# The Chemistry of Glomerular Basement Membrane and Its Relation to Collagen*

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ABSTRACT: The basement membrane from glomeruli of normal dogs was isolated by a procedure involving sonication and differential centrifugation. The amino acid composition of the isolated material in several respects resembled that of mammalian collagen, but contained less glycine, proline, and hydroxyproline, and more hydroxylysine and cystine. The hydroxylysine–hydroxyproline ratio in basement membrane was 0.42, compared with 0.07 in collagen. The total carbohydrate content of basement membrane was about 10% by weight while that of collagen was 0.6%. There were about 0.03 μmole of ester-like groups/mg of basement membrane and 0.05 μmole/mg in collagen demonstrable by the hydroxylamine reaction. X-Ray diffraction gave powder diagrams similar to those of denatured collagen and procollagen, with spacings of 2.8, 4.23, and 10.27 A. The amino acid and carbohydrate composition of solubilized basement membrane was similar to that of the intact material. Soluble material was obtained from basement membrane by reduction of disulfide bonds with mercaptoethanol in 8 M urea followed by alkalation of the sulfhydryl groups with iodoacetate. The soluble product had the following physical constants, $s_{20,w}$ 2.4, $D_{20,w}$ 1.46, and mol wt = 132,000. Disk electrophoresis revealed two fast and one slow component, the latter staining for carbohydrate. Common antigenic determinants were demonstrated between intact basement membrane and its soluble fractions. Precipitin antibodies in antisera prepared by injection of intact or solubilized basement membrane were directed against the carbohydrate-containing protein. It is concluded that normal canine glomerular basement membrane is composed of a collagen-like protein and a glycoprotein.

Basement membrane can be of epithelial cell origin, of mesenchymal (vascular) cell origin, or a combination of both (Kurtz and Feldman 1962; Krakower and Greenspon, 1964; Pierce *et al.*, 1964). The latter is exemplified by the basement membrane of kidney glomeruli, which is adjacent on one side to endothelial cells of the capillary wall and on the other to the epithelial cells of the visceral portion of Bowman's capsule. The presence of large amounts of hydroxyproline in human and dog glomerular basement membrane has led to the suggestion (Goodman *et al.*, 1955) that the membrane is largely composed of collagen, even though collagen fibrils are not seen. Studies by Goodman *et al.* (1955) and Krakower and Greenspon (1964) in dogs and rats suggest that epithelial and mesenchymal basement membranes are related chemically and immunologically in that both possess large amounts of hydroxyproline and carbohydrate, and both contain at least two types of antigen in common, one being related to collagen. However, Mukerjee *et al.* (1965), and Pierce *et al.* (1964), working with mouse-neoplastic epithelial basement membrane, have suggested that epithelial and mesenchymal basement membranes are distinct chemically and immunologically, and that the collagen-like antigen is present only in the mesenchymal basement membrane. Rothbard and Watson (1961), on the other hand, demonstrated that rabbit antibody to rat collagen became localized in the basement membrane of rat glomeruli. The presence of large amounts of hydroxyproline, hydroxylysine, glycine, and carbohydrate have been reported in extracts of human glomeruli by Markowitz and Lange (1964) and by Lazarow and Speidel (1964). The relation between basement membrane and collagen is, thus, still not clear.

Glomerular basement membrane is insoluble in water and in weak concentrations of acids and alkalis. The purpose of the present study has been to develop methods for the solubilization of dog glomerular basement membrane, to determine the physical, chemical, and immunologic properties of the intact basement membrane and of its soluble fragments, and to compare these properties with those of collagen.

## Materials and Methods

### *Preparation of Glomerular Basement Membrane*

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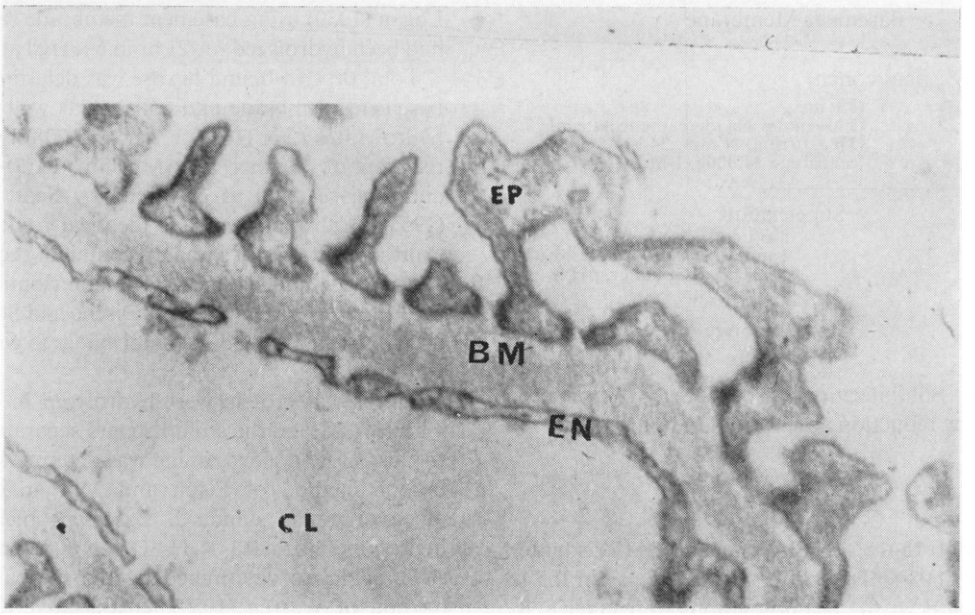


FIGURE 1: Section of canine glomerulus. EP denotes epithelial cell, EN endothelial cell, CL capillary lumen, and BM basement membrane. (Electron microphotograph 47,000×.)

Kidneys were obtained from healthy, mongrel dogs used for acute experiments in the departments of physiology or pharmacology of the University of Illinois College of Medicine. Once removed, the kidneys were immediately placed in sterile, cold 0.85% NaCl solution and kept in a deep freeze at −25°. The basement membrane was prepared according to the method of Krakower and Greenspon (1951). All samples of basement membrane were examined under the light microscope after staining with methylene blue. The cell-free basement membranes appeared as light blue ribbons with some amorphous debris interspersed among them. If the samples appeared satisfactory, they were lyophilized. Certain samples were studied more extensively to establish the purity of the preparations. The most reliable criterion of purity was the examination of the basement membrane preparation under the electron microscope. For this purpose, basement membrane in the amount equivalent to that prepared from a half kidney was stained with uranyl acetate and lead acetate and imbedded in epon according to the method described by Luft (1961). Sections 0.05-$\mu$ thick were cut and placed in grids. The RCA Model EM-U3E electron microscope was used for the study. The basement membrane appeared as a homogeneous narrow cast, free of cells or cell membranes and free of tubular elements (Figures 1 and 2). The width of the basement membrane ranged from 2900 A to 4000 A with an average of 3450 A.

