PHYSICAL PROPERTIES OF THE BOVINE GLOMERULAR BASEMENT MEMBRANE

By

Charles Edward Schwartz

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GLOMERULAR BASEMENT MEMBRANE

Thesis Approved:

Billy D. Hudson
Thesis Adviser

Earl D Mitchell

Dean of the Graduate College

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INTRODUCTION

Statement of Problem

Basement membranes are extracellular matrices found in numerous tissues and existing between epithelial and endothelial cells. These membranes have two important functions: they offer support, as in the case of the lens capsule and Descemet's membrane of the cornea and play an essential role in the differential permeability to molecules across epithelial surfaces and capillary walls, as exemplified by the capillaries of the vascular tree, the alveoli of the lung and the glomerular basement membrane (1, 2).

Most studies on basement membranes have centered on the glomerular membrane because of its important physiological role as the dialyzing membrane of the kidney and the changes that occur in its morphological structure in various disease states and kidney transplants. This membrane thickens two or more times the normal following the onset of such diseases as diabetes mellitus (3, 4), lipoid nephrosis (4), and chronic glomerulonephritis (4). Although its anatomic structure, as revealed by the electron microscope, remains quite similar to the normal, the passage of larger molecular weight proteins through the altered membrane is significantly increased (4). Early thickening of the membrane

has frequently been seen in renal homografts transplanted into modified recipients (5, 6, 7) and changes most closely resembling glomerulonephritis have been observed in long surviving renal homografts (8).

At present only a very limited amount of information is available concerning the basement membrane structure. The amino acid and carbohydrate composition has been determined for a select few and shown to be of glycoprotein nature representing a specialized form of collagen. The types of carbohydrate units present and their mode of attachment to polypeptide chains has been determined only for glomerular basement membrane and lens capsule and the structure of the simpler unit elucidated. No information is available regarding the structure of the complex heteropolysaccharide unit or the site of attachment of both units along peptide chains. Beyond the amino acid composition, nothing, is known about the primary, secondary, and tertiary structure of the protein portion of all basement membranes.

The purpose of this study was to determine some of the chemical and physical properties of the native glomerular basement membrane and its subunits. Structural knowledge of this type is fundamental to an understanding of its physiological role as an ultrafilter and a tissue support, and of chemical alterations that occur in diseases and tissue transplants.

Survey of Literature

The glomerular basement membrane was first isolated for chemical analysis by Krakower and Greenspon (9, 10). The method was further improved by Spiro (11). Simply, the method involves sectioning the kidney cortex into thin slices, followed by the use of molecular sieves

to both disrupt the cortex tissue and separate the intact glomeruli from other elements. Finally the membrane is isolated as an insoluble residue after disruption of glomeruli by ultrasonication.

The chemical composition of the glomerular basement membrane has been determined by several investigators and shown to be of glycoprotein nature (11-18). The amino acid composition is quite similar to collagen and other basement membranes from other tissues as shown in Table I. Both the similarities and dissimilarities between the GB-membrane and collagen are readily apparent. Both are rich in hydroxyproline, proline, hydroxylysine and glycine. However, whereas glycine accounts for just less than a third of the total amino acid residues in collagen, it accounts for about one-fourth in the GB-membrane. In the glomerular basement membrane, just over a tenth of the amino acid residues are attributed to hydroxyproline-proline, while on the contrary these two amino acids account for about one-fourth in collagen. Also striking is the difference in hydroxylysine content, the GB-membrane one being considerably higher. Finally, collagen, unlike the membrane, contains no half-cystine.

The carbohydrate composition of the basement membrane appears in Table II. The sugar residues account for 8.9 percent of the membrane weight which correspondes to 59 sugar residues per 1000 amino acid residues. Glucose and glactose appear in almost equimolar amounts and are responsible for over 70% of the neutral hexose content. Mannose and fucose account for the remainder. Glucosamine, galactosamine and sialic acid are also present.

TABLE I

AMINO ACID COMPOSITION OF CANINE BASEMENT MEMBRANES AND TENDON COLLAGEN[a]

Amino Acid	Glomerulus	Anterior Lens Capsule	Choroid Plexus	Tendon Collagen
	Residues/1000 Amino Acid Residues			
Hydroxylysine	25.0	32.0	9.0	6.0
Lysine	26.0	11.0	41.0	23.0
Histidine	14.4	12.0	16.0	4.0
Arginine	48.2	38.3	61.0	45.0
3-Hydroxyproline	8.5	19.5	Tr.	--
4-Hydroxyproline	56.5	80.0	48.3	86.0
Aspartic	70.0	57.8	68.0	46.0
Threonine	40.5	31.0	39.0	17.0
Serine	49.0	48.0	57.8	33.0
Glutamic	97.0	95.0	94.0	63.0
Proline	69.8	66.0	69.0	140.0
Glycine	229.0	288.0	235.6	329.0
Alanine	65.0	46.1	104.5	112.0
Half-cystine	22.7	18.0	10.0	0.0
Valine	36.0	35.0	37.0	20.0
Methionine	5.0	6.2	9.4	6.0
Isoleucine	28.1	30.0	22.5	10.0
Leucine	60.2	56.5	50.0	21.0
Tyrosine	22.0	11.0	12.0	5.0
Phenylalanine	26.8	29.0	21.5	13.0

[a]Data taken from (1).

TABLE II

CARBOHYDRATE COMPOSITION OF BOVINE GLOMERULAR BASEMENT MEMBRANE

Monosaccharide	Residue Wt. g/100g membrane[a]	Residue/1000 Amino Acid Residues[a]
Sialic Acid	1.12	4.44
Hexosamines	1.95	11.1
Glucose	2.22	16.4
Galactase	2.75	20.2
Mannose	.69	5.15
Fucose	.20	1.55

[a]Spiro (11).

The chemical structure of the carbohydrate portion and its attachment to the polypeptide chain has been elucidated by Spiro (12). The carbohydrate portion is distributed about two different types of units. Their number and composition are given in Table III. One unit is a heteropolysaccharide consisting of sialic acid, fucose, galactose, mannose and hexosamines. It is believed that the detailed structure of this unit is similar to those reported for other glycoproteins such as fetuin (19-21) and thyroglobulin (22), as given in Figure 1, and that it is attached to the polypeptide via carbon 1 of the most internal glucosamine to the amide nitrogen of asparagine.

TABLE III

CARBOHYDRATE UNITS OF BOVINE GLOMERULAR BASEMENT MEMBRANE[a]

	Disaccharide Unit	Heteropolysaccharide Unit
Monosaccharides, moles/mole		
Glucose	1	
Galactose	1	4
Mannose		3
Glucosamine		5
Sialic Acid		3
Fucose		1
Molecular Wt.	324	3500
Percentage distribution by weight of carbohydrate of membrane	50	50
Relative number of units in membrane	10	1
Amino acid likage	Hydroxylysine	Asparagine

[a]Data taken from (12).

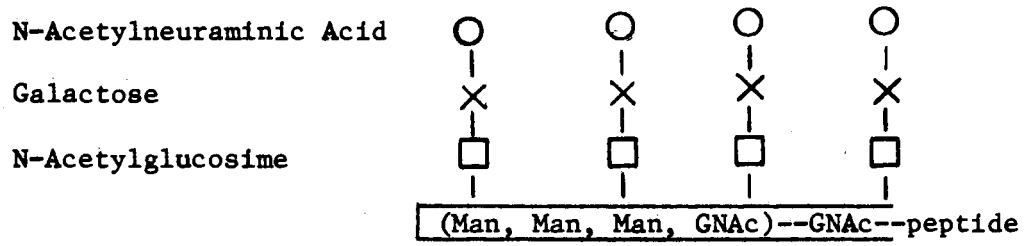

Figure 1. Schematic representation of entire carbohydrate unit, depicting four oligosaccharide chains attached to the core portion. (Taken from studies on fetuin (19-21).)

The second unit is a disaccharide containing glucose and galactose and is linked to the protein through the hydroxyl group of hydroxylysine. The structure is given in Figure 2 and is characterized by a glycosidic bond between glucose and carbon 2 of galactose and a glycosidic linkage between galactose and hydroxylysine.

The glomerular basement membrane, then, belongs to the collagen family of proteins. It is a member by virtue of the presence of hydroxylysine, hydrosyproline and a high amount of glycine even though it differs in relative amounts of these three amino acids when compared to collagen. However, there exists some experimental evidence that the membrane is not a collagen but rather it contains a collagen contaminate (1, 14-16).

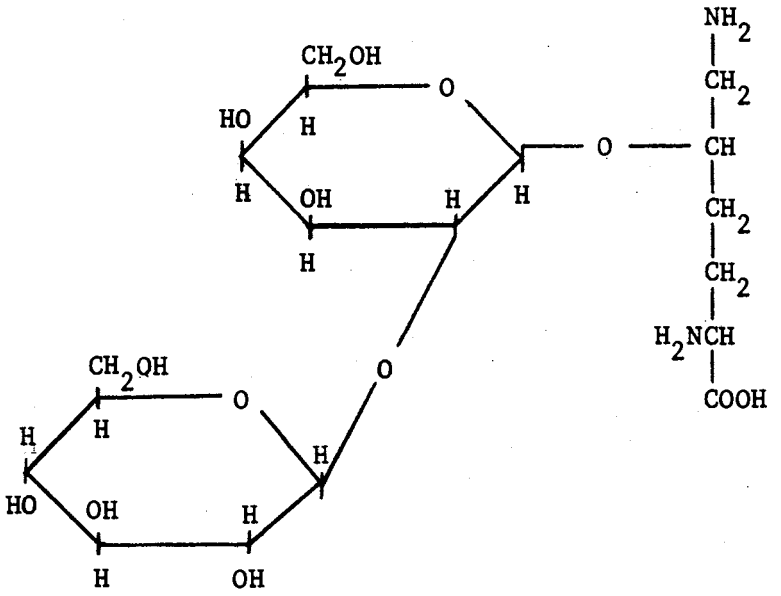

Figure 2. Structure and peptide attachment of the disaccharide unit of the glomerular basement membrane. (2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine). [Taken from Spiro (12)].

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials and Reagents

Sepharoses 4B and 2B, Blue Dextran 2000 and chromatographic columns were obtained from Pharmacea. Cytochrome C (horse heart, Type II), and ovalbumin were purchased from Sigma. Both myoglobin (Equine heart, 2X crystallized) and collagen (calf skin, acid soluble, A grade) were products of Calbiochem and bovine serum albumin came from Mann Laboratories. Chymotrypsinogen was a product of Nutritional Biochemicals Corp. and the acetic anhydride (^{3}H-Ac_2O, 25 mC/mmole) was obtained from Amersham/Searle. The bovine kidneys were obtained from Wilson Certified Foods within one hour of death. Guanidine carbonate came from Eastman and Triton X from Packard.

Two different grades of guanidine HCl were purchased from various sources. The reagent grade was a product of Sigma. Two grades of high purity came from Schwarz-Mann Research Laboratories (Ultra Pure guanidine HCl) and Heico (Extreme Purity guanidine HCl).

Methods

Purification and Preparation of Guanidine HCl

It was unnessary to further purify Schwarz-Mann's Ultra-Pure or Heico's Extreme Purity guanidine HCl. However, the guanidine HCl

obtained from Sigma was further purified by decolorization according to Nozaki and Tanford (23).

When guanidine HCl was unavailable, it was prepared from guanidine carbonate by the method of Nozaki (24), with one slight modification. The guanidine HCl obtained from repeated flash-evaporations of the titrated carbonate solution was dried to ensure correct concentration upon dissolving in water. It was found the guanidine HCl synthesized from the carbonate was sometimes superior to that from Sigma in terms of low absorbance at 280 nm.

Preparation of Myosin

Myosin was prepared from the rabbit muscle by the method of Perry (25) with minor modification. The dorsal and leg muscles were dissected and chilled in ice. When completely cold, the muscle was pushed through a meat grinder at 0° C, weighed and immediately extracted with three volumes of cold KCl-potassium phosphate buffer, pH 6.5. After stirring for 15 minutes, the suspension was centrifuged for 10 minutes at 6000 x g using a GSA rotor. The supernatant was gently filtered through cheese cloth resting over a beaker.

