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HUMAN GLOMERULAR BASEMENT MEMBRANE

HETEROGENEITY OF ANTIGENIC DETERMINANTS

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Summary

Human glomerular basement membrane was solubilized by digestion with proteolytic enzymes and immunoreactive components were quantitated and characterized by using rabbit antibodies raised against the particulate membrane. A number of antigens were demonstrated but they did not separate on gel filtration. However, two antigenic components in a collagenase digest of the membrane could be separated and isolated by Sepharose 6B chromatography. Chemical characterization suggests that both fragments are noncollagenous glycopeptides (molecular weights approx. 1 000 000 and 60 000—200 000, respectively).

Introduction

Contradictory data on the structure and immunoreactivity of basement membranes have been published [1—5]. Consequently, the biosynthesis of basement membranes is presently only partly understood [6—13]. Some of the reasons for the variability are variations in the primary structure of the collagenous and glycoprotein components respectively, the extent and nature of molecular interactions and the alterations brought about by degeneration, regeneration and by ageing. An important problem is to obtain pure basement membrane components for structural studies and for the study of alterations and immunological specificities in certain diseases, notably diabetes and glomerulonephritis [3,14,15].

The purpose of the present study was to investigate if it was possible to prepare pure immunoreactive basement membrane fragments for characterization and use in studies of the pathophysiology of nephritis.

Methods and Materials

Preparation of human glomerular basement membrane and antibodies

Human glomerular basement membrane was prepared according to the method of Spiro [16] as modified by Westberg [14]. Lyophilized human glomerular basement membrane (10 mg/ml) was suspended in 1 M NaCl, mixed with an equal volume of Freund's Complete Adjuvant and injected subcutaneously in the neck of rabbits (1—5 mg doses). Booster doses in Freund's Incomplete Adjuvant were given several times at 4-week intervals. The rabbits were bled 12 days after the last injection and sera were pooled and checked for antibody titer by using electroimmunoassay. The antisera were adsorbed with insolubilised plasma as described by Avrameas and Ternynck [17]. Adsorbed sera reacted immunologically with the soluble fraction of collagenase digested human glomerular basement membrane but did not react with human plasma nor with red cell membrane, platelet or cartilage extracts. Therefore it appears that neither cell structures nor nonspecific connective tissue components react with the antisera. Gamma-globulins were purified according to Steinbuch and Audran [18] and again tested for specificity for human glomerular basement membrane by using immunoelectrophoresis, Ouchterlony double diffusion and crossed immunoelectrophoresis as discussed below.

Immunological methods

Immunoelectrophoresis was performed as described by Grabar and Williams [19] and double diffusion in Agarose gel according to Ouchterlony [20]. Electroimmunoassay according to Laurell [21] was performed in 1% Agarose gel containing 1—2 ml/25 ml of purified antihuman glomerular basement membrane gamma-globulin. Fused rocket immunoelectrophoresis was performed according to Svendsen [22] and crossed immunoelectrophoresis according to Clarke and Freeman [21]. Immunoelectromigration was done according to Grubb [24]. Essentially the same buffer (0.025 M barbital, pH 8.6) was used in all procedures. The plates were washed with saline, dried and stained with Coomassie brilliant blue.

Chemical methods

Hydroxyproline was determined as described by Stegeman and Stadler [25] after hydrolysis of the glomerular basement membrane for 18 h in 6 M HCl at 105°C in sealed tubes. Total phosphorus was measured after digestion of the basement membrane preparation in 70% perchloric acid using the ascorbic acid method of Chen et al. [26].

Total protein was measured as described by Lowry et al. [27] but the protein concentration in chromatographic fractions was usually monitored by their absorbance at 280 nm.

The amino acid composition of glomerular basement membrane and subfractions was determined on a Durrum amino acid analyzer, after the sample had been hydrolyzed for 24 h at 110°C in 6 M HCl under argon. 3- and 4-hydroxyproline were separated on a Biocal amino acid analyzer. The same colour factor of 4-hydroxyproline was used for calculating the amounts of these two amino acids. Contents of neutral sugars were determined by gas chromatography of alditol acetates essentially as described by Axelsson and Heinegård [28].