*Preparation of Collagen.* Small portions of dog Achilles tendon were freed of fascia and were sonicated first in a mixture of chloroform–methanol (1:3, v/v) and then in 0.85% NaCl to remove lipids and vascular debris. The collagen was then prepared according to the method of Einbinder and Schubert (1951). Bovine Achilles tendon collagen was obtained commercially from Nutritional Biochemicals, Ohio.

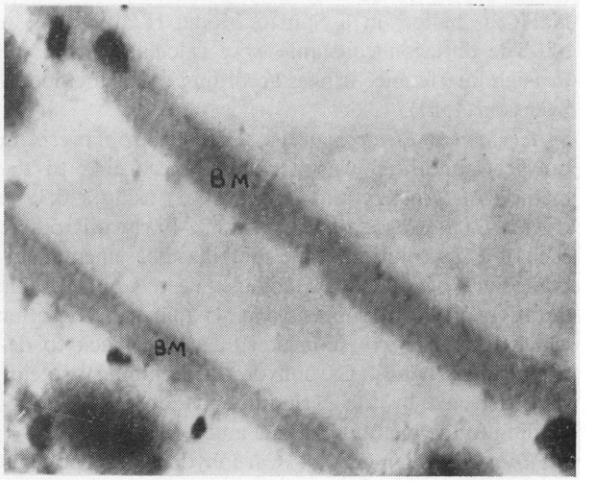


FIGURE 2: Canine glomerular basement membranes devoid of epithelial and endothelial cells. BM denotes basement membrane. (Electron microphotograph 41,000×.)

*X-Ray Diffraction.* X-Ray diffraction photographs were taken with Philips powder cameras, 114.6-mm diameter, using Cuka radiation. The following materials were studied; bovine Achilles tendon collagen fibers, bovine Achilles tendon heat-denatured collagen, and canine glomerular basement membrane.

*Ultracentrifugation.* Sedimentation rate constants were determined for solubilized glomerular basement membrane fractions and solubilized collagen at a con-

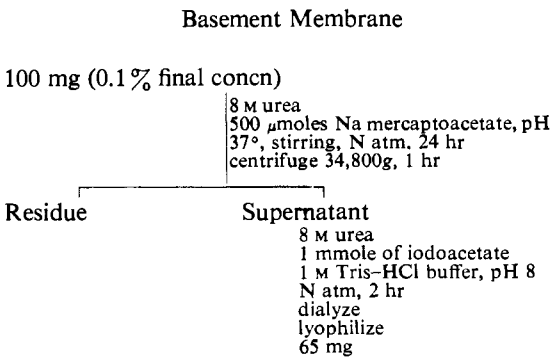


FIGURE 3: Solubilization procedure of basement membrane by reduction and alkylation in 8 M urea.

centration of 0.75–1% in 0.2 M NaCl–0.05 M Tris buffer at pH 8, in 0.05 M Tris buffer at pH 7.5, or in 0.5 M $NaHCO_3$ buffer at pH 8. Photographs were taken at 8-min intervals at a speed of 59,780 rpm. Both the conventional and the synthetic boundary methods were used.

*Diffusion.* Diffusion studies were performed at pH 8 (0.2 M NaCl–0.05 M Tris buffer) and at pH 8 (0.5 M $NaHCO_3$ buffer) in a Spinco Model H apparatus at 5°. The diffusion constants were calculated from the Raleigh interference fringes according to the method of Svensson (1951).

*Acrylamide Electrophoresis.* Disk electrophoresis in polyacrylamide gel was performed according to the method of Ornstein and Davis (1962) using a 0.38 M glycine–0.05 M Tris buffer, pH 8.3. Forty microliters each of a 2% solution of the solubilized basement membrane and of solubilized collagen were applied. The duration of electrophoresis was 45 min at 40 v, after which the gels were stained with amido black to detect protein bands. Carbohydrate-containing proteins were detected by the periodic acid–Schiff reagent according to the method of Uriel and Grabar (1956).

*Paper Chromatography.* Carbohydrate components were identified by paper chromatography following hydrolysis of basement membrane 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) using a Dowex-50 $H^+$ column 0.93 × 5 cm. Aliquots were spotted on Whatman No. 1 paper. Descending chromatography was run in ethyl acetate–pyridine–$H_2O$ system (10:4:3, v/v/v). Chromatograms were sprayed with aniline oxalate to detect reducing sugars (Partridge, 1946).

*Chemical Determinations.* Amino acid analyses were carried out on protein hydrolysates in a Technicon analyzer employing the methods described by Moore and Stein (1954), and by Piez and Morris (1960). Hydrolysis was for 21 hr in 6 N HCl at 110°. No corrections have been applied for amino acid destruction. Hydroxyproline was determined both by automated amino acid analyses and by the colorimetric method of Neuman and Logan (1950) using basement membrane samples which had been hydrolyzed for 21 hr in 6 N HCl at 110°.

Total protein-bound hexose was determined in intact basement membrane and collagen as well as their acid hydrolysates (2 N HCl for 2 hr at 100°) by the orcinol reaction of Weimer and Moshin (1953) and by the anthrone reaction as described by Scott and Melvin (1953). Both methods gave identical results which are expressed in terms of standard containing equal amounts of galactose and mannose. The contribution of fucose determined by the method of Dische and Shettles (1948) was subtracted from the orcinol values to get true hexose values.

Samples of protein were hydrolyzed in 6 N HCl for 4 hr at 100°, and the amino sugars separated according to Boas (1953). Hexosamine was determined essentially by the method of Elson and Morgan (1933) using glucosamine as standard. Basement membrane was hydrolyzed with 0.1 N $H_2SO_4$ at 80° for 2.5 hr and sialic acid was determined by the thiobarbituric acid method of Warren (1959). Quantitative estimation of glucose was carried out on hydrolysates (2 N HCl for 2 hr at 100°) using glucose oxidase as described by Kingsley and Getchell (1960). Galactose was measured quantitatively in basement membrane hydrolysates employing the galactose oxidase method of de Verdier (1962) as modified by Hsia and Inouye (1966). Mannose was calculated from the difference between total hexose, corrected for fucose, and the sum of galactose and glucose determined enzymatically. Total nitrogen was determined by the micro Kjeldahl method. Amide nitrogen was measured by the method of Conway and O'Malley (1942). Sialic acid also subjected to the same procedure released 24% of its nitrogen as ammonia. This necessitated a very small correction for the amide nitrogen value amounting to 0.017 μmole/mg. Acetyl groups were hydrolyzed from protein samples with 2 N HCl for 2 hr in sealed tubes and their content determined by gas partition chromatography using the method of Lehnhardt and Winzler (1964, unpublished data). The presence of ester-like groups was determined by the hydroxylamine reaction under the conditions described by Gallop *et al.* (1959). The colorimetric method of Csasky (1948) for the estimation of hydroxylamine taken up by basement membrane and collagen was used. Disulfide groups were measured by the method of Karush *et al.* (1964) employing the quenching effect of sulfhydryl groups on the fluorescence of fluorescein–mercuric acetate.

