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STUDIES OF NORMAL AND NEPHRITIC RAT GLOMERULAR BASEMENT MEMBRANE

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SUMMARY

Solubilization of the normal glomerular basement membrane with various solvents revealed that the material is held together by hydrogen and disulfide linkages as well as ionic salt bridges which ionize at around pH 10.0. Pronase digestion indicated that differences in susceptibility to enzyme digestion exist between normal and nephritic membrane. Titration of a urea-insoluble material indicated that some alteration must have taken place in the association between various components of the nephritic basement membrane. Chemical analysis of alkali-solubilized fractions suggested that greater alkali susceptibility of the nephritic material may be present. A collagen-like material resembling both tendon and dog basement membrane collagen in its amino acid composition was isolated. It contained 10 % hexose, but in addition to glucose and galactose, mannose was also detected. A glycopeptide fraction obtained by pronase and collagenase digestion has a carbohydrate composition similar to the collagen-like material above. These substances probably represent incompletely digested fragments of the basement membrane.

INTRODUCTION

Since the basement membrane is the only continuous anatomical barrier in the glomerulus between blood and urine, it is believed to play a crucial role in the filtration process [1]. Morphologic alterations in the basement membrane have been observed in a number of diseases such as the nephrotic syndrome, diabetes mellitus and others [2–4]. These usually involve a thickening of the membrane of the glomerular capillary loops and may lead ultimately to an impaired filtration process with the appearance of large quantities of protein in the urine [5].

Basement membranes have been studied from various points of view; morphologic, compositional, structural, immunological and functional [6–24]. Chemical

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studies of the basement membrane from various species showed that it is composed of at least two types of carbohydrate-rich glycoproteins, one a collagen-like, the other a non-collagen glycoprotein [14, 17, 22].

In previous studies, the membrane was found to be partially soluble in 8 M urea, mercaptoethanol, sodium dodecylsulfate and dilute alkali: reduction and alkylation enhanced its solubility [13, 25, 26]. A collagen-like component has been purified and was found to be rich in hydroxyproline and hydroxylysine, it also contained about 10 % carbohydrate by weight [14]. Most of this was found to consist of discrete units of a disaccharide composed of glucose and galactose. These are bound to hydroxylysine by O-glycosidic linkage [27]. Another, more complex heteropolysaccharide composed of galactose, mannose, hexosamine, fucose and sialic acid has also been isolated and characterized [27]. Recently, complete solubilization of the membrane and analysis of the fractionated and enzymatically digested material indicated that the di- and heteropolysaccharide units are not restricted to the collagen and non-collagen glycoproteins, respectively [26].

Studies on diseased glomerular basement membrane have been performed by a number of workers. It appears that different types of chemical changes were found associated with each different disease [11, 18, 28–31].

To date, the structural characteristics of the basement membrane have not been completely elucidated and the nature of the chemical changes associated with nephritis are not entirely clear. Our studies were aimed, therefore, at some of these problems.

MATERIALS AND METHODS

Rat kidneys were obtained from normal or nephritic Sprague-Dawley adult male rats. Glomeruli were isolated essentially by the method of Krakower and Greenspon [32]. Basement membrane was obtained by subjecting isolated glomeruli to ultrasonic vibration for five 1-min bursts using a Biosonic sonicator. Cellular debris was removed by repeated washings. About 1 mg of membrane was obtained from one rat. Each experiment was performed on a homogenized pool of 20–30 mg of material. Nephritis was induced by rabbit anti-rat basement membrane serum [33]. Animals were considered nephritic if they had a daily urinary protein excretion of 200 mg or more 12 days after the first injection of antiserum.