TABLE I

PROTOCOL FOR FRAGMENTATION OF BASEMENT MEMBRANE PREPARATIONS WITH PROTEOLYTIC ENZYMES

Enzyme	Amount of enzyme (mg)	Membrane (mg)	Incubation time (h)	Temp. (°C)	Buffer
Collagenase (CLSPA Worthington)	(0.37 + 0.18 + 0.09)	75	72	37	5 ml 0.001 M HEPES/0.01 M $CaCl_2$, pH 7.5
Pepsin (Sigma)	(0.5 + 0.5)	50	48	21	5 ml 0.01 M acetic acid
Trypsin (Type III, Sigma)	(1)	53	9.5	37	5 ml 0.1 M Tris-HCl, pH 8.0
Pronase (Merck AG)	(1)	31	72	37	5 ml 0.15 M Tris-HCl/0.01 M $CaCl_2$, pH 7.4
Papain (2 × crystallized, Sigma)	(0.1)	10	4	65	2 ml 0.05 M sodium phosphate/0.005 M cysteine-HCl/0.005 M EDTA, pH 6.5

Proteolytic fragmentation of human glomerular basement membrane

Enzymic digestion of basement membrane was performed using the conditions shown in Table I.

All digests were centrifuged at 40 000 × *g* for 1 h at 4°C. Residual pellets were dialyzed against several changes of redistilled water, and were lyophilized. Supernatants were tested for immunoreactive components by using electroimmunoassay with collagenase digested membrane as a standard. Rocket height was shown to be proportional to the amounts of membrane in concentration ranges from 200 μg/ml to 2 mg/ml.

Gel chromatography

Solubilized basement membrane samples were applied to a 1.8 × 180 cm Sepharose 6B column, which had been equilibrated with 0.15 M NaCl/0.05 M Tris-HCl buffer, pH 7.4. Elution was carried out with the same buffer and 8-ml fractions were collected. The effluent fractions were monitored for protein content by their absorbance at 280 nm and tested for contents of antigen by the electroimmunoassay procedure described above. Fractions with identical antigenic activity were pooled (as indicated in Fig. 1) and concentrated by ultrafiltration (Amicon type UM-2 membrane).

Results and Discussion

Purity of the human glomerular basement membrane preparations

The purity of the preparations was studied by using both light and electron microscopy. All preparations appeared to be free of undisrupted glomerulae and cells. The average hydroxyproline content was 7.2%. The total phosphate content was less than 0.1% indicating no, or insignificant, contamination by phospholipids, RNA or DNA. The amino acid, neutral sugar and hexosamine composition of the human glomerular basement membrane preparation is pre-

sented in Table II. The amino acid composition with high relative contents of glycine, proline, hydroxyproline and hydroxylysine are similar to data previously published by others [1,14,31]. The hydrolysate also contained a component which eluted early on the amino acid analyzer (before hydroxyproline) and which was present in a hydrolysate of isolated basement collagen but not in a hydrolysate of guinea pig skin type I collagen. In separate experiments it was shown that a corresponding peak was a major component in hydrolysates of Telomycin (a gift from Bristol Laboratories, U.S.A.) which is known to contain 3-hydroxyproline [29]. It is therefore likely that this component is 3-hydroproline, which is unique for basement membrane collagen. The relative carbohydrate composition is also well correlated to data in the literature [1,14] with approximately equimolar amounts of glucose and galactose, which is typical for basement membrane. The content of mannose, fucose, glucosamine and sialic acid is related to oligosaccharides in the non-collagenous pro-

TABLE II

CHEMICAL COMPOSITION OF INTACT AND FRAGMENTED HUMAN GLOMERULAR BASEMENT MEMBRANE (HGBM)

Amino acids as residues per 1000. Carbohydrates as weight percent. Values in parentheses are weight ratios to glucose.