The crude extract was added to 14 volumes of distilled water in a slow manner, with stirring, and allowed to stand overnight at 0° C in order to bring down the myosin as crystals. The crystals were removed by centrifugation, the resulting gel being transferred to a graduated cylinder using as little supernatant as possible and the volume was noted. Solid KCl was added to bring the ionic strength to 0.5, solubilizing the myosin.

The pH of the solution was adjusted to 6.6 with a small amount of $NaHCO_2$. Water was then added to lower the ionic strength to 0.3, precipitating out any actomyosin. The latter was removed by centrifugation at 27,000 rpm on a Model L with 30 rotor for 1-1½ hours.

The myosin was reprecipitated by lowering the ionic strength to 0.04. Denaturation was avoided by adding the distilled water slowly, while stirring with a glass rod, at 0° C and within 15 minutes. The precipitate was allowed to settle overnight, centrifuged at 7000 rpm, using a GSA rotor, for 20-30 minutes and dissolved up by increasing the ionic strength to 0.5. The pH was adjusted to 6.5 and the reprecipitation procedure was repeated.

After the final precipitation, the myosin was dissolved by increasing the ionic strength to 0.5 and stored as concentrated as possible at 0° C in a flask with a trace of toluene on the stopper and containing minimum air space. The yield from 500 g of minced muscle was a 130 ml solution of a concentration of 24.5 mg/ml as determined by the Lowry method. The preparation was quite pure as checked by 5% SDS-acrylamide gel electrophoresis.

Preparation of Bovine Glomerular Basement Membrane

Isolation of Glomeruli

The isolation procedure was based on the method of Krakower and Greenspon (9) as improved by Spiro (11) with a modification. In place of slicing the cortex from the kidney with a scapel, as had been done in the past, another method was implemented. After the kidney had been taken from storage at -20° C, allowed to thaw and unwrapped from the

foil, the cortex was "peeled" off in long, thin strips by a ordinary potato peeler. The result was cortex shavings of a uniform consistency and a diminished number of traumatized glomeruli. The routine also increased the yield and purity of the preparations. The shavings were subsequently collected on a glass tray and transferred to a 600 ml beaker, kept in ice. A record was made of the amount of cortex obtained.

The cortex was then pushed through a No. 120 sieve in batches of 50 grams. Before the actual pushing, the cortex was "worked up." This was achieved by spreading cortex around with the bottom of a 250 ml beaker--with no applied pressure--until the color changed from its characteristic red to an orange. The material was then pushed through using an edge of the beaker and short strokes across the surface of the sieve, slight downward pressure being applied. The entire sieve surface was utilized. The resultant soup was scraped off the underside with a rubber spatula into a sieve pan resting in ice.

The thick solution was poured into a 4 l flask and diluted to 3 l (for 800-850 grams of cortex) with 0.85% saline solution. The solution was drained through two sieves in 400 ml portions. A No. 140 sieve was placed on top of the sieve pan and a No. 70 on the 140. The solution was poured such that initially it stayed on the No. 70 sieve. It was hastened through by a light double bounce action of the sieve and pan on a rubber mat. Care was taken to use as little bouncing as possible as it decreases yield when in excess. The glomeruli were collected on the No. 140 sieve and the rest of the solution was discarded from the pan.

After all 3 l had been filtered, the white froth on the underside of the No. 70 sieve was washed onto the No. 140. The material on the No. 140 was washed three times with 400 ml of 0.85% saline, using the double bounce technique employed previously. Initially, the sieve pan was filled half way with saline and after each washing was emptied to its original volume.

After the third wash, a slide was made and the purity checked using a phase microscope. Figure 3 displays the glomeruli as they appeared at this stage. A count was made of the number of glomeruli, capsules, tubules and other fragments and, if the impurities totaled less than 10% of the total glomeruli, no further washing was done. The glomeruli were then centrifuged at 1500 rpm for 15 minutes using a SS-1 head on a Sorvall RC-2B. The precipitate was stored at -20° C.

Isolation of Membrane from Glomeruli

Preparations representing 15.3 Kg of cortex were dispersed in a 500 ml beaker and poured into a 500 ml graduated cylinder, kept in an ice bath. The volume was diluted to 342 ml with cold 0.85% NaCl. Again, the pellets were dispersed with a glass rod. A 5 ml sample was taken for future reference. The solution was then diluted to 456 ml with 4M NaCl to give a final concentration of NaCl of approximately 1M.

Sonic disruption was performed on portions of 75 ml in a 100 ml beaker set in an ice bath. A Branson Sonifier, Model S125, was used with the probe terminating 1/2 inch from the top of the solution. The disruption was conducted at No. 6 setting for four one-minute intervals. Extent of glomerular disruption was followed under phase microscope.

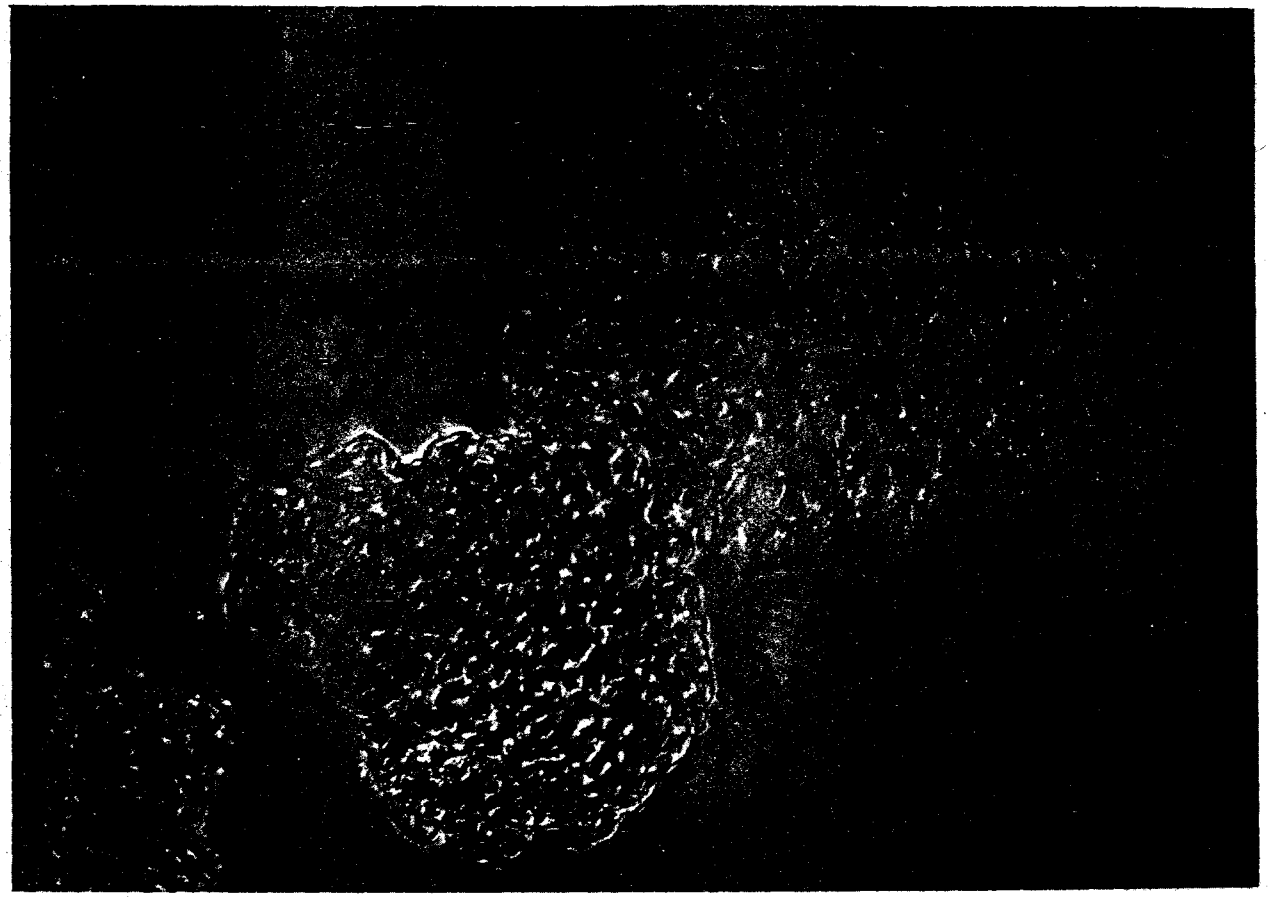

Figure 3. Photomicrograph taken through phase-contrast microscope of bovine glomeruli (x 100).

The sonically treated material was transferred to 12 40-ml centrifuge tubes and centrifuged for 10 minutes at 3000 rpm on the Sorvall RC-2B. The sediment was washed five times with cold 1M NaCl followed by five washings with cold distilled water. The final sediment was suspended in water overnight to allow oil contamination and particles to sediment out. The useable material was suctioned off, suspended in 85 ml of distilled water, and lyophilyzed.

The 5 ml aliquot was washed five times with water in the same manner as the basement membrane and lyophilyzed to yield 0.12092 g of glomeruli. The total weight of basement membrane after lyophilization was 2.25 g which represented a 27.2% yield from washings and sonification.

Preparation of S-Carboxymethylated Standard Proteins

For bovine serum albumin, ovalbumin and chymotrypsinogen, 200 mg were dissolved in 50 ml of 8.0M urea, 0.5M Tris buffer at pH 8.5. Under nitrogen, a 150 fold excess of SH groups of 2-mercaptoethanol (1.14 ml for bovine serum albumin, 0.19 ml for ovalbumin, 0.63 ml for chymotrypsinogen) was added and the flask stoppered and shaken for 12 hours. Another addition of 2-mercaptoethanol (75 fold excess) was made under nitrogen and the flask was again shaken for 12 hours. After 24 hours total reduction, 1.5M Iodoacetic acid in 8.0M urea, 0.5M Tris buffer at pH 8.5, was added in two fold excess of the total amount of 2-mercaptoethanol present (21.8 ml for bovine serum albumin, 3.56 for ovalbumin, 12 ml for chymotrypsinogen). The pH was maintained at 8.5 with the addition of 10N NaOH. Alkylation was allowed to proceed for one-half hour. The entire process was carried out under nitrogen. The mixture was then cooled to 4° C and dialyzed in No. 18

Viking casing against four changes of water for 48 hours. The material was then transferred to a Virtis flask and lyophilyzed and stored in the freezer until used. The reduced and alkylated material was checked by amino acid analysis (6N HCl hydrolysis for 24 hours) on a Beckman 120C.

For myosin, 2.86 g of guanidine HCl was added to 2.5 ml of the 25 mg/ml stock solution, along with 1 ml of 1M Tris buffer, pH 8.5. The resulting mixture was diluted to 6 ml and transferred to a vial. Under nitrogen, 0.04 ml of 2-mercaptoethanol was added. The vial was stoppered and shaken for 12 hours. Under nitrogen, an equal volume of 0.1M Iodoacetic acid in 6M guanidine HCl, 0.2M Tris buffer, pH 8.5, was added and the vial again stoppered. Alkylation continued for one hour. The mixture was dialyzed in No. 18 Viking casing against four changes of water at 4^{o} C for 48 hours. The material was lyophilyzed and stored in the freezer. The reduced and alkylated myosin was checked by amino acid analysis.

The reduction and alkylation procedure for Cytochrome C was essentially the same as that used for myosin with some volume differences, since solid protein was employed. Again the reduction and alkylation was checked by amino acid analysis.

Preparation of S-Carboxymethylated Glomerular Basement Membrane

A third method was employed for the reduction and alkylation of the various bovine glomerular basement membrane samples run on the column. Fifty mgs were dissolved in 5 ml of 6M guanidine HCl, 0.2M Triss buffer at pH 8.5. The buffer had previously been purged with nitrogen. Nitrogen was passed over the top of the vial as a 150 fold excess (of SH groups) of 2-mercaptoethanol (0.14 ml) was added. The

stoppered vial was agitated for 12 hours before another addition of 2-mercaptoethanol (75 fold excess) again under nitrogen. After a further 12 hours of shaking, a two fold excess (of total 2-mercaptoethanol) of 1.5M Iodoacetic acid in 6M guanidine HCl, 0.2M Tris buffer, pH 8.5, (3.6 ml) was added under a nitrogen atmosphere. The pH was maintained at 8.5 with the addition of 10N NaOH. After five and one-half minutes more mercaptoethanol equal to the previous total (0.2 ml) was added, the pH again maintained at 8.5 with 10N NaOH. One ml was dialyzed against four changes of water at 4^{o} C, lyophilyzed and submitted for amino acid analysis.