*Immunochemical Methods.* PREPARATION OF ANTISERA. Antisera were prepared in rabbits against the following antigens: (1) intact canine glomerular basement membrane (BM),[1] (2) an 8 M urea extract of basement membrane (USBM), (3) a soluble fraction of basement membrane obtained by reduction and alkylation in 8 M urea (RABM) (see Figure 3). The designations anti-BM, anti-USBM, and anti-RABM refer to their antisera.

[1] Abbreviations used: BM, basement membrane; USBM, urea extract of basement membrane; RABM, basement membrane from reduction and alkylation in urea.

TABLE I: Amino Acid Composition of Canine Glomerular Basement Membrane and of Canine and Bovine Collagen.

| Amino Acid | Basement Membrane[a] | | | Residues/100 Residues | |
|---|---|---|---|---|---|
| | μmoles/mg (mean ± SE) | (g/100 g) | Residues/100 Residues | Canine[b] Collagen | Bovine[b] Collagen |
| Aspartic | 0.514 ± 0.031 | 6.85 | 7.3 | 4.6 | 4.9 |
| Threonine | 0.280 ± 0.024 | 3.33 | 4.0 | 1.7 | 1.7 |
| Serine | 0.345 ± 0.023 | 3.63 | 4.9 | 3.3 | 3.6 |
| Glutamic | 0.773 ± 0.088 | 11.37 | 11.0 | 6.3 | 8.0 |
| Proline | 0.489 ± 0.022 | 5.63 | 7.0 | 14.0 | 12.5 |
| Glycine | 1.455 ± 0.108 | 10.92 | 21.0 | 32.9 | 31.2 |
| Alanine | 0.469 ± 0.020 | 4.17 | 6.6 | 11.2 | 10.8 |
| Cystine | 0.057 ± 0.001 | 1.38 | 0.8 | 0.0 | 0.0 |
| Valine | 0.332 ± 0.013 | 3.89 | 4.7 | 2.0 | 2.3 |
| Methionine | 0.062 ± 0.000 | 0.92 | 0.9 | 0.6 | 0.5 |
| Isoleucine | 0.200 ± 0.015 | 2.62 | 2.8 | 1.0 | 1.4 |
| Leucine | 0.470 ± 0.016 | 6.16 | 6.7 | 2.1 | 2.6 |
| Tyrosine | 0.125 ± 0.000 | 2.26 | 1.8 | 0.5 | 0.5 |
| Phenylalanine | 0.233 ± 0.002 | 3.85 | 3.3 | 1.3 | 1.4 |
| Lysine | 0.200 ± 0.012 | 2.92 | 2.8 | 2.3 | 2.3 |
| Histidine | 0.100 ± 0.002 | 1.55 | 1.4 | 0.4 | 0.5 |
| Arginine | 0.345 ± 0.017 | 6.01 | 4.90 | 4.5 | 5.4 |
| Hydroxyproline | 0.370 ± 0.030 | 4.85 | 5.7 | 8.6 | 9.9 |
| Hydroxylysine | 0.154 ± 0.011 | 2.48 | 2.2 | 0.6 | 0.70 |
| (Ammonia) | 0.970 ± 0.073 | 1.65 | ... | ... | ... |
| Total Nitrogen | 10.00 | 15.00 | | (18.2)[c] | (18.5)[c] |
| HO-Lysine/HO-proline | | | 0.42 | 0.07 | 0.07 |

[a] Average of 10 runs. [b] Average of 2 runs. [c] N in g/100 g.

Healthy, white rabbits, weighing approximately 4 kg, were immunized with four weekly innoculations. The rabbits were bled by cardial puncture 1 week after the final injection. Ten milligrams of BM and of USBM and 5 mg of RABM were suspended in 1 ml of sterile 0.85% NaCl solution and mixed with 1 ml of Freund's adjuvant for each injection. The first, second, and fourth injections were given intramuscularly in the popliteal region. The third injection was given into the footpad. Ten milliliters of control blood was withdrawn from each rabbit prior to the commencement of immunization. Complement fixation studies were performed by the method of Kabat and Mayer (1961) using 1% suspensions of the antigens sonicated in 0.85% NaCl solution for 10 min to ensure homogeneity.

Immunoelectrophoresis was carried out according to the method of Sheidegger (1955). The antigens, USBM and RABM, were dissolved in 0.5 M $NaHCO_3$ to a final concentration of 1% by weight. A 1% solution in 0.5 M $NaHCO_3$ of a soluble fraction of collagen obtained by extraction with 8 M urea was also used as antigen. Electrophoresis was carried out using 1% agar in 0.5 M Veronal buffer at pH 8.6. Anti-BM and anti-USBM were diffused against each of the antigens following electrophoresis. Following disk acrylamide gel electrophoresis of USBM, the gel column was sectioned into disks of 1–3-mm. thickness. These were placed sequentially on glass slides and covered with 1% agar in 0.5 M Veronal buffer at pH 8.6. A trough was cut into the agar 5 mm from and parallel to the acrylamide disks. The slides were incubated for 24 hr at room temperature and at 4° overnight. Precipitin lines were observed within the first 24 hr.

*Solubilization Procedures.* EXTRACTION WITH 8 M UREA AT 37°. One hundred milligrams of basement membrane was mixed with 100 ml of 8 M urea. The mixture was allowed to stand at 37° with continuous slow stirring for 24 hr. Following this, the mixture was centrifuged at 34,800*g* for 1 hr. The supernatant was dialyzed against large volumes of distilled water for 5 days at 4° and lyophilized. This procedure allowed about 60% of the starting material to be extracted. By further extracting the insoluble residue two more times, almost all of the starting material could be solubilized. The lyophilized material thus extracted with 8 M urea was soluble in distilled water only to an extent of 0.1%. It was soluble to an extent of about 1% in 0.05 M pH 8 Tris buffer; in 0.05 M pH 7.5 phosphate buffer; in 2% $NaHCO_3$; and readily soluble in 4, 6, and 8 M urea.

