

THE STRUCTURE AND METABOLISM OF  
THE GLOMERULAR BASEMENT MEMBRANE

by

Joseph Thomas Hjelle

---

A Dissertation Submitted to the Faculty of the

Department of Pharmacology

through the

COMMITTEE ON PHARMACOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements  
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 7 6

THE UNIVERSITY OF ARIZONA  
GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my  
direction by Joseph Thomas Hjelle  
entitled The Structure and Metabolism of the Glomerular  
Basement Membrane  
be accepted as fulfilling the dissertation requirement for the  
degree of Doctor of Philosophy

Elias Meegan  
Dissertation Director

8/23/76  
Date

As members of the Final Examination Committee, we certify  
that we have read this dissertation and agree that it may be  
presented for final defense.

Klaus Bunde  
Rubyn Bressler  
Michael G. Wells  
Mark R. Haunler

8-23-76  
8-23-76  
8-23-76  
8-23-76

Final approval and acceptance of this dissertation is contingent  
on the candidate's adequate performance and defense thereof at the  
final oral examination.

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED:

Joseph Thomas Hille

To Rita

## ACKNOWLEDGMENTS

I would like to express my deep, personal gratitude to Dr. Elias Meezan, my dissertation director, for his advise, encouragement and support during the course of these experiments.

I would like to thank Dr. Klaus Brendel for his valuable and unique collaboration in this endeavor.

I would like to thank Dr. Edward C. Carlson for generously providing his ultrastructural expertise which significantly facilitated this work.

I would like to thank Dr. Rubin Bressler for assembling a faculty which has shown a desire to actively participate in the training and development of new scientific talent.

I would like to thank my parents for believing that honesty, integrity, concern and involvement can make this a better world.

I would also like to thank Rita, my wife, for being herself.

## TABLE OF CONTENTS

|  | Page |
|--|------|
| LIST OF ILLUSTRATIONS . . . . .                            | vii  |
| LIST OF TABLES . . . . .                                   | xi   |
| ABSTRACT . . . . .   | xii  |
| <br>CHAPTER  |      |
| 1. INTRODUCTION . . . . .                                  | 1    |
| Basement Membrane Definition . . . . .                     | 1    |
| Basement Membrane Composition . . . . .                    | 4    |
| Carbohydrate Attachment to the Basement Membrane . . . . . | 6    |
| Basement Membrane Isolation . . . . .                      | 7    |
| Basement Membrane Fractionation . . . . .                  | 15   |
| Basement Membrane Metabolism . . . . .                     | 23   |
| Basement Membrane Function . . . . .                       | 29   |
| Tissue Support and Repair . . . . .                        | 29   |
| The Basement Membrane in Glomerular Filtration . . . . .   | 30   |
| The Glomerular Basement Membrane in Disease . . . . .      | 34   |
| Rationale and Objectives . . . . .                         | 39   |
| 2. MATERIALS AND METHODS . . . . .                         | 42   |
| Tissue Isolation . . . . .                                 | 42   |
| Rat Glomeruli . . . . .                                    | 42   |
| Rabbit Proximal Tubules . . . . .                          | 45   |
| Bovine Retinal Blood Vessels . . . . .                     | 47   |
| Bovine Brain Blood Vessels . . . . .                       | 48   |
| Bovine Lens Capsule . . . . .                              | 50   |
| Basement Membrane Isolation . . . . .                      | 50   |
| Tissue Preparation for Microscopic Examination . . . . .   | 50   |
| Chemical Analysis of Isolated Basement Membrane . . . . .  | 53   |
| Carbohydrate Analyses . . . . .                            | 53   |
| Amino Acid Analyses . . . . .                              | 54   |
| Metabolism . . . . .                                       | 55   |
| Gluconeogenesis . . . . .                                  | 55   |
| Oxidative Metabolism . . . . .                             | 57   |
| Transport and Incorporation . . . . .                      | 57   |
| Basement Membrane Metabolism . . . . .                     | 59   |
| Incubation Conditions . . . . .                            | 59   |

TABLE OF CONTENTS--Continued

|  | Page    |
|--|---------|
| Basement Membrane Isolation Procedure . . . . .                                | 60      |
| <sup>14</sup> C-Hydroxyproline Determination . . . . .                         | 60      |
| Polyacrylamide Gel Electrophoresis . . . . .                                   | 61      |
| Gel Preparation . . . . .  | 61      |
| Tissue Solubilization . . . . .  | 63      |
| Scintillation Spectroscopy . . . . .   | 63      |
| <br>3. RESULTS AND DISCUSSION . . . . .  | <br>64  |
| Tissue Isolation . . . . .   | 64      |
| Rat Glomeruli . . . . .  | 64      |
| Rabbit Renal Proximal Tubules . . . . .  | 69      |
| Bovine Retinal Blood Vessels . . . . .   | 72      |
| Bovine Brain Blood Vessels . . . . .   | 73      |
| Bovine Lens Capsule . . . . .  | 76      |
| Basement Membrane Isolation . . . . .  | 76      |
| Rat Renal Glomeruli . . . . .  | 76      |
| Rabbit Renal Proximal Tubules . . . . .  | 83      |
| Bovine Retinal Blood Vessel . . . . .  | 83      |
| Bovine Brain Blood Vessel . . . . .  | 86      |
| Bovine Lens Capsule . . . . .  | 86      |
| Basement Membrane Characterization . . . . .                                   | 86      |
| Carbohydrate Analysis . . . . .  | 86      |
| Amino Acid Analysis . . . . .  | 89      |
| Basement Membrane Protein Fractionation . . . . .                              | 92      |
| Effect of Degradative Enzymes on Basement<br>Membrane Ultrastructure . . . . . | 97      |
| Metabolism . . . . .   | 98      |
| Rat Glomerular Gluconeogenesis . . . . .                                       | 98      |
| Rabbit Proximal Tubule Metabolism . . . . .                                    | 107     |
| Glomerular Basement Membrane Metabolism . . . . .                              | 108     |
| Incorporation Experiments . . . . .  | 108     |
| Inhibition Experiments . . . . .   | 137     |
| <br>4. CONCLUSIONS . . . . .   | <br>145 |
| LIST OF REFERENCES . . . . .   | 147     |

## LIST OF ILLUSTRATIONS

| Figure  | Page |
|---|------|
| 1. Diagram of the Anatomical Distribution of Basement Membranes . . . . .   | 2    |
| 2. Structure of the Disaccharide Unit and Its Attachment Amino Acid . . . . .   | 7    |
| 3. Proposed Pathways for the Biosynthesis of the Collagenous and Non-Collagenous Basement Membrane Proteins . . . . . | 25   |
| 4. Diagram of a Portion of the Glomerular Capillary . . . . .   | 31   |
| 5. Iron Oxide Glomerular Isolation Procedure . . . . .  | 44   |
| 6. Microvessel Isolation Procedure . . . . .  | 49   |
| 7. Deoxycholate Basement Membrane Isolation Procedure . . . . .   | 51   |
| 8. Chromatographic Separation of Hydroxyproline and Proline . . . . .   | 62   |
| 9. Rat Glomerulus Fixed <u>In Situ</u> . . . . .  | 65   |
| 10. Iron Oxide Perfused Glomerulus . . . . .  | 66   |
| 11. Foot Processes of Iron Oxide Perfused Glomerulus . . . . .  | 67   |
| 12. Foot Processes of Glomerulus Fixed <u>In Situ</u> . . . . .   | 68   |
| 13. Rabbit Renal Proximal Tubules Obtained by Hand Homogenization and Sieving . . . . .                               | 70   |
| 14. Comparison of Sieved and Collagenase Digested Rabbit Proximal Tubules . . . . .                                   | 71   |
| 15. Isolated Bovine Retinal Microvessels and Basement Membrane . . . . .  | 74   |
| 16. Isolated Bovine Brain Microvessels and Basement Membrane . . . . .  | 75   |
| 17. Rat Renal Glomeruli before and after Deoxycholate . . . . .   | 80   |

LIST OF ILLUSTRATIONS--Continued

| Figure  | Page |
|---|------|
| 18. Glomerular Basement Membrane before and after<br>Deoxycholate Treatment . . . . .   | 82   |
| 19. Isolated Rabbit Renal Proximal Tubule Basement Membrane . . . . .   | 84   |
| 20. Isolated Bovine Retinal Capillary Basement Membrane . . . . .   | 85   |
| 21. A Photomicrograph of the Separation of Basement<br>Membrane Proteins Obtained by SDS-Urea-Polyacryl-<br>amide Gel Electrophoresis . . . . .               | 93   |
| 22. Diagram of the Relative Migration of the Basement<br>Membrane Proteins Isolated by the Deoxycholate<br>Procedure . . . . .                                | 95   |
| 23. $^{14}\text{C}$ -Glucose Formation in Glomerular Tuft Preparations<br>of Varying Bowman's Capsule Content . . . . .                                       | 100  |
| 24. $^{14}\text{CO}_2$ Formation in Glomerular Tuft Preparations of<br>Varying Bowman's Capsule Content . . . . .   | 101  |
| 25. Comparison of Protein Synthetic Activity in Isolated<br>Glomerular Tufts, Glomerular Tufts with Bowman's<br>Capsule, and Glomeruli with Tubules . . . . . | 105  |
| 26. Effect of pH on $^{14}\text{C}$ -Glucose Formation by Glomerular<br>Tufts, Glomerular Tufts with Bowman's Capsule and<br>Isolated Rat Tubules . . . . .   | 106  |
| 27. The Effect of Collagenase on Rabbit Tubule $^{14}\text{C}$ -Glucose<br>Production . . . . .   | 109  |
| 28. The Effect of Collagenase Digestion on Isolated<br>Rabbit Tubule $^{14}\text{CO}_2$ Production . . . . .  | 110  |
| 29. $^3\text{H}$ -Amino Acid Uptake by Isolated Rabbit Tubules . . . . .  | 111  |
| 30. $^3\text{H}$ -Amino Acid Incorporation into TCA Precipitable<br>Protein . . . . .   | 112  |
| 31. $^3\text{H}$ -Uridine Uptake by Isolated Rabbit Tubules . . . . .   | 113  |

LIST OF ILLUSTRATIONS--Continued

| Figure   | Page |
|--|------|
| 32. $^3\text{H}$ -Uridine Incorporation into TCA Precipitable<br>Material by Isolated Rabbit Tubules . . . . .   | 114  |
| 33. The Effect of Collagenase Digestion on the<br>Uptake of $^{14}\text{C}$ - $p$ -Aminohippuric Acid by Isolated<br>Rabbit Tubules . . . . .          | 115  |
| 34. The Effect of Multiple Saline Washes in Removing<br>Saline Extractable $^{14}\text{C}$ -Amino Acids from Incubated<br>Rat Glomeruli . . . . .      | 118  |
| 35. Incorporation of $^3\text{H}$ -Proline into DEOC Insoluble Material<br>Plotted against Protein Concentration . . . . .                             | 123  |
| 36. The Incorporation of $^{14}\text{C}$ -Amino Acids into Rabbit Tubule<br>Basement Membrane Material . . . . .                                       | 125  |
| 37. The Incorporation of $^3\text{H}$ -Glucosamine into Isolated<br>Glomerular Basement Membrane . . . . .   | 126  |
| 38. The Incorporation of $^{14}\text{C}$ -Lysine into Isolated<br>Glomerular Basement Membrane . . . . .   | 128  |
| 39. The Incorporation of $^3\text{H}$ -Glycine into Isolated<br>Glomerular Basement Membrane . . . . .   | 129  |
| 40. The Incorporation of $^3\text{H}$ -Galactose into Isolated<br>Glomerular Basement Membrane . . . . .   | 130  |
| 41. The Incorporation of $^3\text{H}$ -Proline into Isolated<br>Glomerular Basement Membrane . . . . .   | 132  |
| 42. Appearance of $^{14}\text{C}$ -Hydroxyproline in the Soluble and<br>Basement Membrane Fractions Obtained from Isolated<br>Rat Glomeruli . . . . .  | 133  |
| 43. Appearance of $^{14}\text{C}$ -Hydroxyproline in the Soluble and<br>Basement Membrane Fractions Obtained from Isolated<br>Rabbit Tubules . . . . . | 134  |

LIST OF ILLUSTRATIONS--Continued

| Figure  | Page |
|---|------|
| 44. The Effect of Sodium Azide and Cycloheximide on the Appearance of Radiolabel in the Deoxycholate Soluble Fraction of Isolated Rat Glomeruli Incubated with a Mixture of $^3\text{H}$ -Amino Acids . . . . .     | 138  |
| 45. The Effect of Sodium Azide and Cycloheximide on the Appearance of Radiolabel in the Deoxycholate Insoluble Fraction Isolated from Rat Glomeruli Incubated with a Mixture of $^3\text{H}$ -Amino Acids . . . . . | 139  |
| 46. Effect of Colchicine on the Appearance of Radiolabel in the Isolated Basement Membrane Fraction Obtained from Rat Glomeruli . . . . .   | 141  |
| 47. The Effect of $\beta$ -Aminoproprionitrile on the Appearance of Radiolabel in the Glomerular Basement Membrane Isolated from Rat Glomeruli Incubated with $^3\text{H}$ -Proline . . . . .                       | 142  |

## LIST OF TABLES

| Table   | Page |
|---|------|
| 1. Effect of Resonication on Human Glomerular Basement Membrane Content of Selected Amino Acids . . . . .                           | 11   |
| 2. Solubility Properties of Bovine Glomerular Basement Membranes . . . . .  | 16   |
| 3. Effect of Reduction Alkylation and Gel Filtration on Glomerular Basement Membrane Fractionation . . . . .                        | 18   |
| 4. Compositional Similarities of Glomerular Basement Membrane and Tendon Collagen Obtained by Limited Enzymatic Digestion . . . . . | 21   |
| 5. Basement Membrane Dimensions after Deoxycholate Treatment . . . . .  | 81   |
| 6. Carbohydrate Analyses of Isolated Basement Membranes in $\mu\text{g}$ Sugar/mg Dry Weight Basement Membrane . . . . .            | 87   |
| 7. Amino Acid Composition of Isolated Basement Membranes in Residues/1000 Residues . . . . .  | 90   |
| 8. Amino Acid Composition of Isolated Glomerular Basement Membranes in Residues/1000 Residues . . . . .                             | 91   |
| 9. Percent of Tubule $^{14}\text{C}$ -Glucose Production by Isolated Glomerular Fractions (cpm/mg Protein) . . . . .                | 103  |
| 10. Deoxycholate Extraction of Radiolabel from Isolated Glomeruli . . . . .   | 120  |
| 11. Radiolabel Remaining after Multiple Deoxycholate Extractions . . . . .  | 122  |

## ABSTRACT

Basement membranes were isolated from rat renal glomeruli, rabbit proximal tubules, bovine brain blood vessels, bovine retinal blood vessels, and bovine lens capsule by the use of a 4% solution of sodium deoxycholate. The basement membranes isolated by this procedure were ultrastructurally indistinguishable from their in vivo counterparts. Using bovine lens capsule as a reference tissue, the amino acid and carbohydrate composition of the basement membranes obtained by ultrasonic disruption and deoxycholate treatment were identical indicating that deoxycholate treatment did not interfere with chemical analyses. When glomerular basement membranes isolated by the use of deoxycholate were compared to those obtained by ultrasonic disruption, a higher content of hydroxyproline and hydroxylysine was observed in the deoxycholate treated samples. The amino acid content of the various deoxycholate isolated basement membrane samples were found to be similar but not identical. In contrast, the glucose and galactose contents of the isolated basement membranes were found to vary with the tissue source.

Using a highly purified isolated rat glomerular preparation, basement membrane protein synthesis and deposition into the extracellular basement membrane matrix was studied. The incorporation of radiolabeled glucosamine, lysine, glycine, galactose, and a mixture of amino acids was found to be linear during 6 hour incubations. Radiolabeled proline incorporation, however, was found to be linear for the first 4

hours of incubation after which an apparent linear increase in incorporation was observed. This delayed increase in proline incorporation correlated with the appearance of radiolabeled hydroxyproline in the isolated basement membrane. The delayed increase in proline incorporation could be blocked by the collagen crosslink inhibitor  $\beta$ -aminopropionitrile while colchicine, an inhibitor of collagen secretion, had no effect on radiolabeled amino acid incorporation during 6 hour incubations. It was concluded that glomerular basement membrane synthesis and deposition is a two component system characterized by a rapidly synthesized and deposited non-collagenous glycoprotein component and a collagenous component having a 4 - 6 hour delay period before deposition.

In addition to the study of glomerular basement membrane synthesis, the metabolism of other suborgan fractions isolated by techniques developed in our laboratory were examined. Glomerular production of  $^{14}\text{C}$ -glucose from  $^{14}\text{C}$ -succinate,  $^{14}\text{C}$ -pyruvate,  $^{14}\text{C}$ -glutamate, and  $^{14}\text{C}$ -lactate was studied to determine the source of glucose utilized for glomerular glycoprotein synthesis. Using newly developed glomerular separation techniques, radiolabeled glucose production was observed only in glomerular preparations containing Bowman's capsule and not in the glomerular capillary tuft per se. It was concluded that Bowman's capsule or minute quantities of tubule attached to Bowman's capsule was responsible for the radiolabeled glucose production observed in glomerular preparations and that extracellular glucose must therefore be the source of glomerular tuft carbohydrate.

The characterization of a rabbit proximal tubule preparation obtained by gentle hand homogenization and sieving which avoided the use of degradative enzymes was also performed. It was found that  $^{14}\text{CO}_2$  production from  $^{14}\text{C}$ -succinate, radiolabeled amino acid and uridine uptake and incorporation, were significantly depressed in collagenase treated tubules as compared to the non-collagenase treated, sieved tubules. The tissue to medium ratio for  $p$ -aminohippuric acid uptake by the tubules isolated by hand homogenization was 50 while that of collagenase treated tubules was 20. Tubules isolated by the sieving method also formed radiolabeled hydroxyproline and incorporated radiolabel into isolated tubule basement membrane. Thus, the tubules obtained by hand homogenization and sieving are metabolically more active than those isolated with the aid of degradative enzymes.

## CHAPTER 1

### INTRODUCTION

#### Basement Membrane Definition

Basement membranes are specialized extracellular support structures which are found in almost all organs of the body. The term basement membrane was used as early as 1848 by Todd and Bowman (1857) in a description of the extracellular structure to which synovial and serosal epithelial cells appeared to be attached. Subsequently, light microscopic studies observed basement membranes to be interposed between epithelium and connective tissue or between connective tissue and blood vessels (Rinehart, 1930; Lillie, 1952). A more ubiquitous nature was accorded basement membranes with the electron microscopic observations that basement membrane-like structures also surround intramural pericytes of capillaries, nervous tissue, muscle, and fat cells (Fawcett, 1966). Vracko (1974a) has proposed that the basement membrane be defined in light of its ubiquitous nature as an "extracellular scaffold positioned between parenchymal cells and connective tissue". Figure 1 illustrates this definition.

Basement membranes are generally thought to lie adjacent to the cells which secrete them. This is demonstrated by the findings of Pierce, Midgley, and Sri Ram (1963) that parietal yolk sac cells of mouse embryo secrete a layer of basement membrane upon which they become attached. Similar findings have been reported by Hay (1968) and

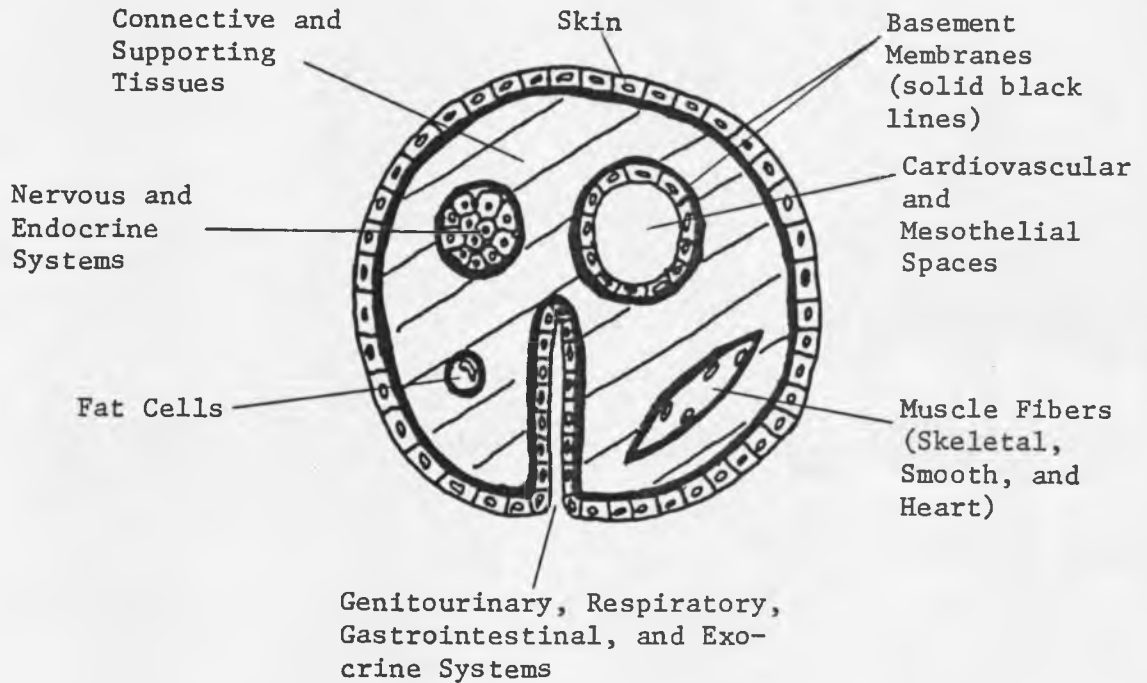


Figure 1. Diagram of the Anatomical Distribution of Basement Membranes

The basement membrane occupies the space between parenchymal cells and the connective and supportive tissues. Parenchymal cells are defined as all epithelial cells of epidermis and epidermal appendages, of the gastrointestinal, genitourinary, and respiratory tracts, endothelial cells of the cardiovascular system, mesothelial cells of body cavities, all exocrine glands, endocrine cells, muscle fibers, fat cells, and cells comprising the central and peripheral nervous systems. The connective and supporting tissues are defined as cartilage and associated cells, bone and associated cells, collagen, elastin, and fibroblasts. Adapted from Vracko (1974a).

McKeehan (1951), in other developing cell systems. Thus, the formation of the basement membrane is followed by a close physical association with the basement membrane forming cells.

Electron microscopic studies of the basement membranes found in the renal glomeruli (Kurtz, 1961; Kurtz and McManus, 1959; Vernier, 1964; Farquhar, 1964; Rhodin, 1955; Yamada, 1955), muscle capillaries (Palade and Bruns, 1964), skin capillaries (Fawcett, 1963; Friederici, Tucker, and Schwartz, 1966), alveolar capillaries (Karrer, 1956; Weibel, 1963), renal tubules (Sjostrand and Rhodin, 1953; Latta, Maunsbach, and Osvaldo, 1967), Descemet's membrane and the anterior lens capsule (Jakus, 1964) show them to all contain a fine feltwork of fibrils 30 - 40 Å in diameter. These fibrils are associated or embedded in an amorphous matrix which varied in width with the various tissue basement membranes. The degree of orientation of these embedded fibrils varied from tissue to tissue. While a high degree of orientation was exhibited by both lens capsule and Descemet's membrane fibrils, the glomerular and muscle capillary basement membranes demonstrated a partial and random fibrillar arrangement, respectively. In addition, Descemet's membrane and anterior lens capsule basement membrane exhibited areas of regular banding with a periodicity of 1100 Å (Jakus, 1964). However, the typical 600 - 700 Å banding of collagen fibrils is generally accepted not to be present in normal basement membranes.

Although the various basement membranes exhibited slight ultrastructural differences, as a group they were observed to be argyrophilic (Rossle and Yoshida, 1909; Rinehart, 1930) suggestive of "reticulin" (collagen) and periodic acid-Schiff positive (McManus, 1948a, 1948b,

1948c; Leblond, 1950) indicative of carbohydrates. In addition, vascular and non-vascular basement membranes were found to be immunologically crossreactive indicating similar antigenic components (Cruickshank and Hill, 1953; Roberts, 1957; Krakower and Greenspon, 1964; Huang and Kalant, 1968; Rothbard and Watson, 1969). In general, the various organ basement membranes are ultrastructurally, histochemically, and immunologically similar.

#### Basement Membrane Composition

Since chemical analysis of the basement membrane requires pure basement membrane, its chemical definition has necessarily relied upon the anatomically distinct characteristics of the structure. Similarity of electron microscopic appearance of isolated basement membrane preparations to in vivo basement membrane is the initial criterion by which basement membrane purity is defined.

Early chemical analyses of isolated basement membrane preparations from bovine lens capsule (Pirie, 1951) and canine renal glomeruli (Goodman, Greenspon, and Krakower, 1955) demonstrated the presence of significant amounts of hydroxyproline, proline, glycine, hexoses, and small amounts of hexosamines. This substantiated the collagenous and glycoprotein nature of the basement membrane that had been inferred from histochemical studies (Lillie, 1947). Subsequently, basement membranes were isolated and chemically analyzed from suborgan fractions of a variety of mammalian species: lens capsule (Dische, 1964; Kefalides, 1969a; Fukushi and Spiro, 1969), renal glomeruli (Lazarow and Speidel, 1964;

Markowitz and Lange, 1964; Kefalides and Winzler, 1966; Spiro, 1967a; Huang and Kalant, 1968; Kefalides, 1968, 1970, 1973; Westberg and Michael, 1970; Wahl, Krezdorn, and Deppermann, 1970; Blau and Michael, 1971; Sachot et al., 1975; Lehotay, 1975), Descemet's membrane (Dohlman and Balazs, 1955; Kefalides and Denduchis, 1969), and renal tubules (Mahieu and Winand, 1970; Ferwerda et al., 1974). These chemical analyses of isolated basement membranes showed them to have similar amino acid contents. Glycine varied between 22 - 28%, hydroxyproline 6 - 10%, proline 6 - 7%, and hydroxylysine 2 - 3.5% of the total amino acid residues present in these isolated basement membranes. A uniquely high 11% of the total hydroxyproline residues were found to be 3-hydroxyproline. Interstitial collagens contain about ten times less 3-hydroxyproline (Kefalides, 1973). Carbohydrates comprised 10 - 15% of the basement membrane weight. Glucose and galactose were found in nearly equimolar amounts in these various basement membranes while the glucosamine, galactosamine, mannose, fucose, and sialic acid contents varied.

Although similar to interstitial collagen in amino acid content, basement membranes contain less than the required 33% glycine and the 22% sum of hydroxyproline and proline to be classified as a collagen protein (Ramachandran, Sasisekharan, and Thathachari, 1962). Two other observations support this view. The first is that the triple helical structure of collagen indicated by a banding periodicity of 640 Å has not been observed in basement membrane specimens. The second is that interstitial collagen contains only glucose and galactose whereas basement membranes additionally contain mannose, fucose, hexosamines, and

sialic acid. These observations led Kefalides and Winzler (1966) to the assumption that collagen was present in the basement membrane, but that the glycine, imido acids, and hexose content were being diluted by non-collagenous glycoproteins also present in the basement membrane. The alternative hypothesis that basement membranes contained collagen-like glycoproteins and no true collagen proteins was supported by Spiro (1967a).

#### Carbohydrate Attachment to the Basement Membrane

Spiro (1976b) digested bovine glomerular basement membranes with collagenase followed by a subsequent digestion with pronase to free the carbohydrate units from the basement membrane peptides. The carbohydrate units were separated by gel filtration and ion exchange chromatography. Two distinct types of carbohydrate units were observed. A disaccharide unit consisting of glucose linked to galactose accounted for half of the carbohydrates present in the basement membrane. The second carbohydrate unit contained galactose, mannose, fucose, hexosamines, and sialic acid. The estimated molecular weight of this heteropolysaccharide was 3,500 daltons. The molar ratio of the disaccharide to the heteropolysaccharide was ten to one. The disaccharide was found to be linked to the peptide chain through a glycosidic linkage with hydroxylysine. Inferential evidence suggested asparagine as the site of attachment for the heteropolysaccharide unit.

In subsequent experiments, Spiro (1967c) showed that the disaccharide with its attachment amino acid had the following structure:

2-O- $\alpha$ -D-glucopyranosyl-0- $\beta$ -D-galactopyranosylhydroxylysine. In addition to being present in the glomerular basement membrane, the disaccharide unit has been observed in numerous isolated basement membranes (Pirie, 1951; Spiro and Fukushi, 1969; Dische, Zelmanis, and Rothchild, 1967; Kefalides, 1969a) and vertebrate collagens (Miller and Matukas, 1974; Nimni, 1973; Cunningham and Ford, 1968). See Figure 2.

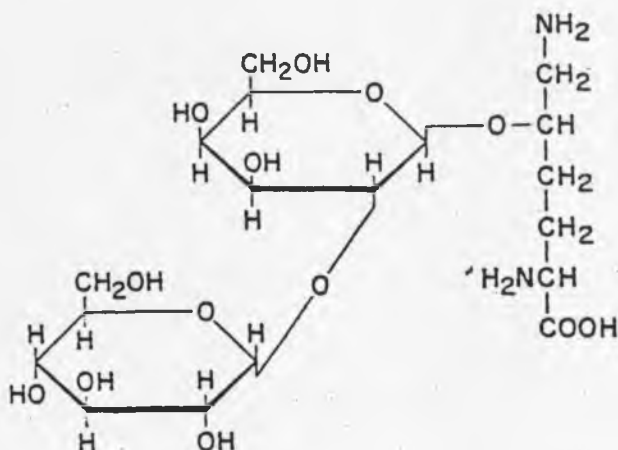


Figure 2. Structure of the Disaccharide Unit and Its Attachment Amino Acid

### Basement Membrane Isolation

Certain tissues such as lens capsule are excellent sources of easily purifiable basement membranes. This is due to the ease with which the lens can be removed from the eye and manipulated and to the macroscopic nature of the lens capsule basement membrane. Other

tissues, while having a relatively high content of basement membrane, are more difficult to utilize as a basement membrane source. This is because of the intrinsic difficulty associated with obtaining purified suborgan fractions from certain organs and the subsequent manipulation of the suborgan fraction to yield basement membrane. These technical problems are magnified when small animals, such as the rat, are used. Renal glomeruli are an example of a basement membrane source, where problems in isolation arise.

In addition to amino acids and carbohydrates, phospholipids (Lidsky, Sharp, and Rudee, 1967; Von Bruchhausen and Merker, 1967), nucleic acids (Krakower and Greenspon, 1951; Lidsky, Sharp, and Rudee, 1967), and cholesterol (Misra and Berman, 1966) have been reported in isolated glomerular basement membrane preparations. However, when Kefalides and Winzler (1966), Spiro (1967a), and Westberg and Michael (1970) examined isolated glomerular basement membrane preparations, no nucleic acids were detected and the presence of phospholipids and cholesterol as components of the basement membrane was questioned. These compositional discrepancies have been related to the purity of the isolated glomerular basement membrane preparation employed (Westberg and Michael, 1970). The isolation of pure glomerular basement membranes is a two step process involving first the preparation of pure renal glomeruli followed by the isolation of the basement membrane from these structures. The most commonly used glomerular isolation procedure is that of Krakower and Greenspon (1951) in which glomeruli are freed from the surrounding renal cortex by forcing pieces of kidney through

stainless steel sieves. The non-glomerular material is disrupted by this technique. Separation of the freed glomeruli from the disrupted tissue was performed by multiple centrifugation. Several modifications of the method of Krakower and Greenspon (1951) have been reported. Most of these modifications involved alternative techniques for the separation of the tissue debris from the freed glomeruli such as differential centrifugation (Richterich and Franz, 1960), filtration through a column of glass beads (Törnblom, 1957), or collection of magnetic iron oxide-filled glomeruli with a magnet (Cook and Pickering, 1958; Misra and Berman, 1966). Similarly, Spiro (1967a) modified the method of Krakower and Greenspon (1951) so as to use the sieves not only to disrupt the tissue but to collect the freed glomeruli. Spiro (1967a) maintained that this modification avoided contamination of the glomeruli with tubules and cell debris during the centrifugation steps used by Krakower and Greenspon (1951). After a purified glomerular preparation was obtained, the cellular material was removed from the glomerular capillary basement membrane by ultrasonic disruption followed by centrifugation to pellet the basement membrane. Ultrasonic disruption followed by centrifugation is the most widely used method for the separation of cellular materials from basement membranes. Thus, Spiro (1967a) feels that by increasing the purity of the intermediate glomeruli the resultant glomerular basement membrane will also be of a higher purity.

