J. Biosci., Vol. 11, Numbers 1–4, March 1987, pp. 245–256. © Printed in India.

Histochemical localization of protein-polysaccharides in renal tissue

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Abstract. The purpose of this study was to investigate the distribution of protein-polysaccharides in the glomerular and non-glomerular regions of the nephron. The techniques used include the digestion of kidney slices with specific polysaccharidases: neuraminidase, hyaluronidase, chondroitinase ABC, and collagenase followed by several cytochemical techniques to identify the glycosaminoglycans and glycoproteins at the light and electron microscope levels. Differential staining of hyaluronic acid and sulphated glycosaminoglycans was accomplished with Alcian Blue at pH 2·5 and pH 0·5, respectively. Sialoproteins were stained with Alcian Blue at pH 2·5. The periodic acid Schiff's reaction technique was employed for the visualization of collagen. At the electron microscope level the polysaccharides were identified with the periodic acid-chromic acid-silver methenamine reaction. Our results indicated that the major polysaccharide components of the glomerular basement membrane were sialoproteins and collagen, with smaller amounts of hyaluronic acid and various sulphated glycosaminoglycans. Hyaluronidase digestion resulted in partial detachment of epithelial processes from the glomerular basement membrane indicating the hyaluronic acid may have a role in the stability of the attachment of these processes. Tubular basement membranes also contain sialoproteins and sulphated glycosaminoglycans but in considerably lower concentrations than the glomerular basement membrane. Bowman's capsule appears to contain mostly sulphated glycosaminoglycans and has a lower concentration of sialoproteins and hyaluronic acid.

Keywords. Kidney; glycosaminoglycans; histochemistry.

Introduction

Mammalian kidneys contain a spectrum of protein-polysaccharides consisting of collagen, sialoproteins, and glycosaminoglycans (GAGs) (Allalouf *et al.,* 1964; Castor and Greene, 1968; Farber and Van Praag, 1970; Murata, 1976; Linker *et al.,* 1981). The structural and functional roles of these glycoconjugates in renal tissue (Pinter, 1967; Kanwar and Farquhar, 1979) and their alterations in disease conditions such as diabetes (Berenson *et al.,* 1970; Malathy and Kurup, 1972; Saraswathi and Vasan, 1982) has been of considerable interest in recent years.

Biochemical studies using solubilized glomerular basement membrane (GBM) have provided considerable information as to the protein-polysaccharide com position of this region in normal and in pathological conditions (Mohos and Skoza, 1969; Kefalides, 1974; Spiro, 1976; Brownlee and Spiro, 1979; Parthasarathy and Spiro, 1980). Other studies using cationic probes (Kanwar and Farquhar, 1979a; b; Reeves *et al.,* 1980) as well as immunological techniques (Carlson *et al.,* 1978; Schienman *et al.,* 1978; Oberley *et al.,* 1979; Madri *et al.,* 1981) have explored the distribution of these materials in various regions of the nephron. The present study

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Abbreviations used: GAGs, Glycosaminoglycans; GBM, glomerular basement membrane; PAS, periodic acid-Schiff's; TBM, tubular basement membrane; BC, Bowman's capsule; LRI, lamina rarae interna.

further examines the distribution of several glucoconjugates in glomerular and non-glomerular regions of the nephron. The techniques employed for this investigation involve the digestion of renal tissue with specific polysaccaridases followed by several cytochemical techniques for GAGs and glycoproteins at the light and electron microscopic levels. Our results indicate that the glucoconjugates are distributed in varying concentrations in different regions of the nephron, with the GBM being rich in sialoproteins, collagen, and sulphated as well as non-sulphated GAGs.

Materials and methods

Protease-free collagenase was purchased from Advanced Biofacture Inc., Lynbrook, New York. Chondroitinase ABC was obtained from Miles Laboratory, Indiana. Leech hyaluronidase was bought from Biomatrix Corporation, Boston, Massachusetts, and Vibrio Cholerae Neuraminidase (Protease free) from Calbiochem, California. Prior to use, the enzymes were tested for activity by standard procedures (Ada *et al.,* 1961; Yuki and Aishman 1963; Saito *et al.,* 1968; Gisslow and McBride, 1975). Proteolytic activity was tested using bovine serum albumin as substrate and indicated that the above enzymes were free of proteases. Hexamethylene tetramine (methenamine) was obtained from Polyscience Lab, Warrington, Pennsylvania.

Adult male Lewis rats, weighing nearly 200 g, were sacrificed under ether anesthesia. The kidneys were removed, stripped of their capsule, and cross sectioned to about 1mm slices. The tissue slices were incubated for 14-16 h, in one of the following enzyme solutions:

(i) Vibrio cholerae neuraminidase in 0·05 M sodium acetate buffer pH 5·6, containing 0·002 M $CaCl_2$.

(ii) Leech hyaluronidase in Mcllwains buffer, pH 5·6.

(iii) Chondroitinase ABC (*P. Vulgaris*), in 0·1 M Tris-HCl buffer, pH 7·8.

(iv) Collagenase in 0·025 M Tris-0·33 M calcium acetate buffer, pH 7·8.

Addition of various enzymes was repeated every 4 h. Control experiments, where the kidney slices were incubated either in physiological saline or the appropriate buffers without the enzymes, were also performed.

