Human Glomerular Basement Membrane. Preparation and Composition*

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ABSTRACT: In order to isolate relatively pure human glomerular basement membrane it was necessary to modify existing preparative methods. Some glomeruli were very resistant to sonication and were removed using a fine mesh sieve. Centrifugation of sonicated glomeruli at higher *g* forces gave increasingly contaminated preparations with decreasing content of 4-hydroxyproline and increasing amounts of phospholipids. A low content of phospholipids was a more sensitive and reliable indicator of the purity of the preparation than light and electron microscopy. Centrifugation in 1 M sodium chloride at a very low centrifugal force (121*g*) gave purer preparations but low yields. Phospholipids were present in low amounts (0.82 μg/mg of lipid phosphorus). The relative composition of phospholipids and cholesterol was very similar in the basement membrane preparations, in basement membrane prepared according to Krakower and Greenspon, in glomeruli, and in cortex. A high amount of serum proteins (about 3.5%) in the extensively sonicated and washed preparations was found after incubation with collagenase in the relative concentration fibrin(ogen) > immunoglobulin G > β-1-*C*-globulin > immunoglobulin M. The chemical composition is similar to that reported for bovine and canine glomerular basement membranes. The concentrations of galactose, 3.33%, glucose, 3.09%, 3-hydroxyproline, 17.3 μM/100 mg, 4-hydroxyproline, 63.3 μM/100 mg, and hydroxylysine, 20.3 μM/100 mg, are higher than previously reported. Sialic acid was found to be present in the membrane in an amount (0.93%) not significantly different from that found in whole glomeruli (0.86%). By histochemical techniques, the sialoprotein in the basement membrane did not bind cationic stains.

Since the description in 1951 by Krakower and Greenspon of a method to isolate GBM,[1] a number of biochemical studies have been reported. The basement membrane has been found to share several characteristics with collagen, but the high ratio of hydroxylysine to hydroxyproline and the high content of carbohydrates are important differences. The ground substance, so characteristic of connective tissue in general, has not been observed in GBM using morphological techniques, and acidic mucopolysaccharides have not been found.

The published biochemical data derived from analyses of GBM from different species show considerable variability, in some cases related to the presence of impurities. In order to understand the changes in the GBM induced by disease, it is essential to establish well-controlled techniques for obtaining GBM preparations that have reproducible biochemical composition.

The present report describes the isolation and composition of relatively pure HGBM.

Material and Methods

Material. In most cases, kidneys were obtained at autopsies performed within 12 hr of accidental death. Kidneys from patients who died of nonaccidental causes were used only if there had been no history of kidney disease, and if kidney function had remained normal until death. Patients treated with cytotoxic or nephrotoxic drugs or corticosteroids were excluded.

Preparation of Glomeruli. The isolation procedure is based on the method of Krakower and Greenspon (1951) as modified by Spiro (1967a). Frozen kidneys were thawed almost completely in a plastic bag immersed in water (22–24°). During the entire procedure care was taken to keep the tissue on ice as much as possible. The medulla was dissected away from the cortex and discarded. The weights of the kidney and of the cortex were recorded. The cortex was cut into very small pieces with a razor blade.

Approximately one-fifth of the cortex was then transferred to a 100-mesh stainless steel sieve (pore size 140 μ). The tissue was forced through the sieve with moderate pressure using the bottom of a small flask and repeated washings with cold 0.85% sodium chloride solution, 0.5–1 l. for one adult-size kidney. The pressing was continued for about 10 min per application or for 1 hr per kidney; material remaining on the screen was discarded. A clean screen was used for each application of tissue. The sieved suspension was then poured through a 60-mesh (pore size 230 μ) sieve, which retained a few bigger tissue fragments, and finally through a 200- or 250-mesh sieve (opening 74 or 61 μ). The material retained on the fine sieve was then extensively washed with cold 0.85% sodium chloride, 1–2 l. per kidney, until nothing but glomeruli and, possibly, some tubular fragments attached to glomeruli

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[1] Abbreviations used are: GBM, glomerular basement membrane; HGBM, human glomerular basement membrane; sem, standard error of mean.

could be seen when the sample was examined by phase contrast microscopy. This material was washed into a beaker, transferred to 50-ml plastic centrifuge tubes, and centrifuged at 1080*g* at 4° for 10 min. A sample of the sediment was examined by phase-contrast microscopy, and the number of nonglomerular fragments per 100 glomeruli counted. In order to estimate the total number of glomeruli, the sediment was carefully suspended in 30 ml of isotonic saline, and with the test tube agitated on a mixer, a 50-μl sample was taken with a micropipet with a broken tip. The sample was spread on a glass plate with a chessboard pattern drawn on its backside. The glomeruli on the plate and those remaining in the pipet were counted under a magnifying glass. From an average of three samples the total number of glomeruli in the preparation was calculated.

In order to prepare glomeruli, the sediment was washed four times in distilled water with centrifugations at 1080*g* at 4° for 10 min and lyophilized.

Preparation of HGBM. The glomerular sediment was suspended in approximately 30 ml of cold 1 M sodium chloride in a plastic tube. The suspension was sonicated in an ice bath using a Blackstone ultrasonicator SS-2 400 with a 0.5-in. stainless steel probe. The sonicator was set at power output "40" and used in 30-sec bursts with 1 min cooling of the probe in ice water between bursts. After a total sonication time of 4 min, and allowing a few minutes for the glomeruli to settle, the suspension was sampled from the bottom of the tube and examined under the phase-contrast microscope. Sonication was continued with frequent microscopic examination until no further disruption of the glomeruli could be seen, but not for a period longer than 7 min.

The suspension was then passed through a 250-mesh stainless steel sieve to remove insufficiently disrupted glomeruli and tubular fragments. After cautious washing of the retained material with 100–200 ml of 1 M sodium chloride, the filtrate was transferred to 50-ml tubes and centrifuged at 121*g* for 15 min at 4°. The supernatant was aspirated, the sediment resuspended in 1 M sodium chloride, and the centrifugation repeated three more times at the same speed for 15 min each time. The supernatants were discarded. The sediment was suspended in 50 ml of distilled water, washed, centrifuged four times at 4340*g* for 5 min, and finally lyophilized.

