PHYSICAL AND CHEMICAL PROPERTIES OF THE BOVINE GLOMERULAR BASEMENT MEMBRANE

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ABBREVIATIONS

AcAm.	Acrylamide monomer
A.P.	Ammonium persulphate
β-Gal	β-Galactosidase
Bis	N,N'-methylenebisacrylamide
BM	Basement membrane
BSA	Bovine serum albumine
CD	Circular dichroism
CTGN	Chymotrypsinogen
Gal	Galactose
GBM	Glomerular basement membrane
Glu	Glucose
GuHCl	Guanidine hydrochloride
mA	Milliampere
M.E	2-mercaptoethanol
M.W	Molecular weight
NANA	N-acetylneuraminic acid
OVAL	Ovalalbumin
S-CM	Carboxymethylated
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

CHAPTER I

INTRODUCTION

A. Statement of Problem

The renal glomerular basement membrane is an extracellular matrix which is located between epithelial and endothelial cells, and is the only continuous membrane barrier between the blood plasma and the glomerular ultrafiltrate. Therefore, it is considered to be the primary ultrafilter of the kidney, being permeable only to small molecular weight metabolites. In a variety of disease states, such as diabetes mellitus, the membrane apparently undergoes alterations which lead to an increase in permeability, hence an impairment of kidney functions.

The whole membrane consists of at least thirteen different polypeptide chains (subunits) ranging in molecular weight from 24,000 to greater than 350,000 daltons. Some subunits are covalently linked by disulfide bonds and aldehyde-derived crosslinks of the type found in vertebrate collagen, while others are held together by non-covalent interactions. A portion of the membrane, 26%, is soluble in 6 M guanidine HCl. The insoluble and soluble fractions represent a gross fractionation of subunits held together by non-covalent interactions. The insoluble portion, which represents 74% of the total membrane weight, closely resembles vertebrate collagen with respect to amino acid composition, presence of hydroxylysine linked mono- and disaccharides, and infrared spectra. In contrast, the guanidine HCl

soluble fraction differs greatly from that of collagen, although it possesses certain characteristics of collagen.

At present, none of these subunits have been isolated in pure form or characterized. Moreover, it is not known which subunits are specifically crosslinked by covalent bonds or held together by non-covalent interaction.

The purpose of this study was to (1) determine the subunit composition of the two membrane fractions held together by non-covalent bonds, the guanidine HCl soluble and insoluble fractions, (2) determine which subunits are crosslinked by intermolecular disulfide bonds, (3) isolate and purify a single subunit and then characterize its chemical and physical properties. The knowledge obtained from these studies is fundamental to the understanding of renal glomerular basement membrane with regard to its overall molecular organization, mechanism of kidney ultrafiltration, and finally, chemical and morphological alterations which are commonly observed in the membrane in various disease states.

B. Survey of Literature

1. General

Basement membranes are extracellular matrices which are distributed in numerous tissues of the animal body; such as, glomeruli and tubules of the kidney, the lens capsule, the alveoli of the lungs, and the capillaries of the vascular tree (1-3).

The renal glomerular basement membrane is most extensively studied, among other basement membranes, because of its prime importance in various states of disease and relative ease of accessibility compared

to more difficult membranes such as the alveolar basement membrane, which resists isolation in pure form due to close association of collagen and elastin tissue (4).

The GBM appears to be the only continuous filtration barrier between the blood plasma and the urine space. Thus, it is considered to play a vital role as a molecular filter of fluids passing through the kidney as well as a supporter of the tissue's structure (5, 6, 7).

Morphological alteration of the GBM has been observed in numerous cases such as diabetes mellitus, various kinds of nephritis, and nephrotic syndromes. It is clear that an understanding of the blood vessel disease and their membrane alteration will depend, to a large extent, on the knowledge of the chemical structure of the basement membrane (8, 9).

Earlier studies have concerned morphological changes of the basement membrane as revealed by the electron microscope. More recently, chemical and immunological studies have been conducted by Spiro (10), Kefalides (11), and Mahieu (12). The amino acid composition and carbohydrate composition of whole basement membrane from various sources have been established. The elucidation of one of the carbohydrate units structures has attracted the research of immunological aspects, since the disaccharide units are the major antigenic site of collagen (13, 14).

Recently, bovine GBM has been fractionated after solubilization by reduction and alkylation. The basic ideas of its subunits and range of molecular weight are obtained (15, 16). The insoluble collagenous portion of basement membrane was also investigated and the presence of collagen was confirmed by isolation of a molecule which consisted of

three identical α chains of the type found in classical collagen (17). However, the isolated collagen was the result of extensive enzymatic digestion of interconnected matrix of the multicomponent system and does not represent the true nature of this component in the native basement membrane.

A better understanding of the overall construction of the GBM will require the analysis of several of the components without subjecting them to extensive enzymatic and/or chemical changes upon isolation. Because of the difficulties arising from its insoluble nature, a pure single component has not been isolated in sufficient analytical quantities, and it may be necessary to wait a few years before any meaningful primary structure can be elucidated. Nonetheless, gel electrophoresis, gas chromatography, and peptide sequential analysis show promise in basement membrane studies.

2. Isolation of Basement Membrane

a. Isolation of Glomeruli

The initial difficulty at this state, which poses great technical problems, is the isolation of glomeruli in pure form from the kidney in large quantities. The first description of a method to isolate glomeruli, in reasonable quantities for chemical analysis, was reported by KraKower and Greenspon (18). Their method, in which minced kidney cortex is disrupted and passed through a fine mesh of stainless steel screen, proved to be the more useful. However, their method relies on repeated sedimentations without the use of sieves and this treatment provides glomeruli of variable quality. Glomeruli can also be collected from the cortex by differential centrifugation (19), or by passing

the disrupted cortex through a small glass column after freezing and thawing (20). Another interesting method was the magnetic attraction of the glomeruli after the kidney was perfused with magnetic iron particles, however, this method is less practical for the purpose of chemical studies.

The best method so far is filtration of disrupted cortex through stainless steel sieves of varying mesh size (10). This method was found to give a preparation of glomeruli almost free of other tissue fragments except for a few tubular and capsule fragments which could be easily eliminated. This method proved so satisfactory that all of the chemical studies reported since 1967 have been done on glomeruli isolated by this sieving method.

b. Isolation of GBM

A cell free glomerular preparation can be obtained by digestion of isolated glomeruli with trichloroacetic acid (21) or with dilute alkali solution (22). These methods were not satisfactory because of the variable quality of membrane were obtained. There is also the possibility that the use of acid or alkaline solution introduces some modification of the polypeptide chain or carbohydrate groups. The gradient centrifugation of homogenized cortex has also been used (23), but these preparations are not clean.

The best result was obtained by Spiro (10) as follows: the glomeruli are subjected to ultrasonic treatment to isolate the GBM from the unwanted cell, followed by careful low speed multiple centrifugation in 1 M NaCl and repeated washings with distilled water. By this method, all the cellular elements can be removed and a preparation can be obtained which is pure based on morphological observation with a

microscope. The chemical analysis showed this membrane preparation is free of lipids, nucleic acids and phosphorus. The yield by this method gives more than 100 mg of purified membrane from one kilogram of kidney cortex. The recovery of GBM is roughly one third of the dry weight of the isolated glomeruli.

3. Solubility Properties of GBM

The basement membranes are insoluble in water at physiological conditions in general. Extensive solubilization of the GBM has been achieved by treatment with 0.1 N NaOH for 8 hours at 37°C (10), or 5% trichloroacetic acid at 90°C (24), or excessive heating by autoclaving at 110°C for three hours in an acetate buffer (25). These methods solubilize GBM up to 80%, by weight. The GBM can also be solubilized extensively by proteolytic enzymes (10, 26). Approximately 65% of the membrane becomes soluble by pepsin or trypsin digestion, and up to 90% solubilization was achieved by treatment with bacteria collagenase at 37°C for 48 hours. Percent solubilization is based on the chemical analysis of hydroxyproline and hexosamine content in the soluble fraction (27). These methods mentioned above are satisfactory when the the purpose of study is chemical or immunological exploration of the fragments derived from a complicated multicomponent membrane.

Recently, extensive studies under mild conditions which do not disrupt the peptide bond have been reported (15, 28). Treatment of the membrane with SDS or 8 M urea, pH 7.0, at 25°C for 36 hours causes the solubilization of 25% of the membrane. The 0.1 M sodium phosphate buffer or 0.5% Triton x-100 in phosphate buffer, can only solubilize less than 5%. Hexose analysis gives slightly higher values for solubilization

than polypeptide measurements in these cases. However, after reducing, alkylation of the GBM with iodoacetic acid, dialysis, and lyophylization, the basement membrane preparation can be obtained with solubility greater than that of the original native membrane (16). Such a preparation is about 50% soluble in neutral phosphate buffer, 70-85% soluble in SDS and the solubility in urea solution, depends on the conditions and determination methods.

4. Chemical Composition

a. Amino Acid Composition

The amino acid composition of GBM has been established by a number of investigators (11, 15, 22, 29-33). The composition of the whole membrane of bovine, human, and canine and the soluble and insoluble portions of bovine membrane are shown in Table I. The tendon collagen is also listed in the same table as an example of typical interstitial collagen for comparison purposes. At a glance, the whole membrane of the three species appear essentially the same in composition within reasonable variation, but the soluble and insoluble portions are considerably different from the original whole membrane.

The similarity between whole basement membranes and collagen can also be seen. In particular, the insoluble portion resembles collagen much more than does the soluble portion (Table VIII). In other words, the basement membrane contains large amounts of glycine and substantial amounts of hydroxyproline and hydroxylysine. This indicates that basement membrane, in general, especially the insoluble portion, belongs to the collagen family of proteins. However, there are many differences, in important respects, from typical collagen. It may be noted that

TABLE I

AMINO ACID COMPOSITION OF BOVINE, HUMAN, AND CANINE GBM AND TENDON COLLAGEN

Amino acid	Bovine basement membrane			Human	Canine	Tendon
	Whole membrane	Soluble	Insoluble	Whole membrane	Whole membrane	collagen
Lysine	26.6	35.4	24.6	26.4	26.0	23.0
Histidine	17.4	21.6	12.2	18.7	14.4	4.0
Arginine	50.8	54.9	46.0	48.3	48.2	45.0
Hydroxylysine	21.8	8.0	21.5	24.5	25.0	6.0
Aspartic acid	65.6	76.4	62.0	70.0	70.0	46.0
Threonine	40.7	48.8	29.5	40.3	40.5	17.0
Serine	51.8	67.6	50.0	54.2	49.0	33.0
Glutamic acid	91.6	106.9	89.4	101.3	97.0	63.0
Proline	77.1	81.7	87.6	64.1	63.0	140.0
Glycine	212.4	150.6	242.3	225.2	229.0	329.0
Alanine	65.2	73.9	72.6	58.6	65.0	112.0
Half cystine	22.0	26.6	14.7	22.0	22.7	none
Valine	36.6	38.0	32.8	36.0	36.0	20.0
Methionine	13.8	13.9	12.2	7.0	5.0	6.0
Isoleucine	30.0	31.3	24.5	28.6	28.1	10.0
Leucine	57.3	66.9	51.5	50.3	60.2	21.0
Tyrosine	16.8	23.4	12.8	20.5	22.0	5.0
Phenylalanine	27.9	31.5	24.3	28.3	26.8	13.0
4-hydroxyproline	77.1	32.6	89.9	53.0	56.5	86.0
3-hydroxyproline	Not listed					none

Numbers are expressed in residues per 1000 amino acid residues.

classical collagen contains 33% glycine while GBM contains less than 23%. The collagen is believed to be free of cysteine and 3-hydroxyproline, but GBM has these amino acid residues. The presence of cysteine in GBM is noteworthy because it plays an important role in stabilizing the basement membrane structure. The basement membrane also differs from collagen in that it has a substantially greater number of aromatic amino acids, dicarboxylic amino acids and other polar amino acids. A large difference is also noted in the amount of hydroxylsine and ratio of hydroxyproline to proline. This means hydroxylation of these amino acids takes place more actively in basement membrane than in collagen. According to Ramachandran et al. (34), a protein is defined as collagen when (1) glycine accounts for about 33%, (2) the sum of proline and hydroxyproline accounts for about 22% of the amino acid composition, (3) the protein exhibits a triple helical structure in which three polypeptide chains are aggregated to form a molecule. However, this definition is far from being perfect with respect to the degree of deviation of many collagen of different species. If the presence of this wide deviation is admitted, the basement membrane could be regarded as basement membrane collagen.

b. Carbohydrate Composition of GBM

The carbohydrate composition has been reported for several species of purified basement membranes (10, 27, 35). The total amount of carbohydrate varies from 8-11%. The composition of bovine and human GBM is presented in Table II. Tendon collagen is also listed for comparison purposes. It is shown that there is some variation in carbohydrate compositions among different species. The variation may be slightly

TABLE II

CARBOHYDRATE COMPOSITION OF GLOMERULAR BASEMENT MEMBRANE AND TENDON COLLAGEN

	gm/100gm		
	Bovine [a]	Human [b]	Tendon Collagen [b]
Hexose	6.29	6.8	0.68
Glucose	2.47	2.5	0.30
Galactose	3.05	2.6	0.40
Mannose	0.77	1.7	0.00
Glucosamine	1.86	2.0	0.40
Galactosamine	0.27		
Fucose	0.22	0.7	0.00
Sialic acid	1.19	1.5	0.06

[a] Spiro (44)

[b] Kefalides (1)

larger than that of the amino acid composition of various basement membranes. Nevertheless, it is obvious that the overall composition is very much alike.

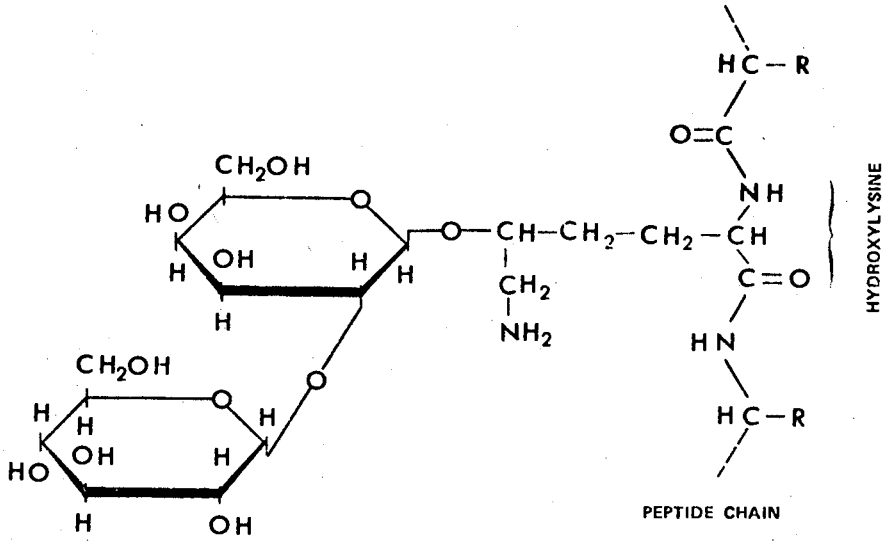
The total content of carbohydrate for basement membrane is much larger than that for collagen, yet there are some similarities, e.g., glucose and galactose occur in the highest amounts. The presence of glucose draws particular interest because this sugar is not a common component of glycoproteins. The glucose has been detected only in collagen and basement membrane as a component of hydroxylysine glycosides. The lens capsule basement membrane, unlike GBM, contains hexuronic acids which are commonly found in mucopolysaccharides. This may suggest that there is some variation in carbohydrate composition among different kinds of basement membranes (24).

5. Chemical Structure of Carbohydrate Units

After digestion of GBM with collagenase and further treatment with pronase, two different groups of glycopeptides were separated by gel filtration. The one which contains only glucose and galactose was purified further by ion exchange column chromatography (36). The structure of this carbohydrate unit, which is also detected in collagen molecule, was investigated by mild acid hydrolysis, glucosidase, periodate oxidation, and methylation experiments. The complete structure of this carbohydrate unit is 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosyl hydroxylysine as shown in Fig 1. The quantitative analysis of the alkaline hydrosylate of the GBM indicates that approximately 77% of the hydroxylysine residues of the membrane are

Figure 1. The Structure of the Disaccharide Unit and a Proposed Conception of the Heteropolysaccharide

DISACCHARIDE UNIT



HETEROPOLYSACCHARIDE UNIT

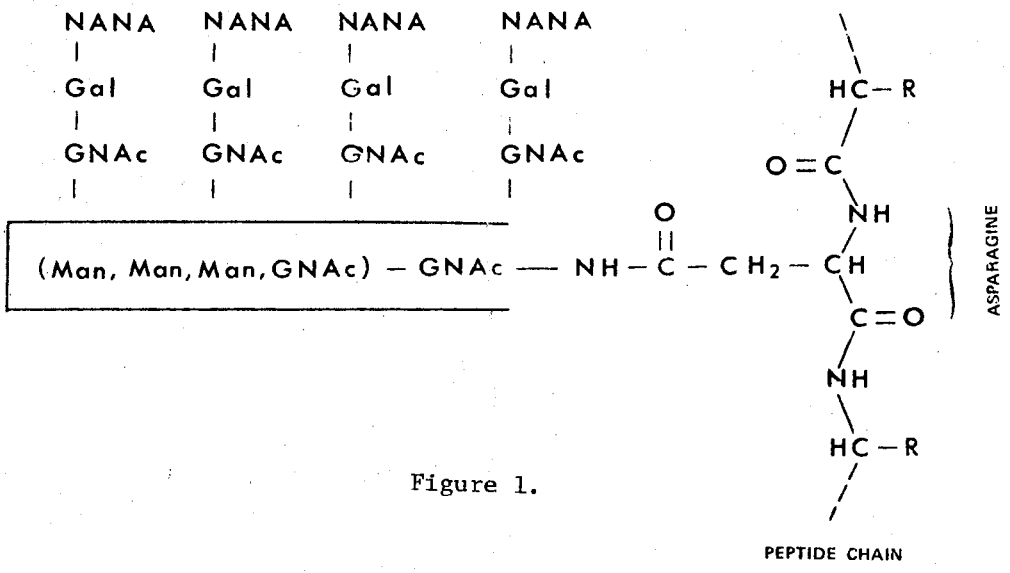


Figure 1.

linked to a disaccharide unit and less than 5% are linked to a galactose residue only.

The second group of glycopeptides were also resolved by gel filtration and ion exchange column chromatography. These glycopeptides differ slightly from each other due to microheterogeneity which is commonly observed in complex carbohydrate units of this type. The average molecular weight of this heteropolysaccharide unit is roughly 3,200 daltons, with an average ratio of 4 galactose, 3 mannose, 5 glucosamine, 3 sialic acid, and 1 fucose residues. Asparatic acid (asparagine) is found as the predominant component attached to the heteropolysaccharide. This suggests that a glycosylamine-type of glycopeptide bond is present, yet detailed structure of this unit is not known. By analogy with some types of carbohydrate units found in globular proteins, the overall structure is speculated as shown in Figure 1. The ratio between disaccharide units and hetropolysaccharide units is roughly 10:1 in bovine GBM.

6. Location of the Carbohydrate Unit on the Polypeptide Chain

The small polypeptide chain containing either the disaccharide unit only or the heteropolysaccharide unit only, were purified after autoclaving, extensive enzymatic digestion, chromatography, and electrophoresis (25). The vicinity of the heteropolysaccharide unit contains aspartic acid, glutamic acid, threonine, serine, proline, and glycine. The amino acid sequence of this peptide portion is not known partly because it is less important with regard to immunological response in some cases of the nephritis patient. However, the disaccharide containing glycoprotein is shown to be one of the major antigenic

determinants of anti-basement membrane autoimmunization. Because of this, the amino acid sequence at the vicinity of the disaccharide was analyzed and compared to the collagen fragment which was previously reported (37, 38). Both sequences are shown below.

Hyl-Gly-His-Arg-Gly in collagen (39)

Hyl-Gly-Gln-Asp-Gly (Asp) in Human GBM (38)

The extent of glycosylation on hydroxylysine in collagen is more extensive than in basement membranes and the amino acid sequences in the vicinity of hydroxylysine in the basement membrane and collagen are totally different, since basic amino acids are found in collagen and acidic amino acids in the basement membrane. However, there is a weak crossantigenic reaction observed in the radioimmunoassay between GBM and the α chains of collagen (38). The correlation of the location between the disaccharide unit and the heteropolysaccharide unit is not known, but the isolated soluble fragment, which shows strong antigenicity, has both a disaccharide unit and a heteropolysaccharide unit. The separation of these saccharide units without extensive enzymatic degradation has not been achieved to date.

7. Subunit Structure

In order to investigate the subunit structure of basement membrane, whole GBM was reduced with mercaptethanol and carboxymethylated. All cysteine residues were shown to be alkylated by this procedure (15, 40). These alkylate subunits become very soluble (70-80% of total membrane) in urea, GuHCl, and SDS solutions. The fractionation of S-CM-membrane components was investigated extensively with various porous agarose gels and DEAE cellulose column chromatography (15, 16). The amino acid

composition and carbohydrate content of each pooled fraction of several agarose gel chromatography were analyzed.

Pronounced differences in the chemical composition of the various fractions were observed. Fractions with a more collagen-like composition tended to be of higher molecular weight and more cationic in nature. These fractions eluted at near void volume and contained a greater number of the amino acids characteristic of collagen, such as hydroxyproline, hydroxylysine, and glycine. These collagen-like subunits were found to contain a high number of disaccharide units with a maximum of 35 units per 1000 amino acid residues. Conversely, other fractions had more abundant amounts of polar amino acids, such as asparatic acid, serine, threonine, tyrosine, lysine, histidine, S-CM-cysteine. As expected, these fractions are relatively rich in heteropolysaccharide units instead of the disaccharide units.

Amino terminal amino acid analysis was carried out and the variation of the relative amount of different N-terminal amino acids in different fractions indicated that these membranes are polymers of several smaller peptide chains. This finding suggests that different types of crosslinks, other than disulfide crosslinks, are involved.

The presence of collagen molecules in basement membrane was reported by Kefalides (41). The basement membrane collagen, which consists of three identical α chains, was isolated after proteolytic enzyme digestion. This collagen cannot be a basement membrane subunit because of the extensive enzymatic digestion used. However, this may prove that a part of the basement membrane subunit is real collagen and a non-collagen-like peptide chain was originally attached to it. In order to gain additional information in regard to subunit structure,

the purification and characterization of the components without enzymatic digestion must be accomplished.