Kefalides (1973), however, disagrees and argues that Spiro's (1967a) modifications of the method of Krakower and Greenspon (1951) to obtain purified glomeruli are not necessary for the isolation of highly

purified glomerular basement membranes. Kefalides (1973) claims that improvements on the method of Krakower and Greenspon (1951) have been due to the increased ultrasonic disruptive ability of more powerful sonicators or sonicating techniques. To demonstrate this point, Kefalides (1969a, 1973, 1974) prepared human glomerular basement membrane from glomeruli obtained by the method of Krakower and Greenspon (1951) by multiple sonication and centrifugation. Table 1 shows the effect of multiple sonications on human glomerular basement membrane amino acid content. Resonication resulted in a conspicuous relative increase in those amino acids typically found in collagen. The basement membrane amino acid content obtained by the resonication of glomerular basement membrane isolated by the method of Krakower and Greenspon (1951) was similar in part to the amino acid content of glomerular basement membrane obtained by the more elaborate isolation methods of Westberg and Michael (1970) or Spiro (1967a). The question of whether resonication does indeed remove non-glomerular basement membrane materials or additionally disrupts the basement membrane itself resulting in the loss of intrinsic basement membrane components has not been answered. In their study of the effects of isolation conditions on human glomerular basement membrane composition, Westberg and Michael (1970) reported that sonication of isolated glomeruli resulted in a partial disruption or fraying of the basement membrane. In contrast to the claims of Kefalides (1973), tubular fragments attached to the glomerular tufts were highly resistant to removal by ultrasonic disruption (Westberg and Michael, 1970). Thus, there is evidence that sonication results not only in a change in the ultrastructural appearance of the resultant isolated glomerular

Table 1. Effect of Resonication on Human Glomerular Basement Membrane Content of Selected Amino Acids

|                  | <u>Residues/1000 residues</u> |                              | Purified Glomeruli               |                                  |
|------------------|-------------------------------|------------------------------|----------------------------------|----------------------------------|
|                  | One Sonication <sup>1</sup>   | Two Sonications <sup>1</sup> | One Sonica-<br>tion <sup>2</sup> | One Sonica-<br>tion <sup>3</sup> |
| 4-hydroxyproline | 53.0                          | 90.0                         | 81.3                             | 84.1                             |
| proline          | 64.1                          | 69.8                         | 57.9                             | 79.4                             |
| glycine          | 225.2                         | 270.0                        | 221.0                            | 220.9                            |
| hydroxylysine    | 24.5                          | 35.0                         | 26.1                             | 21.3                             |
| lysine           | 26.4                          | 20.0                         | 19.5                             | 28.8                             |
| alanine          | 58.6                          | 59.8                         | 59.8                             | 68.9                             |

<sup>1</sup>Abbreviated from Kefalides (1969a).

<sup>2</sup>Abbreviated from Westberg and Michael (1970).

<sup>3</sup>Abbreviated from Beisswenger and Spiro (1970).

basement membrane, but a change in its amino acid composition as well.

In addition to the use of sonication with its attendant difficulties, centrifugation is widely used to separate the disrupted material from the basement membrane. Several reports of basement membrane pellet contamination with cellular debris indicate methodological problems with the centrifugation step as well. Westberg and Michael (1970) pelleted glomerular sonicates at increasing centrifugal forces. As the pelleting forces were increased in increments from 121 x g to 4340 x g, an approximately linear increase was observed in the phospholipid content in  $\mu\text{g}/\text{mg}$  when plotted against 4-hydroxypyroline content in  $\mu\text{g}/\text{mg}$ . Light micrographs of these various pellets indicated a direct relationship between centrifugation force and the presence of cell debris. The ratio of phospholipids to cholesterol in the purest basement membrane preparation (which was obtained at the lowest centrifugation force) was approximately equal to that of whole kidney cortex and whole glomeruli suggesting that both substances were present as extra-glomerular basement membrane contaminants. Spiro (1967a) has reported the presence of small amounts of lipids in bovine glomerular basement membrane preparations. Another indication that substances known to be present in cellular materials may contaminate glomerular basement membrane preparations is shown by the work of Mohos and Skoza (1970). The presence of large amounts of sialic acid on the surface of glomerular epithelial cells by the use of colloidal iron staining has been shown by a number of investigators (Mohos and Skoza, 1969; Rambourg and Leblond, 1967; Groniowski, Biczyskova and Walski, 1969; Jones, 1969). Isolated glomerular basement membranes, however, do not significantly stain with

colloidal iron (Westberg and Michael, 1970). When the centrifugal force used to pellet the glomerular basement membrane was varied from 42 x g to 1200 x g, the amount of sialic acid appearing in the basement membrane pellet increased (Mohos and Skoza, 1970). These authors argued that since the concentration of glucose thought to be indicative of collagen did not change with increasing amounts of sialic acid that sialic acid must be present as a contaminant.

Finally, in addition to nucleic acid, sialic acid, lipid and cholesterol contamination, Westberg and Michael (1970) have reported that when rabbits were injected with human glomerular basement membrane obtained by an extensive procedure of sonication and centrifugation that the resultant antisera contained antibodies directed not only against the basement membrane, but proteins present in human plasma and concentrated urine. These proteins included fibrin(ogen), immunoglobulin M, immunoglobulin G, albumin, and  $\beta$ -1-C-globulin. Thus, it appears likely that basement membrane preparations are also susceptible to contamination by plasma proteins.

The difficulties associated with the widely used method of sonication followed by centrifugation to isolate glomerular basement membrane are summarized below.

1. The process of ultrasonic disruption of the isolated glomeruli results in changes in both the ultrastructural appearance and amino acid composition.
2. The process of centrifugation to obtain a pellet of basement membrane is highly susceptible to contamination by substances known to be present in relatively large amounts in cellular material.

3. Extensive sonication and careful centrifugation are unable to remove contaminants such as plasma proteins from the basement membrane.

Since there is a lack of a precedent for the presence of phospholipids, plasma proteins, large amounts of sialoproteins, or other non-protein materials in the extracellular protein matrices we call basement membranes (Kéfalidés, 1973), one could conclude that these substances are only contaminants of the glomerular basement membrane preparations just discussed. Unfortunately, the question of what constitutes the basement membrane is not easily resolved. Should lipids, cholesterol, plasma proteins or other materials which are not tightly bound and thus extractable from the isolated basement membrane be considered basement membrane contaminants or should they be viewed as mobile closely associated basement membrane components? If these materials play a role (direct or indirect) in basement membrane function, should they be considered functional or modulating components of this structure? Before one can discuss modulating factors of basement membrane function, an understanding of the gross ultrastructural composition of the basement membrane matrix is required. Thus, for the purpose of structural or compositional definition, only those substances which are tightly associated with or covalently bound to ultrastructurally recognizable basement membrane will be considered basement membrane components.

### Basement Membrane Fractionation

Basically two approaches have been used to fractionate the various basement membranes. The first approach involved the use of non-degradative chemical means to solubilize and separate the various basement membrane components. The second approach utilized degradative enzymes to study the collagenous and non-collagenous materials freed from the basement membrane matrix.

Initial attempts to solubilize basement membrane proteins demonstrated these components to be resistant to complete solubilization by non-degradative procedures. Table 2 lists the solubility properties of bovine glomerular basement membranes in various chemical solutions. The basement membrane is nearly insoluble in simple salt solutions. Urea treatment is able to solubilize approximately 30% of the basement membrane indicating the presence of non-covalently bound materials. Reduction and alkylation solubilizes 90% of the basement membrane indicating that the majority of the structure is held together with covalent disulfide bonds. The inability to completely solubilize the basement membrane suggests that covalent bonds other than disulfide bonds are also involved in basement membrane protein crosslinking (Gallop and Paz, 1975; Daniels and Chu, 1975).

Hudson and Spiro (1972a) have reported the appearance of at least eleven proteinaceous components ranging in molecular weight from 30,000 to greater than 220,000 daltons upon SDS-polyacrylamide gel electrophoresis of reduced and alkylated bovine glomerular basement membrane. When the solubilized basement membrane was fractionated by gel filtration

Table 2. Solubility Properties of Bovine Glomerular Basement Membranes

| Ref | Treatment   | % Wt. |      |             |
|-----|---|-------|------|-------------|
|     |   | Time  | Temp | Solubilized |
| a   | 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0   | 24    | 37   | 4 %         |
| b   | 0.5 M NaCl, 0.05 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.5                              | 48    | 4    | 0.5%        |
| a   | Reduced and Alkylated, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0                    | 24    | 37   | 50 %        |
| b   | Reduced and Alkylated, H <sub>2</sub> O   | 48    | 40   | 7.5%        |
| a   | 5% LiCl, 0.1 M Tris chloride, pH 7.0  | 24    | 37   | 14 %        |
| a   | Reduced and Alkylated, 5% LiCl, 0.1 M Tris Cl <sup>o</sup> , pH 7.0                       | 24    | 37   | 72 %        |
| b   | 0.3 M Acetic Acid   | 72    | 4    | 1 %         |
| b   | 0.075 M Na citrate, pH 3.7  | 48    | 4    | 1 %         |
| c   | 0.1 M NaOH  | 8     | 37   | 96 %        |
| a   | 8 M Urea, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0                                 | 24    | 37   | 26 %        |
| b   | 8 M Urea, pH 7.2  | 72    | 40   | 35 %        |
| a   | Reduced and Alkylated 8 M Urea, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0           | 24    | 37   | 74 %        |
| b   | Reduced and Alkylated, 8 M Urea, pH 8.4   | 48    | 40   | 60 %        |
| a   | 1% SDS, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0                                   | 24    | 37   | 24 %        |
| b   | 1% SDS, 3% mercaptoethanol, Tris HCl, pH 8.6  | 48    | 50   | 80 %        |
| a   | Reduced and Alkylated 1% SDS, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0             | 24    | 37   | 75 %        |
| a   | 5% SDS, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0                                   | 24    | 37   | 26 %        |
| a   | Reduced and Alkylated, 5% SDS, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0            | 24    | 37   | 90 %        |
| a   | 0.5% Triton X-100, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0                        | 24    | 37   | 4.4%        |
| a   | Reduced and Alkylated, 0.5% Triton X-100, 0.1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0 | 24    | 37   | 68 %        |

- a. Hudson and Spiro (1972a).
- b. Kefalides (1973).
- c. Spiro (1967a).

and ion exchange chromatography, sixty distinct polypeptide components were collected and analyzed (Hudson and Spiro, 1972b; Spiro, 1976). Although pronounced compositional diversity was observed among these sixty components, all contained hydroxyproline, hydroxylysine, disaccharide units and heteropolysaccharide units (Ohno, Riquetti and Hudson, 1975; Spiro, 1976). Because of his inability to separate hydroxyproline containing collagen peptides from non-collagenous peptides even after reduction and alkylation followed by extensive chromatographic procedures, Spiro (1976) has proposed that the glomerular basement membrane is composed of homogeneous glycoproteins which contain collagen regions in their primary structure. Since the necessary criteria to classify the basement membrane as collagenous have not been met, Spiro (1976) concludes that the basement membrane protein(s) should be classified as glycoproteins with a collagen-like substructure. Spiro (1976) cites the complement protein,  $C1_q$ , as a precedent for such a classification.

Another view of glomerular basement membrane structure is held by Kefalides (1973). When Kefalides and Winzler (1966) solubilized and fractionated canine glomerular basement membrane proteins by reduction and alkylation in the absence of 8 M urea followed by urea-polyacrylamide gel electrophoresis, only four protein bands were observed. During subsequent gel filtration studies of the reduced and alkylated proteins, a glycoprotein fraction was obtained which was devoid of hydroxyproline and glucose while containing those carbohydrates characteristic of the heteropolysaccharide units (Kefalides, 1970) (Table 3). A similar fraction was obtained when only 8 M urea extractable glomerular basement

Table 3. Effect of Reduction, Alkylation and Gel Filtration on Glomerular Basement Membrane Fractionation

| Selected Amino Acids | <u>Residues/1000 Residues</u> |                        |                           |                           |                                |                                |
|----------------------|-------------------------------|------------------------|---------------------------|---------------------------|--------------------------------|--------------------------------|
|                      | Whole GBM <sup>1</sup>        | Whole GBM <sup>2</sup> | Urea Extract <sup>1</sup> | Urea Extract <sup>2</sup> | Urea Gel Fraction <sup>1</sup> | Urea Gel Fraction <sup>2</sup> |
| Hydroxyproline       | 50.0                          | 73.2                   | 50.0                      | 61.4                      | Trace                          | 19.8                           |
| Hydroxylysine        | 20.0                          | 21.0                   | 20.0                      | 21.9                      | Trace                          | 6.8                            |
| Glycine              | 200.0                         | 223.4                  | 200.0                     | 198.8                     | 135.0                          | 121.3                          |
| Proline              | 66.0                          | 78.2                   | 66.0                      | 70.7                      | 83.0                           | 46.1                           |
| Alanine              | 63.0                          | 65.3                   | 63.0                      | 62.0                      | 76.0                           | 73.2                           |
| Lysine               | 28.0                          | 26.1                   | 28.0                      | 24.2                      | 10.0                           | 42.4                           |

1. Kefalides (1966). Bio-Gel P-300.

2. Hudson and Spiro (1972b). Bio-Gel A-15m (fraction 5).

membrane proteins were fractionated (Kefalides, 1972). Gel filtration of glomerular basement membrane material obtained by prolonged collagenase digestion followed by dialysis gave two non-collagenous glycoprotein fractions (Kefalides, 1969b, 1972). A large glycoprotein residue having a molecular weight of over 200,000 daltons contained galactose, mannose, fucose, sialic acid, and hexosamine, but lacked hydroxyproline and hydroxylysine. A more soluble glycoprotein was estimated to have a molecular weight of 30,000 daltons. It also contained galactose, mannose, fucose, and sialic acid, but twice as much hexosamine as the larger glycoprotein. When antisera were prepared against the large and small glycoproteins, both antisera reacted with reduced and alkylated glomerular basement membrane. However, antisera against the large glycoprotein did not react with the small glycoprotein antigen or against hydroxyproline containing fractions obtained by gel filtration fractionation of collagenase treated glomerular basement membranes. Antisera against the small glycoprotein reacted with the hydroxyproline containing fractions but not with the large glycoprotein antigen. Only the antisera to the large glycoprotein reacted with the material extractable in 8 M urea. From these experiments, Kefalides (1973) concluded that two distinct glycoprotein components existed in the basement membrane: a large glycoprotein extractable by urea or reduction and a small glycoprotein which was associated in some manner with a collagen entity.

The presence of a collagen component in various suborgan basement membrane preparations has been demonstrated by Kefalides (1968) and Kefalides and Denduchis (1969). Limited pronase or pepsin digestion of

glomerular basement membranes resulted in the solubilization of a collagenous fraction possessing many of the same characteristics of similarly treated known collagens (Kefalides and Denduchis, 1969); Kefalides and Winzler, 1966; Davison and Drake, 1966). Carboxymethyl cellulose chromatography of the collagen fraction indicated the presence of  $\alpha 1$  chains characteristic of collagen (Kefalides, 1971). The basement membrane  $\alpha 1$  chains had a molecular weight of 110,000 whereas interstitial collagen  $\alpha 1$  chains had a molecular weight of 108,000. Table 4 lists the partial amino acid and carbohydrate composition of the collagen fractions obtained from glomerular basement membrane and tendon upon limited pepsin treatment. Similarities were seen in the glycine and sum of hydroxyproline and proline contents indicating the required composition to be classified as a collagen protein. The carbohydrates characteristic of the glycoproteins found in basement membrane fractions were not detected (Kefalides, 1971). The relatively large amount of disaccharide units found in the  $\alpha 1$  chains of basement membrane collagen accounted for the difference in molecular weight observed between  $\alpha 1$  chains (Kefalides, 1971a). In addition to the larger hexose content of basement membrane collagen, differences in the content of alanine and half-cystine were observed (see Table 4). This indicated not only differences in the amount of post-translational modification of basement membrane collagen as compared to that of interstitial collagen, but a difference in the primary protein structure as well. When the material extracted from lens capsule basement membrane with sodium citrate was precipitated with ATP, electron microscopic filaments with globular ends appeared

Table 4. Compositional Similarities of Glomerular Basement Membrane and Tendon Collagen Obtained by Limited Enzymatic Digestion

| Selected AA         | <u>Residues/1000 Residues</u> |                     |
|---------------------|-------------------------------|---------------------|
|                     | Glomeruli <sup>1</sup>        | Tendon <sup>2</sup> |
| Hydroxyproline      | 152.0                         | 86.0                |
| Proline             | 62.0                          | 140.0               |
| Glycine             | 322.4                         | 329.0               |
| Hydroxylysine       | 42.0                          | 6.0                 |
| Alanine             | 33.4                          | 122.0               |
| Half-Cystine        | 6.0                           | 0.0                 |
|                     |                               | <u>Gm/100 gm</u>    |
| <u>Carbohydrate</u> |                               |                     |
| Glucose             | 5.1                           | 0.28                |
| Glucose             | 5.25                          | 0.40                |

1. Kefalides and Denduchis (1969).
2. Kefalides and Winzler (1966).

(Kefalides, 1973). This was thought to be suggestive of a collagen molecule with a globular terminal portion (Kefalides, 1973). Such filaments are not characteristic of the intact basement membrane matrix.

From these observations, Kefalides (1973) has proposed that basement membranes contain at least two distinct protein molecules. One is represented by the large non-collagenous glycoprotein bound to the basement membrane matrix by hydrogen and disulfide bonds. This molecule is distinct from the collagen  $\alpha 1$  chains. A smaller glycoprotein associated with a collagen entity represents the second component. Since reduction and alkylation does not free the majority of these small glycoproteins so that they can be separated from the collagen containing component, it was thought that the small glycoprotein formed a portion of the primary structure of the collagen component. This hypothesis of a peptide extension being present on the collagen molecules has been supported by the collagen biosynthetic studies of Bornstein et al. (1972).

The finding of a non-collagenous glycoprotein extractable with urea or reduction and alkylation by Kefalides and Winzler (1966) is in direct disagreement with the observations of Hudson and Spiro (1972a) who could not find such a component. The reason for this discrepancy is unknown. It is possible that the method used by Spiro (1967a) to isolate glomerular basement membrane removes the non-collagenous protein that Kefalides and Winzler (1966) observed. The material extracted in urea alone accounts for less than 10% of the basement membrane weight and might represent only a surface protein on the basement membrane

matrix. The polydisperse nature of the glomerular basement membrane proteins obtained by reduction and alkylation observed by Hudson and Spiro (1972a) was not observed by Kefalides and Winzler (1966). No explanation is available. The finding of Spiro (1976) that no pure collagen component could be isolated from reduced and alkylated basement membrane is in agreement with Kefalides (1973) that the small glycoprotein he observes is bound to the collagen component through the primary structure and, as such, is an integral portion of the collagen molecule. The question of whether the basement membrane is composed of glycoproteins which contain collagen regions or collagen molecules with glycoprotein extensions appears to be in part a question of semantics as to what is and what is not "collagen".

#### Basement Membrane Metabolism

Our understanding of the metabolism of the various basement membranes is incomplete. In addition to the transcription and translation processes which are required for protein synthesis, extensive post-translational alteration of the protein structure is known to occur. In the model of the basement membrane proposed by Kefalides (1973), a collagenous component containing a glycoprotein extension and a separate 200,000 molecular weight glycoprotein component constitute the proteinaceous elements of the basement membrane matrix. The glycoprotein component requires the addition of fucose, galactose, hexosamines, sialic acid, and mannose, but does not require hydroxylation of amino acids situated in the peptide chain. The collagen component, however, requires

hydroxylation of both proline and lysine in addition to the glycosylation of the hydroxylysine and the formation of the heteropolysaccharide. Spiro's model of the basement membrane (1976) would require the same biosynthetic modifications as the collagen component of the Kefalides model (1973).

Two types of in vitro systems have been used to study basement membrane protein synthesis. The most active basement membrane synthesizing tissues are of embryonic origin. Whole embryonic chick lens and the cells isolated from them (Grant, Kefalides and Prockop, 1972a) and, more recently, the embryonic rat parietal yolk sac (Clark et al., 1975) have been employed. These rapidly developing systems have several advantages over mature systems: the extracellular matrix is free of most of the extra-basement membrane contaminants, only a single cell type is responsible for basement membrane synthesis (yolk sac only), and most importantly a relatively large percentage of the newly formed protein was basement membrane collagen. The mature tissue in vitro systems for the study of basement membrane formation have the advantage that diseases known to affect basement membrane morphology and structure usually occur in mature systems. Thus, information obtained in these systems is directly applicable to pathological cause and effect relationships.

Because of the similarities between collagen and the basement membrane collagen component, much of the information available about the post-translational modification of the basement membrane protein has been derived from studies with collagen systems (Figure 3). As collagen protein synthesis occurs, the prolyl and lysyl residues in the third

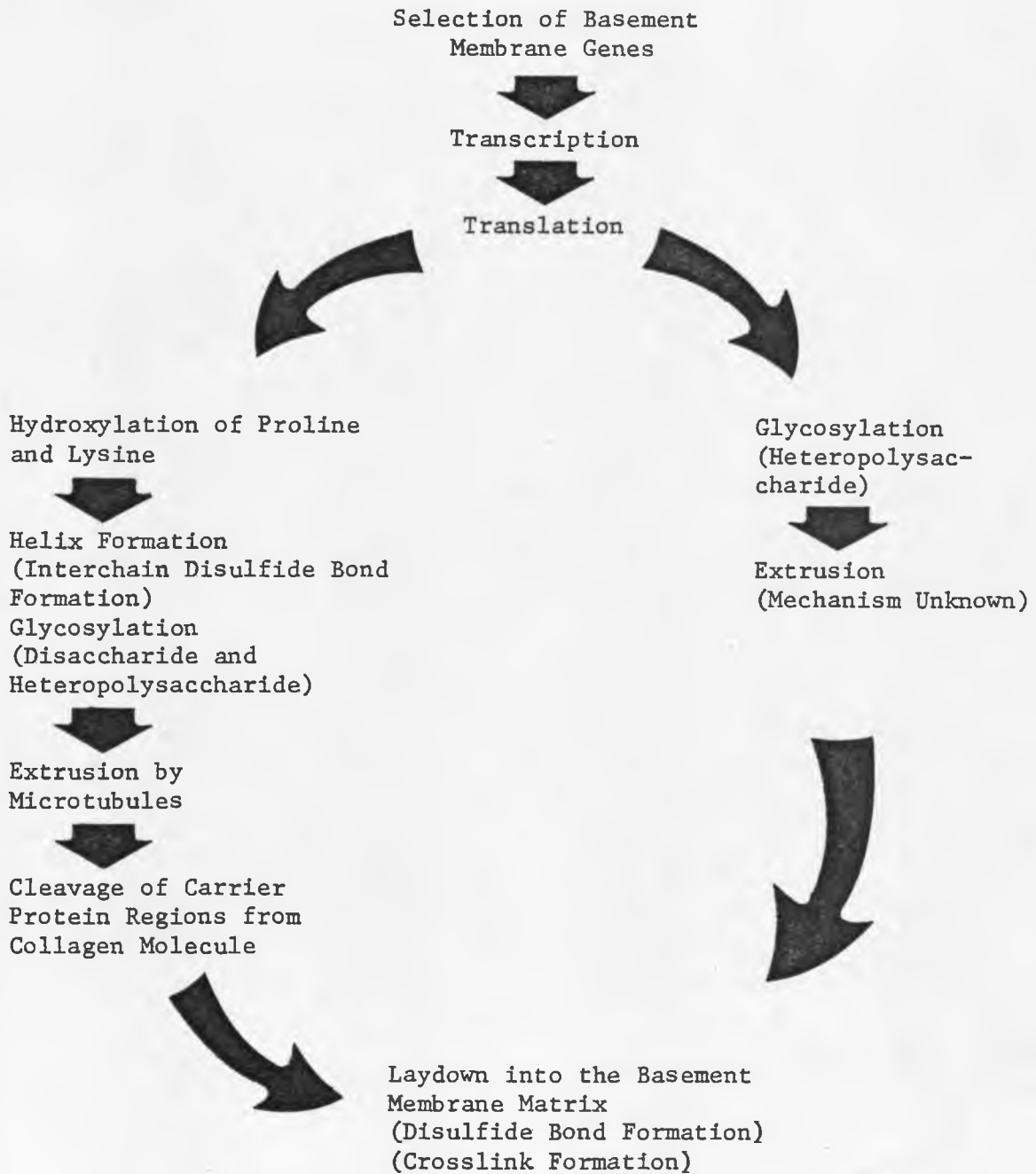


Figure 3. Proposed Pathways for the Biosynthesis of the Collagenous and Non-Collagenous Basement Membrane Proteins

position of what is called the collagen triplet (gly-X-Y) are susceptible to the soluble hydroxylating enzymes peptidyl proline hydroxylase and peptidyl lysyl hydroxylase (Grant and Prockop, 1972; Udenfriend, 1970). These enzymes require  $\alpha$ -ketoglutarate, molecular oxygen, ascorbic acid, and ferrous iron. Hydroxylation occurs before release of the nascent chain from the ribosomes (Lazarides, Lukens and Infante, 1971; Miller and Udenfriend, 1970). It has been found that hydroxylation results in stabilization of the triple-helical conformation characteristic of collagen fibrils (Berg and Prockop, 1973; Jimenez et al., 1973). From this observation of triple-helix stabilization by hydroxylation, it was proposed that triple-helix formation occurs during or shortly after the release of the hydroxylated collagen protein from the ribosomes. It is of interest to note that basement membrane collagen is much more hydroxylated than most vertebrate collagens (Miller, 1971; Kefalides, 1971). Basement membrane collagen contains approximately 190 hydroxylated residues while other collagens contain only about 110 hydroxylated residues.

Although the reason for the difference in the number of hydroxylated residues is not clear, a hypothesis to account for this phenomenon can be suggested. When Grant, Kefalides and Prockop (1972a, 1972b) incubated intact lenses and lens cells with  $^{14}\text{C}$ -proline and monitored the appearance of basement membrane collagen by  $^{14}\text{C}$ -hydroxyproline formation, it was found that a lag time of 60 minutes was required before secretion of the basement membrane collagen was begun. In addition, the molecular weight of the initial radiolabeled basement membrane collagen decreased from 140,000 to 115,000 daltons. In contrast, chick embryo

tendon cells synthesize and secrete collagen in only 20 minutes (Dehm and Prockop, 1971). The molecular weight of newly formed tendon collagen also underwent a conversion to a smaller protein of from 120,000 to 95,000 daltons. If a relatively larger basement membrane collagen was being made which required a longer ribosomal association time before release and helix formation, the soluble hydroxylating enzymes would have a longer period of time in which to hydroxylate susceptible proline and lysine residues than in the case of the smaller tendon collagen molecule.

Attachment of the disaccharide unit to the hydroxylated protein is a two step process. First, UDP-galactose donates galactose to the hydroxyl group of hydroxylysine via the membrane bound enzyme galactosyltransferase (M. Spiro and R. Spiro, 1971). The second step involves the addition of glucose from UDP-glucose to the hydroxylysine-linked galactose by the membrane bound enzyme glucosyltransferase (R. Spiro and M. Spiro, 1971). The number of disaccharide units attached is directly correlated with the number of available hydroxylysine residues present in the collagen molecule (Miller and Matukas, 1974). This suggests that the amount of disaccharide units present in the basement membrane collagen is dependent upon lysine hydroxylation activity. The presence of a glycopeptide extension on the collagen component requires the addition of carbohydrates characteristic of the heteropolysaccharide to this peptide region. At the present time, no information concerning the formation of this heteropolysaccharide is available for basement membrane systems. By analogy from glycoprotein biosynthetic studies,

however, the addition of mannose, galactose, fucose, sialic acid, and galactosamine and glucosamine probably occurs in a stepwise manner involving a nucleotide activated sugar and a specific enzyme transferred system (Spiro, 1969). The carbohydrate transferases are thought to be located on the endoplasmic reticulum (Spiro, 1969).

After synthesis, hydroxylation, glycosylation, and triple helix formation, the basement membrane collagen is secreted from the cell (Grant, Kefalides, Prockop, 1972a; Grant, Harwood, Williams, 1975). The process of collagen secretion is thought to be a microtubule directed phenomenon as indicated by the inhibition of collagen secretion by the microtubule inhibitor, colchicine (Dahlstrom, 1968; Malawista, 1965; Williams and Wolff, 1970). No information is available on the route of extrusion of the non-collagenous glycoprotein basement membrane component. Recent findings by Williams, Harwood and Grant (1976) indicate that the basement membrane collagen component is in the triple helical form and that the individual proteins are covalently linked by sulfide bonds. It is not known whether the disulfide bonds are formed before or after basement membrane collagen secretion.

After extrusion, the basement membrane collagen is enzymatically cleaved to a smaller molecular weight species (Grant, Kefalides, Prockop, 1972a, -b; Grant, Harwood, Williams, 1975; Williams et al., 1976) which is larger than similarly modified collagens (Dehm and Prockop, 1971). The mechanism by which the helical basement membrane collagen components associate with the basement membrane matrix and ultimately become covalently attached to it is unknown.

## Basement Membrane Function

### Tissue Support and Repair

Basement membranes as a class are thought to have two physiological functions. The first and most obvious is that of an extracellular scaffold to which cells that define organ function are attached. This scaffold provides physical support (Murphy and Johnson, 1975) and serves to define and maintain the spatial arrangement of the particular organ system.

In addition to this support function, basement membranes are also involved in tissue repair processes (Vracko, 1974a). When small portions of various organs (lung, muscle, kidney, liver) were injured resulting in cell death, tissue repair occurred by an orderly repopulation of new cells in the injured area. This repopulation process occurred by the growth of new cells along the remaining uninjured basement membrane until the same number of cells, types of cells, and positional arrangement of the cell types was similar to that before the injury. This orderly repair process results in the restoration of physiological function to the injured area. However, if the basement membrane was destroyed during the injury process, orderly tissue repair did not occur, scar tissue was formed and physiological function was lost.

The ability of the basement membrane to properly position the repopulating cell types has been related to a sidedness or polarity for cell types. When skeletal muscle basal lamina segments were excised and reimplanted, the surrounding muscle fibers and capillaries, although

having access to both sides of the implanted basement membrane, invariably preferred to attach and grow on only one side (Vracko and Benditt, 1972). Thus, the basement membrane contains structural information which permits or stimulates repopulation of the proper cell types in the proper numbers in the correct spatial arrangement to maintain tissue structure and function.

#### The Basement Membrane in Glomerular Filtration

In addition to physical support and repair functions, certain basement membranes have been theorized to play a role in other physiological processes. The glomerular basement membrane has been thought to be such a structure. From the electron microscopic tracer studies of Farquhar, Wissig, and Palade (1961) a functional model for the glomerulus and its components was reported. The glomerular basement membrane (GBM) was accorded the role of the main filter of the glomerular capillaries (see Figure 4). The endothelium on the blood side of the capillary was said to be a valve which limited access to the filter (GBM) while the epithelium located on the urinary side of the capillary monitored the passage of filtered protein for recovery. The mesangium was thought to maintain the filtration integrity of the GBM by removing substances which might clog the filter by accumulating against it.