Light microscopy

After the incubations, the tissue slices were rinsed in buffer, and fixed in buffered formalin for 24 h. The tissues were well rinsed in buffer, dehydrated with alcohol, and embedded in paraffin.

Sections (6 μm) were mounted on glass slides, and stained according to one of the following methods: Alcian Blue (8GX 300) at pH 2·5 for non-sulphated poly saccharides (Hyaluronic acid, and sialoproteins); Alcian Blue (8GX 300) at pH 0·5 to visualize sulphated GAGs (Chondroitin sulphate, dematan sulphate, heparin sulphate); periodic acid-Schiff (PAS) for neutral polysaccharides (Collagen). Occasional slides were stained with the combination of Alcian Blue at pH 0·5, followed by Alcian Yellow at pH 2·5, after an intermediate rinse in 0·5 M HCl. All slides were then counterstained with neutral fast red (Revetto, 1964). The staining affinity of enzyme-digested slices was compared with the appropriate control slices.

Electron microscopy

Representative kidney pieces, after incubation, were rinsed in phosphate buffer pH 7·2, fixed in glutaraldehyde (2%), dehydrated with ethanol, and embedded in Araldite 502. Semi-thin sections of about 200 μm were picked up on nickel grids, and reacted with a modified periodic acid-chromic acid-silver methenamine technique for glycoproteins (PA-CA-AgMe) (Rambourg, 1967, 1971; Tesoriero, 1977, 1981). PA-CA acid oxidations were omitted in certain slices to serve as controls. The saline and buffer incubated slices also served as morphological controls. Sections used for morphological comparison were stained with uranyl acetate and lead citrate.

Results

Light microscopy

PAS staining of saline incubated control slices showed the classic distribution of PAS positive material within the glomerulus, Bowman's capsule, and in the tubular basement membrane (TBM) (figure 7). Staining with Alcian Blue at pH 0·5 indicates that sulphated glycosaminoglycans are distributed predominantly in Bowman's capsule (BC) and in the TBM with notably smaller amounts in the glomerulus (figure 5). Alcian Blue staining at pH 2·5 reveals that the non-sulphated polysaccharides are present both in the glomerulus and in Bowman's capsule (figures 1 and 3). A clear distinction could be made between the sulphated and the non-sulphated polysaccharides in the tissue slices stained sequentially with Alcian Blue at pH 0·5 followed by Alcian Yellow at pH 2·5. The glomerulus stains a bright yellow indicating non-sulphated material, while BC stains a contrasting greenish blue, indicatinga predominance of sulphated material with a smaller amount of non-sulphated polysaccharide.

Characterization of the polysaccharides, responsible for alcianophilia under different conditions, was achieved by selective digestion with specific polysaccharidases.

Tissue slices incubated with neuraminidase showed a marked reduction in the Alcian Blue (pH 2·5) staining within the glomerulus and Bowman's Capsule indicating that these regions are rich in sialoproteins (figure 2). After digestion, the GBM resembles a fine line rather than its more robust pre-digestion staining (figures 2 and 4).

Digestion with leech hyaluronidase resulted in a slight to moderate reduction in the Alcian Blue (pH 2·5) stain in the glomerular regions (figure 4). Since this enzyme specifically hydrolyzes hyaluronic acid, the present observation is indicative of the presence of moderate concentrations of hyaluronic acid in the glomerulus.

Following digestion with chondroitinase ABC, the intensity of the Alcian Blue (pH 0·5) stain was reduced in BC and in the TBM (figure 6). The presence of moderate amounts of chondroitin sulphate or dermatan sulphate in these regions is thus indicated since chondroitinase ABC specifically digests these sulphated GAGs. The persistent staining for sulphated polysaccharides, even after the digestion with ABC, probably indicates the presence of heparan sulphate which is not removed by the ABC enzyme.

The digestion with collagenase often showed marked morphological changes in the tissues but only slight reduction in the PAS staining of the glomerulus.

Electron Microscopy

Kidney slices which were incubated in saline for up to 24 h and processed by routine methods for electron microscopy showed only slight changes in the general architecture of the epithelial and endothelial cells of the glomerulus (figure 9). Their attachment to the GBMwas maintained and the general appearance of all basement membranes appeared unchanged.

When semi-thin sections (200 μm) of saline incubated tissue slices were reacted according to the PA–CA–Ag methenamine technique for glycoproteins there was a positive reaction over the GBM, around the processes of the epithelial and endothelial cells, within the mesangial regions, within the endothelial cells of BC and over the basement membrane of BC (figure 10). Occasional isolated fibers of collagen which stand out strongly with this technique were noted on the mesangial side of the GBM.

Following neuraminidase digestion the silver methenamine reaction for glycoprotein was almost totally abolished over the GBM and from the surface of the endothelial cells. A slight reactivity remained around the processes of the epithelial cells. Sialoglycoproteins therefore appear to be the predominant polysaccharides in these regions (figure 11). The reactivity of BC was reduced, and the silver reactions appeared over small, fibrillar, linear structures. The heavier collagen associated with BC remained reactive. These findings suggest that moderate amounts of sialoproteins and collagen are associated with BC (figure 14).

