ANTIGENIC COMPONENTS OF GLOMERULAR BASEMENT MEMBRANE

by

FLORA Y.Y. HUANG
M.Sc. (McGill)

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Dept. of Investigative Medicine
McGill University
Montreal

November 1967

Ph.D. INVESTIGATIVE MEDICINE DEPARTMENT

Flora Y.Y. Huang

ANTIGENIC COMPONENTS OF GLOMERULAR BASEMENT MEMBRANE

ABSTRACT

A study has been made to define the chemical nature of the membrane components responsible for the nephrotoxigenicity of the glomerular basement membrane. Six components from rat glomerular basement membrane were isolated by collagenase digestion, urea extraction and fractionation on Sephadex gels. Antibodies against these components, except two which were solubilized by collagenase and retained on Sephadex G-75, localized to glomerular basement membrane and induced proteinuria in rats. Multiple antigens and common antigenic determinants among the membrane components were demonstrated. All components were glycoprotein containing seven to twenty per cent of carbohydrate, but those solubilized by collagenase digestion contained collagen fragment.

Antibodies to dog glomerular basement membrane localized to rat glomerular basement membrane but were non-nephrotoxic to normal rats. Dog glomerular basement membrane contained several antigens which were similar but not identical to the rat membrane components.

The prior localization of the cross-reacting, non-nephrotoxic antibodies did not prevent the induction of nephrotoxic serum nephritis in rats by nephrotoxic antibodies and subsequent development of proteinuria.

The hypothesis that normal and nephrotoxic glomerular basement membranes are analogous to Sephadex gels of greater and lesser degree of cross-linkage is established. Normal basement membrane produces a molecular sieving effect and acts as a differential filter. In nephrotoxic serum nephritis, the occurrence of proteinuria was postulated as a result of increased permeability of the glomerular basement membrane due to a more open molecular structure of the membrane itself.

ACKNOWLEDGEMENTS

To Dr. J.S.L. Browne, Head of the Investigative Medicine Department, I owe my profound gratitude and respect. I have profited from both his valuable advice and his stimulating discussions.

I am particularly indebted to Dr. N. Kalant, Head of the Lady Davis Institute for Medical Research, Jewish General Hospital, for his continuous supervision, criticism and patent guidance throughout this investigation.

I am grateful to Dr. A. Leznoff, Department of Allergy, Jewish General Hospital, for placing his laboratory facilities at my disposal and for his valuable advice; and to Dr. M. Saffran, Department of Biochemistry, McGill University, for performing the amino acids analyses in his laboratory. My sincere thanks are also due to Dr. M. Richter, Division of Immunochemistry and Allergy, Royal Victoria Hospital, for carrying out the ultracentrifugal analysis in the hospital laboratory; and to Mr. Underdown, of Royal Victoria Hospital, for performing the disc electrophoresis.

The author acknowledges the excellent technical assistance rendered by Mrs. S. Lui and Mrs. D. Sairan; and the help in collection of glomeruli and immunization of rabbits provided by Mr. A. Nowacki and Mr. G. Gerald. Sincere thanks are also due to Mr. N. Feifer for help in the photographic work.

The author gratefully acknowledges the financial assistance obtained from the Jewish General Hospital.

LIST OF ABBREVIATIONS

Ab, Abs	-	Antibody, antibodies
Ag, Ags	-	Antigen(s)
GBM	-	Glomerular basement membrane(s)
NTAb	-	Nephrotoxic antibody
NTN	-	Nephrotoxic serum nephritis
NTS	-	Nephrotoxic serum

TABLE OF CONTENTS

page

A. General Introduction 1

B. Historical Introduction 2

B.1. Structure of Normal Glomerulus 2

B.1.1. Introduction 2

B.1.2. Electron Microscopy of Glomerulus 3

a. Endothelium 3
b. Basement Membrane 4
c. Epithelium 5
d. Mesangium 6

B.2. Glomerular Filtration 7

B.2.1. Introduction 7

B.2.2. Functions of Glomerular Components 8

B.2.3. Glomerular Permeability 12

B.2.4. Proteinuria 14

a. Introduction 14
b. Urinary Proteins 15
c. Mechanisms of Increased Proteinuria 16

B.3. Experimental Production of Nephritis 21

B.3.1. Introduction 21

B.3.2. Nephritis Induced by Chemical Agents 22

a. Aminonucleoside of Puromycin (ANP) 22
b. Tridione 24
c. Polyvinyl Alcohol 24

B.3.3. Glomerulonephritis Induced by Immunological Methods 25

a. Nephrotoxic Serum Nephritis (NTN) 25
b. Foreign Proteins (Ag-Ab complex) Nephritis 26
c. Nephritis Induced by Immunization with Renal Ags 28
d. Nephritis Induced by Immunization with Bacterial Ags 31

Table of Contents (cont'd.)

page

B.3.4. Immunological Aspects of Nephrotoxic Serum Nephritis 33

a. Introduction 33
b. Nephrotoxic Serum Nephritis in Different Species 34
c. Tissue Ags Involved in Producing Nephrotoxic Serum Nephritis 37

i. Renal Tissues 37
ii. Non-renal Tissue Ags 39

d. Physicochemical Characterization and Nephrotoxicity of Anti-kidney Abs 42
e. Localization of Nephrotoxic Abs 43

i. Methods for *In Vivo* and *In Vitro* Localization of NTAbs 43
ii. *In Vivo* Localization of NTAb: Specificity, Rate of Fixation and Persistence in Kidney and Other Tissues 45

f. Localization of Autologous Ab in NTN 48

B.3.5. Conclusion 49

B.4. Nature of Glomerular Basement Membrane 51

B.4.1. Origin and Development of GBM 51

B.4.2. General Microscopic Characterization of GBM 53

B.4.3. Immunological Properties of GBM 55

B.4.4. Chemical and Physical Properties of GBM 58

B.4.5. Conclusion 64

C. Materials and Methods 66

C.1. Experimental Animals 66

C.1.1. Rats 66

C.1.2. Rabbits 66

C.1.3. Dogs 66

Table of Contents (cont'd.)

page

C.2. Isolation of Glomeruli 66

C.3. Preparation of Glomerular Basement Membrane 68

C.4. Preparation of Sephadex Columns 68

C.5. Immunological Procedures 69

C.5.1. Preparation of Rabbit Antisera 69

C.5.2. Determination of Nephrotoxicity of Antiserum in Rats 70

C.5.3. Determination of Antisera Localization by Immunofluorescent Method 71

a. Fluorescein-conjugated Antiserum to Rabbit γ-Globulin 71

b. In Vitro Localization of Antisera 71

c. In Vivo Localization of Antisera 72

C.5.4. Determination of Reactivity of Antisera with Different Ags by Gel Diffusion Method ... 72

C.6. Methods for Chemical Analysis 73

C.6.1. Quantitative Determination of Hexosamines..... 73

a. Introduction 73

b. Hydrolysis Conditions 74

c. Separation of Hexosamines from Amino Acids and Peptides 75

d. Quantitative Determination of Glucosamine and Galactosamine 75

C.6.2. Quantitative Determination of Sialic Acids ... 76

a. Introduction 76

b. General Method 76

c. Purification of Sialic Acids with Ion-Exchange Resins 77

Table of Contents (cont'd.)

page

C.6.3. Determination of Bound Hexoses 78

C.6.4. Determination of Glucose by Enzymatic Method 78

C.6.5. Determination of Hydroxyproline 78

C.6.6. Determination of Total Nitrogen 79

C.6.7. Measurement of α-Amino Nitrogen by Ninhydrin Reactions 80

C.6.8. Measurement of Protein by Folin Reagent 80

C.6.9. Measurement of Protein by Spectrophotometric Analysis 80

C.6.10. Quantitative Determination of Total Amino Acids 80

C.6.11. Determination of Carbohydrate by Paper Chromatographic Method 81

C.7. Methods for Enzymic Digestions 81

C.7.1. Collagenase 81

C.7.2. Pronase 82

C.8. Electrophoretical Analysis 82

C.8.1. Gel Electrophoresis 82

C.8.2. Disc Electrophoresis 83

C.8.3. Immunoelectrophoresis 83

C.9. Ultracentrifugal Analysis 84

C.9.1. Analytical Ultracentrifugation 84

C.9.2. Density Gradient Ultracentrifugation 84

D. Experimental 85

D.1. Immunological Properties and Chemical Nature of Dog GBM 85

Table of Contents (cont'd.)

page

D.1.1. Introduction 85

D.1.2. Collagenase Digestion of GBM and Fractionation of the Membrane Components 85

D.1.3. Immunological Characterization of Dog GBM and Its Components 90

a. Nephrotoxicity of the Antisera 90

b. Localization of Abs in Kidney 92

c. Ab-Ag Reaction in Gel Double Diffusion ... 94

D.1.4. Conclusions 95

D.2. Isolation and Characterization of Antigenic Components of Rat GBM 95

D.2.1. Introduction 95

D.2.2. Preliminary Isolation and Characterization of Rat GBM Components 97

a. Fractionation of the Collagenase-Solubilized GBM and Tendon Collagen 97

b. Immunological Properties 99

c. Chemical Characterization 102

D.2.3. Gel Filtration of Collagenase-Solubilized Membrane on Sephadex G-75 in Acetic Acid 109

D.2.4. Effect of Pronase Digestion on Nephrotoxigenicity of the GBM Component 111

D.2.5. Antigenicity of the GBM Isolated by Mild Alkali Treatment 112

D.2.6. Isolation and Characterization of Antigenic Components of GBM 115

a. Introduction 115

Table of Contents (cont'd.)

page

b. Collagenase Digestion, Urea Extraction and Separation of Solubilized Membrane Components by Gel Filtration 116

c. Nephrotoxicity and In Vivo Localization of Abs to Membrane Components 122

d. Interaction of Membrane Ags and Abs by Gel Double Diffusion 126

e. Chemical Composition of Membrane Components 128

f. Conclusions 133

D.3. Characterization of a Nephrotoxigenic Ag Isolated from Rat GBM 134

D.3.1. Chemical Composition 134

D.3.2. Antigenic Properties 138

D.3.3. Electrophoretical Analysis 141

D.3.4. Ultracentrifugal Properties 141

D.3.5. Conclusions 146

D.4. Effect of Non-Nephrotoxic Abs in Induction of NTN in Rats 148

D.4.1. Introduction 148

D.4.2. Induction of Proteinuria in Rats by Injections of Various Antisera 148

D.5. Molecular Sieving by Normal and Nephrotic GBM 151

D.5.1. Introduction 151

D.5.2. Preparation of Microcolumns of GBM 152

D.5.3. Fractionation of I^{131}-Albumin and C^{14}-Mannose on Microcolumns 153

D.5.4. Conclusions 156

Table of Contents (cont'd.)

	page
E. General Discussion	158
F. Summary	166
Contribution to Knowledge	169
References	171

LIST OF FIGURES

FIGURE		page
1	Gel filtration pattern on Sephadex G-75 of dog glomerular basement membrane solubilized by collagenase digestion	87
2	Localization of rabbit γ-globulin in renal glomerulus in rat injected with antiserum against dog glomerular basement membrane, as demonstrated by immunofluorescent method	93
3	Gel filtration pattern on Sephadex G-75 of collagenase solubilized basement membrane (rat) after prolonged centrifugation	98
4	Gel filtration pattern on Sephadex G-75 of bovine tendon collagen solubilized by collagenase digestion	100
5	Localization of rabbit γ-globulin in renal glomerulus of rat injected with antiserum against rat collagenase solubilized membrane	103
6	Precipitin reactions of rat glomerular basement membrane components and collagen with their antisera, by double diffusion in agar gel	104
7	Paper chromatogram of hydrolysate of collagenase solubilized membrane and monosaccharide standards	107
8	Polyacrylamide gel electrophoresis of collagenase and human serum	108
9	Gel filtration pattern on Sephadex G-75 of rat glomerular basement membrane solubilized by collagenase digestion, eluted in acetic acid (0.5M)	110
10	Localization of rabbit γ-globulin in renal glomerulus of rat injected with antiserum against rat glomerular basement membrane isolated by NaOH treatment	114
11	Gel filtration patterns of rat basement membrane solubilized by collagenase, and of collagenase a. collagenase on Sephadex G-100 (1.1x30 cm) b. CS75A on Sephadex G-100 (1.1x30 cm) c. Collagenase on Sephadex G-200 (1.4x26 cm) d. CS75A on Sephadex G-200 (1.4x26 cm)	117
12	Gel filtration pattern on Sephadex G-75 of rat glomerular basement membrane solubilized by collagenase digestion	119

LIST OF FIGURES (cont'd.)

FIGURE		page
13	Gel filtration pattern on Sephadex G-200 of CS75A	120
14	Gel filtration pattern on Sephadex G-25 of CS75B	121
15	Gel filtration pattern on Sephadex G-200 of decollagenized glomerular basement membrane solubilized by urea extraction	123
16	Isolation procedures of rat glomerular basement membrane components	124
17	Precipitin reactions between rat glomerular basement membrane components and their antisera by double diffusion in agar gel	129
18	Chromatography of glucosamine and galactosamine in acid hydrolysate of glycoprotein (US200A) in Dowex 50 x 8	136
19	The absorption spectra of sialic acid treated with thiobarbituric acid reagent	137
20	Precipitin reactions between glomerular basement membrane components and their antisera by double diffusion in agar gel	139
21	Immunoelectrophoresis of glomerular basement membrane components	142
22	Analytical ultracentrifugal pattern of glycoprotein (US200A) in tris buffer	144
23	Centrifugation pattern of serum γ-globulin and albumin in sucrose density gradient	145
24	Analytical ultracentrifugal patterns of glycoprotein (US200A) in 8M urea	147
25	Elution patterns of albumin-I^{131} and mannose-C^{14} on microcolumns of basement membrane and Sephadex G-25	155

LIST OF TABLES

TABLE		page
1	Relationship of glomerular clearance and molecular weight of some substances	13
2	Production of nephrotoxic serum nephritis in various species	36
3	Extra-renal tissues as antigens for the production of nephrotoxic serum nephritis	40
4	Chemical composition of collagenase solubilized dog glomerular basement membrane and its components	89
5	Immunological properties of dog glomerular basement membrane components	91
6	Nephrotoxic serum nephritis by injection of antiserum against rat glomerular basement membrane components and collagen	101
7	Chemical composition of collagenase solubilized rat basement membrane and its components	105
8	Nephrotoxic serum nephritis induced by injection of antiserum against rat glomerular basement membrane components	125
9	Cross-reaction between rat membrane components and their antisera by gel double diffusion	127
10	Carbohydrate composition of rat glomerular basement membrane components	130
11	Amino acid composition of rat glomerular basement membrane components	132
12	Effect of non-nephrotoxic antibodies on induction of nephrotoxic serum nephritis in rats	150

A. General Introduction

It is well established that heterologous glomerular basement membrane is antigenic to rabbits and that antibodies to glomerular basement membrane induce glomerulonephritis upon injection into animals of the same species. This is the most widely used method for experimental production of nephritis. Numerous investigations have been made to define the renal components responsible for the nephrotoxic antigenicity. However, these attempts have been unsuccessful due to the difficulties inherent in the isolation and solubilization of the glomerular basement membrane.

Chemical studies of the intact glomerular basement membrane of various species have revealed the presence of glycoprotein as well as of collagenous protein. Immunological studies indicate the multiple responses of glomerular basement membrane and cross-reaction of antibody and antigen of glomerular basement membrane among various species. It is the purpose of the present work to combine both chemical and immunological methods to isolate and define the antigenic components of glomerular basement membrane, particularly the specific nephrotoxigenic antigen.

It is now generally accepted that in experimental nephritis induced by nephrotoxic serum the initial fixation of nephrotoxic antibody on glomerular basement membrane leads to the occurrence of proteinuria and development of other clinical symptoms. The interactions of various types of antibodies on glomerular basement membrane in relation to the induction of glomerulonephritis were therefore investigated.

It has been suggested that proteinuria in nephrotoxic serum

nephritis is the result of increased permeability of the glomerular basement membrane. Experiments were therefore made to determine whether in the normal state glomerular basement membrane acts as a differential filter in restricting the passage of serum protein, and whether in nephrotoxic serum nephritis, proteinuria occurs as a result of increased permeability of the membrane itself.

B. Historical Introduction

B.1. Structure of Normal Glomerulus

B.1.1. Introduction

The glomerulus has been the subject of a considerable number of anatomical and functional studies since its discovery. The basic concepts concerning the structure of the renal glomerulus have been reviewed by many authors (1-4).

The glomerulus is a capillary bed originating in the afferent arteriole and terminating in the efferent arteriole. It is essentially a richly branched and interconnected capillary network which is indented at the beginning of the tubular duct. On entering the glomerulus, the afferent arteriole divides into several branches which are the glomerular capillaries. Over them is reflected the basement membrane of Bowman's capsule which, in turn, is continuous with the basement membrane of the tubules. Between the capillaries of a given lobule is a region called the mesangium or intercapillary space.

Electron microscopic investigations (5-7) have demonstrated that the glomerular capillaries develop in situ, i.e., a mass of mesenchymal cells differentiates into capillary endothelial cells. Only one cell type seems to maintain its original mesenchymal structure and functional features (8). This is the so-called mesangial cell.

B.1.2. Electron Microscopy of the Glomerulus

The fine structure of the normal mammalian glomerulus is fairly well established at the present time (1-4,9-11). The glomerular capillary walls are composed of three layers. The endothelial cells form the innermost layer and represent a direct continuation of the endothelium found in the afferent arteriole. A continuous layer of basement membrane establishes the middle component of the capillary wall. The outermost layer, the epithelial cells, covers the surface of the basement membrane and constitutes the visceral layers of Bowman's capsule. In the axial or centrolobular portions of the glomerulus, a fourth component, the mesangial cell, is also noted. This has been referred to as intercapillary or interluminal or deep endothelial cell (11-14). This cell is quite common in the avian glomerulus and can easily be identified in the mouse and rat glomerulus, but with less success in the normal human glomerulus (4).

B.1.2.a. Endothelium

The endothelium of the normal glomerulus is formed by squamous endothelial cells which display a local protrusion into the capillary lumen in the vicinity of the nucleus. The cytoplasm around the nucleus has a few mitochondria, a small Golgi apparatus, and varying numbers of RNP particles and small vesicles. The attenuated periphery of endothelial cells is reduced to a thin cytoplasmic layer occasionally perforated by large pores or "fenestrae" which measure 500-1000Å in

diameter (9,11). Each fenestra is bridged over by a thin diaphragm which measures 70Å in thickness (4,15).

B.1.2.b. Basement Membrane

The basement membrane forms the central continuous layer composed of fine fibrils embedded in a matrix which appears homogeneous at present levels of resolution (10-12,16,17). In human beings, it measures about 1100Å at birth and about 2700Å at maturity (3,7,18,19). It has a variable thickness of about 600Å to 1600Å with an average thickness of 800Å in the rat (3,4).

Earlier studies of the adult basement membrane, utilizing methacrylate embedded tissue, revealed three distinct layers. Hall (20) termed the central dense layer the lamina densa, and the two less dense zones on either side, the lamina rara. Bergstrand and Bucht (19) have stated that the lamina rara (interna and externa) are artifacts. However, Rhodin and others (4,13,14,16) believed that the basement membrane has a multiple-layer structure with a central dense zone and a less dense layer on either side. The central portion of the mature basement membrane has a homogeneous appearance; this section, when stained with heavy metals, demonstrates a network of interconnecting fine filaments of about 40Å in width within the basement membrane (17). These filaments resemble those described within lamina rara interna by Farquhar et al. (12). Neither of the filaments shows periodicity. There is no evidence of structural periodicity in the basement membrane similar to that

exhibited by collagen fibres (12,21).

According to current knowledge, the basement membrane is regarded as a continuous layer in which no pores can be detected. The fine structure reveals that the membrane consists of a network of fine filaments embedded in a homogeneous matrix.

B.1.2.c. Epithelium

The outermost layer of the capillary wall is composed of epithelial cells (or podocytes) with a system of characteristic interdigitating pseudopodia or "foot processes" (pedicels). The nucleus of the mature epithelial cell is surrounded by a fairly large amount of cytoplasm in which lie the Golgi zone and the rough-surfaced endoplasmic reticulum. Several short mitochondria and a large number of vesicular components are found throughout the cytoplasm in addition to numercus RNP particles (4).

The foot processes have a wide base which rests on the basement membrane and becomes narrower away from the basement membrane. The area between the foot processes, usually referred to as the slit pore, has been reported to be 100Å to 400Å wide with an average width of about 250Å (2,4,11). This area is bridged by a membrane 70Å thick, the so-called filtration slit membrane (1,11,15), in which a filamentous central thickening or reinforcement is found.

B.1.2.d. Mesangium

Mesangium, variously referred to as sponge fibres of the stalk or intercellular substances, is located centrally in the capillary tufts (4,13,22-26). The mesangial cells, first recognized by Zimmerman (22), are also termed intercapillary (13) or deep endothelial cells (11,12,25).

Structurally, the nucleus and the cytoplasm of the mesangial cells are almost identical with those of an endothelial cell, except in one aspect. The mesangial cell cytoplasm displays a multitude of short and complicated processes which are surrounded by a spongy material resembling basement membrane, the mesangial matrix (8). The matrix consists of an amorphous substance which fills the irregular spaces between the mesangial cells and the capillary endothelial cells or the true basement membrane.

In conclusion, the organization of the glomerular capillary differs in a number of important respects from that of capillaries found elsewhere in the body (27). The first distinction lies in the degrees of fenestration of the endothelium. The fenestrae of the glomerular endothelium are large and more numerous than those encountered in any other type of capillary. Secondly, the basement membrane of the glomerular capillary is thicker and more compact in appearance than that of most other capillaries. Thirdly, the surface epithelial layer with its foot processes is unique. Fourthly, the glomerulus is characteristically provided with mesangial cells and the discontinuous layer of spongy material which coats

the inner surface of its basement membrane.

B.2. Glomerular Filtration

B.2.1. Introduction

It has been known for years that glomerular ultrafiltration, tubular reabsorption and tubular secretion contribute in varying degrees to urine formation. The process of urine formation is initiated by the separation across the walls of the glomerular capillary loops of an ultrafiltrate of plasma. Driven by the net filtration pressure, the glomerular filtrate flows through the tubules. Many of the constituents of the filtrate are removed from the tubule lumen and returned to the peritubular capillaries to be retained in the body (28).

The filtering function of the glomerular tuft was inferred by Bowman (29) and Ludwig (30) in 1842. Richards and his associates (31) confirmed this view and predicated that the glomerular capsule fluid of amphibians was an ultrafiltrate of plasma. Results obtained by Walker et al. (32) for the mammals were fully in accord with those in the amphibians. It was shown by Hayman (33) that the hydrostatic pressure in the glomerular capillaries is great enough to supply the driving force for separation of the glomerular filtrate from the colloids of plasma.

The rate of glomerular filtration can be related to a series of forces favouring and opposing the filtration process and to the area and

permeability of the membrane across which the filtration occurs. The glomerular capillaries differ from capillaries elsewhere in the body in that they are interposed between the afferent and efferent arterioles. The hydrostatic pressure in the capillary lumen is maintained at a higher level and glomerular capillaries appear to have an appreciably higher permeability (34). Mechanisms of the glomerular filtration will be discussed on the basis of functional studies of glomerular components and the glomerular permeability in the normal state and in renal diseases.

B.2.2. Functions of Glomerular Components

Physiological studies have shown that the permeability of the glomerulus to any substance is related to the molecular dimensions of that substance (35-37). Dextrans of various molecular sizes have been used to study the permeability of the glomerulus and it was found that no dextran of molecular weight in excess of 55,000 appeared in the urine (38,39). Thus, the triple layers of the glomerular capillary wall act as a differential filter determining the molecular size of substances which may pass from blood to urine. Since both the endothelial and epithelial layers are discontinuous, it was suggested that the basement membrane is the only continuous layer and acts as a complete barrier between plasma and its filtrate (1,9-12).

The functional analysis of each of the components in glomerular filtration was first investigated by Farquhar et al. (12,25,40,41). On the basis of their studies on ferritin or thorotrast transfer across the

glomerular capillary wall, they proposed that the basement membrane is the principal filtration barrier, responsible for the differential permeability of the glomerulus to molecules of varying size.

The role of the basement membrane as the principal glomerular filter for large molecules is clearly indicated by the fact that, following ferritin administration, most of the molecules of circulating tracer (diameter 100Å) are retained in the lumen by the membrane and in time gradually pile against its luminal surface (12). The tracer molecules are found either between the basement membrane and endothelium or in the inner third of the membrane. No accumulation of tracer molecules occurs against the endothelial fenestrae or epithelial slits. Hence, their results clearly point to the basement membrane as the principal filter and effectively exclude the endothelium and epithelial slits as alternatives.

The result of the study by Latta and Maunsbach (13,14) on thorotrast penetration of the basement membrane provided direct evidence to support this hypothesis. They have shown that the dense middle layer of the basement membrane traps thorotrast particles which are 90-100Å in size and therefore acts as the main filtration barrier.

The ferritin transfer experiment, by Farquhar et al. (12,41,42) on normal and nephrotic animals suggested that glomerular epithelium may act as a monitor to recover, at least in part, the protein which manages to leak through the basement membrane. The few ferritin particles which penetrate the membrane are taken up by the epithelial cells in small

pinocytic invagination of the epithelial cell membranes at the base of the foot process. These invaginations are pinched off from the cell membrane to form intracytoplasmic vesicles of about 60-80 mμ. In normal animals the pinocytic activity is at a relatively low level. However, in the nephrotic animal, the basement membrane is clearly more permeable and allows passage of increased amounts of ferritin and the pinocytic activity of the epithelium is markedly enhanced (42). The individual ferritin particles are taken into epithelial cells by vesicle formation of plasma membrane and appear in large droplets. Thus, it is apparent that the epithelial cell can assimilate protein material from the glomerular filtrate.

Recently, Graham and Karnovsky reported their observations of glomerular protein transport by the use of peroxidases (43). Intravenously injected horseradish peroxidase (mol. wt. 40,000) passed rapidly through the endothelial fenestrae, across the basement membrane and through the epithelial slit into the urinary space, while human myeloperoxidase (mol. wt. 160,000 to 180,000) was apparently largely retained by the glomerular filter. They demonstrated that myeloperoxidase was impeded at the level of the epithelial slits and claimed that the epithelial slits are the primary filtration barrier responsible for the differential permeability to proteins of varying molecular size. However, in their electron micrograph, it can be seen that membranes of epithelial cells and basement membrane contained myeloperoxidase as well. Thus, it is questionable whether these findings support their conclusions and the suggestion originally made by Hall (2) and later by

Landis and Pappenheimer (44) that the epithelial "slit pores" restrict the filtration of protein and are presumed to impose a more precise restriction than does the basement membrane. Their results differ from those of Farquhar et al. (12) and Latta and Maunsbach (13,14). The actual site of the filtration barrier still remains to be established.

That the mesangial cell in the glomerulus functions in the removal and disposal of filtration residues was pointed out by Farquhar and Palade (25). The ferritin particles which come to lie against the basement membrane are phagocytized by these cells and thereby removed from the glomerular capillary wall. The results that both peroxidases are taken up in large amounts by the mesangial cells (43) lend support to the concept that an important function of the mesangial cells is the incorporation and disposal of glomerular filtration residues.

According to the present concepts, the glomerular filtration process appears to be more complicated than passive filtration through a membrane provided with rigid pores or slit pores. Each of the components of the glomerular capillary wall possibly plays a definite role in the filtration process (11,12,27,43): 1) The basement membrane acts as a differential filter, probably a "coarse" filter to exclude very large molecules and retard somewhat the passage of smaller molecules. The epithelial slits may appear to act as a "fine" filter and thus actually determine the nature of the protein content of the glomerular filtrate. 2) The epithelium may function to monitor the glomerular filtrate and thus compensate for the leakiness of the basement membrane.

3) The endothelium acts as a possible valve which, by varying the number and distribution of its fenestrae, can control access to the basement membrane. 4) The mesangial cells function to remove the filtration residues.

B.2.3. Glomerular Permeability

The original concept of an essentially protein-free filtrate suggested by Ludwig in 1844 (30) has since been modified to take into account the fact that small quantities of protein normally leak into the capsular space and are then reabsorbed by the tubules. The normal glomerulus ordinarily restrains the passage of molecules the size of serum albumin and larger, but allows excretion of smaller-sized proteins (45) or dextrans (38,39).

Experimental results (46-48) support the view that plasma albumin is filtered in very small amounts by the glomeruli of the normal kidney and is largely reabsorbed in the proximal convoluted tubules. Haemoglobin with almost the same molecular weight but a more spherical configuration is estimated to be present in glomerular filtrate at a concentration of about 5% of its concentration in plasma (49). Aggregates of human globin were also found free in Bowman's space within one minute after injection (50). Thus, differential excretion of proteins by glomeruli depends on their molecular weight and shape.

Two types of polysaccharide (inulin and dextran) have been used

to study the permeability of the glomeruli (38,39,51-53). Inulin (mol. wt. approx. 5,200) having a highly elongated molecule and a low diffusibility, passes the glomerular membrane quite readily and the concentration of inulin in glomerular filtrate is the same as that in plasma water (51). The clearance of low molecular weight dextran preparation (15,000 or less) was found to be equal to that of creatinine, indicating that these molecules could freely pass the glomerulus. The clearance of molecules larger than 50,000 to 60,000 was found to be very low, approaching zero, and that of molecules of about 30,000 mol. wt. was 20% of creatinine clearance (38,39,52,53). From the clearance studies with dextran molecules of various sizes, it is clear that their penetration is progressively restricted as their molecular weight increases.

The permeability of normal glomerulus to some protein molecules with decreasing molecular weight is summarized in Table I (43).

TABLE I

RELATIONSHIP OF GLOMERULAR CLEARANCE AND MOLECULAR WEIGHT OF SOME SUBSTANCES

Substance (Reference)		Mol. wt.	Glomerular clearance creatinine = 1
Ferritin	(12)	462,000	-*
Myeloperoxidase	(43)	160,000-180,000	-*
7S gamma globulin	(43)	160,000	-*
Plasma albumin	(37)	69,000	<0.01
Haemoglobulin	(37)	68,000	0.03-0.1
Egg albumin	(37)	43,500	0.22
Horseradish peroxidase	(43)	40,000	-*
Bence Jones protein	(54)	22,000-44,000	-*
Myoglobin	(37)	17,000	0.75
Inulin	(37)	5,500	0.97

* Glomerular clearance data unavailable for these substances.

The glomerular clearance of each of the protein molecules is relative to that of creatinine. The proteins of larger molecular weight have lower clearance. Although no clearance data are available for ferritin and myeloperoxidase, these molecules are not freely permeable to basement membrane and are retained by the normal glomeruli (12,43). However, horseradish peroxidase and Bence Jones protein with smaller molecular weight were reported to pass more freely through the basement membrane (43,54).

Available evidence (35-39,43,50-53) suggests that the normal glomerulus is to some degree permeable to molecules with a mean diameter in the range of 50 to 100Å but there is apparently progressive restriction to passage with increasing molecular weight and average diameter.

B.2.4. Proteinuria

B.2.4.a. Introduction

The occurrence of protein in urine and its relationship with diseases of the kidney was first noted by Bright (55). Protein excretion is considered abnormal in man when total daily excretion exceeds 150 mg or 10-20 mg per cent (56). In some varieties of disease, the urine has a grossly increased protein content, with the daily excretion rising to 20-30 gm or more. Proteinuria tends to remain relatively constant from day to day (57).

Heavy proteinuria (> 4 gm/day) is usually caused by renal

diseases which grossly increase glomerular permeability such as in acute and chronic glomerulonephritis. In the nephrotic syndrome, proteinuria may be increased by a high protein intake (58). Wide variations in protein excretion may occur in association with spontaneous changes in the stage and severity of the underlying renal lesion which are presumably caused by changes in glomerular permeability or tubular transport of protein. There is evidence that the level of plasma albumin and the rate of glomerular filtration also affect protein excretion.

B.2.4.b. Urinary Proteins

It has been shown by electrophoretic fractionation (47,59,60) that two-thirds to three-fourths of the protein in normal urine has the mobility of serum globulin; the remainder, the mobility of albumin. Ultracentrifugal measurements of molecular weight indicate probable identity of the urine and plasma albumin (61). It has been demonstrated also by immunochemical methods that the albumin fraction as well as several globulin fractions in the urine are antigenically closely related to their analogous electrophoretic fractions in normal plasma (47,61,62). There are, in addition, apparently several fractions in normal urine not found in plasma. The most prominent among these is the high molecular weight mucoprotein described by Tamm and Horsfall (63).

In the majority of the diseases associated with significant proteinuria (nephrotic syndrome, acute and chronic glomerulonephritis),

it is now generally agreed that an albumin fraction apparently identical with that in serum, immunologically and electrophoretically, constitutes some 60 to 90% of the total urinary protein (64-66). Much smaller quantities of each of the four major plasma globulins are also excreted. There have been identified in urine small amounts of a lipoprotein which appears to be similar to the ß-lipoprotein in plasma (57). The plasma globulins which are excreted may represent fractions of relatively low molecular weight. The nephrotic urine presumably contains, in addition to intact γ-globulin molecules, the smaller molecular weight fragment of serum γ-globulin.

B.2.4.c. Mechanism of Increased Proteinuria

There are at least four possible mechanisms responsible for the occurrence of proteinuria in renal diseases: 1) Increased glomerular filtration of proteins. 2) Decreased tubular removal of filtered proteins. 3) Presence of abnormal circulating proteins which are more filtrable than normal plasma proteins. 4) Addition of proteins to the urine by renal tubular cells.

The available evidence strongly favours the concept that excessive glomerular leakage of protein is responsible for the heavy proteinuria that occurs in the nephrotic syndrome, and perhaps in all other cases of heavy proteinuria (57,67) in preference to the concepts of depressed tubular functions or the presence in the plasma of abnormal proteins. The following observations and experimental results implicate

the increased glomerular filtration of protein:

1) In almost all situations associated with increased proteinuria, there is anatomical evidence of glomerular involvement (68,69); by contrast, in many disorders characterized by physiological or anatomical evidence of severe tubular damage, proteinuria may be minimal or absent. Ultrastructural correlation with proteinuria will be described later.

2) Chinard et al. (70) demonstrated that following the infusion of human albumin into nephrotic patients, proteinuria may far exceed the estimated maximal rate of filtration of protein in normal subjects. The minimal concentration of albumin in the glomerular filtrate of such patients, calculated from the rate of excretion of albumin and the glomerular filtration rate, may be increased several times over the estimated upper limit for the normal filtrate.

3) Hardwicke and Squire (71,72) have found that the relative clearance of some of the serum proteins in the nephrotic syndrome appears to be roughly inversely proportional to the effective molecular diameter of each protein. They have shown that in nephrotic patients the differential globulin clearance of alpha-1 mucoprotein is equal to 124% of the albumin clearance, and that the β-globulin clearance is 29%, the γ-globulin clearance 27% and the alpha-2 globulin clearance 13% of the albumin clearance (71).

Furthermore, the electrophoretic distribution and the average molecular weight of the proteins separated from normal and nephrotic sera

by ultrafiltration through a semi-permeable nitrocellulose membrane closely resemble those of nephrotic urine (65). These observations strongly suggest that proteinuria in the nephrotic syndrome results from the molecular "sieving" action of an abnormally permeable glomerular filtration.

A similar conclusion would also seem to follow from the fact that the dextran molecules excreted when proteinuric patients are infused with dextran mixtures of varying molecular weight, are considerably larger than those excreted by normal subjects given similar infusions (38).

4) The increased urine proteins appear to consist largely of an albumin fraction chemically and immunologically indistinguishable from that found in normal plasma (64,65). Furthermore, infusion of plasma from proteinuric patients into normal subjects does not produce proteinuria (73). From these considerations, it would follow that if there is increased glomerular filtration of protein, it is mainly caused by increased permeability of the normal glomerular barrier, rather than by the presence in the plasma of abnormal proteins which might be more filtrable than normal proteins.

Some of the morphological alterations in the glomeruli associated with proteinuria have been identified in electron micrographs of glomeruli from renal biopsy material. The earliest recognizable change is the fusion and loss of the foot processes of the epithelial

cells (68,69,74,75). It was demonstrated in puromycin-induced nephrosis, that foot process fusion took place about the time that proteinuria developed (74,75). Further evidence indicates that this lesion can be produced in normal animals by peritoneal injection of both homologous and heterologous albumin (76-78). It is probable that the epithelial changes are the result of increased filtration of protein. Although in some conditions with proteinuria, there is proliferation of the endothelium, no regularly occurring endothelial defects have been demonstrated (79).

Experimental observations indicate that the basic abnormality in various forms of proteinuria lies in the basement membrane itself (11,16,68,69,80-83). Many investigators have found an increase in thickness of the basement membrane and some changes in basement membrane such as focal accumulation of dense material (68), frail appearance and loss of normal texture (16).

Farquhar and Palade (41,42) have used ferritin as a tracer to investigate the glomerular permeability in nephrotic rats. They showed that, in comparison with the control animals, many more ferritin molecules were present in the nephrotic basement membrane, suggesting that penetration of ferritin molecules is freer and deeper in the nephrotic basement membrane. In addition, the pinocytotic activity was marked along the epithelial cell facing the basement membrane. In nephrosis, the reabsorbing activities of the epithelium are greatly enhanced to compensate for the increased permeability of the basement membrane.

Spiro (69,83) reported membrane defects ranging from a few hundreds to a thousand Å units in human nephrosis and suggested that such defects were responsible for proteinuria. Variation in density of the basement membrane, as well as actual defects in the membrane, was observed by Movat et al. (84). A decrease in density presumably associated with hydration and separation of the constituents could account for increased permeability.

Membranous changes are reversed by steroid therapy, especially in children. Subsidence of proteinuria along with reversal of the lesion affords an excellent example of the correlation between basement membrane structure and glomerular permeability as the major mechanism for nephrotic proteinuria (67). Lauson et al. (85) demonstrated a decline in permeability index in children treated with ACTH.

In conclusion, on the basis of the infusion and clearance studies and morphological observations, it seems likely that injury to the glomerulus is the immediate cause of the proteinuria observed in various disease states. The electron microscopic observations in the nephrotic syndrome and glomerulonephritis suggest that the increased glomerular permeability is due to a defect in the molecular organization of the basement membrane which normally appears to be the main differential filtration barrier. The characteristic early changes in the epithelial foot processes are apparently secondary to the increased passage of proteins across the basement membrane. Although proteinuria appears to be mainly the result of increased glomerular permeability to plasma

proteins, a concomitant reduction in the tubular reabsorption of filtered proteins cannot be ruled out.

B.3. Experimental Production of Nephritis

B.3.1. Introduction

The nephrotic syndrome is classically defined as a clinical entity having multiple causes and characterized by increased glomerular permeability that is manifested in massive proteinuria and the excretion of fat bodies. There is a variable tendency toward edema, hypoproteinemia and hyperlipemia (67). Protein excretion rates are usually in excess of 3.5 gm per 24 hours per 1.73 squares metres of body surface area in the absence of depressed glomerular filtration rates.

The common histological findings in the glomeruli of nephrotic patients (e.g. membranous glomerulonephritis) are: the glomerulus is abnormally cellular, many of the cells being polymorphonuclear leucocytes which are absent or rare in the normal glomerulus; the capillary lumina are small and there is blurring, thickening and smudging of the basement membrane in the capillary tufts. In electron microscopy, the earliest changes have been noted in the epithelial cells which show loss of organization of foot processes (68,69,79).

Transient syndromes in animals bearing a clinical and laboratory resemblance to the nephrotic syndrome in humans have been produced in a wide array of species by a variety of techniques. Heymann and Lund (86)

have pointed out the close similarity of the clinical and metabolic features of the experimental diseases in rats to those of the nephrotic syndrome as it occurs in children. Some of these syndromes have appeared to involve primarily an immune mechanism; others a direct nephrotoxicity and still others varying combinations of the two.

