A COLLAGEN OF UNUSUAL COMPOSITION AND A GLYCOPROTEIN ISOLATED FROM CANINE GLOMERULAR BASEMENT MEMBRANE

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In a recent study, Kefalides and Winzler (1964,1965) reported the presence of a glycoprotein associated with a collagen-like protein in glomerular basement membrane of dogs. The basement membrane was solubilized by extraction with 8 M urea or by reduction and alkylation of disulfide bonds in 8 M urea. Amino acid analysis of basement membrane and its soluble fracti revealed the presence of a high hydroxylysine:hydroxyproline ratio (0.42 in basement membrane compared with 0.07 in mammalian collagen). Intact basement membrane contained 5.52% hexose, 1.4% glycosamine, 2.1% sialic acid, and 0.75% fucose. The amino acid and carbohydrate composition of the soluble fractions did not differ significantly from that of intact basement membrane. Dische et al. (1965) reported the presence of glucose and galactose linked to collagen and of a sialofucohexosaminoglycan in human glomerular basement membrane. The present report deals with the separation of two components from canine glomerular basement membrane. One is a glycoprotein and the other a collagen with a much higher hydroxyproline and hydroxylysine content than is found in other mammalian collagens.

Preparation of Glomerular Basement Membrane: Kidneys were obtained from healthy, mongrel dogs. The glomerular basement membrane was prepared according to the method of Krakower and Greenspon (1951).

Extraction Procedures

a) Extraction with 5% TCA: Ten milligrams of lyophilized basement membrane was extracted with 4 ml of 5% trichloroacetic acid for 1 hour at

80°C. After centrifugation at 1400 x g for 15 minutes, the insoluble residue was washed with 5% trichloroacetic acid, centrifuged and the washings added to the extract. Both the extract and the residue were dialyzed for 5 days against distilled water and lyophilized. The residue accounted for about 50% and the extract for about 25% of the starting material by weight. Since a major portion of the carbohydrate in basement membrane was extracted by this method, fractionation by column chromatography was attempted. Bovine Achilles tendon collagen was extracted with 5% TCA in a similar manner.

b) Extraction with 8 M Urea: One hundred milligrams of lyophilized basement membrane was extracted with 8 M urea, adjusted to pH 8.5 with 0.1% acqueous methylamine, at 37°C for 24 hours. The material was centrifuged at 34,800 x g for 1 hour. The supernatant solution was dialyzed against distilled water at 4°C for 5 days, and lyophilized. Approximately 60% of the original basement membrane was thus solubilized.

Column Chromatography: The material extracted from basement membrane with 8 M urea was dissolved in 0.05 M, Tris-HCl buffer, pH 8.4, applied to a Bio-Gel P-300 column (1.1 X 50 cm) and eluted with the same buffer. One ml aliquots were collected at a flow rate of 3 ml per minute. Protein concentration was measured by optical density readings at 280 mμ.

Two major fractions appeared. The first fraction (Fraction A) containing the bulk of the protein emerged with the void volume. The second fraction appeared as a low, broad peak between 52 and 68 ml of eluate. Only Fraction A was analyzed for amino acids and carbohydrate.

Chemical Determinations: Amino acid analysis was carried out on protein hydrolysates in a Technicon analyzer employing the methods of Moore and Stein (1954), and Piez and Morris (1960).

Total protein-bound hexose was determined in intact basement membrane and its fractions by the orcinol reaction of Weimer and Moshin (1953). Samples of protein were hydrolyzed in 6 N HCl for 4 hours at 100°C and the

amino sugars separated according to Boas (1953). Hexosamine was determined by the method of Elson and Morgan (1933).

Results: Table 1 shows the amino acid composition of collagen from bovine Achilles tendon, of basement membrane and its fraction obtained by extraction with 5% TCA. Values are expressed in terms of residues per 100 residues of amino acids. It will be noted that intact basement membrane contains less lysine, hydroxyproline and proline than does tendon collagen. However, basement membrane contains 2.2 residues of hydroxylysine compared to

TABLE 1

AMINO ACID ANALYSIS OF BASEMENT MEMBRANE AND TENDON COLLAGEN*

	Achilles Tendon Collagen	Basement Membrane TCA Extract	Basement Membrane Intact
Aspartic	4.9	5.9	7.3
Threonine	1.7	2.1	4.0
Serine	3.6	3.9	4.9
Glutamic	8.0	8.6	11.0
Proline	12.5	7.1	7.0
Glycine	31.2	31.0	21.0
Alanine	10.8	5.6	6.7
Valine	2.4	2.7	4.7
Cystine 1/2	0.00	1.00	1.6
Methionine	0.45	0.2	0.9
Isoleucine	1.4	1.8	2.8
Leucine	2.6	4.6	6.7
Tyrosine	0.5	0.5	1.8
Phenylalanine	1.4	2.1	3.3
Lysine	2.4	1.3	2.8
Histidine	0.5	0.6	1.4
Arginine	5.4	2.9	4.9
HO-Proline	9.9	15.8	5.3
HO-Lysine	0.7	4.3	2.2
$\frac{\text{HO-Lysine}}{\text{HO-Proline}}$	0.07	0.27	0.42

*Values are expressed in residues per 100 residues.

