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SEPARATION OF GLOMERULAR BASEMENT MEMBRANE SUBSTANCES BY SODIUM DODECYLSULFATE DISC GEL ELECTROPHORESIS AND GEL FILTRATION

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SUMMARY

Beef glomerular basement membranes, solubilized by sodium dodecylsulfate and β-mercaptoethanol, were examined by disc gel electrophoresis and gel filtration. The beef glomeruli were sonicated in either 0.15 M NaCl or 1.0 M NaCl, and the basement membranes sedimented at 1300 × *g*. Disc gel electrophoresis in sodium dodecylsulfate showed that the 1.0 M preparation contained predominantly high molecular weight components; the 0.15 M preparation contained more low molecular weight components. Different amounts of hydroxyproline and DNA were found in the two membrane preparations. Other subunits were obtained when basement membrane was solubilized in 8 M urea or 7 M guanidine thiocyanate.

Gel filtration of solubilized glomerular basement membrane was performed on 6% Agarose in sodium dodecylsulfate–phosphate. Partial separation of the components observed in the disc gel electrophoresis was achieved. Protein and carbohydrate were present in all fractions.

INTRODUCTION

Glomerular basement membrane is the collagen-containing membrane which forms the only continuous barrier between the blood and the urinary space in the kidneys. In protein-losing nephropathies, it seems quite clear that the selectivity of the barrier is damaged. In order to understand any alteration of the basement membrane in disease, it is first necessary to study it in its normal state.

The total chemical composition of the basement membrane has been shown to be complex. While many investigations have used components which were obtained by proteolytic digestion[1–5], a limited number of studies have attempted fractionations of these membranes in which most covalent bonds were probably left intact[6].

Detergents and other dissociating agents have now been widely used to solubilize portions of many membranes[7–10], and it seemed probable that sodium dodecylsulfate–β-mercaptoethanol would partially solubilize the glomerular basement membrane. The subunits could subsequently be observed in disc gel electrophoresis.

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If the method were successful, the disc gel electrophoresis might prove useful in comparing preparative methods and in detecting components which may have been altered in disease. In addition, it should be possible to separate the solubilized components by gel filtration chromatography, a system which fractionates components according to size and shape, as does sodium dodecylsulfate disc gel electrophoresis. The investigations reported in this communication concern the application of these fractionation methods to glomerular basement membrane.

MATERIALS AND METHODS

Preparation of glomerular basement membranes

Beef glomeruli were generally prepared by the Spiro[11] modification of the sieve method[12] with the following alterations. Glomeruli were sedimented at 200 × *g* at 0 °C for 2.5 min. After sonication, the suspension was passed through a 250 mesh sieve[13], and the basement membrane collected as described by Spiro[11].

Chemical analyses

Protein was determined by the biuret procedure[14] and the Miller[15] modification of the Lowry *et al.*[16] assay, utilizing crystalline bovine serum albumin as standard. Anhydrous glucose was employed as the standard in the phenol–sulfuric determination for carbohydrate[17]; crystalline *N*-acetylneuraminic acid in the thiobarbituric assay for sialic acid[18]; and calf thymus DNA in the Croft and Lubran[19] variation of the diphenylamine assay for DNA[20]. Hydroxyproline was measured by the Martin and Axelrod[21] modification of the method of Neuman and Logan[22].

Column chromatography

Samples for gel filtration were prepared by incubating beef glomerular basement membrane (0.21 % protein by biuret) for 3 h at 37 °C in 0.11 M sodium phosphate (pH 7.2) containing 2 % β-mercaptoethanol and 0.4 % sodium dodecylsulfate. The mixture was centrifuged at 60000 × *g* for 30 min, and the supernatant solution dialyzed against the column elution buffer to remove the β-mercaptoethanol. A maximum of 3.5 mg protein was applied to a 6 % Agarose (Biogel A-5m) column[23] at room temperature. Columns were 83 cm × 1.5 cm and were eluted in 0.055 M sodium phosphate (pH 7.2) containing 0.1 % sodium dodecylsulfate. 1-ml fractions from 5 columns were collected at 3–4 ml/h, and protein was determined on 0.1-ml samples by the Miller[15] procedure. Fractions which exhibited similar disc gel electrophoresis patterns were pooled for further analysis.