Experimental nephritis can be produced by chemical agents and by immunological methods. There are several forms of nephritis induced by immunological reactions: first, by nephrotic serum containing heterologous Abs capable of reacting with Ags fixed in the kidney and, second, by foreign proteins forming circulating Ag-Ab complexes, themselves immunologically unrelated to the kidney, which accumulate in the glomeruli as in the case of serum sickness. There is a third form of nephritis which can be induced by immunization with heterologous, homologous or autologous renal Ags. Another experimental approach to induce nephritis is to immunize with bacterial Ags. These various methods will be described separately with emphasis on the nephrotoxic serum nephritis.

B.3.2. Nephritis Induced by Chemical Agents

B.3.2.a. Aminonucleoside of Puromycin (ANP)

In 1955, Frenk and associates (87) produced the nephrotic syndrome in rats by daily subcutaneous injection of 6-dimethylamino-purine 3-amino-D-ribose, an aminonucleoside which represents a hydrolysis product of puromycin. Subsequently, studies of experimental nephrosis

using this technique were reported by many investigators (88-95).

In aminonucleoside nephrosis, a minimum lag period of five to eleven days preceding the onset of proteinuria is regularly noted (88-90). Wilson et al. (89) have reported that ANP produces renal disease in the rat, but not in the dog, rabbit, guinea pig or mouse and they suggested that the substance itself is not nephrotoxic but acts through a derivative which may be a specific antimetabolite (92).

The precise chemical mechanism by which ANP produces the renal lesion has not been completely defined. The nephrotic syndrome was also produced by injection of monomethyl, methylpropyl and diethyl adenosine derivatives but not by adenosine, inosine or dipropyl adenosine derivatives (91). The active derivatives all inhibited a crude adenosine kinase enzyme obtained from yeast. It seems probable that the enzymatic locus of action may be adenosine kinase but action at the nucleotide level is also possible (91,93). Fisher and Gruhn (94) reported a distinctive decrease in succinic dehydrogenase, cytochrome oxidase and TPN and DPN diaphorases in ANP nephrosis and also an apparent direct relation between degree of depletion of tubular enzymes and degree of proteinuria. Giroud et al. (90) noted during the induction of ANP nephrosis that the serum albumin fell before significant proteinuria began and suggested the ANP may inhibit protein synthesis.

The regularity with which a well-defined latent period was observed suggested the possibility that an immune mechanism might be involved in ANP nephrosis. The fact that the disease appears to be

unaffected by adrenal cortical steroids or X-ray irradiation (93,94) refutes this possibility. Evidence obtained by the passive transfer of whole serum, β-globulin and γ-globulin from ANP-treated rats to normal rats and results obtained with cortisone and nitrogen mustard treatment support the view that ANP does not produce nephrosis in the rat by means of an immune mechanism (95).

B.3.2.b. Tridione

Trimethadione, chemically unrelated to the aminonucleoside derived from puromycin, has been reported to produce in rare instances a nephrotoxic syndrome in man (96). It has also been shown that 50% of the rats that were given this compound orally in daily doses, for a period as long as 18 months, developed a nephrotic syndrome (93). None of the rats in which the administration of tridione was discontinued after proteinuria had persisted for several weeks showed evidence of spontaneous subsidence.

B.3.2.c. Polyvinyl Alcohol

Subcutaneous administration of aqueous solutions of polyvinyl alcohol having a molecular weight of 133,000 to rats maintained on a high NaCl diet produced a nephrotic syndrome, characterized by hypertension, edema, ascites, proteinuria and anaemia (97,98). The polyalcohol lodged within the glomeruli, tubular lumens and interstitial spaces. Subsequently, polyvinyl alcohol of three different molecular weights (37,000, 133,000 and 185,000) was used. It was found that the lowest

molecular weight material had no pathological effect, while the other two polymers were apparently too large to be efficiently filtered and hence lodged within the glomeruli causing renal damage (99).

B.3.3. Glomerulonephritis Induced by Immunological Methods

B.3.3.a. Nephrotoxic Serum Nephritis (NTN)

Lindemann in 1900 first demonstrated that heterologous anti-kidney serum could elicit glomerulonephritis upon injection into an animal of the species from which the kidney was originally obtained (100). Since then "nephrotoxic serum nephritis" has been used as an experimental model by investigators concerned with human glomerulonephritis. Masugi (101,102) made a detailed pathological study of NTN in rats and rabbits and noted its similarity to human glomerulonephritis. As the nephritis progressed, animals developed clinical signs and symptoms of impaired renal function, i.e., elevated blood urea nitrogen, persistent urinary abnormalities, hypertension, low serum proteins and hyperlipidemia. Of crucial significance were the experiments of Kay (103,104) who showed the importance of the host response to the injected NTS in experiments using duck anti-rabbit NTS in rabbits.

Pathological studies of NTN by electron microscopy have demonstrated that the glomerulus, particularly the basement membrane, was the site of fixation of NTAb and consequently the site of the main pathological changes. Ultrastructurally the principal lesion was on the luminal side of the basement membrane with irregular deposits of electron-

dense material (23,81). There followed numerous studies describing the immunological events and pathogenetic mechanisms in nephrotoxic serum nephritis. These will be discussed later in detail in another section (B.3.4.).

B.3.3.b. Foreign Proteins (Ag-Ab complex) Nephritis

Chronic glomerulonephritis was first demonstrated in 1913 in the rabbit and the dog by repeated injections of horse serum proteins or egg white protein (105). Hawn and Janeway (106) were able to produce acute glomerulonephritis by a single large injection of bovine γ-globulin into a rabbit. A latent period of one to two weeks occurred before the onset of clinical nephritis characterized by proteinuria and a variety of lesions. It was suggested that the interaction of bovine γ-globulin and host Ab and their localization caused tissue damage in glomeruli and other areas (106).

Hamilton and Fremes (107), in 1954, using reversed passive anaphylaxis, were able to induce nephritis in animals that closely followed the natural history of the disease in man. The animals were first injected with horse serum and shortly thereafter with anti-horse serum. Soon after injection of the anti-horse serum, signs of nephritis began to develop. The earliest renal change was simple, swelling of the glomerular cells; this was followed by glomerular proliferation and changes in the basement membrane.

Germuth (108,109) was the first to demonstrate that, in this

foreign protein nephritis, the renal lesion did not develop until just prior to the appearance of circulating Ab to the foreign proteins. Regression of the lesions begins several days after complete removal of Ag from the circulation. These observations suggest that the circulating soluble Ag-Ab complexes are responsible for the tissue damage.

Subsequently, it was shown that intravenous injections of soluble Ag-Ab complexes could produce serum sickness in mice and rats which was characterized predominantly by glomerulonephritis (110-112). The complexes apparently did not dissociate after their injection in vivo and when injected repeatedly during a 24-hour period induced an acute glomerulonephritis which persisted for two to three weeks.

Recently, Dixon and some collaborators (113-115) made a detailed immunopathological study of rabbits injected daily with heterologous serum proteins and established a relationship between the presence of circulating immune complexes and the development of chronic glomerulonephritis. It was found that the most intense experimental lesions occurred during the immune phase of Ag removal from the body; i.e., during the phase in which circulating soluble Ag-Ab complexes are at a maximum.

Andres et al. (116) were able to reproduce the various types (acute, subacute and chronic glomerulonephritis) of renal lesions in rabbits by repeated injection of bovine serum albumin and confirmed that the ratio of Ag to Ab was the factor determining the development and type of glomerulonephritis. Coincident with the development of nephritis and

throughout the course of the disease, the Ag, host γ-globulin and host complement (β_{1c}-globulin), presumably in complex form, were detected by means of the fluorescent Ab technique as a fine, granular, discontinuous deposit along the glomerular capillary walls (113-117). This pattern appeared to be quite different from the uniform linear deposit of nephrotic Ab along the capillary walls in NTN. Andres et al. (116) demonstrated, ultrastructurally, with the use of the ferritin-Ab technique that Ag-Ab complexes are present in the blood, cross the endothelium and the basement membrane, and accumulate as dense deposits between the basement membrane and the epithelial cytoplasm. Thickened or frayed basement membrane and the fusion of epithelial foot processes were also noted.

In conclusion, glomerulonephritis can be developed in the presence of circulating, soluble complexes of Ab with Ag, immunologically unrelated to renal Ags. Renal lesions may be either acute or chronic, depending upon the level and persistence of the complexes in the circulation. Simultaneously with the development of nephritis, complexes are deposited in the glomerular capillary wall and exert their effects on the glomeruli.

B.3.3.c. Nephritis Induced by Immunization with Renal Ags

Experimental animals may develop anti-renal Abs and glomerular nephritis if immunized to heterologous or homologous kidney Ags. Nephritis has been induced in rats, sheep and rabbits by repeated immunization with heterologous or homologous kidney fractions with or without Freund's

adjuvant.

Heymann et al. (118) reported a well studied, reproducible method for inducing nephritis in rats by intraperitoneal injections of rat kidney proteins mixed with Freund's adjuvant. After six to ten injections (3-5 months), the animals developed proteinuria with hyperlipemia and hypercholesterolemia. The renal disease seemed to be progressive without any further immunization (118). Ultrastructurally, the glomerular basement membrane was thickened and characteristically had numerous electron-dense deposits on the epithelial side (119). Fluorescent Ab studies on nephritic rats demonstrated host γ-globulin and β_{1c}-globulin in small, discrete, irregular beads along the capillary walls (120).

Transfer of this experimental nephritis from diseased rats to normal rats by means of lymphoid cells was demonstrated by Hess et al. (121) and Heymann et al. (122). However, it is necessary to make the animal tolerant at birth to spleen cells from a prospective donor for the successful transfer of disease. It was also demonstrated that nephritis can be transferred by parabiosis (122,123).

Steblay was able to induce glomerular nephritis in sheep (124, 125) and monkeys (126) by immunization to heterologous (human, dog, rat and rabbit) glomerular basement membrane in complete Freund's adjuvant. One to three months after the beginning of immunization, most animals developed a severe and usually fatal glomerulonephritis. Nephritis has been transferred from nephritic sheep to normal sheep by cross-circulation after bilateral nephrectomy of the nephritic sheep (127).

Lerner and Dixon (128) have demonstrated the transfer of nephritis by serum globulins from unilaterally nephrectomized nephritic sheep to normal recipients. Absorption of the globulin to be transferred with sheep glomerular basement membrane removed its nephritogenic effect. By using large amounts of whole plasma from nephritic sheep, Rudofsky and Steblay (129) were able to transfer a progressive disease similar to that in the donor to adult recipients pretreated with Freund's adjuvant.

Glomerulonephritis has also been developed in rabbits by repeated immunization with homologous or heterologous kidney fractions containing basement membrane (130,131). Of the rabbits immunized to homologous kidney fractions with complete Freund's adjuvant, 20 to 50% developed nephritis within 50 to 90 days after immunization (131,132). No higher incidence of glomerulonephritis was obtained by immunization with heterologous mammalian kidney sediment. Autologous basement membranes were found to be not as effective as Ags (132). γ -Globulin from nephritic rabbits, when injected into normal rabbits, could produce a mild and transitory proteinuria in the recipients.

In summary, the experimental results obtained in various species of animal immunized with renal Ags and transfer studies indicate the involvement of auto-antibodies. However, glomerulonephritis has been considered by some to be auto-immune inasmuch as the host's immune response apparently injures his own tissues, even though the eliciting Ag may not be native autologous material. Abs may apparently be directed to those Ags cross-reactive or shared between the immunizing kidney

and that of the host (cross-reactive autoimmunity). The actual pathogenetic mechanisms involved in these nephritides have not been totally elucidated. A circulating Ag-Ab complex lodging in the glomeruli may also be partly responsible for the renal injury in this type of nephritis (133).

B.3.3.d. Nephritis Induced by Immunization with Bacterial Ags

The association of glomerulonephritis with a preceding streptococcal infection has been well documented. One of the first experimental approaches used was that of Duval and Hibbard (134). Rabbits were immunized with streptococci obtained from patients with scarlet fever and challenged later with streptococcal lysates. This resulted in renal lesions which were classified as acute haemorrhagic nephritis. Bell and Clawson (135) injected suspensions of streptococcus viridans intravenously into monkeys over a period of four years. The animal developed chronic glomerulonephritis and died in uremia.

Kelly and Winn (136) inoculated streptococci into an intraperitoneal diffusion chamber in mice, thus allowing only the soluble products of the streptococci to escape into the circulation. Renal lesions developed in mice only when nephritogenic type 12 streptococci were used and occurred about seven days after the initial injection. The result suggested that a hypersensitivity reaction was responsible for the production of the nephritis that developed.

Localization of streptococcal products in animals after intra-

venous injection was noted in renal glomeruli, tubules and the reticulo-endothelial system (137-139). Kaplan (138) injected the M protein from group A streptococci into mice and found it localized principally in the glomerular endothelial cells and in the endocardium. However, the M protein injected was not sufficient to produce visible lesions.

Kantor (140) was able to produce renal lesions in rats and mice by intravenous injections of a large amount of M protein from type 1 streptococci. The lesions were confined to renal glomeruli and characterized by diffuse eosinophilis glomerular lesions, shown to contain streptococcal M protein and fibrinogen. Gradual regression of the morphological lesions was observed during the three weeks following injection. Initial abnormal proteinuria and azotemia returned to control levels by the end of the first week. A second rise in urinary protein excretion and urea retention was demonstrated in some rats coincident with the appearance of antibody to M protein. These results suggest the mechanism of renal localization of streptococcal M protein by means of a complex with fibrinogen which may comprise an initial phase in the pathogenesis of acute post-streptococcal glomerulonephritis (140).

Markowitz and Lange (141,142) have shown that a soluble component from pooled human glomeruli is chemically similar to and immunologically cross-reactive with a soluble Ag derived from the cell membrane of type 12 nephritogenic streptococci.

The presence of streptococcal M protein in renal glomeruli of patients with acute glomerulonephritis has also been reported recently.

Seegal et al. (143,144) employing ferritin-labelled Ag, have demonstrated the presence of streptococcal Ag, 7S γ-globulin and β_{1c}-globulin in the glomeruli of most patients with this disease. Michael et al. (145) pointed out that discrete deposits of β_{1c}-globulin and γ-globulin were present within and along the epithelial surface of the glomerular basement membrane during the acute phase of the disease. The characteristic lesions in acute post-streptococcal glomerulonephritis show some similarity to experimental Ag-Ab complex nephritis. They have also noted the resolution of these deposits during recovery from the disease and their absence from the glomeruli ten years after acute post-streptococcal glomerulonephritis (145).

It is apparent from various studies described that nephritogenic streptococci are involved in immunological reactions within the kidney in the production of glomerulonephritis.

B.3.4. Immunological Aspects of Nephrotoxic Serum Nephritis

B.3.4.a. Introduction

Nephrotoxic serum nephritis (NTN) has been the most extensively studied classic model of glomerular injury in experimental nephritis. In this experimental disease, the serum donor is immunized with the renal tissue from an animal of a different species; the antiserum from the donor is then injected into an animal of the species from which the renal Ag was obtained. A diffuse glomerulonephritis develops which is usually chronic and self-perpetuating (86,100-104). Further investigations have

indicated that NTN is not strictly species-specific (146-148) and non-renal Ags, in some instances, can also elicit nephrotoxic serum (149). The species of recipient animals, the origin of the antiserum, the nature of Ags used in producing NTS, and potency of the antisera are factors in determining the outcome of the disease.

The general character of NTN in different species, antigenecity of kidney and non-renal tissues, the interaction of the NTAb with tissue Ags responsible for renal injury, and the heterologous and autologous Abs involvement in the pathogenesis of NTN, will now be discussed.

B.3.4.b. Nephrotoxic Serum Nephritis in Different Species

The first description of NTN was given by Lindemann (100) in 1900, who induced albuminuria and uremia in rabbits after injection of guinea pig anti-rabbit kidney serum. Pearce (150) studied NTN in dogs with rabbit anti-dog NTS and determined that the renal cortex could be used as the immunizing Ag. Masugi in 1933 described his classical work on experimental nephritis using anti-kidney serum from a rabbit or a duck (101,102). He demonstrated that a single injection of anti-kidney serum given to a rabbit or a rat produced not only an acute nephritis but also a self-perpetuating chronic disease which he suggested to be due to an Ab-Ag reaction within the kidney. Smadel (151-153) confirmed Masugi's work and further reported complete clinical and pathological studies of both the acute and chronic stages of NTN of the rat. He pointed out that the nephrotoxic component of antiserum was a serum globulin.

Studies of NTN in rabbits, by using duck anti-rabbit kidney serum, have demonstrated a latent period of four to ten days (102-104). Kay was the first to show the role of the host's immune response in NTN in rabbits injected with duck antiserum (103,104) and correlated the development of proteinuria with the appearance of circulating host Ab. He demonstrated that prior injection of duck serum eliminated the latent period (103) and X-ray irradiation abolished the development of the persistent proteinuria and suggested that production of Ab by the recipient animal was necessary for renal damage to occur (104).

Subsequently, many investigators have studied NTN in different species. Some of the experiments using different species systems are summarized in Table II. It may be noted that NTN has been studied mainly in rats, rabbits and dogs. The NTS employed for inducing experimental nephritis can be of mammalian (rabbit, guinea pig, goat and sheep) or avian (duck and chicken) origin. Ags used to elicit NTS are mostly of the same species as that of the recipient. However, there are few experiments (146-148,163,172-176) demonstrating species non-specificity in NTN (3 species involved).

Earlier investigators have observed the difference in the onset of the disease induced by mammalian antiserum and avian antiserum. Clinical symptoms (proteinuria and cylindruria) developed immediately after use of mammalian NTS, while a latent period occurred in the case of avian NTS. However, it has been shown that certain high titer duck anti-kidney sera are capable of producing an immediate onset of NTN (155,

TABLE II

PRODUCTION OF NEPHROTOXIC SERUM NEPHRITIS IN VARIOUS SPECIES

Species			
Recipient (NTN-induced)	NTS producing	Ag donor	Investigators and Reference
Rat	Rabbit	Rat	Masugi (101), Smadel (151-153), Heymann (86,154), Baxter (149), Churg (81), Seegal (155), Hammer (156), Unanue (157).
Rat	Duck	Rat	Lange (158), Seegal (155), Stavitsky (159), Hasson (160).
Rabbit	Guinea pig	Rabbit	Lindemann (100).
Rabbit	Goat	Rabbit	Mellors (161,162).
Rabbit	Sheep	Human	Steblay (163,164).
Rabbit	Chicken	Rabbit	Lange (158,165).
Rabbit	Duck	Rabbit	Masugi (102), Kay (103,104), Lange (166,167), Seegal (168).
Rabbit	Duck	Dog	Simonson (146), Markowitz (147).
Dog	Rabbit	Dog	Pearce (150), Seegal (169), Stickler (170), Movat (23).
Dog	Chicken	Dog	Fouts (171).
Dog	Duck	Rat	Simonson (146).
Dog	Duck	Rabbit	Markowitz (147).
Dog	Rabbit	Human	Steblay (148).
Monkey	Sheep	Human	Michael (172), Huang (173), Markowitz (174).
Mice	Rabbit	Human	Arana (175), Blair (176).

157,159,160). It appeared that the clinical severity and onset of NTN was dependent upon the amount of NTS administered and the amount of Ab capable of fixing to the glomeruli.

Unanue and Dixon (157) have shown quantitatively by radioactive labelled Abs that there is a direct correlation between the amount of Ab injected, the amount fixed in the kidney and the degree of severity of the immediate renal injury.

On the basis of various studies on the mechanism of NTN, it has been postulated that the development of this disease depends on the fixation of heterologous anti-kidney Ab to the glomeruli, the synthesis of Ab by the recipient to the heterologous anti-kidney protein and the subsequent interaction of Ab and Ag with resultant injury to the renal glomeruli (103,104,133,155,157,174).

B.3.4.c. Tissue Antigens Involved in Nephrotoxic Serum Nephritis

B.3.4.c.i. Renal Tissues

Since Lindemann (100) first demonstrated in 1900 that heterlologous antiserum to kidney could induce NTN, the major source of nephrotoxic Ags was shortly localized to the cortex of the kidney (150). Solomon et al. (177) further demonstrated that the nephrotoxic Ags resided in the glomeruli. They separated the kidney into glomeruli and tubules and showed the ability of glomerular fractions to preferentially absorb nephrotoxic activity from a rabbit anti-rat kidney serum. Direct

evidence for the antigenicity of glomeruli was provided by Greenspon and Krakower (178) who showed that about 400,000 isolated glomeruli from dog kidney could produce a potent nephrotoxic serum when injected with alumina-gel adjuvant into rabbits. Other cortical components, relatively free of glomeruli, were incapable of doing so.

By studying the antigenic potency of isolated fractions of sonicated glomeruli, Krakower and Greenspon (179) demonstrated that the major nephrotoxic antigen tissue in the kidney was the glomerular capillary basement membrane. They showed that the parietal capsules were relatively non-antigenic. Though antisera to isolated glomerular cells were found to be nephrotoxic, preparations of glomerular basement membrane were 50 times more active than mixed glomerular cells on a nitrogen analysis. Subsequent work demonstrated that the activity of nephrotoxic Ag in immature glomeruli, where glomerular basement membrane is thin, was considerably less than in mature glomeruli (180).

Goodman and Krakower et al. (181,182) presented further evidence to indicate the complex nature of the antigenicity of the glomerular basement membrane. They found that glomerular basement membrane not only produced nephrotoxic Abs, but also induced Abs that cross-reacted with capsular basement membrane, tubular basement membrane and collagenous fibres from tendon and cornea. These latter Abs, however, were proved not to be nephrotoxic. Potent antisera to tubular epithelial cells were produced, but were not nephrotoxic. The results suggested that Ab response to glomerular basement membrane Ags is multiple, only a part being responsible for the production of experimental nephritis.

B.3.4.c.ii. Non-renal Tissue Ags

Many investigators have shown that the kidney is not the sole source of nephrotoxic Ags. In contrast to these results indicating that the nephrotoxic Ags resided only in the glomeruli are the findings that nephrotoxic sera could be produced by using a variety of different organs e.g., lung, placenta, aorta, spleen and liver (149,150,183-192). However, these extra-renal organs were not of equal potency (149). Heterologous antisera to connective tissue Ags of most organs cross-reacted with renal basement membrane, as evidenced by different *in vivo* and *in vitro* methods (191).

Some experiments using extra-renal tissues as Ags to elicit nephrotoxic Abs and subsequently NTN in many species of experimental animals are summarized in Table III. As data accumulated, it became apparent that the nephrotoxic antigenic determinants were present rather diffusely throughout the body. Though non-renal heterologous anti-organ sera rarely produced lesions in organs other than the kidney, they commonly produced glomerulonephritis.

Baxter and Goodman (149) have determined the concentration of nephritogenic Ag among kidney structures and non-renal tissues by their ability to absorb NTAbs *in vitro* and eliminate the nephritogenic potency of the serum. They have placed tissues according to this method in the following order: 1. Glomeruli; 2. Whole kidney or kidney medulla; lung or placenta; 3. Intestine and heart; 4. Other organs. These results led them to suggest that common Ags are present in these tissues and are

TABLE III

EXTRA-RENAL TISSUES AS AGS FOR THE PRODUCTION OF NEPHROTOXIC SERUM NEPHRITIS

Source of Ag	Species of recipient (NTN-induced)	Investigator and Reference
Brain	Rat, Rabbit	Smadel (151), Seegal (168), Baxter (149), Spuhler (186)
Lung	Rabbit, Rat, Dog	Baxter (149), Spuhler (186), Krakower (189), Markowitz (147), Chikamitsu (183), Triedman (190)
Liver Stroma	Rat	Pearce (150), Baxter (149)
Stomach	Rabbit	Hamori (187)
Placenta	Dog, Rat, Mouse	Seegal (169,184), Bevan (188), Baxter (149)
Heart	Dog, Rabbit, Rat	Baxter (149), Krakower (189,192), Katz (191)
Spleen	Rat	Katz (191)
Intestine	Rat	Baxter (149)
Aorta	Rabbit	Strehler (185)
Muscle	Rabbit, Rat	Baxter (149), Spuhler (186); Katz (191)

responsible for the removal of the nephrotoxic Abs for production of NTN.

Krakower and Greenspon (189) have established the amounts of nephrotoxic Ag contained in various tissues by immunization with different quantities of a given tissue fraction necessary to induce a potent NTS. They have shown the relationship between the degree of vascularity and the content of nephrotoxic Ags in various canine tissues. Thus, for the sequence glomeruli, lung, choroid plexus and ciliary, there was strong correlation with the surface area of the capillary vascular bed. While poorly vascular tissues (heart valves, tendon and ligamentum flavum) contained very little antigenic activity, avascular tissues (cartilage, cornea and lens) were devoid of nephrotoxic Ags (189).

The capacity of heterologous anti-tissue-sera to induce nephritis varies greatly. Some antisera such as those directed to rat lung, placenta, heart and spleen may cause immediate npehritis when injected into rats (184,188,190,191). Other antisera, like those directed to the aorta, muscle and liver stroma cause, at most, a delayed nephritis (149,150,185,186,191).

Recently, Katz et al. (191) have attempted to determine the amounts of Ab in such antisera to heart, muscle and spleen; the extent of cross-reactivity among the different tissues and the behaviour of such cross-reacting Ab in the kidney. They have shown that anti-heart and anti-spleen Abs cross-react extensively with the kidney; consequently,

immediate nephritis was induced with these Abs. On the contrary, anti-muscle Abs cross-reacted only to a limited extent with the kidney. Thus immediate nephritis can never develop even with large amounts of these Abs. Heart and spleen absorbed 88.2 and 82.5% respectively of the kidney-fixing Ab from anti-kidney serum, while muscle did not absorb more than 9.4%. These experiments suggested that it is the total amount of kidney-fixing Ab and not its specificity which is of primary importance in inducing immediate nephritis, and the number of cross-reacting antigenic determinants in the glomeruli determines whether enough Ab could be fixed to cause immediate nephritis (191).

B.3.4.d. Physicochemical Characterization and Nephrotoxicity of Anti-kidney Abs

Most studies of NTN have employed rabbit or duck NTS. The rabbit NTAb activity in the long term immunized animals resided wholly in the 7S γ-globulin (157,193,194). However, some Ab could be detected in the macroglobulin fraction of rabbits bled shortly after immunization with rat kidney (157). Baxter and Small (194) demonstrated that 7S γ-globulin isolated from rabbit anti-kidney serum produced immediate and persistent proteinuria when injected intravenously into the rat.

Unanue and Dixon (157) have shown that the antibodies of duck NTS were contained in three apparently distinct immunoglobulins with certain differences in biological behaviour. Of these three immunoglobulins, one was a rapidly sedimentable, fast-migrating γ-globulin apparently equivalent to mammalian γ_{1m}-globulin and the remaining two

were γ_2-globulins with sedimentation rates of 5.8 and 7.4. It was found that the γ_{1m}-globulin was ten times more nephritogenic, on a weight basis, than the γ_2-globulins. The nephritogenic potency of γ_2-NTAb was only partially lost after treatment with mercaptoethanol, while that of γ_{1m}-NTAb was completely lost. However, treatment with mercaptoethanol of γ_2- and γ_{1m}-globulins did not eliminate their ability fo fix to the kidneys.

Small and Baxter (194,195) have investigated the nephrotoxic effects of anti-kidney Ab and of its fragments in rats. Digestion of the NTAb greatly reduced both its nephrotoxicity and its ability to fix complement in vivo. Divalent fragments (pepsin digests of NTAb) in larger doses produced transient proteinuria and the univalent fragments (papain digests) produced no significant proteinuria. The NTAb fragments differed from the intact Ab in fixing little, if any complement in vivo and both NTAb fragments in standard doses consistently failed to prevent renal damage by intact Ab. The rabbit NTAb treated with β-mercaptoethanol still retained all of its nephrotoxicity (195). These results have suggested that NTAb may be able to cause some damage independent of complement fixation but requires divalent molecules.

B.3.4.e. Localization of Nephrotoxic Abs

B.3.4.e.i. Methods for In Vivo and In Vitro Localization of NTAbs

There are three methods to determine the Ab and Ag interaction in vivo and in vitro. The first method introduced by Libby and Madison

(196) consisted of labelling the globulin fraction of antisera with an isotope (usually I^{131}) and injection into animals which were later sacrificed; the content of radioactivity in various organs after being perfused was measured. Thus, the in vivo localization of NTAbs in different organs can be determined quantitatively. The method was quantitative and sensitive but did not provide information on anatomical localization unless it was combined with autoradiography. Pressman and his collaborators (197-199) were the first to label anti-kidney serum with I^{131} and found that NTAb fixed to glomeruli within minutes after intravenous injection.

The second method is the fluorescein labelling of Abs, introduced by Coons et al. (200-202) for histochemical demonstration of specific Abs; this offered excellent anatomical localization in vitro or in vivo but was not quantitative. This has been the most widely used method employing either the direct labelling of NTAb with fluorescein (direct method) or labelling the antibody to the heterologous NTAb (indirect method). The NTAb localized in tissue (in vivo or in vitro) can thus be traced by fluorescence under ultraviolet light. Hill and Cruickshank (203,204) and Mellors et al. (205,206) first employed this technique for the localization of anti-kidney Ab or other NTAbs in the tissues, and demonstrated that glomerular basement membranes were the site of localization of NTAb.

The third method is the conjugation of Abs with ferritin, described first by Singer (207); this provided the method for detecting ultrastructurally the site of Ag-Ab interaction by means of the electron

microscope. Andres et al. (116,208,209) have employed this new technique for studies of experimental nephritis.

B.3.4.e.ii. In Vivo Localization of NTAb: Specificity, Rate of Fixation and Persistence in Kidney and Other Tissues

The localization or fixation of NTAbs in vivo was found mainly in the renal glomerulus of injected animals by autoradiography after injection of NTAb labelled with I^{131} (198,210), by fluorescent methods (155,156,190,205,206) and by immunoelectron microscopy (208, 209,211). Hill and Cruickshank (203,204) have demonstrated in vitro, by direct fluorescent Ab technique (fluorescein conjugated to NTAbs), the specific fluorescent staining on glomerular basement membrane and reticulin when anti-kidney serum was applied to frozen sections of normal rat tissues. However, Mellors and his co-workers (205,206) showed that the same antisera localized almost exclusively on glomerular basement membrane if given in vivo to rats, and persisted in this location for at least three months after injection. A positive fluorescent staining was seen uniformly along capillary walls throughout the entire glomerular tuft shortly after injection of NTS, and subsequently throughout the course of the disease.

The precise anatomical localization of the NTAb in the kidney was difficult to demonstrate by autoradiography employing I^{131}-labelled Ab or by immunofluorescent technique; however, this was accomplished by electron microscopy using ferritin-tagged Ab. Andres et al. (208,209)

applied ferritin conjugated duck anti-rabbit globulin to sections of rat kidneys injected with NTS and observed a heavy concentration of ferritin particles along the glomerular basement membrane which, as discussed before, is the main site of the Ag-Ab interaction. Particles were also found, but in lesser amounts, on the endothelium and on the epithelial foot process. Heavy concentrations were also seen in the glomerular epithelial cell cytoplasm in an amorphous material lining cisternae of endoplasmic reticulum. The results revealed that in nephritic animals, the injected NTAbs localized not only in the glomerular basement membrane but also in the cytoplasm of epithelial and endothelial cells, and further supported the hypothesis of Farquhar et al. (12) that cisternae and glomerular basement membrane possess similar proteins.

The in vivo specificity of NTAbs has been investigated by isotope and immunofluorescent methods. In vivo localization of I^{131}-labelled NTAb took place preferentially in the kidney, but also in the adrenal gland, spleen, lung, ovaries, placenta, gastrointestinal tract and liver (157,198,210,212-214). The relative concentration of NTAb in the kidney and other tissues varied depending on the time elapsed after injection. Immediately following the injection of NTAb there was a relatively high concentration of Abs fixed to extrarenal sites; however, the rate of disappearance of the NTAbs from these extrarenal sites was much more rapid than from the kidney and consequently, the relative concentration of Ab in the kidney increased with time (157).

The first in vivo studies of fixation of NTAb in the kidney were

reported by Sarre and Wirtz (215) who clamped one renal pedicle for 15 to 20 minutes following the injection of duck NTS into rabbits. Severe renal lesions developed in the unclamped kidney as contrasted to mild or no lesions in the clamped one. These results led them to conclude that most of the Abs had been removed rapidly from the circulation during the period of clamping. Pressman et al. (198) determined the rate of fixation of I^{131}-labelled NTAb in mice and found that no Ab persisted in the circulation after 18 minutes, confirming the rapid fixation of NTAb in the kidney.

Further studies, however, have demonstrated that after the initial period of rapid fixation of the Abs, there remained a small but detectable amount of anti-kidney Ab in the circulation for a much longer period (157,216). Blau et al. (216) described two types of rabbit NTAbs in rats; the first, which localized very rapidly, had a half-disappearance time from the circulation of four minutes and the second, which appeared to localize slowly, had a half-disappearance time of 100 minutes. Unanue and Dixon (157) using rabbit and duck NTAb labelled with I^{131}, have determined, in rat, the rate of accumulation in different organs. The kidney showed maximum fixation one hour after injection whereas both liver and lung had maximum fixation at ten minutes after injection, followed by a rapid rate of removal. These authors concluded that NTAbs were directed to different antigenic sites and some of the Abs had low affinity for antigenic sites, remaining in the circulation.

By another approach using fluorescent Ab technique, Seegal et al.

(155) have described the distribution of NTAb in the various tissues and determined the relative concentration of this globulin in acute and chronic nephritis of varying severity. They observed that NTAb bound to glomeruli within 20 minutes and persisted in the glomeruli during progression of the nephritis for more than nine months. The spleen and adrenal also contained small amounts of NTAb up to five months after injection. A direct correlation was demonstrated between the amount of NTAb injected and the degree of acute proteinuria (155,157).

It was also concluded, on the basis of experimental results, that localization of NTAb in glomeruli initiated nephritis; the progression of this disease might be attributable to persistence of the foreign globulin in the glomeruli (157).

B.3.4.f. Localization of Autologous Ab in NTN

The autologous phase of NTN is dependent upon the presence of the host's immune response to the heterologous NTAb (103,104,156). During this phase, the NTAb appears to behave as an Ag, attached to the glomerular capillary walls, to which the host Abs are fixed.

Several days after intravenous injection of NTS, circulating Ab to the heterologous NTAb was detected in the circulation (156,206). Usually coinciding with the presence of the host's circulating Ab, the autologous γ-globulin (Ab to NTAb) was detected in the glomeruli by the immunofluorescent method. Ortega and Mellors (206) provided direct evidence that the recipient animals responded with production of their own

Abs to the NTAbs. Autologous rat globulins were traced by fluorescein-conjugated rabbit anti-rat globulin serum and found localized in the glomerular basement membrane area where NTAbs were fixed.

Unanue and Dixon (217,218) demonstrated that homologous Abs to NTAb passively administered shortly after injection of NTAb promptly localized in the glomerular capillaries in a membranous pattern identical to that of the heterologous, autologous γ-globulins and β_{1c}-globulin. It was also shown that when autologous Ab was actively induced in rat with rabbit NTAb and adjuvant prior to, or simultaneous with, the injection of rabbit NTAb, rat γ-globulin and rat β_{1c}-globulin were found with the injected NTAb in the glomeruli in a membranous pattern (218). These results led them to conclude that continuing Ag-Ab-complement reactions are involved in the progressive glomerular injury.

B.3.5. Conclusion

Experimental nephritis, much akin to the human nephrotic syndrome, can be produced in a variety of animals by well established immunological methods. Although it is difficult to translate the findings to the process in humans, the results of such experiments have given promising insight into the way in which different types of Ag-Ab reactions and their consequences may produce tissue damage.

It is now generally accepted that nephrotoxic serum nephritis occurs in two phases: the primary phase (heterologous phase) which begins within minutes after injection of a nephrotoxic serum, is

due to the fixation of the heterologous Ab in the glomerular basement membrane and is generally manifested by proteinuria and some histological changes; the second phase (autologous phase) begins when host Ab, which is produced against the heterologous ß-globulin, units with the Ab previously fixed in the glomeruli and is usually characterized by proliferative glomerulonephritis and heavy proteinuria. On the basis of the experimental results, it has been claimed that the immediate immunological events which lead to acute injury necessitate the involvement of half or more of the filtration surface of the glomerular capillaries by Abs (157).

There is considerable evidence that complement plays a pathogenic role in experimental nephritis. It is known that serum complement levels are depressed during the second phase and that β_{1c}-globulin representing the third component of complement, are deposited within the glomeruli in the same location as the nephrotoxic and autologous Abs (133,217-219). Although direct evidence of a pathogenic role of complement has not been obtained, a continuing Ag-Ab-complement reaction could explain the progressive glomerular injury. Thus, host complement may mediate, at least in part, the renal injury produced by nephrotoxic Ab. The available evidence suggests that a number of complex immunological reactions appear to be important in the pathogenesis of the renal lesions.

B.4. Nature of Glomerular Basement Membrane

B.4.1. Origin and Development of Glomerular Basement Membrane

The functional importance of the glomerular basement membrane is intimately related to its anatomical characteristics. The electron microscopic studies of the embryological development of glomerular basement membrane have indicated the possibility of two basement membranes early in development, one probably derived from the epithelial cells and the other from the endothelial cells (5,6,7).

Studies by Vernier and Birch-Andersen (7,17) on human foetal glomerulus showed that the earliest recognizable basement membrane consisted of a narrow (500Å) zone between the epithelial and endothelial cells of an immature capillary. This zone is formed by the limiting plasma membrane of these two cells and is nearly devoid of electron-dense material. The basement membrane of the more mature capillary is about 1000Å in width and contains a dense, somewhat fibrillar zone centrally which is about 300Å in width. Fine fibrils about 40Å in width are a major constituent of the central layer. In the mature capillary, the dense central zone is wider and occupies about two-thirds of the space between the limiting membranes. The less dense zones on either side of the central zone contain numerous fine fibrils. During infancy, additional maturation of basement membrane results in an increase in density and width of the central zone due to increased concentration of fine filamentous and granular materials.

Basement membrane can be of epithelial, mesenchymal, or endo-

thelial cell origin (7,12,17,220-232). Glomerular basement membrane is composed of fibrillar elements embedded in a matrix which appears homogeneous at present levels of resolution (12,17,223). The fibrillar elements are of two different types: one type occurs as distinct "tubular" fibrils of approximately 110Å diameter which are visible primarily at the interface between the mesenchymal cell (or deep cell) and the basement membrane (14). The second fibrillar element is much finer and forms a fibrillar felt work of 30-40Å fibrils visible throughout the basement membrane (1,17,27). The source of these fine fibrils is not clear but the epithelial cells have been implicated. These investigators suggested that epithelium may synthesize one or more protein components of the basement membrane.

Vernier and Birch-Andersen (7) have demonstrated that the dispersed fine filaments were found through the cytoplasm of epithelial cells and shown to be chemically related to glomerular basement membrane material by periodic acid and silver methenamine staining techniques. Studies of Farquhar et al. (12) with the immunofluorescent method further suggested that cisternae of the epithelial endoplasmic reticulum contain components of the basement membrane and may be concerned with synthesis, intracellular transport and storage of these components. The validity of this assumption has been substantiated by the findings of Andres and co-workers (209) utilizing the immunoelectron microscopic method (ferritin conjugated Ab). However, in these studies, the epithelial phagocytosis of nephrotoxic globulins was not excluded.

The ability of a specific "anti-epithelial" serum to give

immunofluorescence with normal glomerular basement membrane (222) and the in vivo localization in the glomerulus of antisera directed to the lens capsule which is an epithelial basement membrane product (221), provide further evidence of an antigenic contribution of glomerular epithelium. Present data suggest that such Ags may arise as a result of the ability of the well-formed endoplasmic reticulum of epithelial cells to transport materials into the glomerular basement membrane.

The investigations of Kurtz and Feldman (220) support the concept that the epithelial cell is probably responsible in part for the synthesis and storage of basement membrane. They administered $AgNO_3$ in drinking water to young animals, resulting in deposition of silver throughout the glomerular basement membrane. Upon cessation of $AgNO_3$, the newly formed basement membrane in the growing normal rat and in rats with renal disease (aminonucleoside nephritis) was deposited on the epithelial side of the basement membrane.