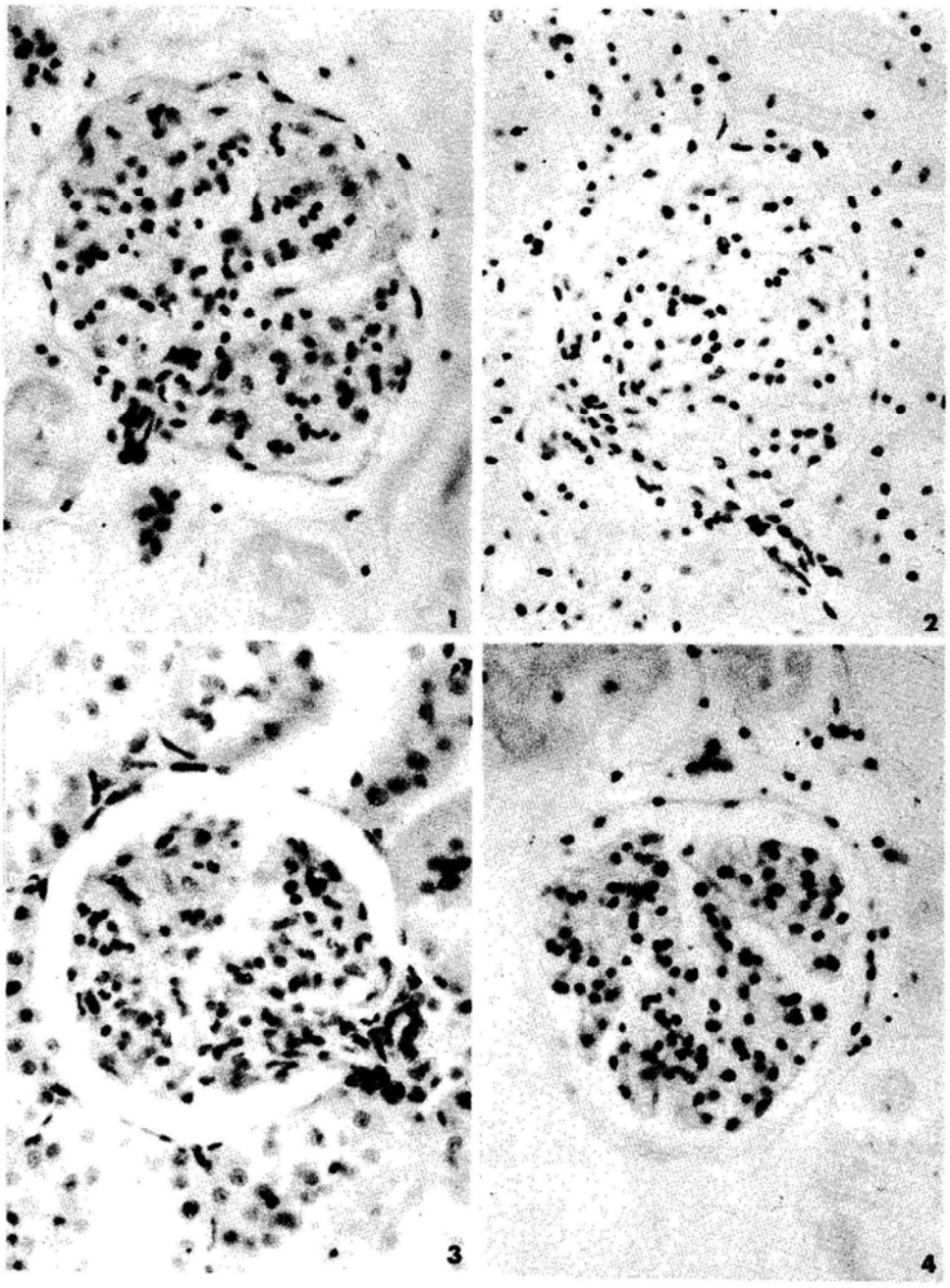
Hyaluronidase digestion resulted in a reduction in the silver methenamine reaction over most of the GBM indicating the presence of hyaluronic acid in this region. The reactivity surrounding the endothelial and epithelial cells and their processes remained unchanged. In the region of the GBM there was an electron lucent and less reactive area within regions of the lamina rarae interna (LRI), which appeared to represent a separation of the epithelial cells from the membrane (figure 12). A similar region was also seen but less consistently on the endothelial side of the GBM. Hyaluronic acid may therefore have a role in the makeup of the GBM and the attachment of these cells to that membrane.

Digestion with chondroitinase ABC resulted in the reduction of silver staining in the lamina rarae externa and LRI of the GBM indicating the presence of some chondroitin and /or dermatan sulphate in these regions (figure 13). BC, the epithelial cell processes, and the lamina dense portion of the GBM were still reactive to the silver stain. These regions appear to contain very little of the chondroitinase ABC digestible GAG.