To determine solubility, basement membranes were suspended in various reagents (5 mg/ml) and agitated by stirring at 4 °C for 24 h three times. The supernatants obtained were pooled, dialyzed and lyophilized. Urea and alkali extracts in 0.1 M NaOH were prepared by a similar procedure, except that they were performed at 37 °C. Hydroxyproline was measured by the method of Woessner [34] after samples were hydrolyzed at 100 °C in 6 M HCl for 6 h. For the determination of neutral sugars, 0.5–1.0-mg samples were hydrolyzed at 100 °C in 2 M HCl for 4 h under N_2, then purified by ion-exchange chromatography using Dowex 1-X8 and Dowex 50W-X4 in a small column. The Dowex 1-X8 was converted to the formate form using 2M sodium formate: the Dowex 50W-X4 was regenerated to the H^+ form. The column eluate containing the sugars was evaporated to dryness to remove all traces of the formate. The residue was redissolved in water and glucose and galactose were measured in these solutions using the Glucostat and Galactostat kits supplied by the Worthington

Biochemical Corp. [35, 36]. Fucose was determined according to the method of Dische and Shettles [37]. Reducing sugars were assayed by the Somogyi technique [38]. Mannose was estimated by measuring the difference between the sum of the individual sugars and the total reducing sugar content. Bound hexose was measured using the phenol-H_2SO_4 reaction of Montgomery [39] and hexosamines were determined by the Cessi and Piliego [40] modification of the Elson-Morgan reaction following hydrolysis of samples at 100 °C in 4 M HCl for 6 h. Sialic acid was released from protein samples by treatment with neuraminidase (obtained from Koch-Light) in pH 5.5 buffer at 37 °C for 4 h. Two units of enzyme were used per milligram of sample. Hydrolysates were assayed directly by the method of Warren [41]. For the determination of amino acids, protein samples were suspended in twice redistilled, constant boiling 6 M HCl (1 mg/ml) then hydrolyzed under an atmosphere of N_2 in borosilicate vials at 110 °C for 24 h and analyzed by an automated procedure based on the method of Piez and Morris [42] using nor-leucine as the internal standard. The small amounts of material available did not permit the performance of multiple amino acid analyses, one was done on each fraction. Thin-layer chromatography of neutral sugars was done by the method of Vomhof and Tucker [43].

Pronase (grade B) was obtained from Calbiochem. Samples were suspended in 0.1 M Tris/acetate, pH 7.8, buffer containing 5 mM calcium acetate. 1 % by weight of the enzyme was added and the mixture incubated at 37 °C for 16 h. An additional 0.5 % of pronase was then added and digestion was continued for another 8 h. Collagenase (SPCA) from Worthington was used by suspending samples in 0.1 M Tris/acetate, pH 7.4, buffer containing 5 mM calcium acetate. Incubation was carried on for 48 h, enzyme (0.8 and 0.1 %, by weight) was added at 0 and 24 h after the start. After enzyme digestion, samples were dialyzed against distilled water at 4 °C and lyophilized.

Optimum conditions of hydrolysis for the release from basement membrane of the different substances to be measured were worked out by comparing release and destruction of these substances under various conditions. Recoveries of different amounts of added hydroxyproline, neutral sugars or hexosamine after hydrolysis and purification, where applicable were better than 90 % with these techniques.

Fractionation of soluble proteins was accomplished by gel filtration on Biogel P-300 or other suitable support material. Usual column size was 1 × 45 cm and 0.5–1-ml fractions were collected. Elution was monitored by measuring ultraviolet absorbance.

For titration, 15–20 mg of material was dissolved in 5 ml of 0.1 M NaOH and 0.1 M HCl was added dropwise with stirring. pH was measured with a Radiometer type PHM 27 equipped with a microelectrode. Total time required from start to endpoint was 70–80 min.

The reproducibility of the various methods used was calculated. The S.D. (S.D. $= \pm \sqrt{(x_1 - x_2)^2/2n}$) of duplicate values in the same assay for hydroxyproline, glucose, galactose, fucose, mannose and hexosamine were $\pm$ 1.8 %, $\pm$ 0.9 %, $\pm$ 2.5 %, $\pm$ 1.6 %, $\pm$ 4.2 % and $\pm$ 3.4 %, respectively. Assaying samples on consecutive days showed that the S.D. values for inter-assay variability for all these tests were between $\pm$ 2.7 and $\pm$ 6.1 %.