Another view of the glomerular filtration mechanism is held by Ryan, Hein, and Karnovsky (1976). They have reported that glomerular filtration barrier function was dependent upon the maintenance of normal blood flow conditions. Two filtration models were proposed by these

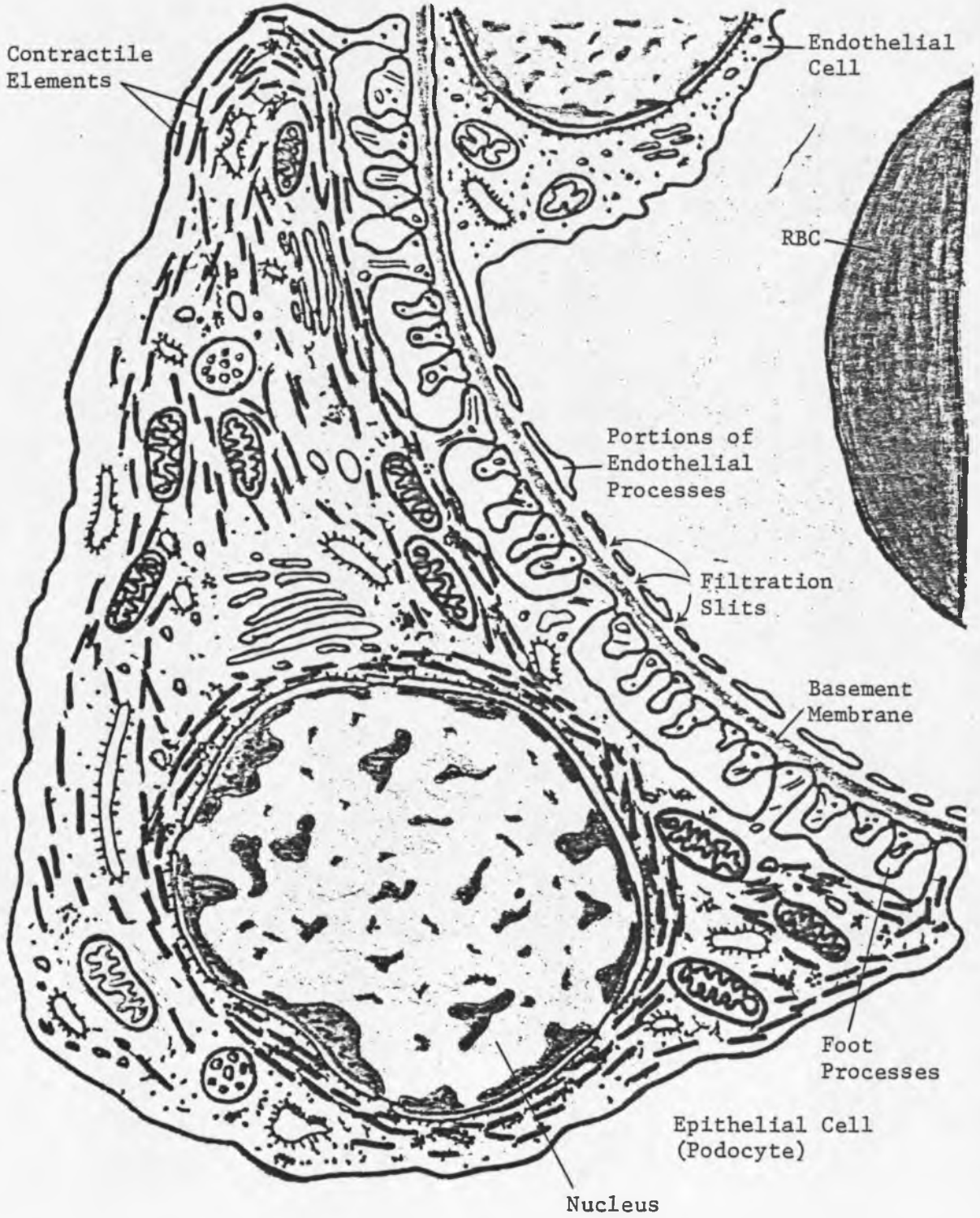


Figure 4. Diagram of a Portion of the Glomerular Capillary

authors to account for their findings. The first proposed mechanism involved the establishment of a "concentration-polarization layer" between the endothelium and GBM. This barrier layer was proposed to arise when large proteins which were held up by the GBM during ultrafiltration inhibited the passage of the smaller blood proteins. The second proposal involved the filtration of proteins not only on the basis of their molecular weight and molecular radius, but charge. Chang et al. (1975) have reported that negatively charged dextrans are held up more than neutral dextrans of the same molecular weight and radius. The location of this charge barrier thought to be in the form of negatively charged glycoproteins was presumed to be either on the endothelial cell surface or the blood side of the basement membrane because plasma proteins were not normally observed to penetrate beyond the endothelial layer (Ryan, Hein, and Karnovsky, 1976). Sialoproteins are also known to coat the epithelial foot processes (Latta, Johnston, and Stanley, 1975). Karnovsky and Ainsworth (1973) have reported electron microscopic tracer studies which suggest that the epithelial slit pores may also possess a filtration capability. In both of their proposed filtration mechanisms Ryan, Hein, and Karnovsky (1976) hypothesize that "the epithelial layer may, by controlling solvent flux, indirectly but significantly modulate the rate of passage of plasma proteins across the GBM".

The administration of puromycin aminonucleoside to rats results in proteinuria within three days and thus provides a model system for studying abnormal glomerular structure and function. When Farquhar and Palade (1961) examined glomerular morphology 9 days after the commencement of daily aminonucleoside injections, they observed not only altered

epithelial structure, but a thickening of the glomerular basement membrane. The glomeruli also exhibited areas of a spongy, less dense material which was presumed to be abnormal basement membrane. Protein tracers were observed to penetrate the glomerular basement membrane of these aminonucleoside treated animals. Farquhar and Palade (1961) reasoned that since the glomerular basement membrane is morphologically altered during puromycin aminonucleoside treatment resulting in proteinuria, the basement membrane is the main filtration barrier. In addition to the observable microscopic changes, Kefalides and Forsell-Knott (1970) have reported an increase in the glucose content of the glomerular basement membrane isolated from rats that had been treated with the aminonucleoside for 11 days. An increased rate of proline incorporation into glomerular basement membrane collagen during aminonucleoside nephrosis had also been observed (Blau and Michael, 1971).

Ryan and Karnovsky (1975) and Fisher and Klein (1963) have examined glomerular structure 3 days after the commencement of aminonucleoside injection. They have reported that while the basement membrane appeared normal the epithelial foot processes were fused and clumped. Michael, Blau, and Vernier (1970) have noted a decrease in the amount of sialoproteins obtained from glomeruli isolated from aminonucleoside nephrotic rats as compared to controls. Interestingly, during proteinuria induced by the injection of large amounts of albumin the sialoprotein content decreased while the epithelial foot processes became fused (Roy, Vernier, and Michael, 1972). The glomerular basement membrane appeared normal during protein load induced proteinuria.

The mechanisms by which aminonucleoside injection or protein load induced proteinuria are not known at the present time. In the case of protein load it appears that proteinuria occurs in the absence of recognizable structural changes in the basement membrane. In the acute stages of aminonucleoside proteinuria, abnormal protein filtration is observed before demonstrable basement membrane changes. In both cases, significant structural alterations are observed in the epithelial foot processes. These findings would favor the Karnovsky model of the sialo-protein coat or epithelial cells as acting as the primary barrier to protein filtration in the glomerulus and not the glomerular basement membrane.

#### The Glomerular Basement Membrane in Disease

The renal glomerulus is the site of numerous disease related processes which result in glomerular basement membrane alterations (Churg and Grisham, 1975). The diseases affecting the glomerular basement membrane fall into two general categories. The first category encompasses diseases which involve a direct attack by extra-glomerular factors on the basement membrane. These are primarily antigen-antibody reactions involving the matrix per se or substances trapped within the matrix. The second category includes diseases in which basement membrane alterations are secondary to changes in glomerular metabolism. The exact nature of these glomerular metabolism changes is not known.

Diabetes mellitus would be classified in this second category. In diabetes the glomerular basement membrane becomes significantly

thicker in width (Østerby and Lundback, 1970). Similar but less dramatic basement membrane thickening is observed in other tissues (Otto, Themann, Watner, 1967; Kilo, Volger, Williamson, 1972; Vracko and Benditt, 1974). Two major questions concerning diabetes and basement membrane alterations have been raised over the years. The first question was how closely does the onset of diabetes correspond to the commencement of basement membrane thickening? Siperstein, Unger, and Madison (1968) examined muscle capillary basement membranes in normal, diabetic and prediabetic patients and found that the basement membrane thickness was smallest in the normal, greatest in the diabetic and intermediate in the prediabetic groups suggesting a hereditary predisposition for basement membrane thickening. More recently, however, more detailed studies of Kilo, Volger, and Williamson (1972) have indicated that the commencement of basement membrane thickening is correlated with the onset and duration of diabetes. Østerby (1973) and Otto, Themann, and Wagner (1967) have also found a correlation between the known duration of diabetes and thickness of capillary basement membrane. Currently, basement thickness is regarded as a function of age and duration of the disease.

The other question that has received considerable attention is whether the glomerular basement membrane composition changes with the duration of diabetes. There is presently a divergence of opinion on this question. Lazarow and Speidel (1964) found an increase in the amount of basement membrane material in glomeruli isolated from human kidneys with long standing diabetes. Although an increase in basement

membrane content was observed, no compositional differences were observed between the normal and diabetic samples. Studies by Kefalides (1974) and Westberg and Michael (1973) have also failed to find any significant differences between normal and diabetic samples although a slight decrease in the half-cystine content was seen in the diabetic samples. Beisswenger and Spiro (1973), on the other hand, have reported finding significantly more hydroxylysine and disaccharide units in diabetic versus normal glomerular basement membrane. A corresponding decrease in the lysine content was also observed. In addition, slight increases in the hydroxyproline and glycine and decreases in the valine and tyrosine contents were reported (Beisswenger and Spiro, 1973; Spiro, 1976). These reported differences in the amino acid content of diabetic glomerular basement membrane samples has not been completely explained. Differences in the duration, severity and control of the diabetic populations sampled might explain the observed compositional discrepancies. Alternatively, the differences in the glomerular basement membrane isolation procedures used by Spiro (1967a) and Kefalides (1966) might also account for the differences observed.

At the present time there are many hypotheses to account for the glomerular basement membrane changes observed in diabetes. Vracko and Benditt (1974) have proposed that diabetic cells are more susceptible to cell injury and destruction than normal cells resulting in an increased diabetic cell turnover. They hypothesize that the cells are programmed to form one quanta of basement membrane during their life span (Vracko, 1974b; Vracko and Benditt, 1970). By increasing the turnover of cells,

basement membrane is formed at a faster rate resulting in a thicker basement membrane (Vracko and Benditt, 1970).

There are many observations, however, which would argue against heredity as being the determinant of basement membrane lesions. Basement membrane thickness correlates with the duration of diabetic metabolic disturbances (Østerby, 1973; Kilo, Volger, Williamson, 1972; Otto, Themann, Wagner, 1967). There is less basement membrane thickening (Jackson et al., 1975; Bloodworth and Engerman, 1973) and a lowered occurrence of nephropathy and retinopathy with good diabetic control (Keiding, Root and Marble, 1952; Hardin et al., 1956; Miki et al., 1969; Pirart, Lauvaux and Eisendrath, 1975; Job et al; 1975). Additionally normal animals in which diabetes was experimentally induced demonstrated microangiopathies. Normal patients affected with pancreatic diseases resulting in a loss of insulin activity also show microangiopathies. Glomerular capillary mesangial lesions resulting from induction of experimental diabetes regressed following pancreatic islet transplantation (Mauer et al., 1975). Taken together, these observations would argue for either a direct or indirect role for insulin in the development of microangiopathies (Spiro, 1976). Peterson, Greene, and Reaven (1971) observed that ribosomes isolated from insulin deficient rat kidney cortex were more active in peptide chain elongation than ribosomes from normal animal kidneys. Mogensen and Andersen (1975) observed that insulin normalized kidney size after a diabetes induced kidney enlargement. These findings suggest that insulin results in decreased protein synthesis. This might account for the increased basement membrane thickness

observed during states of insulin inadequacies (i.e., diatebes). At present it is not known whether insulin itself or an indirect effect of insulin such as on glucose metabolism affects basement membrane metabolism.

Diabetes appears to have a selective effect on certain steps in the basement membrane synthetic pathway. R. Spiro and M. Spiro (1971b) found significantly elevated glucosyltransferase activity in rat kidney cortex samples of diabetic rats as compared to controls. Khalifa and Cohen (1975) have reported increased lysyl hydroxylase activity in glomerular preparations from diabetic rats. These findings would support the hypothesis of increased synthesis of the basement membrane collagen component. Enzymes thought to be responsible for the formation of the heteropolysaccharide were not elevated under experimental diabetic conditions (R. Spiro and M. Spiro, 1971b, 1971c).

Increased hydroxylation followed by a concomitant increase in the disaccharide attachment activity are post-translational processes which may be regulated by blood glucose levels (R. Spiro and M. Spiro, 1971b; Khalifa and Cohen, 1975). The changes in glomerular basement membrane glycine, tyrosine and valine content (Spiro, 1976) are more difficult to explain by alterations in post-translational events. More reasonable would be changes in the rate of formation of different basement membrane component proteins, preferential incorporation of certain basement membrane components, or a selective degradation of certain basement membrane components.

In addition to insulin and blood sugar levels, growth hormone has been implicated in the progression of diabetic microangiopathies (Lundbaek, 1971). Mogensen and Andersen (1975) have reported increased levels of somatotropin in juvenile diabetics. Decreasing the blood levels of somatotropin by pituitary ablation slowed the rate of progression of diabetic retinopathy (Luft and Guillemin, 1974; Ray et al., 1968). Somatotropin-deficient diabetic dwarfs have been found to be resistant to the development of microangiopathies (Merimee et al., 1970). The interrelationship between insulin, blood glucose and growth hormone in the development of basement membrane lesions requires further study.

#### Rationale and Objectives

The study of the basement membrane in health and disease has been hampered by a number of methodological problems. First and foremost has been the lack of methodology for the isolation of morphologically intact highly purified basement membranes from tissues known to be affected in various disease states which result in basement membrane lesions. The question of whether or not the composition of the glomerular basement membrane changes during diabetes may well rest on the development of improved basement membrane isolation techniques. As a corollary to the improvement of basement membrane isolation techniques, the collection of pure suborgan fractions from which to isolate basement membrane is necessary. For all but a few suborgan preparations, the purification of suborgan fractions results in the contamination of the fraction with organ debris or unwanted structures. Consequently, the

first objective of this study will be to develop improved methods for the isolation of pure morphologically intact basement membranes from disease susceptible suborgan fractions. In this regard, Meezan et al. (1973) have developed a procedure for the isolation of a highly purified rat kidney glomerular preparation which is 99% free of tubular contamination. Isolated brain microvessels (Brendel, Meezan, and Carlson, 1974) and retinal blood vessel (Meezan, Brendel, and Carlson, 1974) preparations are also available in high purity. Using these isolated suborgan fractions and appropriate reference tissues, basement membranes will be isolated and analyzed. The structural integrity of the isolated basement membranes will be monitored by light and electron microscopy.

Because of the lack of a procedure for the acquisition of highly purified basement membranes, biochemical studies of basement membrane synthesis have examined primarily non-matrix bound newly formed proteins (Krisiko and Walker, 1974; Williams et al., 1976; Killen, Quadracci, Striker, 1974; Brown and Michael, 1973; Cohen and Vogt, 1975). Since the basement membrane is defined by its anatomical appearance, the use of systems in which the matrix is not purified may be susceptible to non-basement membrane protein contamination. In part due to the difficulty of isolating the basement membrane matrix and the relatively slow turnover of basement membrane proteins (Walker, 1973; Spiro, 1976), the processes associated with the deposition of newly formed basement membrane proteins are poorly defined. Thus, the second objective of this study will be to examine the deposition of newly formed proteins into the basement membrane matrix. The isolated rat glomerular

preparation of Meezan et al. (1973) will be used. This glomerular preparation has been demonstrated to be metabolically active and morphologically intact (Meezan et al., 1973; Brendel and Meezan, 1973). In addition, gluconeogenic activity was observed in this glomerular preparation (Brendel and Meezan, 1973). This finding of gluconeogenic activity in a suborgan fraction known to be altered in diabetes provided an opportunity to study the role of glucose in basement membrane formation. Beisswenger and Spiro (1970) reported that diabetic glomerular basement membranes contained substantially more glucose and galactose than normal glomerular basement membrane samples. Spiro (1971) hypothesized that in diabetes, glucose was shunted to insulin insensitive metabolic pathways. It was implied that the glycosylation of basement membrane proteins was such a pathway (Spiro, 1971). The finding of glucose production in the glomerular preparation raised the question of which pool of glucose might be responsible for this proposed increased glucose utilization. Thus, the third objective of this study will be to determine if glomerular gluconeogenesis is a factor which could influence basement membrane synthesis by increasing the glucose levels in the cells where basement membrane synthesis is occurring.

## CHAPTER 2

### MATERIALS AND METHODS

#### Tissue Isolation

##### Rat Glomeruli

Male Sprague-Dawley rats weighing 250 - 300 gms were used in all metabolic experiments. They were given Wayne Lab-Blox<sup>R</sup> (Allied Mills) and tap water ad libitum. For gluconeogenic experiments, the animals were allowed only tap water for the 24 hours prior to the experiment. Rats were anesthetized with an intraperitoneal injection of 0.1 ml/100 gms Sodium Nembutal<sup>R</sup>. A midline incision was made and the abdominal musculature retracted. The mesenteric artery was ligated. Loose ligatures were placed on the descending aorta above and below the branches to the renal arteries. Cannulation of the descending aorta below the renal arteries followed complete ligation at the upper aortic site. The ascending vena cava was then severed to permit drainage of the perfusate. The kidneys were perfused with 20 ml of Earle's balanced salt solution (116 mM NaCl, 16 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.8 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.6 mM glucose, 1.8 mM CaCl<sub>2</sub>) buffered with HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (pH 7.4) fortified with 0.1% bovine serum albumin and 50 mg/l Penicillin G<sup>R</sup> followed by 10 ml of an iron oxide suspension (0.72 gm/100 ml) consisting of buffer with 4% polyvinylpyrrolidone. Physiological pressures were maintained throughout the kidney perfusion. The kidneys were removed, minced with scissors and homogenized with four up and down strokes with a hand-held homogenizer (Arthur H. Thomas Co.,

Philadelphia, PA), which had a tapered loose fitting teflon pestle in a glass tube.

The homogenate was poured onto a 210  $\mu$  pore size nylon sieve (Kressilk Products Inc., Monterey Park, CA) and washed by spraying with buffer. The material passing through the 210  $\mu$  sieve was then washed through a series of 153  $\mu$ , 110  $\mu$ , and 64  $\mu$  pore size sieves, respectively. The 110  $\mu$  pore size sieve was included in the isolation regimen only when the gluconeogenic activity of the various glomerular fractions was being studied. See Figure 5. The material retained on the 110  $\mu$  and/or 64  $\mu$  sieves was suspended in buffer and held over a permanent magnet. The iron oxide particles lodged in the glomerular capillaries caused the glomeruli to rapidly aggregate near the magnet. The non-magnetic material was decanted off. Well defined, recognizable free tubules made up approximately 80% of the non-magnetic material retained on the 64  $\mu$  sieve with the balance of the material being small, ill defined structures, the majority of which were probably of tubular origin. This fraction was the source of rat kidney tubules used in the gluconeogenic experiments. After several decantation washings, the magnetic material consisted of glomeruli 99% free of tubular contamination.

A further modification of this procedure allowed the separation of glomerular tufts and glomerular tufts with attached Bowman's capsule (Hjelle, Meezan, and Brendel, 1975). This involved suspending the glomeruli, which were freed of tubular contamination by the iron oxide procedure, in Krebs' bicarbonate buffer in a plastic Falcon petri dish. After 5 - 10 minutes those glomeruli with attached Bowman's capsule

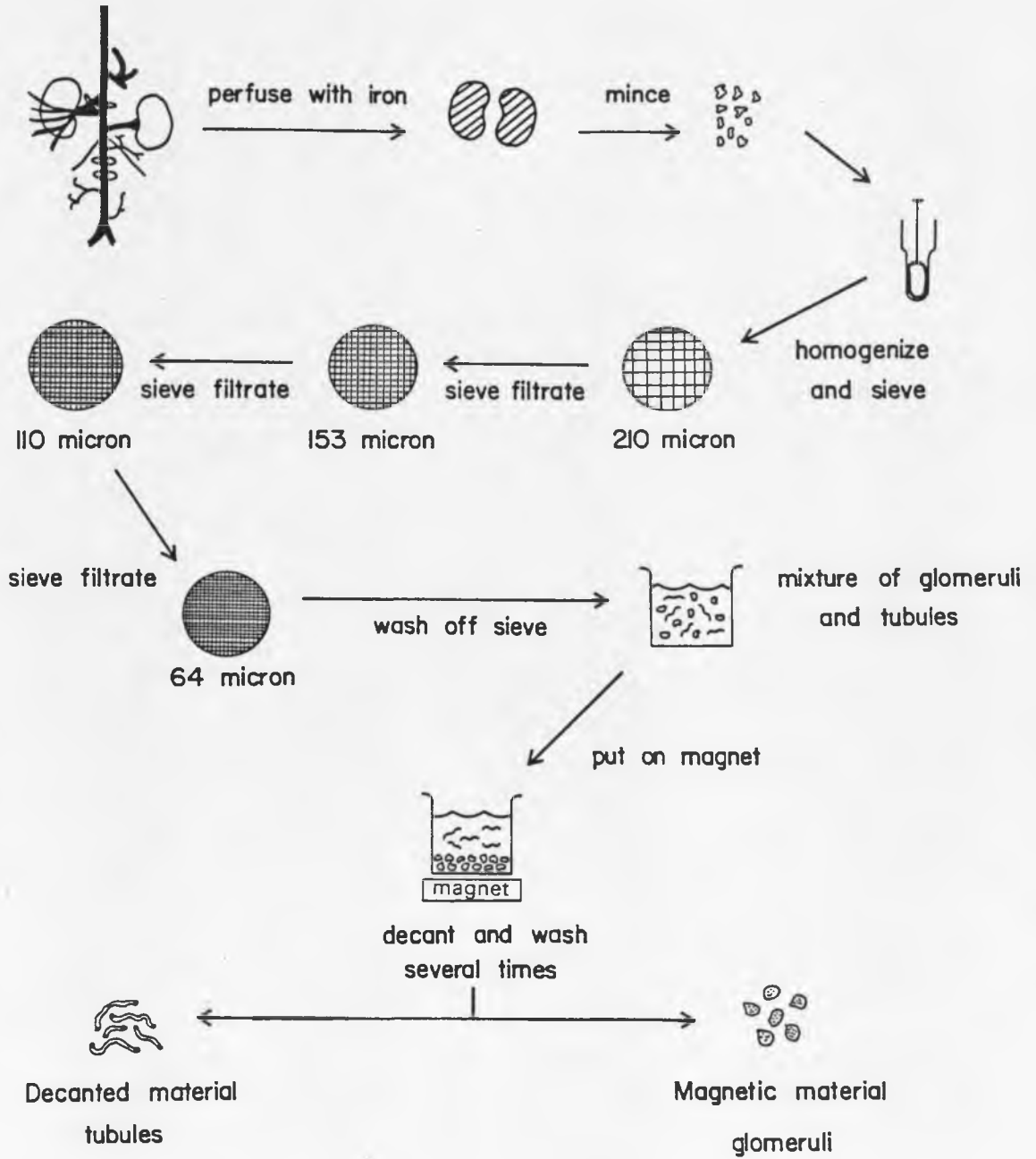


Figure 5. Iron Oxide Glomerular Isolation Procedure

adsorbed to the surface of the petri dish. The glomerular tufts remained unattached and could be collected by decantation. The glomeruli with attached Bowman's capsule could be removed from the petri dish by a mild rinse with albumin containing buffer. The suspended preparation was approximately 95% free of glomeruli with attached Bowman's capsule. Approximately 80% of the glomerular tufts in the non-suspended fraction retained their Bowman's capsule, although this percentage varied among preparations. Further purification of these preparations was accomplished by removing contaminating structures with a drawn pipet while viewing the glomeruli through a dissecting microscope.

#### Rabbit Proximal Tubules

New Zealand white rabbits weighing 2 - 3 Kg were used in all experiments. The rabbits were maintained on Wayne Rabbit Blend<sup>R</sup> (Allied Mills) and tap water ad libitum. The rabbits were given only tap water for the 24 hours prior to gluconeogenic experiments. Initial experiments employed acetaparaldehyde (0.5 mg/kg) given as an intraperitoneal injection followed by diethyl ether to bring the animals to a surgical plane of anesthesia. Subsequent experimentation has shown that killing of the animal with a blow to the head permits the necessary in situ surgery to be performed without adverse effect on the metabolic activity of the isolated tubules. Because of the larger size of the rabbit relative to the rat, the renal arteries could be cannulated directly. The kidneys were perfused under physiological pressures with Earle's balanced salt solution fortified with 0.01% bovine serum albumin and 50 mg/l

Penicillin G<sup>R</sup>. For gluconeogenic experiments, the glucose was omitted from the buffer. After complete removal of blood elements as indicated by a lightening in color of the kidney, a suspension of iron oxide 0.72 gm/l was perfused in Earle's buffer containing 4% PVP. The kidney cortex was then removed with surgical forceps and suspended in buffer in a hand homogenizer equipped with a loose fitting Teflon plunger. Five up and down strokes with the hand homogenizer disrupted the cortical material sufficiently to permit sieving. The homogenate was poured through a 210  $\mu$  pore size sieve and washed by spraying with buffer. The material passing through the sieve was then poured onto a 153  $\mu$  sieve. The material washed through the 153  $\mu$  sieve was placed on a 64  $\mu$  pore size sieve. The material retained on the 64  $\mu$  sieve consisted of 95% tubules with 5% iron oxide filled glomeruli. Following a buffer wash on the 64  $\mu$  sieve, the tubules and glomeruli were washed off the sieve into a plastic beaker and placed over a magnet. The suspended tubules were then decanted into a plastic centrifuge tube while the iron oxide filled glomeruli remained at the bottom of the beaker attracted by the magnet. Centrifugation of the tubule suspension at 2000 x g resulted in a tubule pellet 99% free of any contaminating structures.

To compare the metabolic activity of the tubules obtained by the homogenization and sieving procedure to that of tubules prepared by the classical enzyme digestion procedure of Burg and Orloff (1962), one kidney was cannulated and perfused with Earle's buffer as described above. The contralateral kidney was perfused with 15 ml of Earle's buffer containing 1 mg/ml collagenase type II (Worthington Biochemical Corp.,

Freehold, NJ). The collagenase treated kidney was then minced and suspended in 40 ml of the collagenase solution. After 30 minutes, the kidney mince was filtered through cotton gauze. Subsequent experimentation had demonstrated that the tissue could be more efficiently handled if the cotton gauze was replaced by nylon sieving material. Thus, for the experiments described the cotton gauze used by Burg and Orloff (1962) was replaced with nylon sieves. Extensive washing of the collagenase treated material was required due to the avidity with which the collagenase attached to the tubular basement membrane. The collagenase treated tubules were collected on a 110  $\mu$  pore size nylon sieve.

To compare the effects of collagenase perfusion on the metabolism of the isolated tubules, an aliquot of the tubules isolated by the iron oxide perfusion-homogenization method was suspended in Earle's buffer containing 1 mg/ml collagenase for 30 minutes. The metabolic activities of the non-collagenase treated tubules, the collagenase incubated tubules, and the collagenase perfused and incubated tubules were then measured.

#### Bovine Retinal Blood Vessels

Isolation of bovine retinal blood vessels employed the method of Meezan, Brendel, and Carlson (1974). Freshly enucleated bovine eyes were obtained from a local slaughter house (Jones Meat Packers). The retinas were removed and placed in Earle's buffer. The retinas were kept on ice in Earle's buffer while being transported to the laboratory. Mincing of the retinas with scissors was followed by an initial

homogenization of 10 up and down strokes in a smooth glass hand-held homogenizer equipped with a Teflon plunger. The homogenate was poured onto an 86  $\mu$  pore size sieve and washed by spraying with buffer. After extensive washing, the retained material was rehomogenized with 5 up and down strokes of the hand homogenizer. The homogenate was again poured over an 86  $\mu$  sieve and washed with buffer. The retained material was 99% free of non-vascular material. Figure 6 illustrates this procedure.

#### Bovine Brain Blood Vessels

Following the method of Brendel, Meezan, and Carlson (1974), cortical blood vessels were isolated from bovine brains obtained from a local slaughter house. The brains were kept on ice during transport to the laboratory. Pieces of cortical tissue were removed from the brain with surgical forceps and placed in a hand-held smooth glass tube equipped with a loose fitting Teflon pestle. Hand homogenization with 10 up and down strokes of the cortical pieces suspended in Earle's buffer resulted in the disruption of the majority of non-vascular elements. The homogenate was poured over a 153  $\mu$  pore size sieve which retained the vascular elements but passed the disrupted non-vascular material. The retained vessels were washed with spray of buffer. Complete removal of adhering non-vascular material was accomplished by a second 10 - 15 stroke homogenization of the retained vessels. A resieving of the homogenate and buffer wash gave a highly purified brain vessel preparation.

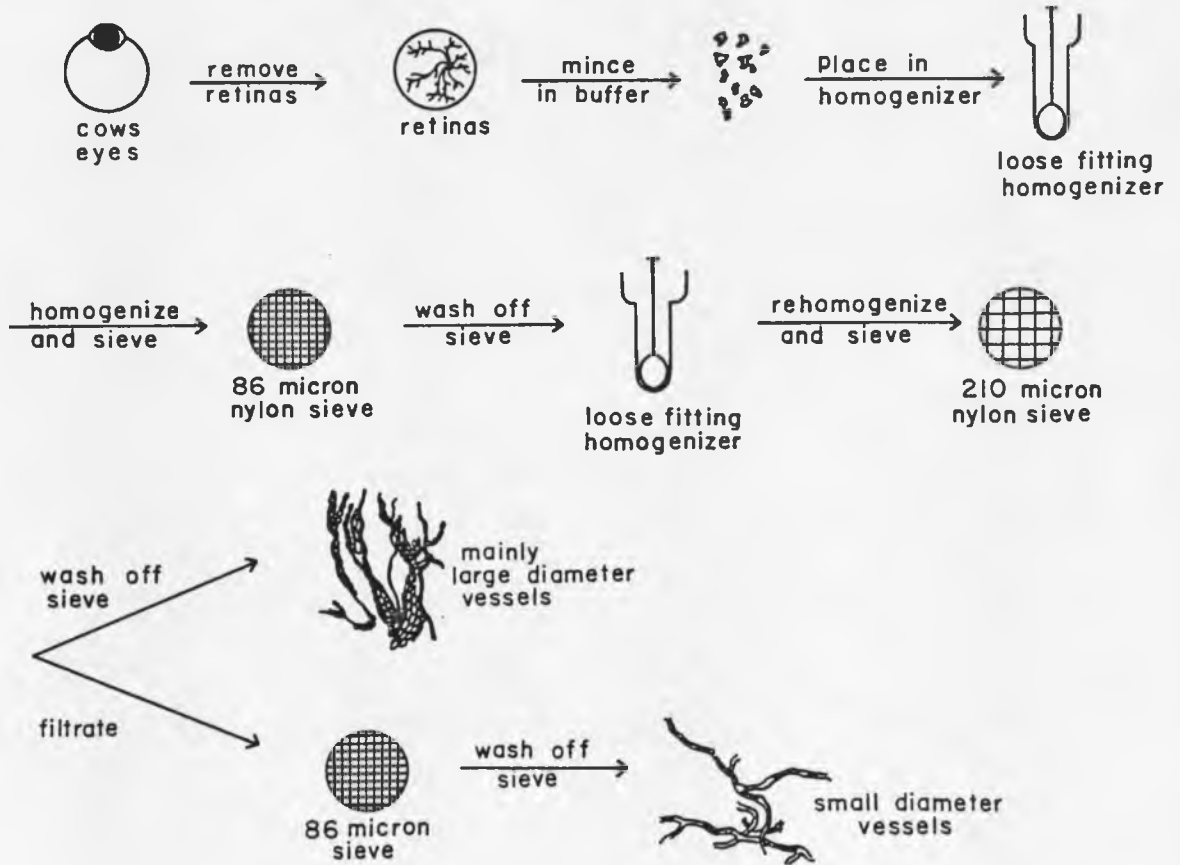


Figure 6. Microvessel Isolation Procedure

## Bovine Lens Capsule

The lens capsule from enucleated bovine eyes was obtained by surgically removing the lens. The lens was then bisected and the lens capsule removed with forceps and washed with a spray of buffer.