8. Polypeptide Crosslinks

The glomerular basement membrane contains 3% cysteine which is not found in typical collagen. The disulfide bonds as well as non-covalent bonds, are primarily important in stabilizing the basement membrane. Recently, other types of polypeptide crosslinks which are known to occur in collagens were also detected in several basement membranes including GBM (42). These crosslinks were detected by reduction of the membrane with $NaBH_4^3$, hydrolysis with acid, or with enzymes, followed by ion exchange chromatography. The results of these studies indicate that; (1) the basement membrane preparations incorporated tritium during $NaBH_4^3$ reduction in amounts similar to interstitial collagen, (2) a large proportion of the radioactivity is present in peaks which correspond to reduced aldehyde and crosslinks, and (3) it is the collagenous proteins of the basement membrane which contain the reduced aldehydes and crosslinks.

Some of the peaks were identified by comparison with a standard collagen sample. The crosslinks aldehistidine, dihydroxylysinonorleucine, hydroxymerodesmosine, and other intermediates were present in the chromatograms, but the histidinohydroxymerodesmosine, found in collagens, did not appear in the chromatograms of the basement membrane. The other group of compounds, N^{ε}-glycosyllysine and N^{ε}-glycosylhydroxylysine, were also present in the basement membrane. The identified crosslinks are presented in Table III. The total basement membrane had a complex chromatographic profile due to unknown radioactive peaks.

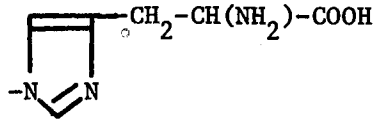
TABLE III

ABBREVIATIONS WHICH ARE USED IN FIGURE 3

Abbreviation	Trivial name	Formula
L	lysine	X-CH_2-CH_2-NH_2
HL	δ-hydroxylysine	X-CHOH-CH_2-NH_2
AL	α-amino adipic acid δ-semialdehyde; allysine	X-CH_2-CHO
HAL	δ-hydroxy, α-amino adipic acid δ-semialdehyde; hydroxyallysine	X-CHOH-CHO
deLNL	dehydrolysinonorleucine	X-CH_2-CH=N-CH_2-Y
LNL	lysinonorleucine	X-CH_2-CH_2-NH-CH_2-Y
ALAL	"aldol";"allysine aldol"	X-CH(CHO)-CHOH-Y
$ALAL_d$	dehydrated"aldol"	X-CH(CHO)=CH-Y
$deHLNL_a$	dehydrohydroxy-lysinonorleucine	X-CHOH-CH_2-N=CH-Y
$deHLNL_b$		X-CHOH-CH=N-CH_2-Y
HLNL	δ-hydroxylysinonorleucine	X-CHOH-CH_2-NH-CH_2-Y
deHLHNL	dehydrohydroxylysino-hydroxynorleucine	X-CHOH-CH=N-CH_2-CHOH-X
HLHNL	hydroxylysinohydroxy-norleucine	X-CHOH-CH_2-NH-CH_2-CHOH-X
deHM	dehydrohydroxymerodesmosine	Y-CH=C(X)-CH=N-CH_2-Y
ALHis	aldol-histidine	Y-CH(Z)-CH(X)-CHO
HisHM	histidino-hydroxy-merodesmosine	Y-CH(Z)-CH(X)-CH=N-CH_2-CHOH-X

X: -CH_2-CH_2-CH(NH_2)-COOH

Y: -CH_2-CH_2-CH_2-CH(NH_2)-COOH

Z: 

These new peaks were found in the neutral and acidic amino acid regions which suggest that these new compounds were not of the general structure found for collagen crosslinks. Since all of these detected compounds are chemically reduced products, in vitro, it is not known that the same kind of reduction of Schiff base, in vivo, takes place in the maturation process of basement membrane as does in collagen. It is not surprising that basement membrane, especially the collagenous type portion, contains similar aldehyde-derived crosslinks which are found in collagen, since the two types of proteins share common features in many respects, such as amino acid composition, carbohydrate composition, and structural role.

9. Proposed Structural Models of GBM

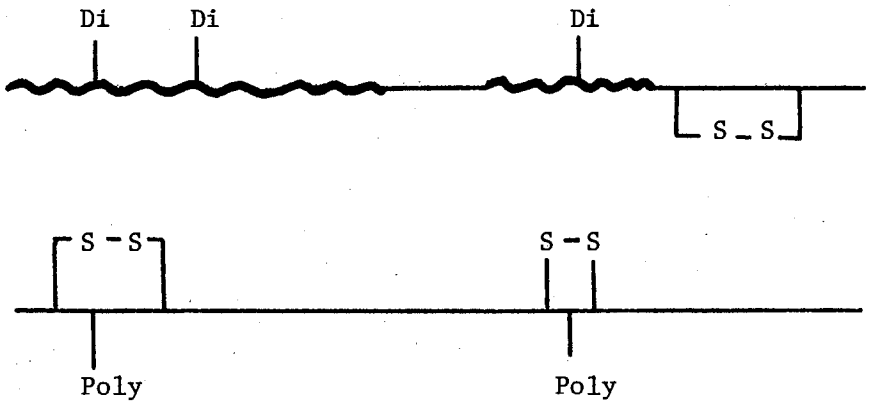
Information concerning the overall structure of GBM is quite limited. The subunit size and chemical composition, distribution of amino acids, the disaccharide unit and heteropolysaccharide unit, and the location and manner of disulfide crosslinks and other crosslinks which are not separable by simple reduction, are major factors of consideration in the hypothetical proposed models (3).

Several models are shown in Fig. 2. These models are drawn under assumption that the disaccharide units are located mainly on the collagen-like portion and the heteropolysaccharide units are located on the more polar region. These assumptions are partially supported by observations in which the vicinity of glycopeptides isolated after extensive enzymatic digestion, showed quite different amino acid compositions (43, 44).

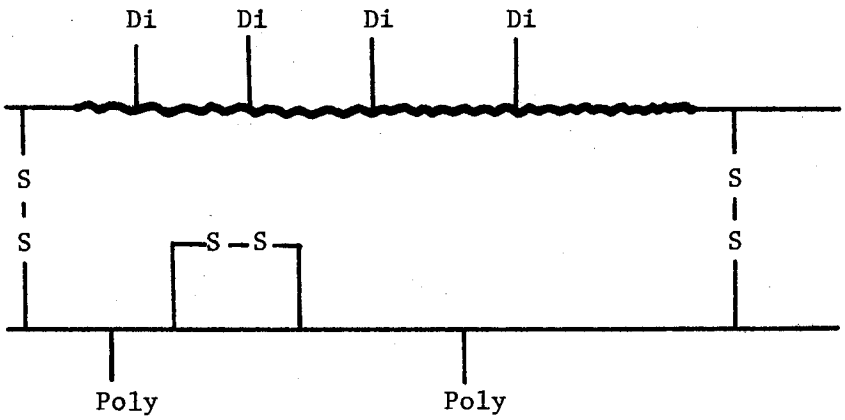
Figure 2. The Glomerular Basement Membrane Models

Representation of three hypothetical models of the GBM showing the possible relationship of the disaccharide (Di) and heteropolysaccharide (Poly) units to each other and the peptide chains. The jagged line represents collagenous portion of the peptide chains while the straight line indicates polar regions. Disulfide bonds are shown and X represent possible lysine-derived crosslinks between the peptide chains.

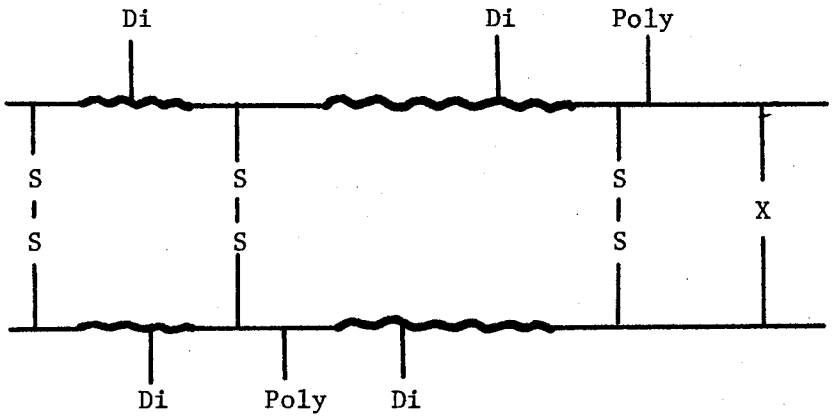
1


Di
Di
Di
S - S
S - S
S - S
Poly
Poly

2


Di
Di
Di
Di
S
S
S
S
S - S
Poly
Poly

3


Di
Di
Poly
S
S
S
S
S
S
X
Di
Poly
Di

In model 1, two distinctly different proteins, a collagen and non-collagen, are held together by non-covalent bonds and disulfide crosslinks. This model was originally hypothesized by Kefalides in 1966 (41). Some difficulties of this model are: (1) the drastic change of solubility of the whole membrane after reduction, (2) all of the urea and guanidine soluble fractions do contain both disaccharide units and heteropolysaccharide units, although the ratio of these carbohydrate units varies considerably.

In model 2, the only difference is the presence of disulfide bonds between the collagen-like peptide and the non-collagen-like peptide. This model seems better than model 1. with respect to solubility change after reduction, but does not explain the fact that reduction alone does not provide either one kind of polypeptide.

The third model indicates that one polypeptide chain which has both a collagen portion and a non-collagen portion is linked to another polypeptide of a similar or dissimilar molecule. The observations, so far, seem to support this scheme because completely reduced and alkylated basement membrane cannot be fractionated into two different fractions, and every fraction always contains two kinds of carbohydrate units.

The situation is complicated even more by the presence of other crosslinks besides disulfide bonds, which was discussed in section 8. The collagen component, which was isolated from the basement membrane after limited enzymatic digestion, consist of three identical α chains, has a molecular weight of 108,000 daltons, has an amino acid composition typical of collagen, and has only glucose and galactose (17). The only difference it has from interstitial collagen is the presence of cysteine. This observation may indicate that a substantial part of

the polypeptide is of a collagen nature. On the other hand, Mahieu isolated a component, after extensive autoclaving with excess mercaptethanol, and the molecular weight of this component is 80,000 (38). The amino acid and carbohydrate analysis show essentially no difference from that of the original whole membrane. In other words, the amount of both disaccharide and heteropolysaccharide units, amount of glycine or hydroxyproline, as well as polar amino acids, are very similar to the original membrane. This observation may indicate that the polypeptide chain at this molecular weight range contains both collagen type and non-collagen type peptides. Both experiments involved extensive degradation of the peptide bond of the basement membrane, therefore, a conclusive answer as to which model represents the true picture of basement membrane cannot be drawn from these observations. The isolation of a single component after reduction without cleavage of the peptide bond may contribute to a portion of the answers of these problems.

10. Biosynthesis of Basement Membrane

The complete biosynthesis of basement membrane requires many steps. The basement membrane biosynthesis is presumably more complicated than interstitial collagen because the basement membrane is a multicomponent system.

These steps are: (1) peptide synthesis by common ribosomal machinery, (2) hydroxylation of non-coded amino acids, (3) attachment of carbohydrate residues in the proper sequence, (4) assembly of the different kind of polypeptides, and (5) establishment of crosslinks after a series of reactions which are necessary to form crosslinks.

It is presumed that the synthesis of the peptide portion takes place prior to the attachment of carbohydrate units, and hydroxylation of proline and lysine is similar to the mechanism which has been shown in the case of collagen synthesis (45, 46, 47).

The nature of collagen hydroxylase and its mechanism or reaction have been extensively studied (48, 49), but the site of hydroxylation is not well established. The attachment of the carbohydrate portion takes place through the action of several specific glycosyl transferases. The galactosyl and glycosyl transferase have been prepared from rat kidney cortex, and these enzymes have been studied extensively (50, 51, 52). The galactosyl transferase can transfer a galactose unit to a hydroxylysine residue of native collagen but cannot transfer a galactose to free hydroxylysine or to a small peptide containing hydroxylysine. The glycosyl transferase can transfer a glucose unit to a hydroxylysine-galactose group either in high or low molecular weight compounds. The enzymes are increased in the kidneys of diabetic rats, possibly reflecting the increased synthesis of basement membrane.

The enzymes responsible for the assembly of the heteropolysaccharide unit have not yet been isolated, but the presence of sialyl and galactosyl transferases have been demonstrated. The mode of the assembly of the heteropolysaccharide unit is speculated to resemble that of the unit in thyroglobulin (53, 54). The enzyme systems of the heteropolysaccharide, notably glucasamine transferase, which is the first enzyme of glycosidation upon asparagine residue of the protein, and mannose transferase, are not known to date.

Basement membranes are produced by epithelial cell and/or cells of mesenchymal origin which include endothelial cells, pericapillary cells or pericytes, where as collagen is produced by fibroblast cells.

The elaborated demonstration of involvement of the epithelial cell in synthesis of the renal glomerular basement membrane was shown some years ago. Silver nitrate was administered in the drinking water of young rats, and a dark silver deposit was observed throughout of basement membrane. Several weeks later, after the silver nitrate was removed from the drinking water, newly synthesized basement membrane free of silver deposit was found to be laid down only on the epithelial side of the membrane (55).

Recently, a soluble component was isolated from parietal yolk sac carcinoma cells in tissue culture. Partial characterization of this component revealed that the component did not contain any hydroxylsine and sialic acid, but large amounts of hexosamine, fucose and galactose were found (56). The amino acid composition, except for the lack of hydroxylysine, resembled the soluble portion of GBM (15). A large amount of polar amino acids, such as aspartic and glutamic acids, which are characteristic of soluble portions of basement membrane were observed. The other epithelial basement membrane collagen was studied both in vivo and in vitro in embryonic chick lens, a tissue in which all of the collagen is in the basement membrane of the lens capsule. The radioactive basement membrane was isolated and partially characterized, but complete amino acid and carbohydrate analysis was not done because of a limited amount. They observed that about 14% of total hydroxyproline was present as the 3'isomer and 85-90% of the lysine was detected as hydroxylysine. The initial form of ^{14}C-membrane synthesized by these systems had a molecular weight of 140,000 daltons and a time dependent conversion to smaller molecular weight (115,000 M.W) was observed. The basement membrane collagen secreted into the medium was

largely resistant to pepsin at 15°C, suggesting that it has a triple helical conformation, but this component in the lens cells was largely digested by pepsin at 15°C, suggesting it was in random coil form. It was also observed that there was a longer delay in the secretion of basement membrane collagen than in the secretion of tendon collagen and it was suggested that this longer lag might be explained by the additional time required to introduce hydroxyl groups and glycosyl groups (45, 46). The study concerning glomerular basement membrane biosynthesis at the molecular level has not been reported to date.

The final assembly of the native BM involves crosslinking of specific polypeptide chains, both disulfide bonds and aldehyde-derived crosslinks. The biosynthesis of the crosslinks in BM is considered to be similar to that of collagen and elastin because some of the crosslinks found in BM have commonly been found in collagen. Crosslinking usually involves residues of lysine or hydroxylysine, that undergo oxidative transformation to their corresponding aldehydes. These aldehyde groups react with the amino group of lysine and lysine derivatives resulting the formation of aldimine (Schiff base) or react with other molecules, i.e., δ-semialdehyde amino acid residue (57), by aldol condensation. Further transformation may occur by oxidative processes as well as reductive reactions. Recently, more complex histidine derivatives were isolated, namely aldohistidine and hystidinohydroxymerodesmosine, which were reported to be derived from a Mechael addition of the imidazole of histidine to the β-carbon of the α,β unsaturated bond of an aldol condensation product which was derived from two molecules of the δ-semialdehyde norleucine (58).

The possible biosynthetic relationships among the various crosslinks and their intermediates as shown in Fig. 3. All pathways are started with oxidation reactions followed by condensation reactions and then finally reduction reactions. These reductive reactions occurring at the crosslinkage of collagen, in vivo, have been considered to be related to the aging process of animals (59). However, it is not known whether the same kind of reductions, in vivo, take place in basement membrane, since all isolated compounds were products of reduced materials by $NaBT_4$. It is not known by what mechanism the specific subunits are organized and crosslinked.

Figure 3. The Possible Biosynthetic Pathway in Formation of Various Crosslinks in Collagen and Basement Membrane

The compounds underlined were not detected in basement membranes. (O) represents oxidative reactions and (H) represents reductive reactions.

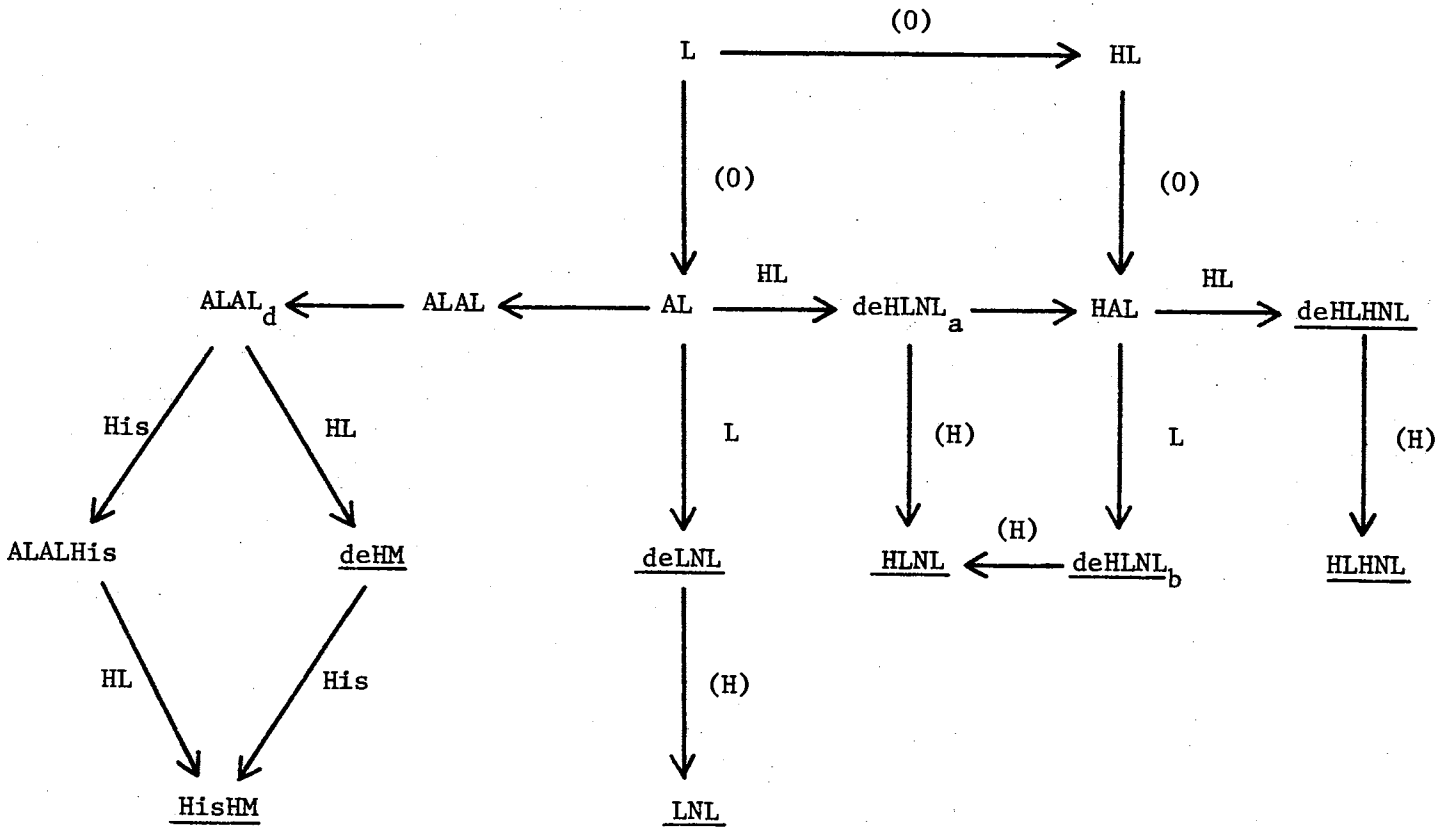

L
(0)
HL
(0)
(0)
ALAL$_d$
ALAL
AL
HL
deHLNL$_a$
HAL
HL
deHLHNL
His
HL
L
(H)
L
(H)
ALALHis
deHM
deLNL
(H)
HLNL
deHLNL$_b$
HLHNL
HL
His
(H)
HisHM
LNL

Figure 3.

CHAPTER II

II. EXPERIMENTAL PROCEDURE

A. Materials and Reagent

Guanidine HCl and sodium dodecyl sulfate (SDS) are purchased from Sigma. Acrylamide monomer, methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine were obtained from Eastman Kodak. The standard proteins were purchased from the following sources: β-galactosidase from Worthington Biochemical Corporation, ovalbumin from Sigma, bovine serum albumin from Mann Laboratory, phosphorylase A (rabbit muscle) and chymotrypsinogen from Nutritional Biochemical Corporation. Myosin was prepared by the method of Perry (60). Biogel A-15 was obtained from Bio-Rad. Dialysis casines were purchased from Union Carbide. All other chemicals used were the best available commercial grades.

The bovine kidneys were obtained from Wilson Certified Foods Company, Oklahoma City, Oklahoma, within one hour after death. The excised whole kidneys were immediately chilled in ice. To avoid contact of kidneys with water, they were placed in plastic bags and transported to the laboratory where they were cleaned and freed of most fat and unnecessary tissues. The treated kidneys were then wrapped in aluminum foil and stored at $-15^{o}C$.

B. Methods

1. Preparation of Bovine GBM

a. Isolation of Glomeruli

The isolation procedure was based on the method of Spiro (44) with a modification. The details of this procedure are also described elsewhere (40). The following procedure was performed at room temperature but all kidney preparations and solutions are kept cold in ice unless otherwise stated. The cortex of the frozen kidney was peeled off in thin strips with a potato peeler carefully avoiding to peel unwanted parts of the under layer of the medulla. The use of the peeler proved successful because the number of broken glomeruli was diminished and the yield and purity of glomeruli were improved. The shavings were collected on a glass plate and transferred to a beaker in ice. The amount of wet cortex was weighed.

The cortex was pushed through No. 120 (1-5-mesh) sieves, a small portion at a time, by applying a gentle grinding motion with the bottom of a beaker until most of the cortex became homogenized. The resulting thick liquid was then pushed through the sieve using the beaker across the entire sieve surface, with slight downward pressure, into a sieve pan. The filtered soup was scraped off the underside with a rubber spatula. This thick filtrate rendered from roughly one kilogram of cortex was diluted to 4 L with 0.85 percent NaCl solution.

A No. 70 (65-mesh) sieve was placed on top of a No. 140 (150-mesh) sieve which has a sieve pan to collect filtrate. The diluted solution was poured onto the top of the stacked sieves portion by portion. As

soon as enough glomeruli were collected on the lower sieve, No. 140, the flow of solution was stopped. It was hastened through by a light double bounce action of the sieves on a rubber mat. The filtrate in the bottom pan was discarded while the top sieve retained substantial glomeruli in the white froth. These glomeruli were filtered onto the No. 140 by 0.85% NaCl solution. The glomeruli on the No. 140 sieve were washed at least three times with the same saline to eliminate unwanted tissue fragments using the double bounce technique.