SOLUBILIZATION OF BASEMENT MEMBRANE BY REDUCTION AND ALKYLATION IN 8 M UREA. The presence of cystine in basement membrane (see Table I) suggested that reduction of the cystine with subsequent alkylation might result in water-soluble fragments. One hundred milli-

TABLE II: Amino Acid Analysis of Basement Membrane and Its Soluble Fractions.

| Amino Acid | Residues/100 Residues | | Residues/100 Residues | |
|---|---|---|---|---|
| | Basement Membrane | 8 M Urea Extract | Reduced and Alkylated Extract | Residue after Reduction and Alkylation |
| Aspartic | 7.3 | 8.0 | 7.6 | 7.0 |
| Threonine | 4.0 | 4.7 | 4.0 | 4.0 |
| Serine | 4.9 | 6.2 | 6.0 | 5.1 |
| Glutamic | 11.0 | 10.6 | 10.0 | 9.9 |
| Proline | 7.0 | 6.6 | 7.0 | 7.0 |
| Glycine | 21.0 | 17.0 | 25.0 | 21.0 |
| Alanine | 6.7 | 6.3 | 6.2 | 6.0 |
| Cystine | 0.8 | 0.7 | 0.0 | 0.5 |
| Valine | 4.7 | 4.9 | 4.0 | 4.0 |
| Methionine | 0.9 | 1.5 | 0.8 | 1.2 |
| Isoleucine | 2.8 | 3.8 | 3.4 | 3.3 |
| Leucine | 6.7 | 7.9 | 7.0 | 6.7 |
| Tyrosine | 1.8 | 1.9 | 1.8 | 1.6 |
| Phenylalanine | 3.3 | 3.3 | 3.3 | 3.1 |
| Lysine | 2.8 | 2.8 | 2.5 | 3.2 |
| Histidine | 1.4 | 1.9 | 1.8 | 1.3 |
| Arginine | 4.9 | 5.2 | 5.0 | 4.6 |
| Hydroxyproline | 5.7 | 5.0 | 7.0 | 7.1 |
| Hydroxylysine | 2.2 | 2.0 | 2.7 | 2.7 |
| Carboxymethylcysteine | ... | ... | 1.9 | ... |
| HO-Lysine/HO-proline | 0.42 | 0.40 | 0.40 | 0.40 |

grams of lyophilized basement membrane was added to a 250-ml flask containing a 100-ml solution of 8 M urea, 500 μmoles of sodium mercaptoacetate, or 500 μmoles of mercaptoethanol. The pH of the solution was adjusted to 7.5 with dilute sodium hydroxide, nitrogen gas was bubbled into the flask for 5 min. The mixture was then allowed to stand in a 37° room for 24 hr with continuous gentle stirring. Undissolved material was removed by centrifugation at 34,800*g*. To the supernatant in a three-necked flask was added dropwise 25 ml of a solution of 0.04 M sodium iodoacetate and 8 M urea, in one of three buffers: (a) 1 M pH 9 glycine, (b) 1 M pH 8 Tris, or (c) 1 M pH 9 sodium bicarbonate. The alkylation was followed from the disappearance of the nitroprusside test for sulfhydryl groups. The solution was stirred gently with a magnetic stirrer and allowed to continue for about 30 min after the nitroprusside test became negative. The solution was then dialyzed against distilled water for 5 days and lyophilized. About 65% of the starting material was recovered in a single extraction. The lyophilized material was soluble to the extent of 1% in distilled water, in 0.05 M pH 8 Tris buffer; in 0.05 M pH 7.5 phosphate buffer, and in 2% $NaHCO_3$. Normal human glomerular basement membrane was subjected to the same solubilization procedure.

*Solubilization of Collagen.* One-hundred milligrams of dog tendon collagen was subjected to the reduction–alkylation procedure outlined in the previous section.

## Results

*Amino Acid Composition.* The amino acid composition of intact canine glomerular basement membrane and of canine and bovine collagen appears in Table I. The data are expressed in three ways, in terms of μmoles per mg of material, in terms of the weight per cent of each residue, and in terms of the number of residues of each amino acid per 100 total residues. Certain similarities and differences between basement membrane and collagen are apparent.

Like collagen, basement membrane is high in hydroxyproline, proline, and glycine. However, glycine accounts for about 33% of the total amino acid residues in collagen, but for only about 20% in basement membrane. In collagen, the combined proline–hydroxyproline content accounts for about 22% of the total residues while in basement membrane it accounts for only about 13%. A striking difference between basement membrane and collagen is the much higher hydroxylysine content in the former (2.2 residues of hydroxylysine per 100 residues in basement membrane compared with 0.7 in collagen). This gives a hydroxylysine–hydroxyproline ratio of 0.42 for basement membrane compared with 0.07 for collagen. Another significant difference between the two materials is in the cystine

TABLE III: Carbohydrate Composition of Basement Membrane, of Its Soluble Fractions, and of Collagen.

| Carbohydrate | Basement Membrane | 8 M Urea Extract (g/100 g) | Reduced and Alkylated Extract | Residue after Reduction and Alkylation | Collagen |
|---|---|---|---|---|---|
| Hexose[a] | 5.52 | 5.70 | 6.20 | 5.57 | 0.60 |
| Glucose | 1.90 | 2.00 | 1.99 | 2.10 | 0.40 |
| Galactose | 2.20 | 2.02 | 2.00 | 2.30 | 0.28 |
| Mannose | 1.41 | 1.21 | 1.40 | 0.80 | 0.00 |
| Hexosamine | 1.40 | 0.90 | 1.02 | 1.13 | 0.04 |
| Sialic acid | 2.10 | 1.76 | 1.60 | ... | 0.06 |
| Fucose | 0.75 | 0.77 | 0.75 | ... | 0.00 |

[a] Corrected for contribution of fucose.

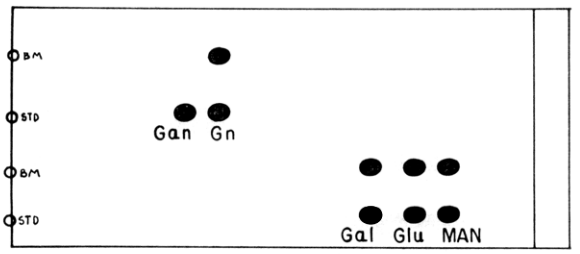


FIGURE 4: Paper chromatogram of carbohydrate components of basement membrane hydrolysate. The amino sugars were first separated from neutral sugars on Dowex-50 $H^+$ columns. Solvent: ethyl acetate–pyridine–water (10:4:3, v/v/v). BM indicates basement membrane hydrolysate, STD standard solutions of galactosamine (Gan), glucosamine (Gn), galactose (Gal), glucose (Glu), and mannose (Man).

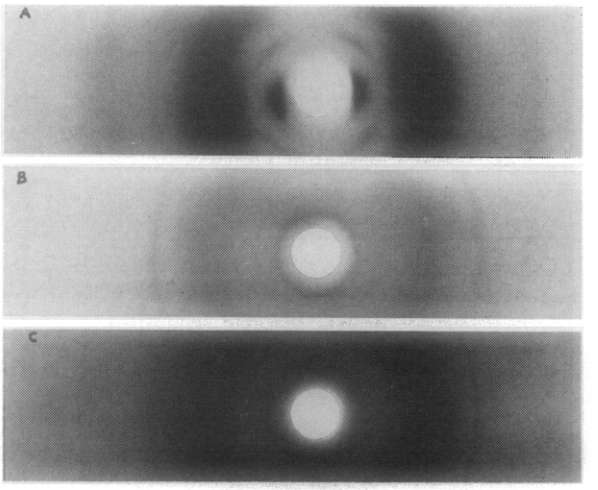


FIGURE 5: X-Ray diffraction patterns of (A) bovine Achilles tendon collagen (native), (B) bovine Achilles tendon (heat denatured), and (C) canine glomerular basement membrane.

content, there being 0.82 residue of cystine per 100 in basement membrane, while none is present in collagen. Thus, there is striking similarity and equally striking differences in the amino acid composition of basement membrane and collagen.