Electron microscopic and immunological studies have indicated a multiple origin of the glomerular basement membrane, one component of which may actually be secreted by the epithelial cells, whereas others arise from connective tissue and capillary endothelium.

B.4.2. General Microscopic Characterization of Glomerular Basement Membrane

The basement membrane appears in the electron micrograph as electron-dense bands which upon higher magnification exhibit network-like interlaced filaments of a thickness of 40-60Å and an interfila-

mentary matrix material (12,17,223). Dische et al. (224) have shown by electron microscopy that pellets of the basement membrane preparation contained two types morphologically of homogeneous material. One type has greater density and a felt-like appearance; the other, presumably the basement membrane, appears lighter and has a slightly granular appearance.

Generally, the basement membrane is stainable by the PAS (periodic acid Schiff) technique (225). The periodic acid-silver methenamine method (226) (analogous to the PAS method) permits electron microscopic-histochemical study of tissue and identification of substances with a high content of 1,2 glycol groups. Thin sections of glomeruli stained with silver methenamine after oxidation by periodic acid disclosed dense deposits of silver in the basement membrane, thus indicating the presence of substances containing 1,2 glycol groups, probably in glycoproteins (7).

The earlier chemical studies of glomerular basement membrane and renal reticulum have shown high content of hydroxyproline (181,227-229), suggesting the major protein component in glomerular basement membrane is a collagenous-type protein. However, basement membrane differs from collagen in its higher content of carbohydrate and lipid (189,224,228) and also in its histochemical characteristics (collagen reacts weakly with PAS and silver impregnation stains). Ultrastructurally, basement membrane lacks the 640A periodicity and fibrils or banded structure which are characteristic of collagen. Windrum et al. (228) suggested that the basement membrane reticulin of kidney was

a lipoglycoprotein. Similar results were reported by Krakower and Greenspon (189) that basement membrane contained a complex glycolipoprotein and a collagenous base.

B.4.3. Immunological Properties of Glomerular Basement Membrane

It has been demonstrated that GBM is antigenic when injected into heterologous species; the Ab elicited is capable of inducing glomerulonephritis and renal lesions when injected intravenously into the original species (179). The complex nature of antigenicity of the GBM has been reported by many investigators (181,182,230).

Goodman et al. (181) and Krakower et al. (182) demonstrated that the GBM has a complex antigenic mosaic. By using ultrasonic vibration and trichloroacetic acid for destruction of cells, they found that GBM could produce nephrotoxic Ab and also Abs that cross-reacted with other tissue Ags suggesting that, in addition to a specific nephrotoxic Ag, the GBM contains "non-nephrotoxic" Ags in common with the capsular basement membrane, the tubular basement membrane and collagen derived from tendon and cornea.

The immunological evidence, demonstrating the precise anatomical localization in tissues of glomerular Ags, has suggested that there are related Ags among GBM and vascular membranes. Cruickshank and Hill (203,204) have shown in vitro by the immunofluorescent method that anti-rat glomerulus and anti-rat lung antisera stained not only the GBM but also the basement membranes of other organs (gastrointestinal

tract, bladder, ovary, etc.) and the reticulum framework of the spleen, lymph nodes, and thymus, the walls of capillaries and arterioles, sarcolemma and neurilemma. These authors postulated a common group of Ags among these different structures. These results have been substantiated by others (230-233).

Scott (230,231) has identified three fibrillary Ags in connective tissues with antiglomerular serum and antisynovial serum. Basement membrane, reticulin fibres and a third fibre found in a variety of sites including granulation tissues, were stained but collagen fibres were not stained by either antiserum. He suggested that GBM contained at least two connective tissue Ags, one distributed in sites corresponding to argyrophilic reticulum and another shared by the vascular and non-vascular basement membranes.

It has been suggested that vascular basement membranes, namely those of capillaries and venules, have a unique, so-called "nephrotoxic Ag(s)" (189,234). Basement membranes, in general, appear to possess at least two types of Ag, one of which is related to collagen, while another Ag is held in common by all these basement membranes. Capillary and venular basement membranes appear to possess a third type of Ag (nephrotoxic) which is not identical but antigenically related to those Ags held in common by basement membranes in general.

There is some evidence showing that GBM is antigenically related to collagen. Antisera to canine tendon agglutinated or precipitated purified GBM (147,181). Rabbit anti-rat collagen sera

contained complement-fixing Ab to rat kidney and fixed *in vivo* to the glomerular basement membrane (235-239). *In vitro* immunofluorescent experiments have demonstrated that the same antisera reacted with glomerular and vascular basement membranes, reticulum and collagen. These results strongly suggest that GBM and collagen have certain common antigenic groupings.

Other experiments using NTAb, however, have not been clear in showing cross-reactivity between GBM and collagen. Antisera to canine GBM agglutinated sonicated or fragmented canine tendon fibres (147,181). However, anti-rat glomerular or anti-rat whole kidney sera did not have complement-fixing Abs to rat collagen (236); similarly, fluorescent conjugated antisera against glomerulus or whole kidney did not stain collagen of normal tissue (203,204,230,232).

Rothbard and Watson (236) demonstrated that antisera to rat collagen alone had no nephrotoxic effect when injected into rats, but renal lesion developed if antiserum was administered with Freud's adjuvant. Another approach, by the absorption method, has also indicated the non-nephrotoxicity of collagen. Krakower and Greenspon (189) found virtually no "nephrotoxic Ag" in collagen. Although tendon fibrils (98% collagen) could absorb Abs from nephrotoxic sera prepared against kidney or lung, Ab eluted from the tendon fibrils was not nephrotoxic (147), confirming the findings of Rothbard and Watson (236). Millazzo (240) prepared a reticulin fraction from renal glomeruli which fully absorbed rabbit antisera to whole glomeruli and suggested that Ab to reticulin was the main component of nephrotoxic sera.

The ability of heterologous antisera to connective tissue Ags of most organs to cross-react with renal GBM and induce glomerular nephritis (Table III) provides further evidence that GBM contains multiple Ags and shares common antigenic groupings with other non-renal tissue Ags which may be non-nephrotoxic.

The nephrotoxic Ags in GBM were not entirely species-specific for they exhibited some degree of cross-reactivity among many mammalian species (Table II). Antisera against GBM from one species can produce NTN in another species. This cross-reactivity between species has also been demonstrated by in vitro approaches, i.e., immunofluorescent (241), agglutination (240), cytotoxicity (242) and complement fixation (148). Gery et al. (243), using isotope-labelled rabbit anti-rat NTAb, demonstrated cross-reactivity between rat and guinea pig, sheep, dog and calf kidneys.

On the basis of immunological and histochemical studies, GBM appears to be a complex antigenic structure possessing specific antigenic sites as well as sites which are the same or similar to those in vascular and epithelial basement membranes, stromal reticulum and also collagen fibres. It is also known that the Ab response to GBM Ag(s) is multiple, only a part being responsible for the production of nephrotoxic serum nephritis.

B.4.4. Chemical and Physical Properties of GBM

The investigation of the chemical structure of GBM from various

species has been recently undertaken in order to understand the role of this membrane in normal and diseased states (224,244-250). It is known that GBM contains multiple Ags and is capable of eliciting nephrotoxic Ab. Earlier investigators attempted to isolate renal nephrotoxic Ags by trichloroacetic acid (TCA), dilute alkali or enzyme (trypsin or pepsin) solubilization, but Ags obtained were not characterized in detail (182,240,251-253).

Immunological and chemical studies have indicated that GBMs of various species contain protein which appears to be collagen and also contain carbohydrate, probably as glycoprotein. The presence of large amounts of hydroxyproline in GBM of dog (181,247), rat (245), beef (249) and human (141,229,244) has led to the suggestion that the GBM is largely composed of collagen, although collagen fibrils are not seen. Recently, the chemical composition and some physical properties of GBM have been reported in more detail. The nature of GBM from different species will be discussed separately.

The chemical composition of normal and diabetic human GBM has been investigated by Lazarow and Speidel (244). They obtained GBM by treatment of isolated glomeruli with 0.05N NaOH for prolonged periods. Basement membrane thus obtained contained 3.4% carbohydrate determined as reducing material. Major component sugars were found to be glucose and galactose with lesser amounts of mannose and fucose. Only small amounts of hexosamine are present in the basement membrane fraction. Complete amino acid analysis and "fingerprint" of the GBM was found to closely approximate that of collagen and reticulin (228). However,

it should be noted that the procedure used for the preparation of GBM and reticulin may result in the loss of sialic acid and other carbohydrate constituents. The chemical analysis suggested that basement membrane is a complex glycoprotein material whose major protein constituent is related to collagen; it can be solubilized by collagenase treatment but not by hyaluronidase (244).

Further studies of the carbohydrate moiety of human GBM by Dische et al. (224) presented evidence that it contained two types of carbohydrate. In contrast to exposing glomeruli to alkali, they obtained GBM by sonification of isolated glomeruli. Collagen was extracted from the membrane by incubation with collagenase or by extraction with 4% TCA. They were able to show that the GBM contained two different sugar polymers, one free of hexosamine and consisting only of glucose and galactose linked to collagen, whereas the other contained hexose, hexosamine, fucose and sialic acid in the form of a glycoprotein which could be separated from collagen by dialysis after collagenase treatment.

The chemical properties of rat GBM in normal and nephrotoxic serum nephritis were investigated by Misra and Kalant (245). They demonstrated that the normal basement membrane contained collagen, lipids and at least two types of glycopeptides which were separable by chromatography on Sephadex G-75 after removal of collagen by collagenase. Sialic acid was found to be associated with collagen and with one of the glycopeptides. Sialic acid was released by neuraminidase to the same extent as by acid hydrolysis, but only half was dialysable,

indicating that the remainder was not present in a terminal position. They have also shown that nephrotic membrane contains less phospholipid and acetyl groups than normal membrane. Their finding of normal amounts of collagen, nitrogen, hexosamine and sialic acid in nephrotic basement membrane, together with the changes in susceptibility to chemical and enzymatic treatment, suggests that the thickening of the membrane is due to a change in structure at the molecular level.

Some physical properties of normal and nephrotic basement membrane have also been reported (246). Electrophoretic studies have shown that mobility of nephrotic basement membrane is significantly lower than that of normal basement membrane. The X-ray diffraction pattern of normal membrane resembles that of collagen with an additional ring corresponding to a spacing at 2.25Å, while that of nephrotic membrane showed several more reflections. It was suggested that the changes in mobility and X-ray diffraction may be the result of loss of phospholipid and an increased ordering within the crystal lattice of both collagen and non-collagen components.

Kefalides and Winzler (247) reported the chemical composition and physical properties of canine GBM and its relation to collagen. Amino acid analysis of GBM in several respects resembled that of mammalian collagen, but contained less glycine, proline and hydroxyproline and more hydroxylysine and cystine. The hydroxylysine-hydroxyproline ratio in basement membrane was 0.42, compared with 0.07 in collagen. The total carbohydrate content (glucose, galactose, mannose, hexosamine, sialic acid and fucose) of basement membrane was about 10% by weight, while

that of collagen was 0.6%. X-ray diffraction of basement membrane gave powder diagrams similar to those of denatured collagen and procollagen.

They have further solubilized the intact basement membrane by extraction with 8M urea or by reduction and alkylation of disulphide bonds in 8M urea. The amino acid and carbohydrate composition of the soluble product thus obtained did not differ significantly from that of intact basement membrane. Some physical constants for the soluble product of the membrane were determined and reported to be: $S_{20,w}$ 2.4; $D_{20,w}$: 1.46 and molecular weight 132,000. Disc electrophoresis of the soluble fraction revealed heterogeneity with two fast components resembling those of the soluble fraction of collagen, and one slow component, the latter staining for carbohydrate, suggesting glycoprotein in nature (247).

Subsequently, Kefalides (248) achieved the separation of two components from canine GBM by 8M urea or 5% TCA extraction. One is glycoprotein and the other a collagen with a much higher hydroxyproline and hydroxylysine content than is found in other mammalian collagens. Amino acid and carbohydrate analysis of glycoprotein and collagen components from basement membrane were reported. Glycoprotein contained 9% hexose and 1.3% hexosamine with traces of hydroxyproline and hydroxylysine. The data on the physical and chemical properties of basement membrane and its components present direct evidence that canine GBM is composed of a collagen-like protein and a glycoprotein (247,248).

Recently, Spiro (249,250) reported the chemical structure of

bovine GMB, its composition, the nature of carbohydrate units and their attachment to the peptide portion of the membrane. Composition analysis indicated that the GBM is a glycoprotein containing approximately 10% carbohydrate in the form of glucose, galactose, mannose, galactosamine, glucosamine and sialic acid (both N-acetyl and N-glycolyl neuraminic acid). The peptide portion of the basement membrane was characterized by the presence of large amounts of glycine (one-fifth of the amino acid residues), as well as by the occurrence of substantial amounts of hydroxyproline (6.5% by weight) and hydroxylysine (2.8%). It appears that bovine and canine GBM have many similarities in carbohydrate and amino acid composition (247,249).

From further chemical studies of glycopeptide isolated after proteolytic digestion (collagenase and pronase) of the bovine GBM, Spiro (250) was able to show that the sugar components are distributed equally between two distinct types of carbohydrate units; a disaccharide consisting of glucose and galactose and a larger heteropolysaccharide unit (mol. wt. 3500). There are approximately 10 disaccharide units for every heteropolysaccharide unit in the membrane. The carbohydrate-peptide linkage of the disaccharide unit was shown to be a glycosidic linkage involving the hydroxyl group of hydroxylysine, whereas the attachment of the heteropolysaccharide unit to the peptide most likely involves asparagine. Periodate oxidation studies indicated that approximately 70% of the hydroxylysine residues of the basement membrane are involved in the linkage of the disaccharide unit (250). Studies on tendon collagen have indicated that carbohydrates linked to hydroxylysine

in this protein are present both as disaccharide units and as a single residue of galactose (254). The similarities of carbohydrate linkage to the peptide portion observed in GBM and tendon collagen indicate the presence of some type of collagen in bovine basement membrane.

In summary, the studies of chemical structure and physical properties of GBM of various species revealed that GBM, in general, is a highly polymerized substance containing at least two major components, one being a glycoprotein and the other a collagen-like protein. It is of interest that two distinct types of carbohydrate unit are present in both bovine and human GBM. These characteristics of the carbohydrate moiety may also exist in the basement membrane of other species. The results suggest similarities among various mammalian species in the chemical composition and primary structure of GBM.

B.4.5. Conclusion

Immunological and embryological studies of GBM suggest multiple Ags in the basement membrane. Chemical composition and physical properties of basement membrane indicate the presence of some type of collagen and glycoprotein in the membrane.

It has been established that GBM is related to collagen in some aspects: interaction of GBM with Ab to collagen; almost complete solubilization of membrane by collagenase; high content of hydroxyproline and hydroxylysine and similar amino acid composition in basement membrane, and also similar X-ray diffraction pattern. However,

GBM differs from collagen in that GBM has a higher carbohydrate content, ultrastructurally lacks 640Å periodicity and fibril structure, histochemically reacts strongly with PAS stain and further contains specific nephrotoxic Ags in GBM.

The similarities observed in the chemical composition and physical properties of GBM of different species suggest the presence of common antigenic groups which may be responsible for the cross-reactivity of basement membrane among various species in producing nephrotoxic serum nephritis.

Principally because of the difficulties inherent in the isolation and solubilization of the different constituents of GBM in pure form, the antigenic composition of the GBM has not been clearly elucidated and characterized.

C. Materials and Methods

C.1. Experimental Animals

C.1.1. Rats

Adult male Sprague-Dawley rats were used throughout all the experimental work. For collection of glomeruli and preparation of GBM, rats of 200-250 gm in weight were used, whereas for testing antisera for nephrotoxicity, rats of 180-200 gm in weight were used.

C.1.2. Rabbits

In all experiments, male New Zealand rabbits weighing 5 lbs were used for immunization purposes.

C.1.3. Dogs

Female mongrel dogs, weighing 18-20 kgs, were used for the collection of glomeruli and basement membrane.

C.2. Isolation of Glomeruli

Two methods have been used for different purposes. The method described by Cook and Pickering (255) for isolation of glomeruli, with some modifications, has been used for all the experiments except that in which the molecular sieving effect of GBM was studied. In this particular experiment, the method of Krakower and Greenspon (179) was used.

The normal rats were anaesthetized with sodium pentobarbital (Abbott, 50 mg/ml) in a dose of 0.1 ml per 100 gm of body weight, injected intraperitoneally. The animals were perfused with 0.9% saline: after exposure of the abdominal aorta, loose knots were tied around the

aorta above the renal arteries and around the great vessels below the renal arteries. The superior mesenteric artery was tied completely. A No. 18 short, bevelled needle attached to a syringe (50 ml saline) was put in the aorta at its bifurcation. The needle so inserted was fixed by tieing the lower loose knot. The upper loose knot was tied firmly, a small nick was made in the inferior vena cava or renal vein to allow drainage of blood and perfusion fluid, and the kidneys were perfused with the saline. When the kidneys appeared free of blood (pale grey), 2% magnetic iron oxide suspension in normal saline, without gum acacia, was infused (approximately 50 ml was required). When the kidneys became uniformly dark grey or black in appearance they were removed and decapsulated. The cortex of the kidney was cut into small pieces and pressed through a 150-mesh sieve. The material emerging on the other side was suspended in cold 0.9% saline solution. The suspensions were centrifuged at 800 r.p.m. for 1-2 min. and the supernatant was decanted. The procedure of resuspension of isolated glomeruli in saline and passage in front of the electromagnet was repeated as described in the original method (255) until the preparation was free from non-glomerular contaminants.

For studies of the molecular sieving effect of normal and nephrotic GBM, glomeruli were isolated by the method of Krakower and Greenspon (179) to avoid the use of iron oxide. Preparations of glomeruli from both methods were of similar purity with less than 10% of non-glomerular elements.

C.3. Preparation of Glomerular Basement Membrane

The isolated glomeruli, suspended in 8% saline (10 ml for the glomeruli obtained from 10 rats) in a 50 ml beaker, were subjected to ultrasonic vibration in an MSE ultrasonic disintegrator (Model No. 3000) at the maximum output (1.3 to 1.5 amp) for a period of 12 minutes. The beaker was kept in an ice bath during this procedure. The disintegrated glomeruli were examined under a microscope for the "glomerular ghosts". The mixtures were centrifuged at 1000 r.p.m. for 10 minutes, the supernatant containing mainly cellular debris was discarded, and the residues containing glomerular ghosts were washed once with 8% saline and twice with distilled water. The GBM preparations suspended in distilled H_2O were stored at $4^{\circ}C$ with a crystal of thymol as preservative, or kept frozen. The basement membrane preparation, on staining with hematoxyline and eosine, showed no nuclear staining, and on staining with PAS, showed a strong positive reaction.

C.4. Preparation of Sephadex Columns

Dry Sephadex of different types (G-25, G-50, G-75, G-100 and G-200) obtained from Pharmacia, Uppsala, Sweden, was suspended in 0.9% saline and Sephadex gel was packed into columns of various dimensions according to the procedure described in the Sephadex literature (256).

The void volume of each Sephadex column was determined by application of Blue dextran 2000 (purchased from the same company). Blue

dextran, dissolved in small quantities of the same solvent, was applied to each column, eluted with the same solvent, and collected with an automatic fraction collector. All fractions were measured for their optical density at 620 mμ and the void volume of each column was determined by referring to the elution volume of the maximum concentration of Blue dextran. The Sephadex columns were then ready for use.

C.5. Immunological Procedures

C.5.1. Preparation of Rabbit Antisera

Dog glomerular basement membrane, rat GBM and their various fractions obtained by different treatments and isolations were used as antigens for immunization of the rabbits. Suspensions of GBM in saline or the solution of different lyophilized fractions dissolved in saline or buffer in a concentration of 1 mg/ml were emulsified with complete Freund's adjuvant (Difco) in the ratio of 1:2 by volume with the aid of an electric mixer until stable emulsions were obtained. Approximately 1 to 1.5 ml of emulsion was given at each injection. For complete immunization (3 injections required) of one rabbit, approximately 1 to 1.5 mg Ag were used.

Three injections are required for producing potent antisera. The routes and schedules of each injection are as follows: the first inoculation was given by intracutaneous injection in the foot pads with a No. 25 or No. 23 needle, and distributed in 5 to 7 sites (0.2

ml emulsion for each site). After 3 weeks, a second injection was given intramuscularly in several sites using a No. 18 or No. 20 needle. One week after the second injection, the third injection was given in the same manner as described for the second injection. One week later, the animals were bled. Approximately 40 to 50 ml of blood was collected by heart puncture under sterile conditions with a No. 20 needle. The blood was left at room temperature for 2 hours and then overnight in the cold. Serum was then separated from the blood cells by centrifugation at 1000 r.p.m. for half an hour and finally transferred to a sealed tube and stored at $-20^{\circ}C$.

Three weeks after the bleeding, the rabbits were given another booster (intramuscularly) and one week later were bled again. This sequence was repeated as often as necessary.

C.5.2. Determination of Nephrotoxicity of Antiserum in Rats

The nephrotoxicity of antiserum was determined by its ability to induce proteinuria in rats (> 200 mg urinary protein/24 hr). Each antiserum obtained was injected intravenously (tail vein) on three consecutive days (1 ml antiserum for each injection) (86). Two weeks after (or even earlier) the first injection, the rats were put in metabolic cages and urine was collected for 24 hours. At the end of collection, the cages were rinsed with distilled water (approximately 50 ml) into the same bottle where the urine had been collected. The volumes of urine and washings were measured, and the diluted urines were filtered. Three ml of filtered urine was used for

determination of urinary proteins by a biuret method (257). Bovine serum albumin was used as standard.

C.5.3. Determination of Antisera Localization by Immunofluorescent Method

C.5.3.a. Fluorescein-conjugated Antiserum to Rabbit γ-Globulin

Three different preparations have been used. The γ-globulin fraction of chicken antiserum to rabbit γ-globulin was labelled with fluorescein isothiocyanate in our laboratory (258). The fluorescein-conjugated γ-globulin (chicken) was further purified on Sephadex G-25 to remove the free fluorescein compound. Just before use, fluorescent Ab solution was twice absorbed with dry rat liver powder (45 minutes each time at room temperature). Another two preparations of fluorescein-conjugated goat antisera to rabbit γ-globulin were obtained from Difco Laboratories, Detroit, and also from Hyland Laboratories, Los Angeles. Both commercial products were used without any previous absorption. The staining results for *in vitro* localization of antisera by these three different preparations were similar.

C.5.3.b. In Vitro Localization of Antisera

The localization of different antisera on normal rat kidney was performed by the modified method of Coons et al. (202); a normal rat kidney, after removal from the animal, was immediately quick-frozen on dry ice. A thin frozen section of kidney was obtained by a microtome with a setting of 6 μ thickness and fixed in acetone for

half an hour. The tissue sections on the microscopic slide were then rinsed three times with phosphate buffered saline (pH 7.1-7.4). The antiserum to be tested was applied on the fixed tissue section and kept for 45 minutes under humid conditions at room temperature; three rinsings with the same buffered saline were used to remove any uncombined Abs from the tissue section. Finally, fluorescein-conjugated anti-rabbit γ-globulin was applied on the section for the same period. After the rinsing, the stained section was mounted with glycerine-saline mixture and a cover glass, and examined for fluorescence under the ultraviolet light microscope.

C.5.3.c. In Vivo Localization of Antisera

The rats, injected intravenously with antisera to be tested, were sacrificed after a few days or two weeks. The kidneys from the injected rats were removed, quick-frozen, sectioned and fixed as described above. Each kidney section was treated with fluorescein-conjugated antiserum to rabbit γ-globulin for 45 minutes, then rinsed and mounted as above. The Ab in the antisera which has already localized on the rat kidney through intravenous injection can be stained by fluorescent Ab. In both staining techniques, normal rabbit serum substituting for the antisera was included as control.

C.5.4. Determination of Reactivity of Antisera with Different Ags by the Gel Diffusion Method

Ouchterlony's double-diffusion method in agar gel was used.

Immuno-plates (pattern G) containing agar gel, obtained from Hyland Laboratories, Los Angeles, were used for this purpose. Antisera to be tested were used without dilution and antigen solution in concentrations of 1-2 mg/ml of saline or tris buffer (pH 8) was used. The plates were kept at room temperature in a humid chamber overnight. The shape and number of precipitin lines were recorded and photographed.

C.6. Methods for Chemical Analysis

C.6.1. Quantitative Determination of Hexosamines

C.6.1.a. Introduction

The classical method for quantitative determination of total hexosamines is the Elson-Morgan reaction (259) which involves the reaction of hexosamine with acetyl acetone in alkaline solution and the subsequent formation of coloured products by reaction with p-dimethylaminobenzaldehyde in HCl (Ehrlich reagent). Subsequent modifications to improve the sensitivity, reproducibility and specificity of the method have been reported by many investigators (260-264). Methods for further quantitative analysis of glucosamine and galactosamine have also been investigated (264-268).

In general, the Elson-Morgan method with slight modification has been used for measurement of total hexosamines without preliminary purification on Dowex-50 (245). However, for the separate estimations of glucosamine and galactosamine, hexosamines were first separated from neutral sugars in the hydrolysate of the glycoprotein, then separated

from amino acids and peptides by acetylation and purification on ion-exchange resins and finally deacetylated (249). The purified hexosamines were then subjected to quantitative separation and measured by the modified Elson-Morgan method (264).

C.6.1.b. Hydrolysis Conditions

Measurement of hexosamines present in bound form requires acid hydrolysis to release the hexosamine. Various materials have different optimal hydrolysis conditions for maximum liberation and minimum destruction. It was found previously that hydrolysis conditions of 2N HCl at 100°C for 5 hours gave the highest value for the hexosamines from GBM (245). Hydrolysis with weaker acid in the presence of Dowex 50 x 8 may lead to less destruction of liberated hexosamines and hence result in an even higher value for hexosamine. Attempts were therefore made to hydrolyze samples with 0.05N HCl or 0.1N HCl in the presence of Dowex 50 x 8 in the $\cdot H^+$ form (2:1, v/v) in a sealed tube, with constant mixing by rotation, at 100°C for periods of 12 to 19 hours. Free hexosamines were eluted from the Dowex with 2N HCl. The HCl in the eluate was removed by evaporation in vacuo in in the presence of NaOH pellets. The residues were taken up in distilled H_2O and analyzed for free hexosamines by the modified Elson-Morgan method (264). Glucosamine-HCl was used as standard (2 µg-6 µg).

It was found that hydrolysis with Dowex 50 x 8 in 0.1N HCl at 100°C for 16 hours gave the highest value for hexosamines. The results

under these conditions were identical to those obtained by hydrolysis in 2N HCl at 100°C for 5 hours. Therefore, the latter conditions were employed for the determination of total hexosamines.

C.6.1.c. Separation of Hexosamines from Amino Acids and Peptides

Protein hydrolysate (2 mg in 0.4 ml) was first diluted with distilled H_2O so that the concentration of HCl was less than 1N, and passed through a column of Dowex 50 x 8 (100-200 mesh, in H^+ form; dimensions 1 x 5 cm). After washing with distilled H_2O (15 ml) to remove neutral sugars, hexosamines and some amino acids were eluted from resins with 2N HCl (10 ml). The HCl eluates were taken to dryness in vacuo, the residues containing hexosamines were dissolved in 1 ml distilled H_2O and acetylated according to the method of Spiro (267). After acetylation, the mixtures were passed through a mixed column (dimensions 1 x 5 cm, upper half: Dowex 2 x 8, formate form, 200-400 mesh and lower half: Dowex 50 x 8, H^+ form, 100-200 mesh) to remove charged compounds, including unacetylated amino acids and peptides; the column was then washed 5 times with 1 ml distilled H_2O; the effluent and washings, containing N-acetylated hexosamines, were evaporated to dryness under vacuum; the residue was hydrolyzed with 0.5 ml 4N HCl for 4 hours at 100°C in a sealed tube to deacetylate the N-acetyl hexosamines; finally the hydrolysate containing free hexosamines was evaporated to dryness.

C.6.1.d. Quantitative Determination of Glucosamine and Galactosamine

The residue containing free purified hexosamines was transferred

to a Dowex 50 x 8 column (0.3 x 32 cm; 250 x 400 mesh, in H^+ form) for further separation of glucosamine from galactoseamine according to the method of Antonopoulos (268). Effluent fractions containing glucosamine and galactosamine were measured by the modified Elson-Morgan method (264).

C.6.2. Quantitative Determination of Sialic Acids

C.6.2.a. Introduction

The presence of sialic acids in biological materials and their chemistry have been reviewed by Gottschalk (269). The most widely used method for quantitative determination of sialic acids is the thiobarbituric acid method of Warren (270). The method measures only unbound sialic acids with some interference from 2-keto-3-deoxy sugar acids. In the present work, sialic acids were measured directly on the acid hydrolysate without any preliminary purification. Svennerholm (271) used ion-exchange resins to purify sialic acid from interfering substances before subjecting it to a colorimetric method. The determination of sialic acid after purification on an ion-exchange resin column has also been attempted.

C.6.2.b. General Method

Sialic acids in the samples were measured after hydrolysis in 0.1N H_2SO_4 at 80°C for one hour by the method of Warren (270). N-Acetyl neuraminic acid (Sigma Chemical Company, St. Louis, from egg crystalline

type III) was used as standard. The absorption spectrum was recorded on each sample and the standard on an automatic recording spectrophotometer (Spectronic 505). A calibration curve was made in the range of 1.6 µg - 8 µg sialic acids.

C.6.2.c. Purification of Sialic Acids with Ion-Exchange Resins

In order to exclude any possible interfering substances in the protein hydrolysate, sialic acids were purified on ion-exchange resins. Dowex 2 x 8 (200-400 mesh) in acetate form and Dowex 50 x 8 (100-200 mesh) in hydrogen form were prepared according to the method of Svennerholm (271). A small column (0.35 x 5 cm) with a wide opening at the top was packed with Dowex 2 x 8 in the upper half and Dowex 50 x 8 in the lower half and washed with 0.1N acetic acid before use. Protein hydrolysate (0.2 ml), or a standard solution of sialic acid were applied to the columns and washed with 0.5 ml distilled H_2O. The neutral sugars and other non-ionic compounds passed through the column into the effluent while cationic substances were taken up by the Dowex 50 x 8. Sialic acids which were adsorbed on the Dowex 2 x 8 resin were then eluted with 0.7 ml of acetate buffer (1M, pH 4.7) followed by washing with 0.7 ml of distilled H_2O. The Dowex 50 x 8 in the lower half of the column removed sodium ions in the acetate buffer which were found to interfere with the thiobarbituric acid assay. The acid eluate and washing containing sialic acid were collected directly in a centrifuge tube and evaporated to dryness *in vacuo*; finally, sialic acids were measured by the thiobarbituric acid method (270).

C.6.3. Determination of Bound Hexoses

Total protein bound hexoses were determined directly without previous hydrolysis by the phenol-H_2SO_4 method (272). Glucose was used as standard for the colorimetric method. The volumes of the reagents were scaled down to one-fifth or one-half those in the original method. Optical density was measured with a microcuvett (20 mm light path) in a Unicam spectrophotometer. A calibration curve was made in the range of 2 to 8 µg glucose.

C.6.4. Determination of Glucose by Enzymatic Method

Quantitative estimation of glucose alone was carried out on the protein hydrolysates (2N HCl for 2 hours at 100°C, evaporated to dryness) using the glucose oxidase method (273). For this purpose, the Glucostat reagent (purchased from Worthington Biochemical Corp., N.Y.) was used on a microscale in which 300 µl of the reagent were added to 200 µl of standard solution or sample hydrolysate containing 1.0 to 8.0 µg of glucose, the mixture was incubated at 37°C for 30 minutes and 1 ml of 7.6N H_2SO_4 was added. After thorough mixing, the colour developed was measured for O.D. at 540 mµ with 20 mm light path microcuvett in a Unicam spectrophotometer.

C.6.5. Determination of Hydroxyproline

Hydroxyproline was determined by the colorimetric method of Neuman and Logan, modified by Leach (274), using L-hydroxyproline as

standard (5 µg to 10 µg/ml). Hydroxyproline in the sample was first released by acid hydrolysis in 6N HCl for one hour at 140°C in sealed test tubes; the hydrolysates were evaporated to dryness in vacuo in the presence of NaOH pellets, the residues were taken up with distilled H_2O and hydroxyproline was measured by the method of Leach (274). Photometric readings were made at 555 mµ with a 10 mm light path. In some cases, when the samples contain Fe_2O_3 particles, which interfere with the determination, iron oxides were removed after hydrolysis as follows: the pH was adjusted to 8.0 with NaOH (saturated solution), and the resulting precipitate of ferric hydroxide was removed by centrifugation. The supernatant solution was analyzed for hydroxyproline.

C.6.6. Determination of Total Nitrogen

Total nitrogen was determined by a micro-Kjeldahl procedure. Protein solutions (containing 20 µg to 50 µg of nitrogen) in 1 ml were first digested in a 100 ml digestion flask with 3 ml of digestion mixture (275) and 2-Hengars selenized granules for one hour after the mixtures became a clear pale green. Twenty-five ml distilled H_2O was added to wash down the side of the flask and 2 drops of 0.5% phenolphthalein were added. The digestion mixture was then subjected to distillation following the addition of 8 ml sat. NaOH solution. Approximately 20 ml of distillate was collected over a period of 10 minutes in 10 ml of 4% boric acid solution (pH 4.8) containing Tashiro's mixed indicator (276). Titration was carried out with N/70 H_2SO_4 using a microburet (capacity 1.0 ml purchased from Manostat Corp., N.Y.). With each series,

a blank (dist. H_2O) and known standards (urea solution 60 µg-120 µg/ml) were run simultaneously.

C.6.7. Measurement of α-Amino Nitrogen by Ninhydrin Reactions

The ninhydrin colorimetric method of Rosen (277) was used for analysis of α-amino nitrogen using leucine as standard (0.02-0.1 µmole). Aliquots of protein solution or enzymatic digest were measured directly for the α-amino group by ninhydrin reagents.

C.6.8. Measurement of Protein by Folin Reagent

The protein content of a solution was determined by Lowry's method using Folin reagent (278). Bovine albumin solutions (20 µg-100 µg/ml) were used as standards for the colorimetric method.

C.6.9. Measurement of Protein by Spectrophotometric Analysis

Protein contents of a solution or effluents were determined in terms of optical density measurements at 280 mµ in a spectrophotometer (Spectronic 505).

C.6.10. Quantitative Determination of Total Amino Acids

For determination of the amino acids, samples were hydrolyzed with 6N HCl in sealed tubes (under vacuum) at 110°C for 22 hours. Hydrolysates were evaporated to dryness *in vacuo* in the presence of NaOH pellets for removal of HCl; the residues were then analyzed for

amino acids by an automatic amino acid analyzer employing the method of Piez and Morris (279). A standard containing 0.1 μmole of each of the amino acids (20 amino acids including hydroxyproline and hydroxylysine) was run in the identical manner. No corrections have been applied for amino acid destruction.

C.6.11. Determination of Carbohydrate by Paper Chromatographic Method

Samples were hydrolyzed in 2N HCl for 4 hours at 100°C; subsequently, chloride ions in hydrolysate were removed by the use of Dowex 1 x 10 in bicarbonate form. After concentration, samples were subjected to one-dimensional descending chromatography on Whatman No. 1 paper. Standards consisting of glucose, galactose, mannose, fucose, ribose, deoxyribose, glucosamine and galactosamine (1 μl of each 1% aqueous solution) were run simultaneously with the samples. The chromatograph was developed with a solvent system of n-butanol-pyridine-distilled H_2O (5:3:2, v/v/v) for a period of 23 to 41 hours. The sugars were located on the chromatogram by the silver nitrate method (280).

C.7. Methods for Enzymic Digestions

C.7.1. Collagenase

Removal of collagen from GBM and solubilization of GBM were achieved by collagenase digestion. Highly purified collagenase (type CLSP-A) from Clostridium Histolyticum was obtained from Worthington Biochemical Corp., New Jersey. The digestions were carried out in tris-

HCl buffer (0.1M, pH 7.3-7.4) containing 0.01M $CaCl_2$ for 19 to 40 hours at 38°C with constant stirring during the incubation. A crystal of thymol was added at the beginning of the incubation to prevent bacterial growth.

In some experiments, further purified collagenase was used. The purification of this commercial product was achieved by gel filtration on Sephadex G-100 with 0.9% saline. The active enzyme fraction was excluded from the Sephadex while the inactive contaminants were retained in the column. The fractions containing only very high collagenase activity were combined, dialyzed and lyophilized. This repurified collagenase was used under the same conditions as previously described. No degradation of enzyme itself occurred during the incubation with GBM and it was found to be still excluded from Sephadex G-100.

C.7.2. Pronase

Proteolytic digestion of GBM component with Pronase (Grade B, Calbiochem) was carried out in phosphate buffer (0.05M, pH 7.13) at 40°C for 24 hours. The ratio of enzyme to substrate was 1:30 by weight.

C.8. Electrophoretical Analysis

C.8.1. Gel Electrophoresis

Horizontal polyacrylamide gel electrophoresis was performed with 7% gel containing 0.2% each of catalyst and ammonium persulphate. Tris-glycine buffer (0.05M, 0.38M respectively, pH 8.4) was used as gel

buffer and electrode buffer. Samples were applied on filter paper (Whatman No. 1, 3 mm) and the paper was inserted into the slits of the gel. Electrophoresis was run for 17 hours at 250 - 300 volts in the cold. The gel was stained for protein with Amido-Schwartz 10B solution (0.04% in mixed solvents: methanol:H_2O:acetic acid, 5:5:1, v/v/v). Excess dye was washed away with the same mixed solvents until the background of the gel was very faint. The gel was stored in the same solvent.

Vertical starch gel electrophoresis (11% gel, at 230 volts for 16 hours in the cold) was performed according to the method of Ferguson (281). Following the electrophoresis, gel was stained as described above.

C.8.2. Disc Electrophoresis

Disc electrophoresis was performed according to the method of Davis (282) except that the stacking gel was omitted. Sample in a 0.1 ml solution (0.6%) was applied and electrophoresis was run for 2 hours with current 4-5 m.a. per tube, after which the gel was stained with 1% Amido black in 7% acetic acid and destained by electrophoresis in acetic acid with the current adjusted to 15 m.a. per tube.

C.8.3. Immunoelectrophoresis

Immunoelectrophoresis was performed according to the method of Grabar (283). Antigen solution (1%) was subjected to electrophoresis in 1% agar in veronal buffer at pH 8.6 for one hour (35 m.a.). Antiserum

was added in the trough and diffused against antigens in a humid chamber for 16 hours. After removal of excess antisera, the slide was stained with Buffalo Black NBR for Ab-Ag reactions.

C.9. Ultracentrifugal Analysis

C.9.1. Analytical Ultracentrifugation

The sedimentation rate constant was determined for GBM component at a concentration of 0.7-1% in 0.05M tris-HCl buffer at pH 8.4 with or without 8M urea. A Spinco Model E ultracentrifuge equipped with a Schlieren optical system was used. Temperature was controlled on all runs at 20°C with speed at 56,000 r.p.m. or 60,000 r.p.m. (in the presence of 8M urea). A single sector cell with Kel-F centrepiece and synthetic boundary cell (Valve type) were used. Pictures were taken at intervals of 8 or 16 minutes.

C.9.2. Density Gradient Ultracentrifugation

Sucrose density-gradient centrifugation was carried out according to the procedure of Martin and Ames (284). A linear gradient covering a range of sucrose concentrations of 5 to 20% was used. The sucrose solutions and sample solution were made up in tris-HCl buffer (0.05M, pH 8.6). Bovine albumin and γ-globulin solution (1%) were used as control. Sample solution (0.2 ml containing 4 mg of protein) was layered over the sucrose solution. Centrifugation was carried out for 17 hours at 39,000 r.p.m. in a Spinco Model L ultracentrifuge with a swinging bucket rotor SW 39L. Following centrifugation, the tubes were

punctured and samples were fractionated by a drop-counting device.