In four separate experiments using four different human kidneys the influence of the centrifugal force on the yield and purity of HGBM isolated from sonicated glomeruli was studied. In each experiment the supernatants after four centrifugations at 121*g* were combined and centrifuged at 480*g* for 15 min in 1 M sodium chloride. The 480*g* sediment was washed three more times and the supernatants were combined, spun in 1 M sodium chloride at 1085*g* for 15 min four times, and the supernatant was again centrifuged in the same way at 4340*g*. The four sediments (121*g*, 480*g*, 1085*g*, 4340*g*) were each washed with distilled water four times with centrifugation at 4340*g* for 5 min, and lyophilized. The final 4340*g* supernatant was dialyzed against distilled water and lyophilized.

The influence of sonication time on the quality of the preparations was studied by sonicating three different glomerular preparations 3, 5, and 7.5 min, respectively.

An attempt was also made to obtain a pure HGBM preparation using 0.5% Triton X-100 (octylphenoxypolyetoxyethanol, Sigma Chemical Company) in 1 M sodium chloride during centrifugation of the sonicated glomeruli. For this experiment, one-third of a pool of sonicated glomeruli from four kidneys was used. Another third was centrifuged in distilled water and the last third in 1 M sodium chloride, in both cases without detergent.

The influence on purity and yield of HGBM of intense pressing of the cortex through a 100-mesh screen for 30 min as compared with more gentle pressing for 5 min was studied in separate experiments.

Light and Electron Microscopy. For electron microscopy the basement membranes were sampled after washing in distilled water. The HGBM was fixed in cold 2% glutaraldehyde buffered in sodium cacodylate at pH 7.4, embedded in Vestopal, and stained with uranyl acetate and lead citrate. The electron micrographs were taken in a Phillips EM-200 electron microscope. Thin sections of sediments fixed and embedded for electron microscopy were stained with toluidin blue for light microscopy. To identify polyanions, preparations of glomeruli and HGBM were stained with alcian blue 8GX at varying pH values between pH 1.0 and 2.5 (Pearse, 1968) and with colloidal iron at pH 1.9 and 2.5 (Mowry, 1963).

Lipid Analysis. Phospholipids were analyzed after extraction at room temperature under nitrogen with an 2-propanol–chloroform mixture, 11:7 (Rose and Oklander, 1965). The extract was dried under nitrogen, dissolved in chloroform–methanol (2:1), and washed according to the method of Folch (1957). Phosphorus was determined on the extract with the Fiske–Subbarow reagent (Bartlett, 1959) after digestion with concentrated sulfuric acid and perchloric acid on a sand bath at 235–260° for 1 hr. Cholesterol was then determined with the method of Zlatkis *et al.* (1953). The extracted phospholipids from cortex, glomeruli, GBM prepared according to Krakower and Greenspon, and GBM preparations obtained with the technique here described were partitioned by thin-layer chromatography on silica gel (silica gel HR Extra Pure, E. Merck, A. G.) using a modification of the method described by Parker and Peterson (1965). The samples, dried under nitrogen, were dissolved in a small volume of chloroform–methanol, 2:1. Duplicates were applied on the heat-activated plate and chromatography was carried out with the solvent mixture chloroform–methanol–acetic acid–water (25:15:4:2). Spots were developed in iodine atmosphere, marked with a needle, and scraped off. Digestion and assay of samples and blanks were performed as for total phospholipids.

Phospholipids were identified by comparison with reference standards (bovine phosphatidylserine, phosphatidylethanolamine, lysolecithin, sphingomyelin, and egg lecithin—from Applied Science Laboratories, Inc. State College, Pa; and bovine phosphatidylinositol from Koch-Light, Inc. Colnbrook, England), and staining reactions using ninhydrin and Dragendorf's reagents. In this chromatographic system the glycerol ether analog of lysophosphatidylethanolamine lies between phosphatidylserine and phosphatidylcholine, a site also occupied by phosphatidylinositol (A. F. Michael and J. Clausen, in preparation; Skipski *et al.*, 1964); those two lipids were not separated and the values are therefore reported together.

Amino Acid Analysis. For the determination of amino acids, the HGBM was hydrolyzed in 6 N hydrochloric acid, 2 ml/mg of HGBM dry weight, at 105° for 21 hr in sealed glass ampoules that had been flushed with nitrogen before sealing. The hydrochloric acid was evaporated in rotating evaporation flasks and the analysis was carried out in a Beckman Model 120B amino acid analyzer. For the acidic amino acids Bio-Rad

Aminex A-4 was used in a column 55-cm long and the sample was eluted with a 0.2 N sodium citrate buffer first at pH 2.93 and then at pH 4.00 at 55°. The basic amino acids were separated on a 15-cm column of Aminex A-5 with a 0.38 N sodium citrate buffer at pH 5.35. Recovery of amino acid standards added to duplicate samples was 99%. The average per cent differences in the concentration (μg/mg) of individual amino acids between replicate analysis of HGBM samples was 8%, and for 4-hydroxyproline, 3%. Methionine and cystine were analyzed according to the method described by Moore (1963) but with lyophilization instead of pervaporation of the oxidized sample. The lyophilized sample was hydrolyzed in 6 N hydrochloric acid for 21 hr at 105°. The acid was evaporated under high vacuum at 40° and the sample dissolved in 200 μl of 0.2 N sodium citrate buffer, pH 2.2, and analyzed on the amino acid analyzer on the 55-cm column, using a 0.2 N sodium citrate buffer, pH 2.93, for elution; 97% of cystine and 93% of methionine were recovered. The results reported are not corrected for recovery. Tryptophan was not analyzed.

Total Nitrogen. Total nitrogen was determined with a micro-Kjeldahl procedure (Koch and McKeekin, 1924) on four samples in duplicates.

Carbohydrate Composition. The neutral sugars were released by hydrolysis in 2 N hydrochloric acid for 2.5 hr at 100° in capped tubes. This hydrolysis time had been found to give the highest values for both glucose and galactose in preliminary experiments. The hydrolysate was neutralized with 2 N sodium hydroxide, passed through an 0.9 × 9.0 cm mixed column of Amberlite IR-45 in the OH^- form and Dowex 50W-X8 in the H^+ form, and eluted with water (Tengström, 1966); 20 ml was collected and lyophilized. The sample was then assayed for glucose (Hsia and Inouye, 1966) with glucose oxidase (Glucostat Special, Worthington Biochemical Corp.) and for galactose (Hjelm, 1967) with galactose oxidase (Galax, AB Kabi, Sweden). Recoveries for glucose and galactose run through the entire procedure were 89 and 87%, respectively.

