Studies on Glomerular Basement Membrane. 1. Isolation and Chemical Analysis of Normal Glomerular Basement Membrane.* (31232)

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Correlation of structure and function ultimately depends on a knowledge of molecular arrangements within the given structure. In the case of glomerular basement membrane (GBM) the limitations of light and electron microscopy have prompted a number of attempts to obtain human GBM in a pure form, *i.e.*, free from cellular, vascular and other contaminants, in order to describe some of its detailed chemical and immunologic characteristics(1,2,3,4). However, the purity of these preparations has not been rigorously demonstrated; and the methods employed to extract the membrane and its constituents from their surroundings raise the serious possibility of destruction and/or change in the chemical and immunologic characteristics of some of its constituents. It is our purpose to describe a method of isolating highly purified GBM with good yields without resorting to chemical or enzymatic treatment. Data obtained from chemical analyses of 5 normal human adult GBM are presented. We define GBM to be the cell-free, homogenous structure obtained by the method described below and illustrated in Fig. 1 and 2.

Material and method. 1. *Source of tissue.* Human kidneys were removed at autopsy from persons dead of non-renal disease within 8 hours of death.

2. *Perfusion of kidney.* We have modified the techniques of Cook and Pickering(5), Goodman *et al* (6) and Misra and Kalant(7) as follows:

The kidney is excised preserving as much of the renal artery as possible and is then perfused *via* the artery with 5-8 liters of cold 0.15 molar saline until the blood has been washed out and the cortex is pale to the naked eye. This perfusion is carried on by means of a cannula in the renal artery and a pump with a flow rate of 50 ml/min. Following this, 3-5 liters of a 2% (V/V) magnetic iron oxide (approximately 300 Å particle size) suspension in 0.15 molar saline, are infused through the same cannula until the cortex appears dark gray (glomerular filling). The iron oxide is prepared in our laboratory by the method of Welo and Baudisch(8). The capsule is removed and the kidneys are cut into halves for visual inspection of the

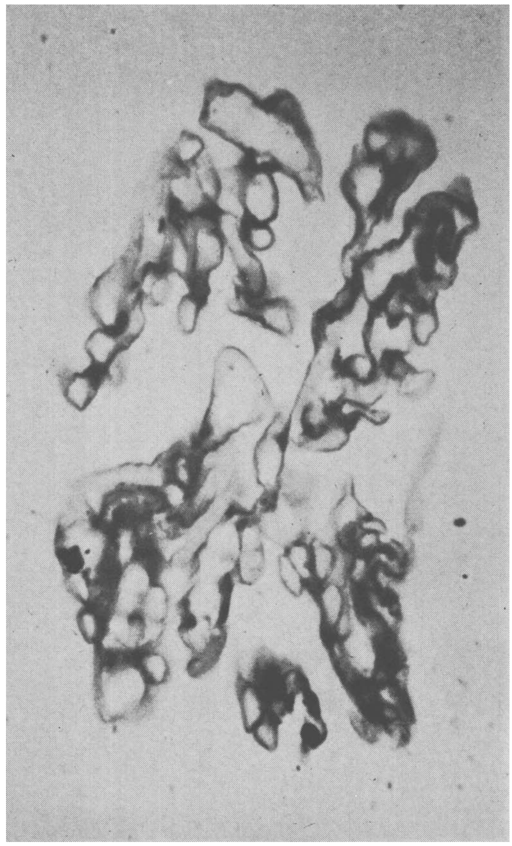

FIG. 1. Light microscopic appearance of isolated cell-free glomerulus (GBM) stained with PAS. Particles of iron oxide are present. Original magnification 320 ×. These membranes are PAS-positive and do not differ in staining characteristics from glomerular membranes of intact kidneys.

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adequacy of glomerular filling. Kidneys are then stored at —20°C, until used.

3. *Isolation of GBM*. The frozen kidney is thawed at 4°C overnight. The kidney is separated by gross dissection into cortical and medullary portions. Small pieces of cortical tissue are suspended in cold 0.15 molar saline and gently homogenized into a thick paste.