	HGBM	Sepharose 6B fractions of collagenase digested HGBM			
		I	IIa	IIb	III
3-Hypro	28.9	n.d.	n.d.	n.d.	n.d.
4-Hypro	98.0	trace	trace	trace	trace
Asp	64.6	89.8	86.2	88.0	91.0
Thr	32.8	49.4	54.8	64.7	60.3
Ser	45.5	77.4	107.1	99.4	102.6
Glu	91.5	127.0	119.0	108.8	118.2
Pro	70.2	61.3	66.2	71.2	65.8
Gly	221.1	189.8	197.7	132.2	149.8
Ala	50.6	78.5	66.7	79.8	76.0
Cys	18.3				
Val	35.2	49.7	48.8	51.9	63.6
Met	9.2				
Ile	31.1	29.5	30.9	40.0	40.2
Leu	62.2	77.5	66.5	78.8	73.1
Tyr	15.2	13.2	15.5	26.8	16.8
Phe	27.0	25.4	28.9	39.2	26.4
Hylys	26.5	20.5	21.0	11.1	trace
Lys	18.2	32.8	24.7	33.2	43.0
His	13.8	21.4	21.0	25.9	27.4
Arg	40.1	56.8	45.2	48.9	45.9
Total hexose	8.2	1.08	1.20	0.74	0.91
Glucose	3.5 (1)	0.36 (0.14)	0.43 (1)	0.24 (1)	0.32 (1)
Galactose	3.8 (1.09)	0.41 (1.14)	0.45 (1.05)	0.33 (1.38)	0.31 (0.97)
Mannose	0.73 (0.21)	0.22 (0.61)	0.21 (0.49)	0.09 (0.37)	0.13 (0.41)
Fucose	0.20 (0.06)	0.05 (0.14)	0.05 (0.12)	0.027 (0.11)	0.075 (0.23)
Glucosamine	1.02 (0.29)	0.6 (1.67)	n.d.	n.d.	0.19 (0.59)
Galactosamine	0.14 (0.04)	0.14 (0.39)	n.d.	n.d.	0.22 (0.69)
Sialic acid	0.64 (0.18)	0.23 (0.64)	0.14 (0.33)	0.20 (0.83)	0.28 (0.88)

n.d., not detected.

teinaceous part of the basement membrane. The analytical data indicate that the basement membrane preparation was pure, with little or no contamination by other proteins, Table II.

Collagenase digestion of human glomerular basement membrane

Approximately 75% of the human glomerular basement membrane and almost all of the collagenous components, as indicated by hydroxyproline, was solubilized by digestion with bacterial collagenase (Table III). The digest was fractionated on Sepharose 6B and the elution profile is shown in Fig. 1. Practically all of the hydroxyproline-containing material was eluted in the most retarded peak from the column (peak IV). The fractions were tested for antigenic activity by fused rocket immunoelectrophoresis, shown in Fig. 1. Two antigens were separated from the bulk of the others, indicated by I and III in Fig. 1. The separation of two fragments with different antigenicity was confirmed by using immunoelectromigration (Fig. 2), double radial immunodiffusion (not shown) and crossed immunoelectrophoresis (Fig. 3). The intermediate fractions (indicated by 'II' in Fig. 1) contained several different antigens. Some of them were shared by several fractions, and also by the material of small size (III in Fig. 1). In conclusion, two antigens in collagenase digested basement membrane can be separated from the others by gel chromatography on a Sepharose 6B column. In addition several other antigens are present but elute in a mixture. It was shown separately that neither of the two components I and III contained detectable amounts of plasma proteins. From calibration chromatograms with plasma proteins it was estimated that the molecular sizes of components I and III (Fig. 1) were in the order of 1 000 000 and less than 200 000, respectively.

The amino acid, neutral sugar, and hexosamine contents of the fractions indicated in Fig. 1 are presented in Table II. Antigenic components (I, II and III) prepared by Sepharose 6B chromatography of collagenase digested base-

TABLE III

ENZYME DIGESTION OF HUMAN GLOMERULAR BASEMENT MEMBRANE

	Collagenase	Pepsin	Pronase	Trypsin
$\frac{\text{OH-proline in supernatant (g/l)}}{\text{Protein in supernatant (g/l)}}$	0.073	0.036	0.063	0.039
$\frac{\text{OH-proline in pellet (mg)}}{\text{Dry weight of pellet (mg)}}$	0.005	0.099	0.043	0.083
$\frac{\text{OH-proline in supernatant (mg)}}{\text{OH-proline in supernatant + pellet (mg)}}$	0.982	0.220	0.931	0.280
Percentage HGBM solubilized	77	44	91	51
Immunological identity (+) or nonidentity (—) of digests				
Collagenase vs.		+	—	+
Pepsin vs.	+		—	+
Pronase vs.	—	—		—
Trypsin vs.	+	+	—	