After sufficient washing, a drop of suspension was placed on a glass slide and the purity was checked by examining under a phase-contrast microscope at 100X magnification. The major impurities are capsules, tubules and other fragments and the total number of these impurities were controlled to less than 5% level by washing. The glomeruli were collected in a centrifuge tube at 2,000 x g for 5 minutes and stored at -15°C.

b. Isolation of GBM from Glomeruli

The glomeruli obtained from 15 Kg of wet cortex were dispersed in approximately 340 ml of 0.85% NaCl. A 5 ml sample was taken for yield calculation. The solution was diluted with 4 M NaCl to give a final concentration of 1 M in NaCl. A Branson Sonifier, Model S125, with 1/2 inch long probe, was used for sonic disruption.

The disruption was conducted for four one-minute intervals with maximum power output, on 75 ml portions of glomeruli suspension in a 100 ml beaker. Care was taken to minimize excess rise in temperature of the solution. Extent of glomerular disruption was followed under the phase-contrast microscope. The sonicated material was transferred to

twelve 40 ml centrifuge tubes and centrifuged for 10 minutes at 3,000 rpm on a Sorvall RC-2B. The sediment was washed five times with 1 M NaCl followed by five washings with distilled water, and then finally lyophilyzed. The 5 ml aliquot was washed five times with water and lyophilyzed. The total weight of the basement membrane was 2.52 g which represents a 30.0% yield.

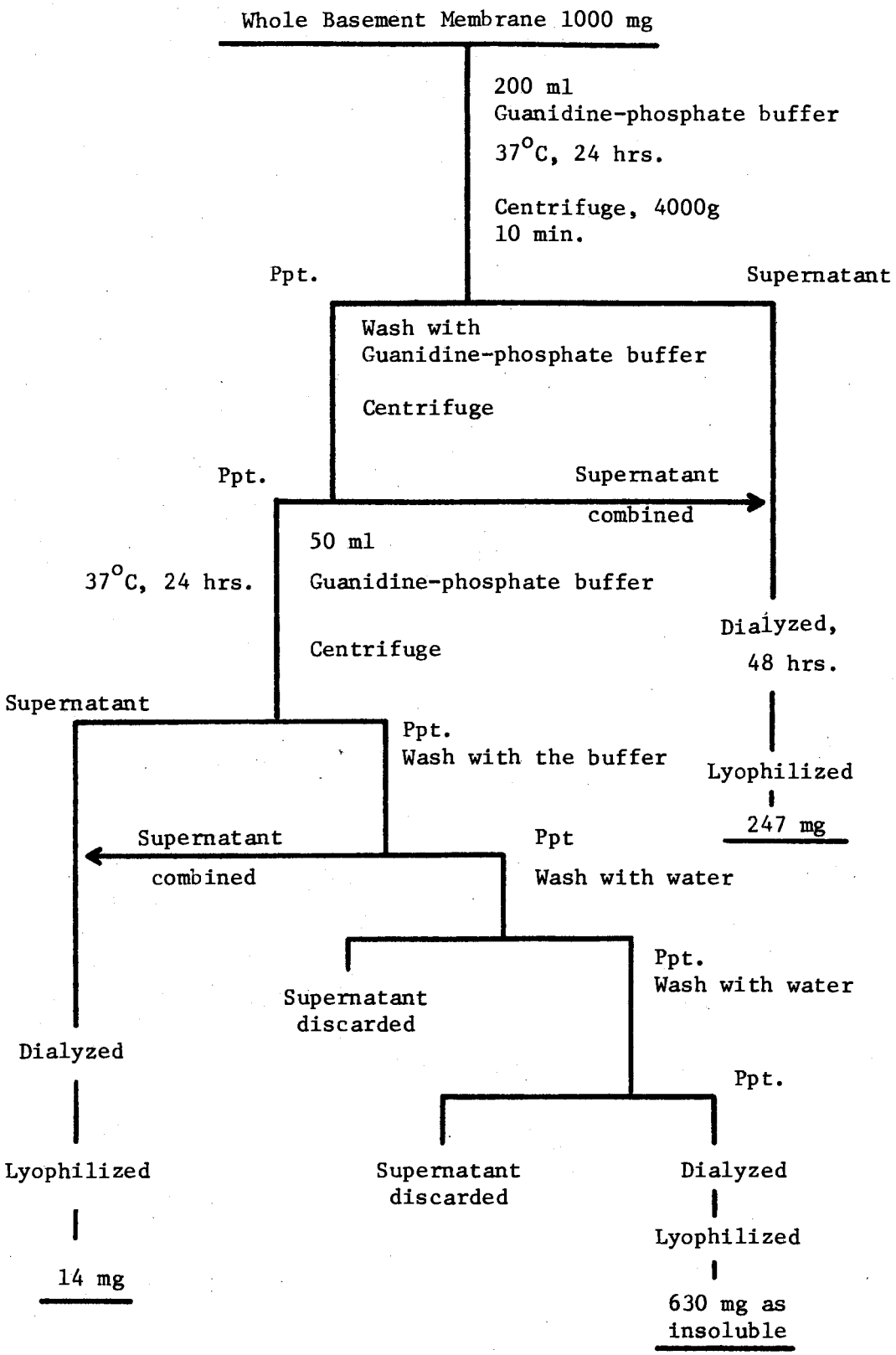
2. Preparation of Guanidine HCl Soluble and Insoluble Fractions

One thousand mg of the whole membrane were dispersed in 200 ml of 6.0 M guanidine HCl, 0.1 M sodium phosphate buffer, pH 7.0. The mixture, in a covered flask, was shaken for 24 hours in a temperature controlled room at 37°C. The soluble portion and the insoluble portion were separated by centrifugation at 4,000 x g for ten minutes.

Only the clear part of the supernatant, 150 ml, was taken out. The remaining precipitate was mixed well with guanidine-phosphate buffer (50 ml) and centrifuged again. The supernatants are combined and put into four dialysis bags (2.7 cm x 40 cm). The dialysis was carried out against 4 L of distilled water which contains 0.02% of NaN_3. The water was changed every eight hours and agitated gently by a magnetic stirrer. After 48 hours, a white precipitate was observed in each dialysis bag. The whole contents of the dialysis bags were transferred into a lyophilyzer bottle and freeze-dried.

The previously obtained precipitate was mixed with 50 ml of guanidine-phosphate buffer and treated in the same manner, as shown in Figure 4. The second crop of soluble portion and the insoluble portion

Figure 4. The Extraction Flow Chart of Whole Basement Membrane with 6 M GuHCl


Whole Basement Membrane 1000 mg
200 ml
Guanidine-phosphate buffer
37°C, 24 hrs.
Centrifuge, 4000g
10 min.
Ppt.
Supernatant
Wash with
Guanidine-phosphate buffer
Centrifuge
Ppt.
Supernatant
combined
50 ml
37°C, 24 hrs.
Guanidine-phosphate buffer
Centrifuge
Dialyzed,
48 hrs.
Supernatant
Ppt.
Wash with the buffer
Lyophilized
247 mg
Supernatant
combined
Ppt
Wash with water
Ppt.
Wash with water
Supernatant
discarded
Dialyzed
Ppt.
Lyophilized
Supernatant
discarded
Dialyzed
Lyophilized
14 mg
630 mg as
insoluble

are consequently obtained. The total yield of the soluble fraction was 261 mg and the insoluble fraction was 630 mg.

3. Gel Electrophoresis

a. SDS System

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described by Weber and Osbon (61) and Hudson and Spiro (16). A stock acrylamide solution was prepared by dissolving 20 g of acrylamide, 0.54 g of N,N'-methylenebisacrylamide in 100 ml of 0.1% SDS, 0.1 M phosphate buffer, pH 7.0. The stock solution of TEMED was prepared by dissolving 0.4 ml of N,N,N',N',-tetramethylethylenediamine in 100 ml of same SDS-phosphate buffer. To prepare twelve 5% gels, 6.0 ml of acrylamide stock solution, and 3.0 ml of TEMED stock solution were mixed in 15.0 ml of 0.1% SDS-0.1 M phosphate buffer, pH 7.0. Eighteen mg of a fresh ammonium persulfate solution was added to the mixture and deaerated for a few seconds after all the ammonium persulfate was dissolved. Clean gel tubes (5 mm x 110 mm), which were coated in advance with column coat purchased from Canalco, were filled with gel mixture with a transfer pipette within ten minutes of mixing. A 3 mm layer of water was immediately placed on top of the gel surfaces to eliminate the meniscus. The gels were usable within four hours, but better results were obtained by leaving gels at room temperature overnight. The water layer was discarded before gel tubes were set in the electrophoresis apparatus. The bottom of the gels were placed at least 3 cm deep in 0.1% SDS-0.1 M phosphate buffer. Bubble formation can be avoided by rotation of the inside apparatus. The top of the gels were

covered by same SDS-phosphate buffer and mixed well inside of the gel tubes to eliminate the remaining distilled water which causes major problems in variation of voltage at early stage of electrophoresis. Pre-electrophoresis for one hour without loading sample eliminated these troubles including the variation of buffer temperature. The sample solution contained, typically, 0.25 to 0.5 mg protein in 0.50 ml of 1% SDS, 0.1 M phosphate, pH 7.0, 15% glycerol, 1% mercaptoethanol, if necessary. The incubation was carried out at 37°C for at least three hours. The size of the sample loaded varied depending on the dye sensitivity, number of components and purpose of gels. Typically, 5-10μ g for one component gives a dark enough band for taking a picture. For photoscanning, the amount of sample should be 1-5μ g per component. The total volume should not exceed more than 200μ 1 without using a discontinuous system, but it should be more than 20μ 1 to ensure a sample solution evenly spread over the gel surface. Usually 25, 50 and 100μ 1 of membrane sample (1 mg/ml) were loaded by constriction pipettes.

Total current for twelve tubes was 96 mA and voltage was 60 V after power drift was stabilized. The duration of electrophoresis was followed by tracking dye (50μℓ of 0.05% bromophenol blue), because it is a function of temperature and gel concentration. Normally, the migration speed was three hours and half per 100 mm of 5% gel at 25°C room temperature.

Fixing and staining were performed according to the method of Fairbank et al. (62). For routine works, No. 2 staining solution was omitted from the procedure to accelerate overall destaining time. This modification is satisfactory for most of the cases except for taking a picture of gels.

When the tracking dye reached the bottom of the gel tubes, the gels were taken out and put in 70 ml of a solution containing 25% isopropanol, 0.025% coomassie blue, and 10% acetic acid, for 12 hours. The gels were transferred to the second staining solution containing 0.0025% coomassie blue in 10% acetic acid for 12 hours. The gels were destained by a charcoal diffusion method and in 10% acetic acid solution.

b. Tris-Glycine System

Gel electrophoresis without SDS in the system was necessary to eliminate the possibility of the presence of size and charge isomer families as described by Hedrick and Smith (63). The system was essentially the same as that reported by Ornstein and Davis (64), but modified by adding glycine to the separating gels and changing the catalizer to ammonium persulfate instead of riboflavin. Each solution was prepared as follows: (1) buffer solution: 3 g Tris, 14.4 g glycine, dilute to 1 L with H_2O, and pH was adjusted to 9.6, (2) AcAm. solution (20%): 20 g AcAm., 0.54 g Bis, and 7.2 g glycine dissolved in 100 ml of buffer solution, and pH was adjusted to 9.6, (3) TEMED solution: 0.46 ml of TEMED dissolved in 100 ml of buffer solution.

The elctrophoresis was done with and without a stacking gel and there was no observable difference in the band widths of membrane components and reference molecules such as BSA. The discontinuous pH between separating gel, stacking gel, or buffer solution produced broad smears. The reason was not known. To prepare twelve 5% gels, AcAm. solution, 6 ml, TEMED solution, 3 ml, and buffer solution, 15 ml, were mixed and then persulfate, 20 mg, was dissolved and the solution was

quickly de-aerated. The solution was transferred into pre-coated gel tubes to same height, and distilled water was layered on the surface. The total procedure, after the ammonium persulfate was added, should be less than ten minutes.

The sample concentration was typically 1 mg per ml in the same buffer soltuion containing 15-20% glycerol. A volume of 30μ l of sample was loaded to each tube. The electrophoresis time was three hours at 96 mA and 130 V for twelve tubes. The staining and destaining procedure was identical to the SDS system.

4. Gel Scanning Method

A UV spectrophotometer, Beckman DU which is equipped with a Gilford Gel Scanning System and Recorder 2,000, was used for this study. The wave length used for Coomassie Blue stained Gel was 550 nm. The slit opening and vernier scale knob were adjusted, with full scale ratio at 0.25 so that absorbance unit, 1.0, is equivalent to the full scale of the recorder chart. The recorder chart speed was eight inches per minute and scanning speed was two cm per minute.

The lower end of the gels were cut in order to place the gels without any strain in a 10 cm cell. The cell was filled with 7% acetic acid and the bubbles were eliminated by using thin plastic tubes.

5. Protein Determination Methods

The method of Lowry et al. (65) was used for most protein determination. When the protein solution contains mercaptoethanol, which produces a dark color with phenol reagent, the method of Geiger et al. (66) was used with some modification. Hydrogenperoxide was added to the sample

solution before the addition of alkaline copper mixture so that complete oxidation of sulfhydryl group is possible. The oxidized mercaptoethanol also produces some color with phenol reagent, but much less than the color intensity which was produced by oxidation of mercaptoethanol-alkaline copper complex. This slight modification enabled measurement of the amount of protein in the presence of more than 25 mM of mercaptoethanol so that extensive dilution of protein solution could be avoided. The standard curve was made as follows: to test tubes (16 x 150 mm) containing 100µ l of 1% SDS, 0.1 M phosphate buffer, 1% mercaptoethanol, various amounts of BSA (up to 200 mg), and water were added to make 1.0 ml. Then, 0.20 ml of 0.3% H_2O_2 was mixed well and heated at 50°C for ten minutes. Alkaline copper reagent, 1 ml, was mixed and heated at 50°C for ten minutes. Finally two ml of phenol reagent was added and allowed to stand for one hour at room temperature before reading the absorbence at 700 nm.

6. Gel Filtration Chromatography of GuHCl Soluble GBM

Bio-Gel A-15 m (200-400 mesh) was packed to a height of 140 cm in a glass column (2.0 x 150 cm) fitted with a 400-mesh nylon filter. The column was equilibrated with 1% SDS, 0.01 M Tris-HCl buffer, pH 7.0, 0.02% sodium azide under a hydrostatic pressure of 30 cm. Prior to use the Bio-Gel was washed with several volumes of the equilibrating buffer system on a sintered glass funnel to remove soluble carbohydrate material, as monitored by the anthrone reaction.

The 6.0 M GuHCl soluble GBM fraction (120 mg), prepared as previously described, was dissolved in 5 ml of 5% SDS, 0.01 M Tris-HCl

buffer, pH 7.0, which contained 0.02% sodium azide as preservative. The mixture was incubated for one hour at 37°C with shaking and then cleared of cloudiness by centrifugation at 4,000 x g for 10 minutes. The sediment was washed with buffer and the wash and the supernatant combined. The total weight of the sediment was less than 3 mg which represents less than 3% of the GBM fraction. The solubilized material (6.7 ml containing 113 mg of GBM) was applied to the column and eluted with the equilibrating buffer under a hydrostatic pressure of 30 cm which permitted a flow rate of 18 ml per hour. Fractions of 6.0 ml were collected and stored immediately in a cold room until further analyzed. The protein content of the fractions was determined by the Lowry method (65). Aliquots of the fractions were subjected to SDS polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol as described below. Fractions which showed bands by the SDS electrophoretic procedure were pooled (Fractions I to VI). These pooled fractions were further subjected to SDS polyacrylamide gel electrophoresis in the presence and absnece of 2-mercaptoethanol. The fractions were finally dialyzed exhaustively anainst distilled water at 4°C, lyophilized and stored at -20°C.

7. Purification of GBM Component VII

Fraction III, 28 mg from the previous chromatographic step, was incubated for 12 hours at 37°C in 1 ml of 1% SDS, Tris-HCl buffer, pH 7.0, 0.02% sodium azide which contained 50μ 1 of 2-mercaptoehtanol. The reduced material was then applied to a small column (1 cm x 90 cm) packed to a height of 80 cm with Bio-Gel A-15m (200-400 mesh). The flow rate was maintained at 3.9 ml per hour under a hydrostatic pressure

of 50 cm. Fractions of 1 ml were collected and aliquots analyzed for protein by the Lowry method (65) and subjected to SDS-polyacrylamide electrophoresis. The fractions which contained only component VII were pooled, dialyzed exhaustively against 40% methanol in water, at room temperature, and finally against distilled water at 4°C, then lyophilized. The total amount of component VII obtained was 11 mg.

8. Ultracentrifugation

All experiments of analytical ultracentrifugation were done by Patricio Riquetti, using a Spinco Model E Ultracentrifuge equipped with Schlieren and Raleigh interference optics and a temperature control unit.

The molecular weight of the membrane component in 6 M guanidine hydrochloride in the presence of 1% 2-mercaptoethanol was obtained by sedimentation equilibrium analysis utilizing the high-speed method of Yphantis (67). The component was dissolved in 6 M guanidine hydrochloride 1% 2-mercaptoethanol with a final pH of 6.5 and dialyzed against the same solvent. The final dialysate was used as the reference solution. Ultracentrifugation was performed at 15-20°C with a 12 mm double sector cell equipped with sapphire windows using 3 mm column heights. Fluorocarbon FC-32 (0.02 ml, Beckman Instruments) was added to the sample sector to provide a flat, transparent cell bottom. Rotor speeds were selected so that the meniscus concentration would be essentially zero at equilibrium. Runs were allowed to proceed until there was no further increase in fringe displacement with time. Following each experiment, a water blank was run without disassembling the cell to correct for cell window distortions. Interference patterns were measured in a Nikon two-dimensional mircocomparator. The blank was

evaluated in the same way to obtain the results of an average fringe. The natural logarithms of the differences between the blank-corrected fringe displacements and the base line (ln X) was plotted against the corresponding radial positions in the cell (r^2). The molecular weight (M.W) was calculated from the equation:

$$M.W = \frac{2RT}{\omega^2 (1-\phi'\rho)} \cdot \frac{d \ln X}{dr^2}$$

in which ϕ' is the effective partial specific volume of the protein, ω is the angular velocity of the rotor, and ρ is the solvent density. The partial specific volume ($\bar{v}$) of the component was calculated as 0.714 from its amino acid composition. In dilute aqueous solutions preferential interactions between solvent and the protein are minimal and thus, $\bar{v}$ can be used in place of ϕ' in the equation. However, the choice of ϕ' for a protein in 6 M guanidine hydrochloride is somewhat uncertain. Hade and Tanford (68) and Castellino and Barker (69) have shown that ϕ' for several proteins may be decreased by as much as 0.01 cm^3/g due to preferential interactions between guanidine hydrochloride and the protein. However, for most proteins examined in guanidine hydrochloride $\bar{v}$ could be used for ϕ' without serious error and we have done this in our calculations.

Sedimentation velocity experiments were run at 59,780 rpm at 20°C with single or double sector 12 mm cells assembled with sapphire windows. Photographs were taken with a phase angle of 60° at eight minute intervals. No corrections to standard condition were made for the sedimentation coefficient values.

9. Gel Filtration Chromtography for M.W Determination

The molecular weight of component VII was determined according to the method of Fish et al. (70) using a Sepharose 4 B column equilibrated and operated in 6 M GuHCl. The component VII and standard proteins were reduced with excess 2-mercaptoethanol and alkylated with iodoacetic acid. The component VII was labelled with either a radioactive or fluorescence compound.

The radioactive method was employed by Schwartz (40) incubating samples with 30μ c ^{3}H-adetic anhydride per 5 mg protein. The mixture was shaken for one hour at room temperature and then dialyzed extensively against 6.0 M GuHCl.

The fluorescein method was employed by Patricio Riquetti. The alkylated sample was incubated with fluorescein isothiocyanate on Celite in GuHCl solution containing 1% $NaHCO_3$ for one hour at room temperature. Excess reagent was destroyed by addition of glycine (71). Elution positions were monitored by absorbance at 280 nm when it is possible, cytochrome C was monitored at 410 nm, Blue dextran at 630 nm, and DNP-alanine at 360 nm. When proteins were ^{3}H-labelled, radioactivity was counted according to a method by Castellino (72). When proteins were labelled with fluorescein, column effluent was monitored with a fluorescence spectrometer. The excitation wavelength was 493.5 nm and fluorescence was measured at 530 nm. The calibration curve was obtained by plotting known molecular weight of each protein vs observed Kd.

The molecular weight of the membrane component was also determined by gel filtration chromatography using SDS as the protein denaturant. Standard proteins and the membrane component were prepared for

chromatography under identical conditions as those used for SDS gel electrophoresis described above. Samples were applied to a 0.6 x 110 cm column packed with Sepharose 6 B in 0.1 M phosphate buffer (pH 7.0) containing 0.1% SDS and 0.02% sodium azide. The column was eluted with the same buffer at a flow rate of 3.2 ml per hour and 1 ml fractions were collected. The fractions were monitored for protein by absorbance measurement at 280 nm.

10. Circular Dichroism

The CD spectra were obtained with a Cary Model 61 Spectrometer using quartz cell of 1 mm pathlength. The concentration of the component VII was 5.6×10^{-5} g/ml in 6 M GuHCl and 5.43×10^{-5} g/ml in 1% SDS, 0.1 M phosphate buffer, pH 7.0. Myoglobin, 1.54^{-4} g/ml in 1% SDS was used for comparison. The proteins were incubated with 1% mercaptoethanol at 37°C for three hours, followed by exhaustive dialysis against an appropriate buffer minus mercaptoethanol.