Solubility Determination of Glomerular Basement Membrane in 6.0M Guanidine HCl

The solubility analysis of the basement membrane was accomplished by suspending varying amounts of the material in 1 ml of 6.0M guanidine HCl (Mann Ultra Pure). The suspensions were agitated for 48 hours, then centrifuged at 10,000 rpm on a Sorvall RC-2B, with SS-1 head, for 20 minutes. The supernatants were taken for analysis, and read at 280 nm on an Hitachi Perkin-Elmer double bean spectrophotometer, model 124, using 1 ml cuvettes. The amount of membrane present was calculated using an $\varepsilon_{280} = 0.814\ cm^{-1}\ mg^{-1}$, derived from the UV spectra of a known amount of guanidine soluble fraction in 6.0M guanidine HCl.

Preparation of Guanidine HCl Soluble and Insoluble Fractions

These fractions were prepared by Mikio Ohno according to the following procedure. Fifty mgs of native basement membrane were dissolved in 10 ml of 6.0M guanidine HCl, 0.1M phosphate, pH 7.0. The solution was shaken for 24 hours at 37^{o} C and the fractions were separated by centrifugation at 5,000 rpm, using a SS-1 rotor on a

Sorvall RC-2B. The insoluble fraction was washed three times with 6.0M guanidine, the washes being pooled with the soluble fraction. This latter portion was dialyzed against water for 48 hours, lyophilyzed and stored at -20° C. The insoluble fraction was washed with water, lyophilyzed and stored at -20° C.

Ultraviolet Spectral Analysis

One-half of a ml of the reduced and alkylated basement membrane was dialyzed against four changes 20 ml of Heico 6.0M guanidine HCl at room temperature for 48 hours. It was then quantitively transferred to a test tube and diluted with Heico 6.0M guanidine HCl to give a final concentration of 0.3 mg/ml.

Collagen and lyophilyzed reduced and alkylated bovine serum albumin were dissolved in Heico 6.0M guanidine HCl to give a concentration of 0.3 mg/ml.

All protein solutions were titrated to acidic and basic pH's and blanked against Heico 6.0M guanidine HCl at the respective pH's. All spectral work was performed on a Hitachi Perkin-Elmer double beam spectrophotometer, Model 124, using 3 ml cuvettes.

Infrared Spectral Analysis

Samples for infrared analyses were prepared by mixing 2 mg of the desired protein with 40 mg of dry KBr. The two were mixed with an agate mortar and pestle set for 10-15 minutes. The powder was then placed in a desiccator containing Drierite and a vaccum was pulled for at least 12 hours. About half of the powder was then used to make a clear pellet using an hydraulic press upon which a vaccum was pulled. The clear pellet, if not immediately used, was returned to the desiccator.

All IR spectra were obtained with a Beckman IR-7.

Circular Dichroism Spectral Analysis

One-half ml of reduced and alkylated basement membrane was dialyzed exhaustively against four changes of 20 ml of Heico 6.0M guanidine HCl for 48 hours at room temperature. It was then quantitively transferred to a test tube and diluted with Heico 6.0M guanidine HCl to a concentration of 0.9 mg/ml as calculated using an ε_{280} of 0.55 cm^{-1} mg^{-1} ml.

Approximately 20 mg of myoglobin was dissolved in 5 ml of water. One-half ml aliquot was then lyophilyzed and dissolved in 10 ml each of Heico 6.0M guanidine HCl and 0.2M NaF. The concentrations were 0.14 and 0.15 mg/ml, respectively, using an ε_{280} of 3.79×10^4 (26).

Circular dichroism data are presented as mean residue ellipticity, $[\theta]$, in degrees x cm^2 x $decimole^{-1}$, which was calculated using the following formula:

$$[\theta] = \frac{\theta^\circ}{10} \frac{M}{\ell C'}$$

where: M is the gram-molecular weight of the sample;

C' is its concentration in grams per cm^3; and

ℓ is the pathlength of the sample solution in cm.

$[\theta]$ has the dimensions of degree-cm^2 per decimole.

The mean residue weights employed were 112.4 for myoglobin (26) and 103 for the basement membrane, calculated from composition data (11). Absorbance spectra were obtained using a Cary Model 61 CD spectropolarimeter and 0.1 cm cells.

Determination of Molecular Weights of Basement Membrane Subunits by Gel Filtration Chromatography in 6.0M Guanidine HCl

Preparation of Column

The fines were removed from the Sepharose 4B by diluting with six volumes of water, swirling the solution and the allowing it to stand. After the gel settled, the supernatant was poured off. This process was repeated four times. To the gel was added an appropriate amount of dry guanidine HCl to make the solvent 6M. The gel was swirled gently until the guanidine HCl dissolved and was then allowed to equilibrate overnight. The gel suspension was degassed just before pouring the column.

A Sephadex chromatography column (90 x 1.5 cm) fitted with Teflon capillary tubing was packed to a height of 84 cm under a pressure head of 15 cm of solvent (27). This head was maintained at all times with a 250 ml Marriot flask. A Swinny filter containing either a prefilter or a disc of Whatman No. 1 filter paper was fitted to the Marriot flask to prevent build up of dust particles on the top of the column. The filter was changed each time a sample was applied. These conditions permitted a flow rate of 2-3 ml/hr. Prior to sample application, the column was permitted to flow for two days.

Calibration of Column with Standard Proteins

The proteins used for standards and their molecular weights are listed in Table IV. The molecular weights used were the best values from the literature.

Blue Dextran 2000 was used to measure the void volume (V_o). DNP-Alanine was used as a marker to determine the total volume accessible to the solvent (V_1).

TABLE IV

PROTEINS USED IN CALIBRATION OF SEPHAROSE 4B COLUMN

Protein	Molecular Wt.	Reference
Myosin	212,000	28
Bovine Serum Albumin	69,000	29
Ovalbumin	43,000	30
Chymotrypsinogen	25,700	29
Cytochrome C	12,400	31

Two separate standard mixtures were prepared from the reduced and alkylated proteins to calibrate the column. One solution contained 10 mg bovine serum albumin, ovalbumin and chymotrypsinogin in 1 ml of 6M guanidine HCl. To this was added DNP-Alanine, blue Dextran and sucrose to make their concentrations 0.05%, 0.3% and 10%, respectively. The second solution had the same concentration of DNP-Alanine and sucrose plus 10 mg of myosin and 5 mg of cytochrome C per ml. For calibration runs, 0.15 ml of solution 1 and 0.3 ml of solution 2 were placed on the column. The solutions were run separately.

To obtain maximum precision in the position of elution for a substance, the method of Fish (27) of weighing the fractions was used. Fractions were collected on a ISCO Golden Retriver set on volume. The fraction size ranged from 0.7 to 0.9 g. The fractions were monitored by absorbance at 280 nm for proteins. Cytochrome C and its heme

containing peptide were monitored at 410 nm. Blue Dextran was followed at 630 nm and DNP-Alanine at 360 nm.

The elution position of each protein was expressed in terms of a distribution coefficient, K_d, commonly used in expressing gel filtration results (32). K_d is equal to $(V_e-V_o)/(V_i-V_o)$ where V_e is the elution position of the substance expressed in grams, V_o is the grams of solvent in the column external to the gel matrix and V_i-V_o is the grams of solvent within the gel matrix.

Estimation of Molecular Weight of the Subunits of the Bovine Glomerular Basement Membrane

The membrane subunit distribution and molecular weight were determined with use of the calibrated Sepharose 4-B column using 6.0M guanidine HCl as eluent. Because of the low solubility of the reduced and alkylated membrane in 6.0M guanidine HCl, it was necessary to monitor column fractions with a more sensitive technique. To accomplish this, membrane was labeled with tritium and column fractions monitored for radioactivity. To an aliquot, 1 to 2.5 ml, of the reaction mixture from the reduction and alkylation of membrane, containing 5 mg protein/ml, was added 5 µl of a 25 mC/275 µl ^{3}H-acetic anhydride solution. The mixture was shaken for one hour at room temperature and then dialyzed extensively against 6.0M guanidine HCl.

For chromatography 0.1 to 0.3 ml of the labeled mixture, 10% in sucrose, was applied to the column and run under identical conditions as standard protein. A small amount of Cytochrome C was sometimes added to check calibration shifts which might have occurred. For determination of tritium in fractions, aliquots (0.4 ml) were pipetted into counting vials to which was added 0.4 ml of water and 10 ml of liquid

scintillation fluid, prepared according to Castellino, et.al. (33) for measurements in guanidine solution. The vials were shaken and allowed to stand until the solution became clear. Radioactive counting was done with a Packard Liquid Scintillation Spectrometer, Model 3320.

Quantitative Analysis of Amino Acid and Carbohydrate Components

Amino Acid Analysis

For amino acid analysis, protein samples were dissolved in 6N HCl at a concentration of 1 mg/ml. Solutions were frozen, evacuated, and flushed with nitrogen. The sample was thawed and the process repeated several times and the tube sealed under reduced pressure. After hydrolysis at 110° C for 24 hours HCl was removed with use of an evapomix and the sample analyzed on the Beckman 120C amino acid analyzer by the Moore and Stein procedure (34).

Neutral Monosaccharide Analysis

Weighed amounts of basement membrane were hydrolyzed in 0.8 ml of 2N sulfuric acid for four hours at 100° C. After hydrolysis, the sample was quantitatively transferred, with four washings of water (1-2 ml), to the top of a Dowex 50 x 4 (200-400 mesh) which was positioned above a Dowex 1 x 8 (200-400 mesh) column. The Dowex 50 column was washed with 8 ml of water four times. The Dowex 1 column was then washed twice with 12 ml of water. The effulent was collected in a 500 ml round bottom flask.

The effluent was lyophilyzed and dissolved in a known amount of water. An aliquot was taken for analysis on a Technicon Carbohydrate Analyzer.

Hexosamine Analysis

A weighed amount of membrane was hydrolyzed in 1 ml of 4N HCl for six hours at 100° C. After hydrolysis the sample was diluted to 5 ml with water and placed on a Dowex 50 column. The column was washed three times with 8 ml of water. The hexosamines were eluted with four rinses of 8 ml of 2N HCl. To the effluent was added guanidino-alanine for use as an internal standard. The solution was taken to dryness and the acid evaporated. The sample was dissolved in a known amount of water and an aliquot was analyzed on a Beckman 120C amino acid analyzer.

Sialic Acid Analysis

The sialic acids of the basement membrane were determined by the tiobartituric acid assay as described by Warren (35), after hydrolysis in 0.1N sulfuric acid at 80° C for one hour.

CHAPTER III

RESULTS

Preparation of Native and S-Carboxymethyl Membrane

Shown in Table V is the composition of the basement membrane--both native and S-carboxymethyl forms. As is evident, the membrane is comparable to that isolated by Spiro (11, 12). Also, the reduction and alkylation procedure resulted in a totally S-carboxymethylated membrane.

Solubility of Membrane in 6.0M Guanidine HCl

The results of the solubility experiment are listed in Table VI and graphically represented in Figure 4 from which two points become apparent. One is the fact that regardless of the initial concentration of the suspension, only about 25% of the native membrane is soluble. The second point is a logical result of the first; that is there is one definite portion of the basement membrane which is soluble in 6.0M guanidine HCl. Thus the membrane can be envisioned as consisting of two distinguishable components, a guanidine HCl soluble portion and a guanidine HCl insoluble portion.

It should be noted that this experiment was not extended to possible saturation point as it becomes extremely difficult to deal with the highly viscous solutions which result at high concentrations.