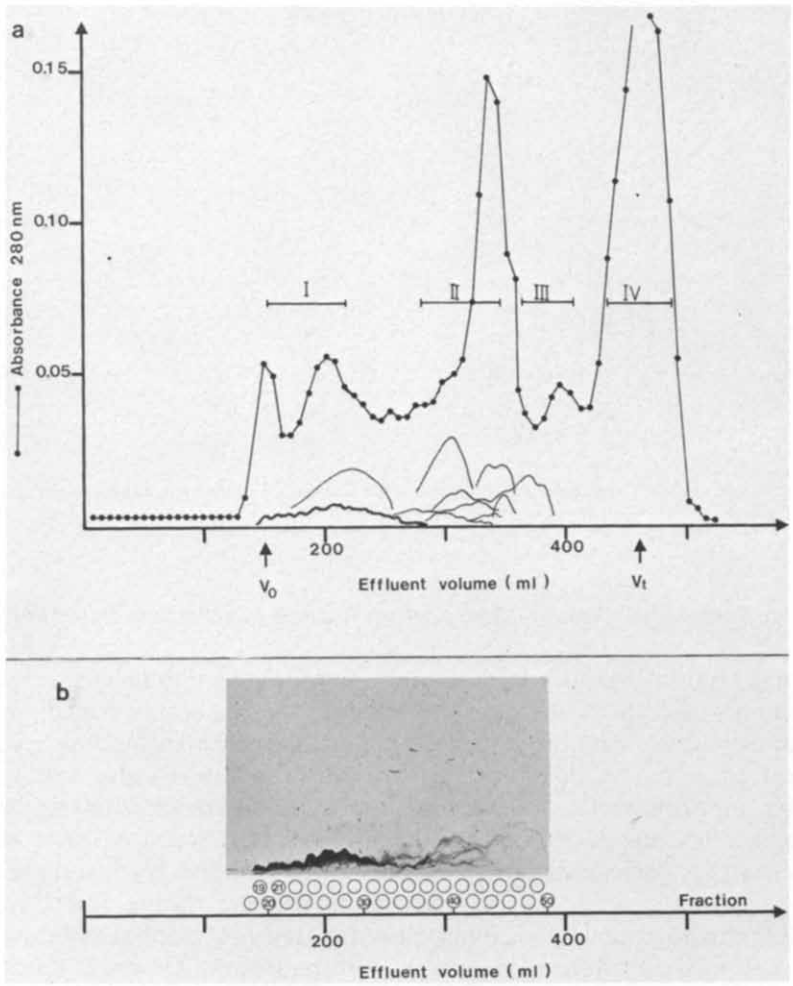

Fig. 1. Analysis of components solubilized by collagenase digestion of human glomerular basement membrane. a. Sepharose 6B elution profile. Insert. schematic representation of fused rocket analysis of fractions (shown in b). b. Fused rocket analysis of Sepharose 6B fractions displaying the interrelation between antigens in different fractions. Continuous precipitation lines are formed for each antigen-antibody system.

ment membrane contained only trace amounts of hydroxyproline and the glycine was significantly lower than in the glomerular basement membrane preparation. Neither cystein nor methionine was found and the hydroxylysine content was low, especially in those antigenic components most retarded in their elution. Therefore, it is likely that these antigens are not of collagenous nature. In support, the carbohydrate composition of the Sepharose 6B fractions indicate lower content of the collagenous part of the basement membrane with lower content of glucose and galactose and higher content of the other carbohydrate residues.

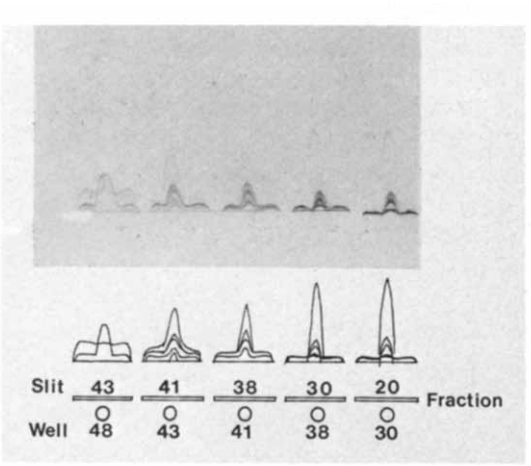

Fig. 2. Immunological identities of Sepharose 6B fractions of collagenase digested human glomerular basement membrane tested by immunoelectromigration analysis.