### Basement Membrane Isolation

The procedure for the isolation of the basement membrane from the various suborgan preparations has previously been reported by our laboratory (Meezan et al., 1975) and is shown in Figure 7. Cell lysis was achieved by stirring the tissue in a large volume (100:1) of distilled water containing 0.1% sodium azide for 1 - 2 hours. After centrifugation the pellet was suspended and stirred in 40 ml of 1 M NaCl containing 2000 Kunitz units of DNAase (Sigma, Deoxyribonuclease I) for 1 - 2 hours. This suspension was centrifugated to obtain a firm pellet. Resuspension of the pellet in a 4% sodium deoxycholate (DEOC) solution containing 0.1% sodium azide was followed by 2 - 4 hours of stirring at room temperature. The DEOC suspension was either pelleted and washed several times with distilled water or poured onto a 44  $\mu$  nylon sieve and extensively washed by spraying with distilled water. This was the procedure for the isolation of basement membranes used for chemical and morphological analyses.

### Tissue Preparation for Microscopic Examination

Phase contrast photomicrographs of bovine brain and retinal blood vessels suspended in phosphate-free saline were obtained with the

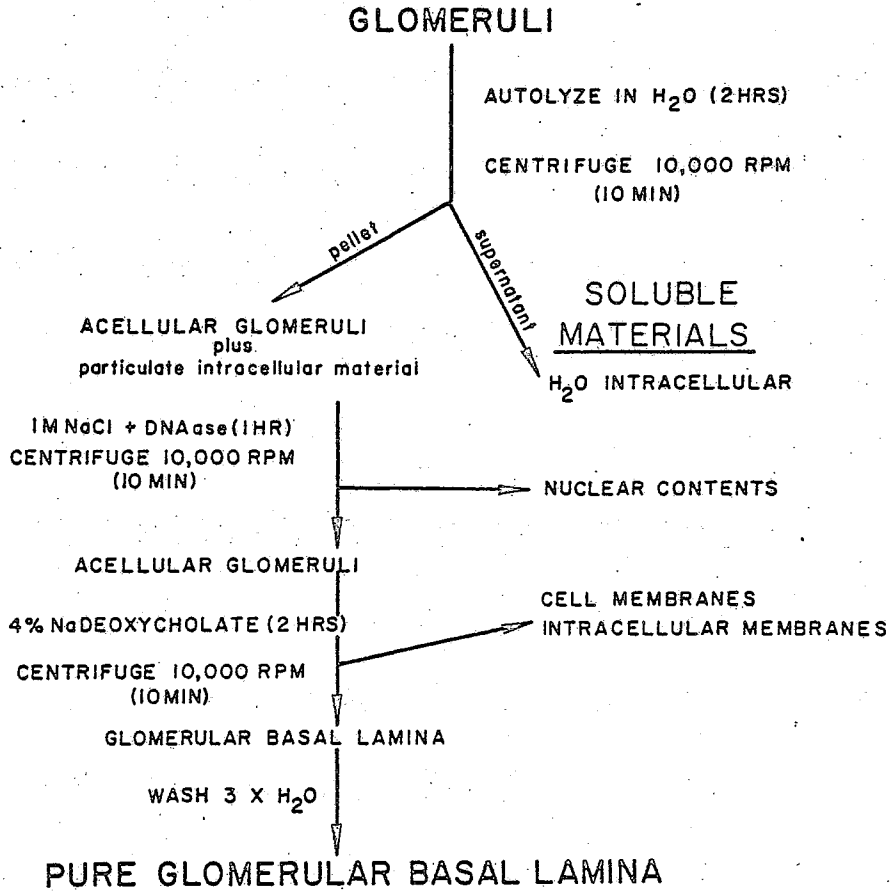


Figure 7. Deoxycholate Basement Membrane Isolation Procedure

Although glomeruli are listed in this scheme, purified basement membrane preparations can be obtained from numerous other suborgan fractions.

use of a Zeiss Ultraphot TT microscope equipped with phase optics.

Transmission and scanning electron microscopic examination of tissue and basement membrane samples were performed by Dr. Edward C. Carlson of the Department of Anatomy, University of Arizona Medical School.

Isolated suborgan fractions and the basement membrane derived from these fractions were fixed 1 hour in cold (4° C) Karnovsky's fixative buffered with 0.2 M sodium cacodylate at pH 7.3 (Karnovsky, 1965). This was followed by post-fixation in 2% OsO<sub>4</sub> buffered with 0.144 M sodium cacodylate buffer. To simplify tissue manipulation, a drop of warm 2% Nobel agar was placed over the pelleted tissue. These pellets and surrounding coagulated agar were dehydrated in an ascending series of graded ethanols (50% to 100%) and propylene oxide before embedding in Epon-Araldite (Anderson and Ellis, 1965). Epoxy blocks were cured overnight at 37° C and 48 hours longer at 60° C. Ultramicrotomy was carried out on a Sorvall MT-2 ultramicrotome equipped with glass or diamond knives. One micron thick sections were stained with toluidine blue (1% toluidine blue in 1% sodium borate) and observed at the level of light microscopy for determination of pellet orientations. Thin sections were mounted on uncoated 200 mesh copper grids and stained with lead citrate (Venable and Coggeshall, 1965) and uranyl acetate (5% in absolute ethanol). Tissue examination was carried out on a Philips EM 300 electron microscope at original magnifications of 5600 - 16000. This procedure has previously been reported (Meezan et al., 1975).

Tissue preparation for scanning electron microscopy followed a different procedure. The suborgan fractions were fixed in 2.5%

glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 24 - 37° C for at least one hour. Following a thorough rinse with phosphate buffer (pH 7.4), the tissue was post-fixed in 1% OsO<sub>4</sub> in 0.1 M phosphate buffer for one hour. Tissue dehydration was accomplished by suspending the tissue in an ascending series of acetone solutions (20%, 40%, 60%, 80%, 100%, 100% and 100%). After critical point drying, the tissue was placed on a specimen stub with double sided tape. Finally, the tissue was coated with evaporated carbon and gold/palladium.

#### Chemical Analysis of Isolated Basement Membranes

##### Carbohydrate Analyses

The isolated basement membrane fractions were hydrolyzed 4 hours in 2 N trifluoroacetic acid at 115° C in 1.5 ml Teflon capped conical tubes. The hydrolysate was evaporated to dryness and dissolved in 1 ml of 0.5 N NH<sub>4</sub>OH; 15 mg of NaBH<sub>4</sub> was added, and the reduction was allowed to proceed for 1 hour. Excess NaBH<sub>4</sub> was then destroyed by dropwise addition of glacial acetic acid and the reaction mixture evaporated to dryness. The residue was taken up in 0.3 ml of glacial acetic acid and 2 ml of acetic anhydride and heated at 120° C for 90 minutes. After evaporation to dryness, the residue was partitioned between 0.5 ml of saturated Na<sub>2</sub>CO<sub>3</sub> and 2 ml of CH<sub>2</sub>Cl<sub>2</sub>. The separated organic phase was removed by evaporation and the residue dissolved in 100 µl of acetone and injected onto a 3% OV 225 gas chromatography column. Peaks of the neutral and amino-sugar alcohol acetates were well separated and eluted

within 30 minutes and were quantitated by cutting and weighing the peaks using 2-deoxyglucose as an internal standard. The above procedure was a modification of that of Grimes (1974).

#### Amino Acid Analyses

The isolated basement membrane fractions were hydrolyzed in glass distilled 6 N HCl under nitrogen for 22 hours at 105° C in vacuum hydrolysis tubes (Pierce Chemical Co., Rockford, IL). Initial analyses were performed by the standard procedure of Moore and Stein (1954) using a Beckman model 118 amino acid analyzer. This resulted in the separation and quantitation of all the sample amino acids except histidine which consistently gave values that were greater than those previously reported. This was probably due to the similar chromatographic characteristics of the crosslinked amino acids known to be present in extracellular matrices (Gallop and Paz, 1975). To avoid this problem and to increase the sensitivity of the analysis, the gas chromatographic amino acid analysis method of Gehrke, Roach, Zumwalt, Stalling and Wall (1968) was employed. This involved evaporation of the hydrolyzate in a warm miniaktor tube (Applied Science, State College, PA) with a gentle stream of nitrogen. Dry butanol:3 N HCl (Regis Chemical Co., Morton Grove, IL) was added to the dry residue. A layer of Teflon tape was placed between the screw cap and the glass miniaktor tube. The residue was suspended in butanol:HCl mixture by placing the capped tube in a sonicator for 1 minute. The tube was then heated in an oil bath for 30 minutes at 100° C. After butylation the tube was cooled and evaporated to dryness with

nitrogen. Trifluoroacetic anhydride (Aldrich Chemical Co., Milwaukee, WI) which had been distilled over phosphorus pentoxide was mixed with dry methylene chloride (1:2) and added to the dry butylated residue. The miniaktor tube was sealed as described above and heated in an oil bath at 145° C for 5 minutes. After the tubes had cooled to room temperature the N-TFA-n-butyl ester derivatives of the sample amino acids were ready for injection. A model 1200 Varian Aerograph with a four-column oven, flame ionization detectors, dual differential electrometers equipped with a Varian CDS-101 integrator and Varian Model 20 recorder was used. The glass columns were packed with either ethylene glycol adipate stabilized (EGA) on Chromosorb W (0.65% x/w/) or 2.0 w/w% OV-17 + 1.0 w/w% OV-210 on Gas Chrom Q (Applied Science, State College, PA). The resins were prepared by the method of Kaiser et al. (1973).

### Metabolism

#### Gluconeogenesis

Rat gluconeogenic experiments were carried out by incubating tubule and glomerular fractions isolated from 24 hour starved rats in Earle's buffer with radiolabeled <sup>14</sup>C-substrate in a disposable plastic vial for 90 minutes at 37° C in a shaking water bath. The method of Brendel and Meezan (1973) was used to assay for <sup>14</sup>C-glucose. The incubation vial was centrifuged and the incubation medium removed and placed on a mixed bed ion exchange column. The column consisted of a mixture of Ag 50 200 mesh hydrogen form and Ag 1 200 mesh bicarbonate form resins. After the column was washed with a 0.17 M glucose solution,

an aliquot of the effluent was counted on a Beckman LS-250 scintillation counter. This represented the total neutral  $^{14}\text{C}$ -labeled material formed. The remainder of the effluent was incubated with glucose oxidase (Glucostat<sup>R</sup>). This converted the  $^{14}\text{C}$ -glucose which does not bind to the column to  $^{14}\text{C}$ -gluconic acid which does. The  $^{14}\text{C}$ -material obtained after application of the Glucostat<sup>R</sup> incubation medium to a mixed bed column and elution with 0.1% glucose solution represented counts per minute other than  $^{14}\text{C}$ -glucose which were eluted through the first column. Subtraction of the post-glucose oxidase counts from the gross glucose counts gave the total  $^{14}\text{C}$ -glucose produced. Disruption of the glomerular tissue by sonication to remove intracellular  $^{14}\text{C}$ -glucose did not significantly increase the amount of  $^{14}\text{C}$ -glucose measured when only the incubation medium was assayed.

Rabbit gluconeogenic experiments were carried out by incubating the proximal tubule preparations isolated from 24 hour starved rabbits in Earle's buffer with radiolabeled  $^{14}\text{C}$ -substrates in glass shell vials (Arthur H. Thomas Co., Philadelphia, PA). After dispensing equal aliquots of each tubule preparation into the dram shell vials,  $^{14}\text{C}$ -substrates were added to each vial and incubated at 37° C. At various times some of the vials were centrifuged and the medium removed and assayed for  $^{14}\text{C}$ -glucose. The pellets were used for protein analysis according to the method of Lowry, Rosebrough, Farr and Randall (1951) or Böhlen, Stein, Dairman and Udenfriend (1973).

## Oxidative Metabolism

The production of  $^{14}\text{CO}_2$  from several  $^{14}\text{C}$ -labeled substrates was used as an index of oxidative metabolism. The collection and measurement of the  $^{14}\text{CO}_2$  produced by the isolated suborgan fractions was facilitated by the use of a continuous monitoring apparatus described by Brendel and Meezan (1974). This apparatus allowed the simultaneous collection of  $^{14}\text{CO}_2$  from 10 different samples. The incubation buffer consisted of Earle's balanced salts buffered with 28 mM HEPES and fortified with 0.01% bovine serum albumin and 50 mg/l Penicillin G<sup>R</sup>. The  $^{14}\text{C}$ -substrates were added directly to the tissue suspended in buffer in 3 ml plastic disposable beakers. The beakers were then attached to the apparatus and incubated at 37° C in a shaking water bath. At 20 to 30 minute intervals, the  $^{14}\text{CO}_2$  collecting vials were replaced and filled with scintillation cocktail. Summation of the  $^{14}\text{C}$  counts per minute gave the time course of  $^{14}\text{CO}_2$  production for each incubation vial.

## Transport and Incorporation

Suborgan fractions were incubated in Earle's buffer at 37° C in a shaking water bath in plastic round bottom centrifuge tubes. At various times after the addition of radiolabeled substrate, multiple aliquots were removed and placed on glass fiber filters and washed with a spray of 0.9% NaCl. Half of the aliquots were washed with cold 10% TCA followed by cold 70% ethanol. The filters were placed in scintillation vials and suspended in 1 ml of 0.1 N NaOH, the filters disrupted by sonication and diluted to 1.5 ml with 0.1 N NaOH. Scintillation

cocktail was added and the vials counted. The saline washed counts represent the total uptake of labeled substrate while the TCA-ethanol treated sample counts represent only particulate of incorporated substrate.

When *p*-aminohippuric acid (PAH) transport was studied, the determination of the ratio of acid labeled PAH taken up into the tissue to that remaining in the medium was necessary. The buffer used in these experiments consisted of 115 mM NaCl, 5 mM KCl, 10 mM sodium acetate, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 1.0 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub> gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The isolated tubules were incubated in buffer with <sup>14</sup>C-*p*-aminohippuric acid. At various times, aliquots were removed and placed on glass fiber filters and washed with buffer. The filters containing the washed tubules were placed in scintillation vials, suspended in 1.5 ml of 0.1 N NaOH, disrupted by sonication, and taken up into scintillation cocktail and counted. This gave the amount of <sup>14</sup>C-PAH taken up by the isolated tubules. To determine tissue volume, an aliquot of the incubate was placed in a graduated cylindrical bottomed tube and centrifuged at 2000 x g for 3 minutes. The pellet height provided an index of tissue volume. An aliquot of the supernatant was then counted. The amount of <sup>14</sup>C-PAH in a volume of supernatant equivalent to that of the tissue volume could thus be calculated. The ratio of the amount of <sup>14</sup>C-PAH in the washed and filtered tubules to the amount of <sup>14</sup>C-PAH in a volume of supernatant equivalent to that of the tissue volume gave the tissue to medium ratio. The tissue to medium ratio provides an index of active transport processes and thus tissue viability.

### Basement Membrane Metabolism

#### Incubation Conditions

The isolated suborgan fraction was incubated in Earle's balanced salt solution fortified with Minimum Essential Medium Vitamins (Grand Island Biological Co., Grand Island, NY) (1.0 mg/l D-Ca pantothenate, 1.0 mg/l choline chloride, 1.0 mg/l folic acid, 2.0 mg/l i-inositol, 1.0 mg/l nicotinamide, 1.0 mg/l pyridoxal HCl, 0.1 mg/l riboflavin and 1.0 mg/l thiamine) and 50 mg/l Penicillin G<sup>R</sup>. When non-radiolabeled amino acids were added to the incubation buffer, Minimum Essential Medium Amino Acid Solution (Grand Island Biological Co., Grand Island, NY) was used. The resulting incubation amino acid concentration was 105 mg/l arginine HCl, 24 mg/l cystine, 292 mg/l glutamine, 31 mg/l histidine, 52.5 mg/l isoleucine, 52.4 mg/l leucine, 58 mg/l lysine, 15 mg/l methionine, 32 mg/l phenylalanine, 48 mg/l threonine, 10 mg/l tryptophane, 36 mg/l tyrosine, and 46 mg/l valine. When radiolabeled carbohydrates were used, glucose was omitted from the incubation medium.

The suborgan fractions were suspended in 4 ml of incubation buffer before the addition of radiolabeled amino acids or carbohydrates. The radiolabel was added in incubation buffer. When inhibitory compounds were used, they were dissolved in incubation buffer before the addition of the tissue. Incubation was carried out in 10 ml plastic centrifuge tubes at 37° C in a shaking water bath.

### Basement Membrane Isolation Procedure

After various times of incubation, aliquots of the incubation medium were placed in 1.5 ml microfuge tubes and centrifuged on a Beckman Microfuge<sup>R</sup>. Glomeruli were microfuged for 30 seconds while non-iron oxide filled tissues were microfuged for 1 minute. The supernatants were removed with a drawn pipet after which the pellets were each twice suspended in 1 ml of saline and microfuged. One ml of 4% DEOC with 0.01% sodium azide was added to each saline washed pellet with vigorous vortexing. After standing overnight at room temperature, the DEOC tissue suspensions were microfuged for 2 minutes and the supernatants removed. The pellets were suspended in 1 ml of saline, vigorously vortexed and microfuged for 2 minutes. Following removal of the supernatant, the resulting pellet was again suspended in 1 ml of 4% DEOC, pelleted, twice suspended in 1 ml of saline followed by microfugation. The final pellet was suspended in 0.1 N NaOH and heated at 60° C overnight to solubilize the basement membrane proteins for protein analysis. A 100 µl aliquot was removed from each microfuge tube for protein determination by the fluorescamine method of Böhlen et al. (1973) using bovine serum albumin as a standard. The remaining 900 µl was neutralized with HCl, taken up into scintillation cocktail and counted.

### <sup>14</sup>C-Hydroxyproline Determination

To measure the formation of <sup>14</sup>C-hydroxyproline from <sup>14</sup>C-proline, suborgan fractions were incubated with <sup>14</sup>C-proline which had been purified by ion exchange chromatography. Purification of the <sup>14</sup>C-proline

was necessary due to the presence of labeled material which co-chromatographed with standard  $^3\text{H}$ -hydroxyproline. After incubation, DEOC soluble and DEOC insoluble materials were separately dialyzed against distilled water. The non-dialyzable material was lyophilized, taken up into 6 N HCl and hydrolyzed at  $110^\circ\text{C}$  for 18 hours. After hydrolysis, the hydrolyzates were evaporated to dryness, taken up into 0.01 N HCl and placed on Ag 50 200 mesh hydrogen form ion exchange columns. The columns were washed with distilled water followed by elution of the radiolabel by concentrated ammonium hydroxide. After evaporation, these residues were dissolved in 0.2 N sodium citrate buffer pH 3.19. Separation of hydroxyproline from proline was effected by injecting a 250  $\mu\text{l}$  aliquot of the citrate buffered sample onto an Aminex-27 anion exchange column (Bio Rad Laboratories, Richmond, CA). See Figure 8.

### Polyacrylamide Gel Electrophoresis

#### Gel Preparation

Two procedures were used for preparation of the polyacrylamide gels. The initial system employed 7% gels which were made by mixing 1 part solution A (48 ml 1 N HCl, 36.3 gm Tris, 0.23 ml TEMED diluted to a total volume of 100 ml with  $\text{H}_2\text{O}$ ), 2 parts solution C (28.0 gm acrylamide, 0.735 gm Bis diluted to 100 ml with distilled  $\text{H}_2\text{O}$ ), 1 part  $\text{H}_2\text{O}$  and 4 parts 0.61 mM ammonium persulfate.

A 5% gel system was prepared by adding 15 ml of buffer consisting of 0.2 M Tris-phosphate, 0.2% SDS (pH 6.8) fortified with 0.4 ml of TEMED to 14.4 gm urea, 0.052 gm Bis and 1.56 gm acrylamide. After

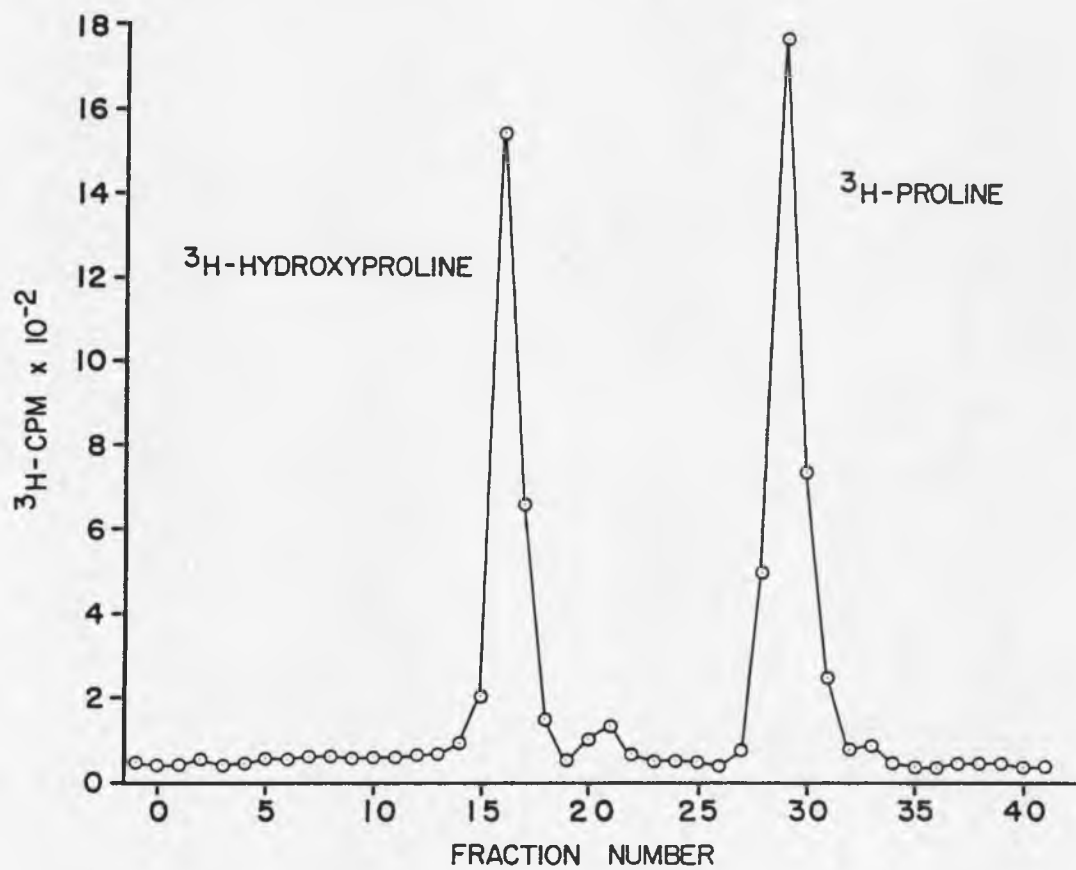


Figure 8. Chromatographic Separation of Hydroxyproline and Proline

the mixture was completely dissolved, 0.2 ml of 0.65 M ammonium persulfate was added.

After the gels were poured they were overlaid with distilled water and allowed to set for 1 - 2 hours before removal of the water. Pyronin Y was employed as the tracking dye.

#### Tissue Solubilization

Experiments utilizing the isolated basement membranes obtained by DEOC treatment employed a solution containing 0.01 M Tris (pH 6.8), 1% SDS, 4 M urea, 10% sucrose, and 1% mercaptoethanol to solubilize basement membrane proteins for electrophoresis. After electrophoresis, the gels were removed from the electrophoresis tubes and fixed in 5% sulfosalicylic acid in 10% TCA for 4 hours. The fixing solution was removed by soaking the gels in 7% acetic acid. The gels were then stained 6 hours in a 0.1% Coomassie blue, 10% acetic acid, 10% isopropanol solution. The gels were destained by soaking in 7% acetic acid.

#### Scintillation Spectroscopy

Kimax<sup>R</sup> scintillation vials were used for all scintillation spectroscopy. The scintillation cocktail consisted of 2 parts of a solution of 22.8 gms of Omnifluor<sup>R</sup> (New England Nuclear, Boston, MA) per gallon of spectral grade toluene (Matheson, Coleman, and Bell, Norwood, OH), and 1 part Triton X-100 (Rohm-Haas Co., Philadelphia, PA). Ten ml of cocktail was diluted with 1.5 ml of aqueous sample. The counting conditions employed resulted in a 25.8% counting efficiency for the full <sup>3</sup>H window and 58% counting efficiency for the narrow <sup>14</sup>C window.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### Tissue Isolation

##### Rat Glomeruli

Glomeruli isolated by the iron oxide perfusion method from male Sprague-Dawley rats have been reported (Meezan et al., 1973; Brendel and Meezan, 1973) to maintain their structural integrity and metabolic activity. Transmission electron microscopic examination of these isolated glomeruli showed the cellular elements of the glomerular capillaries to be intact. The basement membrane of the capillaries appeared normal.

Subsequent scanning electron microscopic inspection of glomeruli prepared by perfusion of iron oxide particles reveals a close structural similarity to glomeruli fixed in situ. The capillary loops are uniform with no evident eruptions or bulges that might indicate excessive iron oxide perfusion pressures (Figures 9 and 10). The spatial arrangement of the capillary loops to the mesangium region of the tuft is maintained in the isolated glomeruli. At higher magnifications (Figures 11 and 12), the epithelial foot processes are well defined in both iron oxide perfused and in situ fixed glomeruli. Loss of foot process detail and epithelial cell blunting is seen in a number of disease states which adversely affect kidney function. Preservation of the fenestrated endothelium lining the glomerular capillaries after iron oxide perfusion has