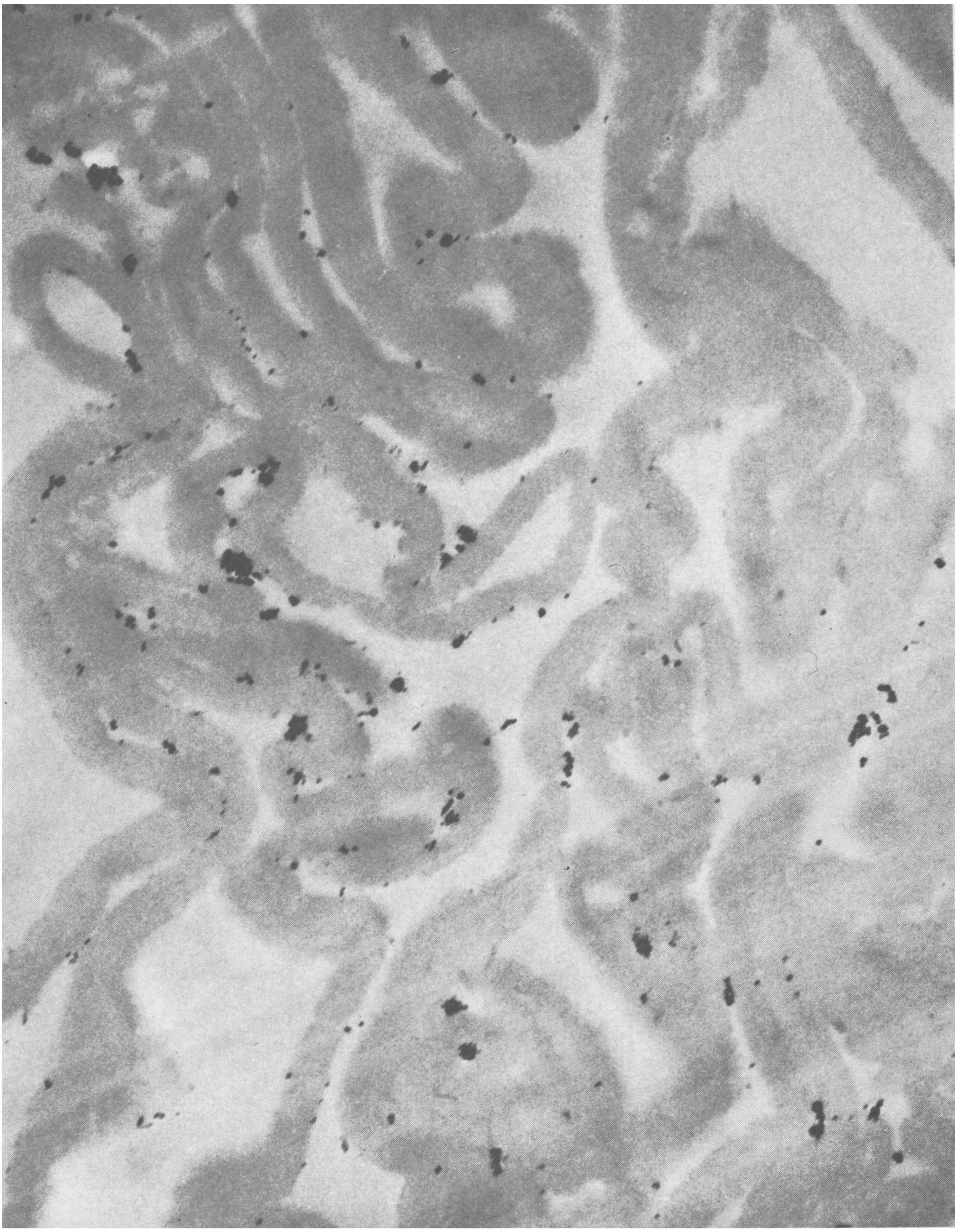

FIG. 2. Electron microscopic appearance of glomerular basement membranes. Particles of iron oxide are present. Original magnfiication 4380 ×.

