Isolation and Characterization of the Collagen from Glomerular Basement Membrane*

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ABSTRACT: The collagen from normal dog glomerular basement membrane was isolated after solubilization with Pronase at low temperatures. This protein, which is separated by precipitation with 15% KCl, contains in terms of residues/1000 residues: glycine, 325; hydroxyproline, 162; proline, 60; hydroxylysine, 40; lysine, 4; and half-cystine, 6. Hexose accounts for 10.4% of dry weight. There is 5.12% glucose and 5.22% galactose. On acrylamide electrophoresis at pH 4.8, the collagen resolves into three fast components, two components with slower mobility, and one with least mobility; all bands stain for protein and carbohydrate. This collagen has a sedimentation constant, $s_{20,w}$, of 3.45 S and an intrinsic viscosity of 5.2 dl/g. Optical rotation and circular dichroism studies revealed a triple-helical structure. The molecular weight is approximately 210,000. Reconstitution of the soluble collagen by precipitation with adenosine triphosphate reveals fibers of variable dimensions. Material obtained by treatment with Pronase at 4° for 24 hr gave SLS-type aggregates measuring about 1660 and 3250 Å, the latter being aggregates of the former.

The data show that basement membrane collagen is a unique mammalian protein rich in hydroxylysine, hydroxyproline, and carbohydrate. Unlike other mammalian collagens its contains cystine. As it is obtained after Pronase treatment of basement membrane, it has about two-thirds the length and molecular weight of ordinary mammalian skin collagen. The possibility that this may result from limited cleavage of the body of the collagen molecule cannot at present be excluded. It is associated with a glycoprotein which is unlike collagen. This association may be responsible for the failure of this collagen to form large aggregates *in vivo* and may be related to the functional requirements of glomerular basement membrane.

The presence of collagen in glomerular basement membrane had been suggested by the presence of significant amounts of hydroxyproline (Goodman *et al.*, 1955; Lazarow and Speidel, 1964; Markowitz and Lange, 1964; Kefalides and Winzler, 1966). Unusually high amounts of hydroxylysine in basement membrane were first reported by Kefalides and Winzler (1964).

The isolation of a collagen from glomerular basement membrane containing higher amounts of hexose, hydroxyproline, and hydroxylysine than tendon collagen was reported earlier in a preliminary communication (Kefalides, 1966).

The presence of fibers or periodic structure within the glomerular basement membrane has not been shown. The suggestion has been made that the collagen in basement membrane fails to form large aggregates by virtue of its excess carbohydrate content and its association with a glycoprotein (Kefalides, 1966). Collagens with large amounts of hexose and hydroxylysine have not been reported in mammalian tissues except for vitrosin, a protein of the vitreous humor of the eye. Vitrosin contains about 10% hexose and 22.5 residues per 1000 residues of hydroxylysine (Gross *et al.*, 1955; Gross, 1963).

It has been reported that Pronase is capable of solubilizing insoluble calfskin collagen at 20° by splitting off extrahelix peptide appendages which participate in intra- and intermolecular cross-links in collagen molecules (Drake *et al.*, 1966). This procedure does not change the main structural features of the collagen molecule. The same authors report that the amino acid composition of the Pronase-digestible material is unlike that of collagen. Since the collagen component of glomerular basement membrane is not extractable with weak solutions of acids or alkalis and when extracted with 5% trichloroacetic acid at 90° (Kefalides, 1966) it is unsuitable for physical characterization, it was decided to use digestion with Pronase at low temperatures in order to remove noncollagen peptides and thus solubilize the collagen.

The purpose of the present study has been to develop methods for the isolation and purification of the collagen component of glomerular basement membrane, to determine some of the physical and chemical properties of this collagen, and to reconstitute it in a fibrous form.

Materials and Methods

Preparation of Glomerular Basement Membrane. Dog kidneys were obtained from normal mongrel dogs. The basement membrane was prepared after isolation of glomeruli from kidney cortices, essentially by the method of Krakower and Greenspon (1951).

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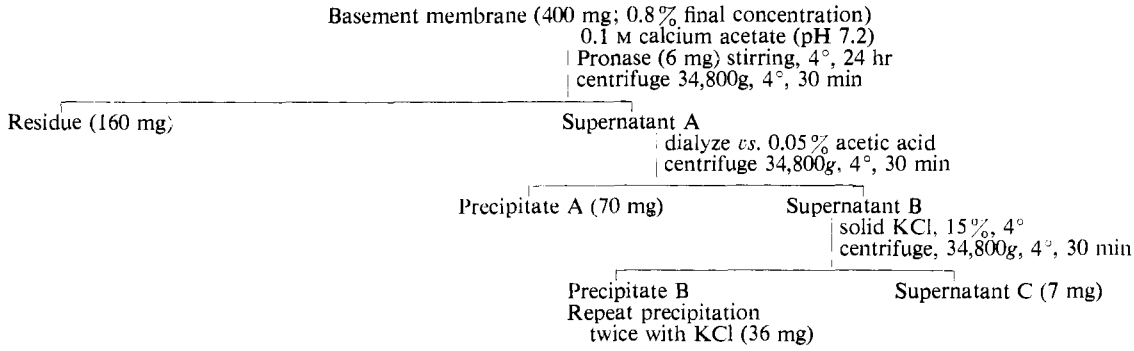

FIGURE 1: Isolation procedure of collagen from glomerular basement membrane.

Isolation of Collagen. Before use, Pronase (Calbiochem, Los Angeles) was dialyzed as 1% solution against 0.1 M calcium acetate (pH 7.2) at 4° overnight. Dialyzed Pronase was sterilized by filtration through a Millipore HA 0.45-μ filter in order to remove viable cells.