11. Quantitative Analysis of Component VII

a. Amino Acid Analysis

Amino acid analyses were determined by hydrolyzing samples with HCl in a sealed evacuated tube with nitrogen for period of 24, 48, and 72 hours at 110°c. The hydrolysates were analyzed on a Beckman Model 120C automatic amino acid analyzer by the procedure of Guire et al. (73). The content of tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (74). The molar ratio between tryptophan and tyrosine was estimated from s value plotted from the

reported observations. The content of methionine sulfoxide was analysed from the alkaline hydrolysate by method of Odell et al. (75), which is also used for hydroxylysine glycoside analysis described in the following section.

b. Quantitative Analysis of Sugar

The galactose, glucose, mannose, and fucose were released by hydrolysis with 2 N sulfuric acid for four hours at 1000°C in sealed tubes. The hydrolysates were passed through a column of Dowex 50 (H+), 200-400 mesh, and Dowex 1 ($HCOO^-$); 200-400 mesh, as described by Spiro (44). The mexosamines were hydrolyzed in 4 n HCl at 100°C for six hours, then determined on the short column of the amino acid analyzer after elution from the Dowex 50 with 2 N HCl (15). Sialic acid was determined by the thiobarbituric assay method of Warren (76) after hydrolysis of glycoprotein with 0.2 ml of 0.1 N H_2SO_4 at 80°C for one hour.

c. Analysis of Hydroxylysine Glycoside

Hydroxylysine glycosides, Glc-Gal-Hyl and Gal-Hyl were analyzed on the amino acid analyzer by the accelerated method of Odell et al. (75). The component VII, 1.50 mg, was placed in alkaline resistant plastic tubes and 0.20 ml of 2 N NaOH was added. The small plastic tube was put inside of a large test tube and evacuated with N_2 gas. The sample was hydrolyzed at 100°C for 24 hours, then neutralyzed with 2 N HCl. The sample was diluted with 0.5 ml of the first buffer and the whole solution was introduced into analyzer.

d. UV Spectrum Analysis

The UV spectrum was obtained with a Varian Spectrophotometer 635 model. In alkaline condition, 0.3 mg of the component was dissolved in 1.0 ml of 0.1 M NaOH. In neutral condition, 6.0 M guanidine-0.1 M phosphate buffer, pH 7.0, was used.

CHAPTER III

RESULT

1. The Extraction of Whole Basement Membrane with GuHCl

The extraction by 6 M GuHCl buffer was almost completed within 24 hours. The amount of the first crop was 247 mg which was 95% of the extractable amount. The second crop was only 14 mg (5%) and this portion may have resulted from the incomplete washings of the insoluble portion in the previous stage because it was difficult to wash the precipitate with a limited volume. The first and second crops were compared by SDS gel electrophoresis and no difference was found. The solubility in 6 M GuHCl, 26%, is very close to the value estimated by protein estimation on supernatant of 5% SDS phosphate buffer (25%) and 8 M urea buffer (27%) as previously reported (16). The insoluble portion was washed by water repeatedly as shown in Figure 4. The yield of insoluble portion was 630 mg, which was considerably lower than the expected value since the total combined weight was 891 mg. The loss of approximately 11% is partly attributed to the fact that the insoluble portion in the high density solution of 6 M GuHCl is a thick jelly-like mixture and complete transfer of this sticky solution from each equipment was extremely difficult. The one possibility may exist that the extract procedure may produce free small molecules which are originally attached to large molecules and these may be lost at dialysis stage.

The water content before extraction was not checked although whole membrane was kept in a desiccator. If this is the case, the soluble fraction is a maximum of 29%.

2. Subunit Composition of GuHCL Soluble and GuHCl Insoluble Fractions

Substantial knowledge of the subunit composition can be obtained from SDS gel electrophoresis since SDS gel electrophoresis primarily separates each component by its molecular weight. The picture of gel electrophoresis is shown in Figure 5. GuHCl soluble portion, gel A, is shown to contain all ranges of components and some large molecular weight components, which stays at the top of the gel. On reduction with mercaptoethanol this large molecular weight component disappears, gel B. The reduced insoluble fraction gives several bands at only the upper part of gel C, which indicates that the insoluble fraction produces only large molecular weight components. The reduced whole membrane, gel D, shows naturally the combined result of gels B and C. The insoluble fraction without reduction does not show any band in gel E, which indicates it is not soluble in the SDS buffer system.

The gel photo scanning pattern of reduced whole membrane, GuHCl soluble and insoluble materials are shown in Figure 6. The whole membrane (Figure 6, A) consists of at least thirteen components ranging in molecular weight from 350,000 to 24,000 daltons. The molecular weight of each component as measured by SDS gel electrophoresis is present in Table IV. The reduced GuHCl soluble fraction consists of all the components present in the whole basement membrane except component I.

Figure 5. The SDS Gel Electrophoresis Pattern of Basement Membrane Components

A: Guanidine Extract without Reduction
B: Guanidine Extract Reduced by Mercaptoethanol
C: Insoluble Portion with Reduction
D: Whole Membrane Reduced by Mercaptoethanol
E: Insoluble Portion without Reduction

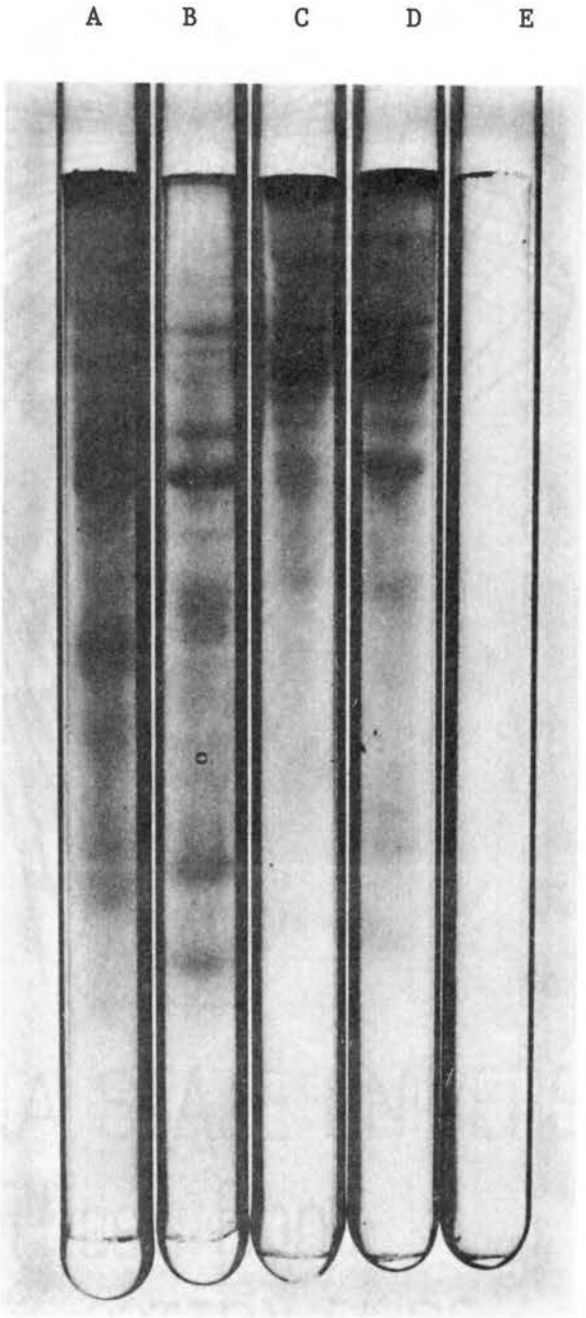


Figure 5.

Figure 6. The Comparision of Photometric Scanning Patterns for the SDS Gel Electrophoresis Reduced Materials

A: Whole Membrane
B: Guanidine Soluble Materials
This is identical to Figure B except reduction of scale to compare other materials.
C: Guanidine Insoluble Materials

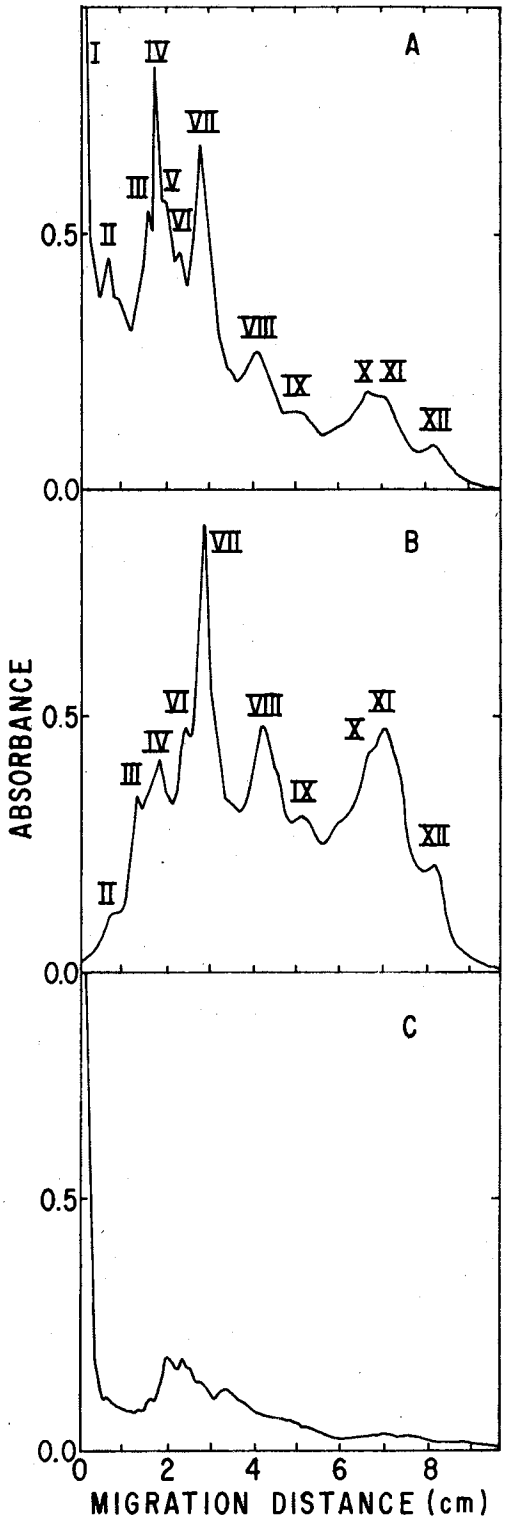

A
B
C
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
0.5
0.0
ABSORBANCE
0
2
4
6
8
MIGRATION DISTANCE (cm)

TABLE IV

MOLECULAR WEIGHTS OF BOVINE GLOMERULAR BASEMENT MEMBRANE COMPONENTS

Component number	Molecular weight [a]
I	350,000
II	260,000
III	225,000
IV	195,000
V	185,000
VI	144,000
VII	140,000
VIII	105,000
IX	79,000
X	59,000
XI	44,000
XII	24,000

[a] Determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The estimation was made under the same condition as shown in Fig 14 using 5% gel.

The major quantitative difference between Figure 6, A and Figure 6, B is relative height of peaks III, IV, V and XI.

3. Estimation of Subunit Crosslink by Intermolecular Disulfide Bond

a. Subunit Composition with and without Mercaptoethanol Reduction

The subunits, which are crosslinked by disulfide bonds, are easily reduced by excess mercaptoethanol in the incubation mixture. The reduced materials are examined by SDS gel electrophoresis and individual peaks were identified. The GuHCl soluble fraction was compared by gel electrophoresis between before and after mercaptoethanol reduction (Figure 7). The reduction of the GuHCl soluble portion changed most of the components except peak VII. The top part of the gel, peak A, completely vanished as shown in the picture and its scanning pattern. The peak E decreased substantially to become a minor component VI, resulted in outstanding protein band of VII. The new components VIII and XI appeared as rather strong bands. These smaller molecules were also observed in the reduced whole membrane. This comparison indicates that some subunits are crosslinked by disulfide bond, however, which of the individual subunit that are crosslinked remain to be determined. Some peaks such as C and IV or F and VII are believed to be identical judging from the migration distances.

b. Fractionation of Components by Gel Filtration Chromatography and Subunit Analysis of Fraction

In an attempt to determine which subunits are crosslinked by disulfide bonds to form higher molecular weight aggregates, the GuHCl soluble portion was fractionated on a Bio-Gel A-15 column, and the fractions then submitted to subunit analysis. The fractionation profile is shown in Figure 8. The elution pattern based on Lowry protein measurement showed a relatively smooth curve. It was thought at the beginning that the separation by this method was incomplete. However, when the effluent of several test tubes were examined by SDS gel electrophoresis, it was found that remarkable separation was observed. The content of all test tubes were examined by SDS gel electrophoresis, loading 50μ 1 or 100μ 1 of each sample onto each gel tube. The relative concentration of individual peaks observed in the gels were compared and similar fractions were pooled as shown at the bottom of Figure 8. The test tubes which contained two groups of proteins at almost equal ratio were two or three tubes, and the total protein in these test tubes were negligible amount because these were located at borderline where two groups of protein content were usually low.

The pooled fraction was again examined by SDS gel electrophoresis with and without mercaptoethanol in incubation mixture before electrophoresis. The result of the staining pattern of each gels were recorded by a gel photo scanner. The scanning pattern of the pooled fraction from I to V are shown in Figure 9. The left column shows the fraction before mercaptoehtanol reduction and the right column shows the fraction

Figure 7. The Photometric Scanning Patterns of the GuHCl Soluble and the Reduced Soluble Fractions

A: Guanidine Extract
B: Guanidine Extract Reduced by Mercaptoethanol

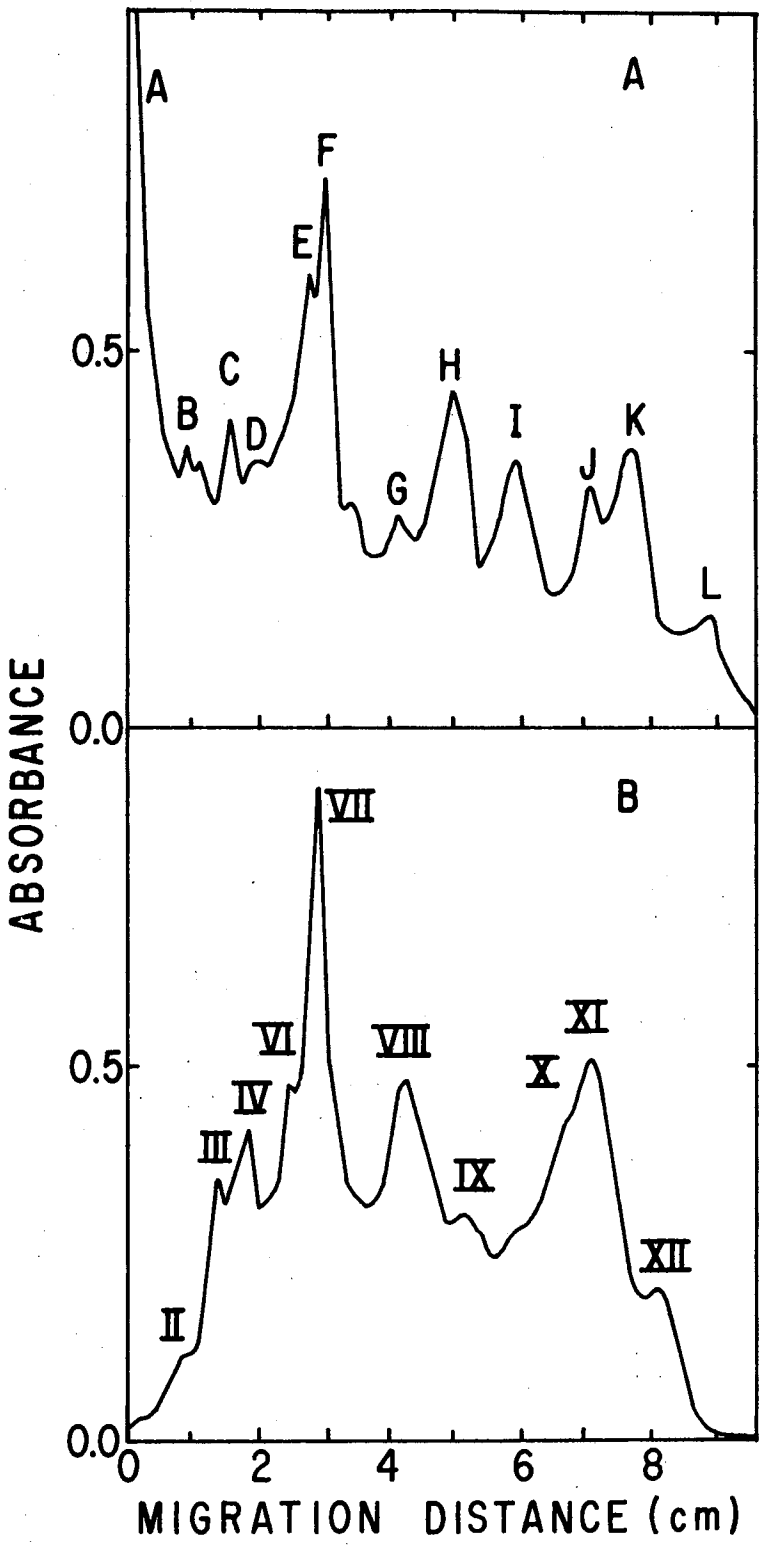
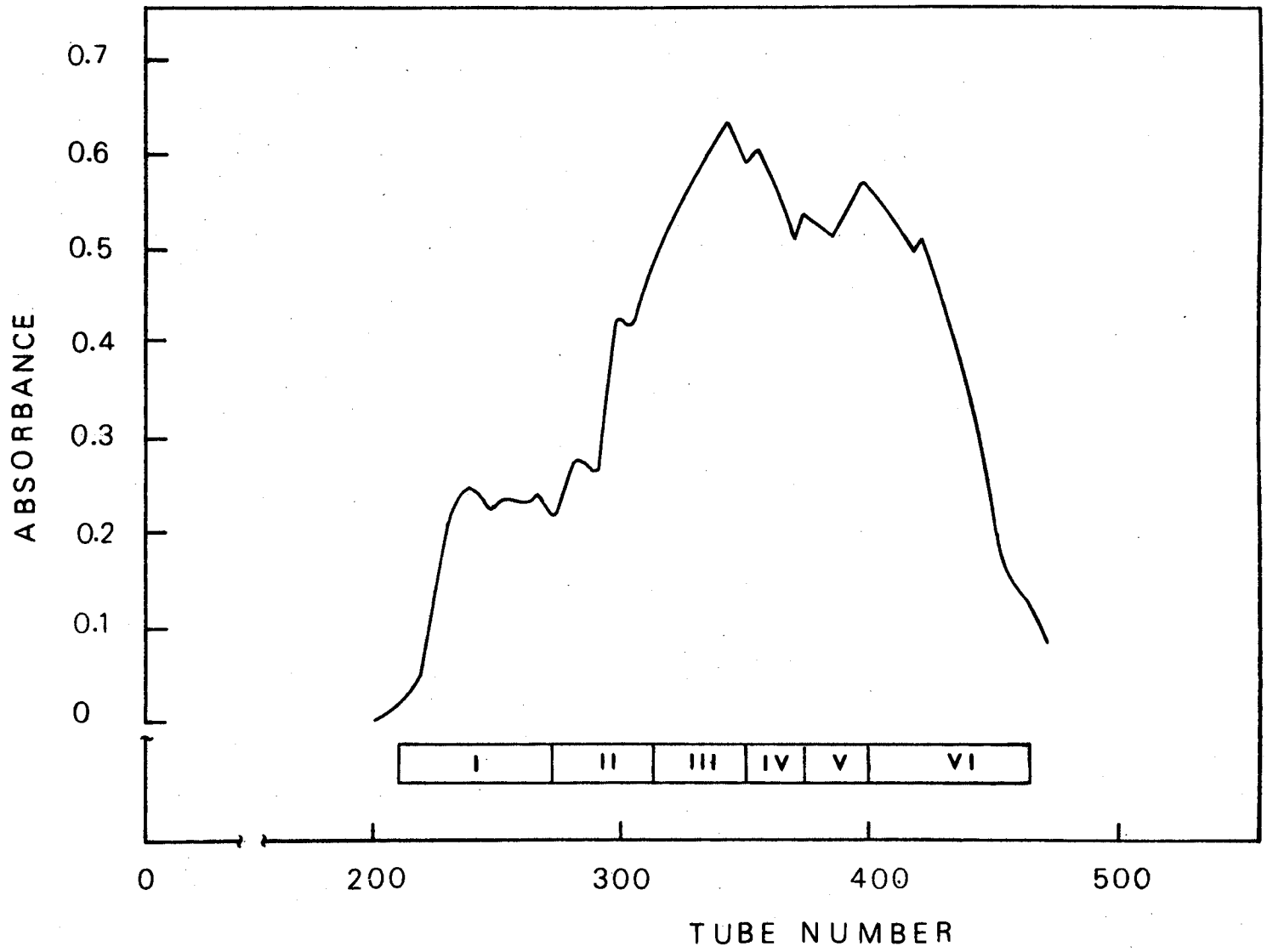

A
A
F
E
C
H
B
D
I
K
J
G
L
0.5
0.0
ABSORBANCE
B
VII
VI
VIII
XI
IV
X
III
IX
II
XII
0.5
0.0
0
2
4
6
8
MIGRATION DISTANCE (cm)

Figure 8. The Elution Pattern of the Soluble Fraction from Bio-Gel A-15m, (First Gel Filtration Column Chromatography)

Numbered areas designate tubes which were pooled for further study.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
I
II
III
IV
V
VI
0
200
300
400
500
TUBE NUMBER

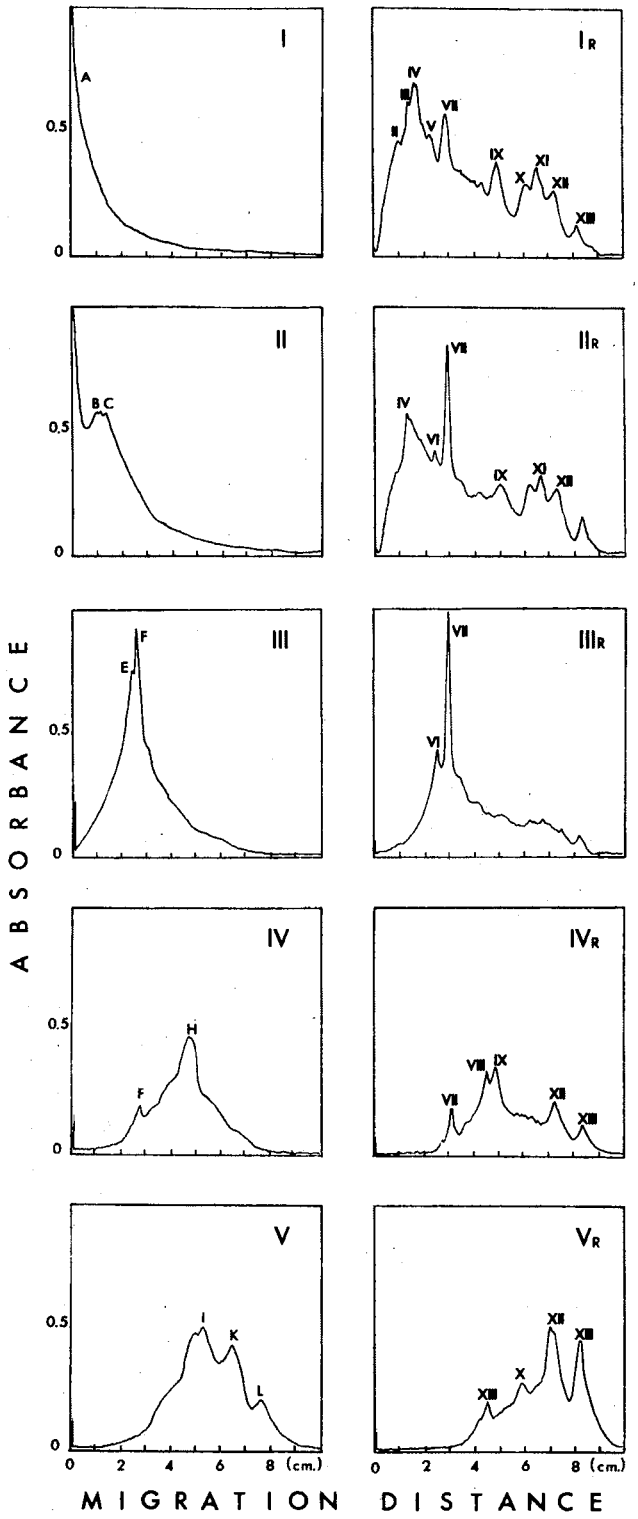
after reduction (R stands for reduction). (The pooled fraction VI was not shown because the result of gel electrophoresis under the same condition did not show meaningful protein band staining, and the application of large amounts of pooled fraction VI on 10% gel, 300μ 1 instead of 50μ 1, gave some smear similar to the pooled fraction V.) It was concluded that the molecular weight of these components were smaller than chymotrypsinogen (M.W 26,000) and these components were difficult to identify under these conditions. The pooled fractions without reduction in gel electrophoresis showed a relatively simple pattern. The reduction of each fraction gave very complicated pattern as shown in Figure 9.