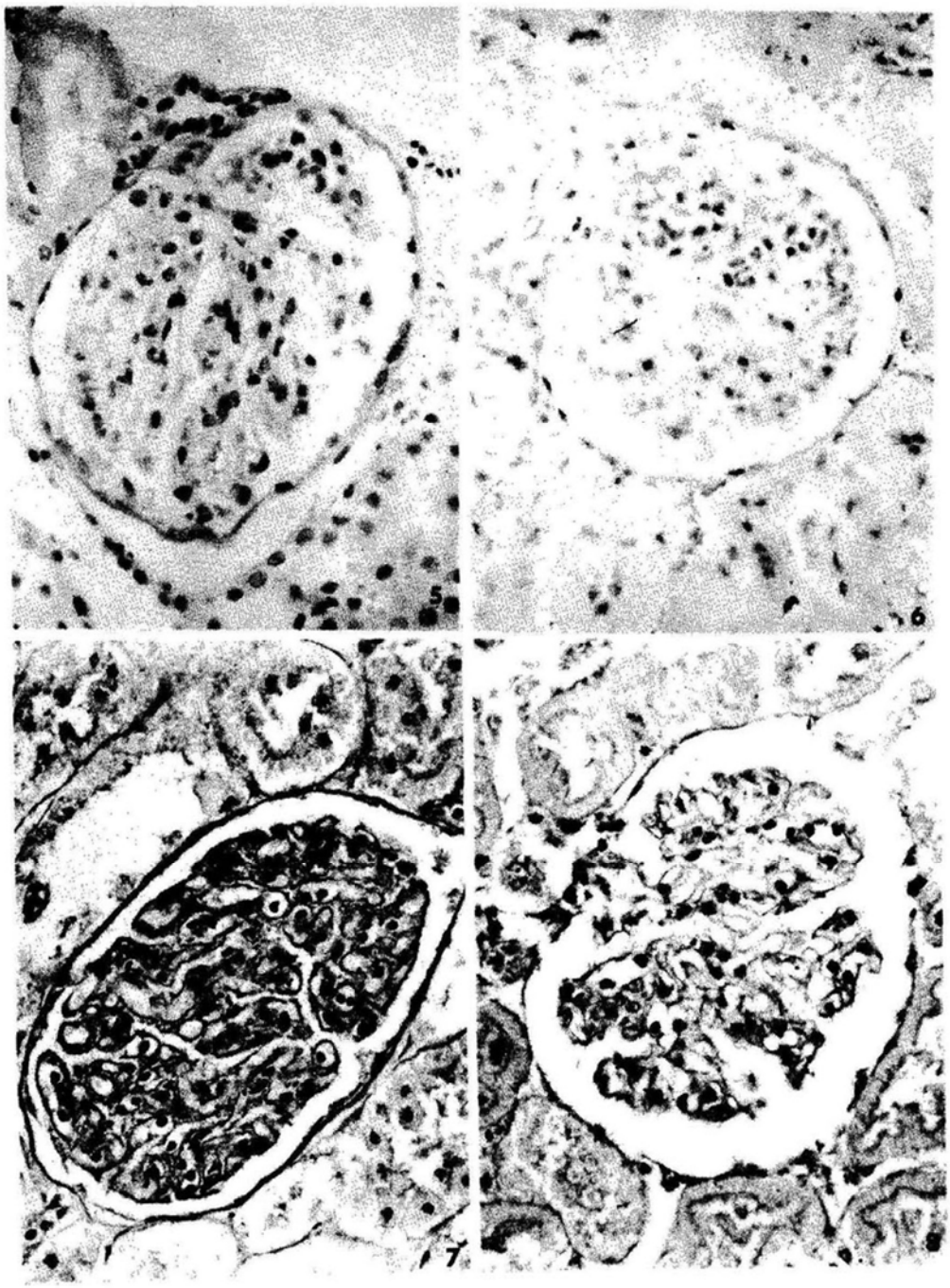
There were major structural changes in the renal tissue following digestion with collagenase but an occasional intact glomerulus could be located. The GBM retained most of its reactivity to the silver stain, as did the epithelial and endothelial cells. BC showed some loss of silver staining, with the notable absence of the highly reactive collagen.