Basement membrane (400 mg) was suspended in 50 ml of 0.1 M calcium acetate (pH 7.2), 0.6 ml of the dialyzed Pronase was added, and the mixture was allowed to stand at 4° with gentle stirring for 24 hr. The mixture was then centrifuged at 34,800*g* for 30 min and the supernatant was dialyzed against ten volumes of 0.5% acetic acid with several changes for 4 days. During dialysis a precipitate appeared in the tubing which was removed by centrifugation at 34,800*g* for 30 min. This precipitate was dialyzed against distilled water and lyophilized. To the supernatant solid KCl was added to a final concentration of 15% at 4° to precipitate undenatured collagen. The precipitate was separated by centrifugation at 34,800*g* for 30 min and then suspended in 10 ml of 0.05% acetic acid and reprecipitated twice with 15% KCl. The final precipitate was suspended in 10 ml of 0.05% acetic acid and dialyzed against 0.05% acetic acid for 5 days. The three supernatant solutions were pooled, dialyzed exhaustively against distilled water, and lyophilized.

Acrylamide Electrophoresis. The method of Nagai *et al.* (1964) which separates the α, β, and γ components of denatured collagen was used. From 150 to 200 μg of protein was applied to each gel. After each run, duplicate gels were stained separately with Amido Black to detect protein bands and with the periodic acid–Schiff reagent to detect protein-bound carbohydrate.

Paper Chromatography. Carbohydrate components were identified by paper chromatography following hydrolysis of basement membrane collagen in 2 N HCl for 2 hr at 100° for neutral sugars and in 6 N HCl for 4 hr at 100° for amino sugars. The neutral sugars were separated from the amino sugars by the method of Boas (1953). Aliquots were spotted on Whatman No. 1 paper. Descending chromatography was run in *t*-amyl alcohol–isopropyl alcohol–H_2O (8:2:3, v/v). Chromatograms were sprayed with aniline oxalate to detect reducing sugars.

Ultracentrifugation. The sedimentation rate constant of the collagen extracted from basement membrane with Pronase at 4° was determined at a concentration of 0.3% in 0.15 M sodium citrate buffer (pH 3.65) at 4°. Schlieren optics were employed. Photographs were taken after attainment of maximum speed of 59,780 rpm.

Viscometry. Intrinsic viscosity was determined in a Ubbelohde suspended-level viscometer at 20°. The solvent was 0.15 M sodium citrate buffer (pH 3.65). Protein concentration was based on nitrogen content using the micro-Kjeldahl method.

Optical rotation was measured in a sodium vapor lamp apparatus using 0.2% solutions of protein in 0.15 M sodium citrate buffer (pH 3.65). Protein concentration was based on nitrogen content.

Circular Dichrosim. The collagen obtained from basement membrane after treatment with Pronase at 4° for 24 hr was subjected to circular dichroism analysis using the Cary Model 6001 spectropolarimeter. Protein was dissolved in 0.05% acetic acid at a concentration of 0.00133 g/ml. The cell path length was 0.1 mm, the scanning speed 50 Å/division, and the wavelength range 250–185 mμ. Analyses were performed at 27°. Native calfskin tropocollagen (obtained from Dr. M. B. Mathews of the University of Chicago) was analyzed similarly at a concentration of 0.002 g/ml. The values of molar ellipticity, $[\theta]$, in units of (deg cm^2)/dmole were obtained from the relation $[\theta] = (\theta/10)/(MRW/lc)$, where θ is the observed ellipticity; MRW, mean residue weight is 100; l is cell path length in centimeters; and c is the concentration of solute in grams per milliliter.

Electron Microscopy. Basement membrane collagen purified by KCl precipitation was dissolved in 0.05% acetic acid at a concentration of 0.5%. To the solution, 1% ATP[1] in 0.05% acetic acid was added until a precipitate was formed. Samples of the precipitate were placed on carbon-shaded grids and stained with 2% phosphotungstic acid (pH 6.5). The Siemens Elmiskop 1 was used.

Chemical Determinations. Samples of basement membrane collagen were hydrolyzed for 22 hr in 6 N HC at 110° and amino acid analyses were performed in a Phoenix analyzer. Early in the study the method of Piez and Morris (1960) was used. Recently, we have been using the method of Moore and Stein (1954) employing the microflow colorimeter in the Phoenix analyzer. With

[1] See *Biochemistry 5*, 1445 (1966).

TABLE I: Amino Acid Composition of Basement Membrane Fractions after Treatment with Pronase at 4°.[a]

Amino Acid	Precipitate B (collagen)	Supernatant C	Precipitate A	Dialysate	Residue
Aspartic	52.0	79.9	57.1	93.8	76.4
Threonine	21.1	62.6	31.5	58.5	40.9
Serine	42.8	71.3	42.0	70.7	65.7
Glutamic	77.0	76.2	79.4	132.8	106.6
Proline	60.0	90.4	64.3	58.3	75.6
Glycine	324.4	189.6	287.2	93.5	266.8
Alanine	33.4	114.0	48.4	99.7	67.7
Valine	27.0	39.8	34.8	62.1	40.2
Half-cystine	6.0	31.0	27.4	4.2	9.4
Methionine	6.5	9.3	6.0	20.5	7.4
Isoleucine	27.3	20.5	26.1	47.4	30.8
Leucine	51.0	36.0	50.2	99.0	65.0
Tyrosine	4.0	9.3	9.8	Trace	0.0
Phenylalanine	25.2	15.5	27.3	2.0	0.0
Lysine	5.0	15.5	15.0	74.0	25.5
Histidine	7.4	13.0	13.3	21.0	14.7
Arginine	25.5	26.0	36.5	64.0	37.5
Hydroxylysine	42.0	27.2	32.0	Trace	21.4
4-Hydroxyproline	144.0	56.3	94.0	Trace	22.1
3-Hydroxyproline	19.0	16.8	18.0	0.0	20.8

[a] See Figure 1. Residues/1000 residues.

the second method, separation of 3-hydroxy- from 4-hydroxyproline can be accomplished.