This paste is pushed through a stainless steel wire mesh (150 wires/inch^2) into a glass tube surrounded by an ice bath. Passage through the wire mesh separates glomeruli from their vascular attachments. The cortical suspension is washed in 0.15 molar saline by centrifugation (800-900 rpm for 2-3 minutes) and resuspended in 0.15 molar saline. The glomeruli are then separated from the cortical suspension by passing the suspension repeatedly in front of an electromagnet. Glomeruli containing magnetic iron oxide are drawn to the electromagnet while the rest of the tissue is washed down. Microscopic observation at this stage reveals glomeruli cleanly separated from other cortical elements. All of the procedures are carried out at 4°C or in an ice bath.

Isolated glomeruli are then suspended in 8% NaCl solution. The glomerular suspension is subjected to ultrasonic vibration for 8-12 minutes at full plate operation (1.5 amps) of the MSE† ultrasonic disintegrator #3000. Differential centrifugation is carried out in 8% saline, at approximately 200 $\times$ g for 10 minutes. Cell-free glomeruli (GBM) are harvested from the bottom layer of the sediment. Washing twice in normal saline and 3 times in distilled water completes the isolation of the basement membrane. The homogeneity of the sample is routinely examined by light microscopy of the wet sediment, and of paraffin embedded basement membrane stained with PAS (Fig. 1) and hematoxylin and eosin. Randomly chosen membrane preparations are further examined by electron microscopy (Fig. 2). Finally, a chemical determination of DNA is made to ascertain the presence or absence of nuclear material (and, presumably, other cellular components) which might contaminate the isolated basement membrane material.

4. *Analysis of basement membrane.* A. Quantitative. Portions of the GBM were analyzed as follows: Total nitrogen by micro-Kjeldahl procedure(9); hexosamine by the method of Boas(10), with and without column purification; hexuronic acid by the method of Dische(11); hydroxyproline by the method of Leach(12), with all of the incubation steps being carried out at 75°C; reducing substances by the method of Somogyi (13); and sialic acid by the method of Warren(14). Determinations of lipid-phosphorus by the method of Bartlett(15) and cholesterol by the method of Zak *et al*(16) were carried out on a lipid extract of GBM obtained by the method of Schneider(17). DNA was determined by the method of Webb and Levy (18) and RNA by the method of Webb(19).

B. Qualitative. Aminosugars and neutral sugars were separated and identified by filter paper chromatography(20,21). Lipid extracts of GBM obtained by the procedure of Folch *et al*(22) were initially separated on an activated silica-gel-G thin layer plate with the following solvent system: Petroleum ether: ethyl ether:glacial acetic acid (85:15:1). Phospholipid spots were further separated by the procedure of Skipski *et al*(23) on a basic silica-gel-G thin layer plate.

C. In order to examine the effects of various contaminants on the results, portions of whole glomeruli containing their full complement of cells were analyzed. In addition, after separation of the glomeruli by the electromagnet, the washings of the remaining cortical mash were concentrated by centrifugation and then handled in the same way as the preparation of GBM. We believe that the bulk of the final material prepared from the cortical mash in the absence of glomeruli represents tubular basement membrane.

Results. 1. *Evidence for purity of the GBM.* A. Light microscopy. Ninety-five-97 per 100 consecutively visualized objects under the low power microscopic field were cell-free glomeruli (GBM) (Fig. 1). Only 3-5 per 100 objects were non-glomerular contaminants, *i.e.*, small bits of tubules and blood vessels. Ten per cent of the glomeruli retained some portion of Bowman's capsule. Paraffin sections of GBM stained with eosin and hematoxylin do not show nuclear stain. The preparations are highly PAS-positive and also stain blue with Masson's trichrome. It may be noted that the staining characteristics of the isolated glomerular basement membrane do not appear to be altered when compared with glomerular membranes *in situ*.

† Measuring and Scientific Equipment Ltd., Spenser St., London SW, Eng.

TABLE I. Clinical Data on 5 Patients Studied.