For the quantitative assay of mannose a method using thin-layer chromatography on cellulose was developed. HGBM (3–4 mg) was hydrolyzed, eluted from ion-exchange columns, and lyophilized as described above for glucose and galactose. The lyophilized sample was dissolved in 50 μl of distilled water and applied to thin-layer cellulose plates (Avicel microcrystalline cellulose powder, E. Merck, A. G.) with duplicates applied to different plates. Two standards containing known amounts of glucose, galactose, mannose, and fucose were run on each plate. Ethyl acetate–pyridine–water (15:5:4) was used as the solvent and the plates were developed three times with drying with a hair drier between each development. The plate was sprayed with an indicator spray (Gardner, 1955) and dried for a few minutes at 70°. The spots were marked under ultraviolet light and scraped from the plate with a razor blade. Blanks from the same height of the plate were also removed; the cellulose from each spot or blank was weighed. The sugars were eluted for 2–4 hr with 2 ml of distilled water with forceful shaking at 37°. The cellulose was retained on a 2-ml sintered glass funnel and washed twice with 1 ml of distilled water, and the sample was analyzed with the ferrocyanide method (Park and Johnson, 1949) with mannose as standard. The recoveries for mannose when applied to the plate was 104% (range 90–112%) and when added to a sample of HGBM 99%. Two different samples analyzed in duplicate showed a difference of 6 and 8%, respectively, between the duplicates.

Fucose was determined by the method of Dische and Shettles (1948) with a 3-min heating period. Sample blanks to which no cysteine was added were included.

Uronic acid was analyzed with the carbazole reaction of Dische (1949) using the modification of Knutson and Jeanes (1968); the influence of neutral hexoses on the reaction was also studied.

Amino sugars were determined after hydrolysis of 2–4 mg of HGBM in 2 ml of 6 N hydrochloric acid for 4 hr at 95°. The samples were evaporated under vacuum, dissolved in buffer, and applied to the 15-cm column of the amino acid analyzer. Elution was carried out with 0.23 N citrate buffer, pH 5.35. Recoveries for glucosamine was 100% and for galactosamine 81% when standards were run through hydrolysis and column separation.

Sialic acid assay was performed according to Warren (1959) after hydrolysis in 0.1 N H_2SO_4 and 1 ml per mg of HGBM at 80° for 60 min.

Deoxyribonucleic Acid. Deoxyribonucleic acid was measured by the method of Paul (1956).

Estimation of Serum Proteins in HGBM. To solubilize the HGBM for the study of serum proteins included within the membrane, a sample was enzymatically hydrolyzed by collagenase (Spiro, 1967b). The hydrolyzed HGBM was then studied for the presence of serum proteins by double diffusion in agar (Ouchterlony, 1958) with and without the use of a Plexiglas template (Wadsworth, 1962) for continuous feeding of the reactants. A quantitative assay was carried out by radial diffusion in agar (Mancini *et al.*, 1965) using rabbit antisera to human IgG (Immunology, Inc.), human IgM (Chaplin *et al.*, 1965), β-1-*C*-globulin (Mardiney and Müller-Eberhard, 1965), human albumin (Armour Pharmaceutical Co.), human fibrinogen (Blombäck and Blombäck, 1956), ceruloplasmin (Behringwerke, A. G.), and α-2-macroglobulin (Hyland).

Water Content. Samples were stored in the lyophilized state in capped vials at −20° and were allowed to equilibrate with room air for several hours prior to weighing and analysis. To determine the water content, approximately 10 mg of the preparations were first weighed in room air and then dried for several days at room temperature in a desiccator containing fresh anhydrous calcium sulfate (Drierite) and phosphorus pentoxide and then reweighed in a dry atmosphere.

Electrolyte Content. Sodium was extracted from lyophilized HGBM (1–3 mg) in 0.2 ml of 2 N nitric acid. The solution was dried on a sand bath at 160° and the amount of sodium was measured by a flame photometer with an internal lithium standard. The amount of chloride was determined by silver nitrate titration. An acidic solution (4 ml) [glacial acetic acid (100 ml)–concentrated nitric acid (6.4 ml)–water (to 900 ml)] was added to 1–2 mg of lyophilized HGBM. Calcium was extracted from 1 to 2 mg of HGBM with 1 ml of a mixture of concentrated acetic acid–72% perchloric acid, (5:1), dried on a sand bath at 260°, and measured with atomic absorption. Test tubes to which extraction media had been added were included as blanks in the methods for sodium, chloride, and calcium.

Results

Preparation of Glomeruli. Approximately 500,000 glomeruli could be isolated from each kidney; the average dry weight of five isolated lyophilized glomerular preparations was 142 mg per kidney (range 61–178 mg). The preparations did not con-

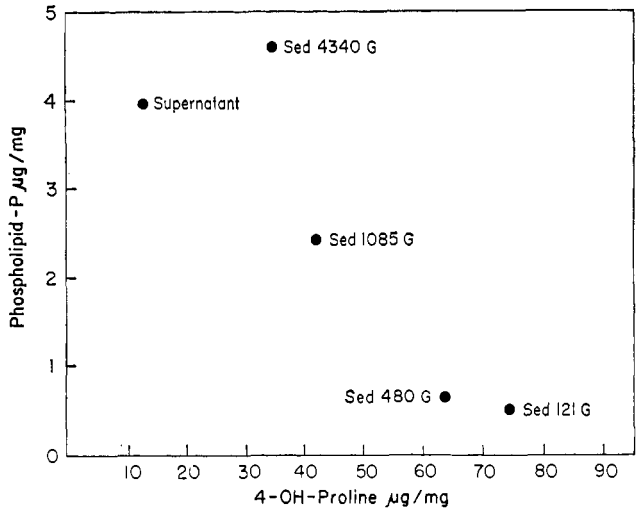

FIGURE 1: Relation between 4-hydroxyproline and phospholipid phosphorus in sediments of sonicated glomeruli, centrifuged with increasing centrifugal forces.