Discussion

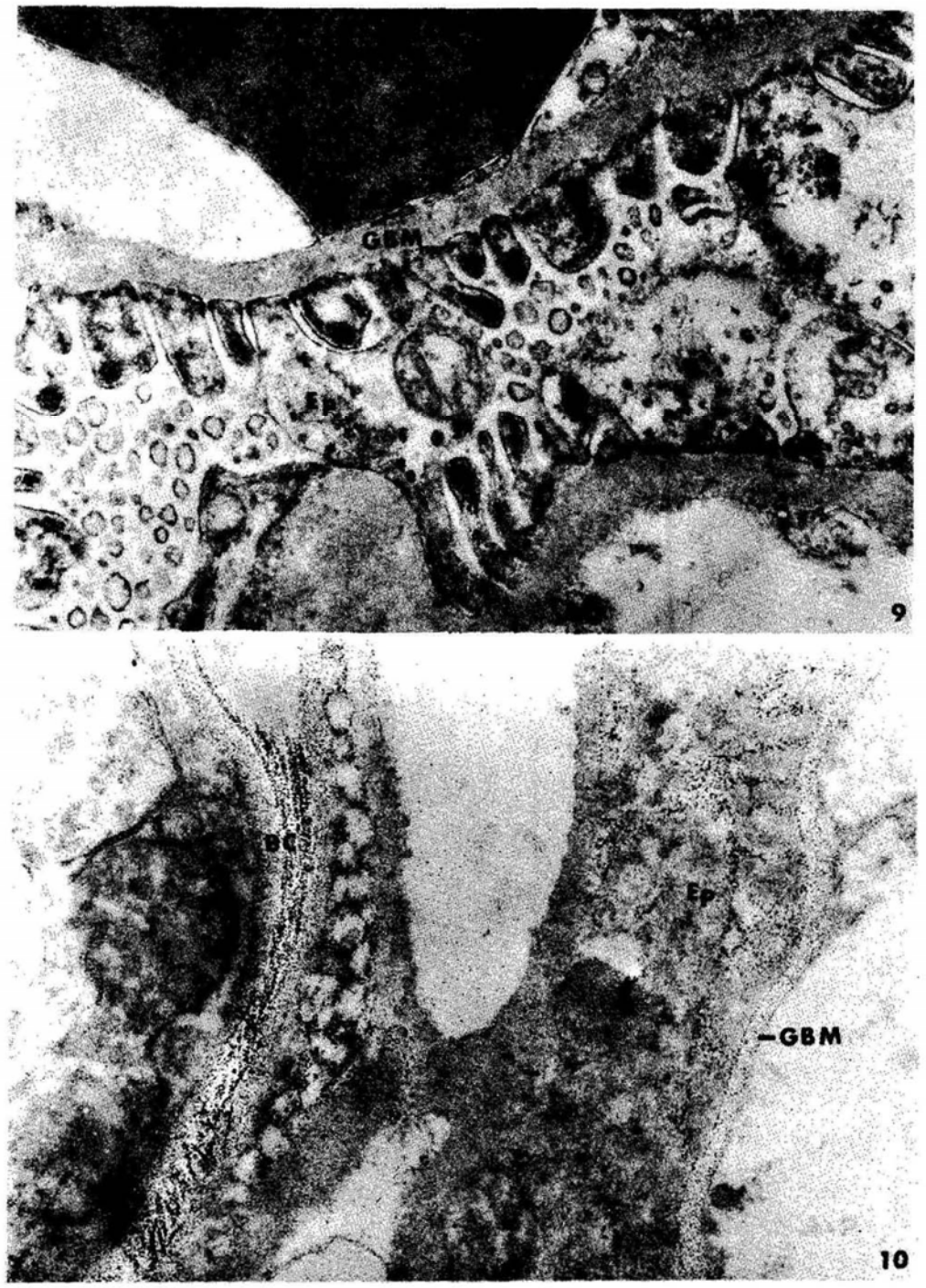
The enzymatic digestion, as well as light and electron microscopic studies, clearly demonstrate the distribution of both sulphated and non-sulphated polysaccharides



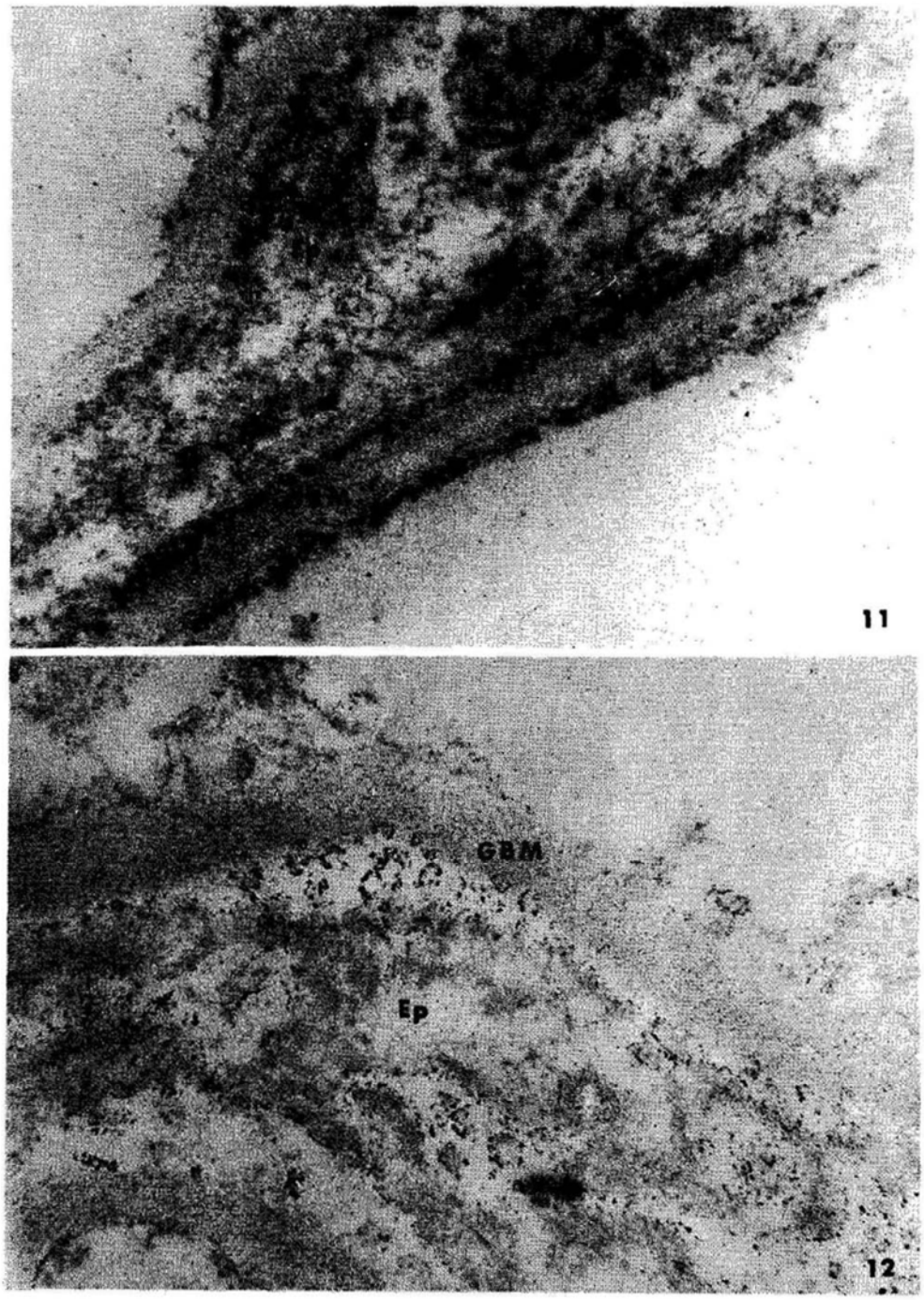
Figures 1-4. 1. Saline incubated control, Alcian Blue, pH 2·5, × 135. **2.** Neuraminidase digestion, Alcian Blue, pH 2·5, × 135. **3.** Hyaluronidase buffer control, Alcian Blue, pH 2·5, × 135. **4.** Hyaluronidase digestion, Alcian Blue, pH 2·5, × 135.