Patient	Age, yr	Sex	Single kidney wt, g	Cause of death
A.M.	78	♀	150	Heart disease
H.M.	70	♂	160	Cardiac arrest
B.J.	83	♂	155	Pneumonia with heart failure
L.C.	39	♂	160	Gunshot wound
A.C.	35	♂	155	" "

B. Electron microscopy. Portions of GBM were fixed in buffered gluteraldehyde and osmium tetroxide. Sections were embedded in Maraglas and stained with lead according to the method of Lever(24). Fig. 2 shows a homogeneous, electron dense structure without evidence of foot processes or cellular material. There was no evidence of easily identifiable RNA particles or remnants of mitochondria.

C. Chemical evidence. The GBM preparation does not contain DNA. However, trace amounts of RNA are present. The nitrogen of RNA accounts for less than 1% of total nitrogen of GBM. Repeated washing with 8% NaCl solution and distilled water at room temperature did not affect the RNA reaction. Extraction of GBM with cold 5% TCA, washing with 95% alcohol and extraction with alcohol and ether mixture (3:1) had no effect on the RNA results. This suggests that RNA is present as a membrane constituent rather than as a contaminant. We have not yet carried out incubation of GBM with RNAase as further documentation of its presence.

2. *Results of chemical analyses.* Clinical data on the 5 patients studied are presented in Table I. None had evidence of renal histologic abnormality by light microscopy. One kidney from each patient was randomly chosen for analysis. The number of glomeruli isolated and pooled from each subject is estimated to be 750,000 (*i.e.*, 75% of one million glomeruli, based on previous unpublished experience).

We have identified the following substances in human GBM.

1) Nitrogen; 2) Hydroxyproline; 3) Hexosamines (glucosamine, galactosamine); 4) Sialic acid; 5) Reducing substances (glucose, mannose, galactose, fucose); 6) Cholesterol; 7) Lipid-phosphorus; 8) RNA.

We have sought but not found: 1) Hexuronic acid, 2) DNA.

The following lipid pattern was obtained on thin layer chromatography with an extract of rat kidney cortex used as a standard.

1) Lipid—cholesterol, free fatty acids, triglycerides and phospholipids

2) Phospholipids—lecithin, sphingomyelin, cephalin, phosphoserine + phosphoinositol.

In Table II, those constituents measured quantitatively are expressed as molar concentration ratios with respect to hydroxyproline. This expression is necessary because we do not have sample weights. Hydroxyproline was chosen as the basis of comparison since the collagen it represents is an extracellular structure and presumably must reside within the GBM. Table II also presents molar ratio data from analysis of whole glomeruli and from tubular basement membrane as defined above.

Discussion. Two points are critical in interpreting the results of this study. The first is the demonstration that the glomerular basement membrane is free of contamination by cellular materials. The evidence for this lies in the homogeneity of the membrane with electron microscopy, the absence of DNA, and the absence of nuclear staining by hematoxylin and eosin. The second point is our assumption that this method of preparation of the glomerular basement membrane has not altered its chemical characteristics, *e.g.*, by washing away some soluble component. Support for this assumption comes from analysis of hydroxyproline in the glomerular basement membrane washings. Of the total hydroxyproline present in the basement membrane plus the washings, we have found less than 3% in the washings. The remaining 97% are present in the membrane itself. The presence or absence of other membrane components in the washings remains to be determined.

The absence of hexuronic acid signifies an absence of mucopolysaccharides from the membrane. In this respect, it differs sharply from the ground substances of cartilage, skin and aorta. The hydroxyproline is present,

TABLE II. Comparison of Chemical Composition of GBM with Tubular Basement Membrane and Whole Glomeruli; Values Expressed as Molar Ratios: Hydroxyproline = 1.