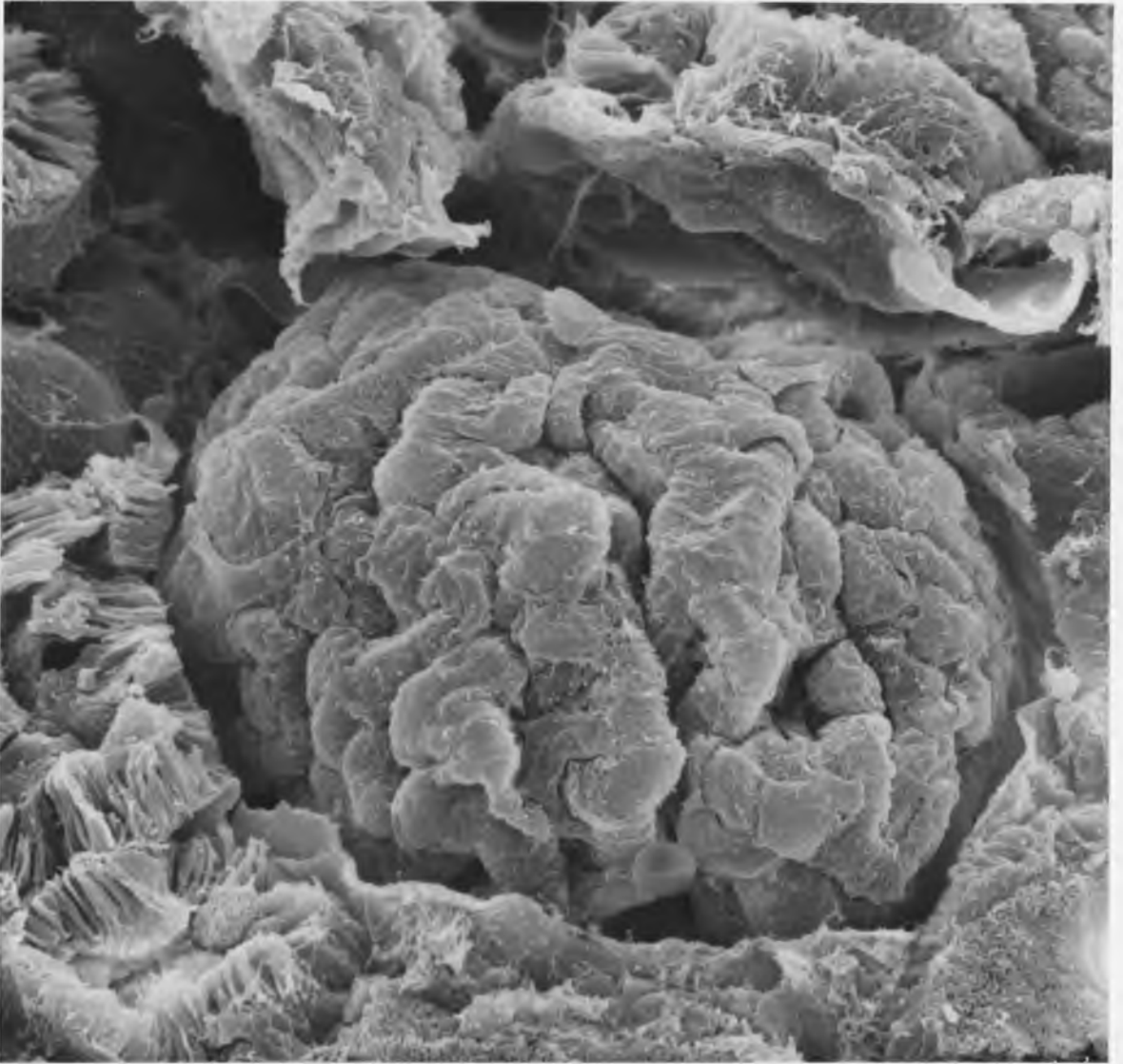


Figure 9. Rat Glomerulus Fixed In Situ

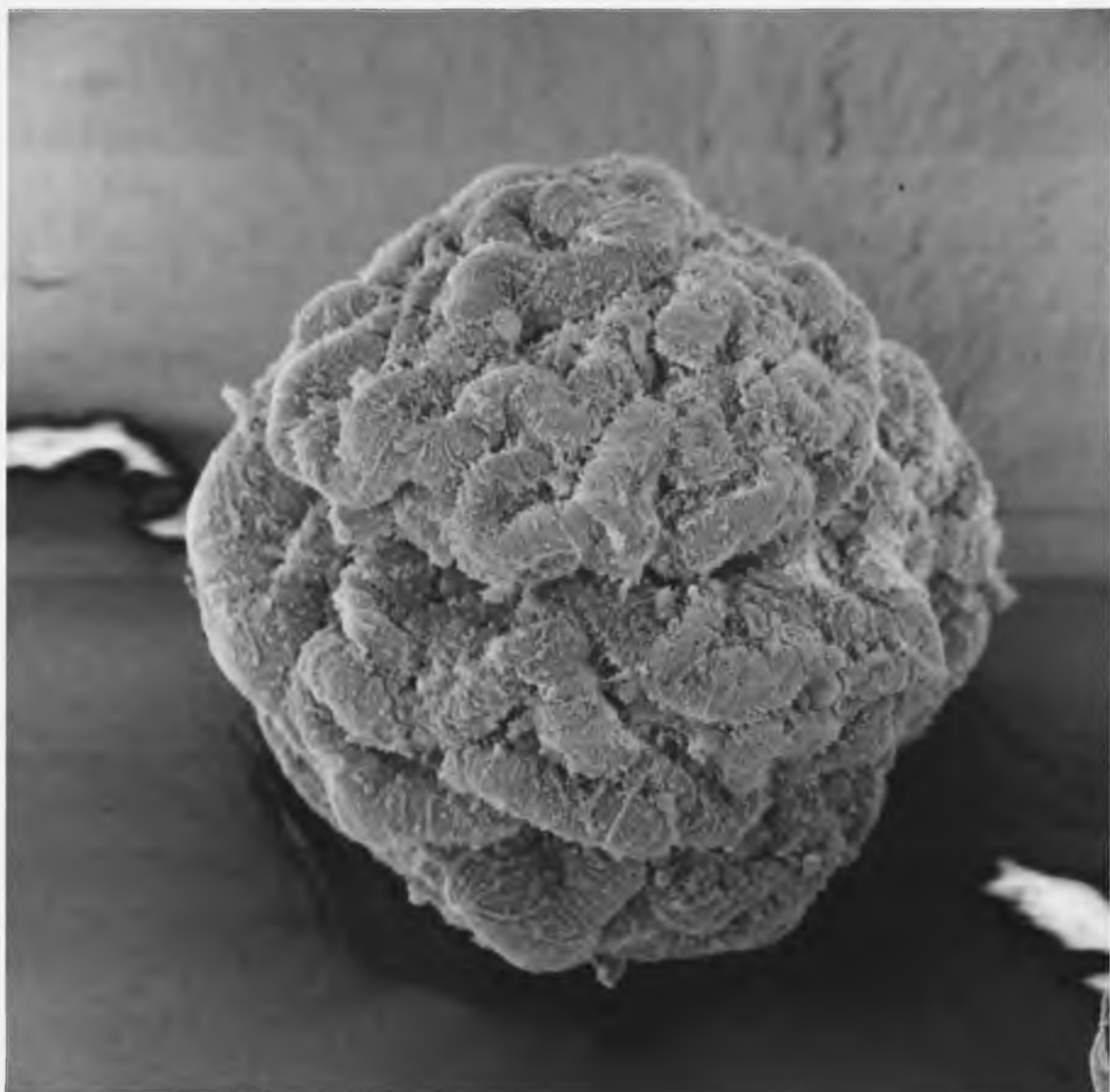


Figure 10. Iron Oxide Perfused Glomerulus

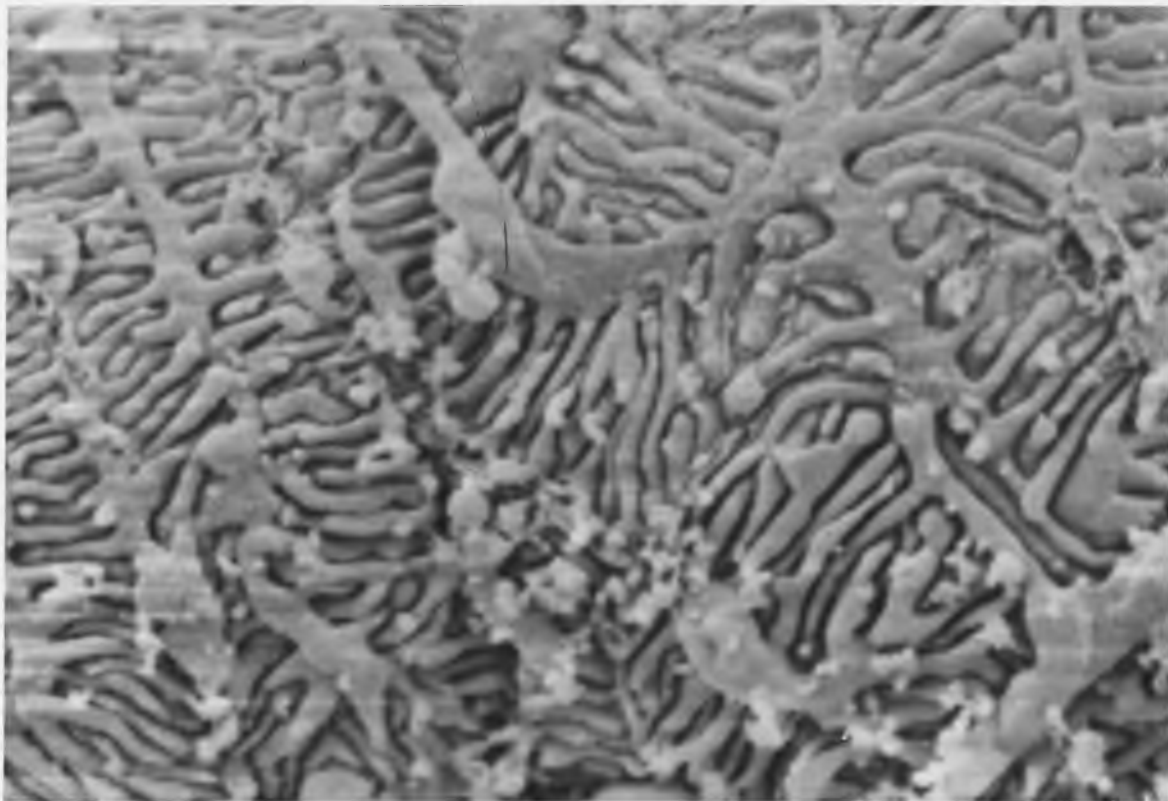


Figure 11. Foot Processes of Iron Oxide Perfused Glomerulus

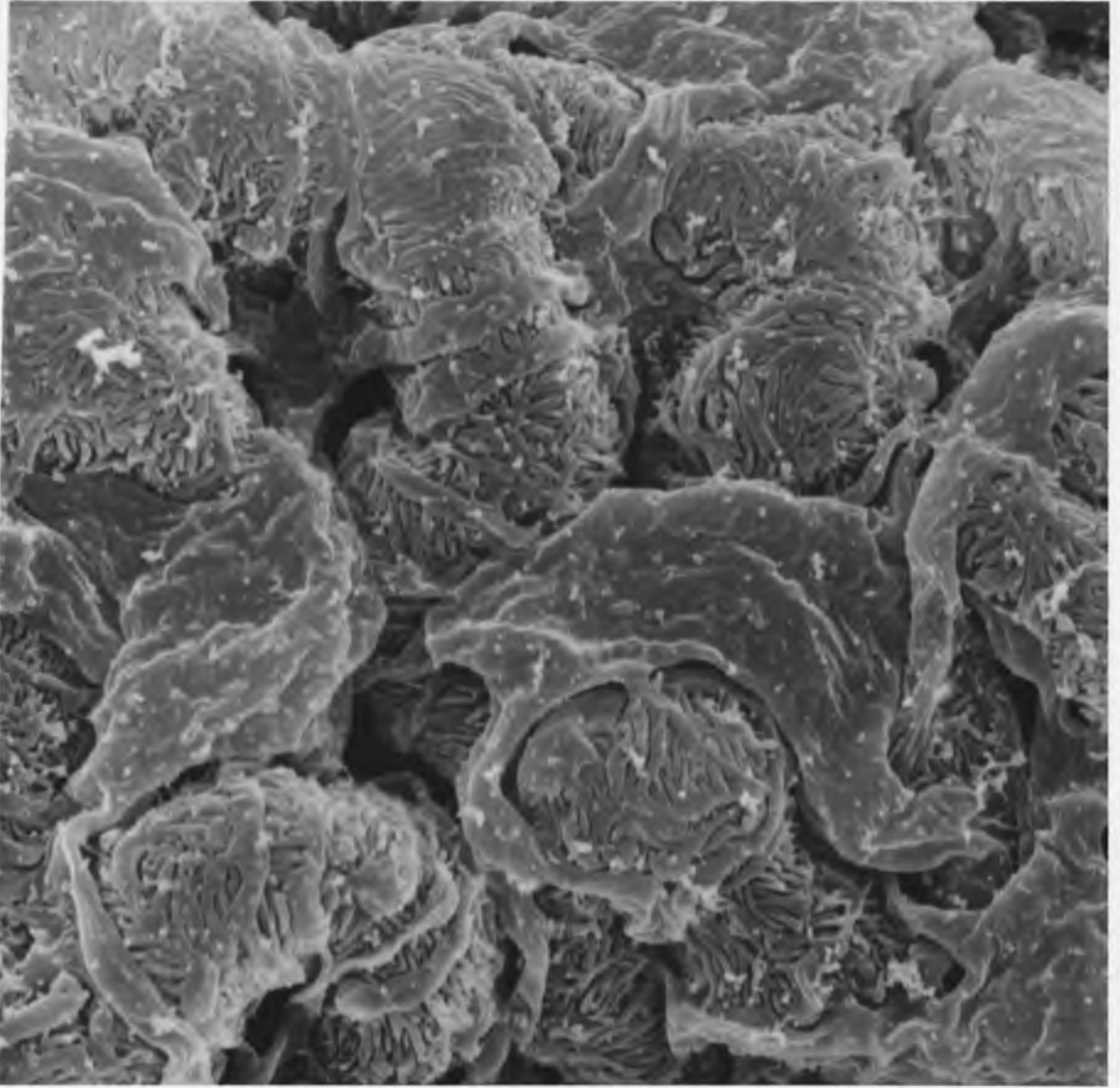


Figure 12. Foot Processes of Glomerulus Fixed In Situ

previously been reported (Meezan et al., 1973). Thus, the rat glomeruli isolated by the perfusion of iron oxide particles followed by hand homogenization, sieving and decantation are morphologically intact and devoid of discernible structural damage which might affect glomerular metabolism.

#### Rabbit Renal Proximal Tubules

The tubules obtained by a modified iron oxide perfusion shown in Figure 13 were shown to be almost entirely proximal convoluted tubules. Figure 13a shows the tubules cut in cross section at the light microscopic level. The tubules maintain their individual identity. Electron microscopic examination of these isolated tubules reveals an abundance of microvilli characteristic of the well-developed brush border of proximal tubules (Figure 13b). The basement membrane surrounds the tubule lumen and is smooth and intact as seen by both transmission (Figure 13c) and scanning (Figure 14a) electron microscopy. The average thickness of the basement membrane was 1500 - 2000 Å. From these examinations of the ultrastructure of the isolated tubules, it was concluded that the isolation procedure does not result in damage to the gross or fine structure of the isolated rabbit tubules (Brendel and Meezan, 1975).

To compare our isolation procedure to the widely used tubule isolation procedure of Burg and Orloff (1962), rabbits were brought to a surgical plane of anesthesia with paraldehyde followed by ether. One kidney was perfused with a solution of 1 mg/ml of Type II collagenase in Earle's buffer while the contralateral kidney was perfused with Earle's

Figure 13. Rabbit Renal Proximal Tubules Obtained by Hand Homogenization and Sieving

- a. A light micrograph of three adjacent proximal tubules stained with toluidine blue. X800.
- b. An electron micrograph of two adjacent isolated tubules. Notice that a layer of basement membrane is present on the outer surface of each tubule. The presence of microvilli on the luminal surface of the tubules indicates a proximal tubular origin. X8,250.
- c. An electron micrograph of interface between two adjacent isolated tubules. The basement membranes are closely applied to the basal surface of the tubule cells. The basement membranes are clear and distinct averaging 1500 - 2000 Å in thickness. X40,000.

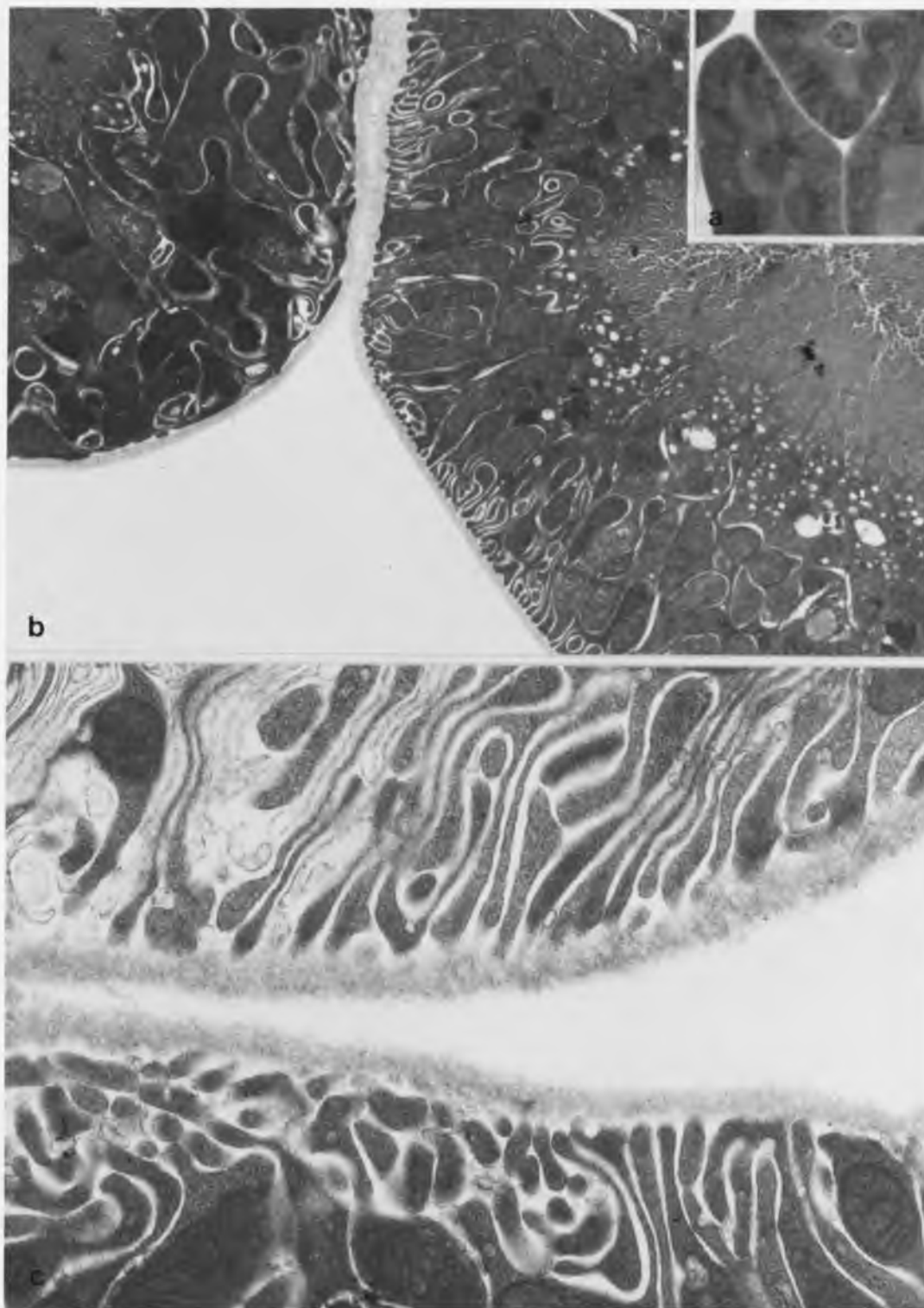


Figure 13. Rabbit Renal Proximal Tubules Obtained by Hand Homogenization and Sieving

Figure 14. Comparison of Sieved and Collagenase Digested Rabbit Proximal Tubules

a. A scanning electron micrograph of isolated tubules obtained by sieving. The basement membrane which forms the outer surface of the isolated tubules is intact. X500.

b. A scanning electron micrograph of tubules isolated by collagenase digestion. The basement membrane is partially or complete digested. Tubule cells can be seen blebbing onto the peritubular surface. Tubules isolated by the sieving technique and subsequently treated with collagenase exhibit a similar structure. X400.



Figure 14. Comparison of Sieved and Collagenase Digested Rabbit Proximal Tubules

buffer followed by the iron oxide suspension. The tubules obtained from the collagenase treatment showed grossly altered morphology (Figure 14b). The basement membrane was partially digested and proximal tubule cells were observed to be blebbing from the luminal to peritubular side of the tubule (Figure 14b). The mitochondria appeared swollen and cellular elements were seen free in the lumen. To compare the effect of collagenase perfusion versus incubation, tubules isolated by the iron oxide perfusion method were incubated with a 1 mg/ml collagenase solution for 30 minutes. These tubules exhibited the same morphological alterations as the tubules obtained from collagenase perfused kidneys. Thus, the treatment of rabbit tubules with collagenase results in gross structural alterations and appears to offer no advantages over the iron oxide perfusion method for the preparation of intact kidney tubules.

#### Bovine Retinal Blood Vessels

Retinal blood vessels were obtained by the method of Meezan, Brendel, and Carlson (1974) which involves the use of gentle hand homogenization of isolated bovine retinas followed by sieving. These authors reported that retinal vessels isolated by this method produced  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -6-glucose,  $^{14}\text{C}$ -2-3-succinic acid, and  $^{14}\text{C}$ -1-oleic acid. Subsequent experiments using this retinal vessel preparation (White, Meezan, and Brendel, 1975) have shown incorporation of radiolabeled amino acids and carbohydrates into proteins. As reported by Meezan, Brendel, and Carlson (1974), light microscopic examination of the isolated retinal vessels shows them to be well defined vessels with

enclosed blood elements. The preparation is homogeneous and remarkably free of non-vascular retinal structures (Figure 15a). Since the vessels are preperfused, blood elements remain in the vessel lumen. Although the width of the isolated retinal vessels may be as great as 50  $\mu$ , the majority of vessels are between 10 - 15  $\mu$  in diameter.

Electron microscopic examination of cross sections through isolated bovine retinal vessels (Figure 15b) shows the general morphological integrity of the endothelial cells to be preserved. One or more layers of intramural pericytes invest the endothelial lining. Beneath the endothelium and surrounding the intramural pericytes is a continuous basement membrane which averages 800 - 1000 Å in thickness. From the evidence available, this retinal vessel isolation procedure yields tissue which is morphologically and metabolically intact.

#### Bovine Brain Blood Vessels

Using the method of Brendel, Meezan, and Carlson (1974), bovine brain blood vessels were isolated. The formation of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -glucose,  $^{14}\text{C}$ -1-oleic acid,  $^{14}\text{C}$ -1-pyruvate,  $^{14}\text{C}$ -glutamic acid, and oxygen consumption by the isolated brain vessels were reported by these authors. Light microscopic inspection of 1  $\mu$  thick sections of isolated brain vessels (Figure 16a) shows them to have a uniform diameter of 7 - 15  $\mu$ . Except for numerous entrapped erythrocytes, the brain vessel preparation was free of non-vascular material (Figure 16b). Brain vessel cell morphology was preserved.

Figure 15. Isolated Bovine Retinal Microvessels and Basement Membrane

a. A light micrograph of bovine retinal blood vessels isolated by gentle hand homogenization and sieving. Erythrocytes are occasionally seen within the vessel lumen. Toluidine blue. X1,500.

b. An electron micrograph of microvessels cut in cross section. Basement membranes are observed to surround the intramural pericytes and underlie the endothelium. A continuous endothelium covers the luminal surface of the basement membrane. Most vessels average 10 - 20  $\mu$  in diameter. X9,250.

c. A low power electron micrograph of bovine retinal blood vessel basement membrane isolated from retinal microvessel similar to those seen in (b). The microvessels have been treated with a 4% solution of sodium deoxycholate to solubilize the cellular materials. X9,200.

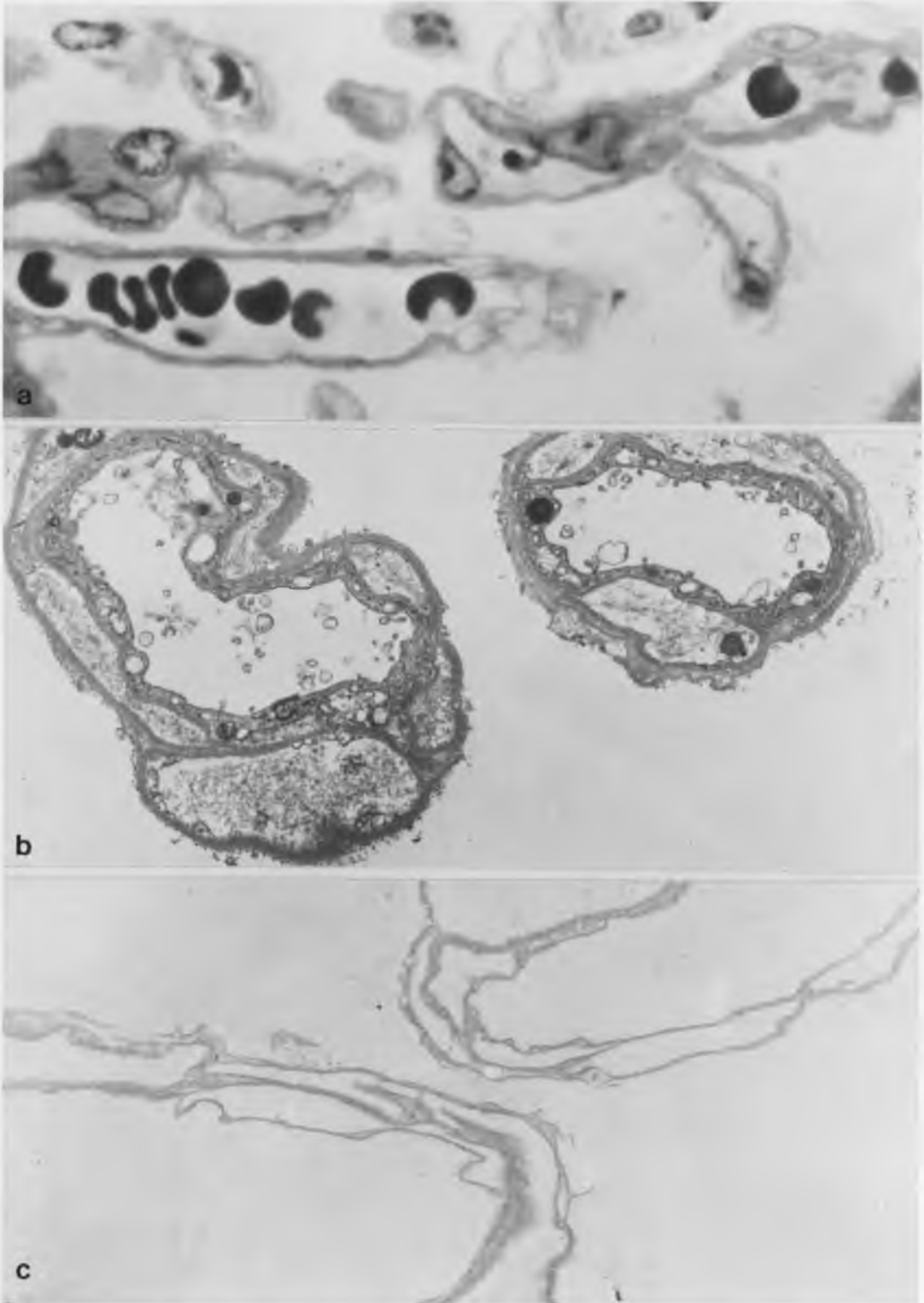


Figure 15. Isolated Bovine Retinal Microvessels and Basement Membrane

Figure 16. Isolated Bovine Brain Microvessels and Basement Membranes

a. A light micrograph of bovine brain vessels obtained by hand homogenization and sieving. The majority of the vessels have diameters less than 15  $\mu$ . Erythrocytes and endothelial cell nuclei are recognizable at this level. Toluidine blue. X700.

b. A phase contrast micrograph of isolated bovine brain microvessels. These microvessels are often long and tortuous with numerous arborizations. Non-vascular tissues are not present. X672.

c. An electron micrograph of isolated bovine brain microvessel basement membrane. Treatment of isolated brain microvessel with a 4% solution of sodium deoxycholate results in the solubilization of the cellular materials. The brain vessel basement membrane is more easily disrupted than those obtained from other suborgan fractions. An average basement membrane thickness of 200 - 500 Å is observed. Occasionally, the basement membrane isolated from bovine microvessels shows unit collagenous fibrils. Recognizable cellular structures are not observed. X47,000.

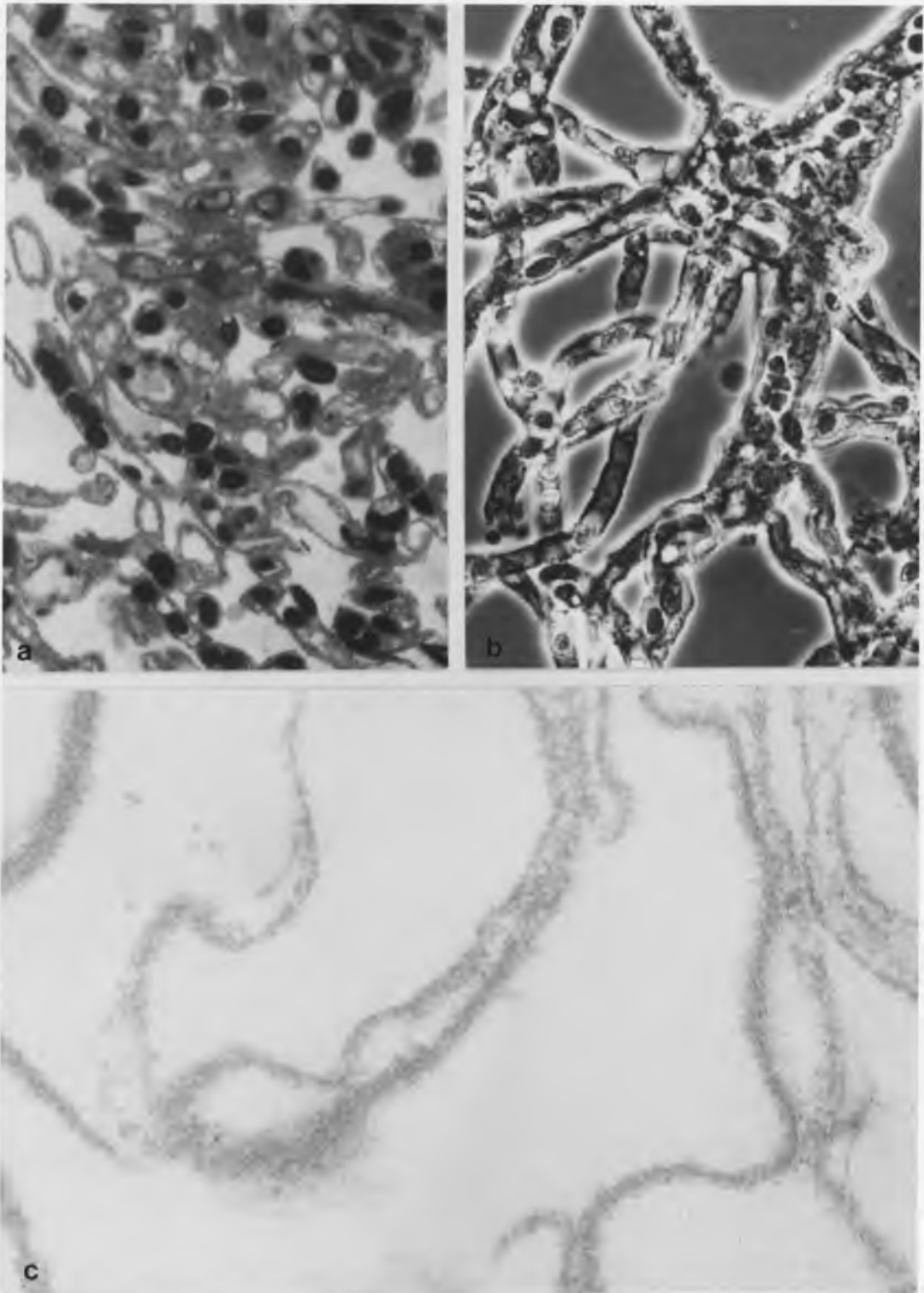


Figure 16. Isolated Bovine Brain Microvessels and Basement Membranes

### Bovine Lens Capsule

Bovine lens capsules were isolated from lens from enucleated eyes by bisecting along the circumference of the lens and removing the sloughed lens capsule with forceps. Because of the relative ease with which this macroscopic tissue could be obtained, extensive ultrastructural examination was not required.

### Basement Membrane Isolation

#### Rat Renal Glomeruli

Initial attempts to isolate rat renal glomerular basement membranes by sonication of glomeruli isolated by the iron oxide perfusion method gave equivocal results. Although the glomeruli obtained by this procedure were 99% free of tubular contamination, the 10,000 x g pellet from this sonicated glomerular suspension consisted of tissue fragments which appeared to be of varying size and shape suggesting a non-uniform disruption. Incubation of the 10,000 x g pellet with a mixture of sodium dodecyl sulfate (SDS) and 1% mercaptoethanol solubilized a portion of this material. SDS-polyacrylamide gel electrophoresis of the solubilized material gave five major and many minor bands. The minor bands were not consistently reproducible between preparations varying in intensity and migration. The non-uniform disruption and the variable band pattern suggested that the isolation procedure was subject to variable contamination of the basement membrane preparations with foreign proteins.

The isolation of glomerular basement membranes by sonication and centrifugation may be subject to a number of technical difficulties. Westberg and Michael (1970) have shown that glomerular basement membranes prepared by sonication and centrifugation were subject to contamination by serum proteins and cell debris. Variations in the centrifugation force applied to glomerular sonicates have been reported to alter the amount of sialic acid obtained in basement membrane pellets (Mohos and Skoza, 1970). Since the available histochemical evidence confirms the presence of relatively large amounts of sialic acid in the cell membranes lining the basement membrane but only trace amounts in the basement membrane itself, the conclusion was that variations in centrifugation force changed the amount of cell membrane contamination in the basement membrane pellet. The presence of serum proteins in isolated basement membrane preparations was found to be resistant to removal by extensive sonication and washing (Westberg and Michael, 1970). The intensity and duration of sonication of isolated glomeruli has been shown to be a critical step in basement membrane isolations. When normal human glomerular basement membranes were obtained by sonication and centrifugation, the ultrastructure of these isolated basement membranes appeared "frayed" (Westberg and Michael, 1970). Kefalides (1973) has reported that multiple sonication of human glomeruli results in a change in amino acid composition of the isolated basement membrane. Interestingly, this compositional change resulted in the relative increase of certain amino acids typically found in collagenous proteins i.e., hydroxyproline, glycine, and hydroxylysine. Obviously, the use of the

sonication and centrifugation procedure requires stringent control and monitoring.

A theoretical question as to the applicability of these procedures to compositional studies of basement membranes in normal and disease states is raised by the disrupted appearance of basement membranes isolated by sonication. If a disease resulted in the labile deposition of a component into the basement membrane which was chemically dissimilar to the normal components, sonication might so disrupt this altered basement membrane that the labile component was preferentially solubilized and thus not detected. Only if a disease resulted in the deposition of a chemically different component which was as tightly incorporated into the basement membrane as the other structural components would a compositional difference be uncovered in the diseased structure. Alternatively, if the deposition process for the incorporation of the normal components of the basement membrane is altered so that certain of these components are only loosely bound, sonication might solubilize these loosely deposited normal components resulting in a compositional change in the pelleted basement membrane. Thus, the intensity and duration of sonication might significantly affect the composition of the basement membrane.

Of course, any treatment or procedure that is employed to remove cellular elements from the basement membrane matrix may be accused of either partially solubilizing a portion of the basement membrane or not completely removing contaminating material. This is in part due to an incomplete chemical definition of the basement membrane. At the

present time, the only conclusive evidence of basement membrane solubilization or contamination is morphological in nature. If the structure of the isolated basement membrane is identical to that of in vivo basement membrane, then the basement membrane is thought to be structurally intact and negligible solubilization of the matrix is presumed to have occurred. The presence of recognizable cellular elements in isolated basement membrane preparations would indicate the incomplete removal of contaminating materials.

Subsequently, a variety of non-disruptive detergent treatments were applied to isolated glomerular preparations in an effort to obtain uniform basement membrane material. When a 4% sodium deoxycholate (DEOC) solution similar to that used by von Bruchhausen and Merker (1967) to remove cellular material from kidney minces was applied to a small number of glomeruli on a microscope slide, the glomeruli appeared to lose their compact structure and seemed to unfold slightly. The capillary loops appeared less opaque and the cellular structures were lost. Treatment of larger preparations of glomeruli with 4% DEOC resulted in the formation of a gel-like material which impeded further tissue manipulations. It was found that autolysis of the glomeruli in distilled water followed by incubation in 1 M sodium chloride which contained deoxyribonuclease (DNAase) eliminated gel formation. The isolation scheme shown in Figure 7 gave pelletable material which was morphologically similar to in vivo glomerular basement membrane (Meezan et al., 1975). Light microscopic inspection of a non-DEOC treated glomerulus shows prominent endothelial and epithelial cell nuclei (Figure 17a)

Figure 17. Rat Renal Glomeruli before and after Deoxycholate

a. A photomicrograph of an encapsulated renal glomerulus demonstrating prominent epithelial and endothelial cell nuclei. Parietal epithelium surrounds the capillary tuft with the visceral epithelium intervening. Toluidine blue. X640.

b. An electron micrograph of a peripheral portion of an isolated encapsulated renal glomerulus. Interdigitating parietal epithelial cells and foot processes of podocytes line Bowman's space. The continuous basement membrane intervenes between endothelium and podocytes. The average basement membrane thickness is 1200 - 2500 Å. X20,000.

c. A photomicrograph of isolated encapsulated glomeruli which have been treated with sodium deoxycholate. Both the parietal epithelial basement membrane and the intraglomerular basement membrane is free of cellular elements. The heavy photodensities indicate deposits of colloidal iron. Toluidine blue. X640.

d. An electron micrograph of isolated glomerular basement membrane obtained by the use of deoxycholate. The histoarchitecture of glomerular extracellular materials is maintained after such treatment. Patches of unstructured basement membrane material is observed in the mesangial area. Cellular debris is not present in these preparations. As in the intact glomeruli, the deoxycholate treated basement membranes had an average thickness of 1500 - 2500 Å.