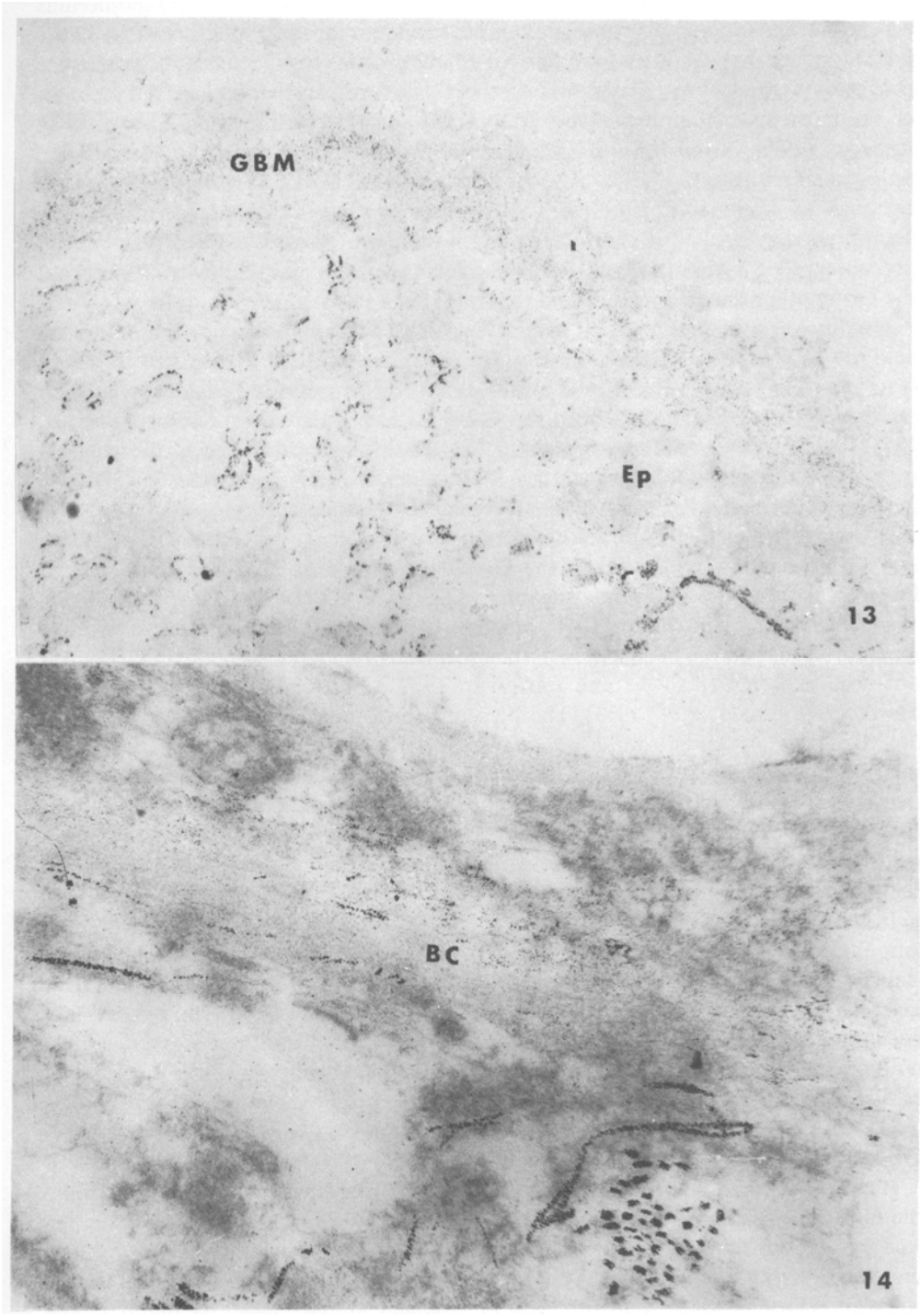
Figures 5-8. **5.** Chondroitinase buffer control, Alcian Blue pH 0·5, × 135. **6.** Chondroitinase digestion, Alcian Blue, pH 0·5, × 135. **7.** Collagenase buffer control, PAS, × 135. **8.** Collagenase digestion, PAS, × 135.