Total protein-bound hexose was determined by the orcinol reaction of Weimer and Moshin (1953). Fucose was measured by the method of Dische and Shettles (1948). Hexosamine was determined by the method of Elson and Morgan (1933) on protein samples that were hydrolyzed in 6 N HCl for 4 hr at 100° and the amino sugars were separated according to Boas (1953). Sialic acid was estimated on protein hydrolysates by the thiobarbituric acid method of Warren (1959) and by the resorcinol method of Svennerholm (1957). Glucose was measured with glucose oxidase using the Glucostat reagent (Worthington) on protein hydrolysates (2 N HCl for 2 hr at 100°). Excess HCl was removed with Dowex 3 (HCO^-_3) followed by evaporation under vacuum. The sample was then dissolved in 1 ml of 0.075 M phosphate buffer (pH 7.0), 1 ml of Glucostat reagent was added, and the solution was incubated at 37° for 10 min. At the end of this period 25 μl of 4 N HCl was added to stop the reaction, and the optical density was read at 400 mμ. Galactose was measured in protein hydrolysates (2 N HCl for 2 hr at 100°) with galactose oxidase using the Galactostat reagent (Worthington). The HCl-free sample was dissolved in 2 ml of 0.075 M phosphate buffer (pH 7.0), 2 ml of galactostat reagent was added, and the solution was incubated at 37° for 1 hr. At the end of this period 6 ml of 0.25 M glycine buffer (pH 9.7) was added to stop the reaction and to stabilize the color. Optical density was read at 425 mμ. Blanks and standards containing glucose or galactose were treated similarly.

Results

Isolation of Collagen. The isolation procedure of basement membrane collagen at 4° appears in Figure 1. In addition to the collagen (precipitate B), four additional fractions were obtained; of these, only the dialysate lacked hydroxyproline and hydroxylysine.

Amino Acid Composition. The amino acid composition of the basement membrane fractions obtained after treatment with Pronase at 4° appears in Table I. The collagen fraction which precipitated with 15% KCl has an amino acid composition which in some respects resembles that of tendon collagen, in that glycine accounts for about one-third of the amino acid residues and that the sum of proline and hydroxyproline residues accounts for about 22% of all the amino acid residues (Table II). Although basement membrane collagen contains 162 residues per 1000 residues of hydroxyproline and 60 of proline compared with 86 and 140 residues, respectively, for tendon collagen, the sum of the residues for these two amino acids is similar in both types of collagen. Significant differences between these two collagens include the large hydroxylysine and low lysine content in basement membrane collagen, the reverse of what is present in tendon collagen. There are six residues of half-cystine, while none is present in tendon collagen. There is significantly less alanine and arginine in basement membrane collagen compared with tendon collagen. Except for the similarity in methionine and tyrosine content, differences exist in the content of the remaining amino acids.

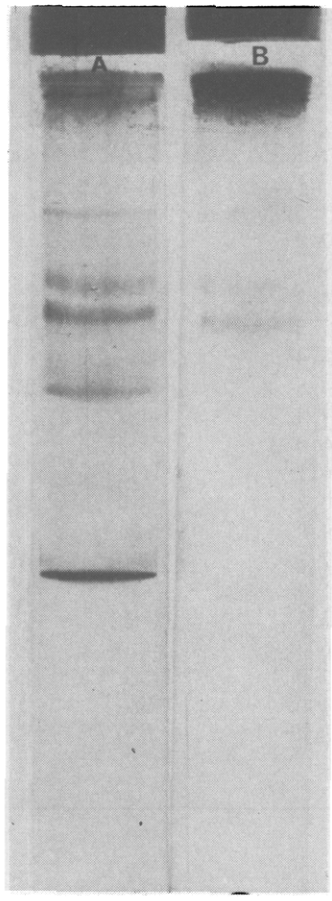

FIGURE 2: Disc electrophoresis of basement membrane collagen precipitated with 15% KCl following treatment of basement membrane with Pronase at 4° for 24 hr, 0.04 M acetic acid–0.23 M glycine buffer (pH 4.0). Protein (150 μg) was applied. Stained with Amido Black (A) and periodic acid–Schiff reagent (B).

TABLE II: Amino Acid Composition of Basement Membrane, Basement Membrane Collagen, and Tendon Collagen.[c]

	Basement Membrane		
	Intact	Collagen (ppt B[a])	Tendon[b] Collagen
Amino acid			
Aspartic	70.0	52.0	46.0
Threonine	40.5	21.1	17.0
Serine	49.0	42.8	33.0
Glutamic	97.2	77.0	63.0
Proline	69.8	60.0	140.0
Glycine	229.0	324.4	329.0
Alanine	65.0	33.4	122.0
Valine	36.0	27.0	20.0
Half-cystine	22.7	6.0	0.0
Methionine	5.0	6.5	6.0
Isoleucine	28.1	27.3	10.0
Leucine	60.2	51.0	21.0
Tyrosine	22.0	4.0	5.0
Phenylalanine	26.8	25.2	13.0
Lysine	26.0	5.0	23.0
Histidine	14.4	7.4	4.0
Arginine	48.2	25.5	45.0
Hydroxylysine	25.0	42.0	6.0
4-Hydroxyproline	65.0	144.0	86.0
3-Hydroxyproline		19.0	

[a] See Figure 1. [b] Kefalides and Winzler (1966). [c] Residues/1000 residues.