D. Experimental

D.1. Immunological Properties and Chemical Nature of Dog GBM

D.1.1. Introduction

Chemical studies of dog GBM indicated that it is composed of a collagenous protein and a glycoprotein (181,247). It is also known that dog GBM is antigenic to rabbits and that rabbit Abs to dog GBM are nephrotoxic when injected intravenously to dogs (169,170). Ab to collagen (rat, mouse, guinea pig and chicken) was found to be non-nephrotoxic in the homologous species (237,238); it is thus probable that the nephrotoxic antigenicity of dog GBM resides in the glycoprotein. Antisera against rat, rabbit and human glomeruli were found to be nephrotoxic in the dog, suggesting cross-reactivities of GBM among various species (146-148). However, it has not been reported whether Abs to dog GBM can induce glomerulonephritis in rats. It was the purpose of the present experiment to determine the nephrotoxicity of rabbit antiserum to dog GBM in rats, and to determine the antigenicity and chemical properties of non-collagen components of dog GBM.

D.1.2. Collagenase Digestion of GBM and Fractionation of the Membrane Components

GBM obtained from 11 normal kidneys was suspended in 45 ml of

tris-HCl buffer solution and incubated at 38°C for 19 hours with collagenase (4 mg divided in 2 additions at 0 hr and 6 hr) as described previously. At the end of incubation, the suspension was dialyzed against cold running water for 3 hours and against distilled H_2O at 4°C for 16 hours and then centrifuged at 1500 r.p.m. for 15 minutes. One ml aliquots of the suspension before and after dialysis and of the supernatant after centrifugation were taken for analysis of hydroxyproline. The clear supernatant was lyophilized and the yield of the solubilized membrane was 43 mg. This material was designated as CSM (collagenase solubilized membrane).

The lyophilized material was subjected to gel filtration. The sample (15 mg) was dissolved in 2 ml of 0.9% saline and the clear solution after removal of insoluble residues was fractionated on a Sephadex G-75 column (1.4 x 28 cm), using 0.9% saline for elution. The eluate was collected in 5 ml fractions. Aliquots of each fraction were analyzed for bound hexose by use of the phenol-sulphuric acid reagent (C.6.3.) and for protein by the u.v. spectrophotometric method. The typical elution pattern is shown in Fig. 1.

The fractions of the appropriate peak were pooled, dialyzed and lyophilized. The materials, designated as CS75A, CS75B and CS75C, were used for chemical analysis and immunological studies. Bound hexose, hexosamine, sialic acid and total nitrogen content of each component were determined as previously described.

The results obtained in the collagenase digestion indicated

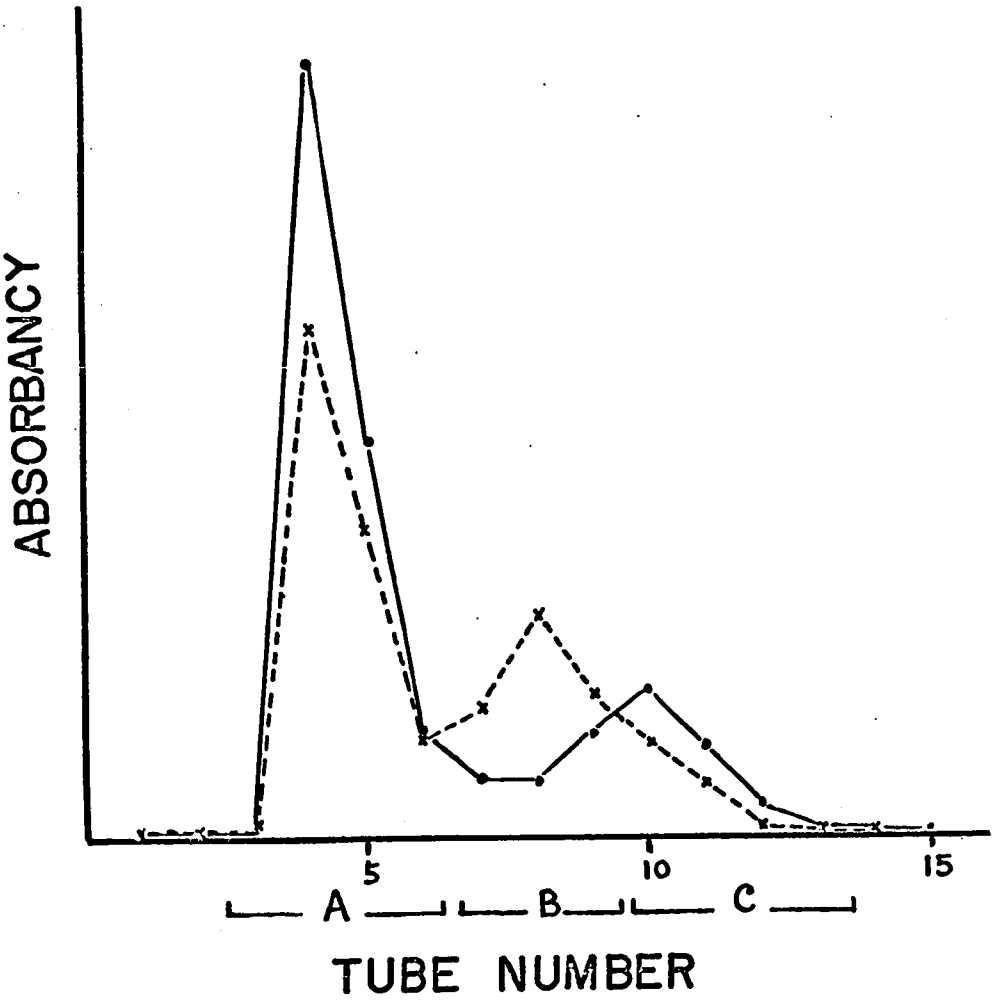

FIG. 1

Gel filtration pattern on Sephadex G-75 of dog glomerular basement membrane solubilized by collagenase digestion.

Column dimension: 1.4 x 28 cm
Volume of each fraction: 5 ml
(—·—·—): absorbancy at 280 mμ; (--x--x--): bound hexose

that GBM was solubilized by collagenase treatment and a large portion of collagen in the membrane was removed after dialysis. Eighteen per cent of the hydroxyproline of the starting material remained in non-dialysable form after enzyme digestion, suggesting that 82% of hydroxyproline of the membrane was solubilized in the dialysable form. The supernatant of the enzyme digest after dialysis still contained 13% of the hydroxyproline of the starting material. This fraction of hydroxyproline might represent the collagen fragments which remained attached to glycoprotein or peptides and which were resistant to collagenase action.

The CSM was separable into three components by gel filtration on Sephadex G-75 (Fig. 1). There were two distinct peaks for protein content and also two peaks for bound hexose content but only one was related to the protein peak. One major peak, which contained more than 50% of the hexose of the sample was eluted with the void volume of the column, suggesting a material with a molecular weight of 10,000 or over. The other two components of smaller molecular size were retained in the gel and subsequently eluted.

It can be noted from the results shown in Table 4 that CSM and its components (CS75A and CS75B) all contained hexosamine, bound hexose and sialic acid. The high carbohydrate content (15% to 25% by dry weight) in these components suggests that they are glycoprotein or glycopeptides of different molecular size. Despite different total carbohydrate contents, the molar ratio of hexosamine:hexose:sialic acid (approximately 3:10:1) is similar among the membrane components. Thus, solubilization

TABLE 4

CHEMICAL COMPOSITION OF COLLAGENASE SOLUBILIZED DOG GBM AND ITS COMPONENTS (% OF DRY WEIGHT)

	CSM	CS75A	CS75B
Hexosamine	2.87 (0.160)*	3.45 (0.193)	5.0 (0.279)
Bound hexose	12.53 (0.693)	9.60 (0.533)	17.36 (0.964)
Sialic acid	1.78 (0.057)	1.88 (0.061)	3.20 (0.103)
Total carbohydrate	17.18 (0.910)	14.93 (0.787)	25.56 (1.346)
Total nitrogen	15.8	12.4	11.2

* The figures in parentheses represent the concentration expressed in μmoles/mg dry weight.

All values are average of two determinations.

of the GBM by collagenase digestion resulted in the degradation of collagen and liberation of glycoprotein or glycopeptides which differed in molecular size but were similar in carbohydrate constituents.

D.1.3. Immunological Characterization of Dog GBM and Its Components

Groups of rabbits were immunized against intact GBM, collagenase solubilized membrane (CSM) and its components (CS75A, CS75B and CS75C) according to the method described in section C.5.1. The resulting antisera were examined for their nephrotoxicity in rats, localization of antibody in the rat kidney and their behaviour in the gel double-diffusion as described previously in sections C.5.2. to C.5.4. The results are summarized in Table 5.

D.1.3.a. Nephrotoxicity of the Antisera

Antisera raised against dog GBM, CSM and its components, when injected intravenously into normal rats (group of 3 rats for each antiserum), all failed to produce proteinuria (Table 5) with the exception of one rat. This animal received anti-CSM and showed moderate proteinuria of 116 mg per 24 hours four weeks after the initial injection of antiserum; the level of proteinuria increased to 276 mg per 24 hours and persisted at a similar level even 11 months after the initial injection. However, all other rats showed urinary protein in the normal range (15 to 40 mg per 24 hrs).

Some of the immunized rabbits were given a booster with the

TABLE 5

IMMUNOLOGICAL PROPERTIES OF DOG GBM COMPONENTS

Antisera	Proteinuria (>200 mg/24 hr)	Localization of Ab**	No. of precipitin lines in gel diffusion (with CSM)
Anti-dog GBM (3)*	-	+++	2
Anti-CSM (2)	-	+++	2-4
Anti-CS75A (2)	-	++	4
Anti-CS75B (1)	-	-	0
Anti-CS75C (1)	-	-	0

* The figures in parentheses indicate the number of rabbits immunized.

** Intensity of fluorescence on GBM is indicated by number of +; - indicates no specific fluorescence.

same Ag as they had received previously. The new antisera were tested again for their nephrotoxicity. None of the rats injected with these antisera (anti-dog GBM and anti-CSM) developed any proteinuria even two months after initial injection. Thus, it can be concluded that rabbit Abs to dog GBM and its components are non-nephrotoxic in rats.

D.1.3.b. Localization of Abs in Kidney

The in vivo and in vitro localizations of Abs on the kidney, as shown by the intensity of the fluorescence, indicate the degree of reaction between Abs and Ags in the kidney. Table 5 presents the results of in vivo localization of Abs against different membrane Ags in kidney. All rats receiving an injection of anti-dog GBM, anti-CSM and anti-CS75A showed positive fluorescent staining for rabbit γ-globulin in GBM, whereas those injected with anti-CS75B and anti-CS75C showed negative fluorescent staining. The positive staining of rabbit γ-globulin by fluorescence is shown in Fig. 2. The typical capillary pattern indicates the specific localization of Abs on GBM. There was no fluorescent staining on BM of tubules or of the Bowman capsule.

The in vitro localization of Abs in a frozen normal kidney section showed results similar to those observed by the in vivo study. However, the positive fluorescent staining for anti-dog GBM, anti-CSM and anti-CS75A on kidney sections was not exclusively on GBM. Tubular BM was also stained by fluorescent Ab but the intensity of the fluorescence was much lower than that observed in GBM. The in vitro localization of

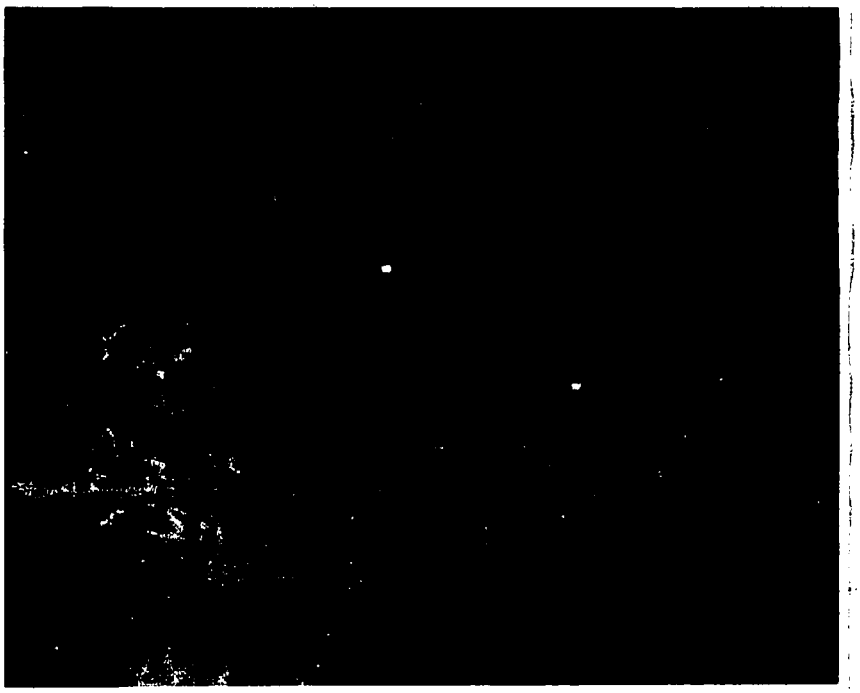

FIG. 2

Localization of rabbit γ-globulin in renal glomerulus in rat injected with antiserum against dog glomerular basement membrane, as demonstrated by immunofluorescent method.

Abs by the immunofluorescent method has also been reported by other investigators to be less specific than the in vivo method (204,285).

The positive localization of Abs against dog GBM on rat GBM provides evidence that GBMs of these species contain common Ag(s). The ability of membrane components (CSM and CS75A) to elicit Abs cross-reactive with rat GBM suggests that it is the glycoproteins in rat and dog GBM which may share the common antigenic determinants. The fact that anti-CS75B and anti-CS75C failed to localize on rat kidney may possibly result from the lack of the antigenic group common to dog membrane components and rat GBM, or from the inability of these components to elicit Abs in rabbit.

D.1.3.c. Ab-Ag Reaction in Gel Double Diffusion

The number of precipitin lines of reaction between Abs and Ags of dog GBM are given in Table 5. By gel diffusion, two precipitin lines were formed between anti-dog GBM and CSM, suggesting that dog GBM contains at least two antigens. Two to four precipitin lines were noted between anti-CSM or anti-CS75A and their Ags. It was demonstrated later that these membrane components were contaminated with collagenase which induced its Ab formation in the rabbit. Therefore, the reaction between collagenase and its Ab contributed to some of the multiple precipitin lines.

It can be noted that no precipitin line developed between anti-CS75B or anti-CS75C and their Ags. The result suggests that these

membrane components of smaller molecular size elicited only non-precipitable Abs or were non-antigenic in rabbits.

D.1.4. Conclusions

a) Chemical studies indicate that dog GBM contains collagenous protein and glycoprotein which can be solubilized by collagenase digestion.

b) Glycoprotein, containing hexosamine, hexose and sialic acid, was separated into three components of different molecular size and immunological properties.

c) Antibodies against dog GBM and its components are non-nephrotoxic in rats despite their localization in GBM.

d) The localization of anti-dog GBM, anti-CSM and anti-CS75A in rat GBM suggests the presence of common antigenic groups in GBM of these species.

e) Double diffusion in gel indicates the presence of multiple antigens in dog GBM.

D.2. Isolation and Characterization of Antigenic Components of Rat GBM

D.2.1. Introduction

It is well established that rabbit antibodies to rat GBM induce glomerulonephritis upon injection into normal rats (101,149,151-157). It has been demonstrated that the GBM has a complex antigenic mosaic (181, 182). GBM induces specific nephrotoxic Ab and also Abs that cross-react

with other tissue Ags, indicating that GBM contains multiple Ags. However, these various Ags in the GBM have not been isolated and characterized.

Several attempts have been made to extract the renal Ag(s) responsible for induction of nephrotoxic Ab. Cole et al. (252) reported that trypsin digestion of rat kidney homogenate released a mucoid substance which precipitated the nephrotoxic Ab from rabbit anti-rat kidney serum but was not antigenic in rabbits. It was thought to be a polysaccharide hapten that would be bound in vivo to trypsin-sensitive protein. This work was supported by several investigators (253,286, 287).

Recently, Dinh (251) reported that a major antigen of rat kidney was isolated from the supernatant of the kidney homogenate in cold saline. The physical and immunochemical properties of this Ag were reported but the nephrotoxicity of this Ag was not investigated. It is apparent that attempts to characterize the nephrotoxic Ag in GBM have proved unsuccessful.

In the present work, attempts have been made to isolate the antigenic components of rat GBM by the use of enzymic digestion, chemical extraction and gel filtration. Membrane components were characterized chemically and immunologically. It is the purpose of this work to define the chemical nature of the antigenic components of membrane and particularly of the nephrotoxigenic Ag.

D.2.2. Preliminary Isolation and Characterization of Rat GBM Components

D.2.2.a. Fractionation of the Collagenase-solubilized GBM and Tendon Collagen

GBM obtained from 140 normal rats was suspended in 50 ml of tris buffer and treated with collagenase (5 mg divided into 2 additions). Ninety per cent of the hydroxyproline in the GBM was removed by solubilization and dialysis. Non-dialyzable components of GBM solubilized by collagenase digestion (CSM) were fractionated on Sephadex G-75 in saline. An elution pattern, similar to that in Fig. 1, was obtained and three components, designated as CS75A, CS75B and CS75C, were separated.

When CSM was subjected to prolonged centrifugation (5,000 r.p.m. for one hour) before application to the Sephadex column (1.4 x 32 cm), the elution pattern (Fig. 3) appeared somewhat different. The major peak (designated as CS75A'), eluted with the void volume of the column, showed a much smaller protein content than before. This probably indicates the removal by centrifugation of some particulate matter of high protein and low carbohydrate content.

Tendon collagen was treated with collagenase (substrate to enzyme ratio, 10:1) under conditions similar to those described for GBM. The loss of hydroxyproline was 88% after dialysis. The non-dialyzable fraction of the enzyme digest of the tendon collagen (designated as CSColl) was fractionated by gel filtration on Sephadex G-75 as previously described. The material was eluted with the void volume of the column as one major peak containing almost all the hexose of the starting

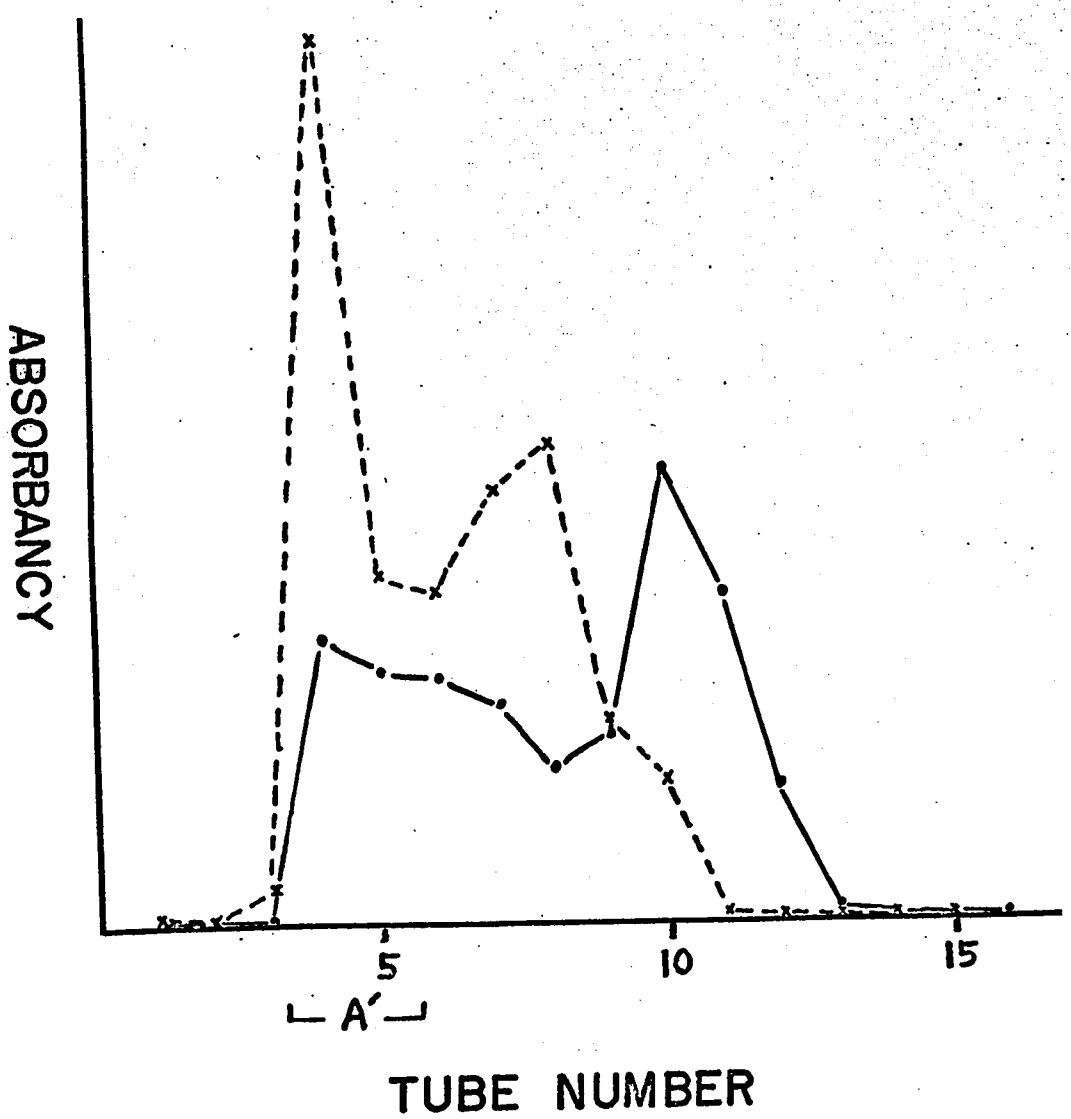

FIG. 3

Gel filtration pattern on Sephadex G-75 of collagenase solubilized basement membrane (rat) after prolonged centrifugation.

Column dimension: 1.4 x 32 cm
Volume of each fraction: 5 ml
(—·—·—): absorbancy at 280 mμ; (---x--): bound hexose

material (Fig. 4).

The results suggest that collagenase degraded tendon collagen into small dialyzable peptides containing 90% of the hydroxyproline of the starting material and larger non-dialyzable peptides containing the remaining hydroxyproline. It was also noted that the non-dialyzable peptides, excluded from Sephadex G-75, were enriched in bound hexose, confirming the results reported by other investigators (288-290).

D.2.2.b. Immunological Properties

The isolated GBM components (CSM, CS75A, CS75B and CS75A'), tendon collagen and its solubilized component (CSColl) were injected into groups of rabbits with complete Freund's adjuvant as previously described. Nephrotoxicity of the resulting antisera and its ability to localize on kidney were investigated.

As can be seen in Table 6, rat GBM solubilized by collagenase digestion (CSM) still retained its ability to induce nephrotoxic Abs. Ab against the component excluded from Sephadex G-75 (CS75A) produced immediate proteinuria upon injection into normal rats whereas Ab against the similar component of lower protein content (CS75A') induced only moderate proteinuria two weeks after initial injection. Since rabbits had been immunized with equal amounts of Ag, it appears that the removal of particulate matter from CSM by centrifugation resulted in an Ag less potent in eliciting nephrotoxic Ab. Anti-CS75B, anti-collagen and anti-CSColl all failed to induce proteinuria in normal rats.

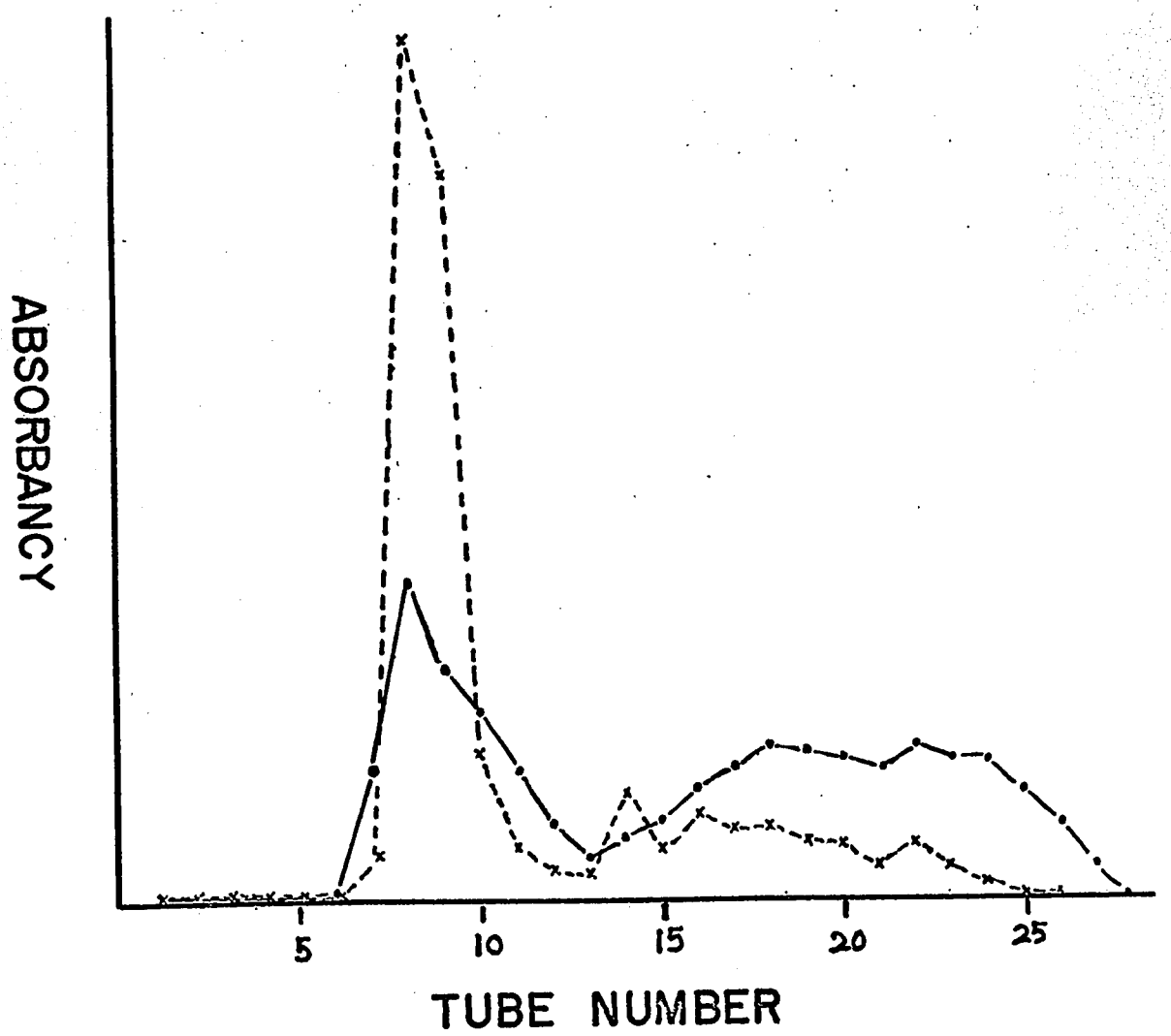

FIG. 4

Gel filtration pattern on Sephadex G-75 of bovine tendon collagen solubilized by collagenase digestion.

Column dimension: 2 x 30 cm
Volume of each fraction: 5 ml
(——·——): absorbancy at 280 mμ; (--x---): bound hexose

TABLE 6

NTN INDUCED BY INJECTION OF ANTISERUM AGAINST RAT GBM COMPONENTS AND COLLAGEN

Antiserum	Proteinuria in no. of rats	Level of proteinuria (mg/24 hr)			Localization of Abs on glomeruli
		Day 6	Day 14	Day 30	
Anti-CSM (3)*	9/9	250-320	-	540-760	+++
Anti-CS75A (2)	4/4	210-320	-	560-970	++
Anti-CS75B (1)	0/2	35-74	-	43	+
Anti-CS75A' (2)	4/6	10-76	180-220	-	++
Anti-CSColl (2)	0/6	9-20	15-35	-	-
Anti-collagen (3)	0/9	22-76	22-110	-	++

* The figures in parentheses indicate the number of rabbits immunized.

Abs against all the GBM components and tendon collagen localized in vivo on kidney GBM (Table 6). Anti-CSM gave the most intensive fluorescent staining on GBM in a uniform linear pattern (Fig. 5), indicating the largest number of Abs fixed to the GBM. Anti-CSColl did not localize on kidney despite the positive fluorescent staining shown by anti-collagen, suggesting that peptides isolated from the collagenase digest of tendon collagen did not contain any common antigenic determinants with GBM.

The gel double diffusion of isolated Ags and its Abs revealed heterogeneity of the membrane components liberated by collagenase digestion (Fig. 6a). Cross-reactions among membrane components were also demonstrated by the multiple precipitin lines formed between GBM components and various Abs. Furthermore, it was found that all components isolated from collagenase digests of GBM and tendon collagen were contaminated with collagenase which induced Abs in rabbits as indicated by the precipitin lines formed between anti-CS75A and anti-CSColl and collagenase (Fig. 6b). It is significant that anti-collagen reacted with CSColl and formed several precipitin lines. However, it did not react with CSM or CS75A (Fig. 6b), suggesting that antigenicity of CSM is not associated with the collagen fragment as such.

D.2.2.c. Chemical Characterization

Chemical analyses of GBM components are given in Table 7. The presence of carbohydrates (hexosamine, hexose and sialic acid) in all

FIG. 5

Localization of rabbit γ-globulin in renal glomerulus of rat injected with antiserum against rat collagenase solubilized membrane as demonstrated by immunofluorescent staining. Specific fluorescence is confined to glomerular basement membrane.

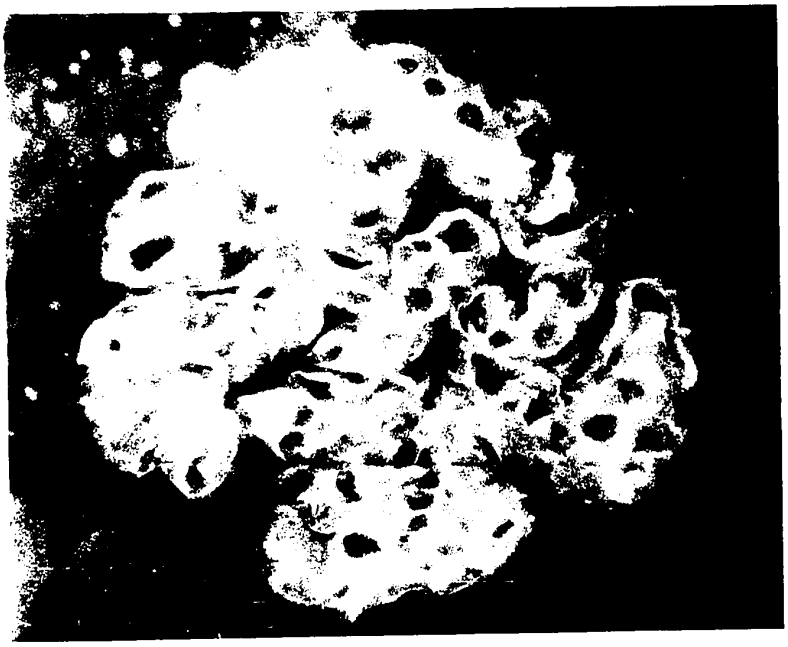

FIG. 5

Localisation of rabbit γ-globulin in renal glomerulus of rat injected with antiserum against rat collagenase solubilized membrane as demonstrated by immunofluorescent staining. Specific fluorescence is confined to glomerular basement membrane.

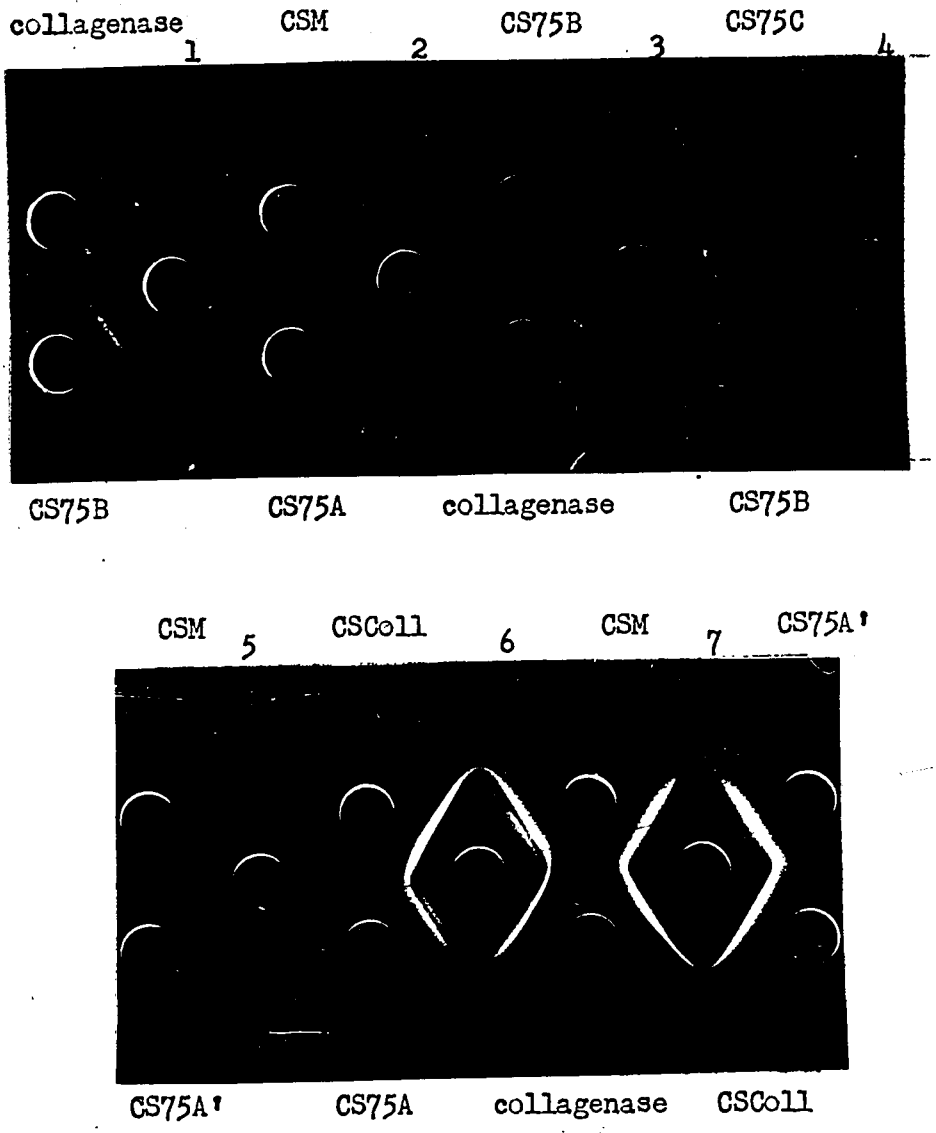

FIG. 6

Precipitin reactions of rat glomerular basement membrane components and collagen with their antisera, by double diffusion in agar gel. Middle wells contain antisera as indicated: (1) anti-CS75A; (2) anti-CSM; (3) anti-CS75A; (4) anti-CS75B; (5) anti-collagen; (6) anti-CSColl; (7) anti-CS75A'. Outside wells contain antigens as indicated.

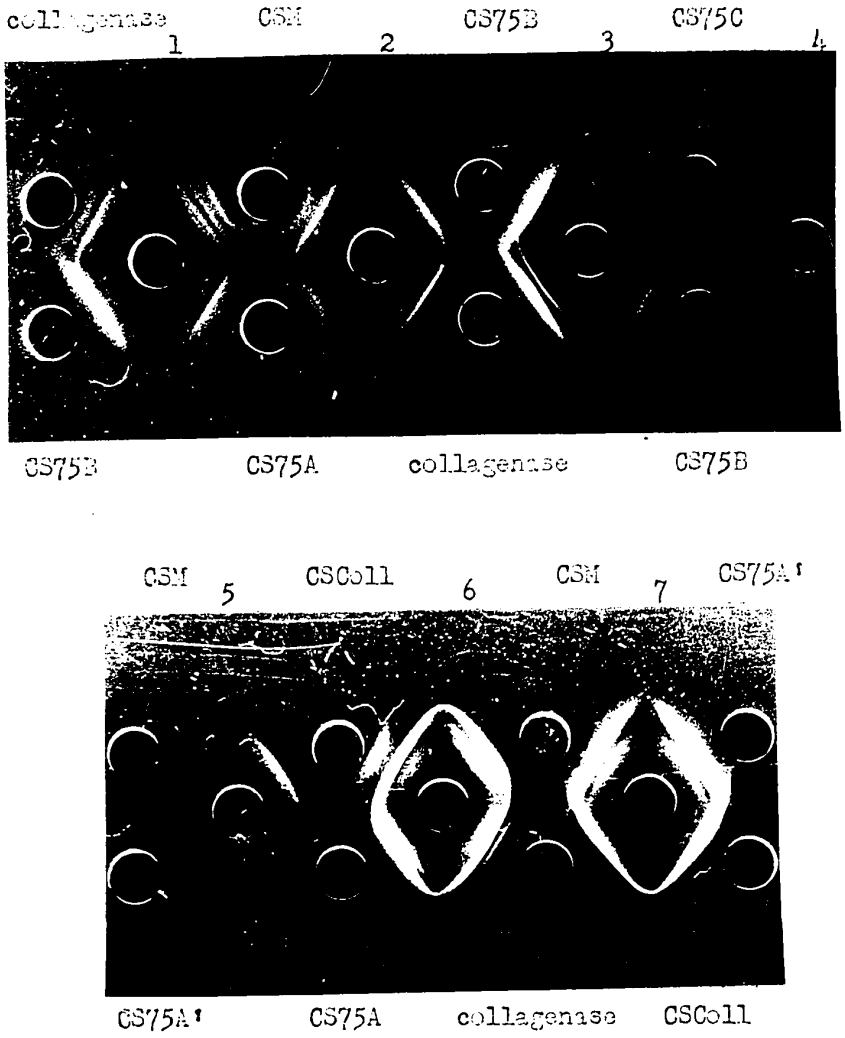

FIG. 6

Precipitin reactions of rat glomerular basement membrane components and collagen with their antisera, by double diffusion in agar gel. Middle wells contain antisera as indicated: (1) anti-CS75A; (2) anti-CSM; (3) anti-CS75A; (4) anti-CS75B; (5) anti-collagen; (6) anti-CSColl; (7) anti-CS75A'. Outside wells contain antigens as indicated.

TABLE 7

CHEMICAL COMPOSITION OF COLLAGENASE SOLUBILIZED RAT GBM AND ITS COMPONENTS (% OF DRY WEIGHT)

	CSM	CS75A	CS75B	CS75A'
Hexosamine	2.95 (0.165)*	2.95 (0.170)	2.37 (0.132)	7.4 (0.413)
Bound hexose	9.8 (0.544)	7.8 (0.433)	10.76 (0.597)	31.0 (1.72)
Sialic acid	1.64 (0.053)	1.59 (0.051)	1.62 (0.052)	1.45 (0.047)
Total carbohydrate	14.39 (0.662)	12.34 (0.654)	14.75 (0.781)	39.85 (2.18)
Hydroxyproline	2.28 (0.174)	- -	1.31 (0.100)	2.36 (0.180)
Total nitrogen	9.2	10.3	9.25	7.8

* The figures in parentheses represent the concentration expressed in μmoles/mg dry weight.

All values are average of two determinations.

the components indicates the presence of glycoprotein or glycopeptides. The presence of hydroxyproline indicates the presence of collagen fragments which might be resistant to collagenase action. The very high carbohydrate content in component CS75A' confirms the result observed in the elution pattern on Sephadex G-75 (Fig. 3).

A paper chromatogram of an acid hydrolysate of CSM obtained by the method described in section C.6.11. indicates that the carbohydrate consisted of glucosamine, glucose, galactose, a small amount of mannose, a trace of fucose and perhaps a trace of galactosamine (Fig. 7).