TABLE V

CHEMICAL COMPOSITION OF BOVINE GLOMERULAR BASEMENT MEMBRANE

Component	Basement Membrane (Spiro, 23, 24)	Basement Membrane	X-Carboxymethylated Basement Membrane
	Residues/1000 Amino Acid Residues		
Aspartic	67.9	71.5	70.0
Threonine	37.4	38.3	37.8
Serine	55.3	48.5	51.9
Glutamic	96.3	108.0	97.5
Proline	68.8	79.6	61.5
Glycine	207.6	216.5	222.2
Alanine	60.7	59.3	61.2
Valine	38.2	39.2	40.3
Methionine	14.2	12.0	10.2
Isoleucine	29.1	30.4	31.0
Leucine	59.3	68.2	73.3
Tyrosine	17.8	18.5	17.4
Phenylalanine	27.8	28.6	31.9
Lysine	27.0	23.6	25.3
Hystidine	16.4	15.5	17.1
Argine	49.0	48.6	50.4
½-Cystine	30.6	25.1	0.0
Hydroxyproline	68.4	72.8	55.4
Hydroxylysines	22.3	20.7	20.8
S-CM Cystine	0.0	0.0	25.1
Sialic Acid	4.44	4.03	4.03
Hexosamine	11.1	9.79	9.79
Glucose	16.4	17.9	17.9
Galactose	20.2	22.2	22.2
Mannose	5.15	5.83	5.83
Fucose	1.55	1.65	1.65

TABLE VI

SOLUBILITY OF GLOMERULAR BASEMENT MEMBRANE IN 6.0M GUANIDINE HCL

Dispersion Concentration	Concentration of Supernatant[a]
mg/ml	
0.90	0.19
1.80	0.48
2.97	0.74
5.82	1.29
10.6	2.30
15.8	3.82

[a]Calculated using ε=.814 $mg^{-1}ml\ cm^{-1}$.

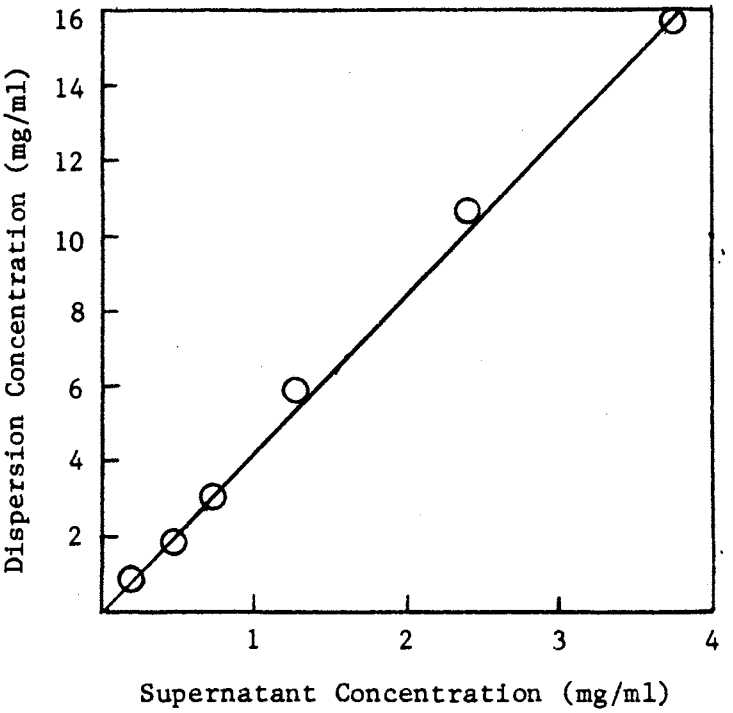

Figure 4. Solubility curve for native basement membrane in 6M guanidine HCl.

Chemical Composition of Guanidine HCl Soluble and Insoluble Fractions

Various chemical studies were performed in order to further characterize the guanidine HCl soluble and insoluble portions. The amino acid and carbohydrate compositions are shown in Tables VII and VIII. Listed for comparison is the composition of the whole membrane.

There exist some very interesting points of dissimilarity in Table VII. For one, glycine (22.6%), hydroxylysine (2.4%) and hydroxyproline (9.6%) are all increased in the insoluble portion while they are decreased in the soluble portion -- 13.2%, 0.84%, and 3.0%, respectively. On the other hand, aspartic, glutamic and leucine show almost the same drastic changes in the reverse direction. It should be noted that some of the amino acids -- methionine, histidine, isoleucine -- show little variance among the three preparations.

The results of the carbohydrate analysis of the fractions, listed in Table VIII, revealed that all the monosaccharides, except for the neutral sugars glucose and galactose exhibit a higher number of residues per 1000 amino acid residues in the soluble fraction than in the other two. It was calculated that the soluble fraction contains 2.8 heteropolysaccharide units per 1000 amino acid residues and only 8.3 dissaccharide units per 1000 amino acid residues which correlates to the drastic decreases in hydroxylysine and increases in aspartic.[1]

TABLE VII

AMINO ACID COMPOSITION OF BASEMENT MEMBRANE FRACTIONS

Amino Acid	Whole Membrane	Guanidine HCl	
		Insoluble	Soluble
	Residues/1000 Amino Acid Residues		
Aspartic	71.5	67.3	88.0
Threonine	38.3	25.9	51.8
Serine	48.5	45.9	67.9
Glutamic	108.0	92.9	125.5
Proline	79.6	65.6	65.2
Glycine	216.5	226.6	132.8
Alanine	59.3	59.4	71.7
½ Cystine	25.1	20.9	26.9
Valine	39.2	35.0	55.7
Methionine	12.0	12.4	14.1
Isoleucine	30.4	28.8	33.6
Leucine	68.2	57.9	75.7
Tyrosine	18.5	15.8	24.3
Phenylalanine	28.6	31.5	33.4
Lysine	23.8	21.8	36.2
Histidine	15.5	16.6	22.0
Arginine	48.6	56.1	36.1
Hydroxyproline	72.8	95.7	29.9
Hydroxylysine	20.7	23.9	8.4

TABLE VIII

CARBOHYDRATE COMPOSITION

Carbohydrate	Whole Membrane	Guanidine HCl	
		Soluble	Insoluble
	Residues/1000 Amino Acid Residues		
Hexosamines			
Glucosamine	9.45	12.1	6.04
Galactosamine	0.43	1.45	0.36
Neutral Sugars			
Glucose	18.4	8.25	21.3
Galactose	22.4	16.9	24.2
Mannose	5.66	8.50	5.35
Fucose	1.73	2.09	1.85
Sialic Acids	4.50	6.94	2.94
Total	62.57	56.23	61.79

Applying the same calculations to the insoluble portion, one finds this fraction accounts for 1.75 heteropolysaccharide units per 1000 amino acid residues and 21.3 dissaccharide units per 1000 amino acid residues. Again, this correlates well with the increase in hydroxylysine and the decrease in aspartic.

UV Spectral Analysis

Listed in Table IX are the extinction coefficients for the basement membrane and bovine serum albumin in 6.0M guanidine HCl. The one for bovine serum albumin is comparable although of slightly lower value than the one reported for phosphate buffer solution (36). Guanidine HCl has been shown to lower the extinction coefficient of lysozyme (37). The

extinction coefficient for the whole membrane is considerably lower than the one for the guanidine HCl extract which only reflects the increase in the number of tyrosine residues/1000 amino acid residues of the latter.

TABLE XI

EXTINCTION COEFFICIENTS IN 6.0M GUANIDINE HCL AT PH 5

Protein	ϵ_{280} mg^{-1} cm^{-1} ml
Bovine Serum Albumin	0.64
Reduced and Alkylated Basement Membrane	0.55
Guanidine HCl, Soluble	0.81
Guanidine HCL, Insoluble	0.54

The spectra of the basement membrane, bovine serum albumin and collagen, at two different pH's, in 6.0M guanidine HCl, are shown in Figure 5. The similarity is evident between the curves of reduced and alkylated membrane and those of reduced and alkylated bovine serum albumin. Both proteins exhibit the characteristic shift toward the longer wavelength upon titration of tyrosine residues. At the same time, it is apparent the ultraviolet spectra of the membrane bears no resemblance to those of collagen. This can probably be explained by the small amount of tyrosine and the lack of tryptophan in collagen (see Table I).

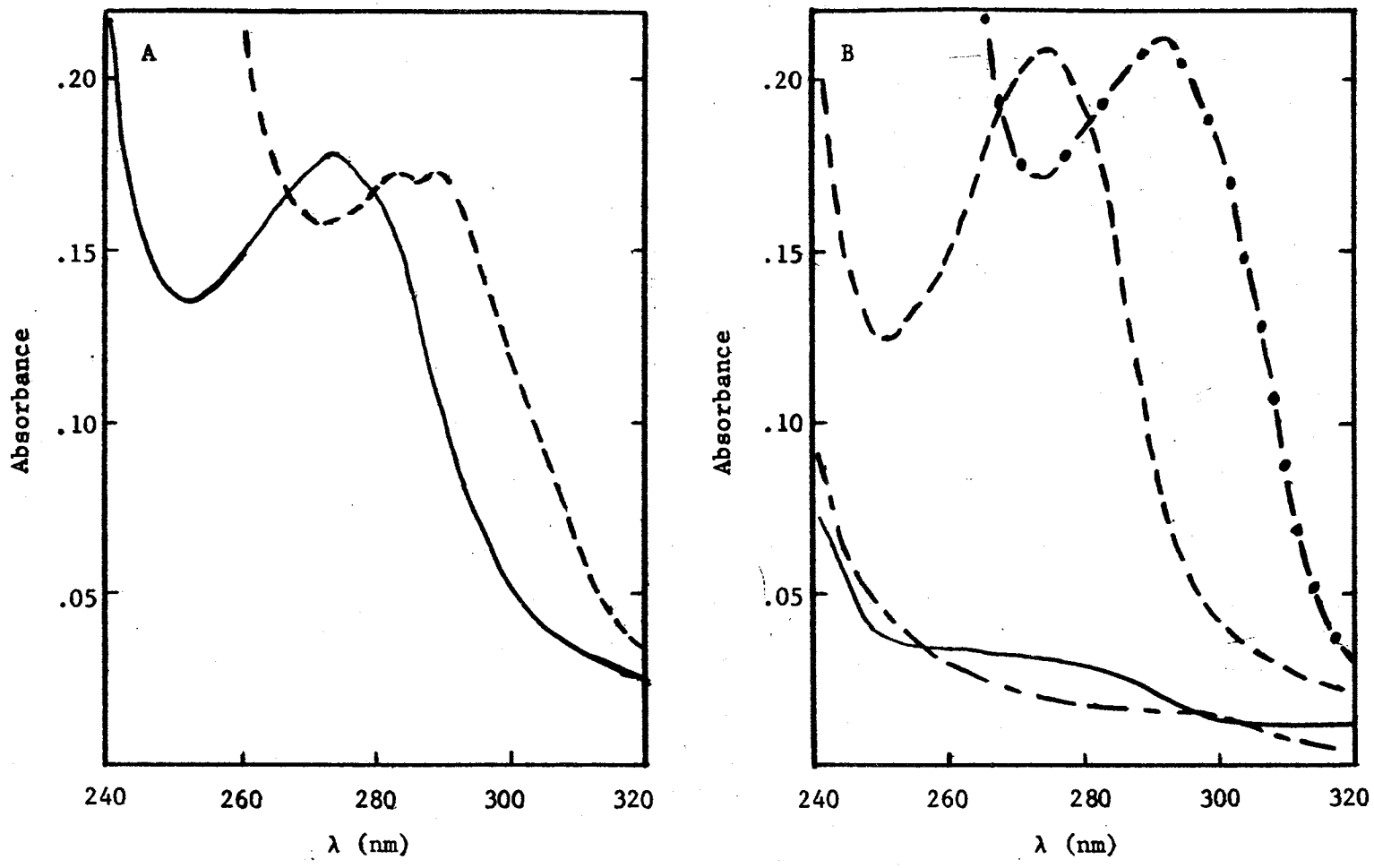

Figure 5. Ultraviolet spectra of reduced and alkylated basement membrane and other proteins at a concentration of 0.3 mg/ml. A. — —, basement membrane, pH 11.1; ———, basement membrane, pH 4.5. B. — · —, bovine serum albumin, pH 11.1; — —, bovine serum albumin, pH 4.2; — - —, collagen, pH 10.5; ———, collagen, pH 4.4.