Carbohydrate Analysis. The carbohydrate composition of basement membrane collagen obtained by Pronase treatment at 4° appears in Table III. There is 10.4% hexose and equimolar amounts of glucose and galactose. There is less than 0.1% hexosamine and no sialic acid or fucose. The hexose content of basement membrane collagen is twice that found in intact glomerular basement membrane (Table III). The carbohydrate composition of precipitate A and supernatant C (Figure 1) is given in Table III for comparison. The hexose content in precipitate A is higher than that of intact basement membrane but there is also a higher amount of hexosamine. This would suggest the presence of a mixture composed of a collagenlike protein containing neutral hexose and another protein rich in hexosamine. Similarly, supernatant C which has a high hexose and hexosamine content must contain a mixture of peptides. The fraction which remained undigested after treatment with Pronase contains 5.2% hexose. The amount of hexosamine has been significantly reduced from 1.4% in intact basement membrane to 0.27%, while the content of sialic acid was reduced by only 50%. The fucose content remains unchanged (0.8%). The dialysate contains about 23% of the neutral hexose originally present in intact basement membrane. About 20% of the hexosamine, 17% of the sialic acid, and 12% of the fucose originally present in intact basement membrane are found in the dialysate.

Paper Chromatography. Paper chromatography of the collagen precipitated with 15% KCl revealed only glucose and galactose.

Acrylamide Electrophoresis. The electrophoretic pattern of glomerular basement membrane collagen isolated by Pronase treatment at 4° appears in Figure 2. There are three fast-moving components, two components with slower mobility and one component having the least mobility. Gel A was stained with Amido Black to detect protein bands and with the periodic acid–Schiff reagent to detect protein-bound carbohydrate. All bands stain for protein and carbohydrate. The fastest moving band stains only faintly for carbohydrate. Although the electrophoretic components have not yet been isolated and characterized, it is possible that they represent three free α chains, two β and one γ components.

Treatment of the collagen with collagenase followed by electrophoresis abolished both the protein and carbohydrate staining.

Physical Characteristics. The physical characteristics

TABLE III: Carbohydrate Composition of Basement Membrane and Its Fractions after Treatment with Pronase at 4°.

Carbohydrate	Basement Membrane (g/100 g)					Dialysate (%)[d]
	Intact	After Treatment with Pronase at 4°[a]				
		Ppt B (collagen)	Ppt A	Supernatant C	Residue	
Hexose[b]	5.52	10.40	9.3	15.0	5.2	23
Glucose	1.90	5.12	3.2	2.5	2.5	
Galactose	2.20	5.22	3.4	1.0	1.9	
Mannose[c]	1.41	0.00	2.7	11.5	0.8	
Hexosamine	1.40	Trace[e]	2.6	3.2	0.3	20
Sialic acid	2.10	0.00	3.4	9.0	1.0	17
Fucose	0.75	0.00	0.1	0.7	0.8	12

[a] See Figure 1. [b] Corrected for contribution of fucose. [c] Value obtained by difference between total hexose and the sum of glucose and galactose. [d] Values represent per cent of dialyzable carbohydrate in intact basement membrane. [e] Less than 0.1%.

of the basement membrane collagen are given in Table IV. This collagen has a specific optical rotation $[\alpha]_D^{24}$ of $-360°$ which agrees with the value obtained for acid-soluble tropocollagen (Cohen, 1955). Drake *et al.* (1966) observed that removal of the telopeptides by dialysis of Pronase-treated tropocollagen restores the specific optical rotation to normal.

The intrinsic viscosity of the basement membrane collagen is 5.2 dl/g. This value is slightly more than half the value obtained with Pronase-treated calfskin tropocollagen (Davison and Drake, 1966) and is consistent with a molecule having about 60% the length of skin tropocollagen.

The sedimentation behavior of the basement membrane collagen appears in Figure 3. The sedimentation coefficient, $s_{20,w}$, determined at 4°, is 3.4 S. This value approaches that observed for Pronase-treated skin tropocollagen of 3.8 S determined at 4° and 3.4 S determined at 20° (Davison and Drake, 1966).

Circular dichroism studies of basement membrane collagen gave molar ellipticity values similar to those obtained with native calfskin tropocollagen (Table IV). The spectra for the two collagens appear in Figure 4. There is a positive band at 223 mμ with an amplitude of 0.46×10^{-4} (deg cm²)/dmole for basement membrane collagen and 0.475×10^{-4} (deg cm²)/dmole for calfskin tropocollagen. A negative band forms at 198 mμ with an ellipicity value of 3.4×10^{-4} (deg cm²)/dmole for basement membrane collagen and 3.7×10^{-4} (deg cm²)/dmole for calfskin tropocollagen. The circular dichroism spectra of the two collagens reported here are almost identical with that for calfskin collagen reported by Timasheff *et al.* (1967). The data are consistent with the presence of a triple-helical configuration in both collagens (Timasheff *et al.*, 1967).

For the estimation of molecular weight the intrinsic viscosity and sedimentation measurements were applied to the formula of Sheraga and Mandelkern (1953). Using a partial specific volume of 0.7 an axial ratio of 1:110 was obtained from viscosity increment calculations (Cohn and Edsall, 1943). The average molecular weight for basement membrane collagen was calculated to be 210,000.

Reconstitution of Collagen. The collagen from basement membrane was dissolved in 0.05% acetic acid and examined in the electron microscope after negative staining with phosphotungstic acid. Only a filamentous structure could be observed. Addition of ATP to basement membrane collagen resulted in the formation of SLS-type aggregates with periodic structure (Figures 5 and 6). Certain fibers measure about 1660 Å (Figures 5A and 6A), while most measure 3250 Å. (Figures 5B and 6B). The latter appear to be aggregates of the 1660-Å fibers.