RESULTS

8 M urea and cysteamine extracted 4 and 8 % of the total hydroxyproline present in the normal basement membrane, respectively, but the concentration of this amino acid changed from 33 μg/mg in the intact basement membrane to 48 and 51 μg/mg in the respective residues (Table I).

TABLE I

SOLUBILITY OF THE GLOMERULAR BASEMENT MEMBRANE IN DIFFERENT SOLVENTS

Salt, 1.0 M NaCl; citrate, 0.15 M, pH 3.6 citrate buffer; urea, 8.0 M urea in 50 mM Tris · HCl, pH 7.4 buffer; cysteamine, 0.2 M cysteamine in 0.6 M NaCl in 0.08 M phosphate, pH 7.0 buffer.

Material	Percent of total hydroxyproline	Concentration of hydroxyproline (nmol/mg)
Basement membrane	100.0	33.0*
Water extract	0.2*	
Salt extract	1.3	
Citrate extract	0.7	
Urea extract	8.2	
Urea-insoluble residue	85.0	48.0
Cysteamine extract	4.0	
Cysteamine-insoluble residue	92.0	51.4

* Mean of duplicate determinations.

Weak alkali treatment (0.1 M NaOH) of a urea-insoluble residue of the normal basement membrane at 37 °C caused solubilization of this material without apparent change in the neutral sugar or amino acid composition except for a significant decrease in the number of cystine residues (Table II; amino acid composition data not shown).

TABLE II

CARBOHYDRATE CONTENT OF UNTREATED AND ALKALI-SOLUBILIZED UREA-INSOLUBLE RESIDUE

The results are expressed as nmol/mg.

	Glucose	Galactose	Fucose	Mannose
Control	110*	103	10.3	77
Solubilized sample	112	104	12.1	74

* Mean of duplicate determinations.

When the basement membrane of rat from normals and from animals with nephritis are compared, the following differences were observed. Pronase digestion of the urea-insoluble residue from normal and nephritic basement membrane followed by gel filtration of the digests gave fractions in which the hydroxyproline, which may

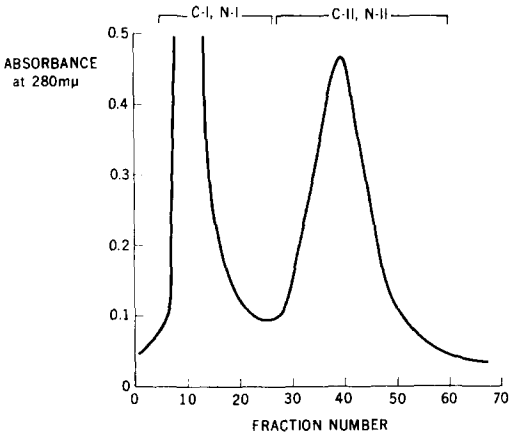

Fig. 1. Elution pattern obtained from normal and nephritic urea-insoluble residue of basement membrane after pronase digestion. Chromatography was on Biogel P-300 column (1 × 45 cm) in 50 mM Tris · HCl, pH 7.4, buffer. C-I and C-II are the larger and smaller fractions obtained from normal basement membrane material, N-I and N-II are the analogous fractions from nephritic rats. The same pattern was obtained from both normal and nephritic materials, so only one tracing is shown.

be taken as an indicator of the presence of collagen, is distributed differently in the normal from the nephritic. Most of the hydroxyproline-containing fragments from normal basement membrane were excluded from Biogel P-300, while a significant proportion of hydroxyproline-containing fragments from the nephritic were smaller and were retarded by the gel.

Treatment of the urea-insoluble residue with alkali resulted in the solubilization of both normal and nephritic material. When the pH of these solutions was gradually lowered by the addition of dilute acid, the titration curve obtained was different in the normal from the nephritic (Fig. 2).