	Total N	Hexosamine	Sialic acid	Reducing substances	Cholesterol	Lipid-P
			Glomerular basement membrane			
No. of kidneys analyzed	5	5	5	3	3	3
Mean	26.0	.164	.034	.68	.04	.036
Range	22.7–30.6	.10–.30	.02–.05	.55–.82	.028–.064	.031–.043
			Tubular basement membrane			
No. of kidneys analyzed	2	2	2			
Mean	34.1	.24	.035			
Range	33.7–34.5	.22–.26	.03–.04			
			Whole glomeruli			
No. of kidneys analyzed	3	3	3	2		
Mean	54.3	.46	.093	.53		
Range	52–57	.44–.48	.09–.10	.48–.58		

probably as part of tropocollagen since mature collagen fibrils are not seen by electron microscopy of GBM. Sialic acid and the hexosamines are considered to be constituents of glycoproteins or glycolipoproteins. The neutral sugars may be part of the glycoprotein and/or the collagen molecules. The nitrogen to hydroxyproline molar ratio of 26 to 1 means that about 60% of the total nitrogen of GBM is accounted for by collagen. The hexosamine, sialic acid and RNA contributions to total nitrogen amount to less than 1%. This leaves about 35-40% of the GBM nitrogen not specifically accounted for. It is reasonable to infer that this fraction is made up of the protein moiety of the glycoprotein. Thus, 3 components, *i.e.,* collagen, glycoprotein and lipids, seem to be responsible for the gross structure of the membrane. The significance of the lipid pattern in GBM is not yet known but, as in plasma membranes of cells, it may play a role in permeability and may even be concerned with GBM regeneration under normal conditions.

Data obtained from analysis of whole glomeruli suggest that the cellular components contribute non-collagen nitrogen as is shown by the increased nitrogen to hydroxyproline ratio of 54. The relative increase of hexosamine and sialic acid may originate from the plasma membranes of the glomerular cells. The important point here is that contamination with cellular elements has a marked effect in a predictable direction on some of the molar ratios of GBM.

The analyses of tubular basement membrane are few in number and we would tentatively suggest only that tubular basement membrane resembles GBM with respect to the constituents shown in Table II.

A comparison with results obtained from human GBM by other authors is presented in Table III. The data have been recalculated as molar ratios with respect to hydroxyproline. Some striking differences in methods and results are apparent. For example, Lazarow and Spiedel (Table III, item 5) using prolonged alkali digestion of isolated glomeruli found no sialic acid and low nitrogen to hydroxyproline ratios. The variability of the 3 sets of data reported by Lange and Markowitz (Table III, items 2, 3, 4) further emphasizes the role that methodology plays in these analyses. The purity and chemical integrity of the membrane preparation becomes crucial when identification of molecular species and immunologic characterization is attempted.

Summary. A method has been described for obtaining isolated human glomerular basement membrane. Evidence for the purity of the isolated GBM includes electron microscopic and chemical data. Some of the membrane constituents, in particular hydroxyproline, aminosugars, sialic acid, neutral sugars, and lipids have been identified. Hexuronic acid is absent as is DNA. Sixty percent of the nitrogen of the membrane can be accounted for by collagen; the remainder may be contributed by the protein moiety of a

TABLE III. Comparison of Data Obtained by Several Authors on Chemical Composition of GBM. All data calculated as molar ratios with hydroxyproline = 1.0.

Item	Authors	Methods to obtain: Whole glomeruli	GBM	GBM extracts	Total N	Hexosamine	Sialic acid	Reducing substances (neutral sugars)
1.	Misra & Berman 1966	Magnetic separation	Ultrasonication	—	26.0	.16	.03	.68
2.	Lange & Markowitz (3) 1965	Diff. centrifugation	?	—	22.4	.12	.04	.77
3.	Lange & Markowitz (3) 1965	Diff. centrifugation	—	Phosphate buffer, pH 8.0 + trifluoro-trichloroethane + trypsin digestion	15.0	.23	.06	1.16
4.	Markowitz & Lange (1) 1964	Diff. centrifugation	—	*Idem*	15.0	.50	.13	1.16
5.	Lazarow & Spiedel (2) 1964	Low speed centrifugation + gravity sedimentation	.05 N NaOH digestion	—	16.6	.03	0	.40
6.	Dische(4) 1965	Low speed centrifugation	Ultrasonication	10% TCA	No data			.47

glycoprotein species.

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