The amino acid analysis of the soluble fractions extracted from basement membrane with 8 M urea, or with reduction and alkylation in 8 M urea, as well as the residue remaining after reduction and alkylation appear in Table II. The values, expressed as residues per 100, are not appreciably different from those of intact basement membrane. No cystine was found in the reduced and alkylated fraction of basement membrane, but an equivalent amount of carboxymethylcysteine appeared in the chromatogram. It is significant that the hydroxylysine–hydroxyproline ratio was the same in all preparations.

*Carbohydrate Composition.* The carbohydrate composition of basement membrane, its soluble fractions, and of collagen appears in Table III. No uronic acid was found. There is 5.52% total hexose by the orcinol reaction (corrected for fucose) in basement membrane compared with 0.60% in collagen. The hexoses were identified as galactose, glucose, and mannose by (Figure 4) paper chromatography. Hexosamine and sialic acid are present in less than 0.10% in collagen while basement membrane contains 1.4 and 2.1%, respectively. The hexosamine was identified as glucosamine by paper chromatography (Figure 4). Fucose is absent in collagen but makes up 0.75% of basement membrane. The sum of the carbohydrate components accounts for about 10% of the basement membrane. Collagen contains 0.4% glucose and 0.28% galactose but no mannose or fucose. Table III also shows that the amounts of the carbohydrate components in the soluble fragments of basement membrane were very similar to intact basement membrane.

*Functional Group Analysis.* Basement membrane and its subfractions were analyzed for amide nitrogen and the results appear in Table IV. There are about 0.4 μmole of amide nitrogen per milligram of intact base-

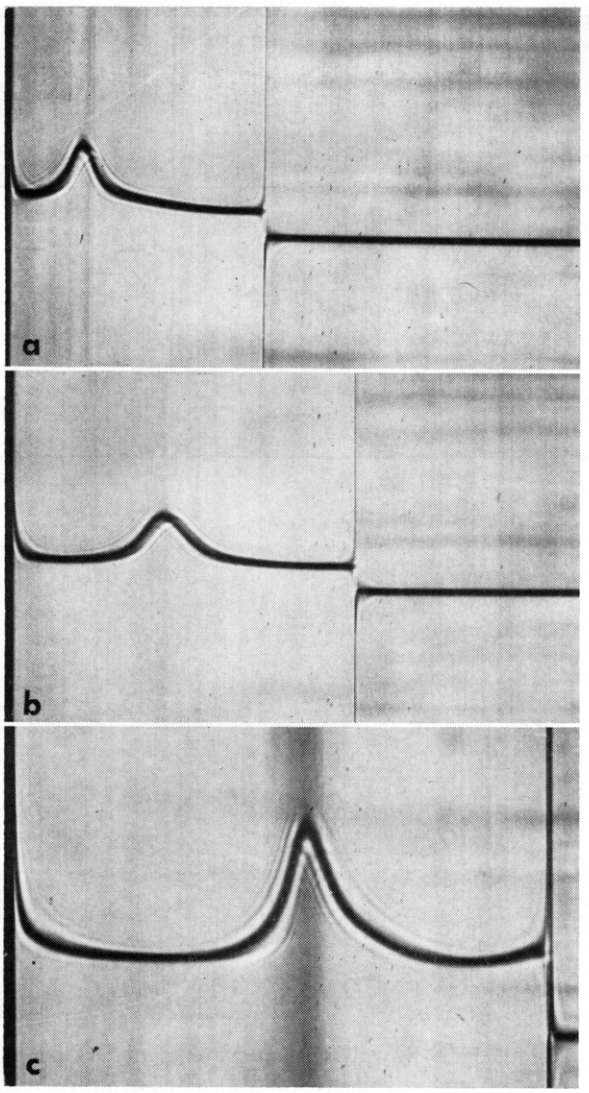


FIGURE 6: Sedimentation analysis of solubilized basement membrane and collagen. (a) 8 M Urea-extractable fraction of basement membrane. Protein concentration 1% in 0.05 M Tris–HCl buffer, pH 7.7. (b) Basement membrane fraction extracted by reduction and alkylation in 8 M urea. Protein concentration 1% in 0.5 M $NaHCO_3$, pH 8. (c) Collagen fraction extracted by reduction and alkylation in 8 M urea. Protein concentration 1% in 0.1 M phosphate buffer, pH 7. Pictures a, b, and c were taken 40 min after attainment of maximum speed of 59,780 rpm.

ment membrane and of its soluble fragments. Similar results were obtained with intact collagen and a fraction extracted by reduction and alkylation in 8 M urea. It has been reported that there are 0.06 μmole of hydroxylamine-sensitive bonds per milligram of collagen (Gallop *et al.*, 1959). About 0.03 μmole of ester-like bonds have now been found in intact basement membrane (Table IV). Table IV shows that there are 0.05 μmole of disulfide groups, determined by the fluorescence quenching method, per milligram of intact basement. This corresponds to the amount of cystine found in basement membrane (Table I). Collagen did not give a detectable reaction. Table IV also shows the value obtained for acetyl groups. The number of micromoles of acetyl groups equals the sum of micromoles of hexosamine plus sialic acid in basement membrane (0.140 μmole/mg compared with 0.148 μmole/mg).

TABLE IV: Functional Group Analysis.

| | μmoles/mg | |
|---|---|---|
| | Basement Membrane Intact | Collagen Intact |
| Amide ditrogen[a] | 0.400 | 0.380 |
| Ester-like groups | 0.034 | 0.054 |
| Disulfide groups | 0.052 | 0.000 |
| Acetyl groups | 0.140 | ... |

[a] Corrected for contribution of sialic acid.

*X-Ray Diffraction.* Although basement membrane shows no definite structural organization by electron microscopy, it was possible that periodicity might be detectable by X-ray diffraction. Examination of the X-ray diffraction patterns of bovine tendon collagen fiber, of thermally denatured bovine tendon collagen, and of intact canine glomerular basement membrane revealed certain similarities and differences. The collagen fiber produced the classical fiber pattern of arcs and broad halos, while thermally denatured collagen and intact glomerular basement membrane produced "powder" diagrams characterized only by halos and lines (Figure 5). The measurements of the reflection angles appear in Table V. The basement membrane exhibits maxima at 10.27, 4.23, and 2.85 A which compare with those of native and denatured collagen.