Chemical analysis of the normal and nephritic materials after further purification by column chromatography on Biogel P-300 gave fractions in which the distribution

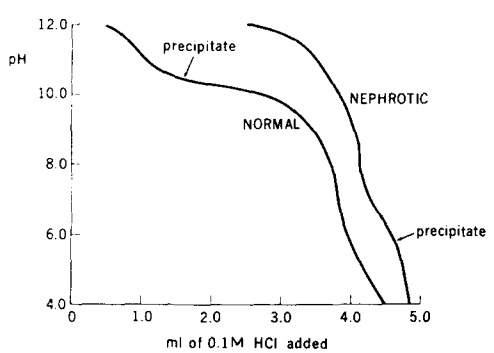

Fig. 2. Materials obtained from a urea-insoluble residue of basement membrane by solubilization with 0.1 M NaOH. For titration, both materials were dissolved in 5 ml of 0.1 M NaOH: 0.1 M HCl was added dropwise with stirring. pH was measured with a Radiometer type PHM 27 equipped with a microelectrode. Total time required from start to endpoint was about 70–80 min.

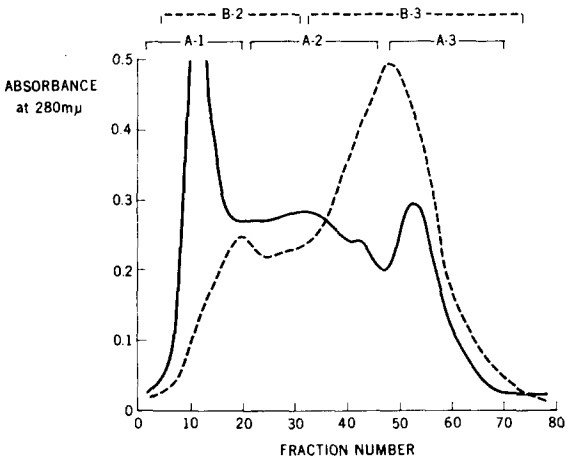

Fig. 3. Materials obtained from a urea-insoluble residue by solubilization in 0.1 M NaOH, precipitation by neutralization and fractionation of the precipitate on Biogel P-300 in 0.1 M NaOH. (—) material from normal rats, (- - -) material from nephritics. Fractions beginning with A are from normal rats, those with B are from nephritics. (B-1 remained in solution after material containing B-2 and B-3 precipitated as the pH was lowered to 5.)

of hydroxyproline and hydroxylysine is quite different in the normal from the nephritic (Fig. 3; amino acid composition data not shown). All of the hydroxyproline and hydroxylysine from the nephritic material was found in the supernatant after a

TABLE III

AMINO ACID COMPOSITION OF BASEMENT MEMBRANE AND OF A COLLAGEN-LIKE MATERIAL OBTAINED FROM IT

(I) Basement membrane (control). (II) Collagen-like material isolated from rat basement membrane by the method of Kefalides [14].

Amino acids	I	II
Hydroxyproline	44.2	112.7
Aspartic acid	72.7	63.4
Threonine	42.5	25.3
Serine	48.6	40.6
Glutamic acid	98.5	91.5
Proline	55.8	56.1
Glycine	177.3	293.7
Alanine	68.2	42.2
Valine	42.2	34.0
Cystine	22.2	15.3
Methionine	17.9	—
Isoleucine	35.9	30.7
Leucine	72.6	43.5
Tyrosine	21.9	9.7
Phenylalanine	32.5	31.6
Hydroxylysine	17.4	37.4
Lysine	41.7	13.3
Histidine	19.8	14.4
Arginine	54.6	40.4

TABLE IV

CARBOHYDRATE COMPOSITION OF COLLAGEN OBTAINED FROM RAT BASEMENT MEMBRANE BY PRONASE DIGESTION AND SALT PRECIPITATION AT 4 °C

Amino acid composition of the material shown in Table III, column II.