Fraction I contained only a large molecular weight component (A) whose molecular weight was estimated more than 350,000 daltons by extrapolation from SDS gel electrophoresis molecular weight determination method. Some tailing was observed, but there was no clear band penetrated inside of the gel. The reduction of I exhibited impressive changes in its gel pattern. Component A disappeared and at least 13 or more components appeared in the gel. Some of the minor band cannot be identified because the scanning of a round gel has several problems by the available photoscanner. Nevertheless, it was possible to correlate each component qualitatively. There are several distinguished peaks in the reduced I. The highest peak IV was observed with several other minor components around IV. The peak VII was clearly separated from others and readily noticeable. The other bands, smaller molecules than IX, are characteristic which are also observed in other reduced fractions.

Fraction II contains a few components which are clearly separated from the large molecule which could not penetrate the 5% gel. By

Figure 9. Photometric Scanning Patterns from the SDS Gel Electrophoresis of the Pooled Fractions and their Reduced Fractions

Each number corresponds to fraction number of Figure 8.
R stands for "reduced".


I
A
0.5
0
IR
II
IV
III
V
VII
IX
X
XI
XII
XIII
IIR
B C
VI
IIIR
E
F
IVR
H
VIII
VR
I
K
L
ABSORBANCE
0 2 4 6 8 (cm.)
MIGRATION DISTANCE

photoscanning only two peaks were observed, but these peaks were so closely located that three sharp protein bands could not be resolved by this method. The pattern was simple and no small molecular weight component was observed which is somewhat similar to I. The reduction of II (II_R) gave a variety of new peaks which are commonly found in I_R although the relative height of each peak was different. Peak VII was the highest peak in II_R and the major difference between I_R and II_R appeared to be the relative amount of peak VII content. The other difference observed between I_R and II_R was the quantitative amount of peaks from II to V although these were not clearly visible by the scanning pattern. Peak C in II and peak IV in II_R seemed to be identical from mobility measurement observation.

Fraction III contained mostly peak F and the closely located peak E and was practically free from other components. The reduction of II indicated that peak E decreased considerably and became a minor component. Peak F which was believed to be identical to peak VII did not change in its relative amount. The minor component, which appeared as a shoulder of peak F, was also decreased. The several smaller molecular weight components which were believed to be peaks VIII to XIII were also observed in I_R and II_R, although the identification of individual peaks were rather difficult. These observations led us to isolate this component VII in pure form in order to study the chemical and physical properties.

Fraction IV showed rather simple scanning patterns because each protein band was not as sharp as the higher molecular weight components. Therefore, overlapping neighboring protein bands made a rather smooth curve after scanning, but the actual protein bands were more complicated

and the individual identification was rather difficult. Peak F was observed in small quantities as was expected due to the incomplete separation of column chromatography. The major components were three borad protein bands, and included H. The reduction of IV gave destruction of these broad bands and yielded several peaks which were rather easily identified. Peak F was not changed by the reduction and became easily recognizable from other components.

Fraction V looked more complicated than IV due to smaller molecular weight components. Peaks I, K, and L and other minor components were overlapped in this fraction and precise identification was rather difficult as previously mentioned in case of IV due to lack of sharpness of each protein band. The same problems were also encountered after reduction, but several peaks were easily recognized. Unlike other fractions, peak IX was not observed in V_R, but peaks XII and XIII were present in rather large amounts relative to other fractions.

The following conclusions could be deduced by the overall observations from these experiments:

(1) The GuHCl soluble fraction consists of relatively large molecular weight components.

(2) The large molecular weight components are held together by non-covalent bonds because these were fractionated.

(3) The large component such as peak A and peak B were completely disintegrated by mercaptoethanol reduction.

(4) The mercaptoethanol reduction of these peaks A and B produced all ranges of molecular weight components and did not produce intermediate products alone, such as peaks IV and VII.

(5) Some large molecular weight components such as peaks C and F were stable to mercaptoethanol reduction, or at least the molecular weight of C and F did not change.

(6) The products that resulted from the reduction of the larger components were not random in molecular weight. Some of the products such as XI, XII, and XIII were always observed after the reduction even if the initial components were not largest in molecular size such as the peaks H, I, and K. This means that peaks XI, XII, and XIII may be the mercaptoethanol reducible smallest size of the membrane.

(7) The intermediate size components such as peaks H and K showed rather broad bands and the reduction of these peaks gave sharper bands. The positions of peak XII is located at the bottom end of gel where protein bands have tendency to become wider than at the position of peaks H and K.

(8) It is possible that the GuHCl soluble fraction contains smaller protein components (~10,000 M.W), but large enough to retain in a dialysis bag. These proteins can be detected by Lowry protein determination, but not by gel electrophoresis because the gel concentration, electrophoresis duration, fixing of protein to avoid diffusion of small molecules, and staining condition are not designed for small molecular weight protein. This may explain why VI cannot be detected by gel electrophoresis. However, the amount of VI is fortunately not large by weight basis.

4. Isolation and Purification of Component VII from III_R

Since III contained only a few components and was rich in component F, it was natural to give attention to this component. It was known that the amoutn of peak E was decreased considerably by the reduction, but peak VII, which seemed to be identical to peak F, was not changed by mercaptoethanol. Furthermore, a very thin protein band, which was recorded as a shoulder of peak F in III, was decreased considerably. Several smaller components were produced after the reduction, but it did not cause any problem to purify component VII since the molecular weight of these smaller molecules were totally different from component VII. The result of rechromatography by using a high L:D ration column was followed by the Lowry protein determination method (Figure 10). The elution pattern indicated the presence of impurities before and after the major peak. The fractions of all test tubes were examined by SDS gel electrophoresis. The fractions which contained only the component VII were pooled. The pooled component VII was compared with III_R by SDS gel electrophoresis in Figure 11. The top figure, A, was III_R which contained VI, IX, XI, XII, and XIII in a small quantity. The bottom, B, was a scanning pattern of the purified component VII which was free of any impurity. The purity of the pooled component VII was checked by several different methods.

(1) The fluorescein labelled component VII was eluted as a single peak from gel filtration column chromatography when the molecular weight was determined.

(2) Component VII showed a single symmetrical peak in Schlieren optical system when sedimentation velocity experiment was done.

Figure 10. The Elution Pattern of Component VII from the Second Gel Filtration Column Chromatography

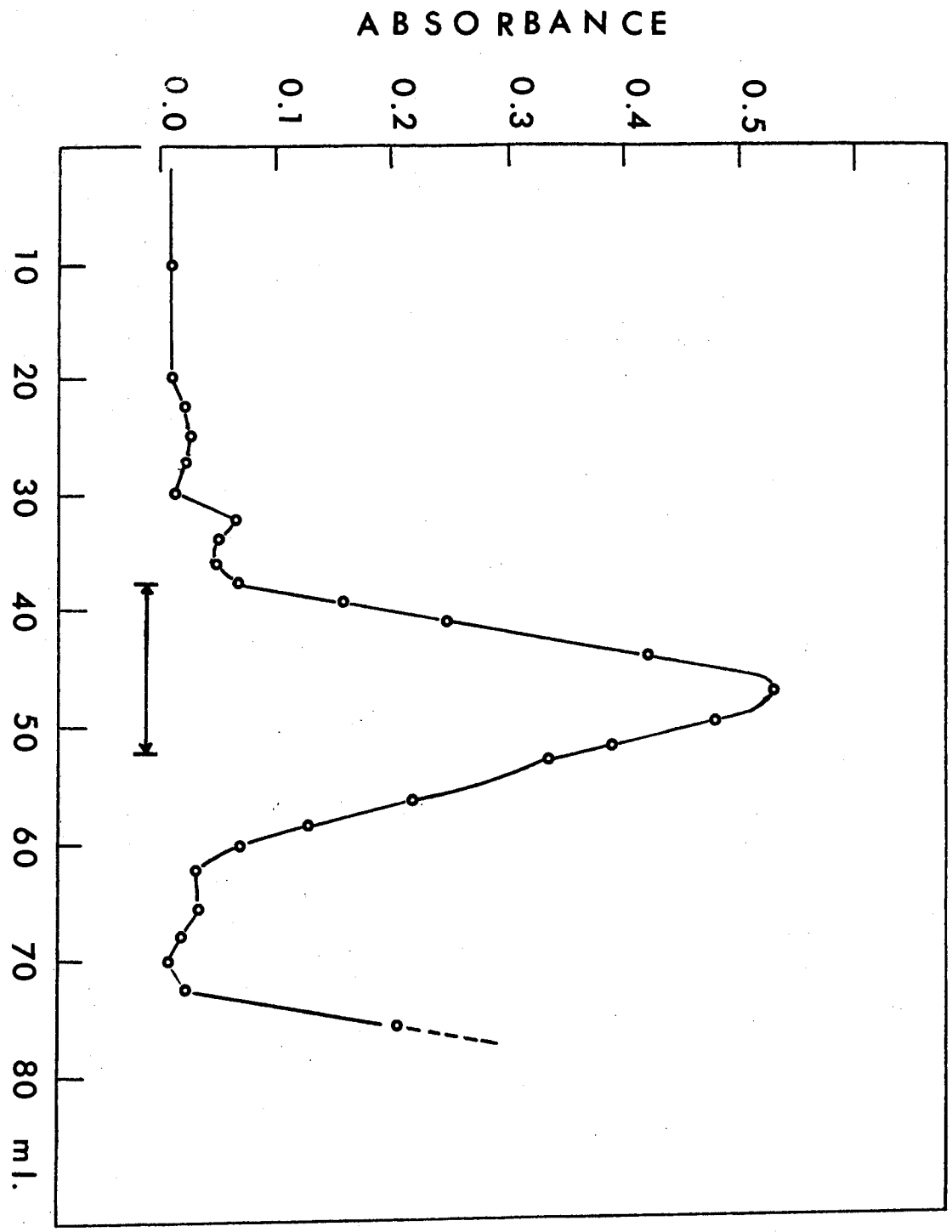
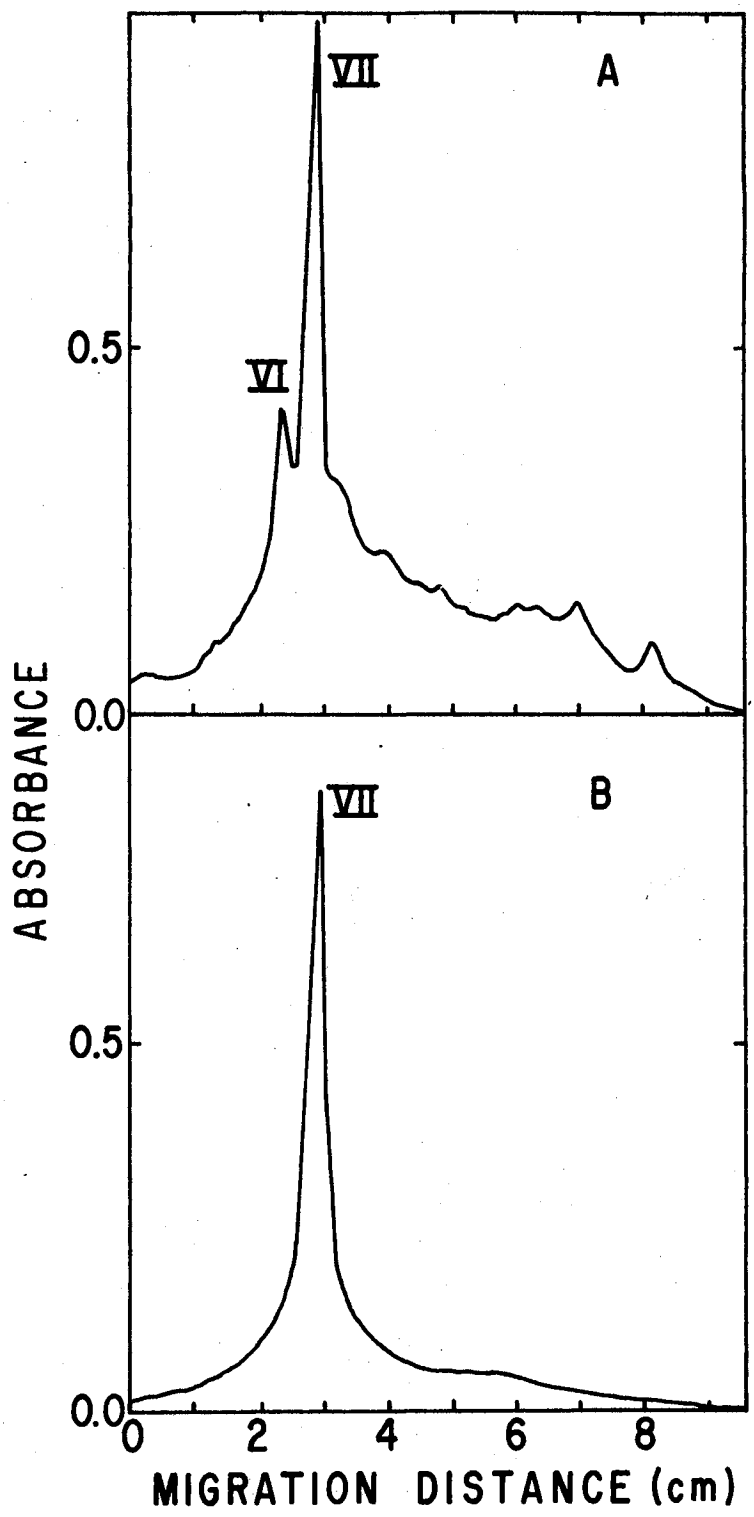

ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
10
20
30
40
50
60
70
80
ml.

Figure 11. Photometric Scanning Pattern from SDS Gel Electrophoresis of Component VII before, A, and after, B, Purification


VII
A
0.5
VI
0.0
VII
B
ABSORBANCE
0.5
0.0
0
2
4
6
8
MIGRATION DISTANCE (cm)

(3) The sedimentation equilibrium experiment was done and high speed meniscus depletion technique was used. The plot of the logarithm of the Raleigh fringe displacement vs. the square of the distance from the center of rotation r^2 was straight to the bottom of the cell.

(4) The single band in SDS gel electrophoresis is not always a single component because there is a possibility of the presence of the size isomer. The gel electrophoresis of component VII without SDS was performed. The gel and buffer contained Tris-glycine buffer at pH 9.0. The possibility of the presence of a charge isomer was eliminated by using different gel concentration. The result of gel electrophoresis is shown in a picture (Figure 12). The mobility of the protein band was also plotted against gel concentration. It was shown to be a straight line as shown in Figure 13.

5. Chemical and Physical Properties of Component VII

a. Molecular Weight Determination

(1) SDS Gel Electrophoresis. The standard line was drawn from observed mobility of standard proteins and their known molecular weight. The mobility of component VII was very close to β-galactosidase which has a molecular weight of 135,000. The estimated molecular weight of component VII from Figure 14 was 139,000 in 5% gel. It has been reported that collagen molecule frequently showed anomalous behavior in SDS gel electrophoresis (94-96). Most of standard proteins are globular proteins and component VII has some characteristic collagen features. The possibility of a different mobility with different gel concentration

Figure 12. Picture of Component VII in Tris-Glycine Gel Electrophoresis of Different Gel Concentrations

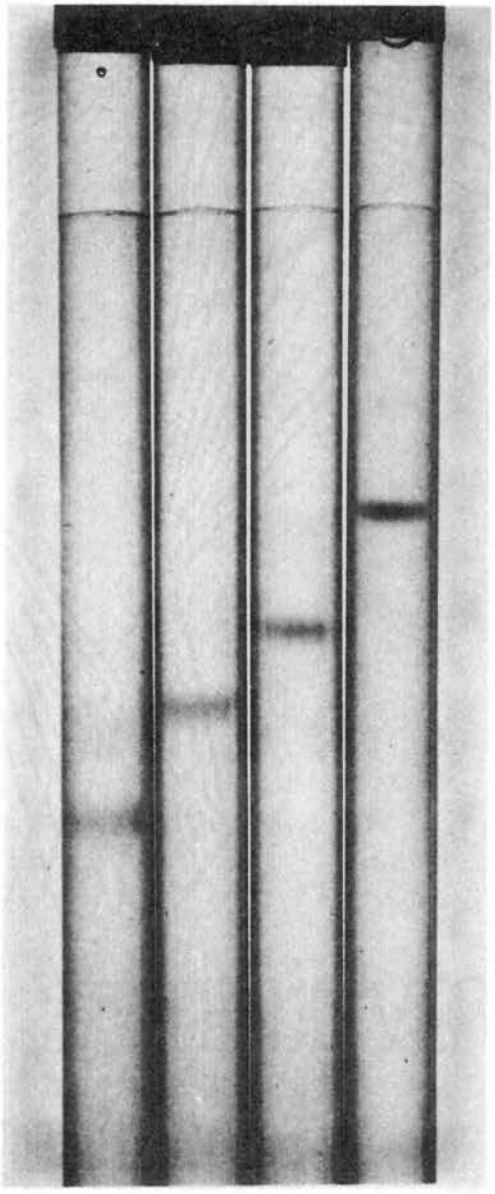


Figure 12.

Figure 13. The Electrophoresis Mobility of Component VII in Different Gel Concentrations

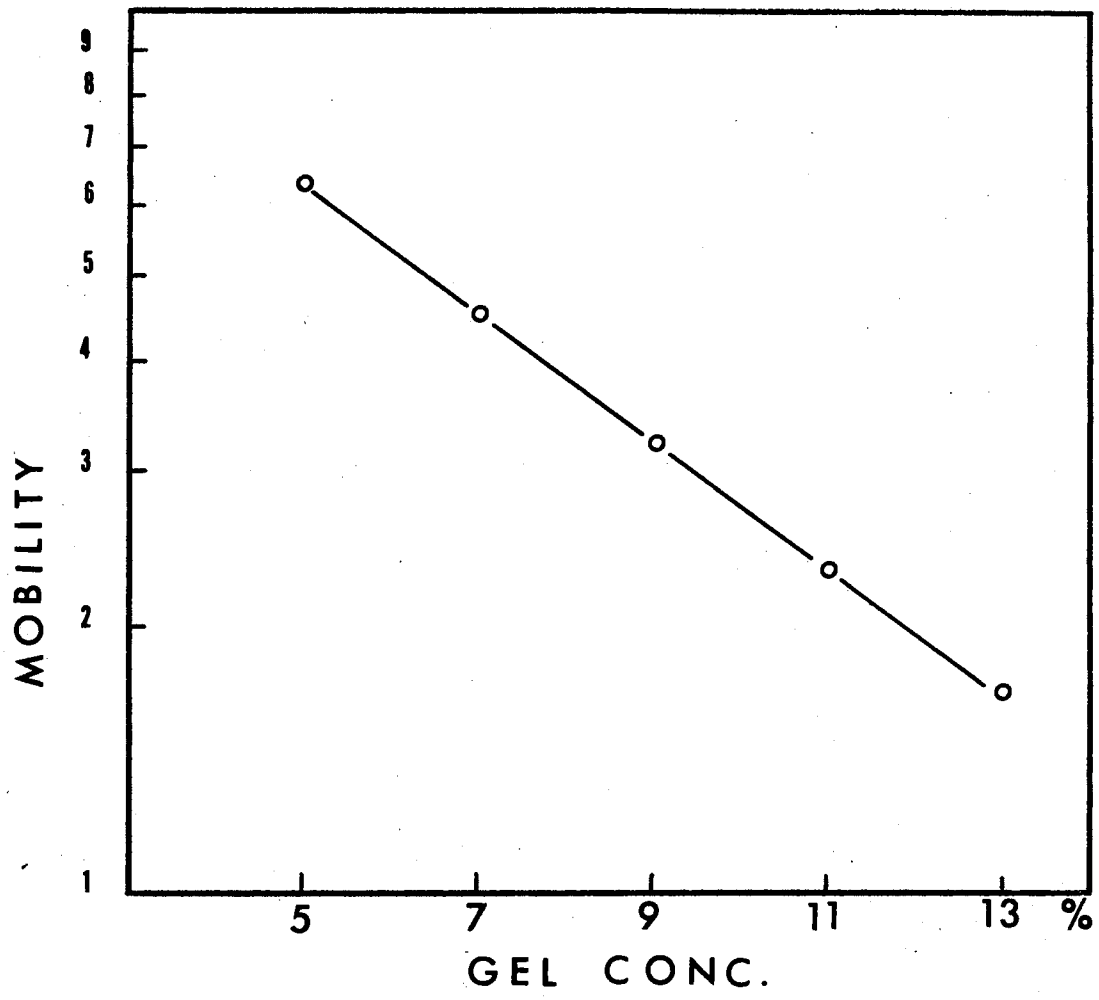
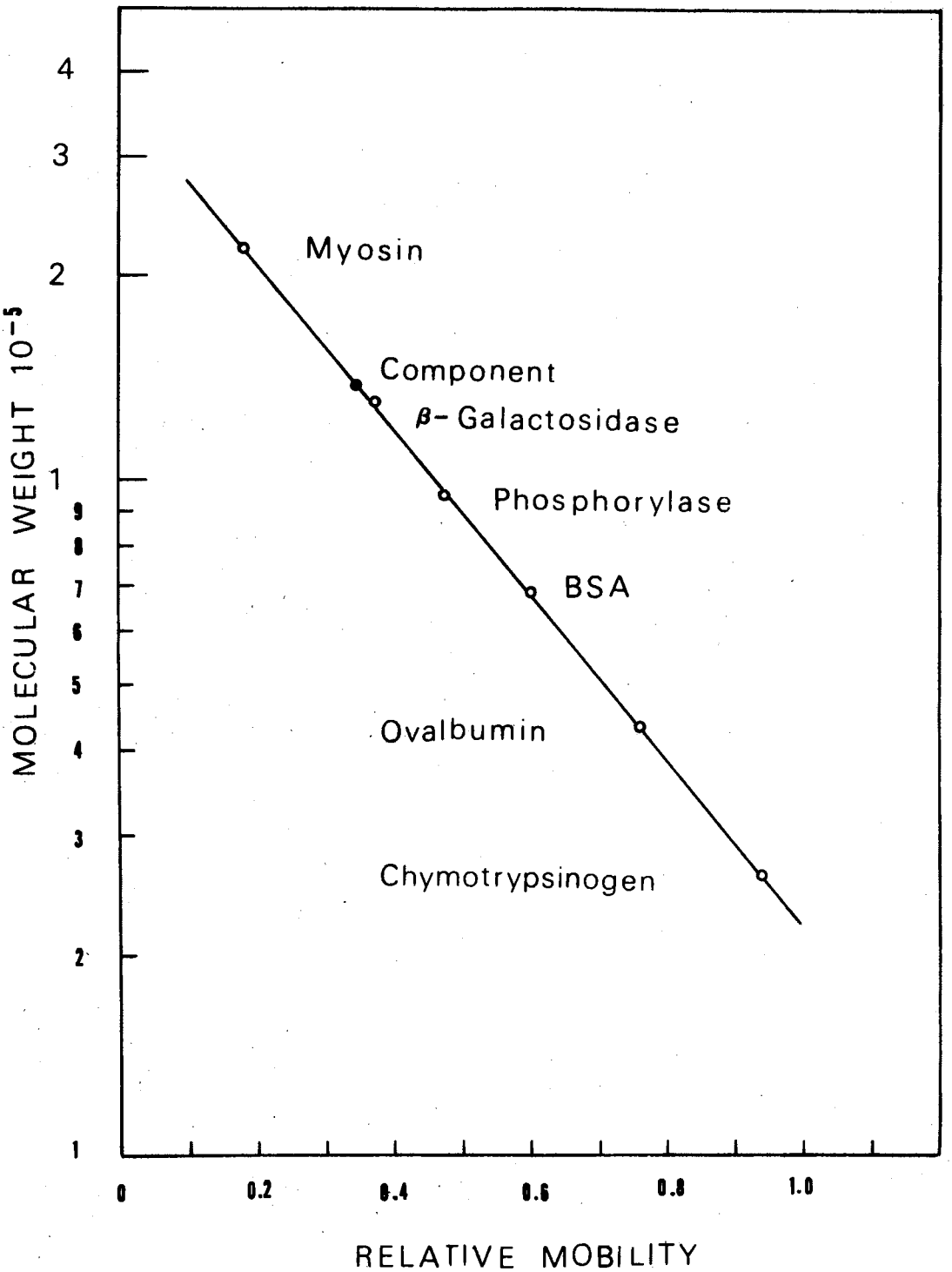

MOBILITY
9
8
7
6
5
4
3
2
1
5
7
9
11
13
%
GEL CONC.