TABLE V: X-Ray Diffraction Measurements.

| Collagen Fiber *d* (A) | | Heat-Denatured Collagen *d* (A) | | Dog Basement Membrane *d* (A) | |
|---|---|---|---|---|---|
| Arc | 11.32 | Halo | 11.62 | Intense halo | 10.27 |
| Arc | 7.25 | Line | 7.62 | | |
| Arc | 5.71 | Line | 6.02 | | |
| Broad halo | 4.28 | Broad halo | 4.18 | Intense halo | 4.23 |
| Broad halo | 2.19 | Line | 2.86 | Line | 2.85 |

*Ultracentrifugation Studies.* The behavior of the soluble fractions of glomerular basement membranes in the ultracentrifuge appears in Figure 6. Synthetic

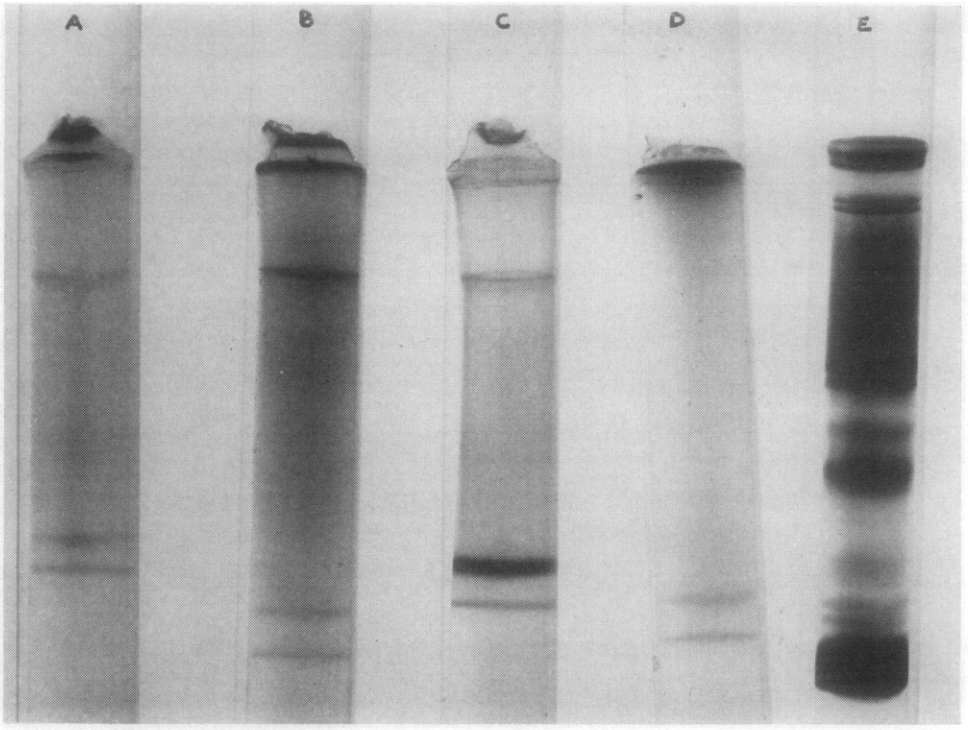


FIGURE 7: Disk acrylamide gel electrophoresis of solubilized basement membrane and collagen, 0.05 M Tris, 0.38 M glycine buffer at pH 8.3. Protein concentration 2% in 0.5 M $NaHCO_3$. (A) Human basement membrane fraction obtained by reduction and alkylation in 8 M urea. (B) Canine basement membrane fraction obtained by extraction in 8 M urea. (C) Canine basement membrane obtained by reduction and alkylation in 8 M urea. (D) Canine tendon collagen fraction extracted with 8 M urea in 0.5 M mercaptoethanol followed by alkylation. (E) Normal canine serum.

boundary runs were carried out at a protein concentration of 1% in 0.05 M Tris–HCl buffer for the fraction obtained by extraction with 8 M urea, and in 0.5 M $NaHCO_3$ for the fraction obtained by reduction and alkylation in 8 M urea. The sedimentation constants ($s_{20,w}$) for the two fractions were 3.5 and 2.4, respectively. It would appear that the reduced and alkylated material exhibits less aggregation. The ultracentrifugation pattern of the soluble collagen fraction appears in Figure 6.

All fractions displayed single peaks with a slight asymmetry. In conventional ultracentrifugal runs the schlieren pattern did not form a typical Gaussian curve and the curves began to flatten within 1 hr after attainment of maximum centrifugal speed. These phenomena suggest that the protein solutions consisted of a mixture of molecular species of varying size.

*Diffusion Studies.* The 8 M urea-extractable fraction had a diffusion constant of 1.33, and the fraction obtained by reduction and alkylation had $D_{20,w}$ = 1.46.

*Polyacrylamide Gel Electrophoresis.* The electrophoretic patterns of various fractions of human and canine basement membrane, collagen, and normal canine serum in acrylamide gel appear in Figure 7. It is seen that both the human and canine basement membrane fractions separate into three components, one slow moving, and two with higher mobilities. The two fast components migrate somewhat slower than canine serum albumin while the slow component migrates in the region of the $\beta$-globulins.

The soluble collagen fraction prepared by reduction and alkylation separated only into two fast moving components with mobilities similar to those of the fast components of basement membrane. The slow moving component was not seen. When the gels were stained for carbohydrate, with the periodic acid–Schiff reagent, only the slow moving component of the basement membrane fractions was visualized. This indicates that a

TABLE VI: Complement Fixation Test.

| Antiserum | Antigen | Antibody Titer |
|---|---|---|
| Anti-BM | BM | 1:128 |
| | USBM | 1:64 |
| | RABM | 1:128 |
| Anti-USBM | USBM | 1:64 |
| | BM | 1:64 |
| | RABM | 1:128 |
| Anti-RABM | RABM | 1:128 |
| | BM | 1:64 |
| | USBM | 1:128 |