Infrared Spectral Analyses

The infrared spectra of glomerular basement membrane is shown in Figure 6 and is compared to the spectra of collagen and myoglobin. The native membrane exhibits a spectra most like that of collagen having a broad absorption band in the region 1600-1700 cm^{-1} with a maxima at 1660 cm^{-1}; whereas, myoglobin has a sharper more intense band with a maxima at 1655 cm^{-1}. In addition the membrane exhibits a shoulder at 1645 cm^{-1}. On reduction and alkylation of the membrane, the maxima shifts from 1660 to 1650 cm^{-1} along with a broadening of the peak in the 1650-1640 cm^{-1} region. Based on the spectral assignments in Table X this spectral change is indicative of an increase in unordered conformation.

TABLE X

INTERPRETATION OF SPECTRA

Peak cm^{-1}	Representation	Reference
1640	β Structure	38, 39
1647	Random Coil	38, 39, 40
1650-1660	α Helix	38, 39, 40
1690	β Structure	38, 39, 41
1630	β Structure	40, 41, 42
1652	α Helix, Random Coil	41

The close similarity in the broadness of the 1600-1700 cm^{-1} absorption band for the native membrane and collagen as contrasted to

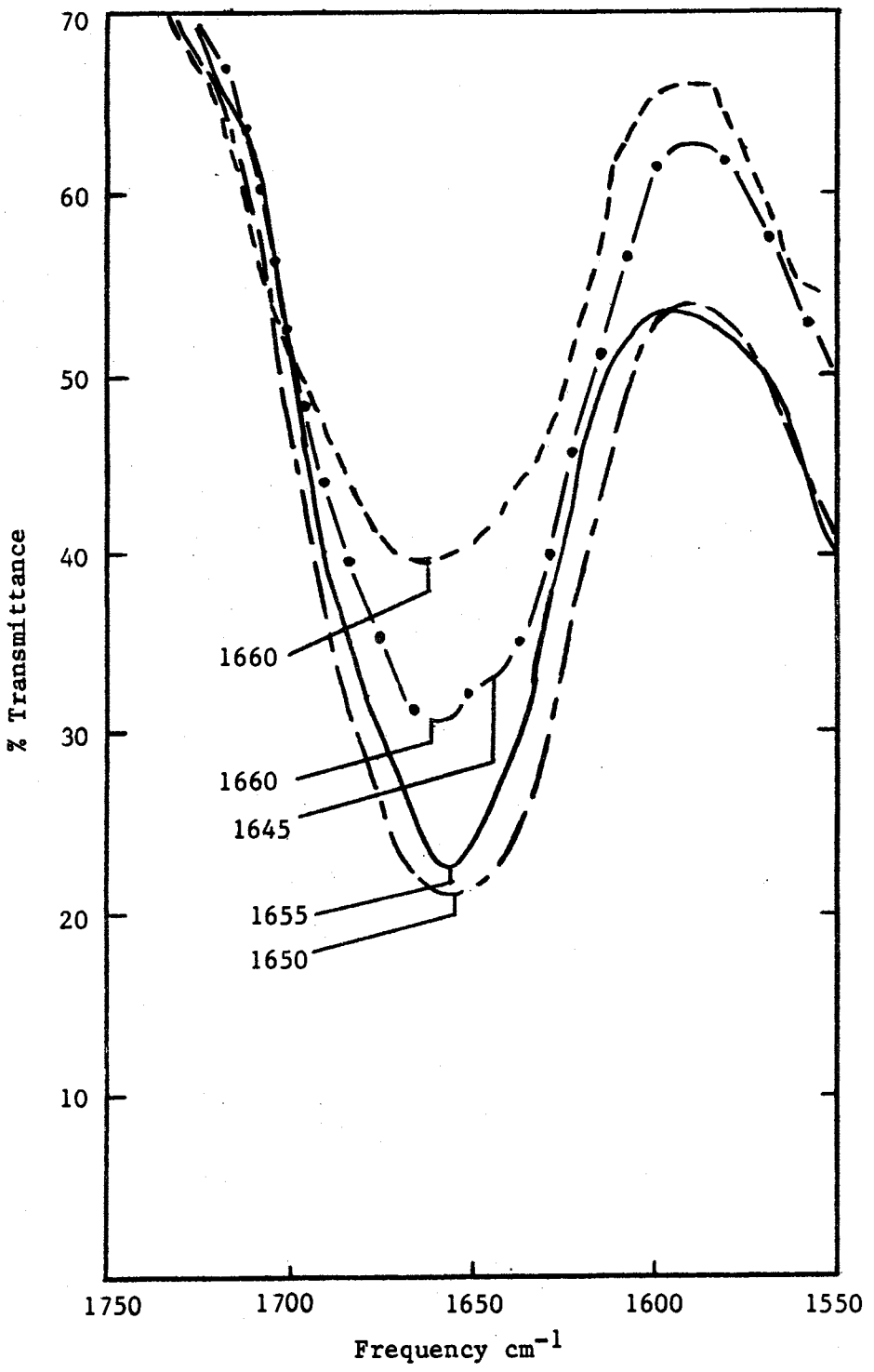

Figure 6. Infrared spectra of basement membrane, with myoglobin and collagen included for comparison; — —, collagen; — · —, basement membrane; —— myoglobin; — – —, reduced and alkylated basement membrane.

myoglobin and cytochrome C is clearly seen by expressing the absorbance at 1630 and 1700 cm^{-1} relative to the absorbance at 1655 cm^{-1}, as given in Table XI. In order to correct for non-standard amounts of sample used in preparing pellets, the intensities were converted to absorbance units with use of the equation $A = \log I_o/I$, where I_o is the intensity at 1800 cm^{-1}, chosen as baseline, and I is the intensity at the specified wave number. These ratios further indicate that reduction and alkylation of membrane causes a decrease in absorption in the 1690 cm^{-1} region. Based on the spectral assignments in Table X, this decrease in absorption may indicate a decrease in the amount of β-conformation.

TABLE XI

COMPARISON OF RATIOS OF PEAK INTENSITIES OF IR SPECTRA

Material	1630/1655[a]	1700/1655[b]
Cytochrome C	0.6130	0.4349
Myoglobin	0.6201	0.5534
Collagen	0.8341	0.5776
Native Basement Membrane	0.7907	0.4732
Reduced & Alkylated Basement Membrane	0.7888	0.3857
Extract	0.6656	0.4453
Insoluble	0.8388	0.8093

[a] $\frac{\log I_o/I_{1630}}{\log I_o/I_{1655}}$ [b] $\frac{\log I_o/I_{1700}}{\log I_o/I_{1655}}$

A comparison of the infrared spectra of the guanidine HCl soluble and insoluble membrane fractions to collagen is shown in Figure 7. It is evident that the insoluble fraction most closely resembles that of collagen and further that it is responsible for the collagen-like spectra exhibited by native membrane. The absorption band has a maxima at 1660 cm^{-1} and is very broad which is reflected in the relatively large absorbance ratios in Table XI. In contrast, the soluble fraction has a sharper absorption band, as is reflected in the low absorbance ratios in Table XI, and further accounts for the shoulder at 1645 cm^{-1} seen in the spectra of native membrane.

Circular Dichroism Spectral Analysis

The circular dichroic spectra of S-carboxymethyl membrane in 6.0M guanidine HCl is compared to that of myoglobin in the presence and absence of this denaturing agent (Figure 8). Myoglobin in 0.2M NaF represents the protein in its native state and exhibits the spectra expected from its α-helical conformation (26).

Myoglobin in 6.0M guanidine HCl exists in an unordered conformation and displays a CD spectra in which significant peaks are absent in the 210-250 nm region. This curve is virtually identical to that reported in the literature and serves as a reference spectra for unordered protein conformation (43). The membrane exhibits a spectra comparable to that of myoglobin which indicates it exists in an unordered conformation in 6.0M guanidine HCl.

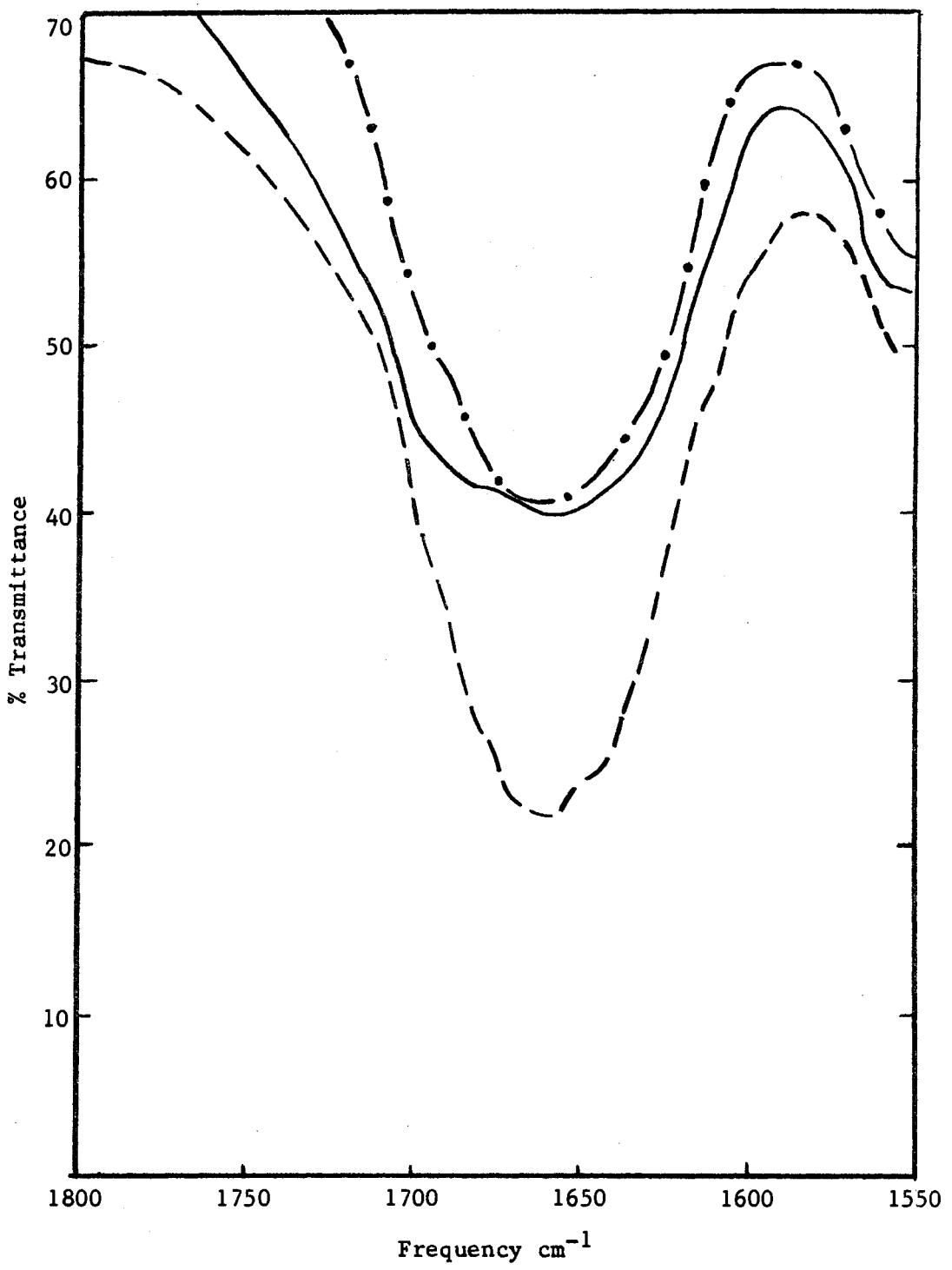

Figure 7. Comparison of the infrared spectra of the collagen, the guanidine HCl soluble and the guanidine HCl insoluble fractions; ——, insoluble; — · —, collagen; — —, soluble.