TABLE IV: Physical Characteristics of Basement Membrane Collagen.[a]

Specific optical rotation (deg)	
$[\alpha]_D^{24}$	−360
$[\alpha]_D^{40}$	−110[b]
Intrinsic viscosity, $[\eta]$ (dl/g)	5.2
Sedimentation coefficient, $s_{20,w}$ (S)	3.4
Molar ellipticity, $[\theta]$ (10^{-4} (deg cm²)/dmole)	+0.46 (+0.475)[c] −3.4 (−3.7)[c]
Molecular weight	
Sedimentation viscosity	210,000

[a] Collagen obtained by treatment of glomerular basement membrane with Pronase at 4° followed by precipitation with 15% KCl. [b] After heating at 40° for 40 min. [c] Values in parenetheses are for native calfskin tropocollagen.

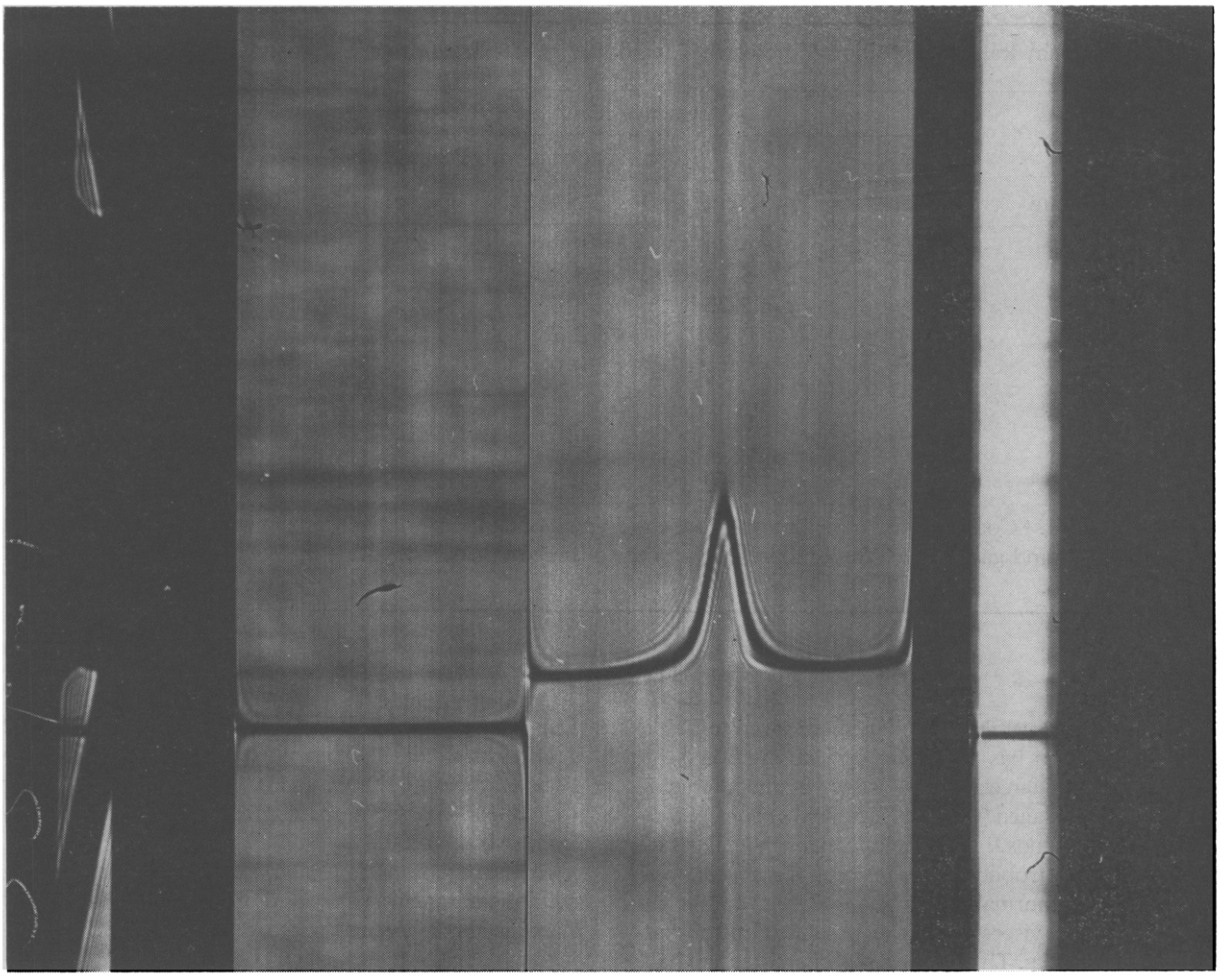

FIGURE 3: Sedimentation analysis of basement membrane collagen precipitated with 15% KCl following treatment of basement membrane with Pronase at 4°. Conditions: 0.15 M sodium citrate buffer (pH 3.65). Protein concentration 0.3%. Sedimentation velocity 59,780 rpm at 4°. Direction of sedimentation is to the right.

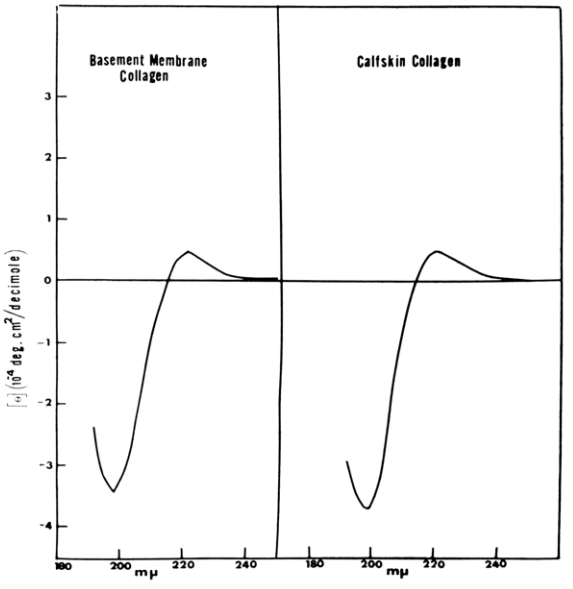

FIGURE 4: Circular dichroism of basement membrane collagen and calfskin tropocollagen. Basement membrane collagen: 0.00133 g/ml of 0.05% acetic acid. Calfskin tropocollagen: 0.002 g/ml of 0.05% acetic acid. Temperature 27°.