Sugar	Concentration (nmol/mg)	Molar ratio
Glucose	233*	21.2
Galactose	174	15.8
Fucose	11	1.0
Mannose	105	9.6
Hexosamine	39	3.6

* Mean of duplicate determinations.

portion of the material precipitated when the pH was lowered to 5.5. In the normal, all the collagen precipitated at pH 10.5 and on subsequent gel filtration on Biogel P-300 the highest hydroxyproline content was found in A-2, the fraction retarded to some extent by the gel. The material that remained in solution after precipitation of the bulk of the protein at pH 10.5 contained negligible amounts of hydroxyproline.

When whole basement membrane is digested with pronase at 4 °C, and a collagen-like material is obtained by salt precipitation, the substance was found to have a high hydroxyproline content (Table III, column II). The material contained approximately equal amounts of glucose and galactose and a significant quantity of mannose (Table IV). This was confirmed by thin-layer chromatography.

Digestion of the urea-insoluble residue from normal basement membrane by collagenase and then by pronase at 37 °C resulted in a mixture of glycopeptides which were fractionated by gel filtration (elution pattern not shown). Three fractions were obtained, but three quarters of the total bound hexose present in the mixture was associated with fraction III. This peptide had a carbohydrate composition in which the molar ratio of hexoses was similar to the carbohydrate ratios of the collagen shown in Table IV (Table V). Its amino acid composition is shown in Table VI. The presence of glucose, galactose and mannose in fraction III was confirmed by thin-layer chromatography.

TABLE V

MOLAR RATIO OF THE CARBOHYDRATE IN VARIOUS PEAKS OBTAINED FROM PROTEASE-DIGESTED UREA-INSOLUBLE RESIDUE

Molar ratio of hexoses taking fucose as 1.0. Urea-insoluble residue of basement membrane was digested with collagenase and pronase, then fractionated on Biogel P-30 and P-6 in pyridine/acetate buffer, 0.1 M, pH 5.0. Fractions are presented in order of decreasing size; Fraction I eluted with the void volume on P-30, fractions II and III separated on Biogel P-6.

Fraction No.	Glucose	Galactose	Fucose	Mannose	Hexosamine	Sialic acid
I	5.0	5.1	1.0	7.0	5.4	1.1
II	0.5	3.8	1.0	6.9	6.2	2.0
III	23.2	18.2	1.0	11.7	1.8	0

TABLE VI

AMINO ACID COMPOSITION OF FRACTIONS II AND III OBTAINED FROM PROTEASE-DIGESTED NORMAL UREA-INSOLUBLE RESIDUE AFTER GEL FILTRATION

Substances obtained as described in the legend to Table V.

Amino acids	II	III
Hydroxyproline	-	84.7
Aspartic acid	99.8	71.8
Threonine	61.9	24.7
Serine	65.2	25.6
Glutamic acid	73.2	111.0
Proline	106.4	121.8
Glycine	157.0	243.2
Alanine	83.8	41.8
Valine	124.7	24.8
Cystine	67.6	22.2
Methionine	-	5.6
Isoleucine	23.3	15.9
Leucine	36.4	30.1
Tyrosine	17.5	1.8
Phenylalanine	15.3	4.4
Hydroxylysine	trace	69.3
Lysine	20.8	62.2
Histidine	15.2	11.9
Arginine	24.3	26.9

DISCUSSION

The solubility properties of rat glomerular basement membrane are similar to those of dog and beef [22, 25]. From Table I it is evident that both cysteamine and urea extraction remove some of the substance of basement membrane, leaving behind a hydroxyproline-enriched material. This suggests that the collagen present is less soluble than other components of the membrane. Also, in agreement with the findings of Kefalides [23] and Spiro [24], it implies that hydrogen bonds and sulfhydryl bridges contribute to the insolubility of the basement membrane.

The data obtained when the alkali-solubilized, urea-insoluble residue was titrated with acid appears to indicate that, in addition to hydrogen and disulfide bonds, there may be ionic salt bridges which contribute to the insolubility of the basement membrane. In the normal membrane these bonds were found to ionize around pH 10.5.