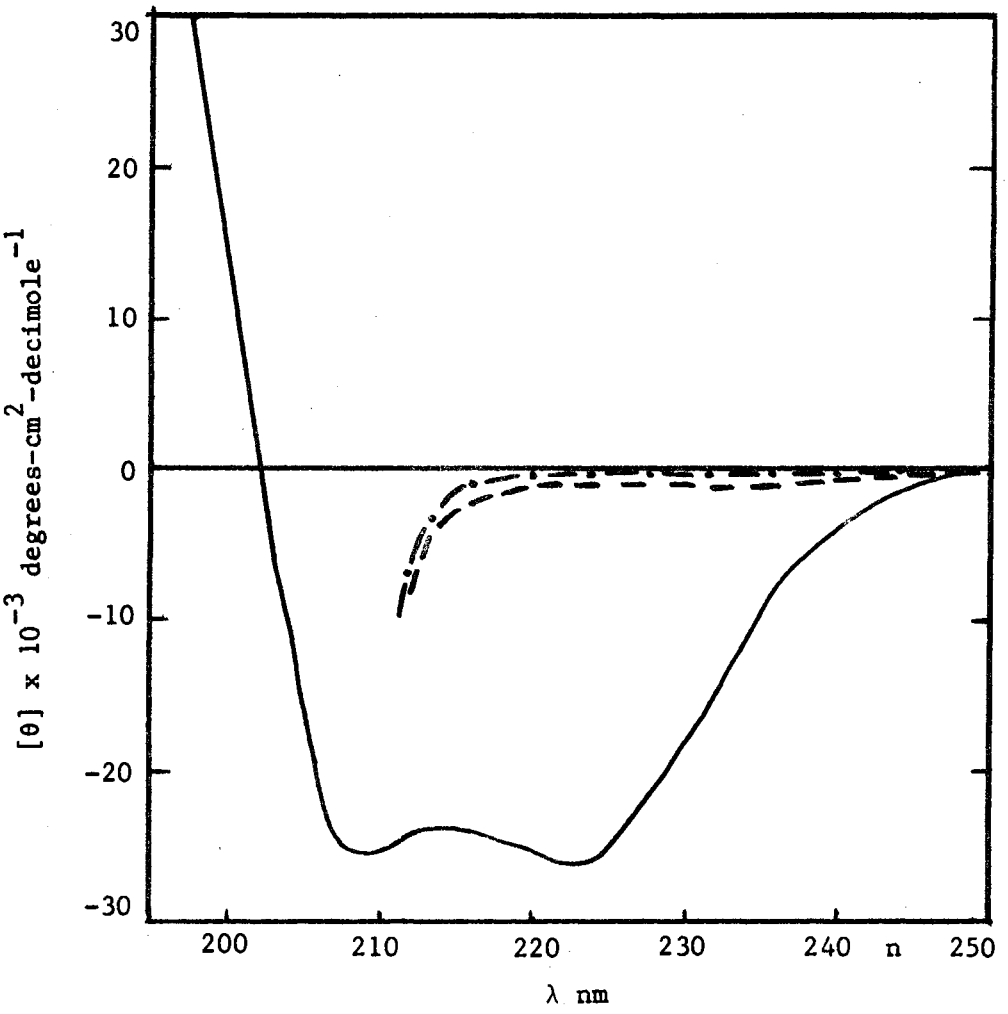

Figure 8. Far ultraviolet circular dichroism spectra of reduced and alkylated basement membrane and myoglobin; ———, myoglobin, 0.2M NaF; — —, myoglobin, 6.0M GuHCl; — · —, membrane, 6.0M GuHCl.

Molecular Weight of Membrane Subunits

An example of the elution profile of S-carboxylmethylated standard proteins from the Sepharose 4B column in 6.0M guanidine hydrochloride is shown in Figure 9A, which illustrates the high resolving power for proteins ranging in molecular weight from 12,500 to 200,000. Figure 9B shows the elution data plotted according to the method of Andrews (44). The data were calculated from elution weights rather than elution volume to obtain maximum precision in accordance with the method of Fish, et.al. (26).

The molecular weights of membrane subunits were estimated by chromatography of S-carboxymethyl membrane in 6.0M guanidine HCl on the calibrated Sepharose 4B column as shown in Figure 10A. Many subunits are present varying in molecular weight from 12,000 to greater than 200,000. However, there are five prominent regions in the profile. These regions along with their molecular weight ranges are indicated in Figure 10A. The most prominent region (I) occurs at the void volume with a molecular weight much greater than 200,000.

It was noticed during the course of dealing with the reduced and alkylated whole membrane, that lyophilyzation altered the solubility. Redissolved, reduced and alkylated membrane is no longer entirely soluble in 6.0M guanidine HCl; only about 90% goes back into solution. Therefore it was felt a gel filtration pattern of the soluble portion would prove informative. The elution profile is given in Figure 10B. As is evident, region I has decreased relative to the sum of regions II-III, thus indicating some of the material in region I is rendered insoluble in guanidine HCl on lyophilyzation.

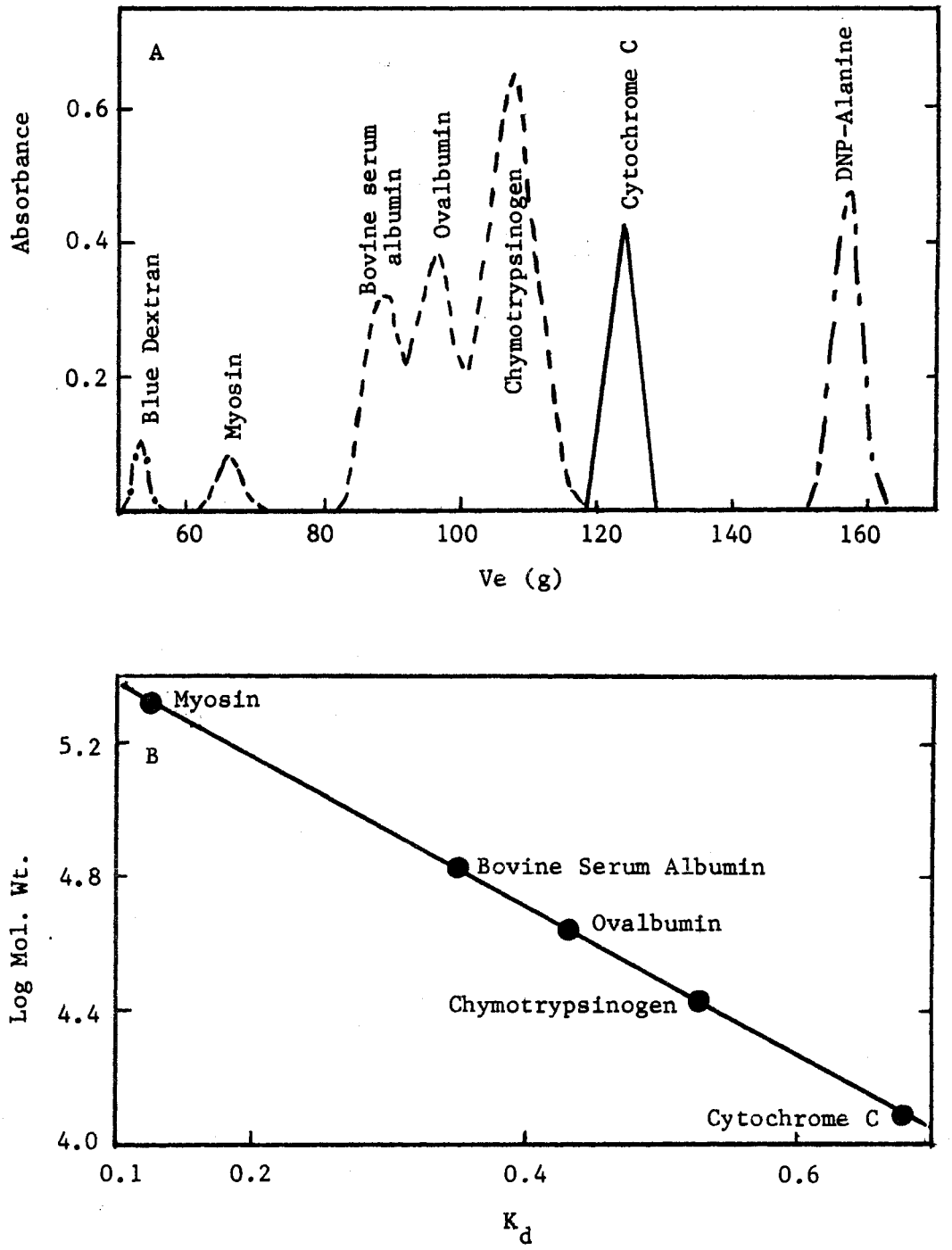

Figure 9. A. Example of typical elution profile obtained from Sepharose 4B column operated in 6.0M guanidine HCL; — · — ·, A_{630}; — — —, A_{280}; ———, A_{410}; — — —, A_{360}. B. Graphical interpretation of elution data of standards, in terms of K_d, obtained for the Sepharose 4B column operated in 6.0M guanidine HCl. A semilogarithmic plot of the molecular weight as a function of the distribution coefficient, K_d.

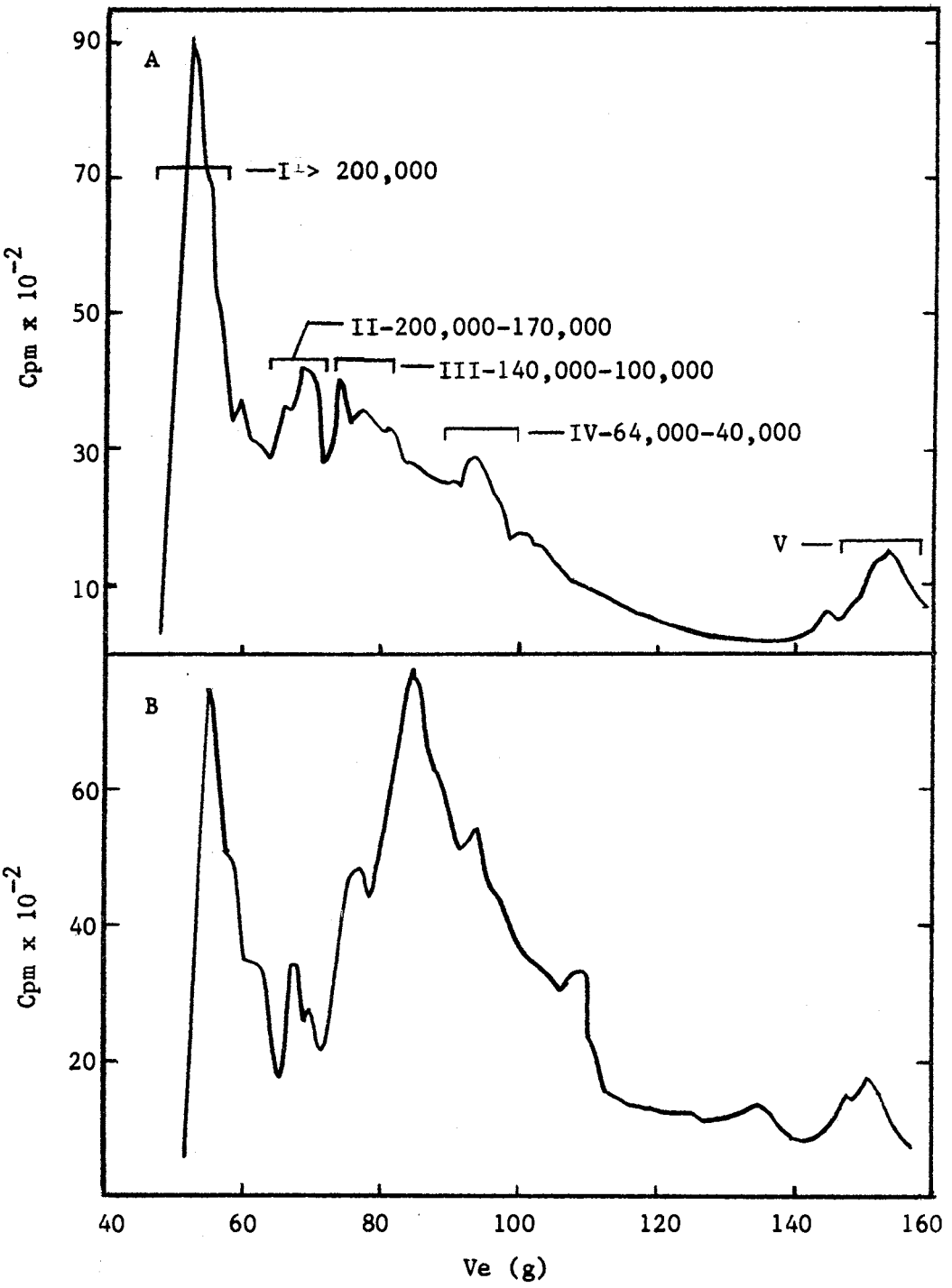

Figure 10. Distribution and molecular weights of membrane subunits. A. Elution profile of S-carboxymethyl whole basement membrane on Sepharose 4B column operated in 6.0M GuHCl. Molecular weights are based on calibration curve in Figure 9A. B. Elution profile of the soluble fraction of redissolved reduced and alkylated whole basement membrane.