Pepsin, trypsin, pronase and papain digestion of human glomerular basement membrane

Both pepsin and trypsin digestion of basement membrane solubilized several antigenic components as indicated by the number of rockets seen on analysis of fractions from Sepharose 6B chromatograms (Figs. 4 and 5). Unfortunately, the concentration of antigens in most fractions was too low to give visible precipitation lines for interpretation of continuity in fused rocket analysis or crossed immunoelectrophoresis. These experiments were not repeated since it could be shown that antigenic fragments released by pepsin and trypsin digestion were immunologically identical to antigens in corresponding fractions from Sepharose 6B chromatograms of collagenase digested basement membrane (summarized in Table III). Prolonged digestion with pepsin or trypsin did not result in destruction of antigenic sites (data not shown). In contrast, upon

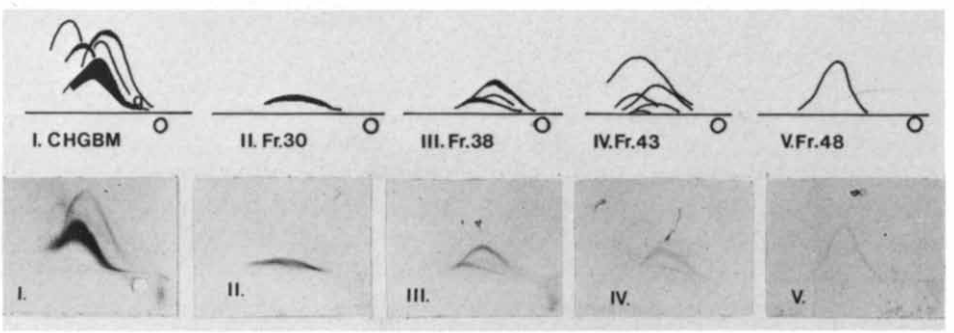

Fig. 3. Crossed immunoelectrophoresis according to Clarke and Freeman of Sepharose 6B fractions of collagenase digested human glomerular basement membrane (as indicated in the figure) against anti-human glomerular basement membrane gammaglobulin.

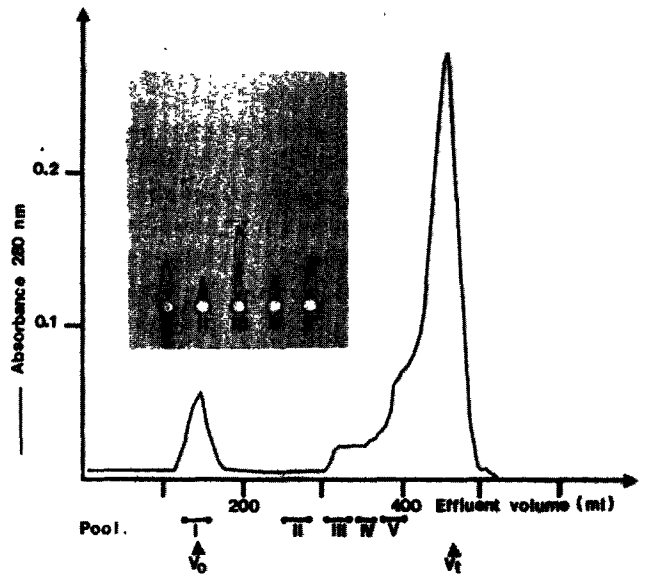

Fig. 4. Sepharose 6B chromatogram of components solubilized by pepsin digestion of human glomerular basement membrane. Insert: electroimmunoassay of pooled fractions. Bars indicate pooled fractions.

pronase digestion, antigens were first released and then successively degraded until at 72 h only one antigen could be identified (Fig. 6). None of the antigens solubilized upon digestion with pronase crossreacted with antigens in collagenase, pepsin or trypsin digests (Table III). Incubation with papain efficiently solubilized the basement membrane but no antigenic activity remained after 4 h of incubation. As with collagenase, digestion with pronase released both the

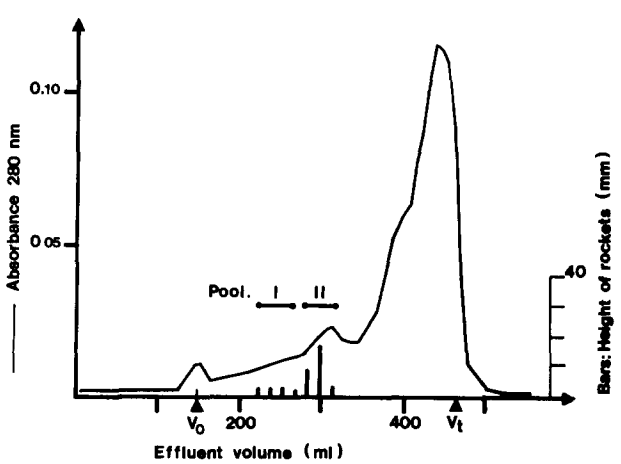

Fig. 5. Sepharose 6B chromatogram of components solubilized by trypsin digestion of human glomerular basement membrane. Vertical bars indicate the rocket height in electroimmunoassay of the fractions.