It was observed that when the urea-insoluble residue from basement membrane was solubilized by weak alkali, then neutralized by HCl, there were differences between normal and nephritic in their solubility at different pH values and in their buffering capacity in the pH range 9–11. These results indicate that some alteration occurs in the relationship between the various components of the basement membrane in nephritic rats. It may be that the diseased basement membrane becomes more susceptible to alkali hydrolysis. This concept is supported by the finding that following alkali solubilization the hydroxyproline-containing fragments from the nephritic basement membrane elute later than normal fragments when subjected to gel filtration on Biogel P-300, indicating smaller molecular size.

Further evidence for the presence of some alteration in the nephritic material was obtained when the urea-insoluble material from basement membrane was subjected to pronase digestion, dialyzed and then fractionated by gel filtration on Biogel P-300. The results imply that the hydroxyproline-containing protein in the nephritic is broken down to smaller fragments, while in the normal most of the hydroxyproline is present in larger molecular weight proteins.

Both the increased alkali susceptibility and the altered mode of degradation by pronase of the nephritic material may be explained by postulating the synthesis of altered basement membrane material. In view, however, of the known events associated with the immune reaction following injection of antiserum, a partial degradation of the basement membrane (as a consequence of the release of proteases during the inflamatory response) seems a more likely possibility. Our data do not allow us to distinguish between these two alternatives. They indicate, however, that the basement membrane responds differently to chemical manipulation in the nephritic from the normal. This may be related to the altered physiological properties of the membrane.

In a previous report Kefalides [14] described the isolation of a collagen-like material from dog basement membrane. We attempted to isolate this material from rat basement membrane. After proceeding in an identical manner, a collagen was obtained which was different. The amino acid composition resembled that of collagen isolated from dog basement membrane: it contained approximately equal amounts of glucose and galactose, but significant amounts of mannose and a small amount of hexosamine were also found.

Since this finding appeared to contradict the earlier contention of Kefalides [22] that the basement membrane collagen contains only disaccharide units while the glycoprotein has the heteropolysaccharides bound to it, another experiment was done to study the glycopeptides that are obtained after collagenase and pronase digestion of the urea-insoluble residue. Three glycopeptides were isolated by gel filtration. Fraction I contained all the hexose units associated with the basement membrane. Fraction II contained all of the sugars, but only a very small amount of glucose. This may correspond to the heteropolysaccharide unit described by Spiro [17] which, as Kefalides [22] postulated, is supposed to be associated with the glycoprotein moiety. Although only about 10 % of the total carbohydrate was present in this fraction, the absence of hydroxyproline and hydroxylysine is compatible with the suggestion that some peptide fragments of the basement membrane may contain the heteropolysaccharide in the absence of any collagen-like material.

In Fraction III, the ratio of glucose, galactose and mannose was almost identical to that found in the collagen isolated from rat basement membrane by the method of Kefalides. The presence of significant amounts of hydroxyproline and hydroxylysine in this fraction is also compatible with the possibility that this material originated from collagen. Although this fraction is retarded on Biogel P-6, which indicates a small molecular size, the complex amino acid composition suggests that a mixture of peptides rather than a single molecular species may be present.

It has been suggested by Kefalides [22] that basement membrane collagen and glycoprotein are distinct entities, each having a characteristic carbohydrate side-chain. Recently, however, Hudson and Spiro [26] described the isolation of a large number of complex fractions from reduced, alkylated and solubilized basement membrane from beef. They found peptides with both di- and heteropolysaccharides occurring in

their digest and claim that the heteropolysaccharides are present on all types of peptide chains found in bovine basement membrane. Kefalides himself has isolated a peptide from the basement membrane of bovine lens capsule with an unusual carbohydrate composition: it contained mannose and hexosamine in addition to glucose and galactose [44].

It appears, therefore, that the structure of the basement membrane is more complex than has been suggested earlier by Kefalides [22]. The data presented above suggest that collagen in the basement membrane may not exist as a separate entity with only one type of carbohydrate side chain attached to it. Rather, it appears to be present as part of a highly cross-linked, insoluble matrix, which requires more drastic methods for complete separation of the various components than those used in this study.

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