The subunit distribution in the native membrane guanidine HCl soluble and insoluble fractions was determined on the S-carboxymethylated form of these fractions by gel chromatography in 6.0M guanidine HCL as shown in Figure 11. For reference the whole S-carboxymethylated membrane is included (Figure 11A). The soluble fraction is void of the subunits of the high molecular weight regions I and II, but contains the subunits of the lower molecular weight regions III to V (Figure 11B). In contrast the insoluble fraction contains the subunits of the former regions I and II, but is void in the later regions III to V, (Figure 11C).

Distribution of Unreduced Membrane Components in 6.0M Guanidine HCl Soluble Fraction

The distribution of components with intact disulfide bonds in the native membrane was examined by gel chromotography in 6.0M guanidine HCL. Eight prominent components were discernable as noted in Figure 12A. On reduction and S-carboxymethylation of this fraction, as shown in Figure 12B, the most pronounced effect is the disappearance of the void volume component (I). In addition the subunits elute as a broad peak with little apparent resolution. The disappearance of the void volume component is indicative of an aggregate of subunits held together by disulfide bonds. The change in the elution profile from one of resolved peaks to a broad peak for the unreduced and reduced fraction, respectively, may be attributed to formation of subunits from the void volume component or other lower molecular weight components in the fraction and to a change in the hydrodynamic radius of subunits which may be present with intramolecular disulfide bonds (45).

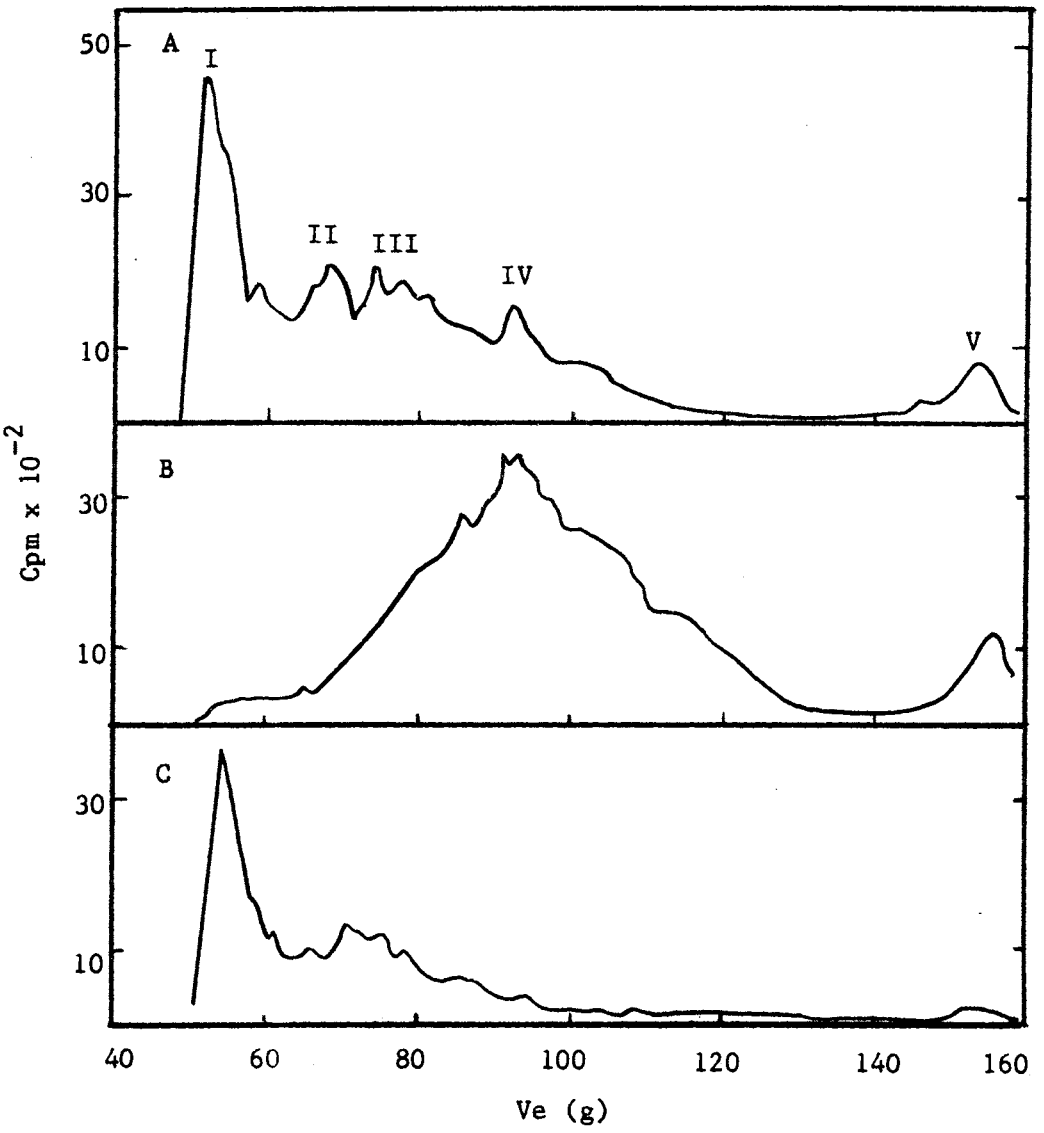

Figure 11. Distribution of membrane subunits in 6.0M guanidine HCl soluble and insoluble fractions. A. Elution profile of S-carboxymethylated whole basement membrane obtained on Sepharose 4B column operated in 6.0M GuHCl. B. Elution profile of S-carboxymethylated guanidine HCl soluble fraction on same column. C. Elution profile of S-carboxymethylated guanidine HCl insoluble fraction on same column.

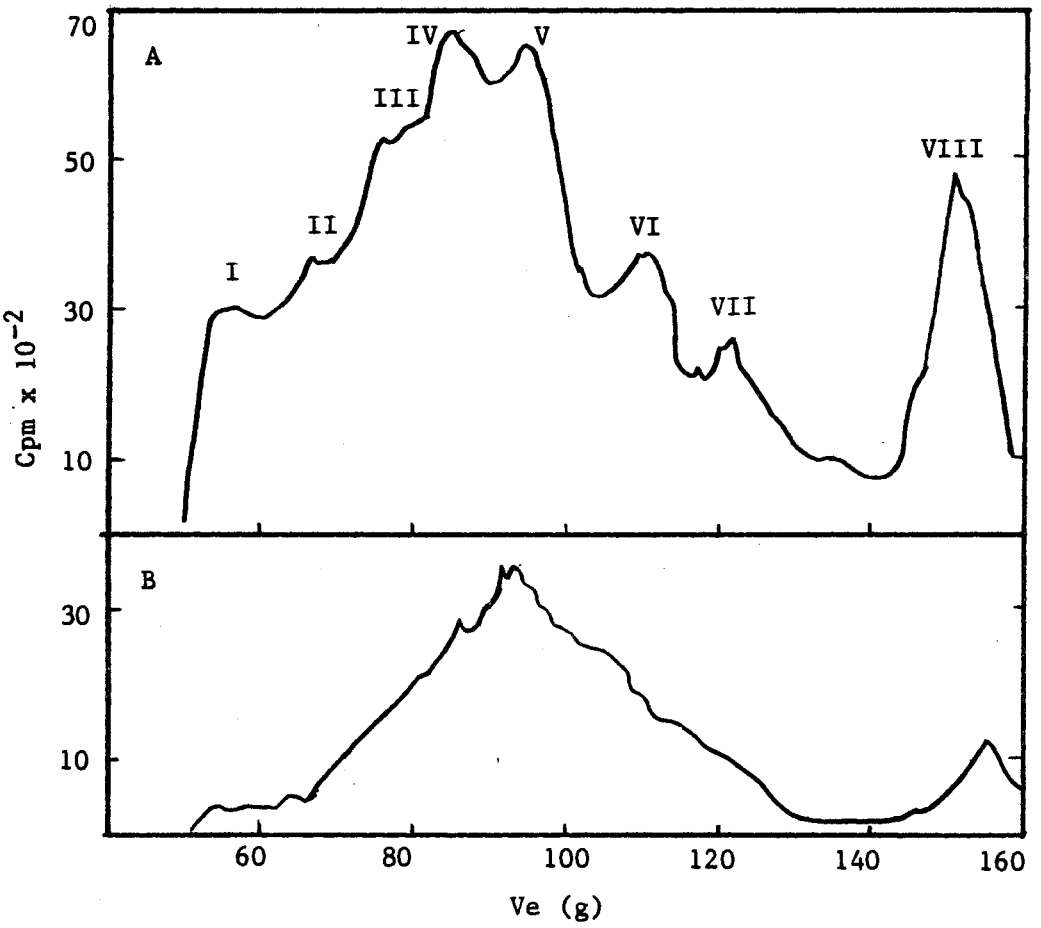

Figure 12. Elution profile of unreduced and reduced guanidine HCl soluble fraction of native membrane. A. Unreduced fraction. B. Reduced and S-carboxymethylated.

FOOTNOTES

[1] The numbers were calculated using the following method:

$$\frac{\text{Residues of Mannose per 1000 amino acid residues}}{\text{3 Mannose Residues per heteropolysaccharide unit}}$$

$$\frac{\text{Residues of Glucose per 1000 amino acid residues}}{\text{1 Glucose Residue per disaccharide unit}}$$

CHAPTER IV

DISCUSSION

The renal glomerular basement membrane is considered to be related to vertebrate collagens on the basis of its amino acid composition in that it contains hydroxylysine, hydroxyproline, and large amounts of glycine (11). Further similarities exist with respect to their carbohydrate moieties in that both contain a disaccharide unit consisting of glucose and galactose (46). However, marked differences exist between the composition of the membrane and the collagens (1). The hydroxylysine content of the membrane is much greater than that of vertebrate collagen whereas the amount of glycine present is less. Moreover, the membrane contains high amounts of half-cystine. Also, the membrane contains 10 times the number of disaccharide units as collagen and, in addition, 50 percent of the carbohydrate in the membrane exists in the form of a complex heteropolysaccharide not found in collagen. The results of this study reveal further that the basement membrane is structurally related to vertebrate collagens in both chemical and physical properties, although marked differences exist.

The membrane is essentially insoluble in buffer solutions in the absence of protein denaturing agents. In the presence of 6.0M guanidine HCl, only 25 percent of the membrane is soluble. However, on

treatment with mercaptoethanol followed by alkylation in the presence of 6.0M guanidine HCl, the membrane is completely solubilized. Furthermore, the alkylated membrane is insoluble in the absence of guanidine HCl and, if lyophilyzed and redispersed in guanidine, it is not completely solubilized. Consequently, several of the physical properties were measured in 6.0M guanidine HCl. Moreover, the guanidine HCl soluble and insoluble fractions of the native unreduced membrane were studied in conjunction with the native membrane because these fractions represent a separation of components which are held together in the native state by non-covalent bonds. Comparison of the chemical composition of the guanidine HCl soluble and insoluble fractions reveals that the latter accounts for the similarities between the composition of the membrane and collagen.

