for chemical characterization, it has been difficult to investigate this relationship. Our immunoelectron microscope study may be taken as evidence that such a relationship exists.

The functional significance of these two collagens in basement membranes is unknown. Classical interstitial collagens presumably provide needed tensile strength in animal tissues (39). Whether they serve other functions is still unknown, though the work of Hay et al. (19) and Hauschka et al. (18) has suggested that they play a role in the development of the cornea and of muscle cells, respectively. Although they do not form fibers as the interstitial collagens do, basement membrane collagens may provide a structural support for other components of the basement membrane. They may also have a function in adhesion and movement of the cells which they underlie. Murray et al. have shown that suspensions of guinea pig epidermal cells, when plated on dishes coated with one of the collagen types I, II, III, or IV will preferentially attach to type IV (32). After attachment, the cells will go on to differentiate into a squamous epithelium in the presence of serum. It is possible that other epithelial cell types including glomerular epithelia preferentially adhere to type IV and/or type AB₂ collagen of the GBM. We have shown that migrating epidermis in culture synthesizes type AB₂ collagen and that continual collagen synthesis is necessary for movement (42). It is conceivable that, during development or in response to injury, renal epithelia may migrate on their basement membranes and that any disturbance of their collagen-synthesizing capacity will lead to functional consequences.

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