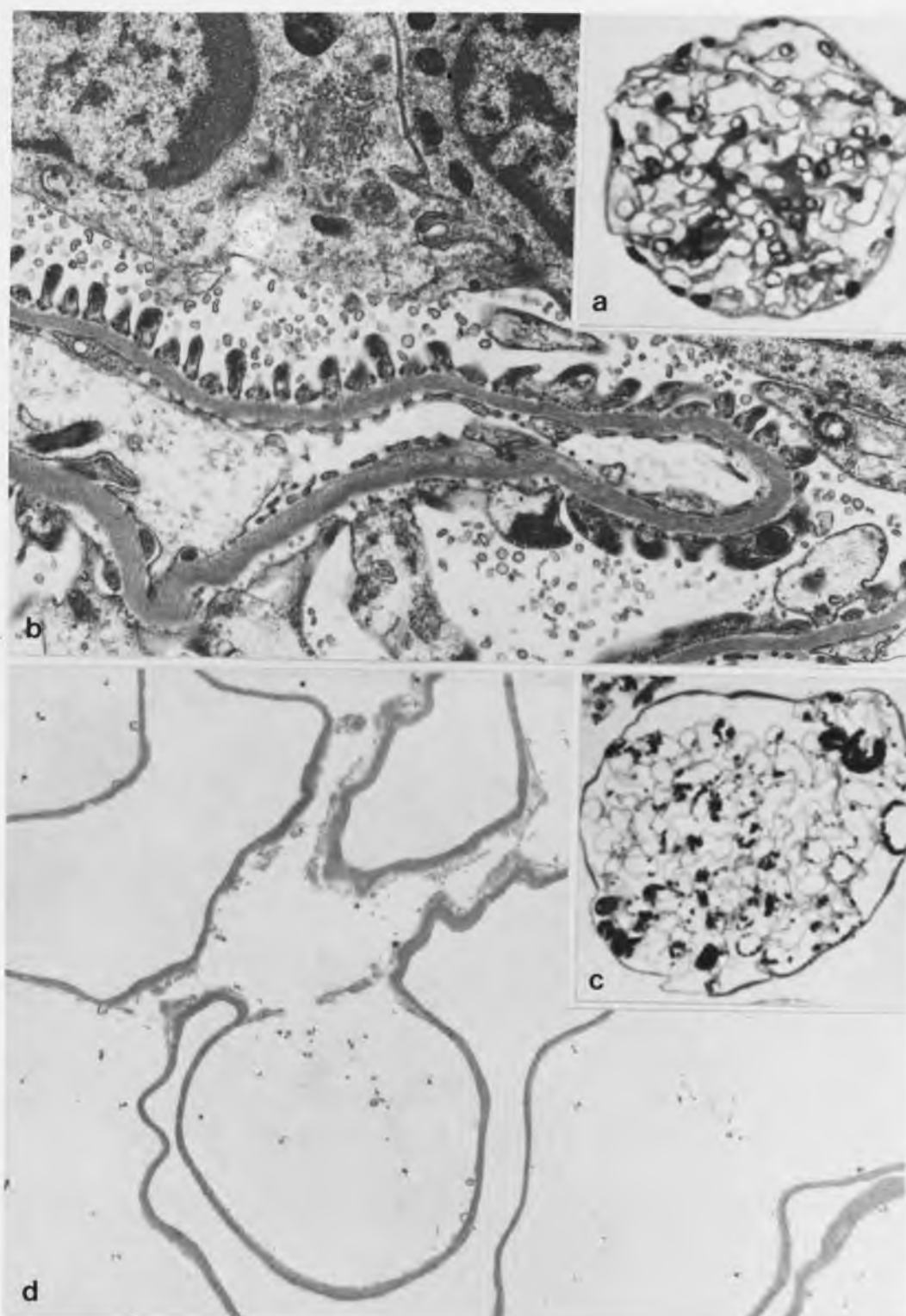


Figure 17. Rat Renal Glomeruli Before and After Deoxycholate

while a DEOC treated glomerulus exhibits only basement membrane and entrapped iron oxide particles (Figure 17c). At the electron microscopic level, no recognizable cellular structures remain after DEOC treatment (Figure 17d). The spatial arrangement of the basement membrane within the glomerular histoarchitecture is preserved. In the mesangial region, the capillary basement membrane is observed to be incomplete with patches of irregular basement membrane (Figure 17d). Table 5 lists the widths of the basement membranes isolated from the tissues used in this study. At high magnification, the basement membrane obtained by DEOC treatment of rat glomeruli is morphologically indistinguishable from in vivo basement membrane (Figure 18). All of the basement membranes isolated by DEOC treatment seem to be composed of fibrillar and amorphous materials; the fibrillar component being an aggregate of 40 - 50 Å fibrils and the interfibrillar matrix material being a flocculent material of medium electron density. The characteristic banding pattern of striated collagen or elastin are seldom observed in the DEOC isolated glomerular basement membrane preparations.

Table 5. Basement Membrane Dimensions after Deoxycholate Treatment

| <u>Width (Å)</u> | <u>Source</u>                 |
|------------------|-------------------------------|
| 1500 - 2500      | Rat Renal Glomeruli           |
| 1200 - 2000      | Rabbit Renal Proximal Tubules |
| 300 - 500        | Bovine Retinal Vessels        |
| 200 - 500        | Bovine Brain Vessels          |

Figure 18. Glomerular Basement Membrane before and after Deoxycholate Treatment

a. An electron micrograph of glomerular basement membrane in an intact rat glomerulus. Interdigitating foot processes of podocytes form the boundary of Bowman's space. The capillary lumen surface of the basement membrane is covered by fenestrated capillary endothelium. X41,000.

b. An electron micrograph of rat glomerular basement membrane isolated by the use of sodium deoxycholate. The basement membrane in the mesangial region is shown. The inner surface of the basement membrane shows unstructured matrix material. The epithelial surface of the basement membrane is smooth and well defined. X41,000.

c. A high power electron micrograph of basement membrane in an intact glomerulus. The glomerular basement membrane is interposed between the flattened capillary endothelium and foot processes of podocytes. The basement membrane is approximately 2000 Å thick and shows a finely granular texture. X135,000.

d. A high power electron micrograph of glomerular basement membrane isolated by deoxycholate. This micrograph is identical to that shown in (c) with respect to the plane of section, magnification, and histological location. The thickness of the basement membrane was approximately 2200 Å. The basement membrane isolated with the use of deoxycholate is morphologically identical to the in vivo control basement membrane shown in (c). X135,000.

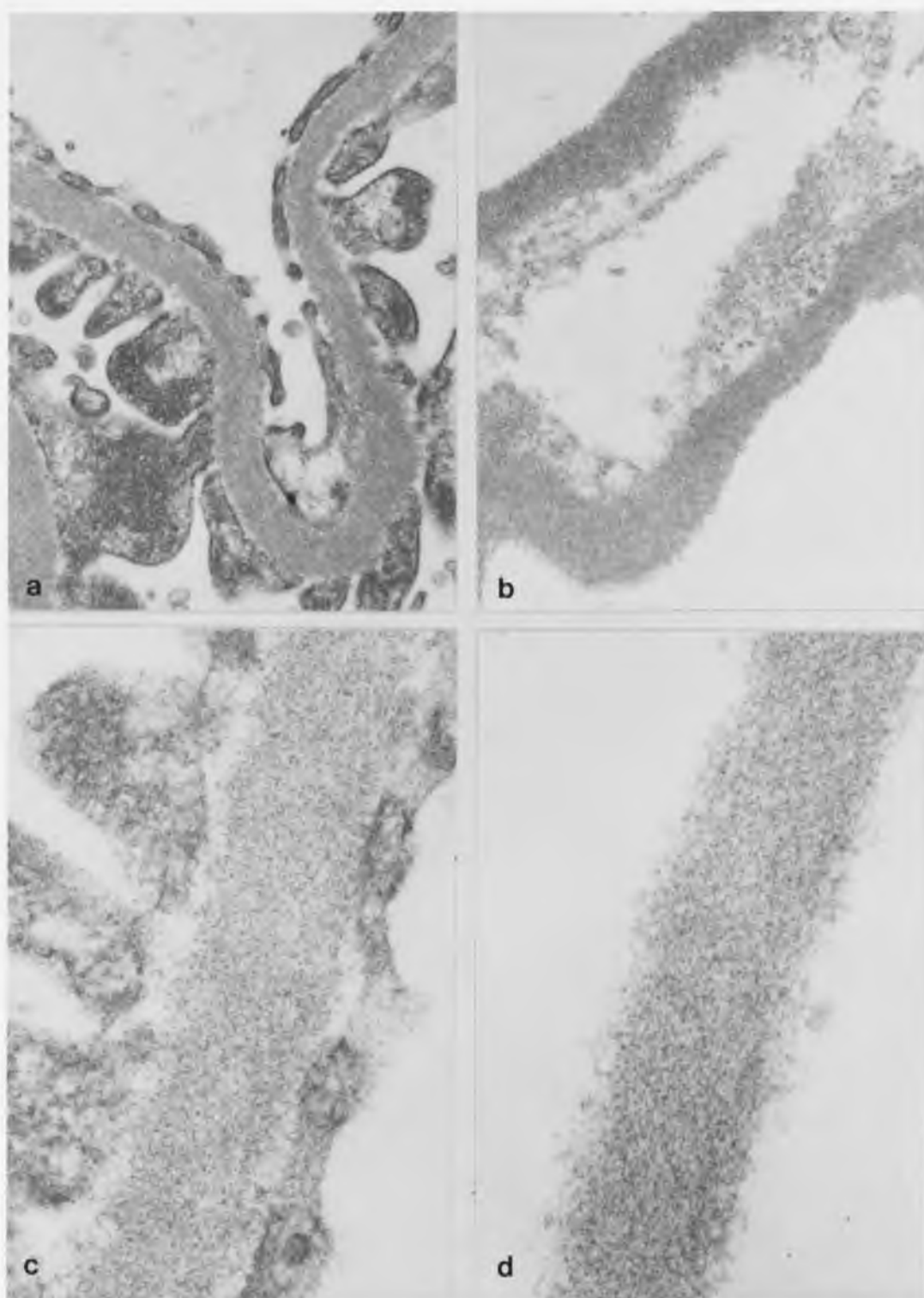


Figure 18. Glomerular Basement Membrane before and after Deoxycholate Treatment

### Rabbit Renal Proximal Tubules

Rabbit renal proximal tubular basement membrane isolated by DEOC treatment is intact and devoid of cellular structures (Figure 19a). Unlike the glomerular basement membrane, the tubule basement membrane is uniform in width throughout its circumference. It is also of a uniform electron density throughout its width (Figure 19b). Occasionally, 200 Å unbanded microfibrils are seen, but striated collagen fibrils are not observed. Comparison of the isolated basement membrane shown in Figure 19a to that of the in vitro tubular basement membrane shown in Figure 13 shows them to be identical.

### Bovine Retinal Blood Vessel

Figure 20 shows the loss of recognizable cellular structures when isolated retinal vessels are treated with a 4% DEOC solution. The isolated vessel basement membrane is intact and has a multilayered appearance. The 300 - 500 Å basement membrane width was independent of vessel diameter. The outer surface of the basement membrane is less distinct than the inner surface. Like the mesangial region of the glomerular basement membrane, the outer surface of the retinal basement membrane has patches of amorphous basement membrane-like material associated with it (Figure 20a). Unlike the tubular basement membrane, the retinal basement membrane appears indistinct at high magnification (Figure 20b).

Figure 19. Isolated Rabbit Renal Proximal Tubule Basement Membrane

a. An electron micrograph of rabbit proximal tubules isolated by the use of deoxycholate. The basement membrane is sometimes associated with small unbanded fibrillar material but native unit collagenous fibrils are almost never observed. Basement membranes isolated by this procedure are indistinguishable from their in vivo counterparts. X19,000.

b. A high resolution electron micrograph of isolated rabbit tubule basement membrane. The basement membrane obtained by the treatment of purified tubules with sodium deoxycholate is homogeneous and sharply defined at its surfaces. In contrast to vascular basement membranes, tubular basement membranes show uniform electron density throughout their thickness. Unbanded 200 Å microfibrils are closely associated with the basement membrane. Very fine fibrils (30 - 40 Å) are embedded in the granular matrix of the basement membrane. X50,000.

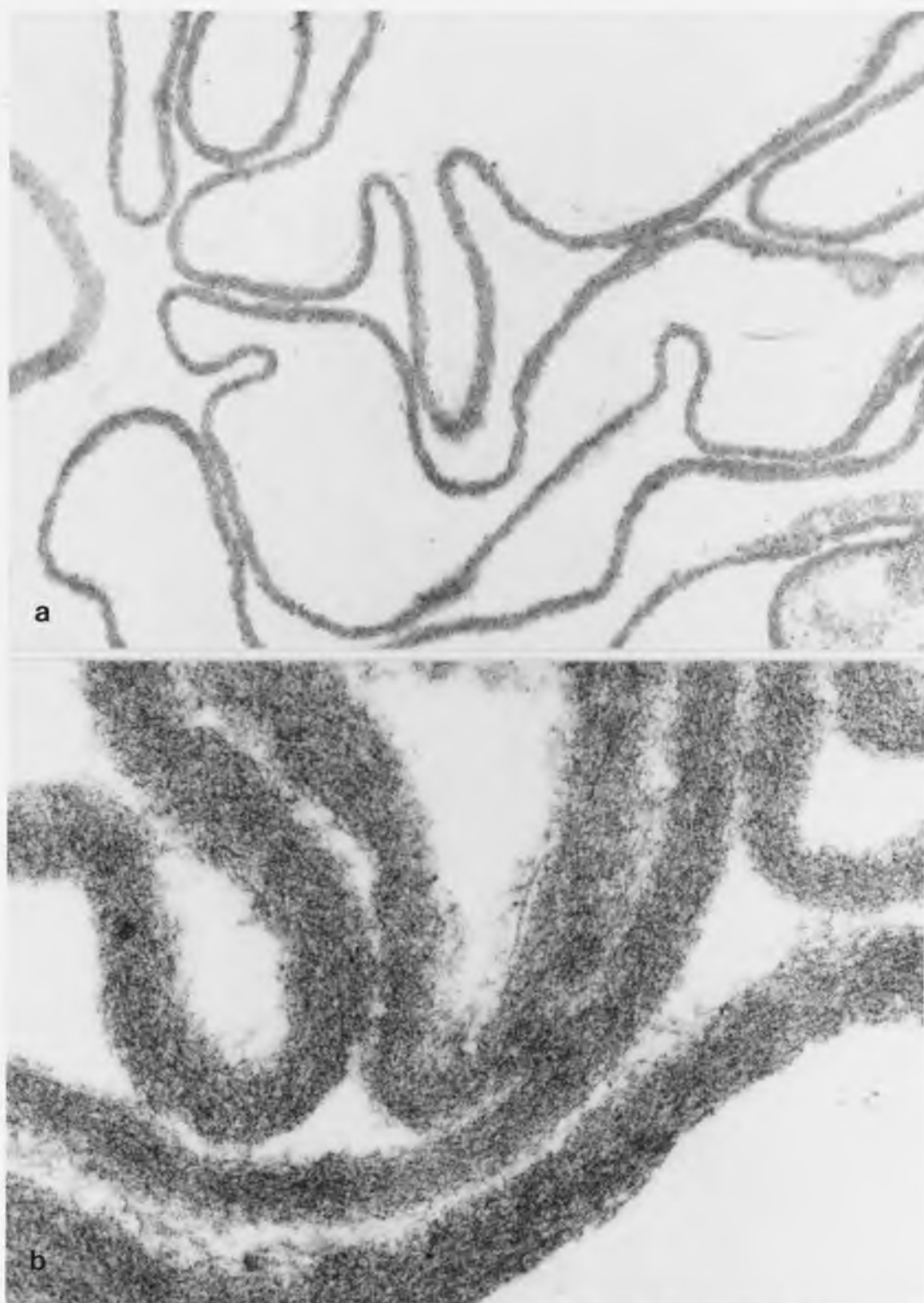


Figure 19. Isolated Rabbit Renal Proximal Tubule Basement Membrane

Figure 20. Isolated Bovine Retinal Capillary Basement Membrane

a. An electron micrograph of bovine retinal microvessels isolated with the use of deoxycholate. The single continuous basement membrane shows a sharply demarcated inner surface and an indistinct outer surface which blends with electron dense material in the spaces between the vessels. A single basement membrane suggests that it is derived from a capillary rather than larger vessels, since the latter would require multiple basement membranes associated with pericytes. The thickness of the basement membrane shown is 800 Å. X25,000.

b. A high power electron micrograph of isolated bovine retinal vessels. The basement membrane shows an electron dense granularity with the highest electron density in the central portion of the basement membrane. The surfaces of the basement membrane appear fuzzy and indistinct. X91,000.

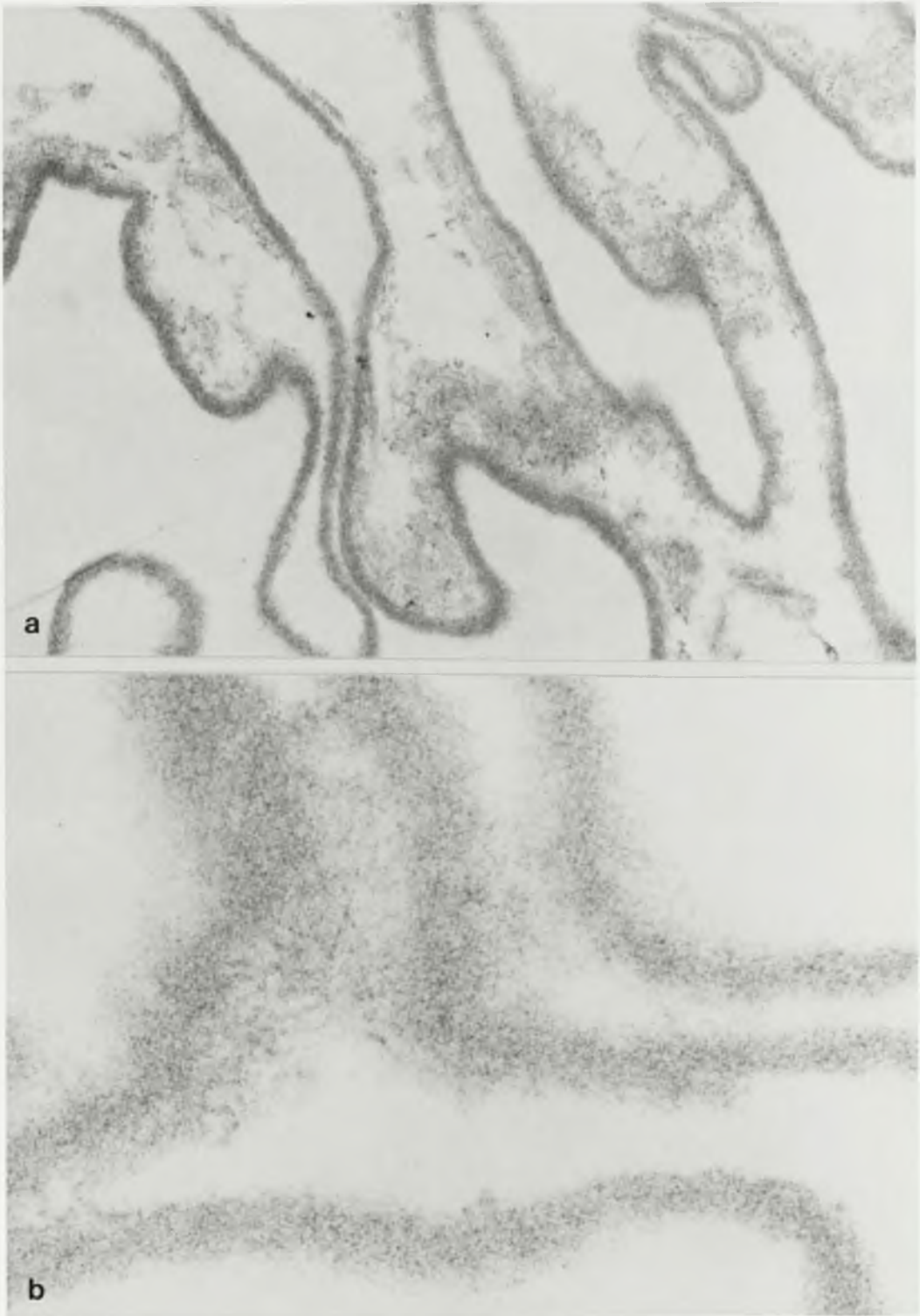


Figure 20. Isolated Bovine Retinal Capillary Basement Membrane

### Bovine Brain Blood Vessel

The brain blood vessel basement membrane obtained by the DEOC treatment of isolated bovine brain vessels is morphologically similar to isolated retinal vessel basement membrane. The brain vessel basement membrane is somewhat more difficult to isolate in ultrastructurally pure and intact form than is that of retinal vessels. When isolated brain vessels of greater than 20  $\mu$  are used as a source of basement membranes, cell debris and collagen fibrils are observed. Use of the smaller 10 - 20  $\mu$  brain vessels avoids this contamination problem and results in morphologically pure basement membrane (Figure 16c).

### Bovine Lens Capsule

Two procedures were used to isolate lens capsule basement membrane. The first method involved sonication in saline or distilled water. The second involved the use of DEOC treatment. The macroscopic material prepared by sonication of DEOC was similar in appearance at the light microscopic level.

### Basement Membrane Characterization

#### Carbohydrate Analysis

Using a variation of the method of Grimes (1974), the carbohydrate content of the various isolated basement membranes was determined. The results are shown in Table 6. Comparison of the carbohydrate composition of the easily isolatable, well characterized bovine lens capsule obtained by sonication to that obtained by treatment with 4% DEOC shows

Table 6. Carbohydrate Analyses of Isolated Basement Membranes in  
 µg Sugar/mg Dry Weight Basement Membrane

| Sugars      | Sonicated<br>Bovine<br>Lens<br>Capsules | Deoxy-<br>cholate<br>Bovine<br>Lens<br>Capsules | Sonicated<br>Rat<br>Glomeruli <sup>2</sup> | Deoxy-<br>cholate<br>Rat<br>Glomeruli | Deoxy-<br>cholate<br>Rabbit<br>Tubules | Deoxy-<br>cholate<br>Bovine<br>Retinal<br>Vessels | Deoxy-<br>cholate<br>Bovine<br>Brain<br>Vessels |
|-------------|---|---|--|---------------------------------------|--|---|---|
| Fucose      | 2.2                                     | 1.49  | 3  | 0.66                                  | 0.87                                   | 2.52  | 1.97  |
| Mannose     | 4.6                                     | 4.47  | 8  | 5.02                                  | 4.09                                   | 4.42  | 2.09  |
| Galactose   | 49.1                                    | 46.41   | 19   | 23.51                                 | 32.07                                  | 26.11   | 9.37  |
| Glucose     | 47.1                                    | 47.60   | 23   | 23.24                                 | 29.90                                  | 26.79   | 11.20   |
| Hexosamines | 6.6                                     | 5.85  | 6.5  | 8.04                                  | 6.89                                   | 6.97  | 6.64  |

1. Taken from Fukushi and Spiro (1969).

2. Taken from Kefalides and Forsell-Knott (1970).

them to be virtually identical. This indicates that the 4% DEOC treatment does not interfere with carbohydrate analysis. Rat glomerular basement membrane isolated by our DEOC procedure was also found to have a carbohydrate content similar to that of basement membrane obtained by sonication. The isolation and characterization of the basement membranes obtained from bovine retinal and brain vessels and rabbit proximal tubules has not been previously described.

Analysis of the basement membranes isolated from suborgan fractions with the use of DEOC revealed the presence of equimolar amounts of glucose and galactose. This is indicative of the glucosyl galactose disaccharide unit which has been observed in all characterized basement membranes (Kefalides, 1973; Spiro, 1967a). Lower disaccharide content was observed in the basement membranes isolated from brain and retinal vessels, renal tubules and glomeruli. The deoxycholate treated basement membranes have less fucose than their sonicated counterparts. The amount of mannose and hexosamines were found to be similar in the basement membranes analyzed.

The functional significance of the varied level of glycosylation is not clear. Lens capsules which provide primarily a structural support for the lens have a relatively high degree of glycosylation. The glomerular, tubular, and retinal vessel basement membranes which are known to be exchange sites for solvent and solute have intermediate levels of glycosylation. Brain vessel basement membrane has the lowest amount of glucose and galactose. The blood vessels of the brain form what has been functionally called the blood brain barrier. Although it

is unlikely that the basement membrane is the permeability barrier, it may serve to align the brain vessel cells into a tight compact arrangement which limits access to the brain. The level of glycosylation may affect cell arrangement along the basement membrane.

#### Amino Acid Analysis

Tables 7 and 8 list the amino acid content in residues/1000 residues for the isolated basement membranes included in this study. Comparison of the composition of the basement membranes obtained by our 4% DEOC treatment to that isolated by sonication and centrifugation shows the lens capsule to have the closest similarity. This is not surprising due to the relative ease with which lens capsule basement membrane can be isolated. However, when rat glomerular basement membrane compositions are compared, a greater variability is observed. There are several explanations which might account for the compositional differences. First, glomeruli are not easily isolated in pure and intact form free of tubular contamination. This isolation difficulty is amplified when small animals are used. Secondly, the preparation of glomerular basement membrane by sonication is subject to contamination with glomerular cell debris and serum proteins (Westberg and Michael, 1970). By using the DEOC procedure to isolate basement membrane from highly purified glomerular preparations obtained by the iron oxide perfusion method, we feel that these difficulties have been minimized. The content of amino acids characteristically found in collagen were found to be slightly higher in the DEOC treated versus sonicated glomerular basement

Table 7. Amino Acid Composition of Isolated Basement Membranes in Residues/1000 Residues

|           | DEOC<br>LC | SON<br>LC | SON <sup>1</sup><br>LC | SON <sup>2</sup><br>LC | Retinal<br>Vessels | Brain<br>Vessels | Tubules |
|-----------|------------|-----------|------------------------|------------------------|--------------------|------------------|---------|
| HOPRO     | 106.1      | 107.3     | 110.7                  | 100.0                  | 98.5               | 88.4             | 92.0    |
| ASP       | 58.2       | 56.8      | 53.8                   | 55.0                   | 58.9               | 55.4             | 64.2    |
| THR       | 30.5       | 29.6      | 28.4                   | 29.0                   | 31.6               | 25.3             | 32.5    |
| SER       | 50.5       | 48.7      | 44.9                   | 42.0                   | 48.0               | 43.2             | 52.4    |
| GLUT      | 88.4       | 91.4      | 87.8                   | 92.5                   | 87.0               | 80.0             | 92.7    |
| PRO       | 71.0       | 70.6      | 71.2                   | 68.0                   | 84.3               | 94.1             | 70.0    |
| GLY       | 253.7      | 260.7     | 267.1                  | 275.0                  | 267.6              | 273.6            | 246.6   |
| ALA       | 41.2       | 41.4      | 39.6                   | 42.8                   | 69.9               | 84.0             | 54.2    |
| VAL       | 25.7       | 28.2      | 29.7                   | 30.0                   | 24.7               | 25.6             | 31.3    |
| CYS/2     | 21.8       | 11.5      | 17.0                   | 28.0                   | 14.6               | 19.2             | 15.9    |
| METH      | 14.6       | 16.6      | 12.5                   | 8.0                    | 8.6                | 3.5              | 9.4     |
| ILE       | 26.5       | 28.5      | 30.8                   | 28.8                   | 21.5               | 19.6             | 29.0    |
| LEU       | 56.6       | 56.8      | 57.2                   | 58.0                   | 43.8               | 37.5             | 56.0    |
| TYR       | 4.1        | 3.0       | 11.1                   | 10.0                   | 5.1                | 2.1              | 6.6     |
| PHE       | 30.1       | 31.1      | 30.9                   | 32.0                   | 25.7               | 21.5             | 46.5    |
| HOLYS     | 52.0       | 51.6      | 44.8                   | 35.0                   | 27.4               | 23.4             | 31.3    |
| HIST      | 16.7       | 15.1      | 11.5                   | 10.2                   | 9.0                | 11.4             | 7.9     |
| LYS       | 12.2       | 12.7      | 12.1                   | 13.2                   | 18.3               | 22.9             | 18.5    |
| ARG       | 40.4       | 36.9      | 35.8                   | 43.0                   | 55.6               | 69.3             | 42.9    |
| HOPRO/PRO | 1.49       | 1.51      | 1.56                   | 1.25                   | 1.16               | .939             | 1.31    |
| HOLYS/LYS | 4.26       | 4.25      | 3.71                   | 2.65                   | 1.49               | 1.021            | 1.69    |
| HOLYS/GLY | .204       | .206      | 1.67                   | .127                   | .102               | 1.021            | .126    |
| HOPRO/GLY | .418       | .411      | .414                   | .363                   | .368               | .323             | .373    |

1. Fukushi and Spiro (1969).
2. Kefalides (1973).

Table 8. Amino Acid Composition of Isolated Glomerular Basement Membranes in Residues/1000 Residues

|           | DEOC<br>Glom | SON <sup>1</sup><br>Glom | SON <sup>2</sup><br>Glom | SON <sup>3</sup><br>Glom | SON <sup>4</sup><br>Glom | SON <sup>5</sup><br>Glom | SON <sup>6</sup><br>Glom | SON <sup>7</sup><br>Glom |
|-----------|--------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| HOPRO     | 70.2         | 44.2                     | 40                       | 58.3                     | 53.0                     | 51.4                     | 61.5                     | 57                       |
| ASP       | 79.8         | 72.7                     | 73                       | 88.8                     | 73.0                     | 82.4                     | 135.5                    | 70                       |
| THREO     | 38.4         | 42.5                     | 45                       | 44.7                     | 40                       | 41.5                     | ---                      | 45.5                     |
| SER       | 58.9         | 48.6                     | 57                       | 55.5                     | 59                       | 42.9                     | 74.4                     | 62.8                     |
| GLUT      | 98.3         | 98.5                     | 103                      | 112.0                    | 110                      | 108.3                    | 111.5                    | 100                      |
| PRO       | 66.3         | 55.8                     | 67                       | 60.2                     | 70                       | 73.2                     | 69.5                     | 62                       |
| GLY       | 202.3        | 177.3                    | 187                      | 191.0                    | 210                      | 176.6                    | 199.4                    | 200                      |
| ALA       | 56.0         | 68.2                     | 62                       | 64.7                     | 67                       | 62.6                     | 77.7                     | 67                       |
| VAL       | 36.0         | 42.2                     | 50                       | 43.7                     | 47                       | 49.4                     | 53.6                     | 43                       |
| CYS/2     | 24.5         | 22.2                     | 20                       | N.R.                     | 16                       | +                        | +                        | 20.4                     |
| METH      | 11.7         | 17.9                     | 2                        | N.R.                     | 9                        | 18.1                     | +                        | 11.0                     |
| ILEU      | 26.8         | 35.9                     | 36                       | 36.9                     | 28                       | 37.8                     | 38.8                     | 30.0                     |
| LEU       | 64.8         | 72.6                     | 86                       | 73.3                     | 67                       | 77.7                     | 80.1                     | 60.0                     |
| TYR       | 17.0         | 21.9                     | 19                       | 16.3                     | 18                       | 22.4                     | 17.3                     | 17                       |
| PHE       | 34.1         | 32.5                     | 29                       | 33.2                     | 33                       | 36.0                     | 33.1                     | 19                       |
| HOLYS     | 23.4         | 17.4                     | 15                       | 18.2                     | 32                       | 21.0                     | ---                      | 21.8                     |
| HIST      | 18.9         | 19.8                     | 24                       | 19.6                     | 28                       | 19.2                     | 21.8                     | 21                       |
| LYS       | 31.6         | 41.7                     | 33                       | 34.9                     | 14                       | 32.6                     | 65.0                     | 40                       |
| ARG       | 41.0         | 54.6                     | 54                       | 49.6                     | 49                       | 46.7                     | 60.0                     | 42.6                     |
| HOPRO/PRO | 1.05         | 0.79                     | 0.6                      | 0.96                     | 0.75                     | 0.69                     | 0.89                     | 0.92                     |
| HOLYS/LYS | 0.74         | 0.43                     | 0.47                     | 0.52                     | 1.16                     | 0.63                     | 0                        | 0.55                     |
| HOPRO/GLY | 0.34         | 0.25                     | 0.21                     | 0.30                     | 0.25                     | 0.29                     | 0.31                     | 0.29                     |
| HOLYS/GLY | 0.11         | 0.10                     | 0.08                     | 0.09                     | 0.15                     | 0.12                     | 0                        | 0.11                     |

1. Lehotay (1975).
2. Sachot et al. (1975).
3. Blau and Michael (1971).
4. McIntosh et al. (1971).
5. Wahl, Krezdorn, and Deppermann (1970).
6. Chow and Drummond (1969).
7. Kefalides (1973).

membranes as indicated by the higher ratios of hydroxyproline to proline, hydroxylysine to lysine, hydroxyproline to glycine and hydroxylysine to glycine. Kefalides (1973) has suggested that increases in the hydroxyproline, hydroxylysine and hexose content of isolated basement membranes is an index of membrane purity.