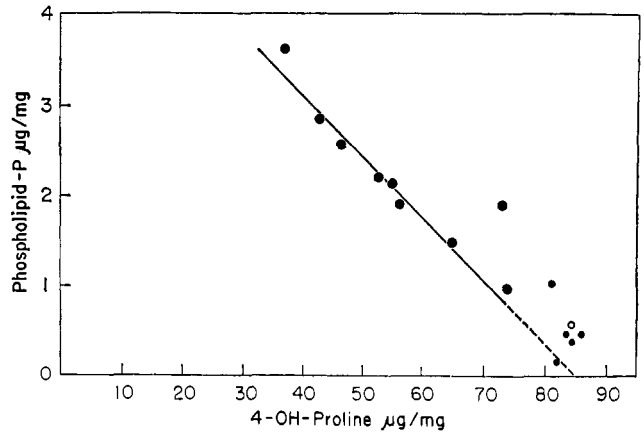

FIGURE 2: Relation between 4-hydroxyproline and phospholipid phosphorus in preparations using the technique of Krakower and Greenspon (1951) (big circles) and in samples obtained with the technique by the authors (small circles). The open circle indicates the average value found in this investigation.

tain any free fragments of tubules or other tissues, but small tubular segments were often attached to the glomeruli (15–25 fragments per 100 glomeruli), representing at the most a few per cent of the total volume of the glomerular preparations. Prolonged pressing of the cortex on the 100-mesh sieve increased the number of tubular fragments. Matrial remaining on the 100-mesh sieve contained many glomeruli when examined microscopically after fixation and staining. When fresh kidneys were used instead of frozen kidneys, there were less tubular contamination and higher glomerular yields. A significant number of glomeruli passed through the 200- and even the 250-mesh sieve during the extensive washing.

Preparation of Glomerular Basement Membrane. INFLUENCE OF CENTRIFUGAL FORCE, TIME, AND DENSITY ON ISOLATION OF HGBM. The effect of using increasing centrifugal forces on the sedimentation of sonicated glomeruli is shown in Figure 1. The concentration of phospholipid increases and that of 4-hydroxyproline decreases in the sediments obtained with increasing *g* force.

In earlier experiments when HGBM was prepared according to the original method of Krakower and Greenspon (1951), a highly variable quality of HGBM was observed. There was a rectilinear inverse relation between the amounts of phospholipids and 4-hydroxyproline (Figure 2). In these experiments glomeruli were isolated by sedimentation without the use of sieves and the centrifugation of sonicated glomeruli was done at a rather low speed, 1500 rpm (270*g*); the variability may have been caused by varying amounts of nondisrupted glomeruli and tubular contaminants in the preparation.

Using the method described for HGBM isolation (*i.e.*, the 121*g* sediment) the average yield of lyophilized HGBM is 38 mg (range 5–120 mg) dry weight per adult-size kidney or 0.25 mg/g of kidney (0.41 mg/g of cortex). In a pool of glomeruli from four kidneys the dry weight was 192 g per glomerulus and the 4-hydroxyproline content 40 μg/mg.

The sediment obtained by using distilled water for the 121*g* centrifugation contained 2.48 μg/mg of phospholipid phosphorus, whereas the sediment obtained by centrifugation in 1 M sodium chloride contained 1.16 μg/mg. HGBM isolated in 0.5% Triton X-100 had the lowest phospholipid phosphorus value—0.18 μg/mg. The yield and quality of preparations obtained after 3, 5, or 7.5 min of sonication were approximately the same when evaluated by microscopy and chemical analysis for 4-hydroxyproline and phospholipids.

Microscopy of Isolated HGBM. Microscopic examination of 1-μ thin sections of sediments obtained at different centrifugal forces revealed striking differences in the degree of contamination corresponding to the amount of cell debris seen (Figure 3 a–d) and the amount of phospholipid present. However, in HGBM preparations obtained by centrifugation of sonicated glomeruli at 121*g*, there was no correlation between the amount of contaminants and the phospholipid content. Electron microscopy indicates that this HGBM is free of cells or recognizable cell fragments (Figure 4) but considerable amounts of debris are seen, probably trapped between the isolated membranes during centrifugation. The nature of this debris is unknown but may be derived from cells, or from aggregated "fibrilles" of basement membrane. As seen in Figure 5, the membranes appear to have become "frayed" during isolation.

The tubular fragments that remained attached to the glomeruli during the preparation showed a high resistance to sonication and a significant number were retained on the 250-mesh screen.

Staining of isolated glomeruli with alcian blue and colloidal iron revealed the presence of a material reactive as a polyanion on the epithelial cell membrane adjacent to the GBM. The isolated HGBM did not stain with colloidal iron at pH 1.9 or 2.5 but stained weakly with alcian blue.

The nondisrupted glomeruli retained on the 250-mesh screen after sonication were subjected to sonication for a total period of 20 min. No disruption was seen, suggesting that these glomeruli may have been hyalinized.

Lipid Composition. Only HGBM preparations obtained with the technique described above and with a phospholipid phosphorus content of less than 1.2 μg/mg are included. Of 33 preparations only one had to be excluded because of high phospholipid content.