Samples to be analyzed for amino acids were first lyophylized, redissolved in a minimal volume of water, and dialyzed 96 h against water with 3 changes. After hydrolysis in 6 M HCl in evacuated, sealed tubes for 24 h at 110 °C, the samples were analyzed in 0.1 M HCl on a Beckman amino acid analyzer, Model 120C.

Disc gel electrophoresis

Polyacrylamide disc gel electrophoresis, in the presence of sodium dodecylsulfate, was conducted essentially as described by Weber and Osborn[24]. It was occasionally necessary to retard gel formation by reducing the concentration of the ammonium persulfate catalyst. A complete gel contained 7.5 % polyacrylamide in the bottom layer (1.7 ml) and 6.5 % polyacrylamide in the upper layer (0.3 ml).

Samples of glomerular basement membrane (maximum protein concentration, 0.7 %) for the disc gel were treated with the dissociating reagent, a 1:4 dilution of the electrophoresis buffer[24] containing 1 % β-mercaptoethanol and 1.25 % sodium dodecylsulfate. Alkylations were performed by the addition of sufficient 1 M Tris–HCl (pH 8.1) to make a given solution 0.05 M in Tris–HCl, and sufficient iodoacetamide to cover any β-mercaptoethanol and cysteine with a maximum 2-fold molar excess. This solution was permitted to sit at room temperature for 1 h with occasional shaking; excess small molecules were removed by dialysis against the diluted electrophoresis buffer or 0.1 M Tris buffer (pH 8.1) in 0.2 M NaCl. Urea (8 M) and guanidine thiocyanate (7 M) were also used to solubilize the basement membrane for disc gel electrophoresis.

Solubilization mixtures were incubated overnight at 37 °C and centrifuged at 200 × g for 10 min. The supernatant solutions (up to 0.2 ml, containing 10–75 μg protein) were mixed with 0.03 ml of 0.005 % bromophenol blue in 20 % sucrose, and were layered on the disc gels. Electrophoresis buffer was diluted 3.3-fold and the gels were run in a Canalco apparatus at room temperature for 4.5 h at 8 mA per disc gel. Gels were fixed overnight in destaining solution (methanol–water–acetic acid, 5:5 1, by vol.), stained for 8 h in 2.5 % Coomassie Blue in destaining solution, destained about 20 h in 100 ml destaining solution, and stored in 7.5 % acetic acid.

RESULTS

Comparison of basement membrane from beef glomeruli sonicated in 1.0 M NaCl and 0.15 M NaCl

Sonication of beef glomeruli in 1.0 M NaCl resulted in a particulate basement membrane suspension, which gave a protein yield (biuret procedure) of about 120 mg/kg kidney cortex. The biuret assay proved most useful since it was the only available protein assay in which the basement membrane appeared to be completely solubilized. If 0.15 M NaCl was the sonication medium, a milky suspension was obtained (biuret protein, about 280 mg/kg of beef kidney cortex). Sodium dodecylsulfate disc gel electrophoresis of glomerular basement membranes and of the sonicate supernatants, from which the membranes were isolated, yielded quite different patterns (Fig. 1). In particular the 1.0 M preparation contained the largest proportion of high molecular weight subunits.

While the identity of these glomerular basement membrane components is of course not known, the highest molecular weight bands were considered to contain collagen and the faster migrating bands to contain other proteins associated with the basement membrane. Using these assumptions and approximate standards (collagens from various sources behave differently on disc gels[25]), the molecular weights of the glomerular basement membrane components, obtained by sonication in 1 M NaCl and solubilization in sodium dodecylsulfate–β-mercaptoethanol, could be estimated from the disc gels[24]. These values are shown in Fig. 2.

The major differences in the composition of the two basement membrane preparations are summarized in Table I. Basement membranes prepared by 1 M NaCl sonication clearly contained less glomerular DNA and more protein than membranes prepared by 0.15 M NaCl sonication The DNA in the latter preparation represents close to 90 % of the total glomerular DNA. The relatively high hydroxyproline content