Acrylamide gel electrophoresis of CS75A and collagenase was performed as previously described in section C.8.1. The results indicate the heterogeneity of both materials. Collagenase migrated towards the cathode in alkaline pH and separated into seven to ten bands (Fig. 8). GBM component CS75A for the most part remained at the origin, though several bands migrated with mobilities corresponding to those of collagenase under the same conditions. This further indicates that membrane components obtained after collagenase digestion were contaminated with collagenase which itself appeared to be heterogeneous.

In summary, collagenase treatment did not alter the nephrotoxic antigenicity of the GBM; the collagenase digestion of GBM resulted in solubilization of glycoprotein or glycopeptides which were separable by gel filtration on Sephadex G-75 into three components. Abs against the membrane components localized to the kidney at GBM but only anti-CS75A

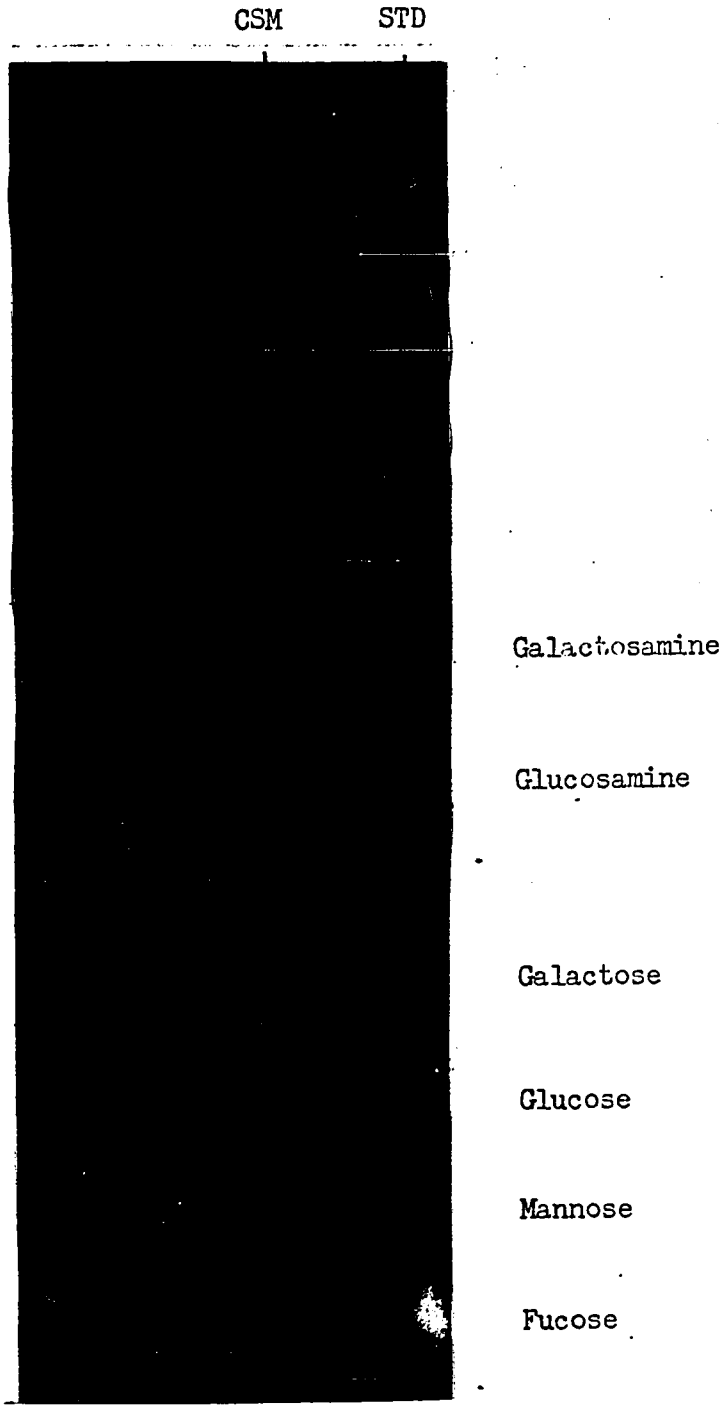

FIG. 7

Paper chromatogram of hydrolysate of collagenase solubilized membrane and monosaccharide standards.

Solvent: n-butanol-pyridine-H_2O (5:3:2 v/v)

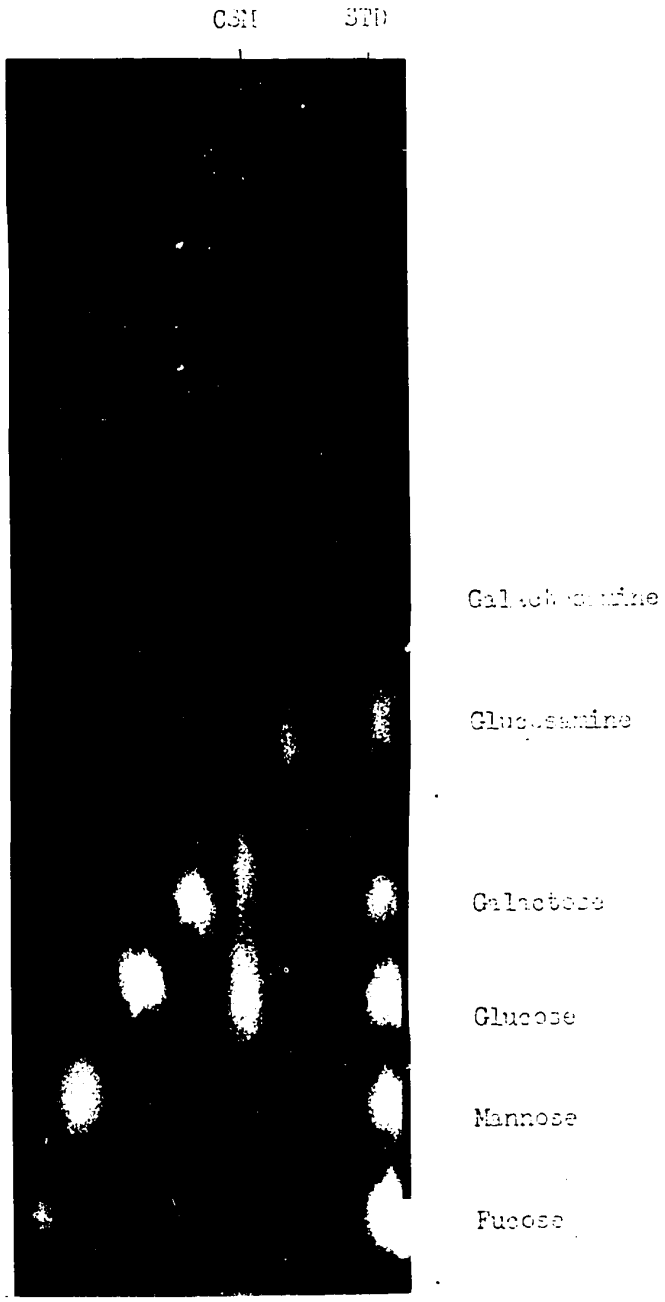

FIG. 7

Paper chromatogram of hydrolysate of collagenase solubilised membrane and monosaccharide standards.

Solvent: n-Butanol-pyridine-H_2O (5:3:2 v/v)

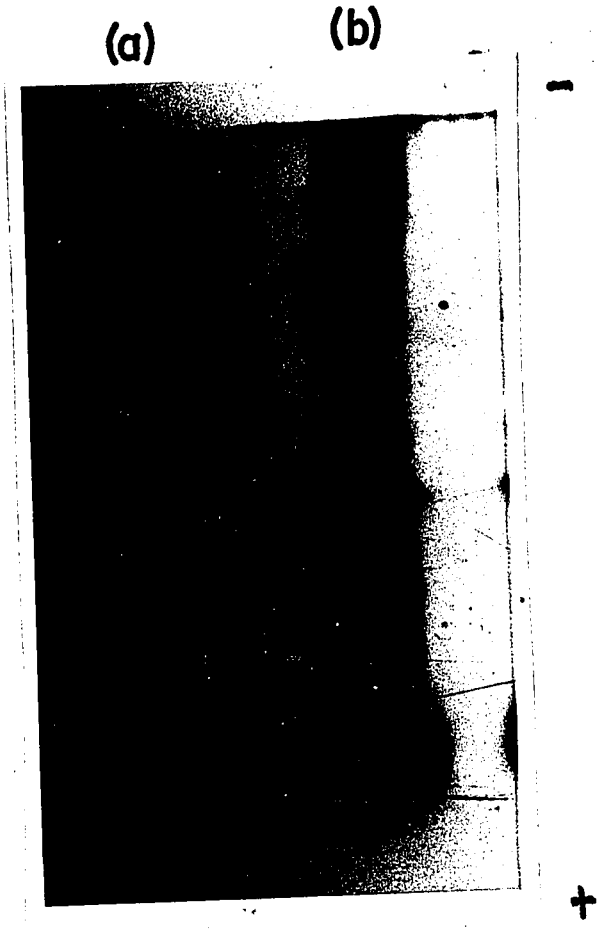

FIG. 8

Polyacrylamide gel electrophoresis of collagenase and human serum.

a) collagenase b) human serum

FIG. 8

Polyacrylamide gel electrophoresis of collagenase and human serum.

a) collagenase b) human serum

or anti-CS75A' were nephrotoxic. Anti-collagen, but not anti-CSColl, localized extensively to rat GBM and both were non-nephrotoxic to rats. The isolated membrane components appeared to be heterogeneous and contaminated with collagenase.

D.2.3. Gel Filtration of Collagenase-solubilized Membrane on Sephadex G-75 in Acetic Acid

GBM was digested with collagenase and dialyzed, and the supernatant obtained after centrifugation was lyophilized as previously described. The lyophilized material (CSM) was found to be only partially soluble in 0.9% saline or tris buffer (0.05M, pH 7.4) but completely soluble in dilute NaOH solution at pH 12 and in acetic acid (0.5M). Therefore, CSM was fractionated on Sephadex G-75 in 0.5M acetic acid to determine whether any better separation would occur.

The column of Sephadex G-75 (1.4 x 28 cm) was washed and equilibrated with excess 0.5N acetic acid. The sample dissolved in the same solvent was applied to the column and eluted with 0.5N acetic acid. Each fraction was measured for protein content by absorbancy at 280 mμ. Different aliquots of each fraction were taken for measurement of bound hexose, sialic acid and hexosamine.

As can be seen from the elution pattern (Fig. 9), CSM was eluted as a major peak with the void volume of the column containing bound hexose, sialic acid and hexosamine, and two minor peaks eluted later. The elution pattern of CSM from Sephadex G-75 in 0.5N acetic

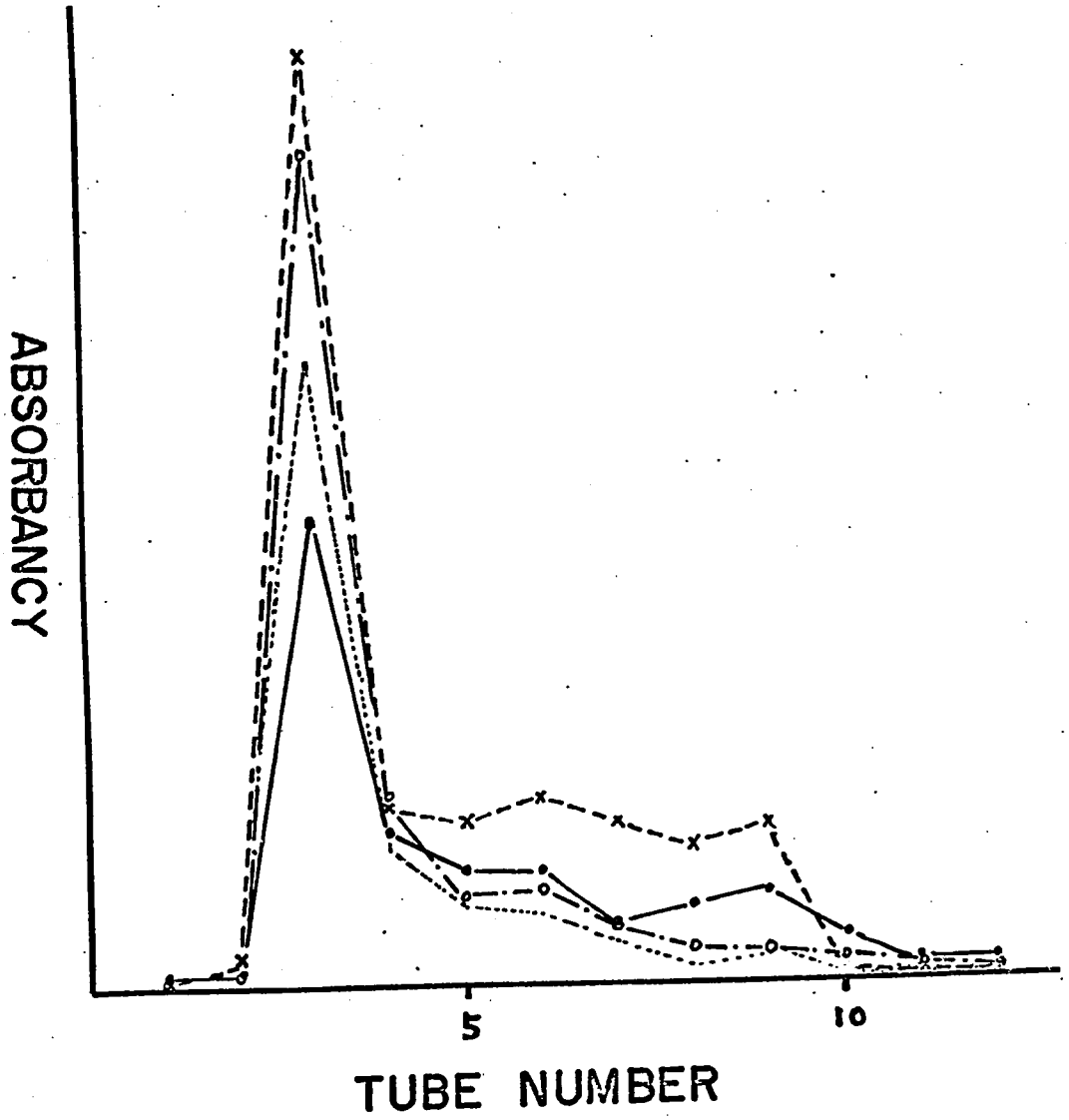

FIG. 9

Gel filtration pattern on Sephadex G-75 of rat glomerular basement membrane solubilized by collagenase digestion, eluted in acetic acid (0.5M).

Column dimension: 1.4 x 28 cm; Volume of each fraction: 5 ml
(—•—•—): absorbancy at 280 mμ; (--x--x--): bound hexose
(——o——): hexosamine; (.....): sialic acid

acid appeared to be slightly different from that in 0.9% saline (Fig. 1) but no better separation of components was achieved.

D.2.4. Effect of Pronase Digestion on Nephrotoxigenicity of GBM Component

In order to determine whether intensive proteolytic degradation of a nephrotoxigenic Ag will alter its antigenicity and whether the antigenic determinants for eliciting nephrotoxic Ab reside on the peptide portion of the glycoprotein, a proteolytic enzyme with broad specificity (Pronase) was used.

GBM component CS75A, which is nephrotoxigenic, was digested with Pronase as previously described (substrate to enzyme ratio = 30:1). At the end of incubation, the mixture was dialyzed against distilled water at 4°C and lyophilized. Thirty-two per cent of protein measured by Folin reagent and almost 100% of bound hexose of the starting material were recovered in tne non-dialyzable fraction of the Pronase digest.

This lyophilized substance was used to immunize one rabbit. The resulting antiserum failed to induce proteinuria in normal rats (4 rats tested). The levels of urinary protein were 32-46 mg per 24 hours at three weeks, and 84.2 mg per 24 hours at 8 months after initial injection. However, the rats injected with this antiserum showed positive localization of Ab on GBM as indicated by immunofluorescent staining. By gel double diffusion, this antiserum developed only a faint precipitin line with CSM but none with its antigen. A possible explanation is that this Ag contained fewer Ab combining sites and formed a non-precipitable complex with Abs.

It may be concluded that Pronase degraded the peptide bonds, whereas carbohydrate prosthetic groups remained linked to some peptides in non-dialyzable form. The glycopeptide was non-nephrotoxigenic, indicating that degradation of the peptide portion of glycoprotein (CS75A) by Pronase action led to the destruction of the molecular structure which may be essential to elicit the nephrotoxic Abs.

D.2.5. Antigenicity of the GBM Isolated by Mild Alkali Treatment

Renal reticulin and GBM have been isolated by the use of dilute alkali solution to solubilize the cellular component of the renal cortex or isolated glomeruli (228,244). The chemical composition of GBM obtained by this procedure differs from that obtained by the ultrasonification procedure (245,247,249) indicating that exposure to mild alkali resulted in destruction of sialic acid and other carbohydrate constituents. It is the purpose of this experiment to determine whether this partial destruction of the carbohydrate moiety of the intact GBM has any effect on immunological properties.

Glomeruli obtained from 50 normal rats were suspended in 100 ml of 0.05N NaOH solution in a beaker, thymol was added as preservative and the mixture was incubated at room temperature with constant stirring. After 24 hours, the supernatant was decanted and the residual glomeruli were resuspended in fresh alkali solution. These processes were repeated for 4 days. The sample at this stage was microscopically examined. The glomerular capillaries were disrupted, cellular components were solubilized

and basement membrane was obtained as glomerular ghosts. The residual GBM was then washed with 0.9% saline until the washing was neutral.

This membrane preparation was injected into three rabbits with Freund's adjuvant as previously described. The resulting antisera were examined for nephrotoxicity, ability to localize and cross-reaction with other membrane components in gel diffusion.

Antisera from three rabbits were injected into 9 normal rats. At one week after injection, only 4 rats developed proteinuria (240-290 mg per 24 hours); however, at 2 weeks after injection, more than 50% of the injected rats (7/9) developed proteinuria at a higher level (200-450 mg per 24 hours) and only 2 rats showed moderate proteinuria (95-105 mg per 24 hours). Thus, it can be concluded that Abs against this GBM preparation are nephrotoxic to rats.

The rats injected with antiserum showed specific localization of the rabbit γ-globulin on GBM as demonstrated by direct immunofluorescent staining. The fluorescence appeared in a typical membranous pattern (Fig. 10). By gel double diffusion, two precipitin lines developed between this antiserum and CSM (membrane digested with collagenase). Anti-kidney serum, when reacted with the same Ag (CSM), also developed two precipitin lines, one of which showed reaction of identity with the previous system.

It can be concluded that the alteration in the chemical composition of this membrane preparation had no effect on the nephrotoxi-

FIG. 10

Localization of rabbit γ-globulin in renal glomerulus of rat injected with antiserum against rat glomerular basement membrane isolated by NaOH treatment, as demonstrated by immunofluorescent staining.

genicity of the GBM. The loss of sialic acid and other carbohydrate constituents resulting from the prolonged exposure of GBM to alkali did not abolish the essential antigenic determinant groups for eliciting nephrotoxic Ab in rabbits. The results obtained in gel diffusion and in vivo localization provide further evidence that this membrane preparation and intact GBM shared some common antigenic groups.

D.2.6. Isolation and Characterization of Antigenic Components of GBM

D.2.6.a. Introduction

The preliminary separation of GBM components indicates both heterogeneity and collagenase contamination of the preparations. Attempts were made to remove residual enzyme from membrane components by employing an immunosorbent method. This involved conjugation of purified rabbit γ-globulin (Ab to collagenase) to EMA polymer (ethylene maleic anhydride) (291). The water-insoluble polyanionic derivatives of Ab (immunosorbent) were mixed with Sephadex G-25 and packed into a column (1.3 x 20 cm). When the sample was applied to the column, the specific absorption of collagenase by the immunosorbent, leaving membrane components in the effluent, was expected. However, the recovery of membrane components was less than 10% and the method was not employed.

A series of gel filtrations of collagenase and CS75A on Sephadex gels of different cross-linkage were performed in order to separate enzyme from membrane component. Previous results indicate that collagenase is heterogeneous and is excluded from Sephadex G-75. When collagenase was

fractionated on Sephadex G-100 in saline, it separated into two distinct peaks; the one containing all the enzyme activity was eluted with void volume of the column and the other, with no enzyme activity, was retarded in the gel (Fig. 11a). CS75A, when fractionated on the same column, was excluded from Sephadex gel as a single peak with a small shoulder (Fig. 11b). Thus, the separation of active collagenase from membrane component was not attainable by gel filtration on Sephadex G-100.

Further fractionation on Sephadex G-200 was attempted. Collagenase was completely retained in the gel and eluted after the void volume of the column as a broad peak (Fig. 11c). However, CS75A was separated into two peaks, one being excluded from the gel and the other being retained in the gel as a broad peak (Fig. 11d). Similar results were obtained by gel filtration on Biolgel P-300. It is apparent that major membrane component can be separated from purified active collagenase by gel filtration on Sephadex G-200.

D.2.6.b. Collagenase Digestion, Urea Extraction and Separation of Solubilized Membrane Components by Gel Filtration

GBMs obtained from 500 normal rats were suspended in 75 ml of tris buffer and incubated with purified active collagenase (first peak excluded from Sephadex G-100) as previously described. The mixture was then centrifuged at 2000 r.p.m. for 15 minutes. The residual membrane was again suspended in buffer and digested with fresh enzyme. These processes were repeated twice. Five mg of purified collagenase were used

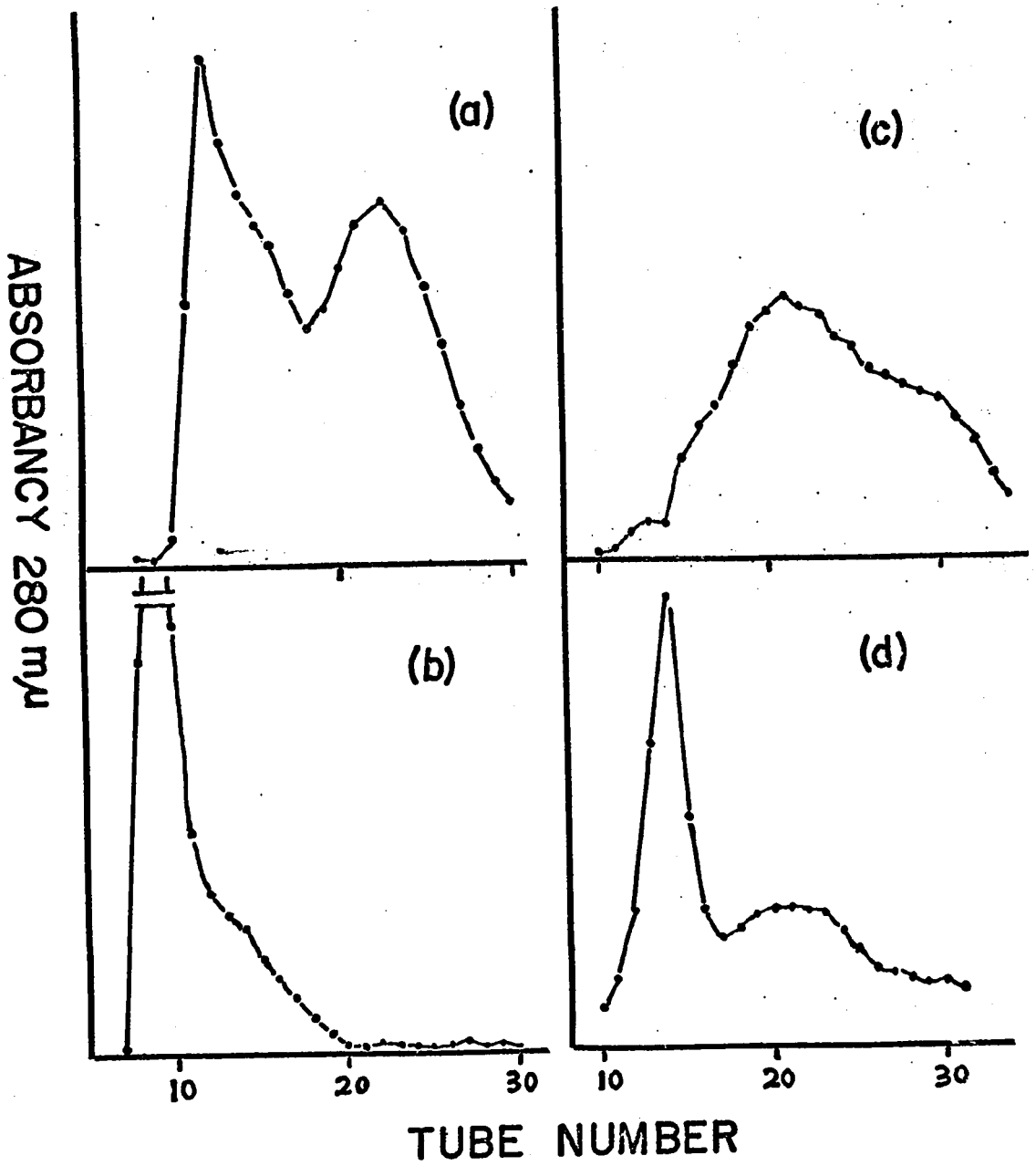

FIG. 11

Gel filtration patterns of rat basement membrane solubilized by collagenase, and of collagenase.

a) Collagenase on Sephadex G-100 (1.1x30 cm)
b) CS75A on Sephadex G-100 (1.1x30 cm)
c) Collagenase on Sephadex G-200 (1.4x26 cm)
d) CS75A on Sephadex G-200 (1.4x26 cm)

for complete digestion during three incubations for a total period of 40 hours. The supernatants from three digestions were combined, dialyzed against distilled water at 4°C and lyophilized (85 mg). This material was designated as CSM (collagenase solubilized membrane).

The residual membrane, after three collagenase digestions, was washed once with distilled water and suspended in 35 ml of 8M urea solution (pH 8.5 adjusted by addition of 2% tris solution). The mixture was allowed to stand at 38°C with continuous stirring for 24 hours. At the end of the extraction, the mixture was centrifuged at 13,000 r.p.m. (34,800 g) for one hour at 4°C. The supernatant was dialyzed against distilled water for 5 days at 4°C with frequent changes of water and finally lyophilized (55 mg). It was noted that heavy precipitation occurred during the dialysis. This material was designated as USM (urea solubilized membrane).

The CSM was submitted to preliminary fractionation on Sephadex G-75 using phosphate saline buffer (0.015M, pH 7.4) for elution (Fig. 12). The fractions of each peak were combined, dialyzed and lyophilized. The peak which was excluded from Sephadex G-75 (i.e. CS75A) was further fractionated on Sephadex G-200 in the same buffer (Fig. 13). Two components were obtained after dialysis and lyophilization (CS200A and CS200B). CS75B, which was retained in Sephadex G-75, was further fractionated on Sephadex G-25 (Fig. 14) and two components (CS25A and CS25B) were obtained. The membrane material, which was solubilized by 8M urea extraction (USM), was fractionated directly on Sephadex G-200 in tris buffer (0.05M, pH 8.4).

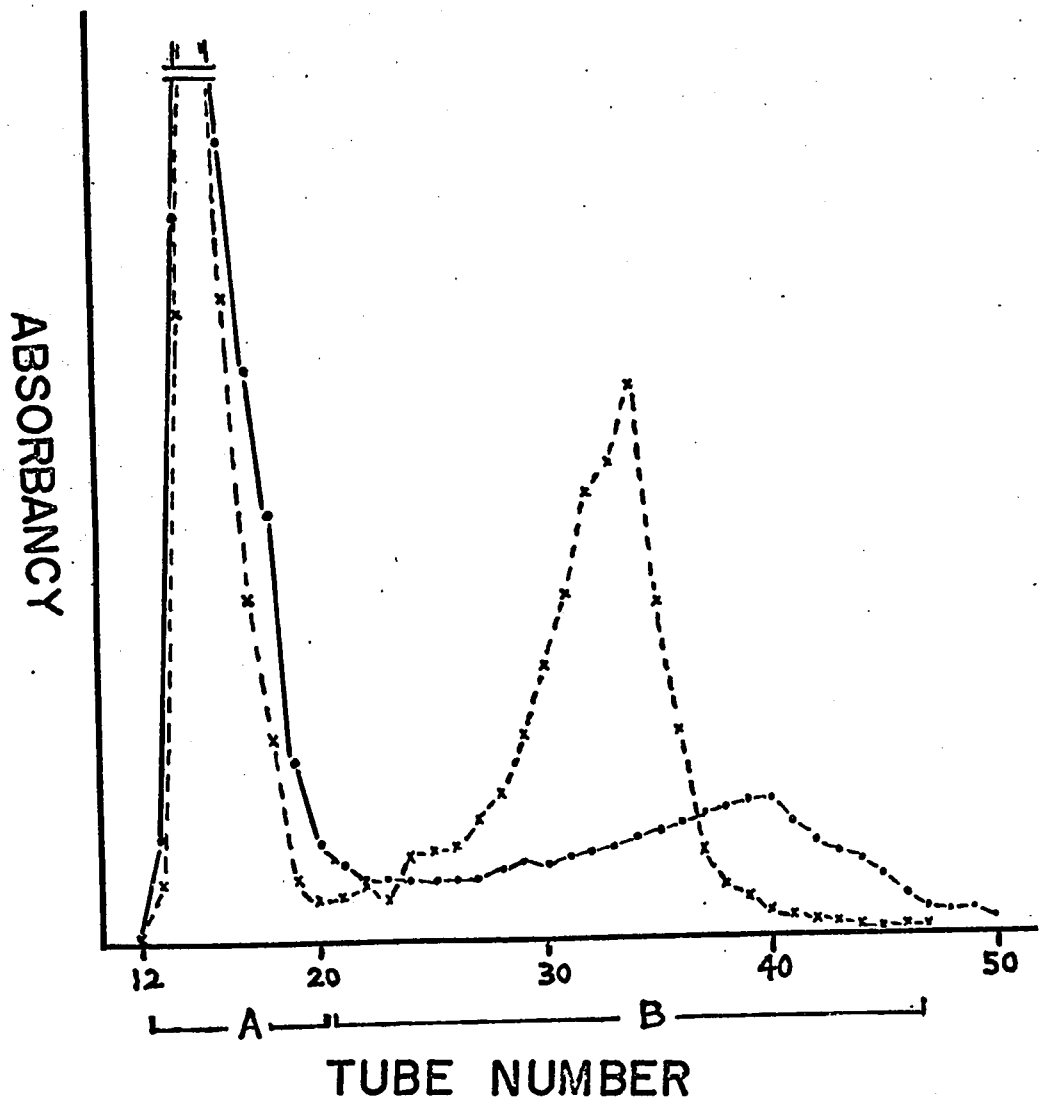

FIG. 12

Gel filtration pattern on Sephadex G-75 of rat glomerular basement membrane solubilized by collagenase digestion.

Column dimension: 2 x 35 cm
Volume of each fraction: 3 ml
(—·—·—): absorbancy at 280 mμ; (--x--x--): bound hexose

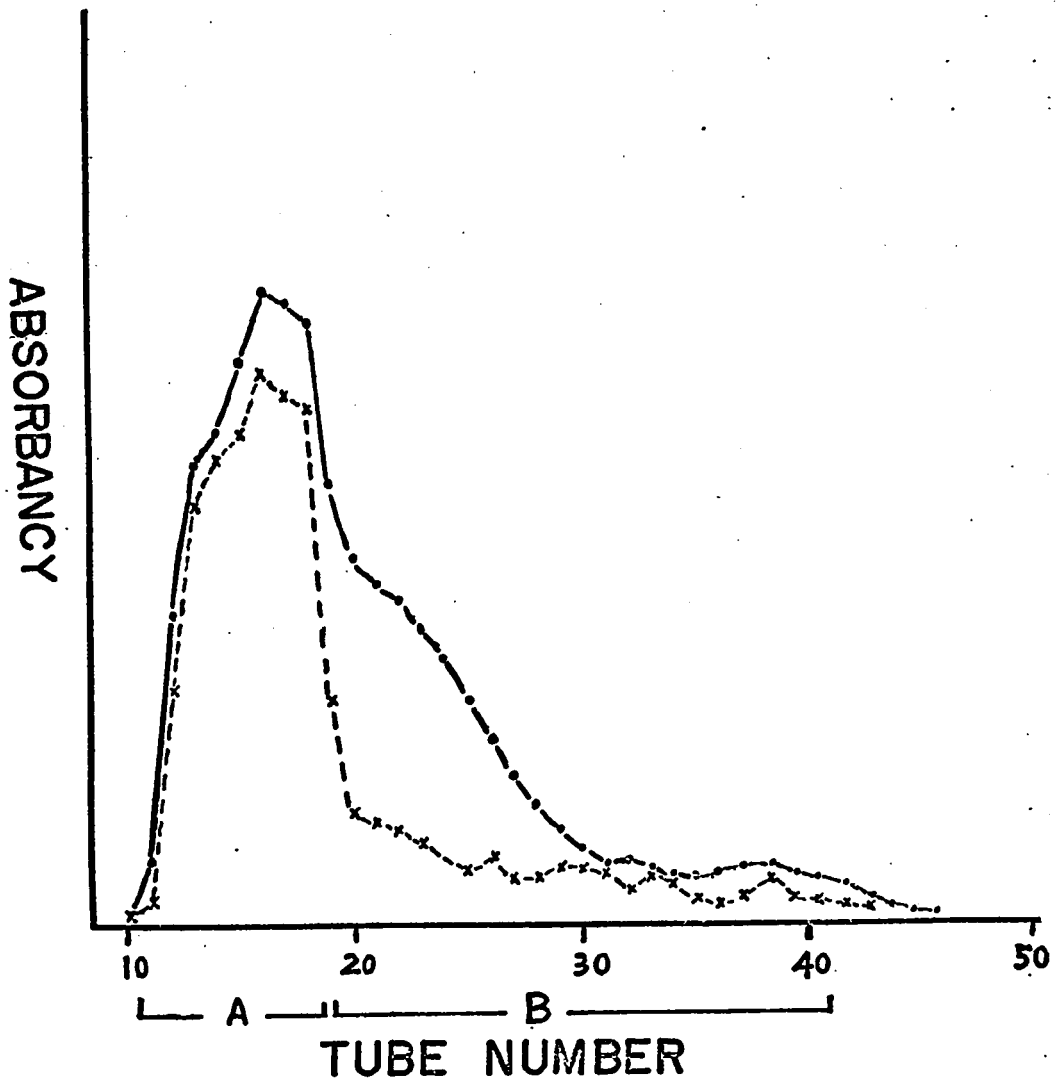

FIG. 13

Gel filtration pattern on Sephadex G-200 of CS75A.

Column dimension: 1.4 x 25 cm
Volume of each fraction: 1 ml
(—·—·—): absorbancy at 280 mμ; (--x--x--): bound hexose

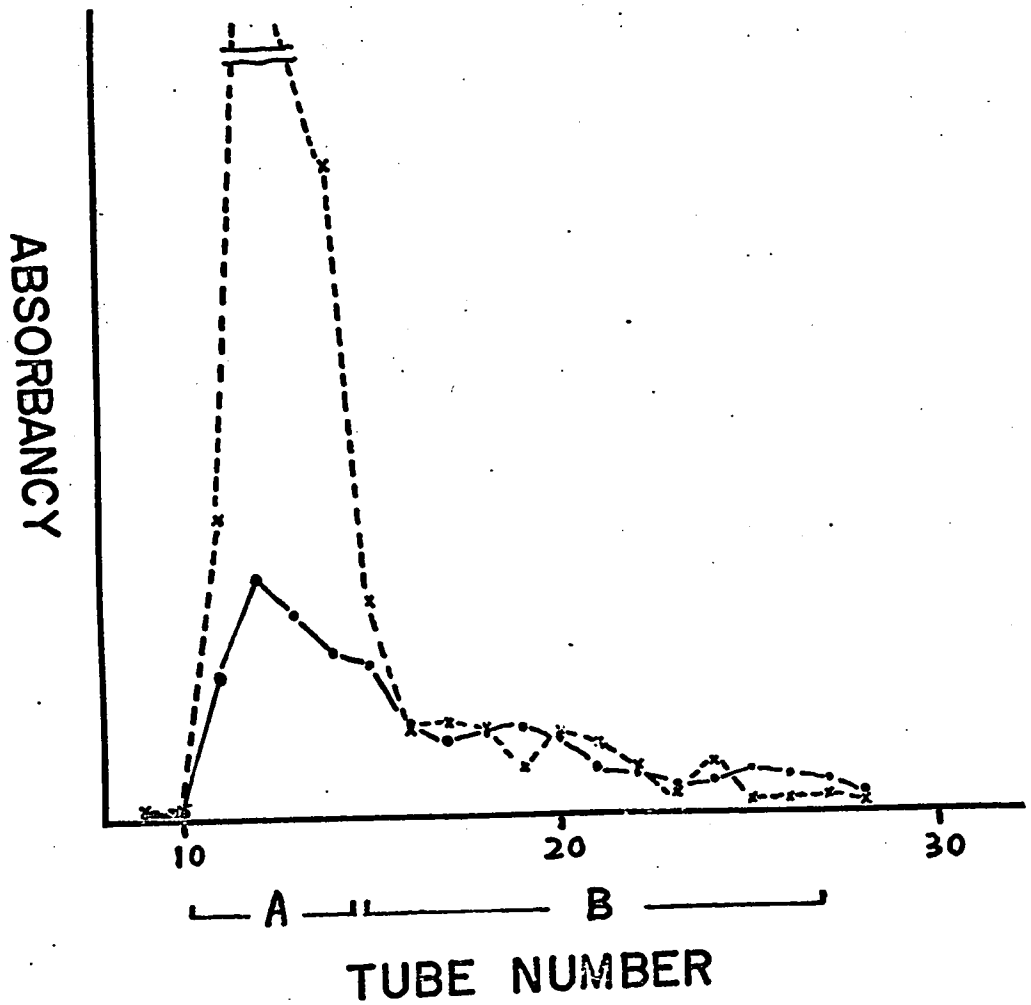

FIG. 14

Gel filtration pattern on Sephadex G-25 of CS75B.

Column dimension: 1.4 x 35 cm
Volume of each fraction: 2 ml
(—·—·—): absorbancy at 280 mμ; (--x--x--): bound hexose

Two components (US200A and US200B) were separated (Fig. 15).

The isolation of the membrane components can be summarized in a flow diagram as shown in Fig. 16. GBM was almost completely solubilized by the combination of collagenase digestion and urea extraction. The solubilized membrane materials were subsequently separated into six components (CS200A, CS200B, CS25A, CS25B, US200A and US200B) which differed basically in molecular size according to gel filtrations.

D.2.6.c. Nephrotoxicity and In Vivo Localization of Abs to Membrane Components

The six membrane components were injected separately into groups of rabbits (two rabbits for each component) with Freund's complete adjuvant as previously described. Nephrotoxicity of the resulting antisera was examined by the ability to produce proteinuria in normal rats. In vivo localization of Abs against each component was investigated in these rats by the immunofluorescent method.