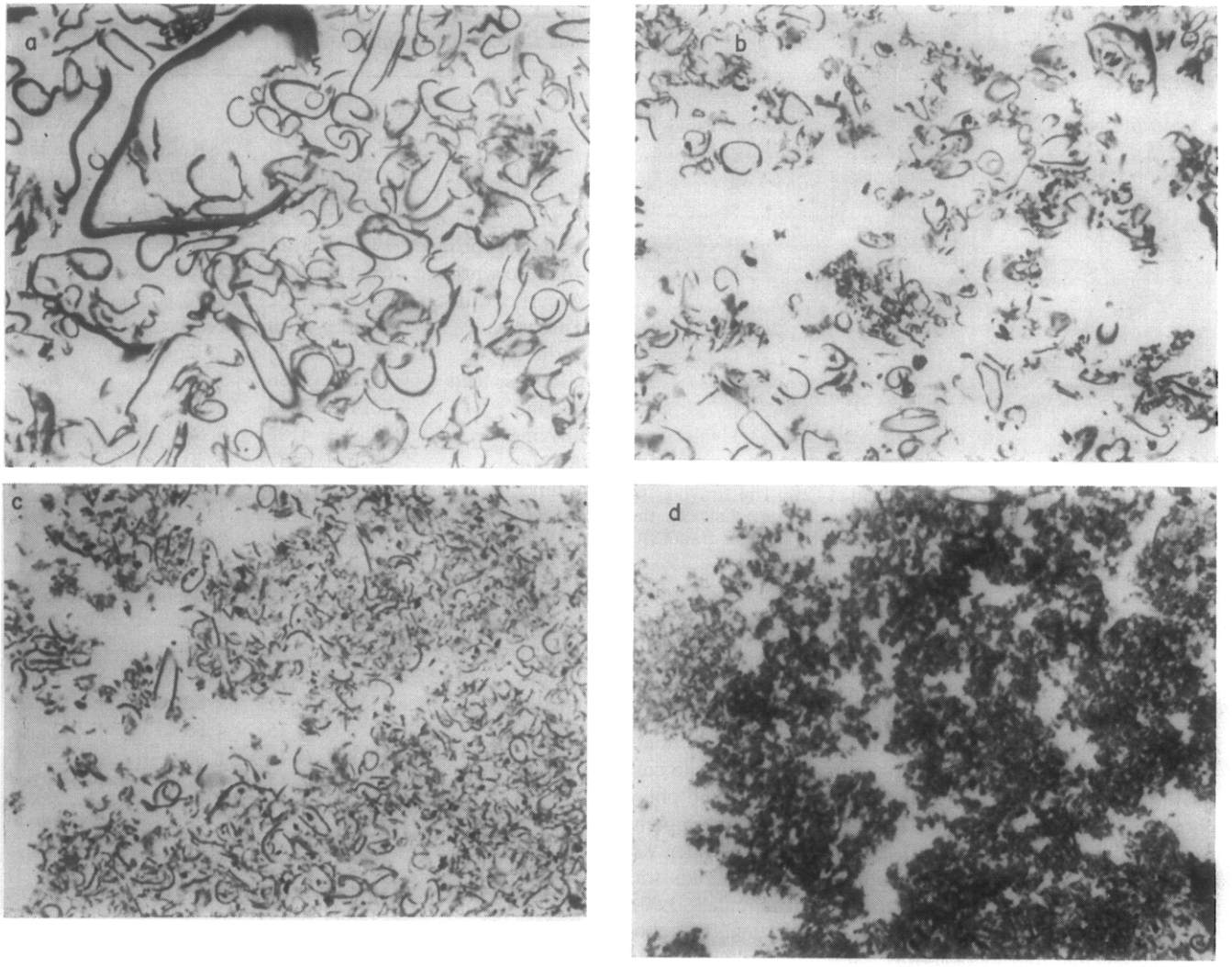

FIGURE 3: Sediments of sonicated glomeruli centrifuged at (a) 121*g*: note absence of recognizable cell fragments. The big heavy structure is thought to be a fragment of Bowman's capsule; (b) 480*g*: the basement membrane fragments are smaller and the amount of debris is greater; (c) 1085*g*: small fragments of HGBM and more debris; (d) 4340*g*: only a few membrane fragments seen in the preparation, which apparently consists mostly of subcellular material (toluidine blue, magnification of approximately 715).

TABLE I: Phospholipid and Cholesterol Composition of Cortex, Glomeruli, "Crude" HGBM, and "Pure" HGBM.[a]

	Cortex (13)[b]	Glomeruli (3)	HGBM Crude[c] (12)	HGBM Pure[b] (3)
Cholesterol (μg/mg)	18.8 ± 4.0	25.3 (23.7–27.8)	18.9 ± 6.5	3.9 (2.6–5.4)
Phospholipid (μg/mg)	69.0 ± 13.4	76.0 (69.8–82.1)	49.4 ± 19.3	10.2 (9.8–10.7)
Lysolecithin (%)	N.E.[d]	3.4 (2.8–5.1)	N.E.[d]	4.3 (2.3–7.0)
Sphingomyelin (%)	16.0 ± 1.8	21.4 (18.0–23.5)	18.8 ± 2.2	23.0 (18.7–26.7)
Lysophosphatidylethanolamine (glycerol ether) and phosphatidylinostol (%)	6.9 ± 2.9	N.E.[d]	7.3 ± 4.3	N.E.[d]
Phosphatidylcholine (%)	36.2 ± 4.5	36.0 (32.7–34.5)	36.5 ± 2.2	39.0 (38.1–40.5)
Phosphatidylserine (%)	11.3 ± 2.2	11.9 (11.0–12.9)	11.9 ± 2.0	13.7 (11.7–14.8)
Phosphatidylethanolamine (%)	23.2 ± 8.0	23.9 (19.6–25.0)	22.4 ± 3.2	12.4 (10.1–14.8)
Recovery (%)	93.6	96.6	96.9	92.4

[a] Mean and standard deviation or range are given. [b] The figures in parentheses indicate the number of preparations analyzed. [c] HGBM crude is prepared by the Krakower–Greenspon method. HGBM pure is prepared as described under Methods. [d] N.E., Not evaluated.

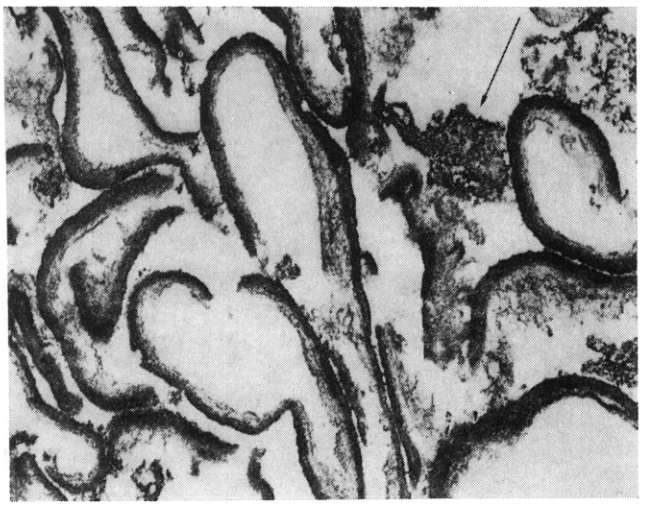

FIGURE 4: Electron micrograph of sediment at 121*g*. The "fraying" of the membrane is clearly visible. The aggregate seen between the membranes (arrow) may be derived from such split-off fragments (×8700).

The average phospholipid phosphorus content of all preparations was 0.82 ± 0.05 μg/mg of dry weight (average ± sem of 32 preparations) corresponding to 20.9 ± 1.3 μg/mg of phospholipid, assuming that phosphorus accounts for 4% of the weight of the phospholipids. There was no correlation between the amounts of 4-hydroxyproline and phospholipids in those preparations. The 4-hydroxyproline content was very constant, 7.15 ± 0.037 g (range 7.01–7.28) per 100 g of residue weight while the amount of phospholipids was highly variable, 2.09 ± 0.13 g per 100 g, (range 0.49–3.00). The amount of cholesterol was 7.5 ± 0.7 μg/mg. The cholesterol:phospholipid phosphorus ratio averaged 10.2 and was of the same magnitude in preparations with low and with high phospholipid content. The cholesterol:phospholipid phosphorus ratio in the experiments with sedimentation at higher speeds averaged also 10.2 with no tendency to change with increasing *g* force. The ratio in earlier experiments using the Krakower–Greenspon method averaged 9.6.