The primary criterion for basement membrane purity, however, is based on morphological grounds. Electron microscopic examination of the basement membrane obtained by the DEOC treatment of highly purified suborgan fractions exhibits a structure indistinguishable from its in vivo counterpart which is, in addition, free of any recognizable cellular elements. Taken together, the structural and chemical composition of the material obtained by the DEOC procedure meet the criteria for a highly purified basement membrane preparation.

#### Basement Membrane Protein Fractionation

Isolated glomerular, tubular, brain vessel, retinal vessel and lens capsule basement membranes and lathyrctic guinea pig skin collagen proteins were solubilized and fractionated by SDS-polyacrylamide gel electrophoresis. Solubilization was effected by treatment with SDS, mercaptoethanol and urea in Tris-phosphate buffer overnight at 37° C. The electrophoretic band pattern of these solubilized basement membrane proteins is shown in Figure 21. The majority of basement membrane proteins migrate similarly to the  $\alpha$  and  $\beta$  bands of lathyrctic guinea pig skin collagen. In all basement membrane samples studied, a band at the top of the gel was observed indicating the presence of a large protein

Figure 21. A Photograph of the Separation of Basement Membrane Proteins  
Obtained by SDS-Urea-Polyacrylamide Gel Electrophoresis

Electrophoretic migration was from left to right as shown in the photograph. Rat renal glomerular basement membrane (GBM), rabbit renal tubule basement membrane (TBM), lathyritic guinea pig skin collagen (COLLAGEN), bovine brain vessel basement membrane (BVBM), bovine lens capsule basement membrane (LCBM), and bovine retinal vessel basement membrane (RVBM).

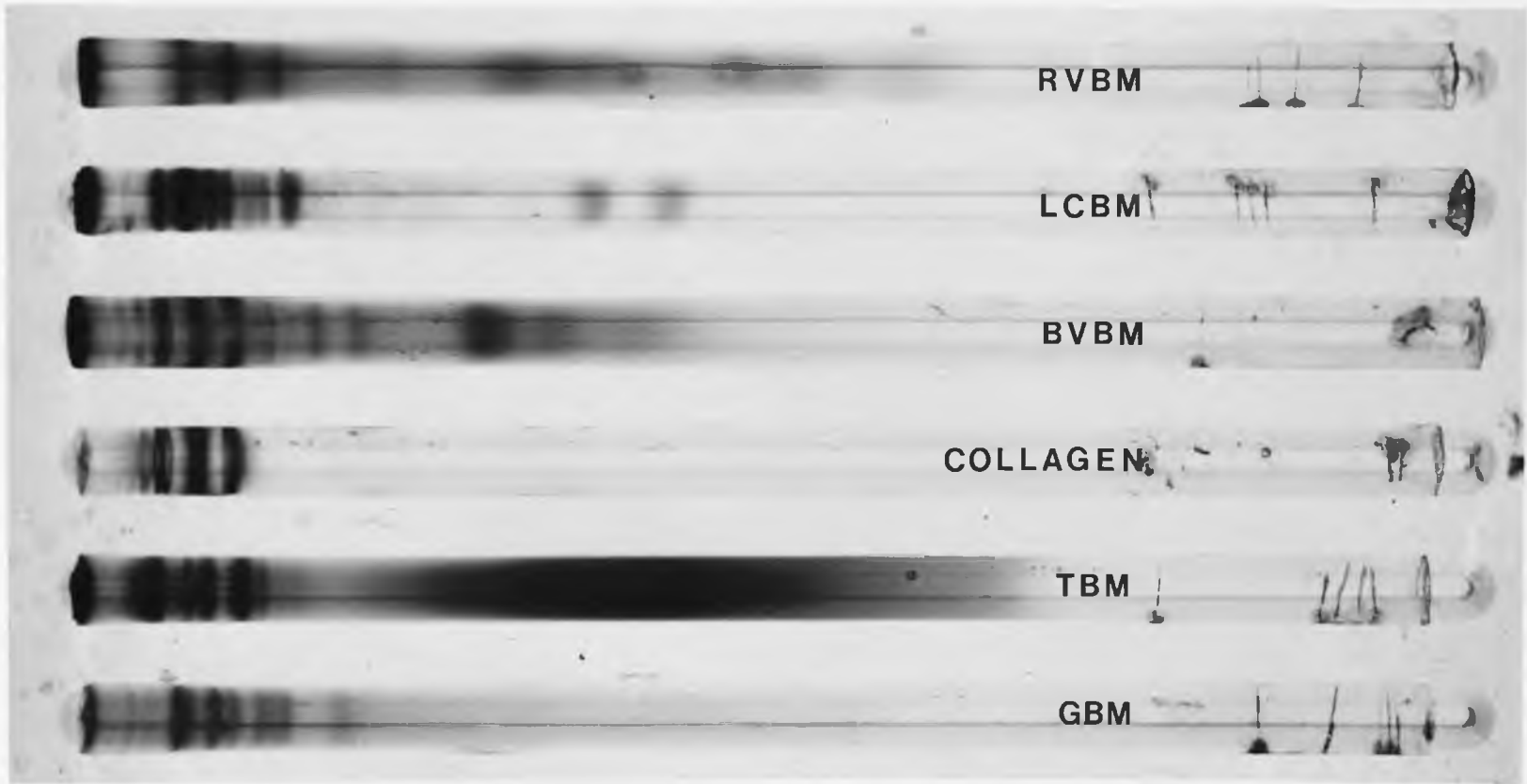


Figure 21. A Photograph of the Separation of Basement Membrane Proteins Obtained by SDS-Urea-Polyacrylamide Gel Electrophoresis

which could not penetrate the gel. This is in agreement with the solubility studies of Kefalides and Winzler (1966), Hudson and Spiro (1972a), and Myers and Bartlett (1972) which indicated that only 80 - 90% of the basement membrane could be solubilized by non-degradative treatments. This also suggests that the basement membrane is held together in part by non-disulfide bonds since reduction did not completely solubilize and allow the fractionation of all basement membrane proteins.

The observation of numerous protein bands supports the findings of Myers and Bartlett (1972) and Hudson and Spiro (1972a) that the glomerular basement membrane contains many separable proteins. Spiro (1976) has proposed that the polydisperse nature of the glomerular basement membrane proteins is caused by the degradative processes associated with basement membrane turnover. The minor bands were thought to be partially degraded matrix proteins. The observation of Kefalides and Winzler (1966) that the glomerular basement membrane contained only four separable protein bands upon solubilization and electrophoresis is not supported by this study. The four bands observed may be related to the major bands observed in the region of the lathyritic collagen bands. The minor bands may not have been observed due to protein underloading of the electrophoretic gels.

To gain a better view of the major bands shown in the gels in Figure 21, an expanded line drawing of the relative migrations of the major protein bands observed in each basement membrane sample is illustrated in Figure 22. Although it is possible to assign each band a relative migration value, the determination of molecular weights of each

Figure 22. Diagram of the Relative Migration of the Basement Membrane Proteins Isolated by the Deoxycholate Procedure

The relative migration axis is on the left hand margin of the diagram. Rat glomerular (GBM), rabbit tubule (TBM), bovine brain vessel (BVBM), bovine lens capsule (LCBM), and bovine retinal vessel (RVBM) basement membrane SDS-urea-polyacrylamide gel electrophoretic relative migrations are shown from left to right. Lathyritic guinea pig skin collagen and a portion of a gel containing molecular weight standard proteins are similarly depicted. The standard proteins shown are thyroglobulin and  $\beta$ -galactosidase. Only the major bands in each basement membrane gel were plotted.

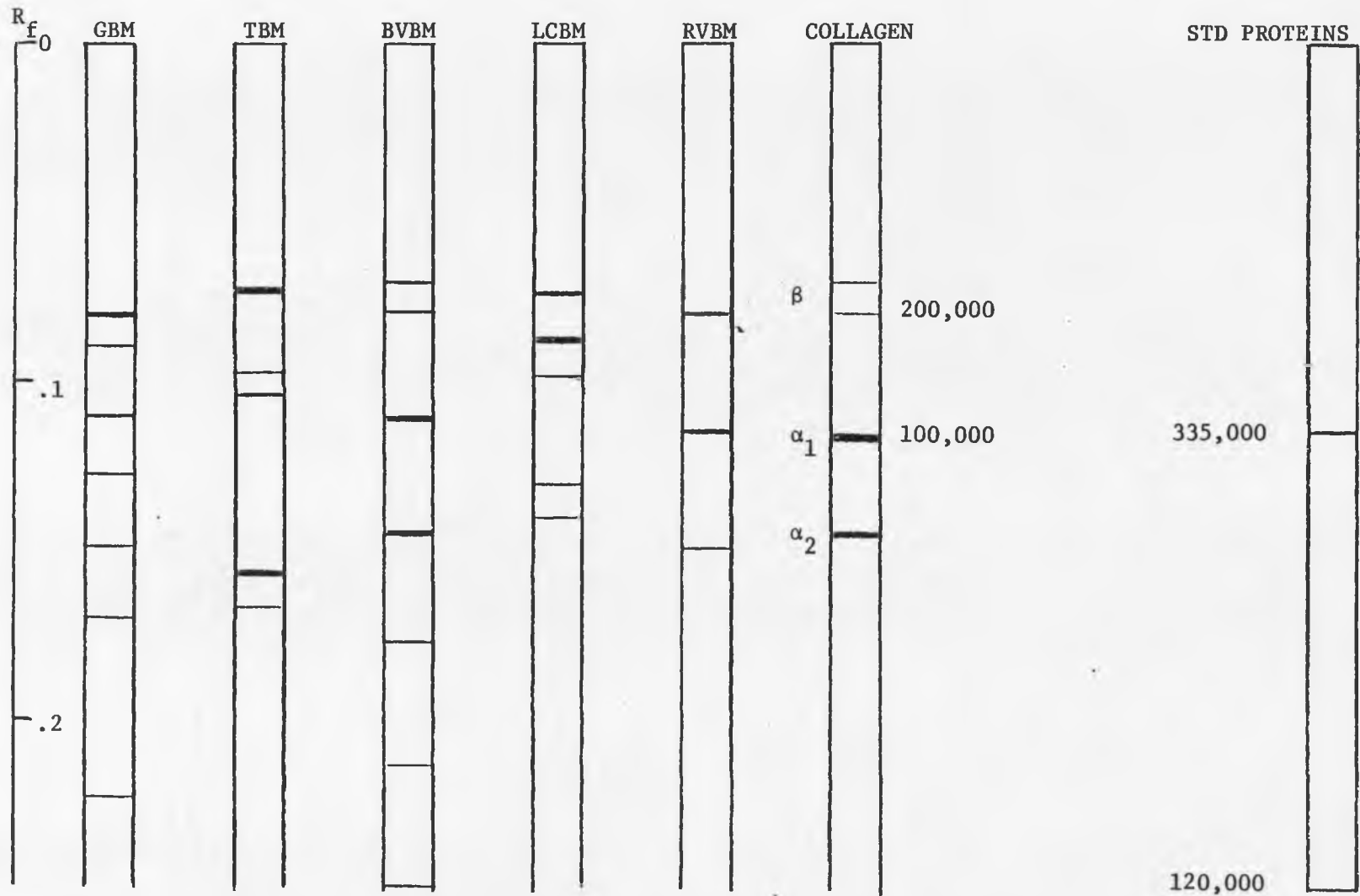


Figure 22. Diagram of the Relative Migration of the Basement Membrane Proteins Isolated by the Deoxycholate Procedure

band is difficult if not impossible with the information at hand. This is because the relative migrations of the standard proteins against which the sample proteins are compared to obtain molecular weights is dependent upon the type of proteins used as molecular weight standards. The collagen standard proteins migrate much differently than do non-collagenous standard proteins. Since the chemical nature of each protein band is not known, it is not possible to predict which molecular weight standards should be applied to which protein bands. The problem of molecular weight assignment may be complicated even further if basement membranes contain proteins which have both collagen regions and non-collagenous glycoprotein regions.

Since collagen (Kefalides, 1973) or collagen-like proteins (Spiro, 1976) are thought to be present in the basement membrane matrix, a comparison of the basement membrane protein bands to those of lathyritic guinea pig skin collagen is appropriate. There are protein bands in each basement membrane sample that migrate in the same region as the  $\beta$  bands of the skin collagen. The glomerular, brain vessel, and retinal vessel basement membrane samples exhibit a similar band in the region of  $\alpha_1$  band of the skin collagen. In the region of the  $\alpha_2$  collagen band, the retinal, brain vessel, and glomerular basement membrane samples again exhibit a similar band. Although there are similar bands present in many of the gels that migrate similarly to the collagen proteins, it is apparent that the basement membranes are more complex than skin collagen. It is also apparent that the basement membrane samples do not have the same band pattern which suggests that the various basement

membranes are not composed of identical proteins. However, certain proteins may be similar between basement membranes obtained from various suborgan fractions.

#### Effect of Degradative Enzymes on Basement Membrane Ultrastructure

Another approach used to study the structure of the basement membrane matrix has been reported by Carlson et al. (1976). Rabbit kidneys were perfused with buffer and iron oxide as described in the Materials and Methods chapter. The proximal tubules isolated from these kidneys were treated with a 4% solution of sodium deoxycholate and the basement membrane isolated. The saline washed basement membranes were then digested with either a 1% solution of pepsin, trypsin or pronase for 30 minutes at 22° C. Electron microscopic examination of the digested basement membranes showed pepsin to more degradative than trypsin which was more active than pronase. When a highly purified collagenase (Worthington CLSPA) was used to partially digest the isolated tubule basement membrane, fine 40 - 50 Å fibrils were preferentially released from one side of the basement membrane matrix. The non-specific proteases also demonstrated a sidedness in their degradation, although it was less dramatic. Hyaluronidase had no demonstrable effect on basement membrane structure. It was not known whether the proteases preferred the same side of the basement membrane matrix as the collagenase. The demonstration that these enzymes preferentially digested one side of the matrix implies that the structure of the basement membrane is different on opposite sides of its surface.

From tissue repair experiments, Vracko (1974a) has proposed that the basement membrane is sided. When muscle capillaries were removed and replanted, the repopulating cells grew on that side of the basement membrane that cells of the same type had previously occupied (Vracko and Benditt, 1972). The present finding that degradative enzymes preferentially digested one side of the basement membrane surface suggests that the structure of the two surfaces of the basement membrane are in some way different. This provides structural evidence for basement membrane sidedness.

Mahieu and Winand (1970) have reported that isolated human tubule basement membrane exhibited a fibrillar periodicity characteristic of collagen. No such structural collagen was observed during extensive electron microscopic examination of the rabbit tubule basement membrane used in this study (Carlson et al., 1976).

### Metabolism

#### Rat Glomerular Gluconeogenesis

Before extensive studies of the metabolism of the basement membrane were begun, it was necessary to know if the glomeruli were metabolically intact. The glomeruli isolated by the iron oxide perfusion method (Meezan et al., 1973) have been reported to form  $^{14}\text{CO}_2$  from a number of  $^{14}\text{C}$ -substrates, incorporated  $^3\text{H}$ -amino acids into TCA precipitable, incorporated  $^3\text{H}$ -uridine into RNA, demonstrated oxygen consumption and formed  $^{14}\text{C}$ -glucose from  $^{14}\text{C}$ -lactate.

It is known that glomerular basement membrane morphology is affected by a number of disease states. In diabetes mellitus, the basement membrane becomes thicker while glomerular filtration function is impaired. It has been proposed (Spiro, 1971) that in diabetes glucose is shunted to secondary pathways which ultimately result in the alteration of glomerular basement membrane metabolism. Since gluconeogenesis is stimulated under diabetic conditions, we were interested in the sources of glucose which might affect the glomerulus.

To study the endogenous formation of glucose by the glomerulus, rat glomeruli were isolated by the method of Meezan et al. (1973) and incubated with various radiolabeled gluconeogenic substrates. During the course of experimentation, the isolation procedure was modified by adding a 110  $\mu$  pore size sieve between the standard 153  $\mu$  and 64  $\mu$  sieves. Introduction of the intermediate screen resulted in the majority of gluconeogenic activity being found in the glomerular fraction retained on the 110  $\mu$  sieve. Light microscopic examination of the 110  $\mu$  and 64  $\mu$  fractions revealed that the glomeruli collected on the larger pore size sieve retained a higher percentage of their Bowman's capsule than did the 64  $\mu$  fraction. The presence of Bowman's capsule was found to correlate with  $^{14}\text{C}$ -glucose production. Decreasing the percentage of attached Bowman's capsule by rehomogenization resulted in a decrease in the production of  $^{14}\text{C}$ -glucose from  $^{14}\text{C}$ -pyruvate (Figure 23). However, the rate of  $^{14}\text{CO}_2$  formation from  $^{14}\text{C}$ -1-oleate (Figure 24) was not changed.

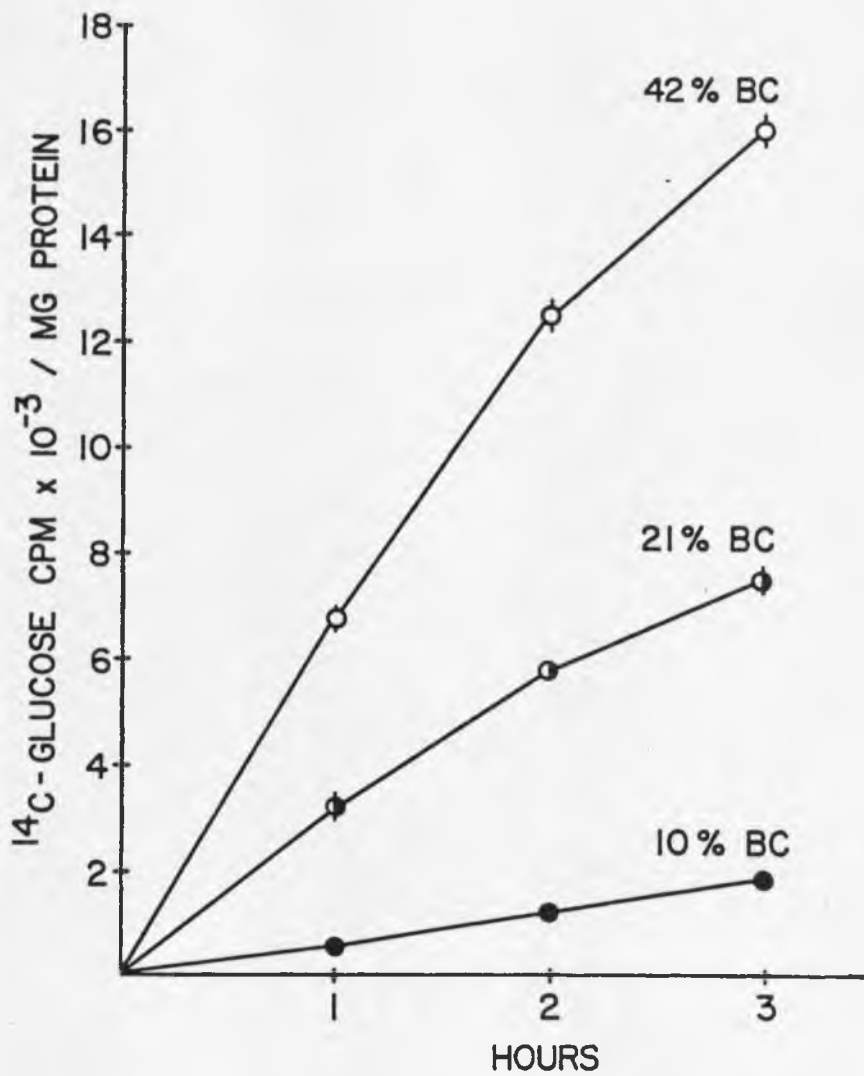


Figure 23.  $^{14}\text{C}$ -Glucose Formation in Glomerular Tuft Preparations of Varying Bowman's Capsule Content

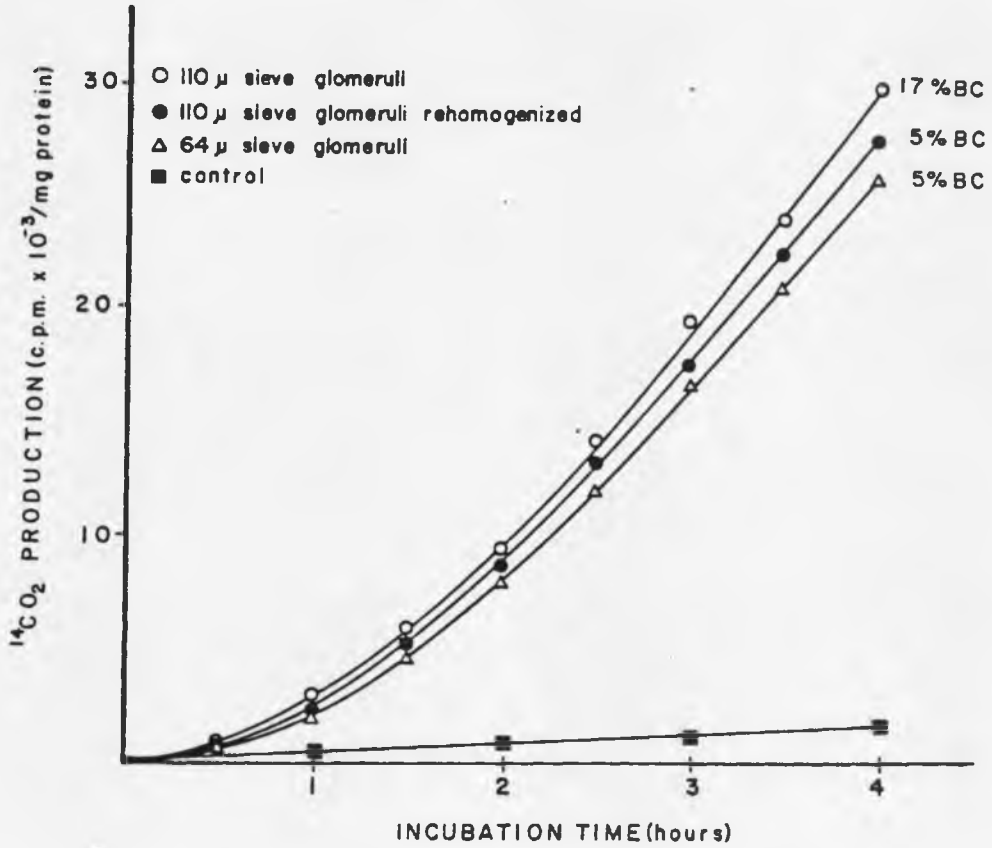


Figure 24.  $^{14}\text{CO}_2$  Formation in Glomerular Tuft Preparations of Varying Bowman's Capsule Content

These findings raised several questions concerning glomerular metabolism. Do isolated glomerular tufts have the metabolic machinery to perform gluconeogenesis? Gluconeogenesis is classically defined as the net flow of carbon atoms from lactate or pyruvate to glucose via a sequence of enzymatic reactions. Thus, the appearance of labeled glucose after tissue incubation with labeled pyruvate may not necessarily mean net carbon flow to glucose but only a sporadic conversion of a small percentage of each gluconeogenic enzyme's substrate pool. However, the appearance of labeled glucose from pyruvate does suggest the potential for net gluconeogenesis. The absence of appearance of labeled glucose in the glomerular tuft preparations may be due to 1) inappropriate incubation conditions which do not stimulate glomerular glucose production, 2) glomerular tuft damage due to the isolation procedure, or 3) the lack of the enzymes or cofactors which define the gluconeogenic pathway.

To investigate these various hypotheses, a number of experimental techniques were developed. Using a modification of the iron oxide perfusion isolation procedure, glomerular tufts, glomerular tufts with attached Bowman's capsule, and tubules were separated and studied.  $^{14}\text{C}$ -glucose production was observed in both glomeruli with Bowman's capsule, and tubules, but only minimally in the glomerular tufts. This small sporadic  $^{14}\text{C}$ -glucose production observed in the glomerular tufts was not significantly above background and was independent of glomerular protein concentration (Table 9). Glomerular tufts and glomerular tufts with attached Bowman's capsule gave similar rates of  $^3\text{H}$ -amino acid

Table 9. Percent of Tubule <sup>14</sup>C-Glucose Production by Isolated Glomerular Fractions (cpm/mg Protein)

|           | Lactate  | Glutamate | Succinate |
|-----------|----------|-----------|-----------|
| Glom - BC | 3.4(4)   | 0.5(20)   | 0.2(18)   |
| Glom + BC | 7.2(4)   | 6.6(13)   | 8.6(13)   |
| Tubules   | 100.0(4) | 100.0(10) | 100.0 (8) |

The number in parenthesis is the number of experimental samples analyzed.

incorporation into TCA precipitable protein indicating that the glomeruli were metabolically active although non-gluconeogenic (Figure 25). Since the Bowman's capsule is continuous with the tuft being linked by a basement membrane, the consistent removal of all Bowman's capsular elements was extremely difficult and beyond the scope of present methodology.

To determine if incubation conditions were optimal for glomerular tuft  $^{14}\text{C}$ -glucose production, the effect of pH on  $^{14}\text{C}$ -glucose formation was measured (Figure 26). The pH optimum for  $^{14}\text{C}$ -glucose formation from  $^{14}\text{C}$ -succinate by tubules and glomerular tufts with attached Bowman's capsule was 7.2. This is similar to the gluconeogenic pH optimum reported by Kurokawa and Rasmussen (1973) for isolated rat kidney tubules. The isolated glomerular tufts devoid of capsular elements gave only trace activity. This trace activity was not dependent on the pH of the incubation medium.

The results obtained from  $^{14}\text{CO}_2$  production, amino acid incorporation and pH optimum experiments are compatible with the hypothesis that Bowman's capsule or minute quantities of proximal tubule attached to Bowman's capsule are responsible for the gluconeogenic activity observed in the earlier preparations (Hjelle, Meezan, and Brendel, 1975). Realizing the difficulty of proving a negative, the possibility that rat renal glomerular tufts do not possess the metabolic machinery to classify them as gluconeogenic is suggested. The observation that significant  $^{14}\text{C}$ -glucose production was observed in known gluconeogenic tissue isolated from the same organ, but not in glomerular tufts would support

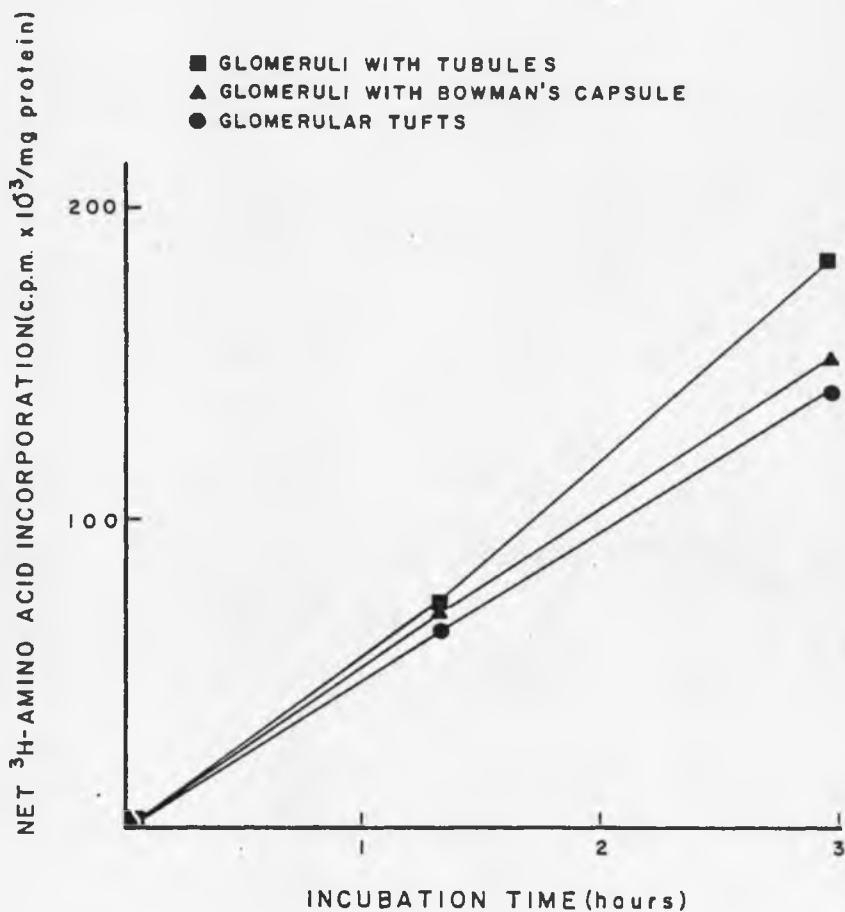


Figure 25. Comparison of Protein Synthetic Activity in Isolated Glomerular Tufts, Glomerular Tufts with Bowman's Capsule, and Glomeruli with Tubules

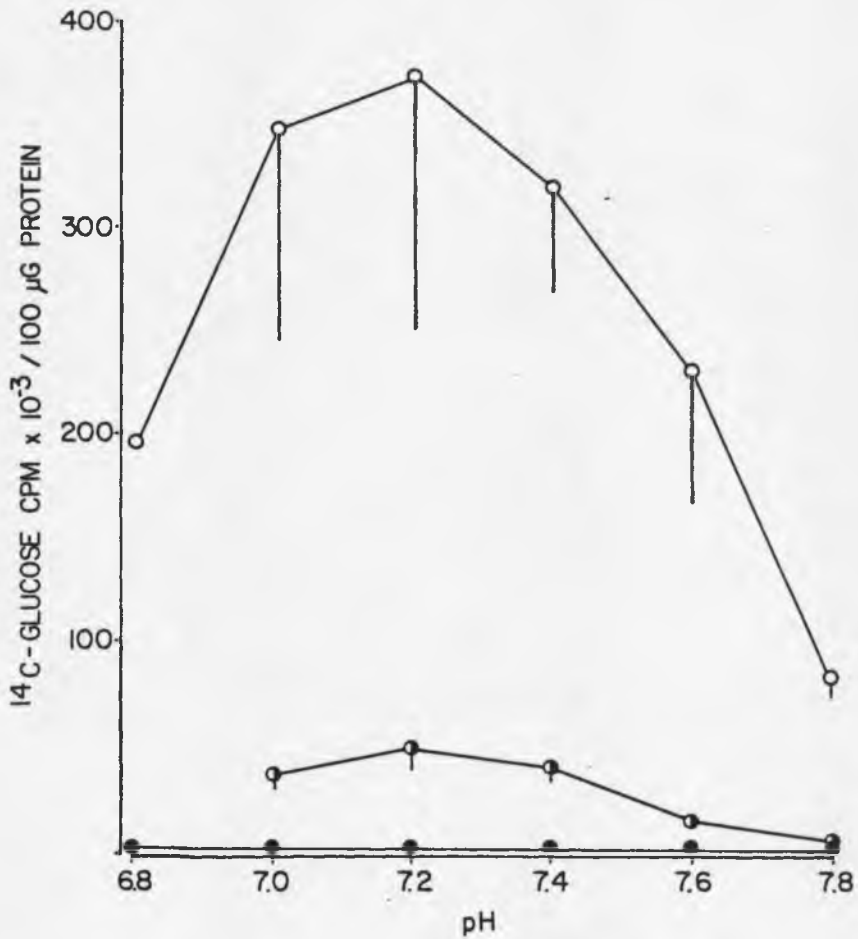


Figure 26. Effect of pH on  $^{14}\text{C}$ -Glucose Formation by Glomerular Tufts, Glomerular Tufts with Bowman's Capsule, and Isolated Rat Tubules

The glomerular tufts ●, glomerular tufts with Bowman's capsule ◐, and isolated rat tubules ○ were isolated from the same rat kidney preparation. Standard error of the mean is shown.

this conclusion. The possibility that perfusion of metal particles under physiological pressure might affect glomerular tuft <sup>14</sup>C-glucose production is extremely difficult to assess. Attempts to isolate glomerular tufts without particle perfusion could not remove tubule fragments, glomeruli with attached Bowman's capsule or tissue debris. A pure glomerular tuft preparation is essential for the characterization of glomerular metabolism due to the diverse structures and their associated functions which contaminate glomerular preparations.