Figure 14. SDS Gel Electrophoresis of Component VII with Several Standard Proteins

The molecular weights of the standard proteins are: myosin, 220,000 (77,78); β-galactosidase, 130,000 (79); phosphorylase A, 94,000 (80); BSA, 96,000 (100); ovalbumin, 43,000 (81); chymotrypsinogen, 25,700 (82).


MOLECULAR WEIGHT 10^{-5}
4
3
2
1
9
8
7
6
5
4
3
2
1
Myosin
Component
β- Galactosidase
Phosphorylase
BSA
Ovalbumin
Chymotrypsinogen
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

was examined. The mobilities of component VII based on chymotrypsinogen as internal standard were essentially constant as shown in Figure 15. An average molecular weight of 136,000±3,100 daltons was calculated as the arithmetical mean value obtained for different percentage of acrylamide.

(2) Other Techniques. The molecular weight of component VII was also determined by ultracentrifugation and gel filtration column chromatography methods by Patricio Riquetti.

The summary of the data is shown in Table V. The molecular weight in SDS system was shown to be 140,000 daltons and 6 M GuHCl system indicated 70,000 daltons.

b. Amino Acid Analysis

Amino acid composition of component VII is shown in Table VI. This analysis revealed that component VII contains not only all of the common amino acids, but also contains hydroxyproline and hydroxylysine which are also found in collagen. The highest amount of amino acid is glycine, 13%, but it is still very low compared to a typical collagen of 33%. The amino acid pattern is listed in Table VIII. Component VII contains a large amount of polar amino acids such as glutamic acid. The total amount of polar amino acids exceed more than 50% which may be partly responsible for its solubility. The amount of acidic amino acids is almost twice that of the basic amino acids. Aromatic amino acids and sulfur containing amino acids are relatively high compared to the insoluble portion and collagen. The amino acid content is low but lysine and hydroxylysine are essentially the same in amount. The amount of

Figure 15. Molecular Weight Determination of Component VII by Different Gel Concentrations of SDS Electrophoresis

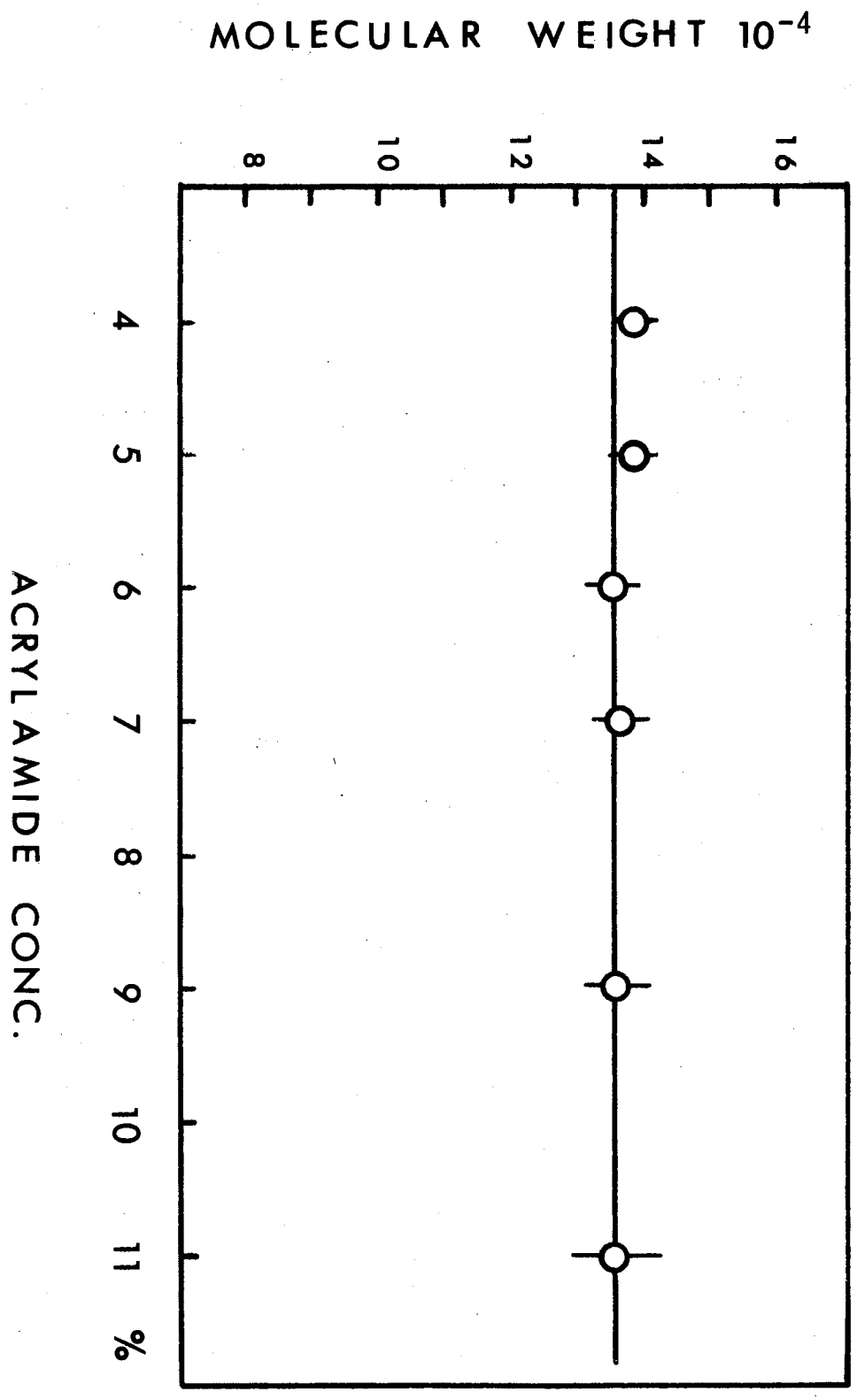

MOLECULAR WEIGHT 10^{-4}
8
10
12
14
16
4
5
6
7
8
9
10
11
%
ACRYLAMIDE CONC.

TABLE V

SUMMARY OF MOLECULAR WEIGHT DATA FOR COMPONENT VII

Method of determination	Solvent	Molecular weight
Sedimentation equilibrium [d]	6 M guanidine hydrochloride-1% mercaptoethanol	68,000 ± 3,000
Gel filtration chromatography [a,d]	6 M guanidine hydrochloride	72,000 ± 2,000
Polyacrylamide electrophoresis	1% sodium dodecyl sulfate-1% mercaptoethanol [c]	136,000 ± 3,100 [b]
Gel filtration chromatography [d]	1% sodium dodecyl sulfate-1% mercaptoethanol	140,000 ± 2,000

[a] Component VII was reduced and carboxymethylated.

[b] Arithmetical mean of values determined with polyacrylamide gels ranging in acrylamide concentration from 4 to 11%.

[c] Protein was dissolved in this solvent and electrophoresis was carried out in 0.1% sodium dodecyl sulfate.

[d] Measured by Patricio Riquetti.

TABLE VI

AMINO ACID COMPOSITION OF COMPONENT VII

Amino Acid	moles	mg	Residue	Residue
	100 mg protein	100 mg protein	1000 amino acids	70,000 daltons
Lysine	24.1	3.09	29.6	16.9
Histidine	17.5	2.41	21.5	12.3
Arginine	44.8	6.99	55.1	31.4
Hydroxylysine	7.0	1.01	8.6	4.9
Aspartic acid	60.0	6.89	73.7	41.9
Threonine [a]	41.8	4.22	51.4	29.3
Serine [a]	65.6	5.70	80.1	45.9
Glutamic acid	94.4	12.19	116.0	66.1
Proline	56.8	5.52	69.8	39.8
Glycine	109.8	6.27	134.9	77.0
Alanine	58.3	4.15	71.6	40.8
Halfcystine	22.9	2.36	28.1	16.1
Valine [b]	49.9	4.96	61.3	35.0
Methionine [c]	9.0	1.19	11.1	6.3
Methioninesulfoxide	3.1	0.51	3.8	2.2
Isoleucine [b]	26.0	2.95	31.9	18.2
Leucine [b]	54.9	7.19	67.4	38.4
Tyrosine	17.4	2.84	21.4	12.4
Tryptophan [d]	9.2	1.72	11.3	6.6
Phenylalanine	23.1	3.41	28.4	16.2
Hydroxyproline	18.2	2.06	22.3	12.7
Total	813.8	87.63	999.3	570.4

[a] Estimated by extrapolation at 0 time hydrolysis at 24,48 and 72 hrs. hydrolysis.

[b] Values are taken from 72 hrs. hydrolysis.

[c] Average of duplication at 24 hrs. hydrolysis.

[d] Estimated from UV absorption spectrum.

hydroxylysine of component VII is comparable to that of collagen but much less than the insoluble portion. The difference between the basement membrane and collagen is apparent in three amino acids. The GBM contains cysteine, tryptophan, and 3-hydroxyproline. These amino acids are not observed in interstitial collagens. Especially the presence of cysteine in basement membrane is very significant in terms of membrane structure because this is a major crosslinking system in the basement membrane. Collagen has different kinds of crosslinkage which was discussed earlier in Chapter I. The hard core insoluble portion of GBM which cannot be made soluble by incubation with mercaptoethanol in 8 M urea and 1% SDS solution may contain some kinds of cross links which are reportedly similar to those found in collagen. These findings from the amino acid analysis alone indicate that component VII shows very little resemblance to interstitial collagen, although the insoluble portion of the GBM is similar to collagen in many respects.

c. Carbohydrate Analysis

The result of the carbohydrate analysis is shown in Table VII. All carbohydrate components found in the whole membrane, insoluble portion and soluble portion are also found in component VII, but the ratio of each carbohydrate residue is considerably different. The total residue of carbohydrate per 1,000 amino acid residues are not much different between the insoluble portion and component VII. The largest amount of carbohydrate found in component VII are galactose and glucosamine, while the insoluble portion or the whole membrane contains mostly glucose and galactose. The amount of galactose is three times that of glucose in component VII while the insoluble portion contains

TABLE VII

CARBOHYDRATE COMPOSITION OF COMPONENT VII

Monosaccharide	Moles 100 mg of protein	mg 1000 of protein	Residues 1000 amino acids[a]	Residues 70,000 M.W[b]
Hexose	22.1	3.57	27.5	15.2
Mannose	4.4	0.72	5.5	2.8
Galactose	13.0	2.10	16.2	9.1
Glucose	4.7	0.75	5.8	3.3
Hexosamine[c]	16.3	3.15	20.4	11.5
Glucosamine	12.8	2.47	16.0	9.0
Galactosamine	3.5	0.68	4.4	2.5
Fucose	1.5	0.21	1.8	1.0
Sialic Acid[d]	5.0	1.53	6.1	3.4
Total	44.9	8.46	55.8	31.1

[a] The calculation was based on data from amino acid composition Table I.

[b] The tentative value 70,000 was employed.

[c] Average of duplicate analysis after hydrolysis with 4 N HCl for 6 hrs. at 100°C and expressed as N-acetyl derivatives.

[d] Average of duplicate analysis by thiobarbituric method.

essentially the same amount of glucose and galactose. The hexosamine content in component VII as well as the sialic acid content indicate that component VII contains a large amount of heteropolysaccharide and only a small amount of glucose-galactose disaccharide units. The presence of glucose in basement membrane is significant because polysaccharide units known to date never contain glucose, and glucose is always found in hydoxylysine linked simple saccharide unit. The presence of heteropolysaccharide unit was reported by Spiro (44). According to his proposal, the heteropolysaccharide unit consists of galactose, mannose, glucosamine, sialic acid, and fucose in ratio of 4:3:5:3:1, respectively. Since glucose is not included in this unit but in the disaccharide unit, the amount of glucose indicates the amount of disaccharide and the amount of fucose indicates the amount of heteropolysaccharide. The ratio between the disaccharide unit and the heteropolysaccharide unit in the whole membrane, insoluble fraction, soluble fraction, and component VII are 10:1, 11:1, 4:1, and 3:1, respectively. Component VII contains much less disaccharide units and much more heteropolysaccharide units. The heteropolysaccharide unit did not account for the presence of galactosamine, however, component VII contains considerable amount of galactosamine and much excess of glucosamine. The probable explanation is that there are other kinds of heteropolysaccharide units which contain galactosamine (S) (see discussion).

d. Quantitative Analysis of Hydroxylysine Glycosides

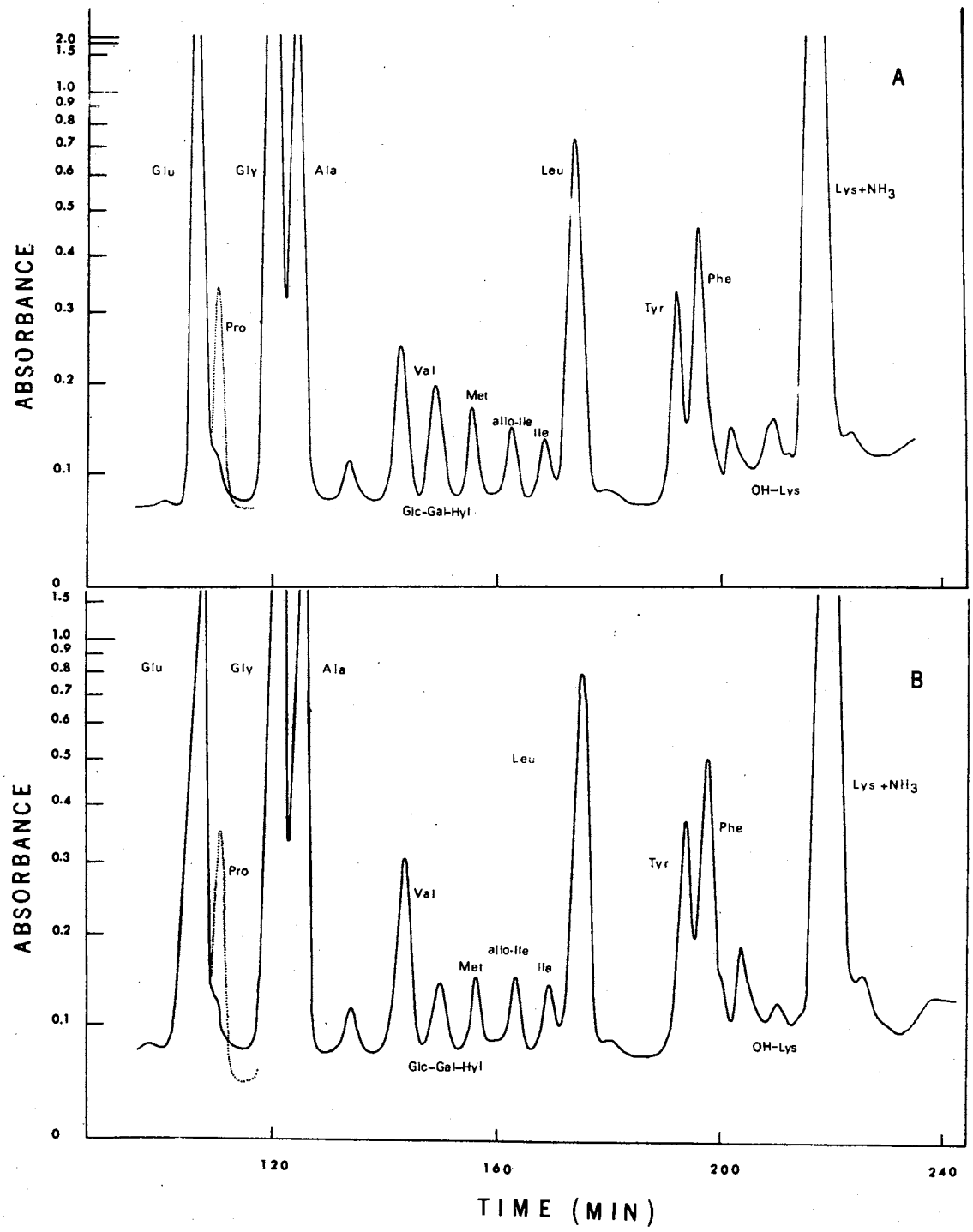
It has been known that the basement membrane contains glucose-galactose carbohydrate unit which is commonly found in interstitial collagen. Since component VII was found to contain glucose, the presence of a disaccharide unit was predicted. The result of amino acid analysis after alkaline hydrolysis of component VII is shown in Figure 16. This rapid analytical method was recently developed by Odell, et al. in our laboratory. The method enabled us to estimate the amount of glucose-galactose-hydroxylysine, galactose-hydroxylysine, and free hydroxylysine quantitatively using leucine as a natural internal standard. The color yields were obtained from separate analysis using known amount of each residue by this method. Component VII was found to contain 7.5 residues of glucose-galactose disaccharide unit per 1,000 amino acid residues and 1.4 residues of free hydroxylysine residue. The amount of galactose-hydroxylysine was almost negligible (0.04 residue). The amino acid analysis from acid hydrolysate previously showed a total of 8.6 residues in the component. The combined hydroxylysine residue from this analysis shows 8.9 residues which is very close within experimental error. This analysis indicated that about 84% of hydroxylysine is glycosylated and only 16% was present as free hydroxylysine in component VII.

e. Circular Dichroism

The circular dichroism spectrum of component VII in both SDS and GuHCl buffer solution was obtained in order to estimate its conformation under these conditions. A globular protein, myoglobin, was used as a

Figure 16. The Chromatogram of Hydroxylysine and its Glycoside

A: Whole GBM Hydrolysate
B: Component VII Hydrolysate


A
B
Glu
Gly
Ala
Pro
Val
Met
allo-Ile
Ile
Leu
Tyr
Phe
OH-Lys
Lys+NH3
Glc-Gal-Hyl
ABSORBANCE
TIME (MIN)
120
160
200
240

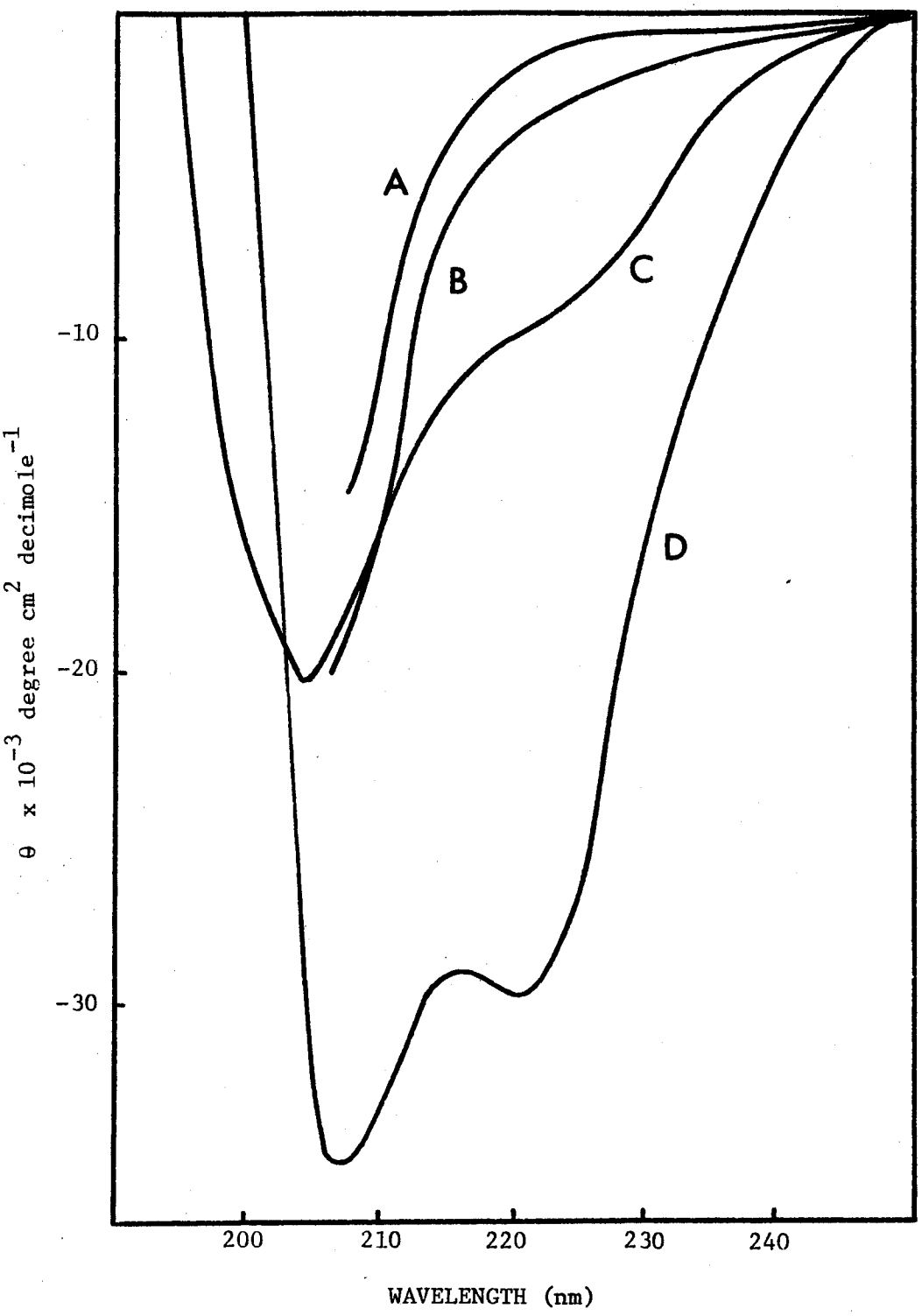
reference protein molecule under same conditions (Figure 17). In the presence of 6 M GuHCl, both component VII and myoglobin exhibited almost an identical simple downward curve with no maximum or minimum point. This indicates that component VII under this condition is in a completely unordered random coil conformation.