Discussion

Solubilization of insoluble skin collagen with proteolytic enzymes at low temperatures has been demonstrated by several investigators (Grant and Alburn, 1960; Oneson and Zacharias, 1960; Rubin *et al.*, 1965). More recently, Drake *et al.* (1966) studied the action of Pronase on calfskin-soluble tropocollagen and insoluble collagen and have shown that peptide appendages with an amino acid composition unlike that of the collagen molecule proper may be split off and that insoluble collagen may be solubilized. Previous solubilization procedures of basement membrane by reduction and alkylation of disulfide bonds in the presence of 8 M urea produced a soluble material which contained in terms of residues per 1000 residues no more than 250 of glycine and 70 of hydroxyproline (Kefalides and Winzler, 1966). It was obvious that a second component, noncollagen in nature was associated with the collagen component in basement membrane.

The data in the present study indicate that Pronase at low temperatures acts on glomerular basement membrane by digesting noncollagen peptides to sizes sufficiently small to dialyze out. Furthermore, as solubilized basement membrane is acted upon by the enzyme,

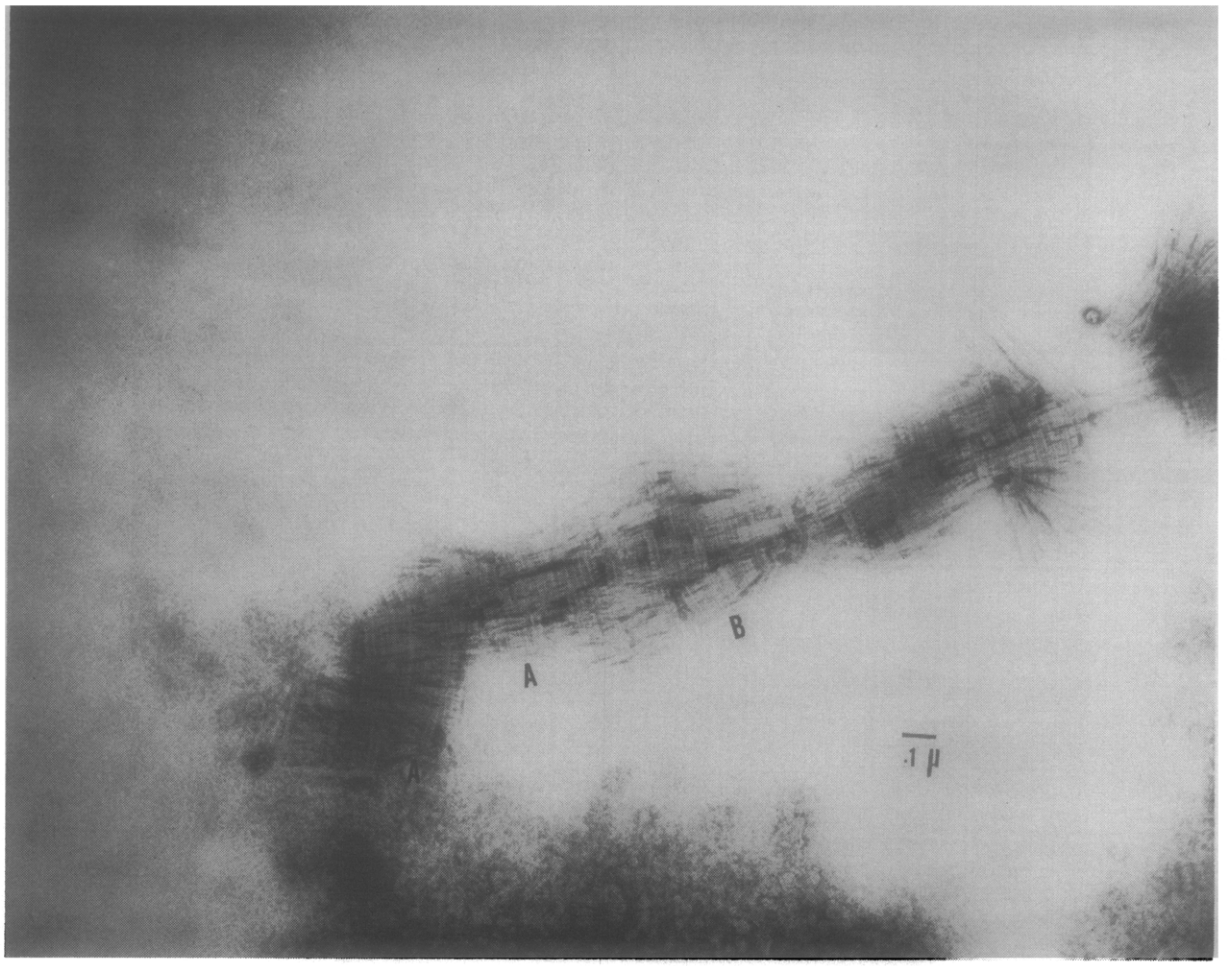

FIGURE 5: SLS agregates of basement membrane collagen precipitated with 1% ATP (basement membrane treated with Pronase at 4° and collagen precipitated with 15% KCl). Negative staining with phosphotungstic acid. Fibers measuring 1660 Å at A and 3250 Å at B (electron micrograph, × 65,000).

collagen is released and remains soluble in acetic acid while the fraction of basement membrane with portions of the noncollagen peptide still attached to the collagen is rendered insoluble at the low pH (Figure 1 and Table I, precipitate A).