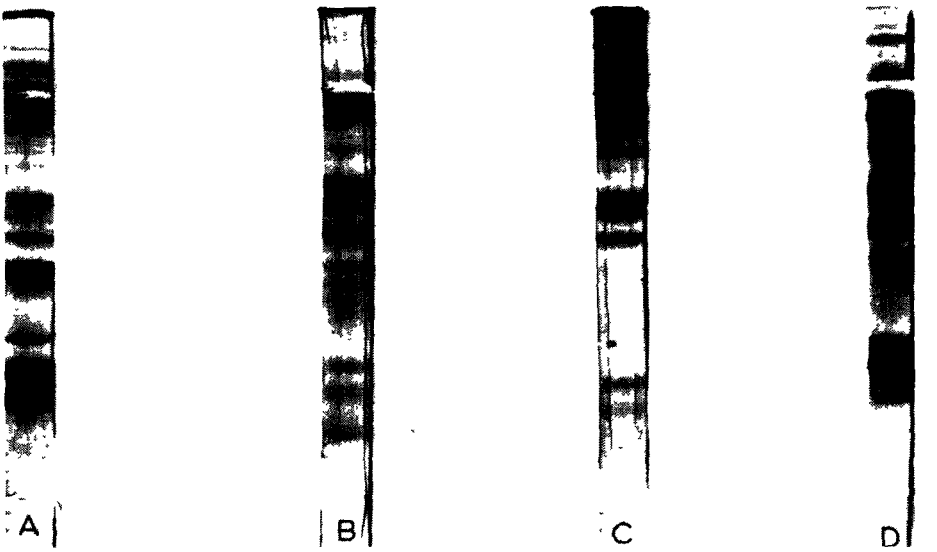


Fig 1 Sodium dodecylsulfate–polyacrylamide disc gel electrophoresis patterns Sonication and electrophoresis were carried out as outlined in Materials and Methods A and B, basement membrane pellet and supernatant, respectively, following glomerular sonication in 0 15 M NaCl, C and D, basement membrane pellet and supernatant, respectively, following glomerular sonication in 1 M NaCl

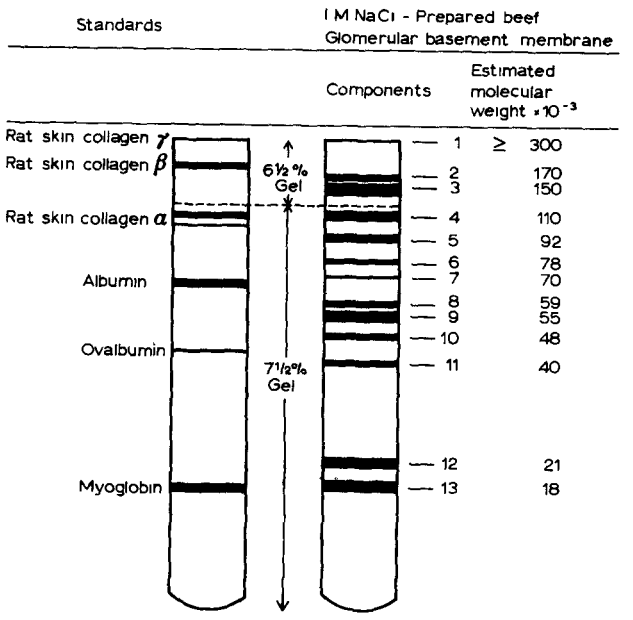


Fig 2 Molecular weight distribution in sodium dodecylsulfate disc gel electrophoresis The collagen standards were obtained by the method of Piez *et al* [26] The other protein markers were purchased from Mann These distributions represent the compilation of many individual disc gels

TABLE I

SOME CHEMICAL DIFFERENCES IN BEEF GLOMERULAR BASEMENT MEMBRANE PREPARED BY 0.15 M AND 1.0 M NaCl SONICATION

Assays are explained in Materials and Methods Results expressed as per cent total weight of lyophilized membrane

NaCl concentration (M)	*Biuret protein*	*Hydroxy-proline*	*DNA*
0.15	73	2.3	15
1.0	85	7.5	3

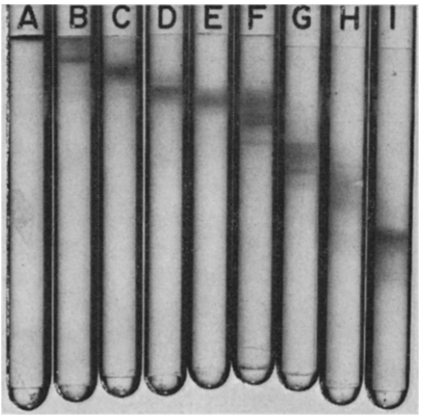
of the 1 M NaCl-prepared membranes, in comparison with that of the 0.15 M NaCl-prepared membranes, clearly suggests a higher concentration of collagen-containing components.