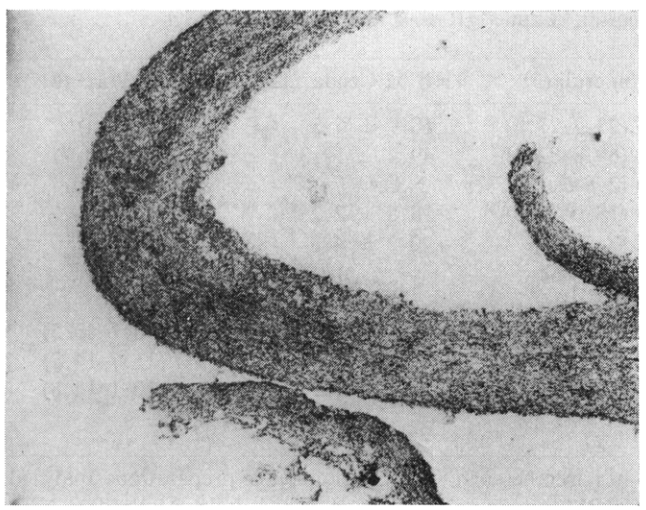

FIGURE 5: Electron micrograph of sediment at 121*g*. Note normal structure and complete absence of cell fragments (×16,400).

TABLE II: Chemical Composition.

	Amounts,[a] μM/100 mg	Residue Weight, g/100 g	Residues per 1000 Amino Acid Residues[b]
3-Hydroxyproline	17.3 ± 3.23	1.96	22.2
4-Hydroxyproline	63.3 ± 0.34	7.15	81.3
Aspartic acid	52.7 ± 2.22	6.07	67.7
Threonine	28.6 ± 2.10	2.89	36.7
Serine	38.1 ± 1.59	3.32	48.9
Glutamic acid	76.6 ± 3.86	9.90	98.3
Proline	45.1 ± 2.93	4.38	57.9
Glycine	172 ± 4.02	9.83	221
Alanine	46.6 ± 5.37	3.30	59.8
Valine	27.7 ± 2.18	2.75	35.6
Methionine	10.3 ± 1.08	1.36	13.2
Isoleucine	24.4 ± 1.98	2.76	31.3
Leucine	50.9 ± 3.19	5.76	65.3
Tyrosine	11.9 ± 0.99	1.96	15.3
Phenylalanine	19.4 ± 1.25	3.09	24.9
Hydroxylysine	20.3 ± 2.61	1.95	26.1
Lysine	15.2 ± 2.24	2.93	19.5
Histidine	11.1 ± 1.51	1.52	14.2
Arginine	29.6 ± 4.15	4.63	38.0
Half-cystine	17.8 ± 0.75	1.83	22.8
	779 ± 1.13[c]	79.4	
Sialic acids[d] (11)[e]	2.99 ± 0.15	0.88	
Glucosamine[f] (10)	6.04 ● 0.14	1.23	
Glucose (8)	17.2 ± 0.72	2.78	
Galactose (8)	18.5 ± 0.67	3.00	
Fucose (3)	1.6 (1.5–1.8)	0.23	
Mannose (4)	3.5 (3.1–4.1)	0.57	
Phospholipids[g] (30)		2.05 ± 0.13	
Cholesterol (15)		0.75 ± 0.07	

[a] Average ± standard error of the mean for 7 samples, with 3 samples analyzed in duplicates (averages used in the calculation of sem). Only 4 samples were analyzed for cystine and methionine. [b] Calculated without regard to tryptophan, which was not analyzed. [c] Molar sum of amino acids analyzed, corresponding to 129.6 μg of N/mg or 97.7% of total nitrogen. [d] Calculated as *N*-acetylneuraminic acid. [e] Number of analyses indicated in parenthesis. [f] Calculated as *N*-acetylglucosamine. Galactosamine was present, but in most samples in an amount of less than 10% of glucosamine. [g] Calculated from phospholipid phosphorus × 25.

No differences were found in the phospholipid partition between cortex, glomeruli, and HGBM prepared according to Krakower and Greenspon (Table I). HGBM prepared by the method described here (HGBM "pure") had a lower concentration of phosphatidylethanolamine.

Although the total phospholipid content of adult human renal cortex is similar to that of isolated glomeruli, it is signif-

TABLE III: Carbohydrate Composition of HGBM.

	Mean ± sem (μg/mg)
Sialic acids[a] (11)[b]	9.25 ± 0.49
Glucosamine[c] (10)	13.3 ± 0.30
Galactosamine (10)	<2
Glucose (8)	30.9 ± 1.3
Galactose (8)	33.3 ± 1.2
Fucose (3)	2.7 (2.4–2.9)
Mannose (4)	6.3 (5.6–7.3)

[a] Calculated as *N*-acetylneuraminic acid. [b] Number of analyses indicated in parenthesis. [c] Calculated as *N*-acetylglucosamine.

icantly higher than that of adult renal medulla and less than that of newborn infant cortex (A. F. Michael and J. Clausen, in preparation). The phospholipid partition values in human cortex and glomeruli show considerable variation but are similar to those reported for rat, pig, sheep, and rabbit kidney (Marinetti *et al.*, 1958; Gray and MacFarlane, 1961; Dawson, 1960; Tinker and Hanahan, 1966).

Amino Acid Composition. Results of the amino acid analysis of preparations of HGBM after 21 hr of hydrolysis are given in Table II.

Total Nitrogen. Total nitrogen of four samples run in duplicate averaged 132.7 ± 1.9 μg of N/mg of HGBM (average ± sem). The amino acids analyzed accounted for 97.7% of this value.

Carbohydrate Composition. By cellulose thin-layer chromatography fucose was found close to the solvent front, followed by mannose, glucose, and galactose. The separation of these sugars was satisfactory and no other sugars were found.

The amount of each sugar is shown in Table III. The concentration of galactose was higher than that for glucose in six of eight samples analyzed. The molar ratio galactose:glucose, 1.08 ± 0.08, was not significantly different from unity. The amount of galactose and glucose in two preparations of human glomeruli was 19.5 μg/mg and 15.0 μg/mg per mg, respectively.

The amount of glucosamine found was 13.3 μg/mg, expressed as *N*-acetylglucosamine, while galactosamine in general was found to be present in trace quantities only (<2 μg/mg). In two samples the galactosamine peak was large enough to be integrated and the values obtained were 1.48 and 1.58 μg/mg expressed as *N*-acetylgalactosamine.