The portion of the solubilized glomerular basement membrane which precipitates with 15% KCl accounts for about 8.5% of the starting material. The compositional data of this protein fit the chemical criteria for mammalian collagens, *i.e.*, 330 residues/1000 residues of glycine and a sum of proline plus hydroxyproline of 220. The existence of collagens having variable amounts of hydroxyproline, proline, and hydroxylysine have been reported in vertebrate and in invertebrate species (Piez and Likins, 1960; Watson and Sylvester, 1959; Gross, 1963). The unique features of the collagen isolated from glomerular basement membrane are the following. (a) It contains an excess of hydroxylysine, hydroxyproline, and neutral hexose. (b) It has a molecular weight and length corresponding to approximately 60% of that found in other mammalian collagens.

The high amounts of hydroxylysine and hydroxyproline suggest that the cells which are involved in basement membrane synthesis must contain a more active hydroxylating system compared with those involved in the synthesis of tendon or skin collagen. The finding of proportionately lower amounts of lysine and proline in basement membrane collagen would support the above view. Since anterior lens capsule of the eye and Bowman's capsule of the glomerulus (both epithelial basement membranes) have similar amino acid compositions and contain 35 and 32 residues per 1000 residues hydroxylysine, respectively, and since the collagen isolated from anterior lens capsule has a very similar amino acid composition as the collagen from glomerular basement membrane (Kefalides, 1968), it would appear that epithelial cells possess these highly active hydroxylating systems for lysine and proline. It should be noted, however, that while the epithelial cells of lens capsule are of ectodermal origin (McKeehan, 1951), the glomerulus and Bowman's capsule develop from differentiating mesenchymal cells (Davies, 1950).

The presence of large amounts of hexose linked to basement membrane collagen is an unusual feature since among vertebrate collagens, only vitrosin contains 10% hexose. It should also be noted that in vitrosin the ratio

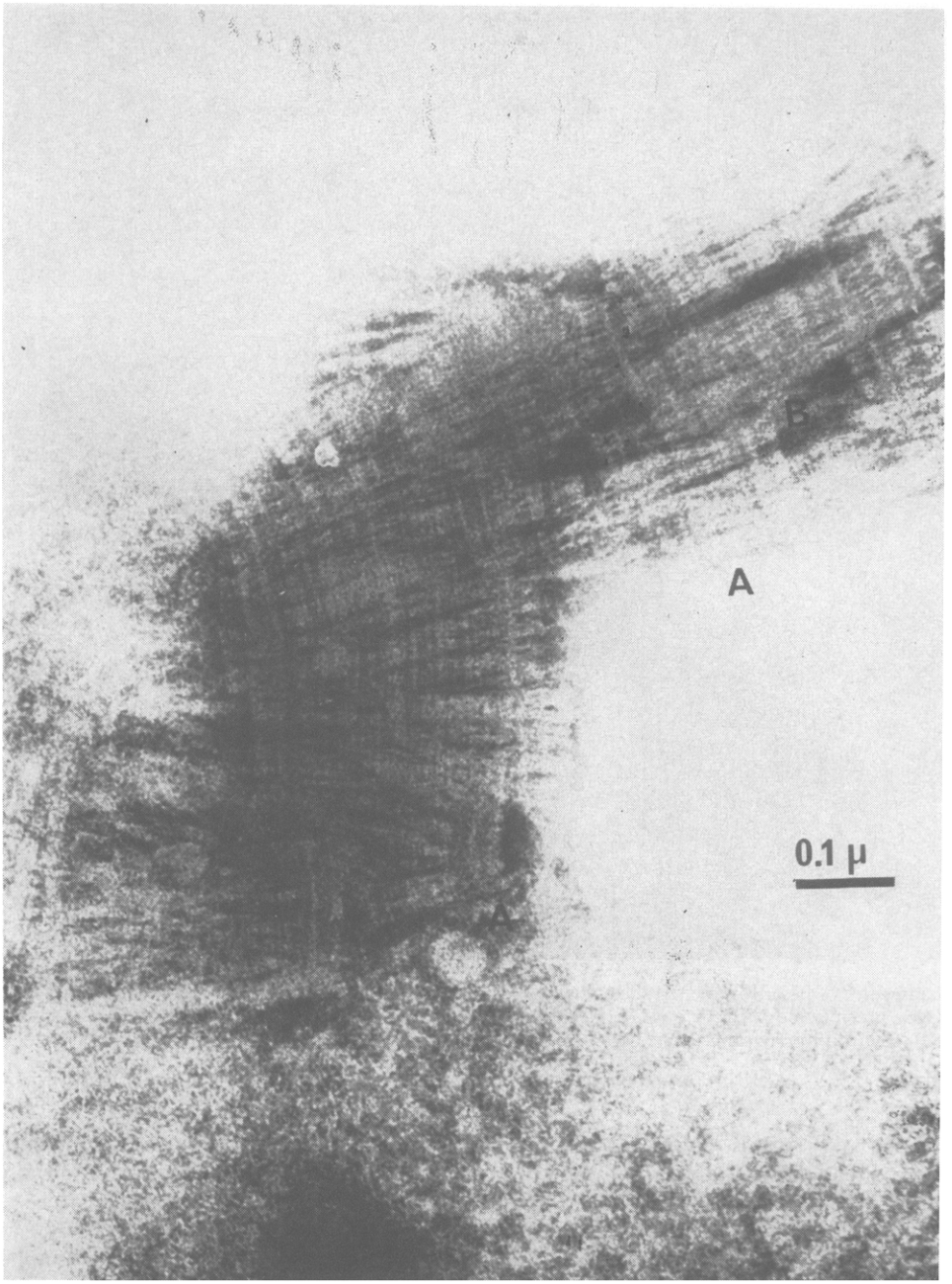

FIGURE 6: Magnification of the left half of Figure 5. SLS aggregates of basement membrane collagen. Negative staining with phosphotungstic acid. Fibers measuring 1660 Å at A and 3250 Å at B (electron micrograph, × 180,000).

of lysine to hydroxylysine is reversed, there being 22.5 residues of hydroxylysine to 13.7 residues of lysine per 1000 amino acid residues (Gross, 1963).