0.7 in collagen. The TCA extract of basement membrane, although resembling tendon collagen in some respects, differs significantly in others. The glycine content is identical in both (31.0 residues in the TCA extract compared with 31.2 in tendon collagen). The sum of hydroxyproline and proline is similar in both proteins (22.9 residues in the TCA extract and 22.4 in tendon collagen). However, there are 15.8 and 7.1 residues of hydroxyproline and proline, respectively, in the TCA extract compared to 9.9 and 12.5 residues, respectively in tendon collagen. Similarly, there is more hydroxylysine and less lysine in the TCA extract than in tendon collagen (4.3 and 1.3 residues respectively in the TCA extract compared to 0.7 and 2.4 residues in tendon collagen).

The amino acid composition of the TCA extract from tendon collagen did not differ from that of intact tendon collagen (data not included in Table I). If it is assumed that all the hydroxyproline in basement membrane is contributed by the collagen component, then it can be concluded that the collagen makes up to about 30% by weight of basement membrane. Column chromatography of the material extracted from basement membrane with 8 M urea resulted in the isolation of a non-collagen component (Fraction A) richer in carbohydrate than the TCA insoluble residue from basement membrane.

Table 2 summarizes the amino acid composition of the fraction which was extracted with 8 M urea and of the non-collagen fraction of basement membrane. Striking similarities are noted between Fraction A and the TCA residue. In both hydroxyproline and hydroxylysine are practically absent. Differences are evident in the content of valine, lysine and glycine. Table 3 shows the hexose and hexosamine content of intact basement membrane and the various fractions. Extraction with 5% TCA removes a large portion of hexose and hexosamine. In contrast, the hexose and hexosamine content of the 8 M urea extract is similar to that of intact basement membrane. Furthermore, separation of the non-collagen component by chromatography in Bio-Gel P-300 from the 8 M urea extract, results in a fraction richer in carbohydrate,

TABLE 2

AMINO ACID ANALYSIS OF BASEMENT MEMBRANE FRACTIONS*

	8 M Urea Extract	Non-Collagen Component Fraction A	TCA Residue
Aspartic	8.00	8.3	8.1
Threonine	4.7	6.0	5.7
Serine	6.2	8.7	7.3
Glutamic	10.6	13.2	11.4
Proline	6.6	8.3	6.8
Glycine	20.0	13.5	12.0
Alanine	6.3	7.6	8.8
Valine	4.9	3.8	5.1
Cystine 1/2	1.4	2.9	2.9
Methionine	1.2	0.9	0.8
Isoleucine	3.8	4.3	3.5
Leucine	7.0	8.9	8.1
Tyrosine	1.9	1.6	2.1
Phenylalanine	3.3	4.2	3.3
Lysine	2.8	1.0	4.0
Histidine	1.9	1.2	1.9
Arginine	5.2	5.4	6.0
HO-Proline	5.0	Trace	0.00
HO-Lysine	2.0	Trace	Trace

*Values are expressed in residues per 100 residues.

containing 0.5 μmoles hexose/mg of protein (9% by weight) and 0.074 μmoles hexosamine/mg of protein (1.3% by weight).

The data suggest that canine glomerular basement membrane is made up of a collagen, unique in its composition. It contains one and a half times as much hydroxyproline and six times as much hydroxylysine, as tendon collagen. The amounts of proline and lysine are reduced by almost 50% in the collagen obtained from basement membrane. The non-collagen component is a glycoprotein which contains 9% hexose and 1.3% hexosamine. In previous communications (Kefalides and Winzler, 1964,1965) we have reported that the X-ray diffraction pattern of basement membrane gives a "powder" diagram with

TABLE 3

CARBOHYDRATE ANALYSIS OF BASEMENT MEMBRANE

μmoles/mg

	Intact	8 M Urea Extract	TCA-Extract	TCA-Residue	Fraction-A
Hexose	0.31	0.32	0.42	0.15	0.50
Hexosamine	0.056	0.06	0.027	0.048	0.074

measurements at 2.85, 4.23 and 10.27 Å, compatible with triple helical, non-oriented structure. If one accepts Ramachandran's criteria (1962) that collagen is a protein with a triple helical structure and whose glycine content makes up about 1/3 of the total amino acid residues and whose hydroxyproline plus proline content accounts for about 2/9 of the total residues, then the fraction extracted from basement membrane with 5% TCA could be classified as a collagen.

The significance of the increased content of hydroxylated amino acids is not as yet clear. It has been shown by Highberger et al. (1951), Schmitt et al. (1955), Wood and Keech (1960), and more recently by Grant et al. (1965) and Mathews (1965), that acid glycoprotein or chondroitin sulfate will affect the reconstitution of collagen fibers. It is conceivable that the type and concentration of collagen and polysaccharide in the extracellular phase may influence the average diameter and rate of fibril formation in vivo. Although no collagen fibers have been seen in basement membrane on electron microscopy, the data presented here indicate that collagen is present and is associated with a glycoprotein. This collagen is unique in its hydroxyproline and hydroxylysine content. Studies are under way to elucidate the possible role that these amino acids might play in the structural orientation of basement membrane.

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