Figures 9 and 10. 9. Saline incubated control, Pb citrate, × 25,000. **10.** Control section, PA-CA-Meth., × 30,000. Ep, Epithelial cells; T, tubule; GBM, glomerular basement membrane.



Figures 11 and 12. 11. Neuraminidase digestion, PA-CA-Meth, × 40,000. **12.** Hyaluronidase digestion, PA-CA-Meth., × 40,000. Ep, Epithelial cells; GBM, Glomerular basement membrane.



Figures 13 and 14. 13. Chondroitinase ABC digestion, PA-CA-Meth., × 40,000. **14.** Neura minidase digestion, Bowman's capsule, PA-CA-Meth., × 40,000. Ep, Epithelial cells; GBM, Glomerular basement membrane.

in the glomerular and non-glomerular regions of the rat nephron. The glomerulus was found to contain considerable amounts of sialoproteins located primarily in the GBM, and on the surface of epithelial and endothelial cells. Studies using enzyme perfusion methods, as well as cationic probes have previously identified the presence of sialoproteins on epithelial and endothelial surfaces (Mohos and Skoza, 1969; Andrews, 1978; Kanwar and Farquhar, 1979a, 1980) but failed to identify the sialoproteins within the GBM. Kanwar and Farquhar (1980) have reported that the removal of sialic acid from the above regions, by means of perfusion with neuraminidase, resulted in the detachment of these processes from the GBM. We did not observe such alterations in the ultrastructure of the glomerulus resulting from the enzymatic removal of sialic acid (figure 11).

Identification of sialoproteins within the GBM has been primarily based on the analysis of solubilized GBM (Sato *et al.,* 1975; Spiro, 1976). Recently, the use of specific antibodies has (Madri *et al.,* 1981) identified the presence of sialoproteins such as fibronectin and laminin within the GBM in mesangial regions (Oberley *et al.,* 1979; Courtoy *et al.,* 1980; Madri *et al.,* 1981).The present study provides additional data which demonstrates the presence of these anionic polysaccharides in GBM by means of enzymatic digestion and histochemical staining.

In addition to sialoglycoproteins, we found the glomerulus to contain considerable amounts of GAGs. Even though the GAGs have been isolated and characterized from renal tissue by many investigators (Allalouf *et al.,* 1964; Castor and Green, 1964; Farber and Van Praag, 1970; Murata, 1976) the distribution of most of these macromolecules in the nephron, with the exception of heparan sulphate in GBM, has not been explored (Kanwar and Farquhar, 1979; Cohen 1980; Parthasarathy and Spiro, 1980; Linker *et al.,* 1981). The present results indicate that the GAGs which are digestible by chondroitinase ABC are predominantly located in the lamina interna, and lamina externa of GBM. The presence of chondroitin sulphate and dermatan sulphate in these regions, as indicated by this observation, has not hither to been reported. The GBM still retained some reactivity to Alcian Blue (pH 0·5) stain for sulphated GAG, even after digestion with chondroitinase ABC, indicating the presence of heparan sulphate.

In addition to the sulphated GAGs, the GBM was also found to contain small amounts of hyaluronic acid, as indicated by the reduction of silver-methenamine stain, upon incubation with hyaluronidase. The presence of this GAG in GBM extracts has been suggested previously (Kanwar and Farquhar, 1979), but has not been confirmed until now. The reduction in the silver-methenamine stain was most notable at the interface of the GBM and the endothelial process. Upon the removal of hyaluronic acid, this region was often found to be unstable indicating that this GAG may have a role in the stability of the attachment of these processes to the GBM.

Heparan sulphate has been implicated as the anionic site of the GBM, regulating the filtration of anionic plasma proteins (Kanwar and Farquhar, 1979a). While the role of chondroitin sulphate and dermatan sulphate in GBM is not clearly understood, these sulphated GAGs are known to influence the fibrillogenesis of collagen (Obrink, 1973). The presence of collagen in the GBM, as noted in the electron microscopic studies as well as by the earlier reports (Westberg and Michael, 973; Trelstad and Lawlye, 1977). suggests that this protein and the GAGs may have an important role in the structure of the glomerulus.

Although collagen has been identified in isolated GBM and BC, it is not usually demonstrable in electron micrographs. The PA–CA–Ag methenamine technique is very reactive for collagen and we have found isolated strands of collagen fibers located on the epithelial and mesangial side of GBM, and throughout the width of BC (figure 14).

The present study also demonstrates the polysaccharide composition of TBM and BC. As has been reported earlier (Munakata *et al.,* 1978) in isolated TBM, our study indicated the presence of sialoglycoproteins in this region. The concentration of this macromolecule was found to be somewhat lower in this region than the GBM which is in accord with earlier biochemical studies (Langeveld and Veerkamp, 1981). In addition to sialoproteins, TBM also contained sulphated GAGs, which were mostly digestible by chondroitinase ABC. It is not clear whether these glucoconjugates of TBM have a role in the tubular reabsorption process during urine production

BC has a lower content of sialoproteins and hyaluronic acid than GBM. This region appeared to contain predominantly sulphated GAG, especially HS. The significance of this finding is not immediately understood. Nevertheless, a knowledge of the distribution of various polysaccharides at the ultrastructural level will be valuable in future studies on the role of these macromolecules in normal and pathological renal tissues.