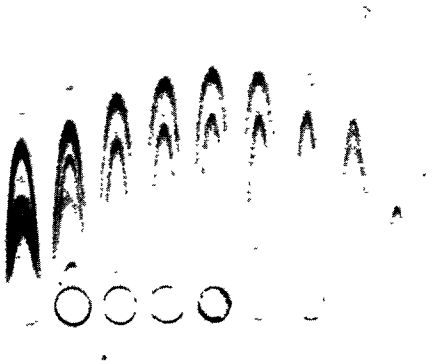

Fig. 6. Components solubilized from human glomerular basement membrane by pronase digestion. Electroimmunoassay of samples at: (from left to right) less than 1 h, 1, 2, 3, 4, 5, 7, 24 and 72 h of digestion.

collagenous and the noncollagenous component of the basement membrane while trypsin digestion primarily released noncollagenous components. Pepsin digestion gave intermediate results (Table III).

General Discussion

In recent years numerous investigators have shown that the chemical structure and the molecular organisation of glomerular basement membrane is very complex [30—33]. The extensive crosslinking of the components by covalent disulphide bonds and the collagen type of crosslinks as well as other less known interactions render the membrane quite insoluble. Therefore, procedures in which such bonds, or more often peptide bonds, are cleaved have to be used, and a wide variety of heterogenous fragments have been solubilized. An additional factor for increasing the complexity may be limited in vivo proteolysis of the proteins [3,30]. Only few basement membrane subunits have been isolated in a form homogenous enough to make chemical characterization possible and the yields have been low (Ohno et al. [32] and Kefalides [33]).

When this investigation was started it was presumed that some of the difficulties encountered in chemical analysis might be resolved by using immunochemical methods. Since the first report by Krakower and Grenspoon [34] on the nephritogenic property of dog glomerular basement, the interest in basement membrane immunology has been centered around the problem of autoimmunisation against membrane components in glomerulonephritis (for review, see Refs. 36 and 5). Usually antigens have been solubilized by means of enzymic digestion, and in a few instances by mechanical or chemical extraction [5,35,36]. Kefalides [37] has suggested that there are three antigenic sites in basement membranes in general. Two sites are considered to be located in a procollagen-like molecule: one in the triple helical portion and the other in the

non-helical extension of the molecule. A third site is supposed to be located in a large molecular weight matrix glycoprotein.

This interpretation is probably an oversimplification, since the total number of antigens detected rises when resolution is enhanced by using for instance crossed immunoelectrophoresis [38].

From analyses of the amino acid and sugar contents of fractions from the Sepharose 6B chromatogram of basement membrane preparations which had been digested with various proteases, it appears that fractions containing antigenic fragments did not contain any collagenous components. It is likely then, that the antigenic sites demonstrated are mainly located in the non-collagenous, non-triple-helical portions of the basement membrane.

It is of interest to note that it was possible to cleave the membrane in such a way that fragments containing single antigenic sites could be identified. However, the large number of molecules within each antigenic class makes isolation by methods based on size or electrical charge difficult. It was hoped as an alternative that some of the various solubilization procedures used would liberate more homologous antigens into solution, but this was not the case with any of the methods tested. Enzymatic cleavage by several enzymes did not yield fragments with fewer antigenic sites. On the other hand, the quantitative electroimmunoassay and the fused rocket technique made it possible to study the enrichment of the antigens in different fractions and, as discussed above, two antigenic fragments isolated by Sepharose 6B chromatography of collagenase digested membrane could be separated from the bulk of the others. The two fractions isolated are still chemically heterogeneous or polydisperse. The smaller molecular weight material, most retarded on Sepharose 6B, seems to be immunochemically homogeneous but chemically heterogeneous or polydisperse. This antigen crossreacts immunologically with an antigen isolated from normal human urine, while the determinant located in the large fragment is never demonstrated in urine [39], whether normal or pathological. Further information on the composition of human glomerular basement membrane may be gained from studies of degradation products in the urine.