Gel filtration of beef glomerular basement membranes on 6% Agarose gel

In order to test the possibility of separating the subunits which had been observed in disc gel electrophoresis, gel filtration on 6% Agarose A-5m in sodium dodecylsulfate was undertaken. Basement membrane prepared from the sonication of glomeruli in 1 M NaCl was used for the separation since both the hydroxyproline content and the high molecular weight subunit content were high. That is, it appeared that this preparation would be more interesting in terms of obtaining the collagenous substructures of the basement membrane. In addition, preliminary column chromatography on both preparations produced a clearer separation of the 1 M NaCl-obtained subunits.

Centrifugation of the solubilized basement membrane suspension at 60000 $\times$ *g* for 30 min yielded a supernatant solution containing 60% of the initial biuret protein (centrifugation at 850 $\times$ *g* for 10 min yielded 70% of the starting protein in the supernatant solution, but this solution did not enter the Agarose gel readily). The sodium dodecylsulfate concentration in the column elution buffer was decreased to one-quarter the amount employed by other workers[23], and any β-mercaptoethanol in the sample was removed by dialysis. In this way it was possible to remove most of the extraneous absorbance at 230 nm which appeared in the buffer after it passed through the column. Preliminary investigations suggested that the extraneous material was carbohydrate and therefore solubilized column material (all tubing and connections used appeared to be inert).

The degree of subunit separation was most evident from the disc gel analysis of the fractions (Fig. 3). An elution pattern is also shown (Fig. 4). Chemical analyses, performed as outlined in Methods (on undialyzed column fractions in order to maintain soluble components), produced assays containing a slight turbidity. Nevertheless the following information was obtained. DNA was found primarily in the fraction containing the highest molecular weight component (first column fraction, Component 1) with small amounts in fractions containing Components 2 and 3. The presence of carbohydrate and protein was demonstrated in all fractions. The carbohydrate: protein ratio (0.08–0.16) was somewhat higher in fractions containing Components 1–5 than in the later fractions. Sialic acid determinations were negative. The amino acid



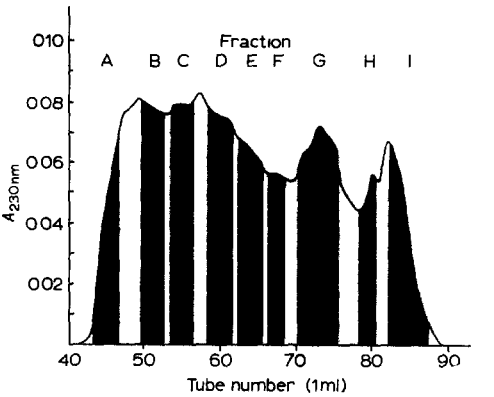


Fig 3 Sodium dodecylsulfate–disc gel electrophoresis patterns of selected beef glomerular basement membrane fractions after 6% Agarose gel filtration. A, Component 1; B, Component 2; C, Component 3, D, Component 4, E, Component 5; F, Components 6 and 7; G, Components 8, 9 and 10, H, Components 11, and I, Component 12 and 13

Fig 4 6% Agarose gel elution pattern of 1 M NaCl-prepared glomerular basement membrane after solubilization as outlined in Materials and Methods

analyses of selected fractions (Fig. 3, A, C, D, G, I) showed the presence of hydroxyproline and hydroxylysine in the fractions containing Components 1, 3 and 4, although not in quantities as high as expected.

Effects of various solubilizing systems on the disc gel patterns of beef glomerular basement membranes

The disc gel electrophoresis patterns in Fig. 5 show the effects of treating beef glomerular basement membranes (isolated from 1 M NaCl-sonicated glomeruli) with a

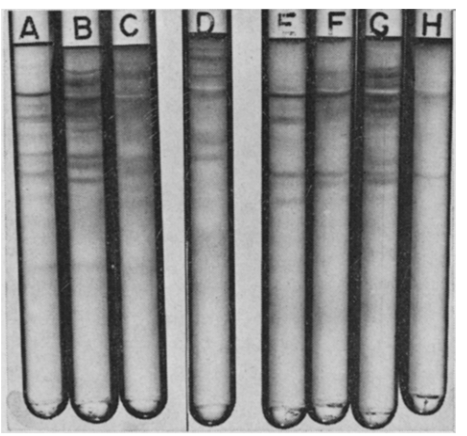


Fig 5 Disc gel electrophoresis patterns of beef glomerular basement membranes subjected to different solubilizing agents and procedures A, solubilized in 1 25% sodium dodecylsulfate in phosphate buffer, B, as in A with 1% β-mercaptoethanol added, C, as in B and dialyzed against 0.1 M Tris in 0 2 M NaCl, D, solubilized in 7 M guanidine thiocyanate; E, solubilized in 8 M urea, F, as in E with 0 1% β-mercaptoethanol added; G, as in E with 5% β-mercaptoethanol added; H, as in G and dialyzed against 0 1 M Tris in 0 2 M NaCl