Glucose and galactose exist in equimolar amounts and account for all the carbohydrate in this collagen. The nature of the neutral hexose unit in bovine glomerular basement membrane has been elucidated by Spiro (1967) who found a disaccharide glycosidically linked to hydroxylysine having the sequence: glucosyl-galactosyl-hydroxylysine. A similar disaccharide linked to hydroxylysine has been found by Butler and Cunningham (1966) in guinea pig skin tropocollagen and by Kefalides (1967, 1968) in anterior lens capsule collagen. The occurrence of this disaccharide appears to be common to basement membrane collagens irrespective of the source. It has been isolated from human, rat, and dog glomerular basement membrane as well as alveolar basement membrane and choroid plexus of the brain (unpublished data).

The sequence and linkage of the oligosaccharide which contains galactose, mannose, hexosamine, fucose, and sialic acid are still unknown. Spiro (1967) has suggested that the oligosaccharide could be linked to asparagine by virtue of the fact that the glycopeptide bond of the oligosaccharide is alkali resistant and that aspartic acid occurs as one full residue. The fraction which

precipitates during dialysis of the Pronase digest against 0.05% acetic acid (Figure 1, precipitate A), contains increased amounts of neutral hexose, hexosamine, and sialic acid. Although this fraction can be considered to be collagenlike because of its high content of glycine, hydroxyproline, and hydroxylysine, the presence of large amounts of half-cystine, hexosamine, and sialic acid suggest that it might be associated with a noncollagen peptide fraction. Further treatment of this fraction with Pronase at 4° for 24 hr resulted again in a precipitate after dialysis against acetic acid but failed to yield a KCl-precipitable collagen. The observations that the hexosamine content of basement membrane collagen after treatment with Pronase at 18 and 25° varies between 1.2 and 0.2%, respectively, that after digestion of glomerular basement membrane with collagenase followed by dialysis more than 98% of hexosamine and sialic acid remain in the undialyzable residue, while 97% the neutral hexose and 90% of the hydroxyproline are found in the dialysate (unpublished data), strongly suggest the presence of at least two protein components in basement membrane—one being the collagen, containing glucose and galactose, and the other a noncollagen protein containing an oligosaccharide composed of galactose, mannose, hexosamine, fucose, and sialic acid. The mode of interaction of these two proteins *in vivo* is still unknown. The presence of disulfide bonds in basement membrane (Kefalides and Winzler, 1966) and the high half-cystine content in the fractions after Pronase treatment suggests that one type of linkage might involve disulfide bonds.

The possible role that the carbohydrate might play in the interaction of these protein components is not yet clear.

Physical characterization of the basement membrane collagen by circular dichroism and optical rotation studies indicates the presence of a triple-helical structure (Timasheff *et al.*, 1967). The values for molar ellipticity obtained with this collagen compare with those for native calfskin tropocollagen obtained in this study and the ones reported in the literature (Timasheff *et al.*, 1967).

The intrinsic viscosity value of 5.2 dl/g found for basement membrane collagen is about half of that reported for calfskin tropocollagen treated with Pronase at 20° (Davison and Drake, 1966). However, the sedimentation constant of 3.4 S is similar to that found by the above authors for Pronase-treated calfskin tropocollagen. These data, the calculated axial ratio of 1:110, and the molecular weight of 210,000 are consistent with a collagen molecule in basement membrane having about 60% the length and molecular weight of other mammalian collagens. The presence of SLS-type fibers on electron microscopy (Figures 5 and 6) measuring about 1660 Å is in agreement with a molecule having such dimensions. The question arises, however, whether these are the physical parameters of the collagen as it exists *in vivo* or whether the shorter molecule might result from limited clevage of the collagen molecule proper. Further studies are under way to elucidate this point.

It is of interest to note that although *in vivo* basement membrane shows no fibrous or periodic structure, the collagen which is isolated from it can be aggregated to form SLS-type fibers with ATP. SLS-type aggregates in some way similar to those obtained in this study have been observed by Olsen (1965) in ATP precipitates of vitrosin which was previously treated with trypsin. In lens capsule, as in glomerular basement membrane, the collagen molecules do not form large aggregates as seen in the electron microscope. This property could be related to the functional requirements of the basement membrane. If we were to ascribe two functions to the basement membranes, (a) supportive or elastic accommodation as in the lens capsule, and (b) selective permeability, as in the glomerular capillary, then if collagen molecules were permitted to aggregate into fibers, they would offer resistance to change in shape and size of the capsule, on one hand, and interfere with selective permeability in the case of the glomerular filtration. Such functional requirements could be met either by virtue of association of the collagen molecules with a noncollagen-type protein (Kefalides, 1966) or by their containing excess carbohydrate. Either alternative could alter the aggregation properties of the collagen molecules.

The present study demonstrates that glomerular basement membrane contains a collagen which differs from other mammalian collagens in that it contains large amounts of carbohydrate, hydroxylysine, and hydroxyproline. As it is obtained, after Pronase treatment at 4°, it has a molecular weight and length about 60% of that found for other mammalian collagens, however, it exhibits a triple-helical structure. Although it does not aggregate *in vivo* to form fibers, *in vitro* after solubilization with Pronase, it can be aggregated by the addition of ATP. It is proposed that the chemical and structural characteristics of this collagen could be related to the functional integrity of the basement membranes.

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