The insoluble fraction, which accounts for 75% of the whole membrane, is as rich in hydroxyproline (9.6%) as is collagen (9.9%) and almost as well endowed with glycine, 22.6% as compared to 31.2%. As for hydroxylysine, the insoluble fraction contains more than collagen, 2.4% as compared to 0.79%.

In contrast, 25% of the whole membrane, as represented by the soluble fraction, differs from collagen in its chemical composition. Although it may be considered collagen-related because of the presence of hydroxylysine, in an amount (0.8%) similar to that for collagen, and its content of hydroxyproline (3.0%) and glycine (13.3%), although their amounts are considerably reduced from the levels in collagen.

The increase in hydroxylysine content in the insoluble fraction and the decrease of this amino acid in the soluble fraction should reflect corresponding changes in the amount of disaccharide units

present in each portion. This is indeed the case. The insoluble fraction has about 21 units while the soluble has about 8. At the same time the heteropolysaccharide units show inverse changes, increasing to about 3 in the soluble fraction and decreasing to about 1 in the insoluble. Thus the ratio of heteropolysaccharide units to disaccharide units is 12 to 1 in the insoluble fraction and only 3 to 1 in the soluble fraction. Both of the ratios differ significantly from the 10 to 1 ratio present in the membrane.

The ultraviolet absorption spectra of the S-carboxymethylated membrane in 6.0M guanidine HCl bears little resemblance to that of collagen but closely resembles that of soluble proteins, as exemplified by serum albumin. This lack of similarity between membrane and collagen may be attributed to the larger content of tyrosine, phenyalenine, and tryptophan in the membrane. The guanidine HCl soluble membrane fraction has a larger molar extinction coefficient than the insoluble fraction which reflects the larger amount of tyrosine in the former.

The infrared spectra of native membrane is quite similar to collagen in that both exhibit broad absorption bands in the region 1600 to 1700 cm^{-1} with a maxima at 1660 cm^{-1}. However, the membrane differs from collagen in that its band is slightly sharper and a shoulder is present at 1645 cm^{-1}.

The guanidine insoluble membrane fraction is responsible for the broadness of the absorption band and, further, its spectra is almost identical to collagen. On the other hand, the soluble fraction is responsible for the shoulder at 1645 cm^{-1} and its rather sharper band in the 1600-1700 cm^{-1} region accounts for the narrowness of the whole membrane's spectra as compared to collagen.

The CD spectral analysis of the reduced and alkylated whole membrane in 6.0M guanidine HCl indicates the membrane polypeptide chains retain no ordered structure. Hence, all non-covalent interactions, including those that might lead to association and aggregation between chains, have been disrupted and therefore one can realiably look at the subunit structure of the membrane in this solvent.

The membrane is composed of several dissimilar subunits of varying molecular weight, as evidenced from gel filtration chromatography in 6.0M guanidine HCl of the fully reduced and S-carboxymethylated membrane. The subunits vary in molecular weight from much greater than 200,000 to 12,000 daltons. Notable is the presence of one or more polypeptide chains of the former molecular weight. Since only one polypeptide chain of molecular weight greater than 200,000 has been reported in the literature (47), a strong possibility exists that other covalent crosslinks are present in the high molecular weight subunits of the membrane. Moreover, as structural similarities exist between the membrane and collagen, these crosslinks may be of an aldehydic nature similar to those reported for collagen (48).

As with other analyses, a comparison of the gel filtration patterns of the S-carboxymethylated guanidine HCl insoluble and soluble fractions reveal which parts of the whole membrane profile are contributed by each fraction. The pattern of the insoluble fraction is similar to that of the native membrane on the high molecular weight regions and most surely accounts for all of the greater than 200,000 region. The soluble fraction's profile reveals it is probably responsible for the molecular regions below 100,000.

Intermolecular disulfide bonds must play a large role in the crosslinking of subunits in the native membrane as may be inferred from the chomatic effect of mercaptoethanol on solubility of the membrane. However, more direct evidence is that reduction of guanadine soluble fraction of the native membrane causes a high molecular weight component to disaggregate into small-sized subunits. However, at present it cannot be decided which subunits are crosslinked by disulfides or how many intramolecular bonds occur.

In conclusion, the glomerular basement membrane closely resembles vertebrate collagen in both chemical and physical properties, although marked differences are apparent. The membrane consists of two distinct fractions which are held together by non-covalent bonds; the guanidine HCl soluble and insoluble fractions representing 25 and 75 percent of the total protein, respectively. In almost all respects, the chemical and physical properties of the insoluble fraction are almost identical to that of collagen. Moreover, the membrane consists of several polypeptide chains varying in molecular weight from much greater than 200,000 to 12,000 daltons, some of which are crosslinked by disulfide bonds and probably by crosslinks of the type in vertebrate collagens.

CHAPTER V

SUMMARY

Several physical and chemical studies were performed on the bovine glomerular basement membrane in an attempt to elucidate its three-dimensional structure.

The basement membrane, in the presence of 6.0M guanidine HCl proved to be 25% soluble. Upon chemical analysis it was found these two fractions, the soluble and insoluble, to be vastly different. The larger fraction representing 75% of the whole membrane was similar to collagen in its hydroproline and glycine content. It contained a greater amount of hydroxylysine than collagen. As for the distribution of carbohydrate, the ratio of disaccharide to heteropolysaccharide units was 13 to 1, an increase over the 10:1 ratio in the whole membrane. In contrast, the soluble fraction, while containing hydroxyproline and glycine, their relative amounts were low compared to collagen. Its hydroxylysine content was almost equal to that of collagen. The distribution of carbohydrate about the units was such as to give a 3 to 1 ratio of disaccharide to heteropolysaccharide units. However, its total amount of carbohydrate was about the same as for the insoluble fraction and whole membrane.

The ultraviolet spectra of the reduced and alkylated membrane exhibited similarities to those of a standard protein, serum albumin, while exhibiting no similarities to those of collagen. This latter

may be accounted for by the low amount of tryptophan and tyrosine in collagen as contrasted to those in the membrane.

Infrared spectra of the native membrane indicated a broad band similar to that of collagen although a shoulder at 1645 cm^{-1} existed in the former and not in the latter. It was further determined the broadness was contributed by the guanidine HCl insoluble fraction while the shoulder was attributed to the soluble fraction.

The circular dichroism studies of the S-carboxymethylated basement membrane in 6.0M guanidine HCl revealed that the protein existed in an unordered and unaggregated state in the presence of this denaturing solvent. Thus studies of the membrane subunit structure were undertaken with the use of gel filtration in 6.0M guanidine HCl.

Gel filtration of the reduced and alkylated basement membrane on a sepharose 4B column operated in 6.0M guanidine HCl resulted in numerous subunits ranging in molecular weight from 200,000 to 12,000 daltons. A portion of the membrane was eluted with the void volume indicating the presence of a subunit or subunits of very large molecular weight. Gel filtration patterns of the S-carboxymethylated guanidine HCl insoluble indicated this large molecular weight region to be in the insoluble portion and raised the possibility of aldehydic crosslinking similar to those in collagen to be present. A comparison of the profiles for the soluble and S-carboxymethylated soluble fractions indicated the presence of intermolecular disulfide bonds between popypeptide chains.

A SELECTED BIBLIOGRAPHY

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

(2) Spiro, R. "Chemistry and Metabolism of the Basement Membrane." Diabetes: Theory and Practice. Eds. M. Ellenberg and H. Rifkin. New York: McGraw Hill, 1970.

(3) Farguhar, M.G., J. Hopper, and H. D. Moon. Am. J. Path., 35 (1959), 721.

(4) Caulfield, J. B. New England J. Med.,,270 (1964), 183.

(5) Calne, R. Y., Loughbridge, et.al. Brit. Med. J., 2 (1963), 645.

(6) Merril, J. P., et.al. J. Am. Med. Assn., 185 (1963), 347.

(7) Porter, K. A., et.al. Brit. Med. J., 2 (1963), 639.

(8) Merril, J.P., and J. E. Murray. Pediat. Clin. N. Amer., 13 (1966), 381.

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

(10) Goodman, M., S. A. Greenspon and C. A. Krakower. J. Immunol., 75 (1953), 96.

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

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

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

(14) Kefalides, N., and R. J. Winzler. Biochemistry, 5 (1966), 702.

(15) Kefalides, N. Biochemistry, 7 (1968), 3103.

(16) Kefalides, N. In Proceedings of the Vth Congress of the International Diabetes Foundation. Amsterdam: Excerpta Medica International Congress Series, 1967.

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

(18) Westburg, N. G., and A. F. Michael. Biochemistry, 9 (1970), 3837.

(19) Spiro, R. J. Biol. Chem., 237 (1962), 382.

(20) Spiro, R. J. Biol. Chem., 237 (1962), 646.

(21) Spiro, R. J. Biol. Chem., 239 (1964), 567.

(22) Spiro, R. J. Biol. Chem., 240 (1965), 1603.

(23) Nozaki, Y., and C. Tanford. Methods in Enzymology, XI. New York: Academic Press, 1967.

(24) Nozaki, Y. Personal Communication.

(25) Perry, S. V. Methods in Enzymology, II. New York: Academic Press, 1955.

(26) Straus, J. H., et.al. Eur. J. Biochem., 11 (1969), 201.

(27) Fish, W. W., et.al. J. Biol. Chem., 244 (1969), 4989.

(28) Gershman, L. C., et.al. J. Biol. Chem., 244 (1969), 2726.

(29) Tanford, C. Advan. Protein Chemistry, 23 (1968) 121.

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

(31) Eck, R. B., and M. O. Dayhoff. Atlas of Protein Sequence and Structure, Silver Spring, Maryland: National Biomedical Research Foundation, 1966.

(32) Gelotte, B. J. J. Chromatography, 3 (1960), 330.

(33) Castellino, F. J., et.al. J. Biol. Chem., 245 (1970), 417.

(34) Spackman, D. H., W. H. Stein and S. Moore, Anal. Chem., 30 (1958), 1190.

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

(36) Reynolds, J. A., et.al. J. Biochemistry, 6 (1967), 937.

(37) Castellino, F. J., W. W. Fish and K. G. Mann. J. Biol. Chem., 245 (1970), 4269.

(38) Choules, G. L., and R. F. Bjorklund. Biochemistry, 9 (1970), 4759.

(39) Wallach, D. F. H., et.al. Arch. Biochem. Biophys., 131 (1969), 322.

(40) Susi, J., el.al. J. Biol. Chem., 242 (1967), 5460.

(41) Wallach, D. F. H., and P. J. Zahler. Proc. Nat. Acad. Sci., 56 (1966), 1552.

(42) Green, D. H., and M. R. Salton. Biochem. Biophys. Acta, 211 (1970), 139.

(43) Dearborn, D. G., and D. B. Wetlaufer. Biochem. Biophys. Research Communications, 39 (1970), 314.

(44) Andrews, P. Biochem. J., 96 (1965), 595.

(45) Mann, K. G., and W. W. Fish. Methods in Enzymology (in press).

(46) Spiro, R. G. J. Biol. Chem., 244 (1969), 602.

(47) Gwynne, J. T., and C. Tanford. J. Biol. Chem., 245 (1970), 3269.

(48) Zimmermann, B. K., et.al. Eur. J. Biochem., 16 (1970), 217.

VITA

Charles Edward Schwartz

Candidate for the Degree of

Master of Science

Thesis: PHYSICAL PROPERTIES OF THE BOVINE GLOMERULAR BASEMENT MEMBRANE

Major Field: Biochemistry

Biographical:

Personal Data: Born in New York City, New York, February 24, 1948, the son of Mr. and Mrs. Gabriel Schwartz.

Education: Graduated from Roslyn High, Roslyn, New York in 1966; graduated from Colgate University, Hamilton, New York, with honors in Chemistry in 1970; completed requirements for Master of Science degree in May, 1972.

Professional Experience: Served as research assistant in Biochemistry at Oklahoma State University from 1970 to 1972.