variety of dissociating reagents. When the β-mercaptoethanol was omitted from the incubating solution, solubilization was not as complete as in its presence (Fig. 5). If the sodium dodecylsulfate–β-mercaptoethanol-solubilized basement membrane was dialyzed extensively against 0.1 M Tris (pH 8.1) in 0.2 M NaCl, most of the material remained in solution and the subunits observed in electrophoresis were very similar to those observed in electrophoresis before dialysis (Fig. 5). Alkylation of the sodium dodecylsulfate–β-mercaptoethanol-solubilized basement membrane resulted in disc gel patterns which were very similar to those of the non-alkylated material. The same similarity to non-alkylated material was also observed when the alkylated fraction was dialyzed extensively against 0.1 M Tris (pH 8.1) in 0.2 M NaCl.

A 0.2% beef basement membrane suspension in 7 M guanidine thiocyanate was incubated overnight at 37 °C, but the solubilized membrane could not be run directly on the disc gel due to the formation of a precipitate on the disc gel. Prior dialysis of the solubilized membranes against the diluted electrophoresis buffer, however, eliminated this difficulty, and disc gel electrophoresis patterns were obtained These patterns (Fig. 5) were not identical with those of the sodium dodecylsulfate–β-mercaptoethanol-solubilized basement membrane, but they did suggest that guanidine thiocyanate solubilization might provide a distinct set of relatively high molecular weight components.

Basement membranes (0.2% suspension) may also be solubilized in 8 M urea. The presence of 0.1 % β-mercaptoethanol in the urea did not greatly affect the degree of solubilization, but the presence of 5 % β-mercaptoethanol considerably increased the extent of solubilization (Fig. 5) When the reduced sulfhydryl groups on the solubilized basement membrane were not alkylated, and the material was extensively dialyzed against 0.1 M Tris (pH 8.1) in 0.2 M NaCl, many solubilized subunits no longer remained in solution (Fig. 5).

DISCUSSION

Variations in the physicochemical properties of the same tissues from different species at different ages and in varying states of health make it highly improbable that unique methods can be developed for obtaining specific components. However, the analytical disc gel is a convenient method of assessing some of the variables among solubilizable components. In the studies reported here, disc gel electrophoresis is shown to be most useful in elucidating not only differences among solubilizing systems but also differences between methods of processing the kidney tissue. It has also been observed in this laboratory that the search for a biochemical defect within a complex system must encompass not only isolated structures of possible interest but also the milieu from which they are derived: normal and aminonucleoside rat glomerular basement membrane (sonicated in 0.15 M NaCl) show essentially identical disc gel patterns while the sonicate supernatant solutions show the absence of several high molecular weight species in the aminonucleoside supernatant when compared with the normal supernatant.

While the separation of basement membrane components on Agarose gel did occur on the columns used in these experiments, it was incomplete. A preliminary separation was made on two columns (2.5 cm diameter), which were connected in series and contained a total Agarose gel bed length of 195 cm. The separation of

components was only somewhat better than that achieved by the use of the single smaller column. Still, the primary difficulty with these Agarose columns was the apparent partial solubilization of the gel by the sodium dodecylsulfate followed by the appearance of carbohydrate in the column fractions.

It was hoped that carbohydrate analysis of the column fractions would reveal the association of carbohydrate with a limited number of the solubilized subunits. Carbohydrate was associated, however, with all the fractions. Since basement membrane contains heterogeneous heteropolysaccharides as well as disaccharide units which are specifically attached to collagen[27], this ubiquitous presence of carbohydrate is not unexpected.

Finally, the amino acid content of the column fractions analyzed was sufficiently varied to suggest that the components which these fractions contained could not merely be exhibiting different degrees of covalent association of a basic subunit.

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