As can be seen in Table 8, antisera against CSM, CS200A, USM and US200A, all induced proteinuria in almost all the rats by the eighth day after initial injection. The proteinuria persisted and increased to higher levels as time elapsed. Several animals injected with these antisera died within one to two weeks and showed edema, another evidence of nephrotoxicity (292). Anti-CS200B from one rabbit induced high proteinuria after one week, in all rats injected; however, antiserum from another rabbit induced only very mild proteinuria even one month after initial injection. Anti-US200B induced proteinuria in only half of the

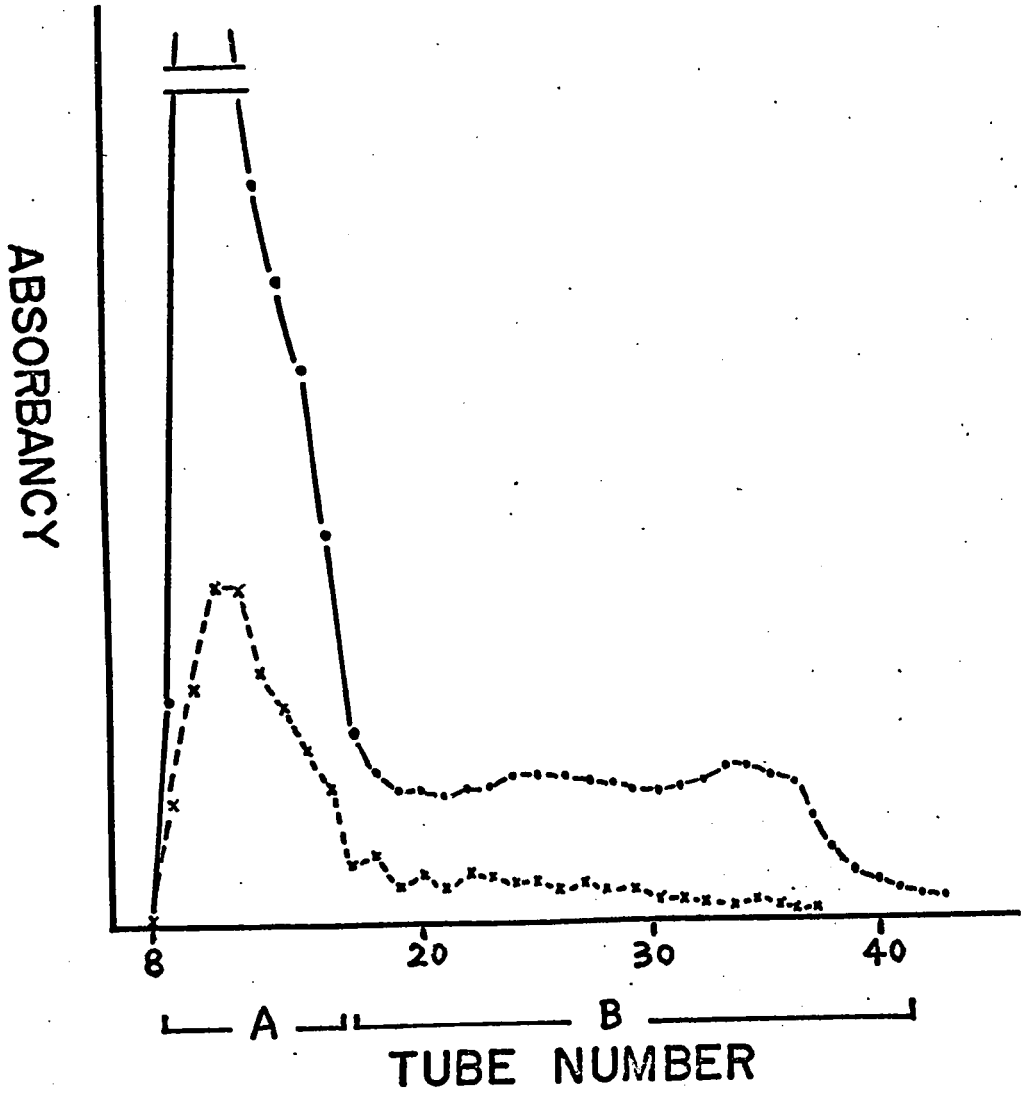

FIG. 15

Gel filtration pattern on Sephadex G-200 of decollagenized glomerular basement membrane solubilized by urea extraction (USM).

Column dimension: 2 x 30 cm
Volume of each fraction: 3 ml
(—·—·—): absorbancy at 280 mμ; (--x--x--): bound hexose

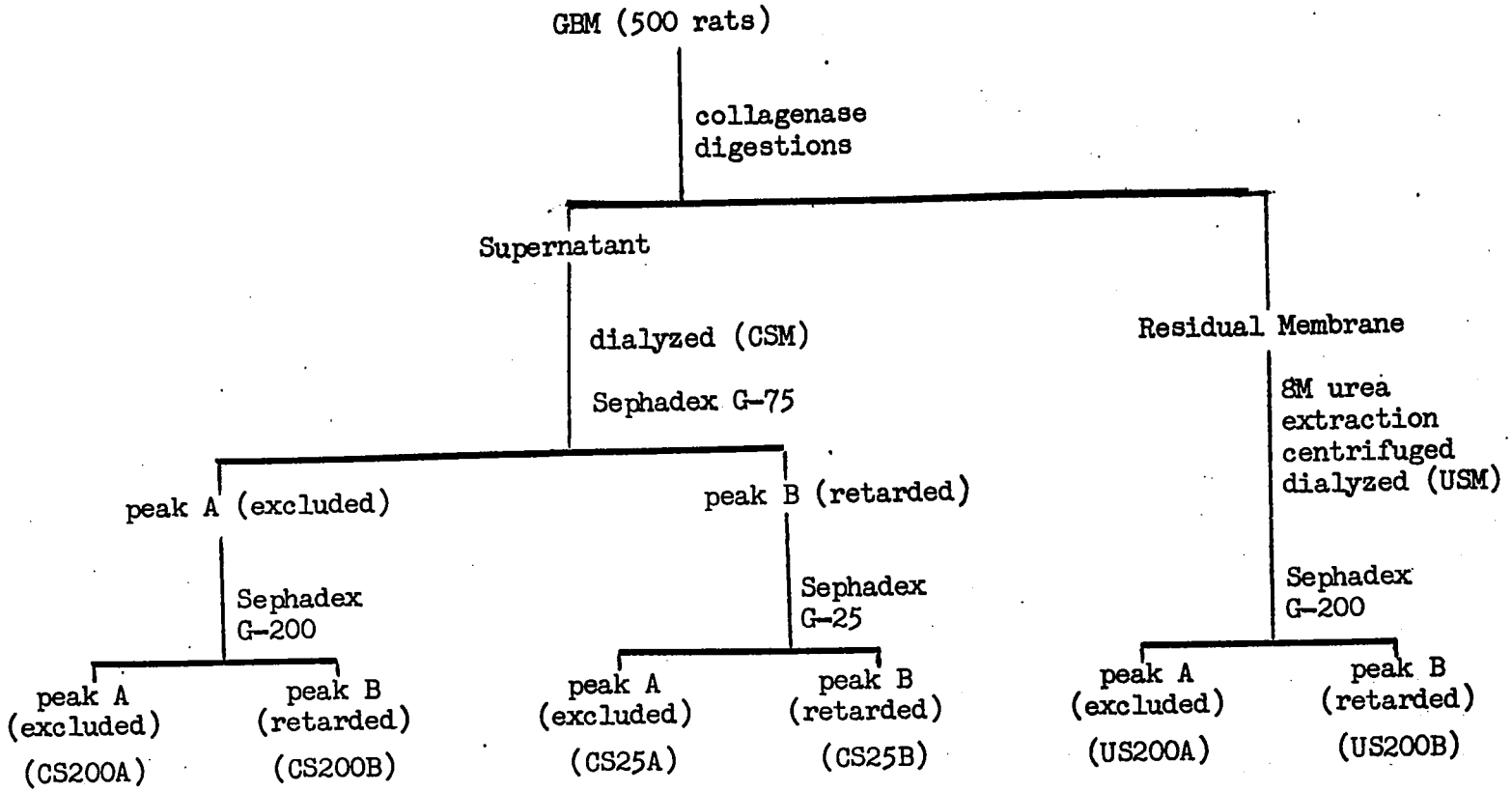

FIG. 16

Isolation procedures of rat glomerular basement membrane components.

TABLE 8

NTN INDUCED BY INJECTION OF ANTISERUM AGAINST RAT GBM COMPONENTS

Antiserum	Proteinuria in no. of rats	Level of proteinuria (mg/24 hr) Day 8	Day 15	Localization of Abs on glomeruli
Anti-CSM	6/6	300-380	-	+++
Anti-CS200A	3/6 (3)*	275-330	-	+++
Anti-CS200B	3/6	290-310 20-40	380 85-106	++
Anti-CS25A	0/6	35-46	33-46	+
Anti-CS25B	0/6	18-32	6-32	-
Anti-USM	5/6 (1)	335-405 62-77	- 170-372	++
Anti-US200A	5/6 (1)	212-270 58-124	324-382	+++
Anti-US200B	2/6 (2)	54-250	63-438	++
Normal serum	0/3	-	20-48	-

* The figures in parentheses indicate the number of rats which died during the first two weeks following injection.

rats injected, two weeks later. It can be concluded that the two membrane components (CS200A and US200A) are potent Ags, whereas CS200B and US200B are less potent, but all are capable of inducing nephrotoxic Abs in rabbits.

It can be noted also that none of the rats injected with anti-CS25A and anti-CS25B developed proteinuria even one month later. The urinary protein content in 24 hours was in the same range as that of rats injected with normal rabbit serum. It is apparent, in confirmation of previous findings, that these two membrane components, which were retained in Sephadex G-75, are non-nephrotoxigenic.

All antisera, except anti-CS25B, localized to GBM in vivo as indicated by fluorescent staining (Table 8). It was noted that the animals from the same group exhibited similar intensity of fluorescence despite different degrees of proteinuria developed. It is evident that all membrane components, except one, induced localizing Abs in rabbits but only some of them were nephrotoxic to rats.

D.2.6.d. Interaction of Membrane Ags and Abs by Gel Double Diffusion

Gel double diffusion of membrane components and their Abs indicate the presence of multiple Ags (Table 9). Common antigenic determinants among the components were demonstrated by cross-reactions between one Ag and antisera against another Ags. It can be noted that two components of smaller molecular size (CS25A and CS25B) did not develop any precipitin line with their own or other antisera. However,

TABLE 9

CROSS-REACTION BETWEEN RAT MEMBRANE COMPONENTS AND THEIR ANTISERA BY GEL DOUBLE DIFFUSION (NO. PRECIPITIN LINES)

Antiserum	Antigen							
	USM	US-200A	US-200B	CSM	CS-200A	CS-200B	CS-25A	CS-25B
Anti-USM	3	3	2	3	3	-	-	-
Anti-US200A	3	3	1	3	2	-	-	-
Anti-US200B	2	2	1	1	-	-	-	-
Anti-CSM	2	2	1	3	3	2	0	0
Anti-CS200A	2	2	-	3	3	2	-	-
Anti-CS200B	1	-	-	2	-	2	0	-
Anti-CS25A	0	-	-	1	0	1	0	0
Anti-CS25B	0	-	-	0	-	-	0	0
Anti-rat kidney	2	2	2	2	2	1	-	-
Anti-collagenase	0	0	0	2	0	2	-	-

anti-CS25A cross-reacted and formed a precipitin line with CSM. A possible explanation for this is that CS25A, containing fewer Ab combining sites, forms a non-precipitable complex with Abs, whereas CSM, being a larger molecule with multiple valences, forms larger precipitable aggregates with anti-CS25A. There was no precipitin line formed between anti-collagenase and any membrane component, except CS200B, indicating that collagenase was present only in CS200B (Table 9).

Cross reactions in both directions between components solubilized by collagenase and by urea extraction indicate the presence of common antigenic determinants in both components and partial immunologic identity (Fig. 17a). When isolated components were reacted with anti-kidney serum, only one or two precipitin lines were developed (Fig. 17b). The collagenase digestion and urea extraction of intact GBM thus appear to liberate hidden antigenic determinants. It can also be noted that one of the precipitin lines formed between anti-kidney serum and various membrane components showed a reaction of identity as demonstrated by fusion of the precipitin lines. The result suggests that isolated membrane components share common antigenic determinants with the kidney Ags, as expected.

D.2.6.e. Chemical Composition of Membrane Components

Carbohydrates and amino acid compositions of the membrane components were determined as previously described. As can be seen in Table 10, all components contained hexosamine, bound hexose and sialic acid as carbohydrate constituents. Components solubilized by collagenase

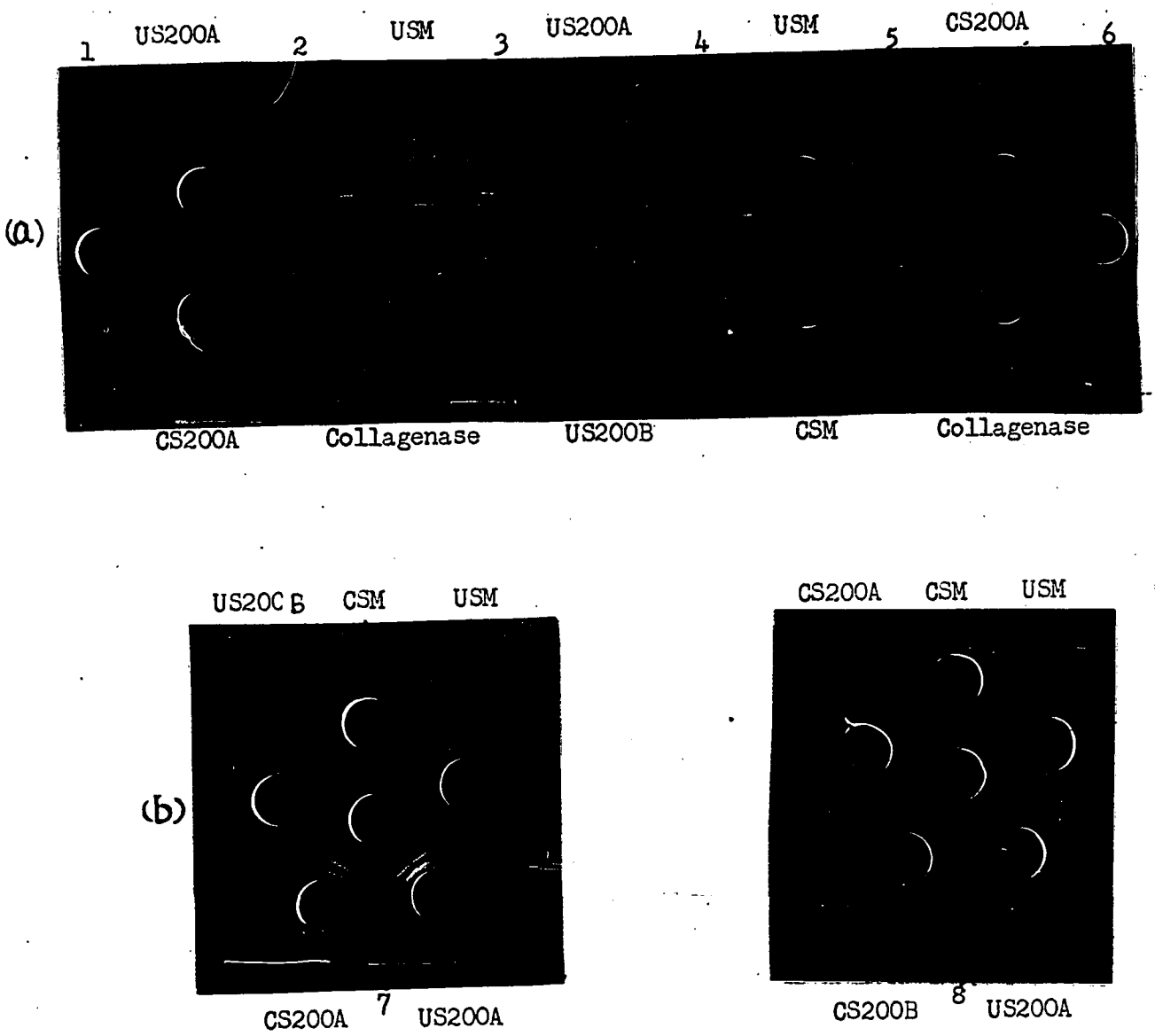

FIG. 17

Precipitin reactions between rat glomerular basement membrane components and their antisera, by double diffusion in agar gel. Middle or centre wells contain antisera:(1) anti-CS200A; (2) anti-US200A; (3) anti-CSM; (4) anti-USM; (5) anti-rat kidney; (6) anti-CS200A; (7) anti-USM; (8) anti-rat kidney. Outer wells contain antigens as indicated.

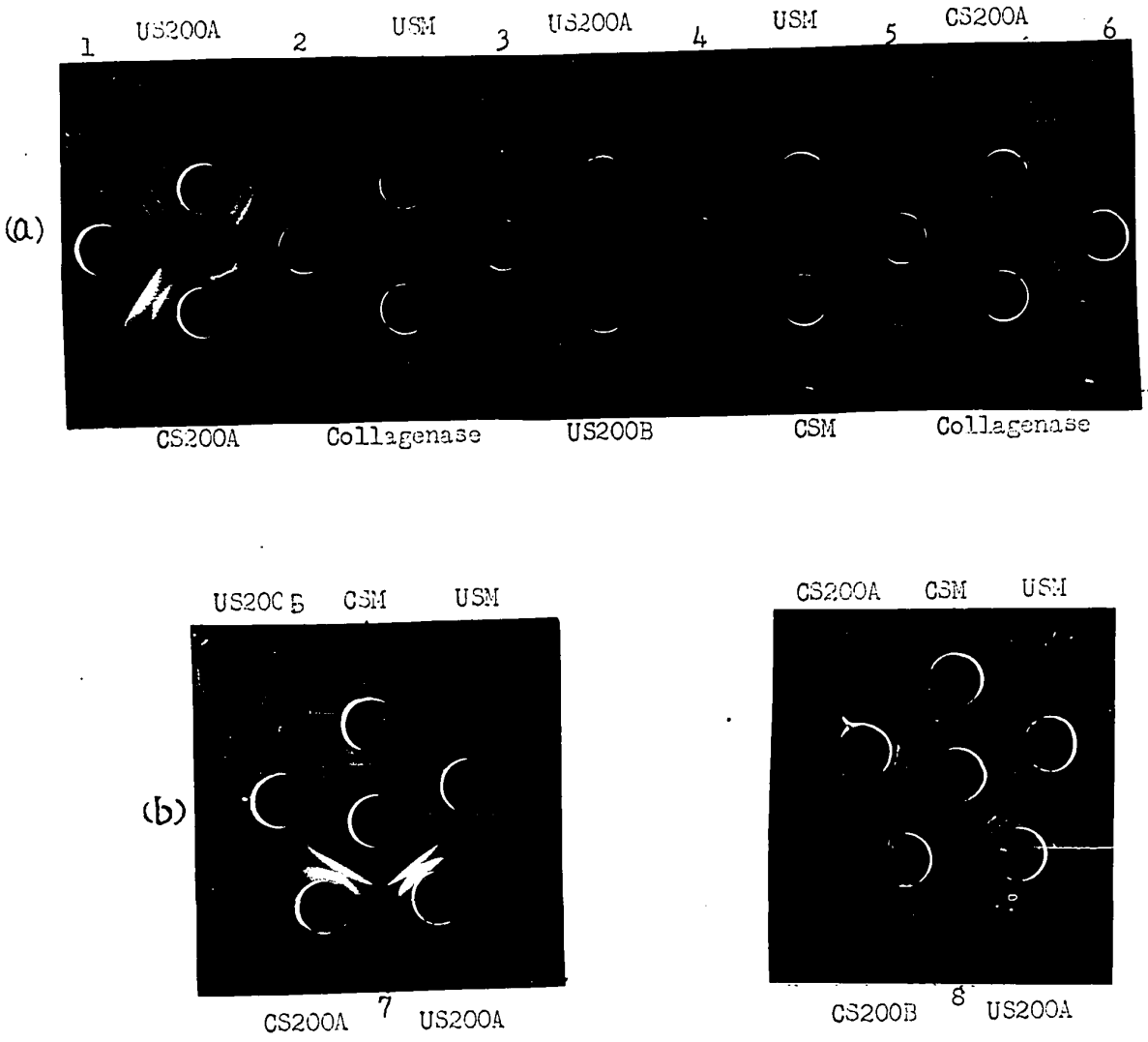

FIG. 17

Precipitin reactions between rat glomerular basement membrane components and their antisera, by double diffusion in agar gel. Middle or centre wells contain antisera: (1) anti-CS200A; (2) anti-US200A; (3) anti-CSM; (4) anti-USM; (5) anti-rat kidney; (6) anti-CS200A; (7) anti-USM; (8) anti-rat kidney. Outer wells contain antigens as indicated.

TABLE 10

CARBOHYDRATE COMPOSITION OF RAT GBM COMPONENTS
(% OF DRY WEIGHT)

	CS200A	CS200B	CS25A	US200A	US200B
Hexosamine	5.2 (0.290)*	2.76 (0.154)	3.46 (0.193)	4.37 (0.244)	2.40 (0.134)
Bound hexose	12.2 (0.677)	4.12 (0.228)	15.0 (0.833)	5.18 (0.287)	4.56 (0.253)
Sialic acid	1.2 (0.035)	0.72 (0.023)	0.99 (0.032)	1.11 (0.036)	0.45 (0.014)
Total carbohydrate	18.7 (1.002)	7.60 (0.405)	19.45 (1.058)	10.66 (0.567)	7.41 (0.401)
Total nitrogen	10.53	12.08	7.28	10.40	12.88

* The figures in parentheses represent the concentration expressed in μmoles/mg dry weight.

digestion, except CS200B which was partially contaminated with collagenase, showed much higher bound hexose content than those solubilized by urea extraction. It has been reported that a fraction of larger peptides enriched in content of esters and hexose was isolated from a collagenase digest of ichthyocol (290). It is possible that collagenase digestion of GBM may result in the solubilization of similar collagen peptides enriched in hexose content.

The hexosamine and bound hexose were identified by paper chromatography as glucosamine, galactosamine, glucose, galactose and mannose for components CS200A, CS200B and US200B. However, glucose was not detected in US200A.

Table 11 presents the amino acid composition of various components. The data were expressed in terms of the numbers of residues of each amino acid per 100 total residues. Nineteen amino acids were determined quantitatively. Striking differences can be noted between components solubilized by collagenase digestion and those by urea extraction in that the latter components (US200A and US200B) contained practically no hydroxyproline or hydroxylysine, and had a lower glycine content suggesting the absence of collagen in these components.

It is of interest that the component solubilized by collagenase digestion (CS200A) still contained 4.88% of hydroxyproline of the total amino acid residues as compared to 7.33%, 6.84% and 5.7% for

TABLE 11

AMINO ACID COMPOSITION OF RAT GBM COMPONENTS
(RESIDUES PER 100 RESIDUES)

	CS200A	CS200B	US200A	US200B
Aspartic acid	8.95	9.20	9.57	10.51
Threonine	5.39	6.46	6.57	5.65
Serine	6.45	6.31	5.39	5.40
Glutamic acid	10.35	11.12	11.93	12.84
Proline	5.69	5.48	6.49	5.70
Glycine	17.19	11.58	9.49	8.28
Alanine	5.86	6.39	6.89	7.48
Valine	4.33	5.27	5.60	6.10
Cystine	3.05	3.64	4.46	1.98
Methionine	-	2.02	1.98	1.88
Isoleucine	2.97	3.97	3.97	5.01
Leucine	6.75	7.40	8.11	8.92
Tyrosine	3.39	3.46	3.29	2.92
Phenylalanine	3.52	3.86	3.69	4.16
Lysine	2.93	4.55	4.10	5.40
Histidine	2.16	2.34	2.64	2.43
Arginine	4.33	4.58	5.80	5.30
Hydroxylysine	1.78	trace	0	0
Hydroxyproline	4.88	2.34	0	0

intact GBM of rat, beef and dog respectively (245,247,249). Hydroxylysine content in this component was 1.78 residues per 100 amino acid residues, whereas those in bovine and canine GBM were reported to be 2.23% and 2.2% respectively (247,249). The hydroxylysine to hydroxyproline ratio in CS200A was 0.37 which is between the ratios (0.32 and 0.42) reported for bovine and canine GBM respectively.

Despite the occurrence of substantial amounts of hydroxyproline and hydroxylysine in CS200A, the amino acid compositions of this component and others were very dissimilar to that of tendon collagen (247). In addition, contents of aspartic acid, threonine, serine and glutamic acid, which are characteristic amino acids of glycoproteins, were higher in the membrane components than in tendon collagen or intact GBM. The results suggest that isolated membrane components are glycoprotein in nature with collagen fragments attached to some of the components.

D.2.6.f. Conclusions

Six membrane components were isolated by collagenase digestion, urea extraction and fractionation on Sephadex gels. Abs against these components, except two which were solubilized by collagenase and retained in Sephadex G-75, localized to the GBM and induced proteinuria in rats. Multiple Ags and common antigenic determinants among the membrane components were demonstrated by gel double diffusion. All components were characterized as glycoprotein containing 7% to 20% of

total carbohydrate, consisting of galactose, mannose, glucose, glucosamine, galactosamine and sialic acid. The components solubilized by collagenase digestion still contained collagen fragments but those solubilized by urea extraction were free of hydroxyproline and hydroxylysine.

D.3. Characterization of a Nephrotoxigenic Antigen Isolated from Rat GBM

The antigenic components isolated from GBM were not all nephrotoxigenic. It has been demonstrated that collagenase treatment did not alter the nephrotoxigenecity of the GBM and localizing Ab against tendon collagen failed to produce proteinuria in rats. This provides evidence that collagenous protein in GBM was not responsible for production of nephrotoxic Abs. It is, therefore, concluded that the specific nephrotoxigenic Ag derives from glycoprotein of the GBM. A component solubilized by urea extraction (US200A) was free of collagenous protein and may represent the specific glycoprotein of the GBM. This component was further characterized.

D.3.1. Chemical Composition

Amino acid and carbohydrate compositions of the component US200A as given previously in Tables 10 and 11 suggest that this is a glycomacroprotein with a carbohydrate content of 10.66% of the dry weight or 56.7 μmoles of monosaccharide per 100 mg. The carbohydrate content of this material was slightly higher than that of intact GBM

(9% and 10% for bovine and canine BM respectively) (247,249). The molar ratio of bound hexose:hexosamine:sialic acid was found to be 8:7:1 in this glycoprotein as compared with the ratio of 10:2.5:1 reported for intact bovine GBM (249) and with the ratio of 3.4:1 for hexosamine:sialic acid in intact rat GBM (245).

Carbohydrate constituents of this glycoprotein were further characterized. Individual estimations of glucosamine and galactosamine were performed according to the method described in section C.6.1. Complete separation of galactosamine and glucosamine on a Dowex-50 column was achieved (Fig. 18). A molar ratio of 6.35:1 for glucosamine and galactosamine was obtained for this glycoprotein which is in close agreement with the ratio reported for bovine GBM (87:13 or 6.7:1) (249).

Sialic acid content in this glycoprotein was determined quantitatively on an acid hydrolysate and also after purification on ion-exchange resins as described in section C.6.2.b. The values for sialic acid obtained by these methods agreed fairly well (1.28% of dry weight by the resin method and 1.11% by the direct method). Sialic acid in the acid hydrolysate of the glycoprotein, with or without purification on ion-exchange resin, when reacted with thiobarbituric acid reagent gave absorption spectra identical to those given by standard N-acetylneuraminic acid (Fig. 19). The result indicates that direct measurement of sialic acid in the acid hydrolysate by the thiobarbituric acid method is specific and reliable.

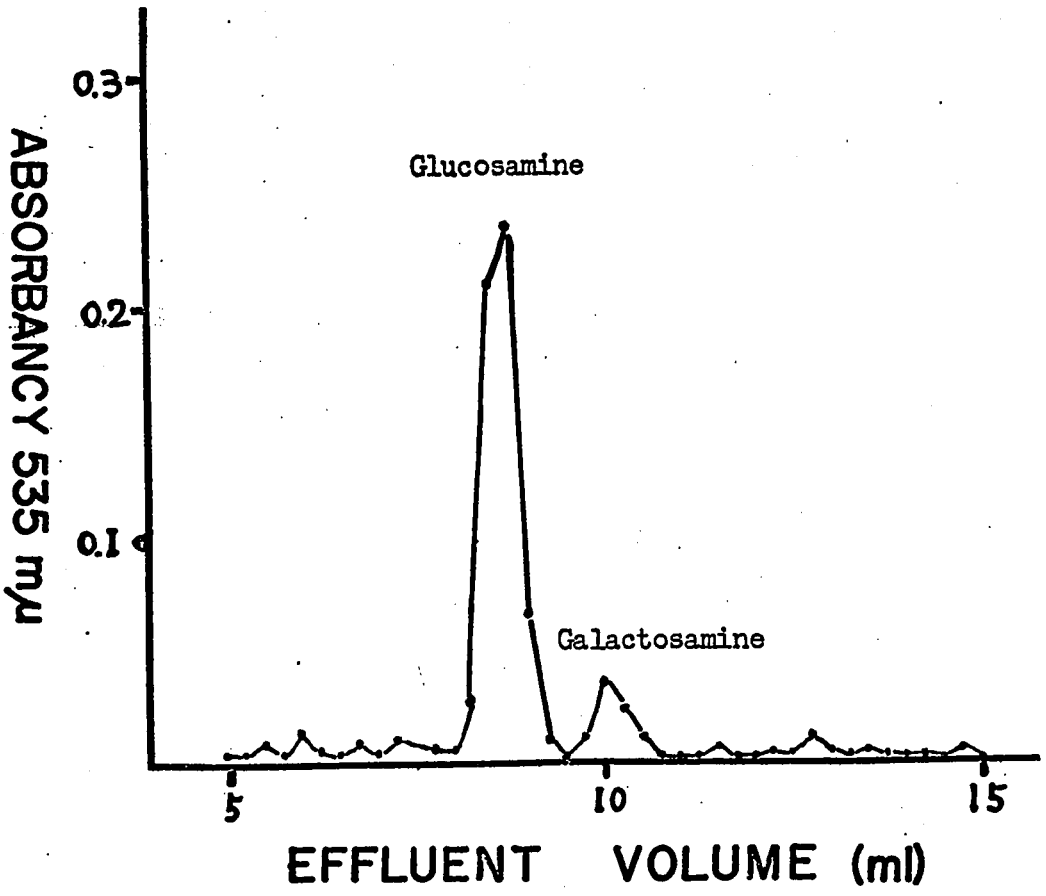

FIG. 18

Chromatography of glucosamine and galactosamine in acid hydrolysate of glycoprotein (US200A) on Dowex 50 x 8 (column 3 x 320 mm).

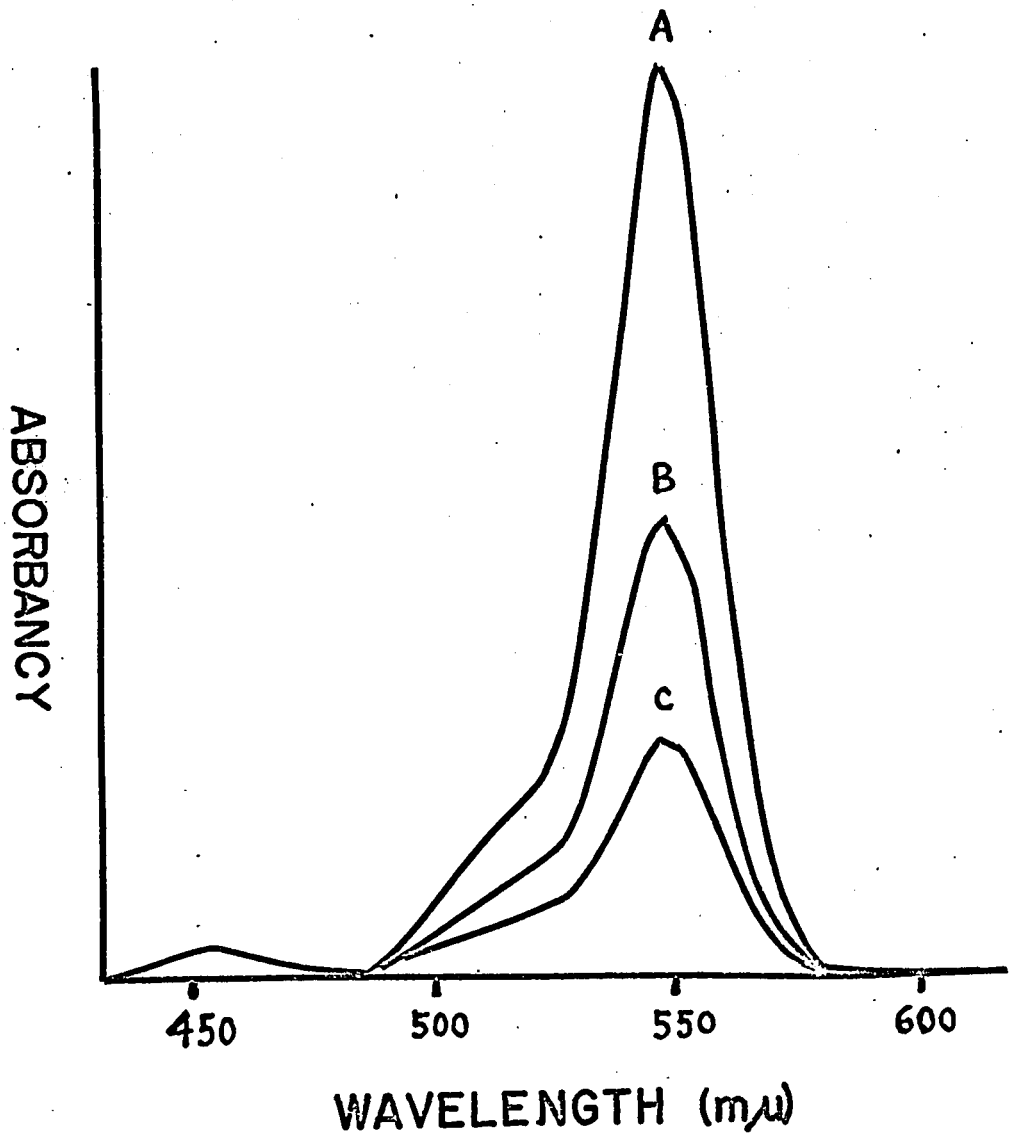

FIG. 19

The absorption spectra of sialic acid treated with thiobarbituric acid reagent.

a) N-acetylneuraminic acid standard
b) Sialic acid in acid hydrolysate of glycoprotein (US200A)
c) Sialic acid in acid hydrolysate of US200A after purification on ion-exchange resins.

The neutral sugars were identified as galactose and mannose by paper chromatography. Virtual absence of glucose in this glycoprotein (< 0.2% by weight) was further confirmed by the quantitative micro-method with the use of glucose oxidase. It has been reported that bovine GBM contains two types of sugar polymers; a disaccharide having glucose and galactose is linked to collagen through hydroxylysine and an oligosaccharide containing galactose, mannose, hexosamine and sialic acid is linked through asparagine to glycoprotein (224,250). The absence of hydroxylysine in the peptide portion and of glucose in the carbohydrate moiety of glycoprotein US200A is compatible with such a concept and further suggests that this specific nephrotoxigenic Ag may contain only the oligosaccharide unit of the GBM.

D.3.2. Antigenic Properties

It has been demonstrated that this glycoprotein (US200A) elicited potent localizing Ab which induced NTN upon injection into rats (Table 8). Gel double diffusion of this glycoprotein and other compounds with their Abs indicated multiple Ags and the presence of the common antigenic determinants among the isolated components (Table 9). It is of interest that this glycoprotein (US200A) developed only a single precipitin line with antiserum against GBM isolated by NaOH treatment (anti-GBM alk.), whereas it formed two precipitin lines with anti-kidney serum (Fig. 20a). CS200A developed two precipitin lines with both antisera.

FIG. 20

LEGEND

1. Anti-GBM-alk.
2. Anti-rat kidney serum
3. Anti-US200A
4. Anti-US200A absorbed with CS200A
5. Anti-CSM absorbed with CSM
6. Anti-CS200A absorbed with US200A
7. Anti-CS200A
8. Anti-dog CSM
9. Anti-rat kidney
10. Anti-dog GBM
11. Anti-US200A

Outer wells contain antigens as indicated.

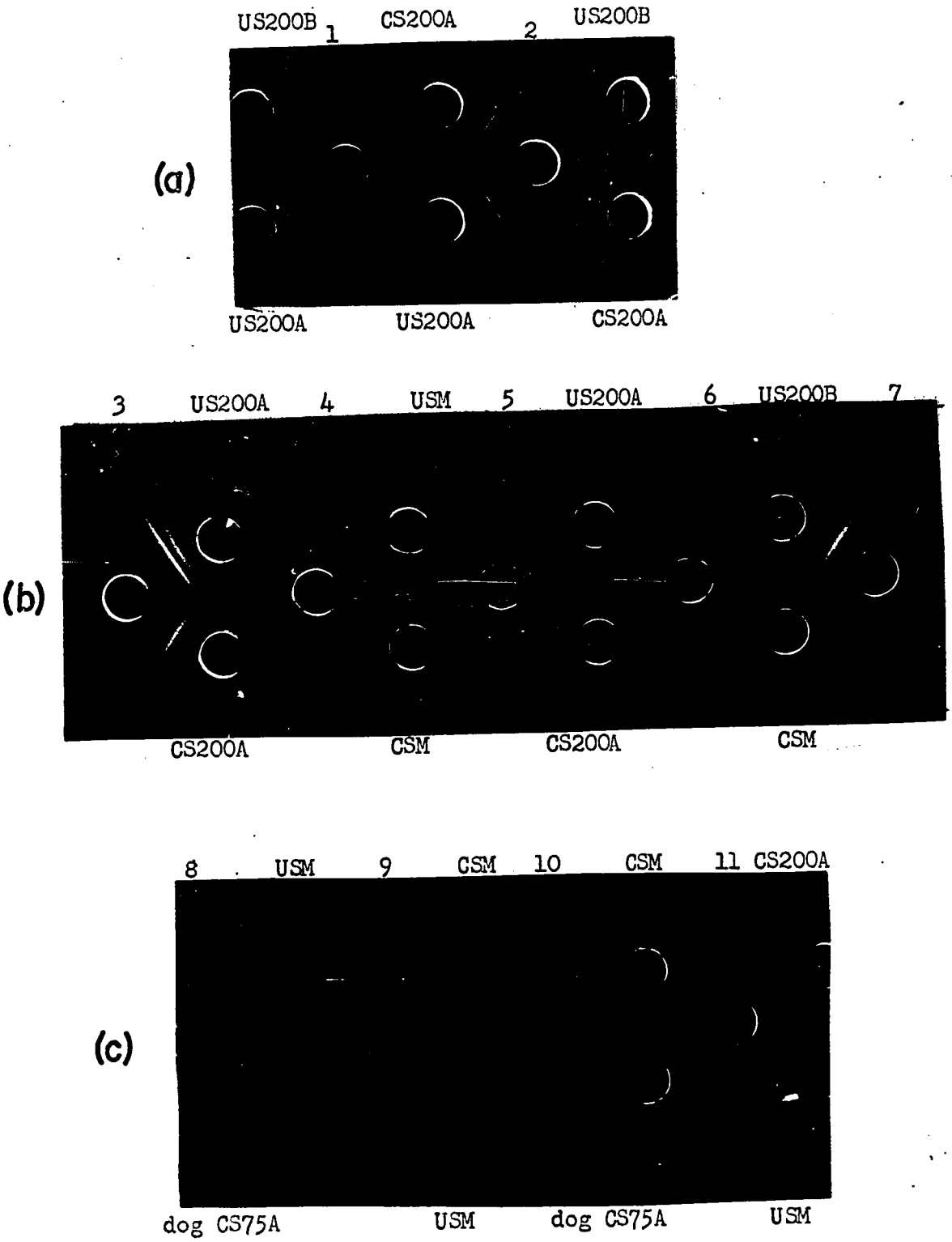

FIG. 20

Precipitin reactions between glomerular basement membrane components and their antisera by double diffusion in agar gel. Middle wells contain antisera (see covering page for legend).

The cross-reaction of membrane components (US200A and CS200A) was further demonstrated by absorption experiments. The extent of cross-reactivity between these two Ags was determined as follows: Ag solution (CS200A) was added to anti-US200A. The mixture was incubated at room temperature for one hour with occasional stirring and one hour more at 4°C. The heavy precipitate formed was removed by centrifugation. The supernatant, containing non-precipitated Ab, was reacted with its Ag (US200A) or the absorbing Ag (CS200A) in a gel diffusion plate. No precipitin line developed, indicating that all the Ab against US200A was absorbed completely by CS200A (Fig. 20b).

It was also demonstrated that Ab against CS200A could be completely precipitated by US200A, indicating the complete cross-reactivity of these membrane components (Fig. 20b). Despite the different precipitin lines developed between anti-US200A with US200A (3 lines) and anti-US200A with CS200A (2 lines), anti-US200A was completely removed by CS200A, suggesting that Ab was directed toward common antigenic determinants which may reside in the glycoprotein of the membrane. The multiple precipitin lines may represent the reaction between Ab and various Ag aggregates which contained the same Ab combining sites.