Acknowledgements

This work was supported by the New York Diabets Association and by NIH 5-SO7RRO5393. The authors wish to thank Ms. Kimberly Alexander and Ms. Deborah Shelton, who were supported by the Rockefeller Aoundation-Howard University Program, for their technical assistance.

References

Ada, G. L., French, E. L. and Lind, P. E. (1961) *J. Gen. Microbiol,* **24,** 409.
Allalouf, D., Ber, A. and Sharon, N. (1964) *Biochim. Biophys. Acta,* **83,** 278.
Andrews, P. M. (1978) *Am. J. Anat.,* **153,** 291.
Berenson, G. S., Ruiz, H., Dalferes, E. R., Dugan, F. A. and Radhakrishnamurthy, G. (1970) *Diabetes,* **19,** 161.
Brownlee, M. and Spiro, R. G. (1979) *Diabetes,* **28,** 21.
Carlson, E. C, Brendel, K., Hjelle, J. T. and Meezan, E. (1978) *J.Ultrastruct. Res.,* **62,** 26.
Castor, C. W. and Greene, J. A. (1968) *J. Clin. Invest,* **47,** 2125.
Cohen, M. P. (1980) *Biochem. Biophys. Res. Commun,* **92,** 343.
Courtoy, P. J, Kanwar, Y. S., Hynes, R. O. and Farquhar, M. G. (1980) *J. Cell Biol,* **87,** 691.
Farber, S. J. and Van Praag, D. (1970) *Biochim. Biophys. Acta.,* **208,** 219.
Gisslow, M. T. and McBride, B. C. (1975) *Anal. Biochem.,* **68,** 70.
Kanwar, Y. S. and Farquhar, M. G. (1979a) *J. Cell Biol.,* **81,** 137.
Kanwar, Y. S. and Farquhar, M. G. (1979b) *Proc. Natl. Acad. Sci.,* **76,** 1303 4493.
Kanwar, Y. S. and Farquhar, M. G. (1980) *Lab. Invest,* **42,** 375.
Kefalides, N. A. (1974) *J. Clin. Invest,* **53,** 403.
Langeveld, J. P. M. and Veerkamp, J. H. (1981) *Comp. Biochem. Physiol,* **B68** 31.
Linker, A., Hovingh, P., Kanwar, Y. S. and Farquhar, M. G. (1981) *Lab. Invest,* **44,** 560.
Madri, J. A., Roll, F. J, Furthmayr, H. and Fodiart, J. M. (1981) *J. Cell Biol,* **86,** 682.
Malathy, K. and Kurup, P. A. (1972) *Diabetes,* **21,** 1162.
Mohos, S. C. and Skoza, L. (1969) *Science,* **164,** 1519.
Munakata, H., Sato, T. and Yoshizawa, Z. (1978) *Tohoku. J.Exp. Med.,* **125,** 77.

Murata, K. (1976) *Connect. Tiss. Res.,* **4,** 131.
Nevins, T. E. and Michael, A. F. (1981) *Kid. Int.,* **19,** 553.
Oberley, T. D., Mosher, D. F. and Mills, M. D. (1979) *Am. J. Pathol.,* **96,** 651.
Obrink, E. (1973) *Eur. J. Biochem.,* **34,** 129.
Parthasarathy, N. and Spiro, R. G. (1980) *J. Biol. Chem.,* **256,** 507.
Pinter, G. G. (1967) *Experientia.* **23,** 100.
Rambourg, A. (1967) *J. Histochem. Cytochem.,* **15,** 409.
Rambourg, A. (1971) *Int. Rev. Cyt.,* **31,** 57.
Reeves, W. H., Kanwar, Y. S. and Farquhar, M. G. (1980) *J. Cell Biol,* **85,** 735.
Revetto, C. (1984) *J. Histochem. Cytochem.,* **12,** 44.
Saito, T., Yamagata, T. and Suzuki, S. (1968) *J, Biol. Chem.,* **243,** 1536.
Sato T., Munakata, H., Yoshinaga, K. and Yoshizawa, Z. (1975) *Tohoku. J. Exp. Med.,* **115,** 229.
Saraswathi, S. and Vasan, N. S. (1982) *Biochim. Biophys. Acta.,* **755,** 237.
Scheinman, J. I., Fish, A. J., Matas, A. J. and Michael, A. F. (1978) *Am. J. Pathol.,* **90,** 71.
Spiro, R. G. (1976) *Diabetologia,* **12,** 1.
Tesoriero, J. V. (1977) *J. Histochem. Cytochem.,* **25,** 1376.
Tesoriero,J. V. (1981) *Anat. Rec,* **199,** 254.
Trelstad, R. L. and Lawley, K. R. (1977) *Biochem. Biophys. Res. Commun.,* **76,** 376.
Westberg, N. G. and Michael, A. F. (1973) *Med. Scand.,* **194,** 39.
Yuki, H. and Fishman, W. H. (1963) *J. Biol. Chem.,* **243,** 1877.