References

1 Spiro, R.G. (1972) in Glycoproteins: Their Composition, Structure and Function (Gottschalk, A., ed.), 2nd edn., Part B, pp. 964—999, Elsevier, Amsterdam
2 Spiro, R.G. (1973) N. Engl. J. Med. 288, 1337—1342
3 Berman, L.B. and Misra, R.P. (1972) Am. J. Med. 53, 701—703
4 Kefalides, N.A., Tomichek, E. and Alper, R. (1974) in Protides of Biological Fluids (Peeters, H., ed.), Vol. 22, pp. 349—356, Pergamon Press, Oxford
5 Bardos, P. and Muh, J.-P. (1975) Ann. Biol. Clin. (Paris) 33, 335—349
6 Spiro, R.G. and Spiro, M.J. (1971) J. Biol. Chem. 246, 4899—4909
7 Spiro, R.G. and Spiro, M.J. (1971) J. Biol. Chem. 246, 4919—4925
8 Spiro, M.J. and Spiro, R.G. (1971) J. Biol. Chem. 246, 4910—4918
9 Grant, M.E., Kefalides, N.A. and Prockop, D.J. (1972) J. Biol. Chem. 247, 3539—3544
10 Grant, M.E., Kefalides, N.A. and Prockop, D.J. (1972) J. Biol. Chem. 247, 3545—3551
11 Johnson, L.D. and Starcher, B.C. (1972) Biochim. Biophys. Acta 290, 158
12 Minor, R.R., Clark, C.C., Koszalka, T.R., Brent, R.L. and Kefalides, N.A. (1974) in Protides of Biological Fluids (Peeters, H., ed.), Vol. 22, pp. 357—361, Pergamon Press, Oxford
13 Clark, C.C., Tomichek, E.A., Koszalka, T.R., Minor, R.R. and Kefalides, R.R. (1975) J. Biol. Chem. 250, 5259—5267
14 Westberg, N.G. and Michael, A.F. (1970) Biochemistry 9, 3837—3846

15 Mohos, S.C. and Scoza, L. (1970) Exp. Mol. Pathol. 12, 316—323
1 Spiro, R.G. (1967) J. Biol. Chem. 242, 1915—1922
1 Avrameas, S. and Ternynck, T. (1969) Immunochemistry 6, 53—66
18 Steinbuch, M. and Audran, R. (1969) Arch. Biochem. 134, 279—284
19 Grabar, P. and Williams, C.A. (1953) Biochem. Biophys. Acta 10, 193—194
20 Ouchterlony, O. (1962) Prog. Allergy 6, 30—154
21 Laurell, C.B. (1966) Anal. Biochem. 15, 45—52
22 Just Svendsen, P. (1973) Scand. J. Immunol. 2, Suppl. 1, 69—70
23 Clarke, H.G.M. and Freeman, T. (1967) in Protides of Biological Fluids (Peeters, H., ed.), Vol. 14, pp. 503—509, Pergamon Press, Oxford
24 Grubb, A. (1972) Scand. J. Clin. Lab. Invest. Suppl. 124, 59—65
25 Stegemann, H. and Stadler, K.H. (1967) Clin. Chim. Acta 18, 267—273
26 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Axelsson, I. and Heinegård, D. (1975) Biochem. J. 145, 491—500
29 Sheehan, J.C., Whitney, J.G. (1962) J. Am. Chem. Soc. 84, 3980
30 Sato, T. and Spiro, R.G. (1976) J. Biol. Chem. 251, 4062—4070
31 Kefalides, N.A. (1975) J. Invest. Dermatol. 65, 85—92
32 Ohno, M., Riquette, P. and Hudson, B.G. (1975) J. Biol. Chem. 250, 7780—7787
33 Kefalides, N.A. (1971) Biochem. Biophys. Res. Commun. 45, 226—234
34 Krakower, C.A. and Greenspoon, S.A. (1951) Arch. Pathol. 51, 629—651
35 Naruse, T. and Shibata, S. (1972) Immunology 22, 925—932
36 Marquardt, H., Wilson, C.B. and Dixon, F.J. (1973) Kidney Int. 3, 57—65
37 Kefalides, N.A. (1972) Connect. Tissue Res. 1, 3—13
38 Henriksen, K. and Jensen, H. (1975) Scand. J. Immunol. 4, 699—706
39 Bygren, P.G., Wieslander, J. and Heinegård, D. (1979) Biochim. Biophys. Acta 553, 255—261