This glycoprotein component of rat GBM was further examined for its cross-reactivity with dog GBM components. By gel double diffusion, precipitin lines developed between Abs and Ags of rat and dog membrane components (Fig. 20c). The cross-reactions in both directions suggested

the presence of common antigenic determinants. However, a reaction of identity was not demonstrated among the precipitin lines formed, indicating that only partial immunological identity existed between rat and dog GBM components.

D.3.3. Electrophoretic Analysis

In order to determine the electrophoretic behaviour of US200A, starch gel and acrylamide gel electrophoreses were performed using 0.2% protein solution in tris buffer (pH 8.4) according to the method described in section C.8. The glycoprotein did not migrate but remained at the origin with very slight trailing toward the anode at pH 8.5. When 100 µl of 0.6% solution was subjected to disc electrophoresis, the glycoprotein was precipitated out at the surface of the gel. Electrophoresis in acidic buffer was not feasible due to the low solubility of the sample in acidic medium.

Immunoelectrophoresis was attempted. A precipitin line near the well containing Ag was obtained by reacting with anti-kidney serum or anti-US200A (Fig. 21). It is apparent that US200A and CS200A did not migrate during electrophoresis, but developed similar precipitin lines with each antiserum, suggesting partial immunological identity despite different chemical composition.

D.3.4. Ultracentrifugal Properties

Ultracentrifugation of glycoprotein in buffer was performed

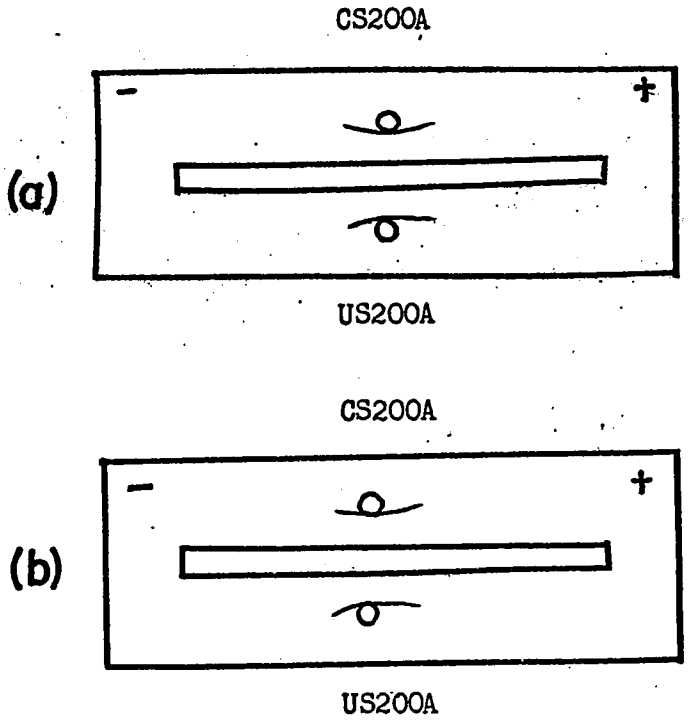

FIG. 21

Immunoelectrophoresis of glomerular basement membrane components in 1% agar gel. 0.05M veronal buffer, pH 8.6.

a) trough contains anti-rat kidney serum

b) trough contains anti-US200A serum.

as described in section C.9.1. In its ultracentrifugal pattern (Fig. 22), this glycoprotein (US200A) appeared heterogeneous, having two peaks with sedimentation coefficients of 5.93S and 3.49S in relative amounts of 65% and 35% respectively. Smaller S_{20} values were reported for the canine GBM fractions extracted by 8M urea (3.5S) and by reduction and alkylation in 8M urea (2.4S). It was therefore postulated that the fast sedimenting peak (5.93S) of the glycoprotein may represent the aggregate form of the slow moving peak (3.49S).

Further separation of these two ultracentrifugal peaks of glycoprotein was attempted by the method of centrifugation in sucrose density gradient as described in section C.9.2. After the centrifugation, the sample was fractionated in aliquots of 7 drops (approximately 0.1 ml). Protein concentration of each fraction was measured in terms of absorbancy at 280 mμ. A solution containing bovine albumin and γ-globulin was subjected to centrifugation in the identical manner.

As can be seen in Fig. 23, the albumin peak was separated from the γ-globulin peak within the range of sucrose gradient (5% to 20%). However, glycoprotein (US200A) failed to separate into two peaks as expected and sedimented as a pellet at the bottom of the centrifuge tube. This might have resulted from use of too dilute a solution of sucrose (density = 1.0810 for 20% sucrose solution) or from the aggregation of glycoprotein in the presence of sucrose.

In order to determine whether aggregation of glycoprotein is actually responsible for the earlier results of ultracentrifugation in

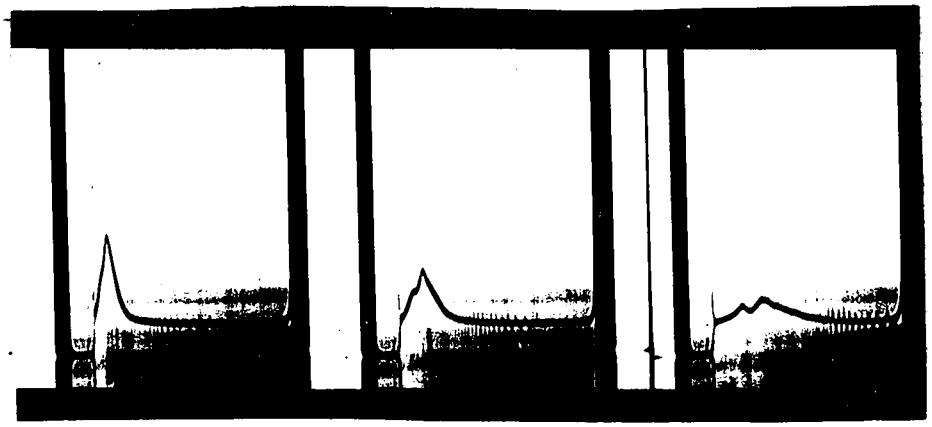

FIG. 22

Analytical ultracentrifugal pattern of glycoprotein (US200A) in tris buffer (0.05M, pH 8.6) at 56,000 r.p.m., 20°C, performed in a single sector cell. The pictures shown were taken at 1, 9 and 17 minutes after attainment of maximum speed (diaphragm angle 70°).

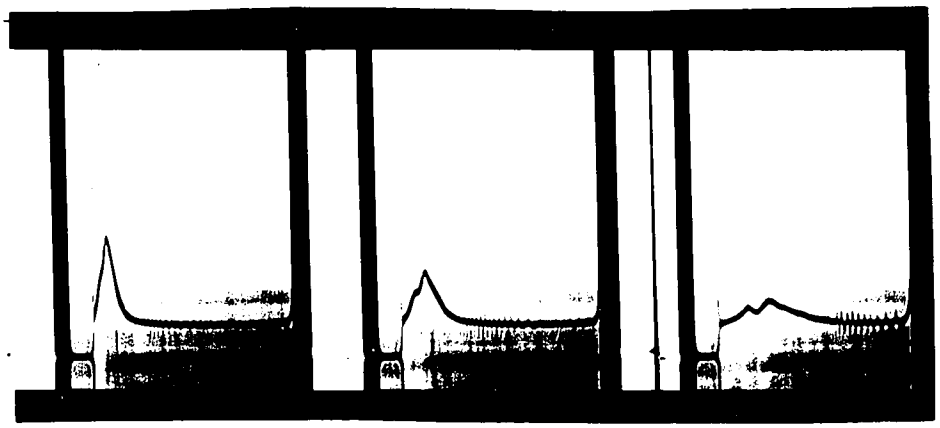

FIG. 22

Analytical ultracentrifugal pattern of glycoprotein (US200A) in tris buffer (0.05M, pH 8.6) at 56,000 r.p.m., 20°C, performed in a single sector cell. The pictures shown were taken at 1, 9 and 17 minutes after attainment of maximum speed (diaphragm angle 70°).

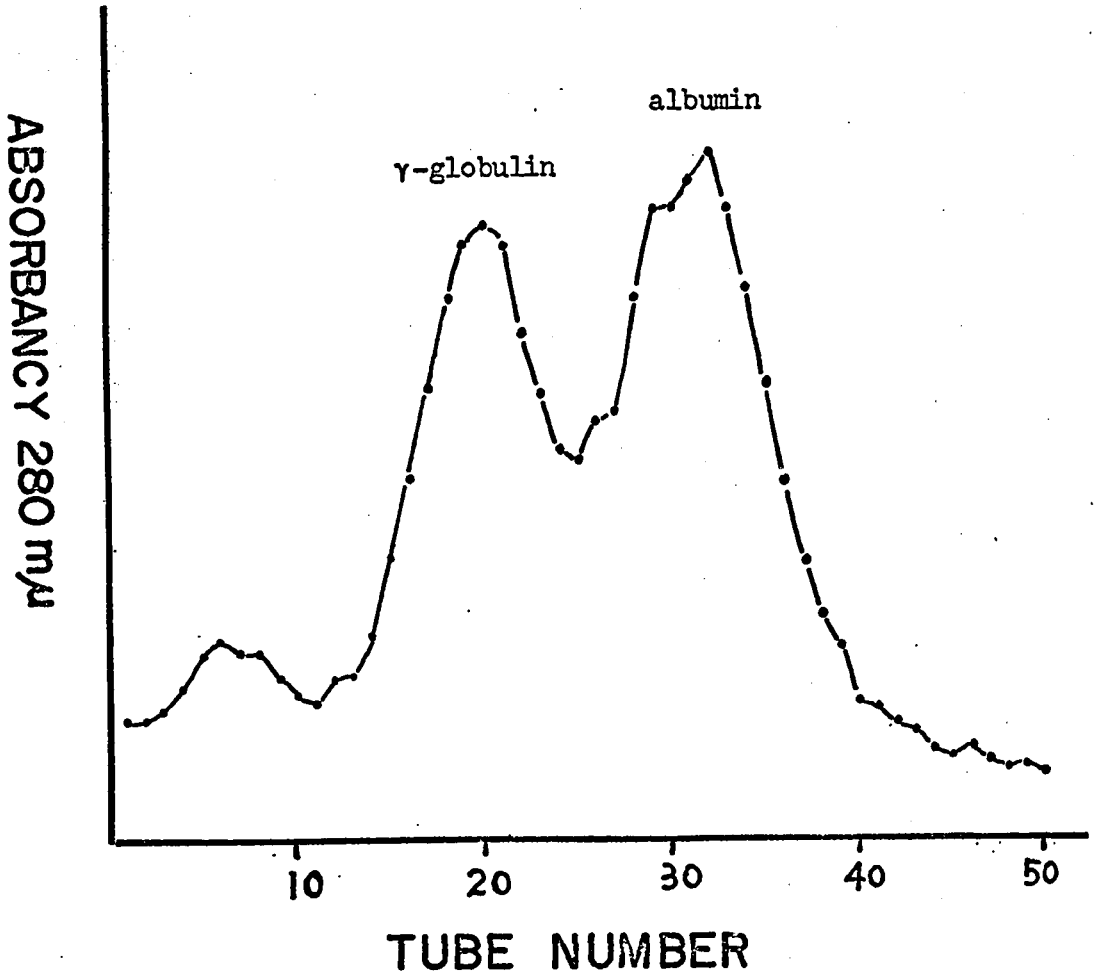

FIG. 23

Centrifugation pattern of serum γ-globulin and albumin in sucrose density gradient.

buffer, ultracentrifugation in 8M urea was attempted. As can be seen in Fig. 24, glycoprotein appeared as a single symmetrical peak in 8M urea medium, with sedimentation constants of 1.33S (obtained in synthetic boundary cell) or 1.428S (obtained in single sector cell). The ultracentrifugal patterns suggest that glycoprotein apparently disaggregated into one smaller molecular species in urea medium and the heterogeneity observed in the tris buffer may have been due to the presence of different aggregates of the glycoprotein monomer.

D.3.5. Conclusions

A nephrotoxigenic Ag, isolated from decollagenized GBM by urea extraction and gel filtration on Sephadex G-200, was characterized as a glycoprotein containing 10.66% of carbohydrate consisting of galactose, mannose, glucosamine, galactosamine and sialic acid. Antigenically, it cross-reacted with other rat membrane components and dog GBM and appeared to have multiple antigenic groups. It did not migrate in the electrical field at pH 8.6 in starch gel or acrylamide gel. It appeared heterogeneous in the analytical ultracentrifuge, giving two peaks with 5.93S and 3.49S in the tris buffer and appeared as a single symmetrical peak with lower S_{20} value (1.33S) in urea medium. Heterogeneity of this Ag as shown by gel double diffusion and the ultracentrifugation pattern in buffer was therefore interpreted as the result of aggregation of glycoprotein.

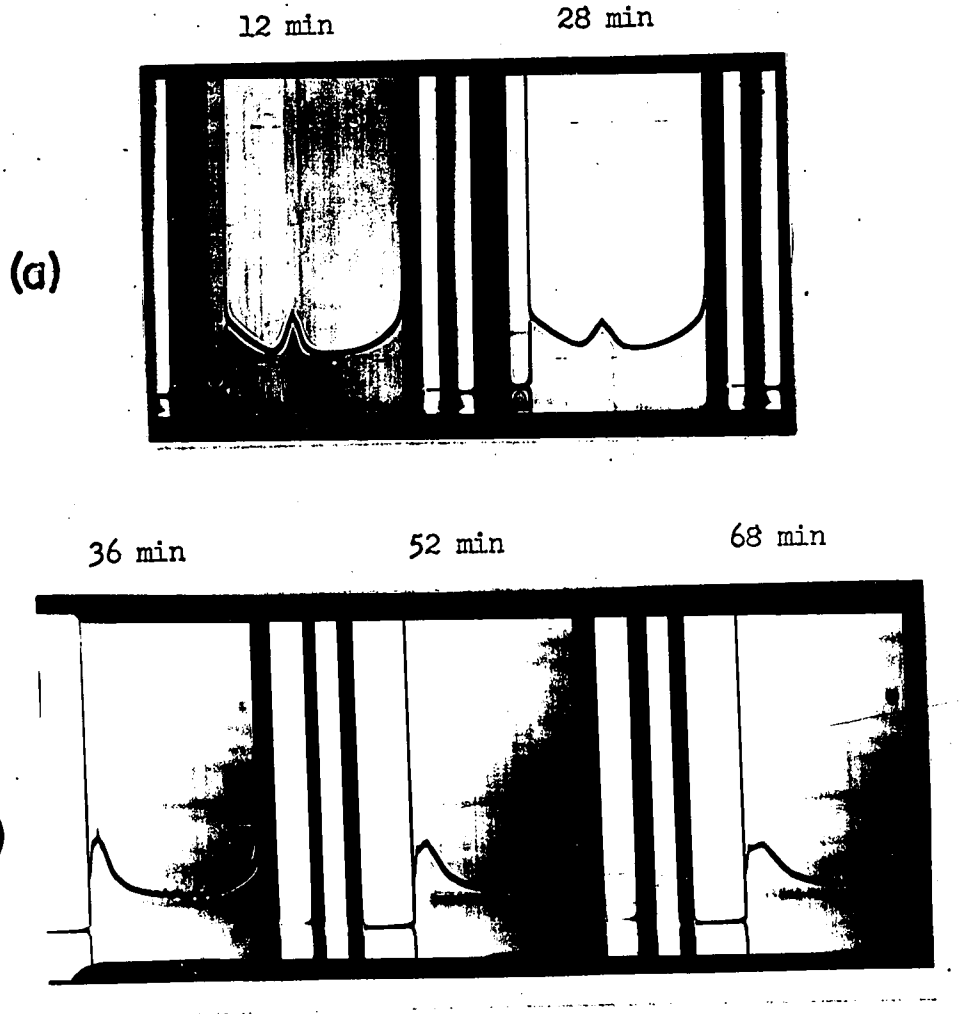

FIG. 24

Analytical ultracentrifugal patterns of glycoprotein (US200A) in 8M urea at 60,000 r.p.m., 20°C.

a) performed in a synthetic boundary cell (S_{20} = 1.330S)

b) performed in a single sector cell (S_{20} = 1.428S).

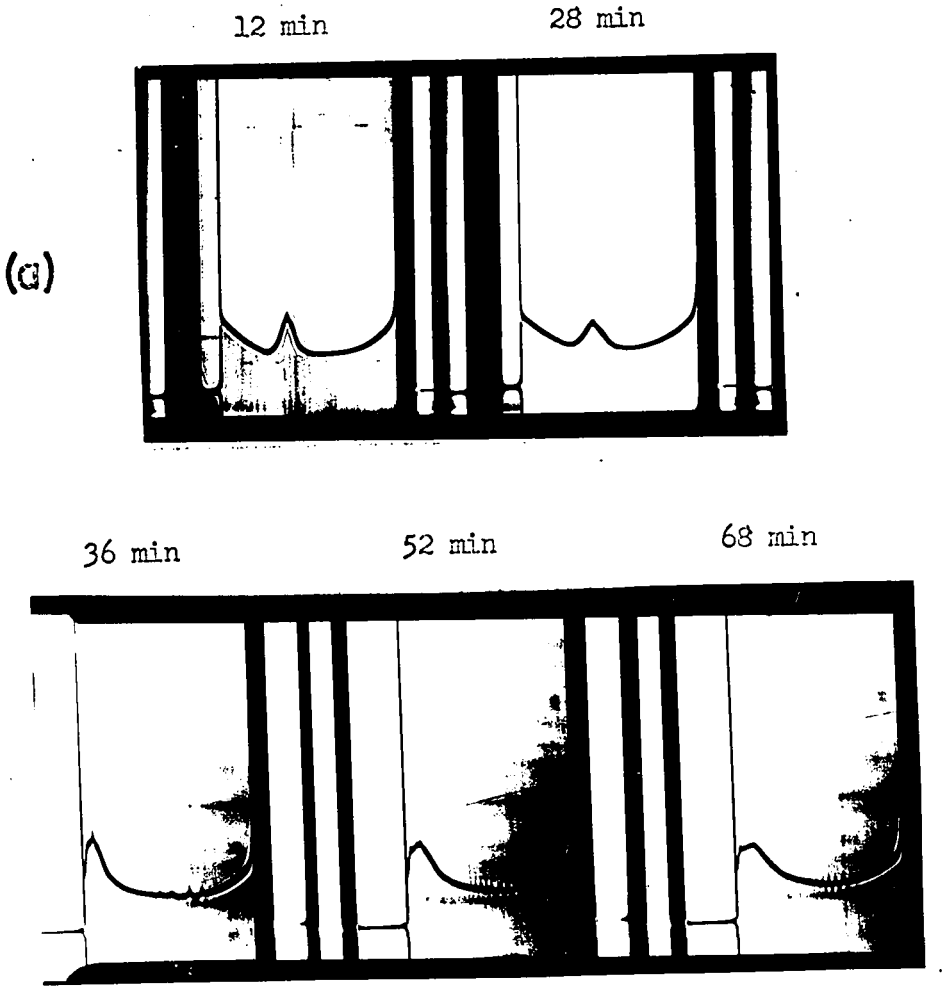

FIG. 24

Analytical ultracentrifugal patterns of glycoprotein (US200A) in 8M urea at 60,000 r.p.m., 20°C.

a) performed in a synthetic boundary cell (S_{20} = 1.330S)

b) performed in a single sector cell (S_{20} = 1.428S).

D.4. Effect of Non-nephrotoxic Abs in Induction of NTN in Rats

D.4.1. Introduction

It has been demonstrated that GBM exhibited a complex antigenic mosaic and multiple immunological responses (181,182). The results obtained in the previous experiments indicate that various membrane components of rat GBM elicited different types of Abs, some of which were nephrotoxic while others only localized to GBM and exerted no nephrotoxic effect. It was also shown that anti-collagen and anti-dog GBM localized to rat GBM but were not nephrotoxic. Localization of various Abs to rat GBM and cross-reaction among Ags and Abs in gel diffusion suggest the presence of common antigenic determinants. It is the purpose of this study to determine whether non-nephrotoxic Abs and NTAb share common combining sites in GBM.

It is generally accepted that fixation of NTAb to GBM initiates the glomerulonephritis and occurrence of proteinuria (133). Nephrotoxic serum nephritis was studied in rats injected with one type of antiserum or both localizing Abs and NTAbs in order to establish whether prior injection of localizing non-nephrotoxic Abs has any effect on induction of NTN by NTAbs.

D.4.2. Induction of Proteinuria in Rats by Injections of Various Antisera

Antisera against tendon collagen, dog GBM and rat BM component (CS25A) were employed as localizing, non-nephrotoxic Abs, whereas anti-

sera against CSM, CS200A and US200A from rat BM were employed as NTAbs in this study. Three sets of experiments (anti-CS25A with anti-CSM; anti-collagen with anti-CS200A and anti-dog GBM with anti-US200A) were performed (Table 12). Each set consisted of three groups (2 rats in each group) including rats receiving injections of a single antiserum as controls. Rats were injected with non-nephrotoxic antiserum first on two consecutive days (3 ml) followed by nephrotoxic antiserum in two injections (2 ml) (Table 12). One week and two weeks after the first injection of NTAb, rats were placed in metabolic cages and evaluated for proteinuria.

As can be seen in Table 12, animals from groups 1, 4 and 7 receiving non-nephrotoxic Abs only, did not develop proteinuria whereas those from groups 3, 6 and 9 receiving NTAbs developed high proteinuria one week later, confirming the previous findings. Animals from groups 2, 5 and 8 receiving two types of antisera also developed high proteinuria one week later. Once the proteinuria was developed, it persisted and increased to a higher level. However, the rats which failed to develop proteinuria (groups 1, 4, 7) at one week remained at the normal level even three weeks later. It is apparent that the prior injection of localizing non-nephrotoxic Abs did not prevent the induction of NTN by NTAbs.

After urinary protein had been measured, the animals were sacrificed at different times for determination of localization of the injected Abs by the immunofluorescent method. Kidney sections of the

TABLE 12

EFFECT OF NON-NEPHROTOXIC ABS ON INDUCTION OF NTN IN RATS

Group	Injection of antisera				Level of proteinuria (mg/24 hr)			Localization of Abs
	1st 1.5 ml	2nd 1.5 ml	3rd 1 ml	4th 1 ml	Day 7	Day 12	Day 20	
1	Anti-collagen	Anti-collagen	-	-	89 42	29 35	-	+++
2	Anti-collagen	Anti-collagen	Anti-CS200A	Anti-CS200A	123 166	187 270	300	+++
3	-	-	Anti-CS200A	Anti-CS200A	186 175	431 297	322	+++
4	Anti-dog GBM	Anti-dog GBM	-	-	19 39	22 65	48	+++
5	Anti-dog GBM	Anti-dog GBM	Anti-USM	Anti-USM	175 96	281 250	340	+++
6	-	-	Anti-USM	Anti-USM	25 204	201 293	228	+++
7	Anti-CS25A	Anti-CS25A	-	-	54 47	43	50	++
8	Anti-CS25A	Anti-CS25A	Anti-CSM	Anti-CSM	216 192	198	260	+++
9	-	-	Anti-CSM	Anti-CSM	302 278	300	246	+++

animals from all the groups showed intense fluorescent staining on GBM in a typical capillary pattern. However, a slight difference in intensity of fluorescence was noted in different groups (Table 12). It appeared that localization of non-nephrotoxic Abs did not prevent the subsequent fixation of NTAbs.

Cross-reacting Abs such as anti-collagen and anti-dog GBM were previously shown to contain common antigenic determinants with rat GBM components (collagen fragment in CS200A and glycoprotein in US200A). However, the renal antigenic sites for these cross-reacting Abs apparently differ from those for NTAbs. Thus, it can be concluded that the prior injection and localization of cross-reacting Abs did not prevent the subsequent fixation of NTAbs which resulted in development of proteinuria.

D.5. Molecular Sieving by Normal and Nephrotic GBM

D.5.1. Introduction

As discussed in the Historical Introduction, the glomerular capillary wall consists of endothelial cells, basement membrane and epithelial cells. Of these three layers, the basement membrane appears to be the only complete barrier between the plasma and the glomerular filtrate. The studies on ferritin transfer across the glomerular capillary wall provided evidence that GBM is the major filtering mechanism (10,12,27). However, it was proposed later that the epithelial slits are the primary filtration barrier (43). It is thus still not established

whether the GBM acts as a differential filter.

Nephrotoxic serum nephritis is characterized by increased permeability of the glomeruli to serum protein and consequent proteinuria. Paradoxically, the permeability is associated with an increase in thickness of the GBM (16,42,68). It was suggested that in acute glomerulonephritis, a decrease in density of the BM, presumably associated with hydration and separation of the constituents, could account for the increased permeability (23). Similarly, on the basis of chemical composition and x-ray diffraction patterns of normal and nephrotic BM (245,246), it was concluded that the membrane thickening in NTN might be due to molecular rearrangement of the membrane components, so that the interstices between molecules become larger and permit passage of large molecules such as plasma proteins.

If this explanation is correct, the normal and nephrotic membranes are analogous to dextrans of greater or lesser degree of cross-linkage (e.g. Sephadex G-25 or G-200). To test this hypothesis, a study was made on the behaviour of microcolumns packed with segments of BM to determine whether these columns produce a molecular sieving effect comparable to that of dextran gels.

D.5.2. Preparation of Microcolumns of GBM

Rat GBM was prepared according to a modification of the procedure of Krakower and Greenspon (179). NTN was produced by three daily injections of rabbit antiserum against rat GBM. The animals which

showed high proteinuria (> 200 mg per 24 hours), at two weeks after the first injection, were used for the experiments.

Microcolumns of normal and nephrotic basement membrane were prepared as follows: GBM from 10 rats suspended in 1 ml of a 0.9% saline was transferred to a small chromatographic tube (inner diameter, 1.5 mm) which was pulled to a fine tip at one end and plugged with glass wool. Membrane segments were allowed to settle by gravity but occasional stirring was required to obtain uniform sedimentation. After the membrane was packed to the desired length (60 mm), excess saline (1-2 ml) was passed through the column to stabilize it. The flow rate of the column was approximately 10 µl per 20 minutes. A microcolumn of Sephadex G-25 (1.5 x 60 mm) was also prepared and used for comparison.

D.5.3. Fractionation of I^{131}-Albumin and C^{14}-Mannose on Microcolumns

In order to determine the molecular sieving effect of GBM, mannose and serum albumin were chosen as two representative molecules, differing distinctly in molecular size, one being freely filtered through the glomerular capillary wall and the other being largely retained in the capillary lumen, under normal conditions.

A 10 µl aliquot of a solution containing 2 µc of albumin-I^{131} (sp. activity = 0.191 mc/mg) and 5 µc of mannose-C^{14} (sp. activity = 5 mc/mM) was applied to each column. The columns were then eluted with 0.9% saline. The eluate was collected in fractions of 10 µl with capillary glass tubes. One-half ml of a solution containing carrier albumin and

mannose (0.5% each in saline) was added to each fraction. Albumin was separated from mannose by precipitation with addition of an equal volume of 20% TCA (0.5 ml). Precipitates were washed once with 10% TCA solution, then dissolved in 2.5N NaOH solution (1 ml). The resulting solution was assayed directly for I^{131} in a well scintillation counter. One ml aliquots of combined supernatant and washing (TCA), containing mannose-C^{14} were added to 15 ml of counting solution (50 gm naphthalene, 5 gm PPO and 0.125 gm POPOP dissolved in 500 ml p-dioxane). Radioactivity (C^{14}) was assayed in a liquid scintillation counter. Radioactivity was expressed as c.p.m.

The elution patterns of protein and mannose from columns of Sephadex G-25 and of normal and nephrotic GBMs are given in Fig. 25. As anticipated, albumin, being excluded from the Sephadex G-25, was eluted well ahead of the mannose. The column of normal BM produced a small but definite retention of the mannose relative to the albumin. Thus, BM from normal glomeruli produced a molecular sieving effect.

When BM from nephrotic rats was used, complete overlap of the albumin and mannose peaks occurred, indicating that albumin was retarded to the same extent as mannose. The retardation of albumin was therefore greater than that shown by normal membrane, suggesting that exclusion of albumin from the nephrotic membrane was not as complete. Consequently, it can be inferred that the pores or channels in nephrotic membrane are larger than those of normal membrane.

It may be noted that the elution volume of albumin from the

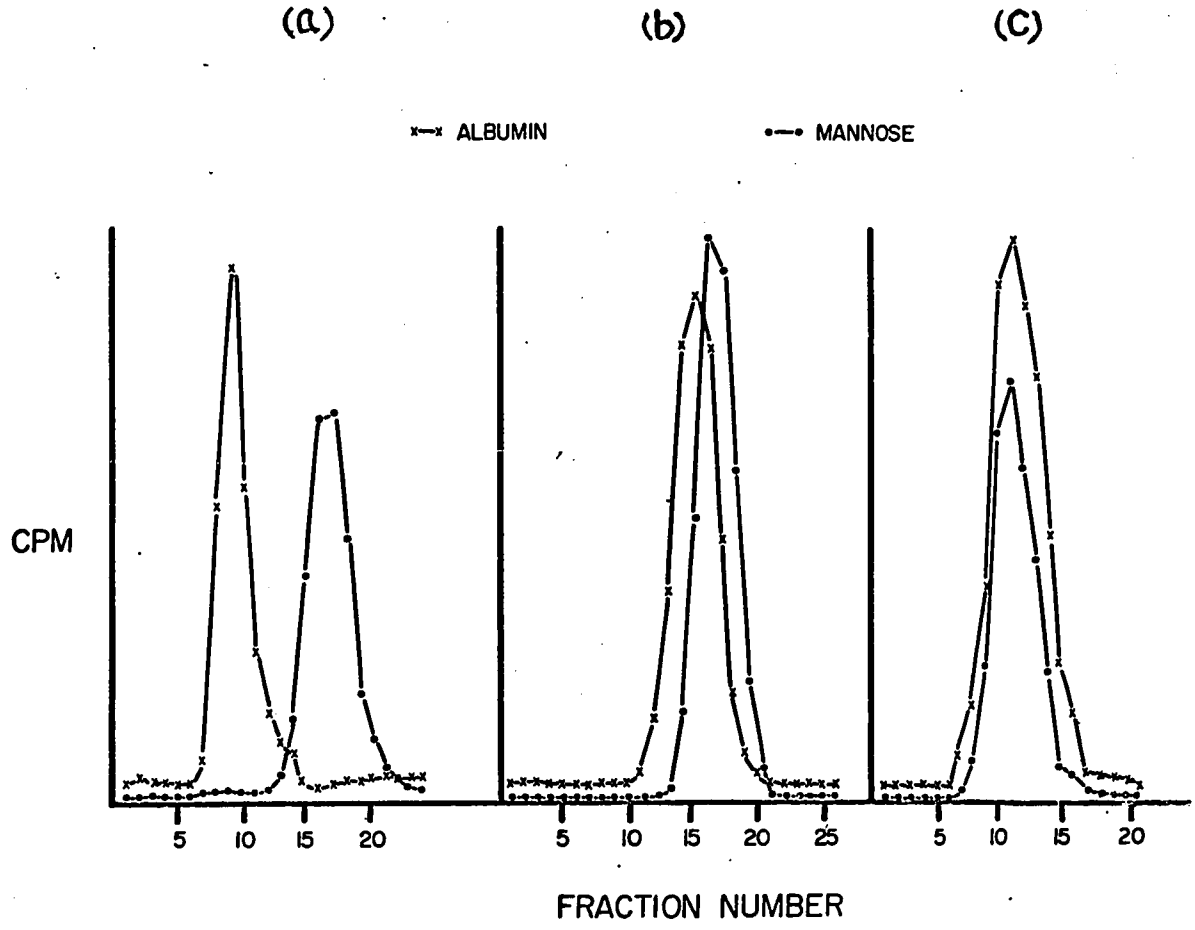

FIG. 25

Elution patterns of albumin-I^{131} and mannose-C^{14} on microcolumns of basement membrane and Sephadex G-25.

a) Sephadex G-25 column

b) Normal glomerular basement membrane column

c) Nephrotic glomerular basement membrane column.

column of nephrotic membrane was smaller than that from the column of normal membrane. Attempts to determine the void volume of membrane columns using "Blue dextran 2000" were unsuccessful since the dextran was adsorbed by the membrane. It is known that the void volume of Sephadex columns varies with the degree of cross-linkage and that the void volume of a Sephadex G-25 column may be equal to or even greater than the elution volume of a material which is retained on a column of less highly cross-linked Sephadex. This phenomenon probably explains the small elution volume for the column of nephrotic membrane.

When microcolumns of normal whole glomeruli or BM mixed with Supercel (to improve the flow rate of the column) were used, similar results to those shown in Fig. 25 were obtained. The same direction and degree of separation were achieved, confirming the result obtained by normal BM and suggesting that the BM is responsible for the action of the glomeruli.

These results are compatible with the hypothesis that renal GBM acts like Sephadex in separating large and small molecular species and that in glomerulonephritis induced by NTAbs, the BM behaves like a more porous, less highly cross-linked Sephadex. It seems likely, therefore, that BM in vivo acts as a differential filter and that in NTN, the BM is more permeable because of a more open molecular structure.

D.5.4. Conclusions

The results obtained in this study lead to the conclusion that

the renal glomerulus acts like beads of Sephadex in separating large and small molecular species, that BM exerts the same action and is therefore responsible for the action of the whole glomeruli, and finally that nephrotic BM behaves like a more porous, less tightly cross-linked Sephadex. The results provide evidence that BM in vivo acts as a filtering membrane. Under normal conditions, the BM is relatively impermeable to serum proteins; however, in NTN the membrane appears to be more permeable because of a more open molecular structure of the membrane itself, resulting in leakage of serum protein (proteinuria).

E. General Discussion

The present study concerns the experimental nephritis induced by injection of heterologous nephrotoxic antiserum. Ags employed to elicit NTAb are mostly of the same species as that of the recipient. However, such specificity is not essential for production of NTN (146, 147). The present results indicate that dog GBM and its components are non-nephrotoxigenic to rats. Abs to dog GBM localized to rat GBM but failed to induce NTN. It should be noted that antiserum to whole rat glomeruli was nephrotoxic to dogs (146). However, in the present study, the cross-reactivity and partial immunological identity of the GBM of the two species have been clearly demonstrated by the localization of anti-dog GBM on rat GBM and the precipitin lines developed between Abs and Ag of the two species in the gel double diffusion procedure.

Chemical studies of the GBM have provided further evidence indicating similarities between BM of these two species. In a preliminary isolation of GBM components, collagenase solubilized the collagen component to the same extent and resulted in liberation of glycoprotein which had similar fractionation patterns by gel filtration on Sephadex G-75. Recent reports on the chemical composition of intact GBM indicate that the BM of various species consists of collagen and glycoprotein (224,245,247,249). The overall similarity in chemical structure of GBM of dog and rat observed in the present study suggests that common antigenic groups are responsible for the cross-reactivity of

these BMs. The difference observed in the nephrotoxicity of Abs to these BMs may be related to their ability to bind complement.

During the experiments on BM, several incidental observations were made on the nephrotoxicity of tendon collagen. Collagenase action on tendon collagen or GBM did not effect complete degradation of collagen into a dialyzable form. The present finding that non-dialyzable collagen fragments enriched in carbohydrate can be solubilized from tendon collagen by collagenase digestion is in agreement with that reported by Franzlau et al. (290). The non-nephrotoxicity of Ab to tendon collagen in rat, reported by Rothbard and Watson (236), is also confirmed in the present study. Anti-collagen localized in vivo on rat GBM. However, it is of interest that Abs to non-dialyzable collagen fragment obtained by collagenase digestion of tendon collagen did not localize to rat GBM, suggesting that common antigenic determinants present originally in tendon collagen and GBM are disrupted by collagenase action which is known to degrade the crystalline region of the collagen into dialyzable tripeptides (293). Nevertheless, the non-dialyzable collagen fragment still reacted and formed precipitin lines with anti-tendon collagen indicating the presence of other antigenic determinants in collagen which appear to be resistant to collagenase degradation.

In preliminary attempts to isolate rat GBM components, it was found that the collagen component was not an essential nephrotoxigenic Ag, while glycoprotein solubilized by collagenase digestion produced NTAb. Proteolytic digestion of glycoprotein with pronase resulted in

destruction of the molecular integrity necessary to elicit NTAb. Studies on the antigenicity of the rat GBM isolated by mild alkali treatment provide further information on the nature of the nephrotoxigenic Ag. It has been reported that GBM thus isolated contains much less total carbohydrate and practically no sialic acid (244). The present results indicate that the partial destruction of the carbohydrate constituents did not affect the nephrotoxigenicity of the GBM. Abs against this chemically altered BM induced NTN in rats and cross-reacted with intact membrane components. It is apparent that the essential antigenic structure for eliciting potent NTAb is more closely related to the peptide portion than to the carbohydrate moiety of the glycoprotein.

Subsequently, six components were isolated from rat GBM by collagenase digestion, urea extraction and gel filtration of solubilized materials on Sephadex G-25 through Sephadex G-200. The chemical analyses indicated that the components solubilized by collagenase were glycoprotein containing collagen fragments enriched in bound hexose, whereas those solubilized by urea extraction of decollagenized BM were glycoprotein free of collagen. On the basis of the chemical composition it was not possible to determine whether the glycoprotein solubilized by collagenase digestion is identical to that solubilized by urea extraction; however, immunological evidence obtained in gel double diffusion and absorption experiments strongly suggests that the two glycoproteins are identical or at least contain common antigenic groups.

The membrane components, as separated by gel filtration,

differ quantitatively but not qualitatively in chemical composition. In gel diffusion, cross-reactions among membrane components have also been demonstrated. It is, therefore, possible that some of the material eluted in the void volume might have been degraded to material which was retarded. The nephrotoxigenicity of the membrane components appears different and depends on the molecular size; components of larger molecular size, i.e., excluded from Sephadex G-200 were more potent in eliciting NTAbs.

It is not possible on the basis of present evidence to draw firm conclusions regarding the heterogeneity of the major glycoprotein extracted from BM (US200A). The description "homogeneous" is used to indicate that a preparation is pure in the sense that all extraneous material has been successfully removed by fractionation, but the material may nevertheless be polydisperse. Homogeneity is asserted with respect to the measurable parameters such as electrophoretic mobility, sedimentation coefficient, elution patterns on column chromatography or gel filtration and immunological reactivity.

In the present study under the conditions employed, electrophoretic analysis of this Ag was unsuccessful, since the material showed no mobility. This may have been due to incorrect choice of buffer, or to exclusion of the macromolecular glycoprotein from the supporting gels.

Gel double diffusion has been employed for determining the homogeneity of this glycoprotein by immunological criteria. In general, each Ag-Ab system in a mixture forms a separate band of precipitin in

a different position. The appearance of multiple lines is usually evidence of a heterogeneous preparation. However, there are a number of conditions which may result in double lines from a homogeneous Ag (294). Moreover, it has been stated that a homogeneous Ag bearing two or more antigenic sites may give rise to more than one precipitin band (295,296).

In the present study, collagenase was found to be a good Ag and engendered its specific Ab. By preliminary purification of collagenase and by appropriate treatment of membrane components, collagenase contamination of these components was eliminated from all but one fraction. The nephrotoxigenic glycoprotein fraction was devoid of collagenase but formed two or three precipitin lines with its own antiserum or with anti-kidney serum in gel double diffusion. This suggested heterogeneity of the antigen, the presence of multiple antigenic sites or the presence of different aggregate forms of glycoprotein.

Sedimentation analysis suggested the last of these possibilities and indicated that this glycoprotein may have been polydisperse. In tris buffer, this Ag appears as two peaks whereas in the same buffer containing 8M urea, it appears as a single peak with lower sedimentation coefficient. Thus, it is probable that the glycoprotein consists of ultracentrifugally "homogeneous" subunits which can be reaggregated to form larger polymers. It is possible that the polydispersity of this glycoprotein may have been induced by degradation during urea solubilization.