The sialic acid value of 9.25 μg/mg was not significantly different from the amount found in whole glomeruli, 8.59 μg/mg (four preparations analyzed).

No uronic acid was detected in HGBM (<2 μg/mg). The slight brown color that developed could be accounted for by the neutral sugars known to be present in the sample.

Deoxyribonucleic Acid. DNA was not found in the HGBM preparations (<0.3 μg/mg).

Estimation of Serum Proteins. When a collagenase extract of HGBM was examined by double diffusion in agar, the antisera detected the presence of albumin, immunoglobulins G and M, β-1-*C*-globulin (C3), and fibrinogen (or fibrin). α-2-Macroglobulin and ceruloplasmin were not found. The immunodiffusion plate demonstrating the presence of IgG, albumin, and fibrinogen is shown in Figure 6. The use of a Plexiglas template for continuous feeding of reactants was necessary to prove the presence of IgM. The precipitin lines between the extract of HGBM and the monospecific antisera showed a reaction of nonidentity, indicating that the antisera

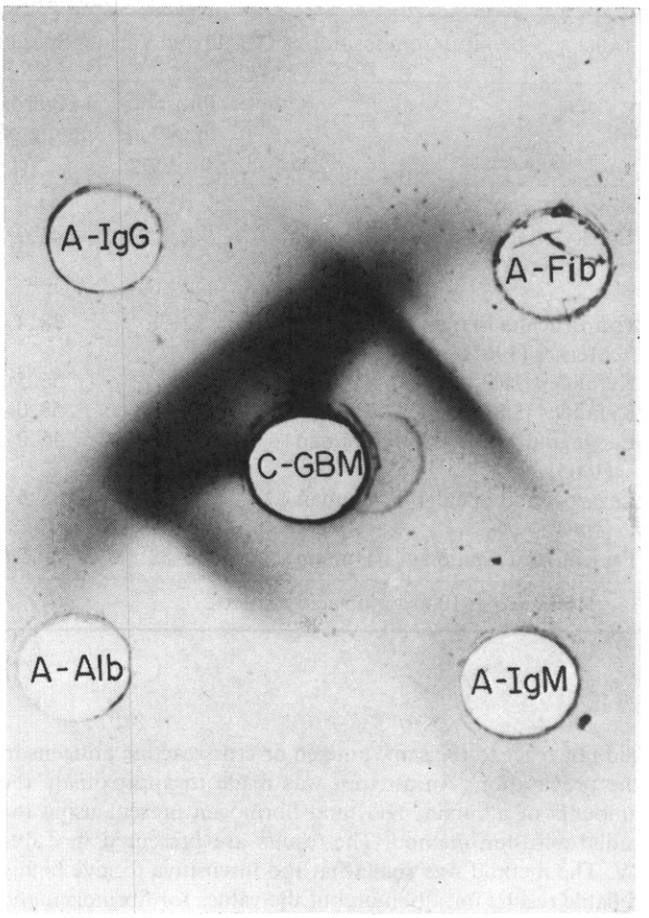

FIGURE 6: Immunodiffusion of agar, showing the reaction of a collagenase extract of HGBM (C-GBM) with antisera to human albumin (A-Alb), human IgG (A-IgG) with human fibrinogen (A-Fib). A reaction with antisera to human IgM could be seen only when a Plexiglas template was used.

TABLE IV: Serum Proteins in Basement Membrane Preparations

	μg/mg of HGBM		
	Albumin	IgG	Fibrinogen
Pool of crude HGBM from 12 kidneys[a]	1.5	4.5	15.3
Pool of pure HGBM from 6 kidneys	3.1	7.5	26.6
Pure HGBM from 1 kidney	1.5	2.0	10.8

[a] Average of two determinations.

TABLE V: Chemical Composition of GBM from Various Species.

Reference	Species	Phospho-lipids, μg of P/mg	4-Hydroxyproline, μg/mg of Residue Weight	Total Nitrogen, μg/mg	Sialic Acids, μg/mg	Hexos-amines, μg/mg	Glucose, μg/mg	Galactose, μg/mg
Spiro (1967a)	Bovine	0.22	64.7 (68.4[a])	159	11.9	21.3	24.7	30.5
Lidsky *et al.* (1967)	Bovine (acellular glomeruli)	2.3	41.9[a]	120	9.4		6.2	
von Bruchhausen and Merker (1967)	Rat	2.36	38.4	116				
Kefalides (1969)	Dog		56.5[a]		20	14.0	24	25
Kefalides (1969)	Human		53.0[a]		15	20	25	26
Lange and Markowitz (1965)	Human		56.0	134	5.5	9.5		
Lazarow and Speidel (1964)	Human		103.5	175		3	Total carbohydrate 34 μg/mg	
Present investigation	Human	0.82	71.5 (81.3[a])	133	9.3	13.3	30.9	33.3

[a] Residues per 1000 amino acid residues.

did not react to the same antigen or crossreacting antigens in the preparation. An attempt was made to approximate the amounts of albumin, IgG, and fibrinogen present using the radial diffusion method. The results are presented in Table IV. The method was somewhat too insensitive to give highly reliable results for albumin, but the values for fibrinogen and IgG were well within the optimal range of the method. No attempt was made to look for serum proteins in the collagenase-extracted residue which represented about 15% of the dry weight of the HGBM.

Water Content. Weight losses in five samples varied from 5.1 to 8.6% with an average of 7.7%. Similar results were obtained after drying the samples in the oven at 105° overnight. All analytical results in this report are adjusted for a water content of 7.7%.

Electrolyte Content. In three samples where both sodium and chloride was measured, the amount of sodium averaged 0.36 μequiv/mg of HGBM of (range 0.23–0.49 μequiv/mg) and of chloride 0.34 μequiv/mg (range 0.20–0.65 μequiv/mg). This corresponds to a sodium chloride content of 2.1%. Calcium was found in trace quantities only, <0.3 μg/mg of HGBM (<0.015 μequiv/mg).

Discussion

Since the original work by Krakower and Greenspon was published in 1951, there have been many studies concerned with the preparation and composition of glomeruli and glomerular basement membrane. Table V summarizes some of the reported data for rat, bovine, and human GBM. The chemical composition reported is highly variable. Some of the differences may be caused by species variation or the analytical methods used but the main differences are probably related to the method of preparation. Spiro (1967a) introduced the use of sieves to remove contaminating tissue fragments and prepared bovine GBM free from DNA and RNA and with a very low content of lipid phosphorus. We have adapted and modified this technique for human kidneys obtained at autopsy.