On the other hand, component VII in 1% SDS buffer exhibited a broad shoulder around 215 nm and a turning point of this shoulder was found somewhere around 222 nm. The minimum ellipticity was observed at 205 nm and the cross over point at 195 nm. Myoglobin in the same buffer showed ellipticity minima at 207 and 222 nm and maximum at 215 nm. This curve indicates that myoglobin under this condition still retains a large amount of α-helix. The component VII in SDS is not a totally random coil from this observation although the difference in two buffer systems is not so clear cut as in the case of myoglobin.

Several attempts were made to calculate the percentage of α-helix content empirically using several globular proteins whose secondary and tertiary structures are known by x-ray analysis (83). These equations are not always satisfactory due to a wide deviation of average values. A tentative calculation based on Chen and Yang was attempted. The calculated values of α-helix content of component VII in SDS buffer solution are 23.8% at 220 nm and 26.5% at 222 nm. If the maximum deviation is included, these values are estimated to be somewhere between 16.6% and 33.8%. The same calculation is applied to myoglobin in SDS buffer. The result showed that the myoglobin molecule still retains 89% of original α-helix content. The empirical equation was based on small globular molecules so that application of these coefficients to a collagen-type molecule may lack some certainty. For

Figure 17. CD Spectrum of Component VII and Myoglobine

A: Component VII in 6 M GuHCl
B: Myoglobin in 6 M GuHCl
C: Component VII in 1% SDS
D: Myoglobin in 1% SDS


A
B
C
D
-10
-20
-30
θ x 10^-3 degree cm^2 decimole^-1
200
210
220
230
240
WAVELENGTH (nm)

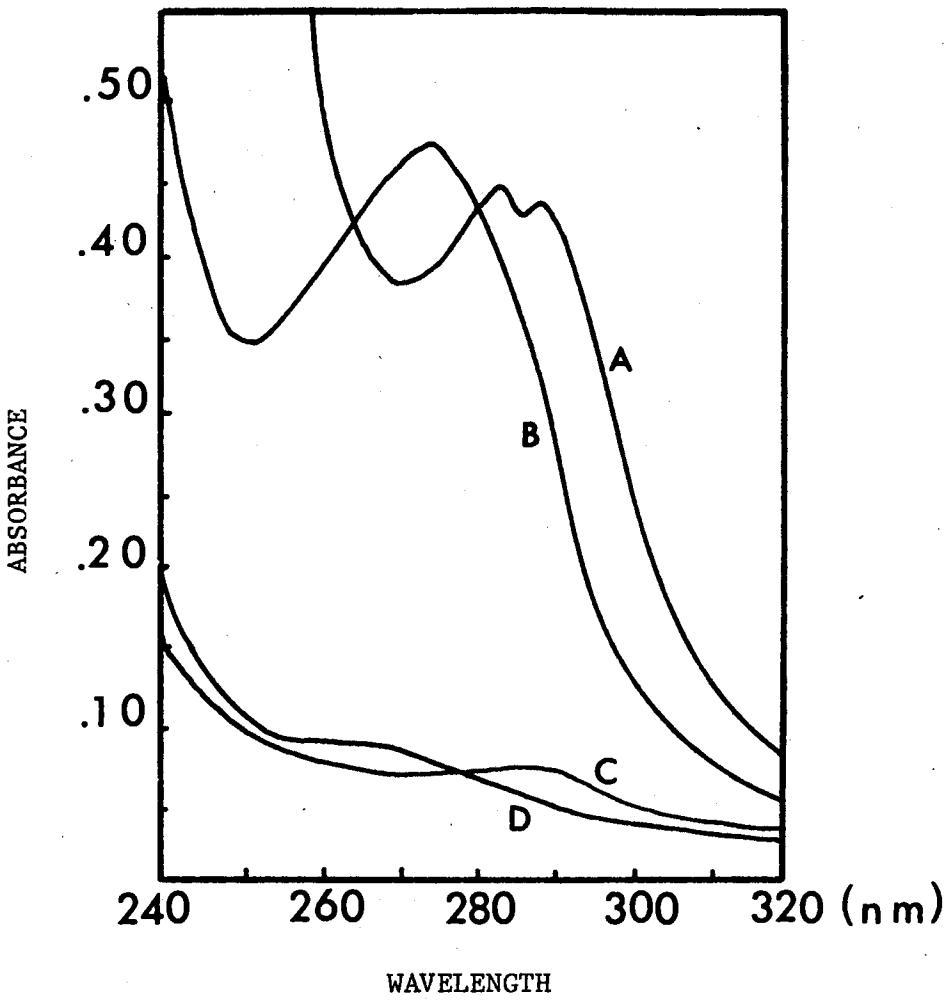
our experiments, the validity is not important to the overall conclusion in this case.

f. Ultraviolet Absorption Spectrum

One of the differences between interstitial collagen and the basement membrane is the amount of aromatic amino acids. The amino acids mainly responsible for the absorption of ultraviolet light at 280 nm are tyrosine and tryptophan. Tendon collagen contains only 5 residues of tyrosine per 1,000 amino acid residues and totally lacks tyrptophan, while component VII contains 21 residues of tyrosine and a substantial amount of tryptophan. The hydrolysis of a protein using strong acidic condition destroys most of tryptophan residues. Therefore, the amount of tryptophan cannot be obtained from the acid hydrolysis while optical method gives satisfactory results in tryptophan analysis without consumption of samples. The UV spectrum of component VII in neutral pH and alkaline pH are shown in Figure 18. Skin collagen was also included for comparison. Component VII at neutral pH showed a simple curve with an absorption maximum at 275 nm. When component VII was in alkaline pH (0.1 N, NaOH), the absorption spectra showed typical bathochromic effect, shifted to longer wavelength, and the maximum peak was split into two peaks due to ionization of tyrosine. Two maxima were found at 290.3 nm for tyrosine and 284.5 nm for tryptophan. The tyrosine-tryptophan molar ratio and then each amount of tyrosine and tryptophan can be calculated from a slope of two peaks and absorption maximum by method of Bencze and Schmid (74). The amount of tryptophan was found to be 9.2μ moles per 100 mg, which is equivalent to 11.3 residues per 1,000 amino acid residues. The amount of tyrosine was also

Figure 18. UV Spectrum of Component VII

A: Component VII in 0.1 N Na OH
B: Component VII in 6 M GuHCl, pH 7.0
C: Calf Skin, Acid Soluble Collagen in 0.1 N NaOH
D: Calf Skin, Acid Soluble Collagen in 6 M GuHCl, pH 7.0


.50
.40
.30
.20
.10
ABSORBANCE
A
B
C
D
240
260
280
300
320 (nm)
WAVELENGTH

calculated from acid hydrolysis and the result from UV analysis by this method was satisfactory. This analysis revealed that component VII has little similarity to the collagen molecule because the collagen contains only a small amount of aromatic amino acids while GBM contains a substantial amount.

CHAPTER IV

DISCUSSION

The renal GBM has attracted the study of many investigators because of the prime importance of its physiological function and the direct relationship of its morphological changes observed in many disease states, such as diabetes (84, 85, 86). The major problem in the study of the basement membrane is the difficulty of obtaining pure membrane in a suitable amount for detailed investigations. Fortunately, the renal GBM is relatively easy to isolate from kidney without using drastic conditions, such as alkaline extractions to separate it from other tissues (22). The improved techniques, developed by Spiro (44), were successfully used and whole membrane in a gram quantities were obtained. The purity of the membrane is one of major criteria in this field, and since it is not soluble the purity has to be checked on a morphological basis with a microscope. The chemical analysis indicated that the possibility of contamination of cellular membrane was limited, but other components, which are similar to GBM, would be extremely difficult to detect (87). Nevertheless, it is believed that the whole membrane preparation was pure enough for most cases. Some of the analyses, which have been done for each membrane preparation routinely, were amino acid analysis, carbohydrate analysis, and SDS gel electrophoresis. Several different membrane preparations gave identical

analytical values and gel patterns. These were indications that the membrane preparations were pure or at least consistent in its qualities.

The insoluble nature of GBM is another problem for investigating the membrane. The whole membrane cannot be solubilized without using strong hydrophilic agents, such as 8 M urea and 6 M GuHCl or SDS (22). A portion of the membrane (26%) is soluble and extractable with 8 M urea or 6 M GuHCl. Extracts with both 8 M urea and 6 M GuHCl are identical based on the observations from the SDS gel electrophoresis with and without mercaptoethanol. Apparently these components (subunits) are held together by non-covalent bonds. The soluble portion contains many components with molecular weights ranging from 350,000 to less than 24,000 daltons. Since the reduction of the soluble portion yielded smaller molecules, and the large molecular weight components, 350,000 daltons, had disappeared, then this indicated that the large molecular weight components are held together mainly with disulfide bond(s). It is interesting to note that the reduction by mercaptoethanol produced small molecular weight components. Similar components were also found in the non-reduced GuHCl extracted portion. In other words, the reduction did not produce smaller molecules at random. These findings were also confirmed by reduction of each pooled fraction after gel filtration column chromatography. Fraction I to V, all showed the same behavior, i.e., the reduction of the large components such as B, E, and H, produced smaller components such as IX, X, XI, etc.. There was one exception in these components; component F, which was supposedly identical to component VII based on its migration distance upon SDS gel electrophoresis, was not changed by mercaptoethanol reduction. If there is any change, such as the reduction of intramolecular bond(s),

the gross structures of the molecule and the molecular weight appeared to be the same.

Component VII is one of the products of the reduction of the larger components, such as components A and B in Fig. 9. Component E has a similar molecular weight to component F (VII) based on their electrophoretic migration distances, however, the amount of component E was diminished considerably upon reduction. Component VII was not produced from the reduction of the insoluble fraction, rather the reduction of the insoluble fraction produced only larger components, such as component III and IV, or similar molecular weight components, but almost negligible amount of components smaller than component VI were formed. It is known that the major crosslinks in basement membrane are disulfide bonds which are not found in interstitial collagen in general. It is also known that the basement membrane contains lysine-derived crosslinks, but to a lesser extent than those found in collagen. It is not known if the lysine-derived crosslinks of basement membrane are reducible, such as a Schiff base or the partly non-reducible crosslinks which are often observed in collagen and elastin (88, 89), since the isolated and identified crosslinks in basement membrane are chemically reduced by $NaBT_4$. This can be determined with a mass spectrometer by the observation of relative intensities of (m-2) peaks against (m) peaks of the isolated crosslink derivatives which are reduced by $NaBT_4$ (90-93).

If component VII contains such lysine-derived crosslinks, it is natural that the reduction by mercaptoethanol does not produce any smaller components like IX or XI.

If wild speculations are allowed, they would be as follows: (1) since the amino acid and carbohydrate compositions of component VII are

very similar to the total GuHCl soluble fraction, but very different from the insoluble portion, which is rather similar to collagen molecules, then component VII may be the most important intermediate in the formation of the non-collagenous portion of the GBM, which is formed from disulfide crosslinkings, (2) there may be a fundamental polypeptide chain with a molecular weight similar to or slightly smaller than that of chmotrypsinogen (26,000 M.W). These chains may be polymerized by lysine-derived crosslinks forming higher molecular weight components in a stepwise fashion. Some of the protein bands showed small differences in their migration distances in SDS gel electrophoresis. This can be explained by the different degrees of glycosylation of the disaccharide and heteropolysaccharide units. The smaller intermediates are not fully glycosylated, therefore, relative content of hexose per unit of protein is quite low compared to the larger components (16), (3) peak IV may be the non-collagenous portion which is linked to a large collagen type molecule by disulfide bond(s).

The reasons for these proposals are: (1) peaks III and IV or same molecular weight components were observed in the GuHCl soluble portion without reduction as minor stable components and relative amount was not changed after mercaptoethanol reduction, (2) peaks III and IV were also produced from large soluble components as shown in fraction I, component A in Fig. 9, (3) the insoluble fraction produces components III and IV or at least equivalent molecular weight components after mercaptoethanol reduction.

The whole membrane became mostly soluble (72%) after reduction in 1% SDS. The hard core portion, 28%, cannot be detected by the Lowry method in supernatant of incubation mixture of the whole membrane which

contains mercaptoethanol because it is precipitated out. Kefalides called this portion "basement membrane collagen" because the glycine content is 33% which is the same as the amount found in interstitial collagen, however, marked differences are obvious in the cysteine content, carbohydrate content, etc., which will be discussed later. The whole insoluble portion contains only 22% glycine and the whole soluble portion contains 13% glycine which is the same amount found in component VII.

These data indicate that the insoluble portion must contain large amounts of the non-collagenous portion. It is estimated, by simple calculation from relative glycine contents and solubility experiments, that the amount of the non-collagenous portion of the whole insoluble portion is somewhere between 36% and 45%. This calculation is based on rather naive assumptions, i.e., (1) all non-collagenous components only contain 133 glycine residues per 1000 amino acid residues and the hard core insoluble portion (or basement membrane collagen) contains 330 glycine residues, (2) the contribution of the carbohydrate portion is neglibible, (3) the Lowry protein determination method always gives proportional amounts of protein based on the standard curve of BSA. Obviously, all three assumptions are not always valid; nevertheless, the difference between 36% and 45% is reasonably close. The overall distribution of each group of components is estimated as follows: GuHCl soluble, 26$\pm$1%; GuHCl insoluble but soluble after reduction in 1% SDS 41$\pm$4%; hard core insoluble 28$\pm$4%.

It is not likely that one type of cell produces three groups of components separately at almost the same time in a large amount. The origin of these components in GBM is speculated from the available

knowledge of other kinds of basement membrane systems as follows: (1) the probasement membrane (M.W, 140,000) which is similar to pro-collagen, are divided into two peptides, enzymatically, in vivo, collagenous peptide (m.W, 120,000) and non-collagenous peptide (M.W, 20,000). The molecular weights above were assumed using a data reported by Grant et al.(101, 102, 103) based on SDS system; (2) the collagenous peptide which still contains a telopeptide is extensively crosslinked through a lysine-derived compounds, histidine derivations, and N-glycosyl-lysine derivatives (42). These are hard-core insoluble portions and high M.W components, which are more than million daltons; (3) the non-collagenous peptides (M.W, 20,000) were crosslinked much less extensively. The major crosslinks are disulfide bonds and the smallest component does not have intermolecular disulfide bonds; (4) some intermediate components (M.W, 200,000) formed from non-collagenous peptide were linked by disulfide bonds to the hard core insoluble portion. These speculations will be discussed later.

These speculations and experimental results serve as a basis for the proposal of a new version of a membrane model which will be presented later on Fig. 19.

In order to elucidate the structure of native membrane, the complete chemical analysis of the individual components is desirable and will be helpful to construct the original membrane models. It was assumed that such purification of any single component would be extremely difficult, judging from the results of SDS gel electrophoresis of whole basement membrane. It was actually very difficult and tedious to isolate pure component VII in a large enough amount for chemical and physical analyses.

The reasons why component VII was chosen for isolation are the following: (1) although it was not the richest component, it was easily detected by SDS gel electrophoresis. On the other hand, component IV is surrounded by several other minor components, such as III and V, and it was difficult to pinpoint a single band due to overlapping these components. (2) The GuHCl soluble fraction contains components VII as the highest peak in the gel electrophoresis scanning pattern and component VII was well separated from the other groups of proteins. (3) The mercaptoethanol reduction did not change the amount of component VII but most of component E, which is slightly larger molecule than component VII, was diminished by reduction.

Because of the above, GuHCl extraction followed by gel filtration column chromatography were necessary steps. The Bio-Gel A-15m was chosen since previous experiments reported by Hudson (15) indicated that Bio-Gel A-15m and A-50m gave most successful separation of reduced and alkylated GBM, but poorly separated by A-150m and A-1.5m. Since the soluble fraction contained a maximum molecular weight of around 3~5 million, then A-15m was suited for the separation of these components rather than A-50m. The second purification step with the same procedure, except the column having a high L/D ratio, showed that component VII eluted with a minor component followed by component VII. The final yield was expected to be about 16 mg, however, because of this minor component, the actual yield was 11 mg. All criteria for the purity of component VII was satisfied by several observations: (1) labeled component VII from gel filtration column chromatography was eluted as a single peak and no other labeled protein was observed, (2) the sedimentation equilibrium experiment of component VII showed that the change of fringe displacement

vs the square of the corresponding radial position (r^2) showed a straight line, (3) the sedimentation velocity experiment showed a single symmetrical peak, (4) the gel electrophoresis of several different concentrations of the gel showed a single band and the plot of migration distance vs gel concentrations was straight, with and without SDS.

The minimal molecular weight of component VII was estimated by sedimentation equilibrium centrifugation, gel electrophoresis, and gel filtration column chromatography. These methods rely on different principles to estimate the molecular weight. The SDS gel electrophoresis was employed, initially, and a molecular weight of 137,000 - 140,000 daltons was obtained from several different gel concentrations (Fig. 13). It has been known that some proteins, such as collagen and sialo-glycoprotein, behave anoumalously in SDS system (94-97). The apparent molecular weight of collagen is estimated much higher than the values obtained by other techniques.

Because of this reason, ultracentrifugation works were applied in 6 M GuHCl and a value of 68,000 daltons was obtained, which was almost half of the value obtained from the SDS gel electrophoresis. In the sedimentation equilibrium data alone, ambiguity exists as to whether the protein with a given mass is completely dissociated. Therefore, it was necessary to use the gel filtration column method, based on a column calibrated by several proteins, which measures the effective hydrodynamic radius. The value obtained from SDS system was 140,000 daltons and GuHCl system gave 72,000 daltons. These discrepancies can be a result of (1) charge effects due to a decrease charge per unit molecular length, (2) a conformation having a larger hydrodynamic radius than the

standard proteins used in calibration of the gels, or (3) dimer formation.

It is not likely that the charge effects are responsible because in a gel filtration method, which the amount of charges is less important, and, in gel electrophoresis, which depends on charge per unit mass, gave same value. Furthermore, the relative mobility of component VII is independent of the acrylamide gel concentrations (Fig. 15), which usually influences the mobility of the protein if it were the charge effects (63). The formation of a dimer is not likely because dissociation to monomer was not observed in any conditions and two peaks of monomer and dimer were not observed in any experiments. The possibility of having a more disordered conformation than a standard protein was examined by CD spectra. Myoglobin and component VII in 6 M GuHCl showed spectra indicative of a totally random conformation (Fig. 17). However, myoglobin retained large amounts of α-helix in 1% SDS buffer and component VII showed some ordered conformation, but much less than that of myoglobin. This indicates that component VII in 1% SDS behaves as a more unordered protein than typical standard proteins which still retain large amounts of secondary conformation.

Since the molecular weight is proportional to the hydrodynamic radius of a protein when it is completely random, then the value, 70,000 daltons, obtained from 6 M GuHCl systems is more likely a true molecular weight than that from SDS systems. It is not surprising that component VII behaves somewhat similar to the interstitial collagen, because there are some similarities in their chemical compositions.

The patterns of the amino acids and the carbohydrates of component VII is compared with previously listed data in Table VIII. All chemical

compositions of component VII are very much similar to GuHCl soluble fraction in general. The amino acid composition is almost identical in all respects, however, the carbohydrate composition are different in some cases, such as hexosamine content. Previous reports have indicated that smaller molecular weight component may contain less carbohydrate composition and each component may contain different amounts of carbohydrate. Another problem of the carbohydrate analysis is that there is no common internal standard for each different kind of analytical method. However, the ratios of the carbohydrate compositions within a same group of sugars, such as neural sugars or hexosamines, should be reliable since each group of sugars was analyzed at same time. Most of the amino acids were analyzed at same time, therefore, when the amino acid content was expressed by per cent basis, each content was expressed by build-in internal references.

Even if there are some differences in the chemical analysis, it might be safe to say that component VII represents the whole soluble fraction. The chemical analysis of component VII indicated that this component is much less similar to interstitial collagen than the insoluble portion or whole membrane. Some of the major differences were that component VII is rich in acidic amino acids, cysteine and aromatic amino acids. On the other hand, it is low in glycine, hydroxylysine, and hydroxyproline. Most of the amino acid content of the whole membrane showed roughly the weight average value of the soluble and insoluble fractions within reasonable errors (Table VIII).