The mechanism by which urea denatures proteins has not yet been firmly established. Undoubtedly urea disrupts hydrogen bonds. In addition, however, it has been suggested, on the basis of solubility studies of amino acids and peptides (297), that the polypeptide organization is disrupted because of the solubilization of the non-polar side chains which presumably participate in hydrophobic bonding (298,299). It has been reported that urea of high concentration exerts a depolymerization effect on the erythrocyte membrane (300), on β-galactosidase (301) and on urinary glycoprotein (302) and leads to dissociation of the highly polymerized material into subunits. On removal of urea, reaggregation of monomers into various polymeric forms occurs. In the present study a similar action of urea is suggested.

The nephrotoxigenic Ag (US200A) was characterized as glycoprotein, free of hydroxyproline and hydroxylysine, containing 10.7% carbohydrate (galactose, mannose, galactosamine, glucosamine and sialic acid). The chemical composition is similar to that of a glycoprotein isolated by urea extraction of intact bovine GBM (248), except that a higher hexose content (9%) and lower hexosamine (1.3%) content were found in the bovine glycoprotein than in the rat glycoprotein. These differences may be due to the difference in species.

The composition of the glycoprotein in the present study, however, differs from that of a fraction isolated from GBM by trypsinization (142,303). It was reported that a soluble fraction obtained by trypsinization of human GBM had higher carbohydrate content (13.6% of

hexose and 2.5% of hexosamine) and higher concentrations of glycine, hydroxyproline and hydroxylysine than the intact GBM (142). Very recently, a nephrotoxigenic Ag was also isolated from a trypsin digest of rat cortex homogenate (303); it showed a chemical composition (12.45% of hexose, 1.52% of hexosamine and 37.76% of glycine) similar to that reported for the human GBM preparation. The high content of glycine, hydroxyproline and hydroxylysine suggests that trypsinization has cleaved a portion of the glycoprotein, leaving the carbohydrate-containing moiety of the glycoprotein together with a collagen-like protein enriched in hexose.

In the present study, it was found that Abs to GBM components and collagen almost all localized to GBM in vivo; however, not all localization of Abs leads to the development of glomerulonephritis. The further findings that prior localizations of non-nephrotoxic, cross-reacting Abs have no effect on subsequent induction of glomerulonephritis by NTAb suggests that NTN is possibly induced only by specific fixation of NTAb on GBM.

The reaction of NTAb with glycoprotein on the BM may initiate a conformational change of the macromolecular structure, resulting in abnormality in the BM and occurrence of proteinuria (246). This hypothesis is supported by the finding obtained in the study of molecular sieving by GBM. Normal GBM produced a molecular sieving effect similar to that of a tightly cross-linked dextran (e.g. Sephadex), indicating that the renal GBM in vivo can act as a differential filter, restricting the filtration of serum proteins. In glomerulonephritis induced by

NTAb, the BM is more permeable and behaves like a more porous, less tightly cross-linked dextran.

F. Summary

1. Dog glomerular basement membrane and collagenase-solubilized components were antigenic to rabbits and elicited antibodies which localized to rat glomerular basement membrane but failed to produce proteinuria. Chemical study indicates that dog glomerular basement membrane contains collagen and glycoprotein with hexosamine, bound hexose and sialic acid as carbohydrate constituents of the latter.

2. The cross-reactions between glomerular basement membrane components of dog or rat and their antibodies in gel double diffusion and the localization of anti-dog glomerular basement membrane on rat glomerular basement membrane provide evidence that glomerular basement membranes of the two species contain multiple antigens and share common antigenic determinants.

3. Treatment of rat glomerular basement membrane with collagenase did not alter the nephrotoxic antigenicity of the membrane. Further proteolytic digestion with pronase resulted in loss of ability to elicit nephrotoxic antibody. Rat glomerular basement membrane prepared by mild alkali treatment, still retained the nephrotoxic antigenicity.

4. Antibody to tendon collagen localized to rat glomerular basement membrane but had no nephrotoxic effect on normal rats. Antibody to collagen fragment obtained from collagenase digestion failed to localize on rat glomerular basement membrane but still

cross-reacted with tendon collagen in gel diffusion.

5. Six components were isolated from normal rat glomerular basement membrane by collagenase digestion, urea extraction and fractionation on Sephadex gels. Antibodies against these components, except two which were retained on Sephadex G-75, localized to glomerular basement membrane and induced proteinuria in rats. One of the components retained on Sephadex G-75 produced localizing, non-nephrotoxic antibodies, while the other produced no localizing antibodies. Multiple antigens and common antigenic determinants among membrane components were demonstrated by gel double diffusion.

6. Chemical analysis indicates that all the components were glycoprotein containing seven to twenty per cent carbohydrate. The components solubilized by collagenase digestion still contained collagen fragments but those solubilized by urea extraction were free of hydroxyproline and hydroxylysine.

7. A specific nephrotoxigenic antigen from rat glomerular basement membrane was characterized as a macromolecular glycoprotein containing 10.7% carbohydrate, present as galactose, mannose, N-acetylneuraminic acid, glucosamine and galactosamine. In analytical ultracentrifugation in tris buffer it gave two peaks with sedimentation constant of 5.93S and 3.49S while in 8M urea it appeared as a single symmetrical peak with lower sedimentation constant (1.33S), suggesting the presence of

aggregate forms of the glycoprotein.

8. The prior injection and localization of non-nephrotoxic antibodies had no effect on the subsequent induction of glomerulonephritis in rats by nephrotoxic antibody.

9. Particles of basement membrane from normal rats exerted a molecular sieving effect comparable to that of Sephadex, while membrane from nephrotic rats failed to exert such an effect. This indicates that the normal basement membrane alone can act as a differential filter in vivo and that the increased permeability of the membrane could account for proteinuria in nephrosis.

Contribution to Knowledge

1. Dog glomerular basement membrane and its glycoprotein components induce antibodies which localize to rat glomerular basement membrane but exert no nephrotoxic effect.

2. Treatment of rat glomerular basement membrane with collagenase does not alter the nephrotoxigenicity of the membrane, but further treatment with pronase abolishes this antigenicity. However, partial destruction of carbohydrate constituents of glomerular basement membrane by mild alkali treatment does not affect the nephrotoxigenicity.

3. The ability of tendon collagen to elicit antibody which localizes on rat glomerular basement membrane is abolished by collagenase treatment.

4. Collagenase digestion of rat glomerular basement membrane leads to the solubilization of glycoprotein containing collagen fragment enriched in carbohydrate. Antibodies against membrane components of large molecular weight (excluded from Sephadex G-75) are nephrotoxic to normal rats.

5. Complete solubilization of glomerular basement membrane can be achieved by collagenase digestion followed by urea extraction. A specific nephrotoxigenic antigen which is a macromolecular glycoprotein can be isolated from rat glomerular basement membrane by this procedure.

6. Glomerular basement membranes of dog and rat contain multiple antigens and exhibit partial immunological identity of the glycoprotein component.

7. The prior localization of non-nephrotoxic antibodies does not prevent the induction of nephrotoxic serum nephritis in rats by subsequent administration of nephrotoxic antibodies.

8. It has been demonstrated that normal glomerular basement membrane produces a molecular sieving effect *in vitro* and may thus act as a differential filter *in vivo*. In nephrotoxic serum nephritis, the membrane shows increased permeability to macromolecules *in vitro*; this may account for proteinuria *in vivo*.

REFERENCES

1. Yamada, E. J. Biophys. Biochem. Cytol. 1, 551 (1955).

2. Hall, B.V. Am. Heart J. 54, 1 (1957).

3. Mueller, C.B. Am. Heart J. 55, 304 (1958).

4. Rhodin, J.A.G. in Diseases of the Kidney. Ed. Strauss, M.M. and Welt, L.G. Little Brown and Company, Boston (1963). p. 1-29.

5. Hall, B.V. and Roth, L.E. Proc. Stockholm Conf. on Electron Microscopy (1956).

6. Kurtz, S.M. Exper. Cell. Res. 14, 355 (1958).

7. Vernier, R.L. and Birch-Andersen, A. J. Pediat. 60, 754 (1962).

8. Suzuki, Y. Keio J. Med. 8, 129 (1959).

9. Pease, A.C. Anat. Rec. 121, 701 (1955).

10. Rhodin, J.A.G. Exper. Cell. Res. 8, 572 (1955).

11. Farquhar, M.G. Proc. 1st Inter. Congr. Nephrology, Geneva/Evian, 1960. p. 357 (1961).

12. Farquhar, M.G., Wissig, S.L. and Palade, G.E. J. Exper. Med. 113, 47 (1961).

13. Latta, H., Maunsbach, A.B. and Madden, S.C. J. Ultrastruct. Res. 4, 455 (1960).

14. Latta, H. J. Ultrastruct. Res. 5, 364 (1961).

15. Takahi, E., Ishikawa, E. and Suzuki, T. Proc. 1st Inter. Congr. Nephrology, Geneva/Evian, 1960. p. 662 (1961).

16. Kurtz, S.M. Lab. Invest. 10, 1189 (1961).

17. Vernier, R.L. in Small Blood Vessel Involvement in Diabetis Mellitus. Am. Inst. Biol. Sci., Washington, D.C. (1964). p. 57.

18. Bloom, P.M., Hartmann, J.F. and Vernier, R.L. Anat. Rec. 133, 251 (1959).

19. Bergstrand, A. and Bucht, M. Ztschr. f. Zellforsch. 48, 51-73 (1958).

20. Hall, B.V. Proc. 6th Ann. Conf. on Nephrotic Syndrome. Ed. Metcoff, J. National Kidney Disease Found., New York (1955).

21. Pak Poy, R.K.F. Australian J. Exper. Biol. Med. Sci. 37, 153 (1959).

22. Zimmerman, K.W. Ztschr. Micro. Anat. Forsch. 32, 176 (1933).

23. Movat, H.Z., Steiner, T.W. Am. J. Clin. Path. 36, 289 (1961).

24. Jones, D.B., Mueller, C.B. and Menefee, M. Am. J. Path. 41, 373 (1962).

25. Farquhar, M.G. and Palade, G.E. J. Cell. Biol. 13, 55 (1962).

26. Mautner, W., Churg, J., Grishman, E. and Dachs, S. Lab. Invest. 11, 518 (1962).

27. Farquhar, M.G. in Small Blood Vessel Involvement in Diabetis Mellitus. Am. Inst. Biol. Sci., Washington, D.C. (1964). p. 31.

28. Berliner, R.W. in Diseases of the Kidney. Ed. Strauss, M.B. and Welt, L.G. Little Brown and Company, Boston (1963). p. 30.

29. Bowman, W. Trans. Roy. Soc. London 132, 57 (1842).

30. Ludwig, E. Handworterbuch der Physiologie II. Bramsweig (1844).

31. Richards, A.N. Proc. Roy. Soc. London, Series B, 126, 398 (1939).

32. Walker, A.M., Bott, P.A., Oliver, J. and MacDowell, M.C. Am. J. Physiol. 134, 580 (1941).

33. Hayman, J.M. Am. J. Physiol. 79, 389 (1927).

34. Pappenheimer, J.R., Reukin, E.M. and Borrero, L.M. Am. J. Physiol. 167, 13 (1951).

35. Bott, P.A., Richards, A.N. J. Biol. Chem. 141, 391 (1941).

36. Marshall, M.E. and Deutsch, H.F. Am. J. Physiol. 163, 461 (1950).

37. Pappenheimer, J.R. Klin. Wochschr. 33, 362 (1955).

38. Wallenius, G. Acta Soc. Med. Upsaliensis Suppl. 4. (1954). p. 1-91.

39. Arturson, G. and Wallenius, G. Scandinav. J. Clin. and Lab. Invest. 16, 81 (1964).

40. Farquhar, M.G. and Palade, G.E. Anat. Rec. 133, 378 (1959).

41. Farquhar, M.G. and Palade, G.E. J. Biophys. and Biochem. Cytol. 7, 297 (1960).

42. Farquhar, M.G. and Palade, G.E. J. Exper. Med. 114, 699 (1961).

43. Graham, R.C., Jr. and Karnovsky, M.J. J. Exper. Med. 124, 1123 (1966).

44. Landis, E.M. and Pappenheimer, J.R. Handbook of Physiology. Ed. Hamilton, W.F. and Dow, P. Washington, D.C. (1963). p. 1017.

45. Bayliss, L.E., Kerridge, P.M.T. and Russell, D.S. J. Physiol. 77, 386 (1933).

46. Terry, R., Hawkins, D.R., Church, E.H. and Whipple, G.H. J. Exper. Med. 87, 561 (1948).

47. Rigas, D.A. and Heller, G.G. J. Clin. Invest. 30, 853 (1951).

48. Oliver, J., MacDowell, M. and Lee, Y.C. J. Exper. Med. 99, 589 (1954).

49. Lathem, W. J. Clin. Invest. 38, 652 (1959).

50. Menefee, M.G., Mueller, C.B., Bell, A.L. and Meyers, J.K. J. Exper. Med. 120, 1129 (1964).

51. Hendrix, J.P. Westfall, B.B. and Richards, A.N. J. Biol. Chem. 116, 735 (1937).

52. Brewer, D.B. Proc. Roy. Soc. Med. 44, 561 (1951).

53. Squire, J.R. Brit. Med. J. 2, 1389 (1953).

54. Meyer, F. and Putnam, F. J. Exper. Med. 117, 573 (1963).

55. Bright, R. Reports of Medical Cases: Longmans, Rees, Orme, Brown and Green (1827).

56. White, H.L. and Rolf, D. Am. J. Physiol. 152, 505 (1948).

57. Relman, A.S. and Levinsky, N.G. in Diseases of the Kidney. Ed. Strauss, M.B. and Welt, L.G. Little Brown and Company, Boston (1963). p. 80-87.

58. Bing, J. Acta Med. Scandinav. Suppl. 76 (1936).

59. Boyce, W.H., Garney, F.A. and Norfleet, C.M., Jr. J. Clin. Invest. 33, 1287 (1954).

60. Webb, T., Rose, B. and Sehon, A.H. Can. J. Biochem. and Physiol. 36, 1159 (1958).

61. Webb, T., Rose, B. and Sehon, A.H. Can. J. Biochem. and Physiol. 36, 1167 (1958).

62. Rowe, D.S. and Soothill, J.F. Clin. Sci. 21, 75 (1961).

63. Tamm, I. and Horsfall, F.C. J. Exper. Med. 95, 71 (1952).

64. Gitlin, D. and Janeway, C.A. J. Clin. Invest. 31, 223 (1952).

65. Rowe, D.S. Biochem. J. 67, 453 (1957).

66. Broch, O.J. and Brodwall, E. Acta Med. Scandinav. 160, 353 (1958).

67. Schreiner, G.E. in Diseases of the Kidney. Ed. Strauss, M.B. and Welt, L.G. Little Brown and Company, Boston (1963). p. 335-444.

68. Farquhar, M.G., Vernier, R.L. and Good, R.A. J. Exper. Med. 106, 649 (1957).

69. Spiro, D. Am. J. Path. 35, 47 (1959).

70. Chinard, F.P., Lauson, H.D., Eder, H.A., Greif, R.L. and Hiller, A. J. Clin. Invest. 33, 621 (1954).

71. Hardwicke, J. and Squire, J.R. Clin. Sci. 14, 509 (1955).

72. Squire, J.R. Advances in Inter. Med. 7, 201 (1955).

73. Hayman, J.M. Jr. and Bender, J.A. Arch. Int. Med. 51, 447 (1933).

74. Vernier, R.L., Papermaster, B.N. and Good, R.A. J. Exper. Med. 109, 115 (1959).

75. Harkin, J.C. and Recant, L. Am. J. Path. 36, 303 (1960).

76. Post, R.S. Proc. 11th Ann. Conf. on Nephrotic Syndrome. Ed. Metcoff, J. National Kidney Disease Found. New York (1960). p. 222.

77. Fisher, E.R. and Hellstrom, H.R. Lab. Invest. 11, 617 (1962).

78. Vernier, R.L. in CIBA Foundation Sympos. on Renal Biopsy. Little Brown and Company, Boston (1961). p. 4.

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

80. Vernier, R.L. J. Pediat. 58, 620 (1961).

81. Churg, J., Grishman, E. and Mautner, W. Am. J. Path. 37, 729 (1960).

82. Churg, J., Mautner, W., Grishman, E. and Eisner, G.M. Arch. Int. Med. 109, 97 (1962).

83. Spiro, D. Proc. 11th Ann. Conf. on Nephrotic Syndrome. Ed. Metcoff, J. National Kidney Disease Found. New York (1960). p. 171.

84. Movat, H.Z., Steiner, J.W., and Huln, D. Lab. Invest. 11, 117 (1962).

85. Lauson, H.D., Forman, C.W., McNamara, H., Mattan, G. and Barnett, H.L. J. Clin. Invest. 33, 657 (1954).

86. Heymann, W. and Lund, H.Z. Pediatrics 7, 691 (1951).

87. Frenk, S., Antonowicz, J.M., Craig, J.M. and Metcoff, J. Proc. Soc. Exper. Biol. and Med. 89, 424 (1955).

88. Fiegelson, E.B., Drake, J.W. and Recant, L. J. Lab. and Clin. Med. 50, 437 (1957).

89. Wilson, S.G.F., Hackel, D.B., Horwood, S., Nath, G. and Heymann, W. Pediatrics 21, 963 (1958).

90. Giroud, C.J.P., Kalant, N., Despointes, R.H. and DasGupta, D. Rec. Progress in Hormone Res. 17, (1961). p. 353.

91. Wilson, S.G., Heymann, W. and Goldthwait, D.A. Pediatrics 25, 228 (1960).

92. Recant, L., Borowsky, B.A. and Kessner, D.M. J. Clin. Invest. 37, 924 (1958).

93. Heymann, W. J. Pediat. 58, 609 (1961).

94. Fisher, E.R. and Gruhn, J. Arch. Path. 71, 129 (1961).

95. Alexander, C.S. and Hunt, V.R. J. Lab. and Clin. Med. 62, 103 (1963).

96. Bernett, H.L., Simons, D.J. and Well, R.E. Am. J. Med. 4, 760 (1948).

97. Hall, C.E. and Hall, O. Am. J. Path. 41, 247 (1962).

98. Hall, C.E. and Hall, O. Lab. Invest. 11, 826 (1962).

99. Hall, C.E. and Hall, O. Proc. Soc. Exper. Biol. and Med. 112, 86 (1963).

100. Lindemann, W. Ann. Inst. Pasteur 14, 49 (1900).

101. Masugi, M. Beitr. Path. Anat. 91, 82 (1933).

102. Masugi, M. Beitr. Path. Anat. 92, 429 (1934).

103. Kay, C.F. J. Exper. Med. 72, 559 (1940).

104. Kay, C.F. Am. J. Med. Sci. 204, 483 (1942).

105. Longcope, W.T. J. Exper. Med. 18, 678 (1913).

106. Hawn, C.V. and Janeway, C.A. J. Exper. Med. 85, 571 (1947).

107. Hamilton, J.D. and Fremes, N.E. Am. J. Path. 30, 127 (1954).

108. Germuth, F.G. Jr. J. Exper. Med. 97, 257 (1953).

109. Germuth, F.G., Pace, M.G. and Tippett, J.C. J. Exper. Med. 101, 135 (1955).

110. McCluskey, R.T. and Benacerraff, B. Am. J. Path. 35, 275 (1959).

111. Miller, F., Benacerraff, B., McCluskey, R.T. and Potter, J.L. Proc. Soc. Exper. Biol. and Med. 104, 706 (1960).

112. McCluskey, R.T., Benacerraff, B. and Miller, F. Proc. Soc. Exper. Biol. and Med. 111, 764 (1962).

113. Dixon, F.J., Feldman, J.D. and Vezquez, J.J. J. Exper. Med. 113, 899 (1961).

114. Dixon, F.J. in Immunopathology - Immunopathologie. Graber, P. and Mieschen, P. Benno Schwabe, Basel (1962). p. 71.

115. Dixon, F.J. Harvey Lectures Ser. 58, 21 (1963).

116. Andres, G.A., Seegal, B.C., Hsu, K.C., Rothenberg, M.S. and Chapeau, M.L. J. Exper. Med. 117, 691 (1963).

117. Kniker, W.T. and Cochrane, C.C. J. Exper. Med. 122, 83 (1965).

118. Heymann, W., Hackel, D.B., Harwood, S., Wilson, S.G. and Hunter, J.L.P. Proc. Soc. Exper. Biol. and Med. 100, 660 (1959).

119. Heymann, W., Hunter, J.L.P. and Hackel, D.B. J. Immunol. 88, 135 (1962).

120. Watson, J.I. and Dixon, F.J. Proc. Soc. Exper. Biol. and Med. 121, 216 (1966).

121. Hess, E.B., Ashworth, C.T. and Ziff, M. J. Exper. Med. 115, 421 (1962).

122. Heymann, W., Hunter, J.L.P., Hackel, D.B. and Cuppage, F. Proc. Soc. Exper. Biol. and Med. 111, 568 (1962).

123. Glassock, R. and Watson, J.T. Fed. Proc. 25, 659 (1966).

124. Steblay, R.W. Clin. Res. 8, 289 (1960).

125. Steblay, R.W. J. Exper. Med. 116, 257 (1962).

126. Steblay, R.W. Nature 197, 1173 (1963).

127. Steblay, R.W. Fed. Proc. 23, 449 (1964).

128. Lerner, R.A. and Dixon, F.J. J. Exper. Med. 124, 431 (1966).

129. Rudofsky, J. and Steblay, R.W. Fed. Proc. 25, 2655 (1966).

130. Milgrom, F., Centeno, E., Shulman, S. and Witebsky, E. Proc. Soc. Exper. Biol. and Med. 116, 1009 (1964).

131. Unanue, E.R. Fed. Proc. 25, 659 (1966).

132. Unanue, E.R. and Dixon, F.J. J. Exper. Med. 125, 149 (1967).

133. Unanue, E.R. and Dixon, F.J. Advances in Immunology 6, 1 (1967).

134. Duval, C.W. and Hibbard, R.J. J. Exper. Med. 44, 567 (1926).

135. Bell, E.T. and Clawson, B.J. Am. J. Path. 7, 57 (1931).

136. Kelly, D.K. and Winn, J.F. Science 127, 1337 (1958).

137. Schmidt, W.C. in Streptococcal Infection. Ed. McCarty, M. Columbia University Press, New York (1954). Chapter 6.

138. Kaplan, M.H. J. Exper. Med. 107, 341 (1958).

139. Miller, F. Proc. Soc. Exper. Biol. and Med. 108, 539 (1961).

140. Kantor, F.S. J. Exper. Med. 121, 861 (1965).

141. Markowitz, A.S. and Lange, C.F. J. Immunol. 92, 565 (1964).

142. Lange, C.F. and Markowitz, A.S. Biochem. Biophys. Acta 101, 217 (1965).

143. Seegal, B.C., Andres, G.A., Hsu, K.C. and Zabriskie, J.B. Fed. Proc. 24, 100 (1965).

144. Andres, G.A., Accini, L., Hsu, K.C., Zabriskie, J.B. and Seegal, B.C. J. Exper. Med. 123, 399 (1966).

145. Michael, A.F. Jr., Drummond, K.N., Good, R.A. and Vernier, R.L. J. Clin. Invest. 45, 237 (1966).

146. Simonson, M. Acta Path. et Microbiol. Scandinav. 32, 85 (1953).

147. Markowitz, A.S. Immunology 3, 117 (1960).

148. Steblay, R.W. and Lepper, M.H. J. Immunol. 87, 627 and 636 (1961).

149. Baxter, J.H. and Goodman, H.C. J. Exper. Med. 104, 467 (1956).

150. Pearce, R.M. Univ. Pennsylvania Med. Bull. 16, 217 (1903).

151. Smadel, J.E. J. Exper. Med. 64, 921 (1936).

152. Smadel, J.E. and Farr, L.E. J. Exper. Med. 65, 527 (1937).

153. Smadel, J.E. J. Exper. Med. 65, 541 (1937).

154. Heymann, W. and Hackel, D. J. Lab. and Clin. Med. 39, 429 (1952).

155. Seegal, B.C., Hsu, K.C., Rothenburg, M.S. and Chapeau, M.L. Am. J. Path. 41, 183 (1962).

156. Hammer, D.K. and Dixon, F.J. J. Exper. Med. 117, 1019 (1963).

157. Unanue, E.R. and Dixon, F.J. J. Exper. Med. 121, 697 (1965).

158. Lange, K. Proc. 9th Ann. Conf. on Nephrotic Syndrome. Ed. Metcoff, J. National Nephrosis Foundation, New York (1958). p. 13.

159. Stabitsky, A.B., Heymann, W. and Hackel, D.B. J. Lab. and Clin. Med. 47, 349 (1956).

160. Hasson, M.W., Bevan, M. and Seegal, B.C. Arch. Path. 64, 192 (1957).

161. Mellors, R.C., Arias-Stella, J., Siegel, M. and Pressman, D. Am. J. Path. 31, 687 (1955).

162. Mellors, R.C., Siegel, M. and Pressman, D. Lab. Invest. 4, 69 (1955).

163. Steblay, R.W. Fed. Proc. 22, 216 (1963).

164. Steblay, R.W. Fed. Proc. 23, 216 (1964).

165. Lange, K., Wenk, E.J., Wachstein, M. and Noble, J. Am. J. Med. Sci. 236, 767 (1958).

166. Lange, K. Proc. 3rd Ann. Conf. on Nephrotic Syndrome. Ed. Metcoff, J. National Nephrosis Foundation, New York (1952). p. 16.

167. Lange, K., Slobody, L.B. and Wenk, E.J. A.M.A. J. Dis. Child. 92, 474 (1956).

168. Seegal, B.C. 9th Ann. Conf. on Nephrotic Syndrome. Ed. Metcoff, J. National Nephrosis Foundation, New York (1958). p. 1.

169. Seegal, B.C., Hasson, M.W., Gaynor, E.C. and Rothenberg, M.S. J. Exper. Med. 102, 789 (1955).

170. Stickler, G.B., Wakim, K.G. and McKenzie, B.F. J. Lab. Clin. Med. 48, 866 (1956).

171. Fouts, P.J., Corcoran, A.C. and Page, I.H. Am. J. Med. Sci. 201, 313 (1941).

172. Michael, A.F. Jr., Venters, H., Jr. and Good, R.A. Fed. Proc. 20, 408 (1961).

173. Huang, C.C., Chang, S.C., Lin, Y.C., Chen, K.H. and Hsu, K. J. Lab. Clin. Med. 62, 201 (1963).

174. Markowitz, A.S., Clasen, R., Nidus, B.D. and Ainis, H. J. Immunol. 98, 161 (1967).

175. Arana, J., Nidus, B., Blair, J. and Kaplan, M.H. Fed. Proc. 23, 509 (1964).

176. Blair, J.D., Arana-Stella, J.A. and Kaplan, M.H. Fed. Proc. 24, 682 (1965).

177. Solomon, D.H., Gardella, J.W., Fanger, H., Dethier, F.M. and Ferrebee, J.W. J. Exper. Med. 90, 269 (1949).

178. Greenspon, S.A. and Krakower, C.A. A.M.A. Arch. Pathol. 49, 291 (1950).

179. Krakower, C.A. and Greenspon, S.A. A.M.A. Arch. Path. 51, 629 (1951).

180. Krakower, C.A. and Greenspon, S.A. A.M.A. Arch. Path. 58, 401 (1954).

181. Goodman, M., Greenspon, S.A. and Krakower, C.A. J. Immunol. 75, 96 (1955).

182. Krakower, C.A., Greenspon, S.A. and Warren, H.N. Exper. Cell. Res. 13, 230 (1957).

183. Chikamitsu, H.S. Folia endocrinal Japonica 16, 85 (1940).

184. Seegal, B.C. and Loeb, E.N. J. Exper. Med. 84, 211 (1946).

185. Strehler, E. Schweiz. Med. Wchnschr. 81, 104 (1951).

186. Spuhler, O., Zollinger, H.U. and Enderlin, M. Schweiz. Med. Wchnschr. 81, 904 (1951).

187. Hamori, A. and Olah, F. Lancet 260, 586 (1951).

188. Bevan, M., Seegal, B.C. and Kaplan, R. J. Exper. Med. 102, 807 (1955).

189. Krakower, C.A. and Greenspon, S.A. A.M.A. Arch. Path. 66, 364 (1958).

190. Triedman, R.S., Metzger, H., Hsu, K.C., Rothenberg, M., Seegal, B.C. and Urquhart, A. Am. J. Path. 41, 95 (1962).

191. Katz, D.H., Unanue, E.R. and Dixon, F.J. J. Immunol. 98, 260 (1967).

192. Krakower, C.A. and Greenspon, S.A. Am. J. Path. 43, 55 (1963).

193. Yagi, Y. and Pressman, D. J. Immunol. 86, 431 (1961).

194. Baxter, J.H. and Small, P.A. Jr. Science 140, 1406 (1963).

195. Small, P.A. Jr. and Baxter, J.H. J. Immunol. 95, 282 (1965).

196. Libby, R.L. and Madison, C.R. J. Immunol. 55, 15 (1947).

197. Pressman, D. and Keighley, G. J. Immunol. 59, 141 (1948).

198. Pressman, D., Eisen, H.N., Siegel, M., Fitzgerald, P.J., Sherman, B. and Silverstein, A. J. Immunol. 65, 559 (1950).

199. Pressman, D., Korngold, L. and Heymann, W. Arch. Path. 55, 347 (1953).

200. Coons, A.H., Creech, H.J., Jones, R.N. and Berliner, E. J. Immunol. 45, 159 (1942).

201. Coons, A.H. and Kaplan, M.H. J. Exper. Med. 91, 1 (1950).

202. Coons, A.H., Leduc, E.H. and Connolly, J.M. J. Exper. Med. 102, 49 (1955).

203. Hill, A.G.S. and Cruickshank, B. Brit. J. Exper. Path. 34, 27 (1953).

204. Cruickshank, B. and Hill, A.G.S. J. Path. and Bact. 66, 283 (1953).

205. Mellors, R.C., Siegel, M. and Pressman, D. Lab. Invest. 4, 69 (1955).

206. Ortega, L.G. and Mellors, R.C. J. Exper. Med. 104, 151 (1956).

207. Singer, S.J. Nature 183, 1523 (1959).

208. Andres, G.A., Morgan, C., Hsu, K.C., Rifkind, R.A. and Seegal, B.C. Nature 194, 590 (1962).

209. Andres, G.A., Morgan, C., Hsu, K.C., Rifkind, R.A. and Seegal, B.C. J. Exper. Med. 115, 929 (1962).

210. Pressman, D. J. Immunol. 63, 375 (1949).

211. Arhelger, R.B., Gronvall, J.A., Carr, O.B. Jr. and Brunson, J.G. Lab. Invest. 12, 33 (1963).

212. Pressman, D. and Korngold, L. J. Immunol. 78, 75 (1957).

213. Bale, W.F. and Spar, I.L. J. Immunol. 73, 125 (1954).

214. Spar, I.L., Bale, W.F., Wolfe, D.H. and Goodland, R.L. J. Immunol. 76, 119 (1956).

215. Sarre, H. and Wirtz, H. Deutsches Arch. Klin. Med. 189, 1 (1942).

216. Blau, M., Day, E.D. and Pressman, D. J. Immunol. 79, 330 (1957).

217. Unanue, E.R. and Dixon, F.J. J. Exper. Med. 119, 965 (1964).

218. Unanue, E.R. and Dixon, F.J. J. Exper. Med. 121, 715 (1965).

219. Cooper, N.R. and Fogel, B.J. J. Pediat. 70, 982 (1967).

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

221. Krakower, C.A. and Greenspon, S.A. Proc. Exper. Biol. Med. 116, 301 (1964).

222. Pierce, G.B. Jr., Beals, T.F., Sri Ram, J., and Midgley, A.R. Jr. Am. J. Path. 45, 929 (1964).

223. Bruchhausen, F.V. and Merker, H.J. Naungn. Schmeidebergs Arch. Exp. Path. u. Pharmak. 251, 1 (1965).

224. Dische, R.M., Pappas, G.D., Grauer, A. and Dische, Z. Biochem. Biophys. Res. Commun. 20, 63 (1965).

225. Gersh, I. and Cathpole, H.R. Am. J. Anat. 85, 457 (1949).

226. Marionozzi, V. J. Biophys. Biochem. Cytol. 9, 121 (1961).

227. Barclay, J.A. and Singh, I.D. Acta Med. Scandinav. 154, 483 (1956).

228. Windrum, G.M., Kent, P.W. and Eastoe, J.E. Brit. J. Exper. Path. 36, 49 (1955).

229. Bonting, S.L., DeBruin, H. and Pallak, V.E. J. Clin. Invest. 40, 177 (1961).

230. Scott, D.G. Brit. J. Exper. Path. 38, 178 (1957).

231. Scott, D.G. Ann. Rheumatic Diseases 18, 207 (1959).

232. Boss, J.H. Lab. Invest. 12, 322 (1963).

233. Boss, J.H. Exper. Mol. Path. 4, 416 (1965).

234. Krakower, C.A. and Greenspon, S.A. in Small Blood Vessel Involvement in Diabetis Mellitus. Am. Inst. Biol. Sci., Washington, D.C. (1964). p. 161.

235. Watson, R.F., Rothbard, S. and Vanamee, P. J. Exper. Med. 99, 535 (1954).

236. Rothbard, S. and Watson, R.F. J. Exper. Med. 109, 633 (1959).

237. Rothbard, S. and Watson, R.F. J. Exper. Med. 113, 1041 (1961).

238. Rothbard, S. and Watson, R.F. J. Exper. Med. 122, 441 (1965).

239. Rothbard, S. and Watson, R.F. J. Exper. Med. 125, 595 (1967).

240. Millazzo, S.C. J. Path. Bact. 73, 527 (1957).

241. Hill, G.S., Cruickshank, B. and Crossland, A. Brit. J. Exper. Path. 34, 27 (1953).

242. Liu, C.T., McCrory, W.W. and Flick, J.A. Proc. Soc. Exper. Biol. Med. 95, 331 (1957).

243. Gery, I., Yagi, Y. and Pressman, D. J. Immunol. 94, 950 (1965).

244. Lazarow, A. and Speidel, E. in Small Blood Vessel Involvement in Diabetis Mellitus. Am. Inst. Biol. Sci., Washington, D.C. (1964). p. 127.

245. Misra, R.P. and Kalant, N. Nephron. 3, 84 (1966).

246. Kalant, N., Misra, R.P., Manley, R.St.J. and Wilson, J. Nephron. 3, 167 (1966).

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

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

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

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

251. Dinh, B.L. Can. J. Biochem. 45, 781 (1967).

252. Cole, L.R., Cromartie, W.J. and Watson, D.W. Proc. Soc. Exper. Biol. Med. 77, 498 (1951).

253. Goodman, H.C. and Baxter, J.H. J. Exper. Med. 104, 487 (1956).

254. Butler, W.T. and Cunningham, L.W. J. Biol. Chem. 240 3449 (1965).

255. Cook, W.F. and Pickering, G.W. Nature 182, 1103 (1958).

256. Sephadex - Gel filtration in theory and practice. Pharmacia Fine Chemicals. Uppsala, Sweden (1966).

257. Gornall, A.G., Bardawill, C.J. and David, M.M. J. Biol. Chem. 177, 751 (1949).

258. Marshall, J.D., Eveland, W.C., and Smith, C.W. Proc. Soc. Exper. Biol. Med. 98, 898 (1958).

259. Elson, L.A. and Morgan, W.T.J. Biochem. J. 27, 1824 (1933).

260. Boas, N.F. J. Biol. Chem. 204, 553 (1953).

261. Roseman, S. and Daffner, I. Anal. Chem. 28, 1743 (1956).

262. Cessi, C. and Serafini-Cessi, F. Biochem. J. 88, 132 (1963).

263. Neuhaus, O.W. and Letzring, M. Anat. Chem. 29, 1230 (1957).

264. Gardell, S. Acta Chem. Scand. 7, 207 (1953).

265. Ludoweig, J. and Benmaman, J.D. Anal. Biochem. 19, 80 (1967).

266. Glowacka, D., Kopacz-Jodczyk, T. and Popowicz, J. Anal. Biochem. 19, 1 (1967).

267. Spiro, R.G. J. Biol. Chem. 234, 742 (1959).

268. Antonopoulos, C.A. Arkiv. For Kemi. Bd. 25, 243 (1966).

269. Gottschalk, A. The chemistry and biology of sialic acids and related substances. Cambridge University Press (1960).

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

271. Svennerholm, L. Acta Chemica Scand. 12, 549 (1958).

272. Montgomery, R. Biochem. Biophys. Acta 48, 591 (1961).

273. Washko, M.E. and Rice, E.W. Clin. Chem. 9, 594 (1963).

274. Leach, A.A. Biochem. J. 74, 70 (1960).

275. Campbell, W.R. and Hanna, M.I. J. Biol. Chem. 119, 1 (1937).

276. King, E.J. and Wootton, I.D.P. Micro-analysis in medical biochemistry. 3rd edition. J.A. Churchill, London (1956). p. 50.

277. Rosen, H. Arch. Biochem. Biophys. 67, 10 (1957).

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

279. Piez, K.A. and Morris, L. Anal. Biochem. 1, 187 (1960).

280. Trevelyan, W.G., Procter, D.P. and Harrison, J.S. Nature 166, 444 (1950).

281. Ferguson, K.A. and Wallace, A.L.C. Nature 190, 629 (1960).

282. Davis, B.J. "Disc Electrophoresis" Part II. Canal Industrial Corp., Bethesda, Md. (1962).

283. Grabar, P. and Williams, C.A. Jr. Biochem. Biophys. Acta 17, 67 (1955).

284. Martin, R. and Ames, B. J. Biol. Chem. 236, 1372 (1961).

285. Myers, J., Frei, J.V., Cohen, J.J., Rose, B. and Richter, M. J. Immunol. 11, 155 (1966).

286. Glynn, L.E. and Holborow, E.J. Lancet 2, 449 (1952).

287. Yagi, Y., Korngold, L. and Pressman, D. J. Immunol. 77, 287 (1956).

288. Grassmann, W., Hofmann, V. and Wolf, K. in Connective tissue. Ed. Tunbridge, R.E. Council International Organization of Med. Sci. Symposium. Blackwell Ltd. Oxford (1957). p. 157.

289. Gross, J., Durnsha, B. and Glazer, N. Biochem. Biophys. Acta 30, 294 (1958).

290. Franzblau, C., Gallop, P.M. and Seifter, S. Biopolymers 1, 79 (1963).

291. Levin, Y., Pecht, M., Goldstein, L. and Katchalski, E. Biochem. 3, 1905 (1964).

292. Kalant, N., Das Gupta, D., Despointes, R. and Giroud, C.J.P. Am. J. Physiol. 202, 91 (1962).

293. Gallop, P.M. in Connective tissue: Intercellular Macromolecules. Little Brown and Company, Boston (1964). p. 79.

294. Ouchterlony, O. Progr. in Allergy 6, 30 (1962).

295. Crowle, J. Ann. Rev. Microbiol. 14, 161 (1960).

296. Finger, I., Kabat, E.A., Bezer, A.E. and Kidd, A. J. Immunol. 84, 227 (1960).

297. Nozaki, Y. and Tanford, C. J. Biol. Chem. 238, 4074 (1963).

298. Bruning, W. and Holtzer, A. J. Am. Chem. Soc. 83, 4865 (1961).

299. Kauzmann, W. Advan. Protein Chem. 14, 1 (1959).

300. Bakerman, S. and Wasemiller, G. Biochemistry 6, 1100 (1967).

301. Shifrin, S. and Steers, E. Jr. Biochem. Biophys. Acta 133, 463 (1967).

302. Maxfield, M. in Glycoproteins, their composition, structure and function. Ed. Gottschalk, A. Elsevier Publishing Company. B.B.A. library. Vol. 5. (1966). p. 446.

303. Shibata, S., Naruse, T., Nagasawa, T., Takama, T. and Miyakawa, Y. J. Immunol. 99, 454 (1967).