The method for preparing GBM from glomeruli was modified in two respects. First, it was necessary to use a low centrifugal force (121*g*) for the sedimentation of sonicated HGBM in 1 M sodium chloride solution, even though a preparation with a low concentration of phospholipids could be obtained from bovine kidneys by Spiro using a speed of 3000 rpm. Secondly, it was found imperative to remove undisrupted and hyalinized glomeruli from human kidneys to obtain a preparation that was free of glomeruli and cells and contained only cell-free HGBM. This was effectively achieved by passage of sonicated glomeruli through a 250-mesh screen. A low yield of HGBM was obtained. Assuming a total number of 10^6 glomeruli per kidney and that the 4-hydroxyproline in the glomerulus is present only in the GBM, the average percentage yield of GBM would be 41%.

The question whether phospholipids are an integral part of the HGBM itself or only a contaminant is not conclusively answered by this study. The striking similarity in the ratio of phospholipid to cholesterol and especially the ratio of the separated phospholipids (Table III) in the cortex, glomeruli, crude HGBM, and pure HGBM lend support to the idea that the phospholipids are mostly contaminants. The lower concentration of phosphatidylethanolamine and slightly higher concentration of sphingomyelin in the pure HGBM may indicate a unique lipid constituent or contaminant of the membrane. Misra (Misra and Berman, 1966) believes that lipids along with collagen and glycoprotein are responsible for the gross structure of the membrane. Because of the technique used in preparing GBM, their data were reported only in molar ratios to hydroxyproline and not in absolute values which makes comparison with our data difficult. Other authors (Lidsky *et al.*, 1967; von Bruchhausen and Merker, 1967) also report high values for phospholipid phosphorus and low values for 4-hydroxyproline. Without careful evaluation of preparative techniques any changes in the amounts of

GBM lipids in diseased kidneys must be interpreted with great caution.

It is surprising that soluble plasma proteins are trapped strongly enough not to be removed during the extensive sonication and washing of the HGBM. The presence of serum glycoproteins (*e.g.*, orosomucoid or haptoglobin) in significant amounts, could possibly influence the biochemical results. Serum proteins in the GBM may be important to the immunologist who makes antisera to GBM in a heterologous system. We have found that rabbit antisera to HGBM, raised against a preparation corresponding to crude HGBM, have strong antibodies against proteins in human plasma and concentrated normal urine. It is to be expected that some plasma proteins may be present in the GBM *in vivo*, as plasma proteins normally occur in the urine. The plasma proteins were found in isolated HGBM in the relative concentrations: fibrin(ogen) > immunoglobulin G > albumin > β-1-*C*-globulin > immunoglobulin M, while α-2-macroglobin and ceruloplasmin were not found. This order of concentration, which is different from that in plasma, might be explained partly by the differences in shape of the molecules, the long fibrinogen molecule having the greatest difficulties in escaping from the glomerular basement membrane lattice. Whether or not these proteins have any role in the function of the membrane is unknown.

The amounts of sodium and chloride found in the preparations were very variable, probably indicating that the membranes had not been washed well enough during isolation. That calcium was present, if only in trace amounts, may indicate that divalent ions are bound in the membrane.

The amino acid values presented here are similar to those reported by Kefalides (1969) for human GBM and Spiro (1967a) for bovine GBM. HGBM—in comparison with bovine GBM—has a higher content of 3- and 4-hydroxyproline, hydroxylysine, and leucine and a lower concentration of proline, lysine, arginine, and cystine. Compared with the data reported by Kefalides (1969) for HGBM our study shows much higher values for 3- and 4-hydroxyproline and methionine with lower values for lysine, histidine, arginine, and tyrosine. Those differences may be explained by differences in technique and in purity of the preparation. We have repeatedly found lysine, histidine, and arginine to be present in higher amounts in impure preparations. The presence of significant amounts of 3-hydroxyproline in amounts higher than previously reported (Lidsky *et al.*, 1967; Kefalides, 1969) is of interest. The importance of this amino acid, present in higher concentration in the HGBM than anywhere else in the body, is not clear.

Carbohydrates (Table III) were present in significant amounts with a distribution similar to that found in bovine material (Spiro, 1967a) but with a lower amount of hexosamines and higher values for glucose and galactose. There are more striking differences with the data of Kefalides (1969) for HGBM, who found less glucose and galactose and more of the components of the heteropolysaccharide moiety (Spiro, 1967b)—sialic acid, mannose, fucose, and hexosamine.

It is of interest to note that the sialic acid content in HGBM is similar to or higher than that found in the glomerulus, indicating that sialic acid is part of the HGBM and not a contaminant. Mohos and Skoza (1969) used a colloidal iron stain at pH 2 for the localization of sialic acid containing proteins in glomeruli, and found positive staining mainly on the epithelial cell membranes and podocytes. On no occasion did they see staining of the basement membrane itself. Nolte and Ohkuma (1969) on the other hand, found weak deposition of colloidal iron in the basement membrane. We have found that the isolated HGBM did not react with colloidal iron, and very weakly with alcian blue, although the epithelial cell membrane in isolated glomeruli stained strongly for polyanions. The reason why the sialic acid in the HGBM does not react with the colloidal iron is not clear, but could possibly mean that the charge of the sialic acid is influenced by the way it is bound in the molecule.

The results of this study are compatible with an overall structure of the HGBM similar to collagen, but with much higher amounts of hydroxyproline, hydroxylysine, and carbohydrates. No evidence for a mucopolysaccharide ground substance has been found. One possible explanation for the molecular sieve effect of the GBM (Huang *et al.*, 1967), would be the existence of a polyanion, capable of attracting cations and so influence the "pore size" of the structure to a great extent. The only sufficiently charged molecule found is sialic acid, and in concentrations seemingly too low to be of importance in this regard, if the polyanion is not organized in a particularly efficient way, *e.g.*, as a thin coating on the surface of the membrane. This seems less likely in view of the negative results with the colloidal iron staining.

It is, on the other hand, possible that the GBM is acting as a filter only for the very largest molecules. The epithelial cell membrane may instead be the critical barrier. This concept is supported by the electron microscopy studies by Farquhar *et al.* (1961) using ferritin, and Graham and Karnovsky (1966) and Venkatachalam *et al.* (1969) using peroxidases of different sizes. If this is the case, the "impermeability factor" may be present in the glomerular cells and not in the basement membrane.

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