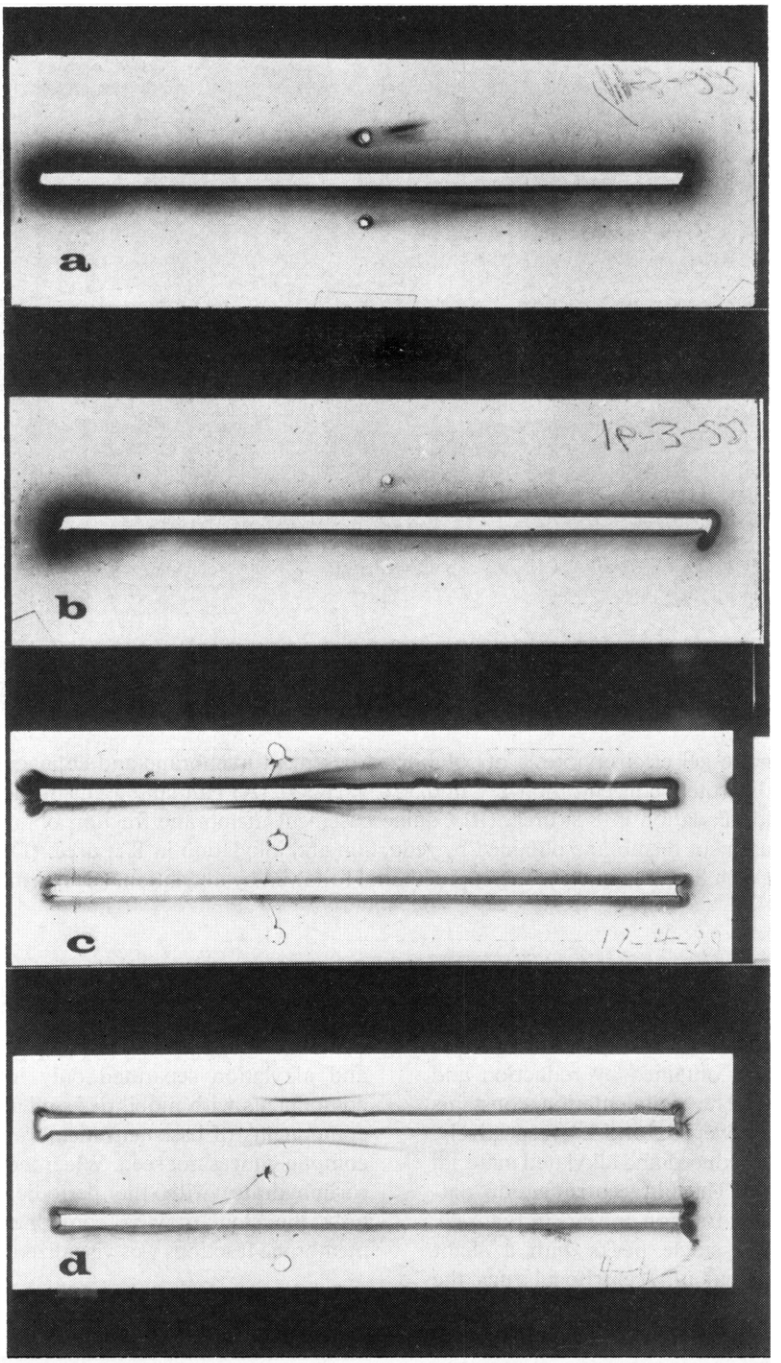


FIGURE 8: Immunoelectrophoresis of soluble fractions of basement membrane in 1% agar, 0.05 M Veronal buffer, pH 8.6. A current of 3 ma was passed per slide for 90 min. (a) The upper well contains normal canine serum, and the lower well the 8 M urea-extractable fraction of basement membrane. The trough contains rabbit anti-BM serum. (b) The upper well contains basement membrane fraction obtained by reduction and alkylation in 8 M urea, and the lower well normal rabbit serum. The trough contains anti-BM serum. (c) All wells contain 8 M urea-extractable basement. Upper trough contains rabbit anti-USBM serum, lower trough contains normal rabbit serum. (d) All wells contain basement membrane fraction obtained by reduction and alkylation in 8 M urea. Upper trough contains rabbit anti-USBM serum, lower trough contains normal canine serum.

carbohydrate-containing protein is present in basement membrane and that it migrates independently of the other two fast moving protein components.

*Immunochemical Studies.* COMPLEMENT FIXATION. Complement fixation was carried out in 0.05 M pH 7.48 Veronal buffer using 1% antigen concentrations. It is seen in Table VI that all three antigens (BM, USBM, RABM) fixed complement in their respective antisera

and in the antisera against the other two antigens. The titers ranged from 1:64 to 1:128. These data suggest that basement membrane and its soluble fractions are antigenic in rabbits and that they contain common antigenic determinants.

*Immunoelectrophoresis.* Immunoelectrophoretic patterns appear in Figure 8. A precipitin line was obtained by treating the antiserum to intact basement membrane with its two soluble fractions (USBM and RABM). Similar precipitin lines were formed with antisera prepared against the individual soluble fractions. No precipitin lines were produced when soluble collagen was treated with anti-BM. These data demonstrate the presence of common antigenic determinants in intact canine glomerular basement membrane and its soluble fractions.

*Disk Immunoelectrophoresis.* Figure 9 shows the results of disk immunoelectrophoresis. After disk electrophoresis of USBM the acrylamide column was cut into 30 1–2-mm disks and each examined by immunodiffusion against anti-USBM. Precipitin lines were given by disks 2 through 6, corresponding to the upper section of the gel where the glycoprotein migrates. No precipitin lines were observed in the remaining portions of the gel and particularly in the region of the two fast moving protein components.

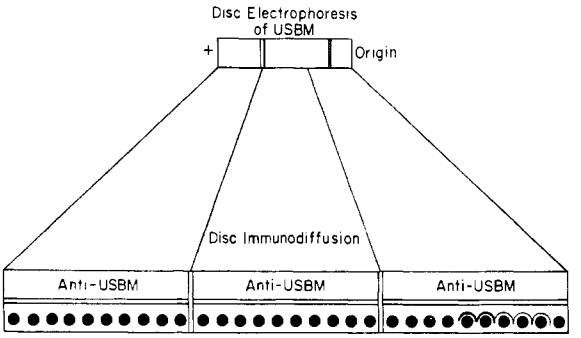


FIGURE 9: Disk immunoelectrophoresis of 8 M urea-extractable basement membrane against its antiserum. After electrophoresis, the unfixed gel column was cut into 1–2 mm disks and imbedded on slides with 1% agar in 0.05 M Veronal buffer, pH 8.6, and diffusion allowed to proceed. The troughs contain antiserum against the 8 M urea-extractable fraction of basement membrane. The slow moving components on the acrylamide gel, which give the precipitin reaction, stain with the periodic acid–Schiff reagent.

## Discussion

The present study was designed to establish the physical, chemical, and immunochemical properties of canine glomerular basement membrane and compare them with those of collagen, and to develop methods for its solubilization. The data on the physical and chemical properties of basement membrane obtained in the present study strongly suggest that there is a collagen-like component associated with a glycoprotein in glomerular basement membrane. The X-ray diffraction pattern of intact basement membrane is superimposable on that of heat-denatured collagen and the spacings are similar to those of collagen.

The electrophoretic behavior of the soluble fractions of basement membrane also suggest that a separable glycoprotein component is present. The two non-carbohydrate-containing protein components observed on acrlyamide gel resemble in electrophoretic mobility those of the soluble fraction of collagen. It will be necessary to isolate each of these components in sufficient quantity for chemical study to resolve this question.