The similarities in the chemical compositions of the insoluble portion to the interstitial collagen are typically illustrated by such examples as a high amount of glycine and proline, the presence of

TABLE VIII

COMPARISON OF THE AMINO ACID PATTERNS

	Bovine basement membrane			Tendon collagen	Component VII
	Whole membrane	Soluble	Insoluble		
Glycine	208	150.6	242.3	329	133.9
Hydroxy amino acid	210	180.4	203.7	163	205.9
Acidic amino acid	164	183.3	151.4	114	209.0
Basic amino acid	115	119.9	104.3	78	105.0
Aromatic amino acid	52	54.9	37.1	18	61.0
Sulfur containing amino acid	45	40.5	26.9	6	39.0
Hydroxylysine	22	8.0	21.5	6	8.6
Lysine and hydroxylysine	49	43.4	50.9	29	37.9
Hydroxyproline	68	32.6	89.9	86	45.0
Proline	69	81.7	87.6	140	69.3
Imino acid	137	114.3	177.5	226	114.3

Numbers are expressed in residues per 1000 amino acid residues.

hydroxyproline, hydroxylysine, hydroxylysine glycosides, and lysine-derived crosslinks. The differences in the chemical composition of the insoluble portion from the collagen are the presence of cysteine, tryptophan, 3-hydroxyproline, and large amounts of carbohydrates.

When a comparison is made of component VII with the soluble fraction, the differences between component VII and collagen are greater than the similarities. The amount of glycine is relatively high in component VII, but only 40% of collagen level compared to 80% level of the insoluble fraction. The acidic amino acids (e.g., glutamic acid) and hydroxyproline, are twice that of the collagen level, while the insoluble portion contains comparable amounts to collagen. The only similarity between component VII and tendon, but not the insoluble fraction, is the hydroxylysine content which is found to have 8 residues in component VII, 6 in collagen, and 22 in the insoluble fraction.

The total carbohydrate residues in basement membrane is much higher than that of interstitial collagen, usually more than ten fold. Furthermore, the carbohydrates in collagen are mostly (90%) glucose and galactose (3). On the other hand, basement membrane contains mannose, hexosamine, fucose, and sialic acid which are components of a heteropolysaccharide as well as glucose and galactose. The total amounts of these carbohydrates in the soluble and insoluble fractions were essentially the same. However, the insoluble portion contains large amounts of glucose and galactose and much less other sugars.

As for carbohydrate composition, component VII contains all carbohydrate residues present in whole GBM. However, the total amount of carbohydrates among component VII, soluble and insoluble fractions, are roughly the same (8-11%) which are ten fold higher than that of

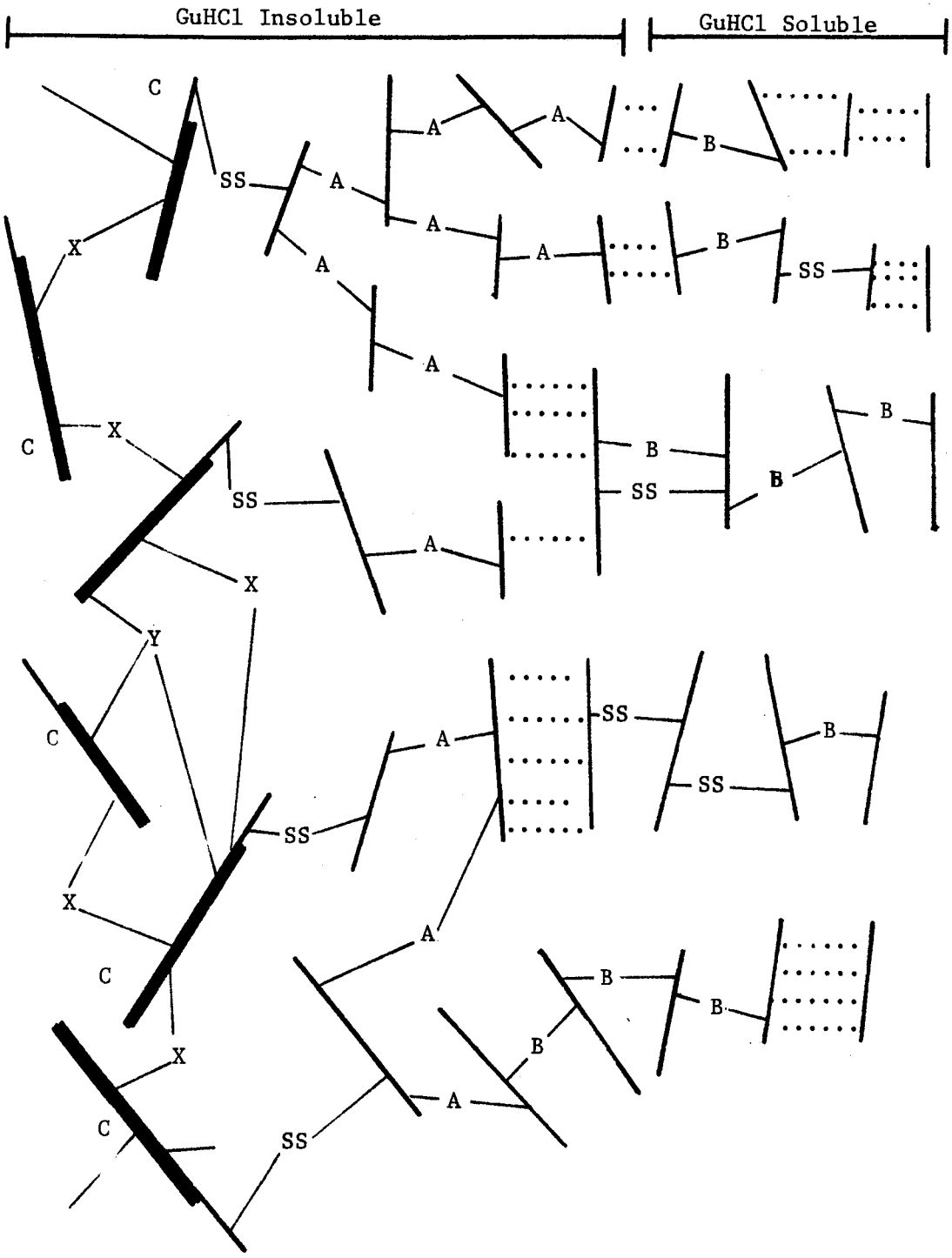
interstitial collagen. If the amount of glucose represents the number of disaccharide units, the ratio aof the amount of disaccharide units in the insoluble fraction and component VII is 4:1 in molar basis. The components of the heteropolysaccharide unit was reported by Spiro (10), as mentioned in the literature review. If the amount of fucose represents the number of heteropolysaccharide units, then the ratio of disaccharide units and heteropolysaccharide units in component VII is 3:1. Whole membrane showed the ratio, 10:1, therefore, component VII is shown to be rich in heteropolysaccharides and less in disaccharide unit since the total amount of carbohydrates is essentially the same. It is noteworthy that pure component VII contains both the disaccharide units and the heteroploysaccharide units on apparently the same polypeptide chain.

Since the proposed heteropolysaccharide unit did not include galactosamine, the direct estimation of the amount of heteropoly-saccharide is somewhat difficult. Component VII contains one hetero-polysaccharide unit per estimated molecular weight of 70,000 daltons, however, the glucasamine, 4 residues, and galactosamine, 2.5 residues, were found to be in excess. This may indicate the presence of another carbohydrate unit which contains glucosamine and galactosamine.

Several schematic diagrams of GBM were proposed by Kefalides and Spiro (43, 44), previously mentioned in the introduction. The actual structure of GBM cannot be expressed by these simple diagrams. Recently, Kefalides (99) proposed the overall structure of GBM in a simple but sophisticated diagram in which explains most of present knowledge of GBM. However, there are several problems which cannot be explained even with this scheme because of the following facts: (1) the

presence of a small molecular weight glycoprotein in the soluble fraction without reduction, (2) the reduction of the large molecular weight soluble components produces smaller components, (3) the large molecular weight components are produced by reduction of the insoluble fraction, (4) the lack of periodicity in GBM unlike collagen, (5) the collagen molecule, if it is not extensively crosslinked, can be soluble when the size of the smallest molecule is roughly a trimer of α_1 chain, and (6) the GuHCl soluble portion and so-called basement membrane collagen, $(\alpha_1(IV)_3)_n$, differ in chemical compositions. In order to satisfy the above facts, one may consider a somewhat different version of the structure of basement membrane, as illustrated in Fig. 19. The organization of subunit construction of the proposed basement membrane model is as follows: (1) the basement membrane collagenous portion, C, is visualized to contain a telopeptide which possesses the hetero-polysaccharide unit. This portion also contains cysteine residue(s); (2) the collagen portion is inter-connected mostly by lysine-derived weak covalent bonds, such as Schiff's base, illustrated as X. Some of the crosslinks are trifunctional crosslinks such as aldol-histidine, Y; (3) The arrangement of collagen portion is not polarized so that there is no periodicity as observed in interstitial collagen. However, these arrangements determine the "pore size" of the basement membrane for filtration. The non-collagenous proteins are connected mainly by disulfide bonds to collagenous peptides. Sometimes, other unknown cross-links are also involved. Some molecules do not change their molecular weight by disulfide bond reduction alone because of the involvement of unknown crosslinks. (4) Some intermediate size (M.W, 200,000), the non-collagenous component, can be freed from C by disulfide bond (SS)

Figure 19. Schematic Representation of Proposed Glomerular Basement Membrane


GuHCl Insoluble
GuHCl Soluble
C
X
SS
A
A
A
A
A
A
B
B
SS
A
C
X
SS
A
B
SS
B
B
X
Y
C
X
C
X
SS
A
SS
SS
B
A
B
B
B
A
C
X
SS

reduction. GuHCl soluble and the insoluble portions are also bonded non-covalently expressed as (.....). GuHCl soluble components may have some covalent bonds (B), but most of covalent bonds are disulfide bonds so that the reduction of larger components can produce smaller subunits and few components does not change its size by mercaptoethanol reduction alone.

CHAPTER V

SUMMARY

Glomerular basement membrane is soluble to the extent of 26% in 6.0 M guanidine hydrochloride. The soluble fraction contains at least 13 different polypeptide subunits as revealed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis after reduction with 2-mercaptoehtanol. The apparent molecular weight of these components ranges from 24,000 to greater than 350,000 daltons.

On the other hand, the insoluble fractions consist of large molecular weight components. The subunits, which are obtained from the insoluble fraction by mercaptoethanol reduction, were shown by SDS gel electrophoresis to have rather large molecular weights, ranging from 190,000 to 225,000 daltons.

The GuHCl soluble fraction contains several components. Subunit analysis of these components was performed by fractionation on a Bio-Gel A-15m gel filtration column followed by SDS gel electrophoresis of mercaptoethanol reduced fractions. The analysis revealed that: (1) some of these larger molecular weight components disappear after mercaptoethanol reduction to yield smaller molecular weight subunits, (2) certain components are not changed from their original molecular weight by reduction. It is apparent that most of subunits are held together by disulfide bonds to form larger molecular weight components and certain components are crosslinked by other bonds, possibly lysine-

derived crosslinking. One of the components of the guanidine soluble fraction, named component VII, was isolated in a pure form by repeated gel filtration column chromatography on Bio-Gel A-15m. The purity of isolated component VII was established by polyacrylamide gel electrophoresis with and without SDS using different gel concentrations, by ultracentrifugation and by gel filtration column chromatography.

The molecular weight of component VII was analyzed by several different systems which depend on different principles. The experiments showed the molecular weight of component VII to be 70,000 daltons, but the SDS system indicated 140,000 daltons due to an atypical behavior commonly observed with this type of molecule. The amino acid analysis showed that component VII has some similarities to interstitial collagen, but that it has much smaller amounts of glycine and proline compared to collagen and the GuHCl insoluble fraction. Component VII contains a large amount of acidic amino acids and aromatic amino acids. It also contains cysteine, tryptophan, and 3-hydroxyproline which are not generally found in interstitial collagen.

Carbohydrate analysis of component VII showed that it contained glucose, galactose, mannose, fucose, glucosamine, galactosamine, and sialic acids. The total carbohydrate content in component VII is ten fold higher than that of interstitial collagen. Glucose and galactose, which are constituents of the disaccharide unit, are found to be low compared to the insoluble portion and collagen. The component contains a high amount of hexosamines which are constituents of the heteropolysaccharide unit. The quantitative carbohydrate data indicate that component VII possesses one heteropolysaccharide unit and three dissaccaride units per molecule.

SELECTED BIBLIOGRAPHY

1. Kefalides, N. A. (1969) Arthritis and Rheumatism 12, 427.

2. Spiro, R. (1970) Diabetes: Theory and Practice (Ellenberg, M., and Rifkim, H., eds) pp. 210-229, McGraw-Hill, New York.

3. Kefalides, N. A. (1970) Chemistry and Molecular Biology of the Intracellular Matrix (Balazs, E. A., ed) pp. 535-574, Academic Press, New York.

4. Gallop, P. M., Blumenfeld, O. O., and Seifter, S. (1972) Annu. Rev. Biochem. 41, 617.

5. Venkatachalam, M. A., Karnousky, M. J., Fahimi, H. D., and Cotran, R. S. (1970) J. Exp. Med. 132, 1153.

6. Mahieu, P., and Winand, R. J. (1970) Eur. J. Biochem. 12, 410.

7. Venkatachalam, M. A. (1970) J. Exp. Med. 132, 1153.

8. Kefalides, N. A. (1970) Biochem. Biophys. Acta. 203, 62.

9. Beisswenger, P., and Spiro, R. G. (1970) Science 168, 596.

10. Spiro, R. G. (1967) J.Biol. Chem. 242, 1915.

11. Kefalides, N. A. (1971) Int. Rev. Exp. Patholo. 10, 1.

12. Mahieu, P., and Winand, R. J. (1970) Eur. J. Biochem. 12, 12.

13. Mahieu, P. (1972) Kidney 1, 115.

14. McPhaul, J. J., and Dixon, F. J. (1970) J. Clin. Invest. 49, 308.

15. Hudson, B. G., and Spiro, R. G. (1972) J. Biol. Chem. 247, 4229.

16. Hudson, B. G., and Spiro, R. G. (1972) J. Bio. Chem. 247, 4239.

17. Kefalides, N. A. (1971) Biochem. Biophys. Res. Commun. 45, 226.

18. Krakower, C. A., and Greenspon, S. A. (1951) Am. Med. Assn. Arch. Pathol. 51, 629.

19. Richterich, R., and Franj, H. F. (1960) Nature, 188, 498.

20. Thornblom, N. (1957) Acta. Med. Scand. 159, 369.

21. Lidsky, M. D. (1967) Arch. Biochem Biophys. 121, 491.

22. Lazarow, A., and Speidel, E. (1964) "Small Vessel Involvement in Diabetes Mellitus," (Siperstein, M., ed) Am. Inst. Biol. Sci., Washington, D. C., p. 127.

23. Bruchhausen, F. V., and Merker, P. (1967) Histochemie 8, 90.

24. Dische, R. M., Pappas, G. D., Grauer, A., and Dische, Z. (1967) Biochem. Biophys. Res. Commun. 20, 63.

25. Mahieu, P. M., Lambert, P. H., and Maghum-Rogister, G. R. (1973) Eur. J. Biochem. 40, 599.

26. Kefalides, N. A. (1968) Biochemistry 1, 3103.

27. Kefalides, N. A. (1969) Biochemistry 8, 4613.

28. Myers, C., and Bastlett, P. (1972) Biochem. Biophys. Acta 290, 150.

29. Blan, E. B., and Michael, P. (1971) J. Lab. Clin. Med 77, 97.

30. Gang, N. F., and Kalant, N. (1970) Lab. Invest. 22, 531.

31. Mahieu, P. M., and Winard, R. J. (1970) Eur. J. Biochem. 15, 520.

32. Misra, R. P., and Berman, L. B. (1966) Proc. Soc. Exp. Biol. Med. 122, 705.

33. Wahl, P., and Depperman, D. (1970) Klin. Wochenschr. 48, 653.

34. Ramachandram, G. N., Sasisekharan, V., and Thathachari, Y. T. (1962) Collagen (Ramachandram, G. N., ed) Interscience, New York, p. 81.

35. Beisswenger, P. J., and Spiro, R. G. (1970) Science 168, 596.

36. Spiro, R. G. (1967) J. Biol. Chem. 242, 4813.

37. Cunnigham, L. W., Ford, J. D., and Segrest, J. P. (1967) J. Biol. Chem, 242, 2570.

38. Mahieu, P. M., Lambert, P. H., and Maghuin-Rogister, G. R. (1973) Eur. J. Biochem. 40, 599.

39. Morgan, P. H., Jacobs, H. G., Segrest, J. P., and Cunnigham, L. W. (1970) J. Biol. Chem. 245, 5042.

40. Schwartz, C. E. (1972) Master Thesis, Oklahoma State University.

41. Kefalides, N. A. (1966) Biochem. Biophys. Res. Commun. 22, 26

42. Tanzer, M. L., and Kefalides, N. A. (1973) Biochem. Biophys. Res. Commun. 51, 775.

43. Spiro, R. G., and Fukushi, S. (1969) J. Biol. Chem. 244, 2041.

44. Spiro, R. G. (1967) J. Biol. Chem. 242, 1923.

45. Grant, M. E. (1972) New Engl. J. Med. 286, 194.

46. Blumenkrantz, N., Rosenbloom, J., and Prockop, D. (1969) Biochem. Biophys. Acta 192, 81.

47. Vuust, J., and Piez, K. A. (1972) J. Biol. Chem. 247, 856.

48. Cardruale, G. J., Rpoad, R. E., and Udeufriend, S. (1971) Biochem. Biophys. Res. Commun. 43, 537.

49. Miller, R. L., and Udeufriend, S. (1970) Arch. Biochem. Biophys. 139, 104.

50. Spiro, R. G., and Spiro, M. J. (1971) Diabetes 20, 641.

51. Spiro, R. G., and Spiro, M. J. (1968) Fed. Proc. 27, 345.

52. Spiro, R. G., and Spiro, M. J. (1968) Diabetes 17, 311.

53. Spiro, M. J., and Spiro, R. G. (1968) J. Biol. Chem. 243, 6520.

54. Spiro, M. J., and Spiro, R. G. (1968) J. Biol. Chem. 243, 6529.

55. Kurtz, S. M., and Feldman, J. D. (1962) J. Ultrastruct. Res. 6, 19.

56. Johnson, L., and Stacher, B. C. (1972) Biochem. Biophys. Acta 290, 158.

57. Davis, N. R. (1973) Biochem. Biophys. Res. Commun. 54, 914.

58. Tanzer, M. L., Housley, T., and Berube, L. (1973) J. Biol. Chem. 248, 393.

59. Viidik, A. (1973) Int. Rev. Connect. Tissue Res. 6, 197.

60. Perry, S. V. (1955) Method in Enzymology II, Academic Press, New York.

61. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406.

62. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606.

63. Hedrick, J. L., and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155.

64. Ornstein, L., and Davis, B. J. (1962) Disc Electrophoresis, Distillation Products Industries, Rochester.

65. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265.

66. Geiger, P. J., and Bessman, S. P. (1972) Anal. Biochem. 49, 467.

67. Yphantis, D. A. (1964) Biochemistry 3, 297.

68. Hade, E. P., and Tanford, C. (1967) J. Amer. Chem. Soc. 89, 5034.

69. Castellino, F. J., and Barker, R. (1968) Biochemistry 7, 2207.

70. Fish, W. W., Mann, K. G., and Tanford C. (1969) J. Biol. Chem. 244, 4989.

71. Mann, K., and Fish, W. W. (1972) Methods Enzymol. 26, 28.

72. Castellino, F. J. (1970) J. Biol. Chem. 245, 417.

73. Guire, P., Riquetti, P., and Hudson, B. G. (1974) J. Chromatogr. 90, 350.

74. Bencze, W. L., and Schmid, K. (1957) Anal. Chem. 29, 1193.

75. Odell, V., Wegener, L., Peczon, B., and Hudson, B. G. In printing.

76. Warren, L. (1959) J. Biol. Chem. 234, 1971.

77. Gershman, L. C., and Dreizen, P. (1969) Biophys. J. 9, A235.

78. Slayer, H. S., and Lowey, S. (1967) Proc. Nat. Acad. Sci., U.S.A. 58, 1611.

79. Ullmann, A., Goldberg, M. E., Perrin, D., and Monod, J. (1968) Biochemistry 7, 261.

80. Seery, V. L., Fisher, E. H., and Teller, D. C. (1967) Biochemistry 6, 3315.

81. Castellino, F. J., and Barker, R. (1968) Biochemistry 7, 2207.

82. Rutishauser, V., Cunningham, B. A., Bennett, C., Konigsberg, W. H., and Edelman, G. (1968) Proc. Nat. Acad. Sci., U.S.A. 61, 1414.

83. Chen, Y. H., and Yang, J. T. (1971) Biochem. Biophys. Res. Commun. 44, 1285.

84. Westberg, N. G., and Michael, A. F. (1972) Diabetes 21, 163.

85. Westberg, N. G., and Michael, A. F. Acta Med. Scand. In press.

86. Hudson, B. G. In press.

87. Westberg, N. G., and Michael, A. F. (1970) Biochemistry 9, 3837.

88. Gallop, P. M., Blumenfeld, O., and Seifer, S. (1972) Annu. Rev. Biochem. 41, 617.

89. Davis, N. R. (1973) Biochem. Biophys. Res. Commun. 54, 914.

90. Bailey, A. J., and Peach, C. M. (1971) Biochem. J. 121, 257.

91. Mechanic, G., Gallop, P. M., and Tanzer, M. L. (1971) Biochem. Biophys. Res. Commun. 45, 644.

92. Paz, M. A., Henson, E., Blumenfield, O. O., Seifer, S., and Gallop, S. (1971) Biochem. Biophys. Res. Commun. 44, 518.

93. Franzblau, C. (1969) Biochemistry 8, 2833.

94. Nelson, C. A. (1971) J. Biol. Chem. 246, 3895.

95. Sergest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971) Biochem. Biophys. Res. Commun. 44, 390.

96. Furthmayr, H., and Timple, R. (1971) Anal. Biochem. 41, 510.

97. Jirgensons, B., and Capetillo, S. (1970) Biochem. Biophys. Acta 214, 1.

98. Spiro, R. G. (1972) Glycoprotein, Elsevier Publishing Company, New York.

99. Kefalides, N. A. (1973) Int. Rev. Connect. Tissue Res. 6, 63.

100. Tanford C., Kawahara, K., and Lapanje, S. (1967) J. Amer. Chem. Soc. 89, 729.

101. Grant, M. E., Kefalides, N. A., and Prockop, D. J. (1972) J. Biol. Chem. 247, 3539.

102. Grant, M. E., Kefalides, N. A., and Prockop, D. J. (1972) J. Biol. Chem. 247, 3545.

103. Grant, M. E., Schofield, J. D., Kefalides, N. A., and Prockop, D. J. (1973) J. Biol. Chem. 248, 7432.

104. Grant, M. E., and Prockop, D. J. (1972) New Engl. J. Med. 286, 242.

105. Grant, M. E., and Prockop, D. J. (1972) New Engl. J. Med. 286, 291.

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