Chemical analyses reveal the presence of large amounts of hydroxyproline, glycine, and proline as well as hydroxylysine. Except for hydroxylysine, the amounts of the above amino acids are found in higher proportions in collagen than in basement membrane. On the other hand, the hydroxylysine content of basement membrane is three times that found in most collagens. This might suggest that either (a) basement membrane is a collagen-like polymer of similar subunits of unusual composition, or (b) that it is a polymer of dissimilar subunits one of which may be collagen-like and one noncollagen but rich in carbohydrate. In the latter case, the presence of a second noncollagen component, containing no hydroxyproline or hydroxylysine and smaller amounts of glycine, would decrease by dilution the amounts of hydroxyproline, hydroxylysine, and glycine contained in the collagen of basement membrane. In favor of the second hypothesis is preliminary work carried out in our laboratory. We have isolated a collagen-like protein and of a glycoprotein from basement membrane. The glycine accounted for $^1/_3$ of the total amino acid residues in this collagen and the sum of hydroxyproline and proline for about $^2/_9$ of the total residues. Hydroxylysine accounted for 4.3% of the total residues. The glycoprotein contained no hydroxyproline, a trace of hydroxylysine, and had 8.7% hexose by weight. In none of the vertebrate collagens examined so far has the hexose content been shown to be higher than 1%, whereas basement membrane contains almost 6% hexose. The conclusions of Mukerjee *et al.* (1965), and Pierce *et al.* (1964), that neoplastic epithelial basement membrane of the mouse is chemically distinct from collagen, is based on quantitative differences of glycine, hydroxyproline, proline, tyrosine, and cystine between the two proteins. However, when one compares the amino acid composition of their purified neoplastic basement membrane (Mukerjee *et al.*, 1965), one finds a striking similarity between it and that of the glomerular basement membrane determined in the present study. More recently, Dische *et al.*, (1965) reported the presence of collagen-bound hexosamine-free hexose noncovalently bound to hexosamine-bound hexose in 4% trichloroacetic acid extracts and collagenase hydrolysates of human glomerular basement membrane.

The lack of fibrous organization in basement membrane as seen in the electron microscope might argue against the presence of a true collagen. Farquhar (1964), and Vernier (1964,; Vernier and Birch-Anderson,

1962), however, claim that small filaments 60–100 A in diameter can be seen in glomerular basement membrane. Collagen, however, may exist *in vivo* and *in vitro* in its protofibril form or polymerized only to a small extent without formation of fibers. Wood (1960) and Wood and Keech (1690) showed that there are two phases of fiber formation *in vitro*. The first is a nucleation phase, roughly corresponding to the lag phase of fiber formation (Gross and Kirk, 1958), in which tropocollagen molecules aggregate to form nuclei which then grow to form collagen fibers by the aggregation of soluble collagen in the growth phase. Low concentration of polyanions (0.022–0.005%), such as chondroiton sulfate A or C and keratosulfate, accelerates the nucleation phase and rate of fiber formation, while heparin and deoxyribonucleic acid retard it. The polyanions which accelerate fibril formation also induce the development of thin fibrils, and it is possible that the type and concentration of polysaccharides in the extracellular phase may influence the average diameter and rate of fibril formation *in vivo*. It should be noted here that orosomucoid, a highly charged negative polyelectrolyte, was found to affect the spacing of the repeating units of collagen fibers formed from soluble collagen *in vitro* (Gross *et al.* 1952; Schmitt *et al.* 1953). It would make an attractive hypothesis, therefore, to suggest that the glycoprotein in basement membrane may form some type of cross-linkage between collagen molecules and, hence, determine their orientation. Work to elucidate this point is now under way.

It can be said, therefore, that the data obtained here suggest that glomerular basement membrane is a highly polymerized substance containing at least two major components, one being a glycoprotein, the other a collagen-like protein. Soluble fragments can be obtained by treatment with 8 M urea or by reduction and alkylation of disulfide bonds in 8 M urea. The data also demonstrate that basement membrane and its soluble fractions are antigenic in rabbits, and that the soluble fractions share common antigenic determinants with the intact basement membrane.

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# The Absolute Configuration of Biotin*

James Trotter and Jean A. Hamilton

ABSTRACT: The structure and the relative configuration at each of the asymmetric centers of biotin have been determined previously by an X-ray crystallographic analysis of the bis-*p*-bromoanilide of $CO_2$-biotin. By studying the anomalous dispersion of X-rays by crystals of this derivative, the absolute stereochemistry of biotin has now been established.

The structure of the bis-*p*-bromoanilide of carbon dioxide biotin has been determined by X-ray crystallographic analysis (Bonnemere *et al.*, 1965), and, as is usual in this type of analysis, only the relative configuration at each of the asymmetric centers of biotin was obtained. The results are in good agreement with those obtained from an X-ray study of unsubstituted biotin, where again only the relative configurations were determined (Traub, 1956, and personal communication, 1964). It is, however, possible, by careful measurement of the anomalous dispersion of X-rays (Bijvoet *et al.*, 1951), to determine the absolute configuration, and the present paper describes a series of such measurements for the bis-*p*-bromoanilide of $CO_2$-biotin, which establishes the absolute stereochemistry of biotin.

Structure factors were calculated for all the *hkl* the $\bar{h}\bar{k}\bar{l}$ reflections of the $CO_2$-biotin derivative from the final positional and isotropic thermal parameters (Bonnemere *et al.*, 1965, Table I). The scattering factors used for the two bromine atoms and for the sulfur atom were of the form

$$f = (f_o + \Delta f') + i\Delta f''$$

with $\Delta f'$, $\Delta f''$ for Cu K$\alpha$ radiation (International Tables for X-Ray Crystallography, 1962). Many of the reflexions showed significant differences between $|F_c(hkl)|$ and $|F_c(\bar{h}\bar{k}\bar{l})|$, and several of the planes with the largest indicated differences were chosen at random and the intensities were measured on a General Electric XRD-5 spectrogoniometer. This instrument gives more accurate measurements and permits the detection of small intensity differences with more reliability than the photographic recording used in the original X-ray analysis. It would be possible to remeasure all the intensities and refine the structure further but, since the analysis of Bonnemere *et al.* was considered to be a sufficiently detailed one, only a few reflections were measured to deduce the absolute configuration. For these measurements a single crystal was chosen from the same sample that was used in the original X-ray analysis, the crystal was carefully aligned on the goniostat, and the various instrumental settings were precomputed from the measured cell dimensions (Bonnemere *et al.*, 1965). The integrated intensities were then measured with a $\theta$–$2\theta$ scan, using a scintillation counter and approximately monochromatic Cu K$\alpha$ radiation (nickel filter and pulse height analyser), and corrections for background counts were made.

The results of these measurements are given in Table I, $I_o$ being measured intensities and $|F_c|$ calculated structure amplitudes. The results indicate unambiguously that $I_o(hkl)/I_o(\bar{h}\bar{k}\bar{l}) \approx |F_c(\bar{h}\bar{k}\bar{l})|^2/|F_c(hkl)|^2$, and hence the true absolute configuration is the optical enantiomorph of that shown in all the diagrams in the paper by Bonnemere *et al.* The correct configuration is obtained by referring the positional parameters of Bonnemere *et al.* (Table I of their paper) to a *left-*

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