Because of the lack of gluconeogenic activity in the glomerular tuft, gluconeogenesis did not appear to be a factor which could significantly influence glomerular basement membrane metabolism. It also suggested that if glucose altered basement membrane metabolism that the glucose must be derived from extracellular pools.

#### Rabbit Proximal Tubule Metabolism

The isolated proximal tubule preparation was of interest to us because it provided a one cell type system to study basement membrane metabolism. The isolated glomerular system contains at least three different cell types. The role of these three cell types in basement membrane metabolism is poorly understood.

Before basement membrane studies were begun, however, it was necessary to determine the viability of the isolated tubules prepared by hand homogenization and sieving. The metabolic activity of tubules obtained by the classic collagenase digestion procedure of Burg and Orloff (1962) was used as a standard of tissue viability.

When  $^{14}\text{C}$ -glucose production from  $^{14}\text{C}$ -succinate was measured in tubules isolated by the described methods from 24 hour starved rabbits, a significant difference was observed. The proximal tubules isolated by the iron oxide perfusion technique were significantly more active in forming  $^{14}\text{C}$ -glucose than were the collagenase treated preparations regardless of whether the tubules were initially isolated from the iron oxide perfused or collagenase perfused kidney (Figure 27). Collagenase digestion also diminished  $^{14}\text{CO}_2$  production from  $^{14}\text{C}$ -succinate (Figure 28). Amino acid uptake (Figure 29), protein synthesis (Figure 30), uridine uptake (Figure 31) and RNA synthesis (Figure 32) were all found to be depressed in those tubule preparations which were treated with collagenase. The rate of  $\rho$ -aminohippuric acid uptake was found to be substantially greater in the non-collagenase treated tubules as shown in Figure 33. The ability of tubules to transport  $\rho$ -aminohippuric acid is regarded as an index of their viability. Because of the depressed metabolism and altered morphology of the tubules following the collagenase treatment, tubules isolated by hand homogenization and sieving were employed in the basement membrane experiments.

#### Glomerular Basement Membrane Metabolism

##### Incorporation Experiments

In order to employ the deoxycholate method of basement membrane isolation in the study of the formation of the basement membrane, it was necessary to know if the rate of synthesis and laydown of de novo

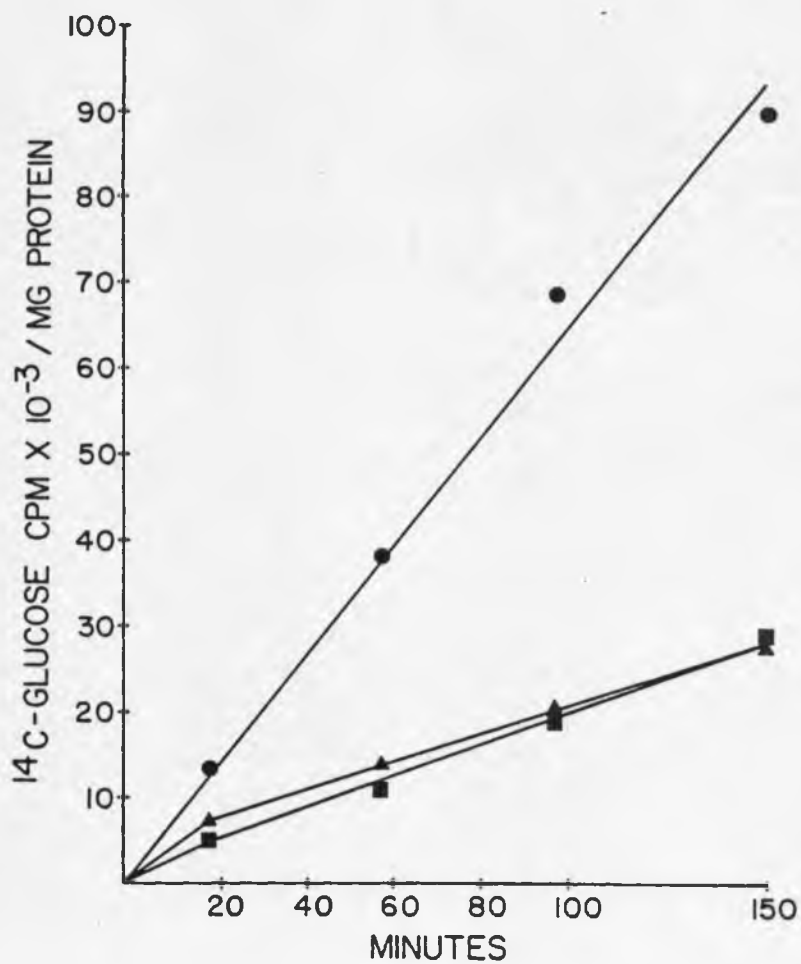


Figure 27. The Effect of Collagenase on Rabbit Tubule <sup>14</sup>C-Glucose Production

No collagenase ●.

Isolated with the use of collagenase ▲.

Isolated by sieving followed by incubation with collagenase ■.

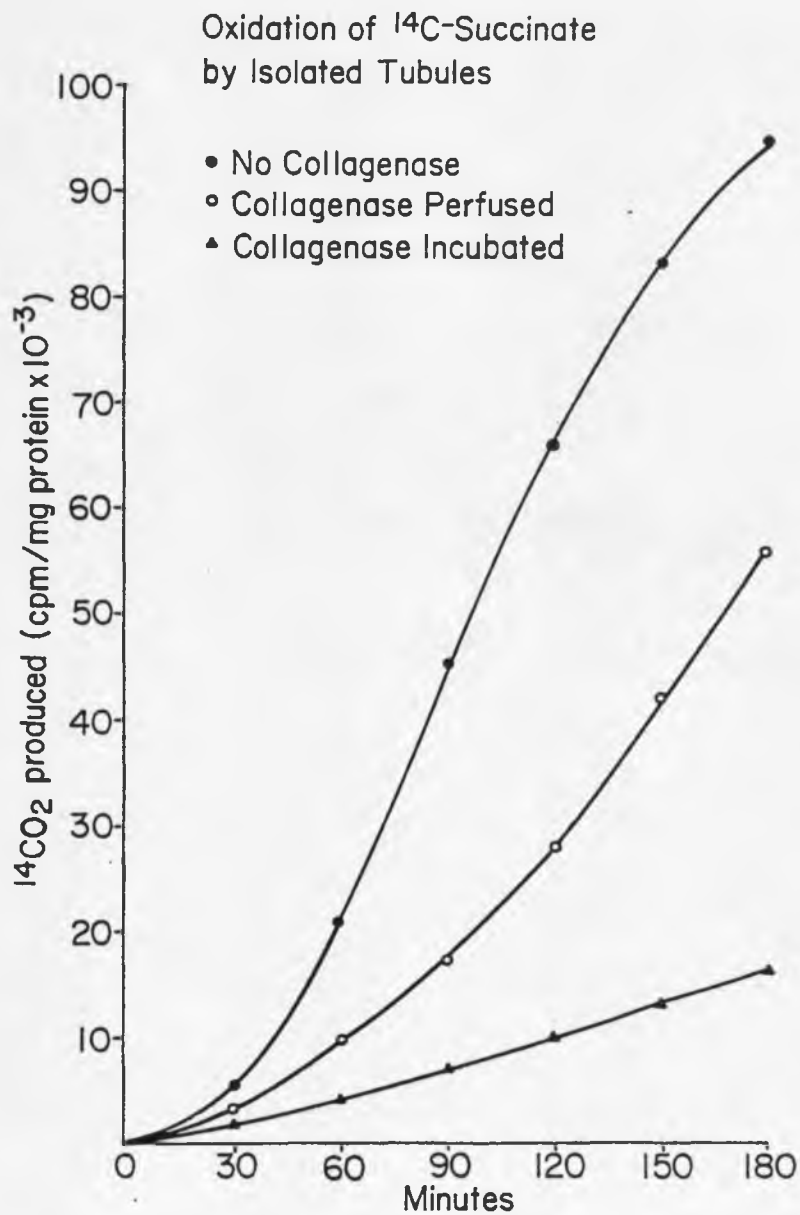


Figure 28. The Effect of Collagenase Digestion on Isolated Rabbit Tubule  $^{14}\text{CO}_2$  Production

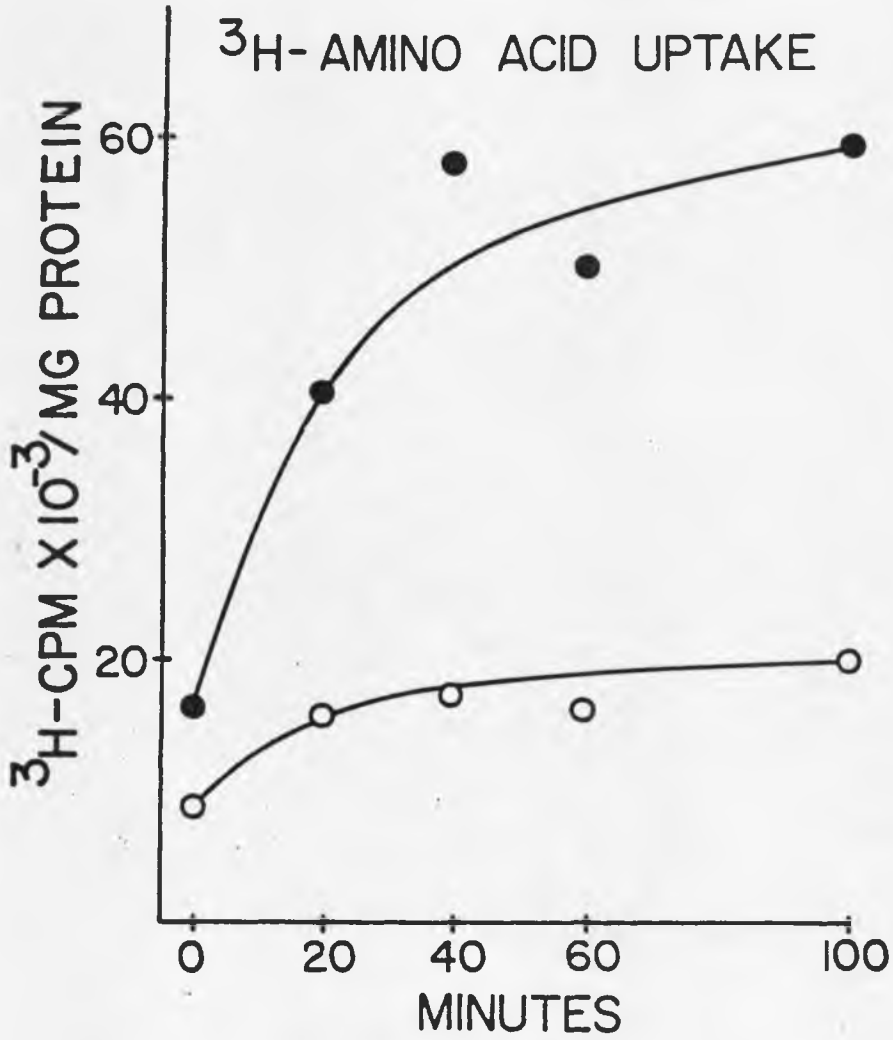


Figure 29.  $^3\text{H}$ -Amino Acid Uptake by Isolated Rabbit Tubules

No collagenase ● .

Collagenase treated ○ .

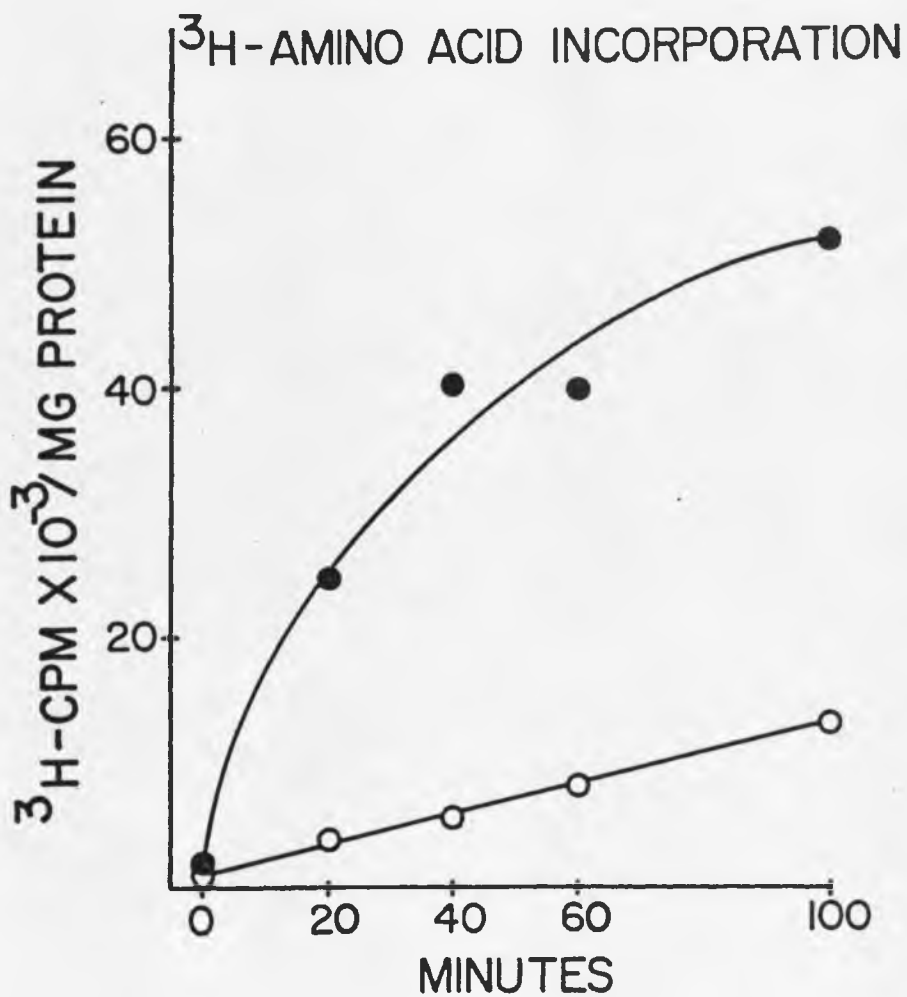


Figure 30. <sup>3</sup>H-Amino Acid Incorporation into TCA Precipitable Protein

No collagenase ● .  
Collagenase treated ○ .

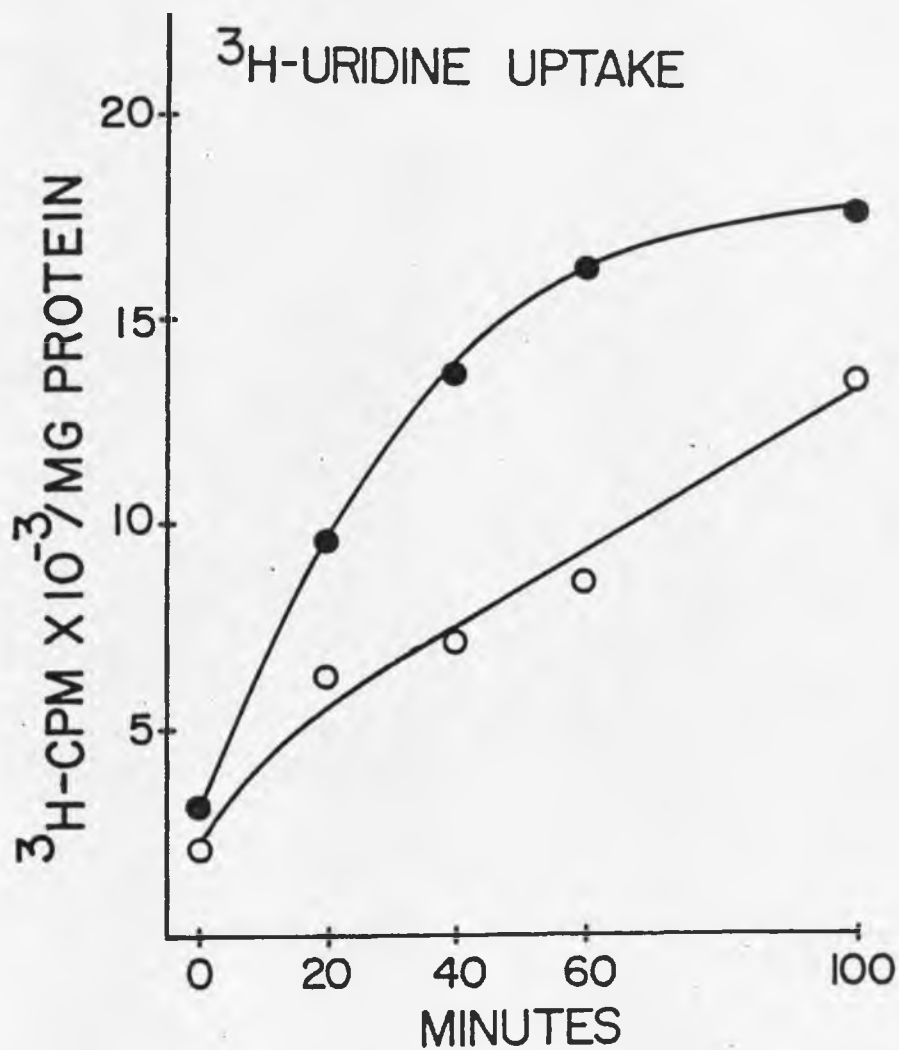


Figure 31. <sup>3</sup>H-Uridine Uptake by Isolated Rabbit Tubules

No collagenase ● .  
Collagenase treated ○ .

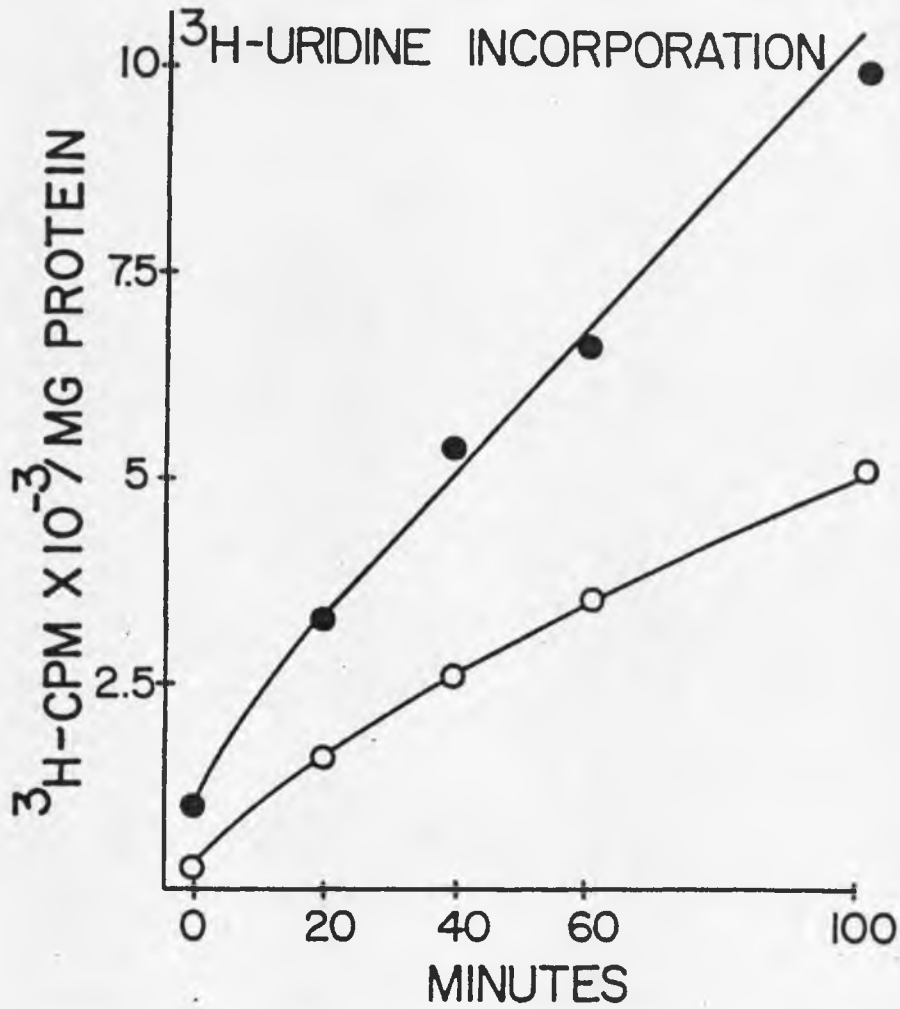


Figure 32. <sup>3</sup>H-Uridine Incorporation into TCA Precipitable Material by Isolated Rabbit Tubules

No collagenase ● .  
Collagenase treated ○ .

### $^{14}\text{C}$ -PAH Uptake by Isolated Tubules

- No Collagenase
- ▲ 10' Collagenase
- 20' Collagenase
- 30' Collagenase

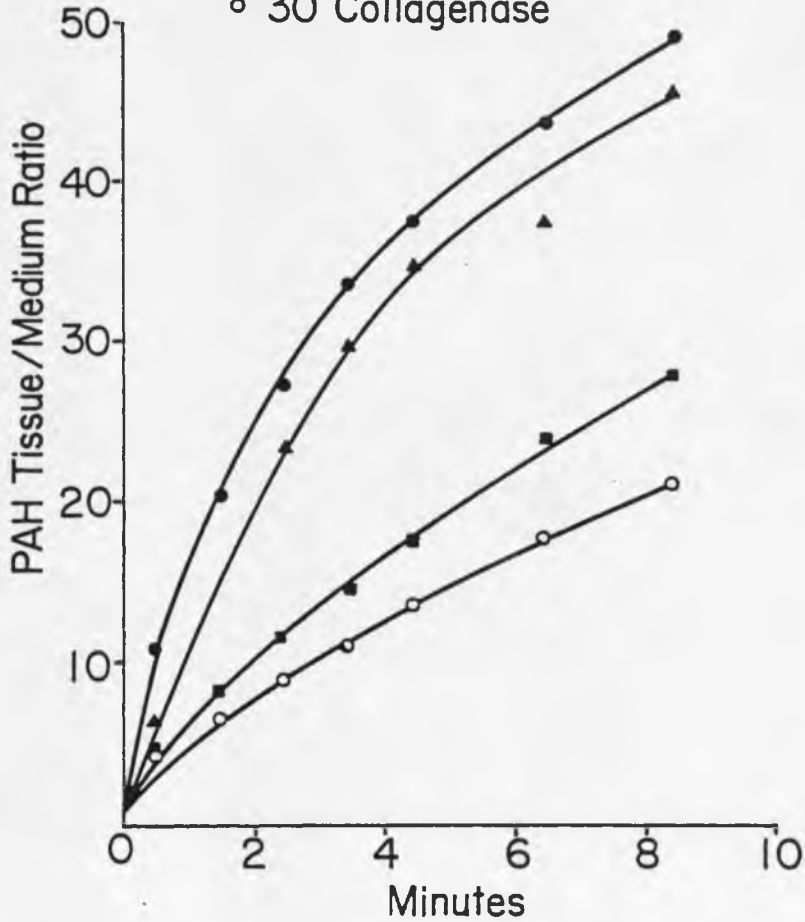


Figure 33. The Effect of Collagenase Digestion on the Uptake of  $^{14}\text{C}$ - $\rho$ -Aminohippuric Acid by Isolated Rabbit Tubules

basement membrane protein was of sufficient velocity to permit accurate measurement. Several independent studies had suggested that the turnover of the basement membrane was a slow process taking several months (Walker, 1973; Spiro, 1976). However, Meezan et al. (1973) had demonstrated that isolated glomeruli incorporated radiolabeled amino acids into TCA precipitable proteins. Grant, Harwood, and Williams (1975) subsequently reported that isolated glomeruli incorporated proline and lysine into non-diffusible material, but that basement membrane protein formation accounted for less than 5% of the total incorporation. Using a preparation of isolated glomeruli, Cohen and Vogt (1975) have reported that radiolabeled lysine was incorporated into material which pelleted with the sonicated basement membrane fragments. Initial experiments using isolated glomeruli incubated with radiolabeled amino acids and carbohydrates from which the basement membranes were isolated by sonication and centrifugation resulted in the appearance of radiolabel in the pelleted protein.

To study de novo basement membrane formation, glomeruli isolated by the iron oxide perfusion method were incubated with radiolabeled amino acids and carbohydrates. By using deoxycholate to isolate the basement membrane matrix, it was hoped that the non-matrix newly formed proteins could be removed and the newly synthesized deposited matrix proteins obtained. To determine the feasibility of such procedures, glomeruli were incubated with a mixture of  $^{14}\text{C}$ -amino acids in Earle's basic salts buffered with HEPES fortified with Penicillin G<sup>R</sup>. After 150, 200, and 400 minutes of incubation, multiple aliquots were removed and

microfuged for 15 seconds. The supernatants were removed by a drawn Pasteur pipet while the tubes containing the magnetic iron oxide filled glomeruli were held over a permanent magnet. This avoided loss of glomeruli during supernatant removal. To monitor the removal of extracellular free  $^{14}\text{C}$ -amino acids from the incubated glomeruli, the pelleted glomeruli were suspended in 1 ml of 0.9% saline, microfuged and the supernatant removed and prepared for scintillation spectrometry. The glomerular samples were washed three times with saline in this manner.

Determination of the radiolabeled material in the saline supernatants provided information as to the rate of removal of extracellular free amino acids which would interfere with the measurement of intracellular amino acid uptake and utilization. Figure 34 shows the loss of radiolabeled material from predominantly extracellular portions of the glomeruli. The decrease in variability with increased number of washings suggested that the washing procedure could be performed in a reasonably consistent manner and that only a small amount of radiolabel remained after three washings. The small amount of radioactivity obtained in the third wash might be due to either residual extracellular saline soluble material or leakage from intracellular sites.

To determine the effect of passive DEOC treatment versus a rigorous DEOC treatment, the saline washed glomerular samples were each suspended in 1 ml of a 4% DEOC solution with vigorous vortexing. Half of the samples were then sonicated while the other half were only vortexed.

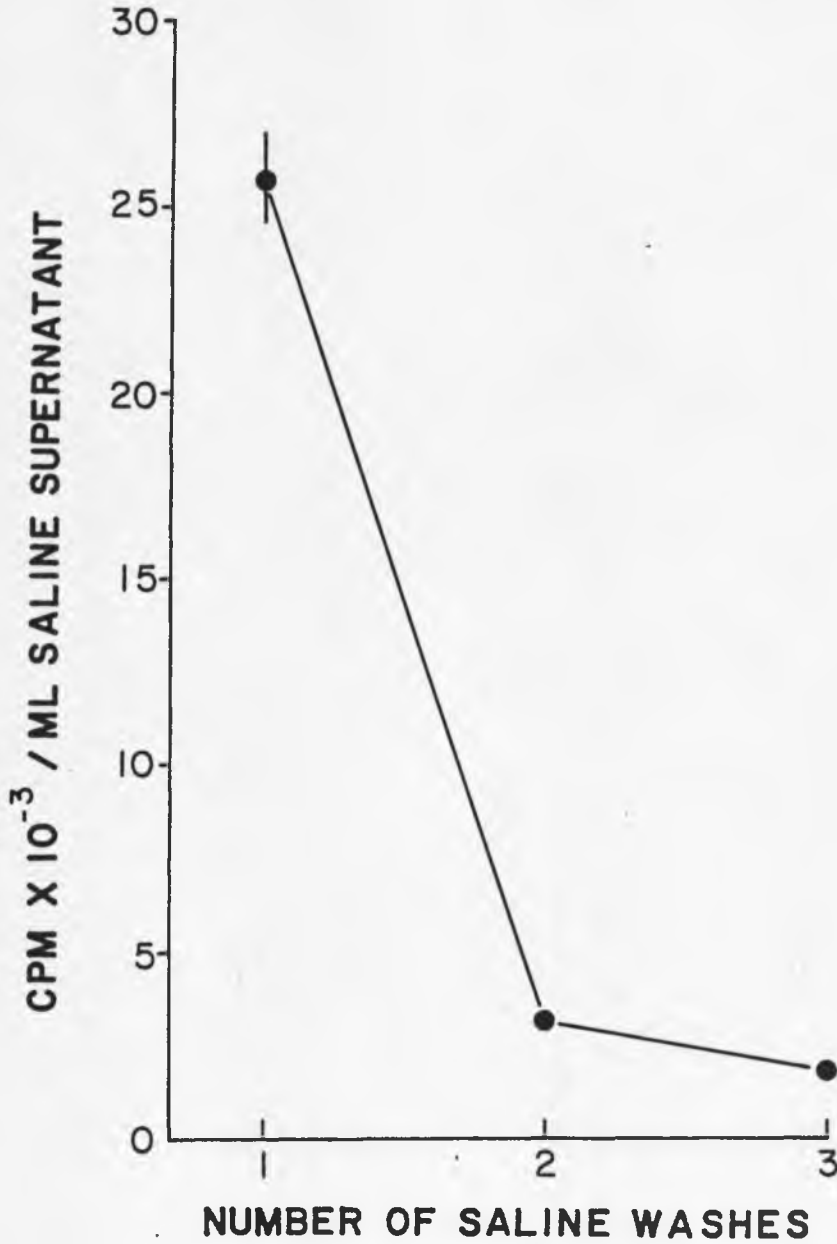


Figure 34. The Effect of Multiple Saline Washes in Removing Saline Extractable  $^{14}\text{C}$ -Amino Acids from Incubated Rat Glomeruli

After standing overnight at room temperature, the samples were microfuged and the supernatant removed and prepared for scintillation counting. The pellets were twice resuspended in 1 ml of DEOC, vortexed, microfuged, and the supernatants removed and counted. Table 10 depicts the loss of intracellular radioactivity from sonicated and non-sonicated DEOC treated saline washed glomeruli. An asymptotic decrease in the DEOC soluble radioactive material with each subsequent DEOC extraction was observed. Interestingly, the amount of radioactivity extracted in all the DEOC washes increased with increased incubation time. This increase could be due to 1) uptake of amino acids into intracellular spaces, 2) intracellular protein synthesis, 3) radiolabeled secreted proteins which were beginning to associate with extracellular structures, or 4) residual radiolabeled amino acids bound nonspecifically to extracellular sites. Since sonication in the detergent, sodium deoxycholate, did not significantly increase the small amount of radioactivity removed in the third DEOC wash relative to the non-sonicated preparation, it was presumed that the majority of non-DEOC extractable radioactivity was tightly bound to the extracellular material. The three times DEOC treated glomeruli were subsequently washed with saline resulting in the extraction of only a small amount of radioactivity. When this insoluble material was again washed with DEOC, no significant radioactivity could be removed. The remaining DEOC insoluble material was suspended in 1 ml of 0.1 N NaOH and heated for 18 hours at 60° C. A 100 µl aliquot was removed from each conical tube for protein assay by the fluram method. The remaining 900 µl was neutralized with HCl and

Table 10. Deoxycholate Extraction of Radiolabel from Isolated Glomeruli

| Incubation Time (Min) | Wash # | <u>Deoxycholate Soluble Material</u> |   |       |                          |   |     |
|-----------------------|--------|--------------------------------------|---|-------|--------------------------|---|-----|
|                       |        | Vortex Only<br>(CPM/ML Deoxycholate) |   |       | Sonication and<br>Vortex |   |     |
| 150                   | 1      | 44,240                               | ± | 789   | 45,478                   | ± | 490 |
|                       | 2      | 825                                  | ± | 146   | 604                      | ± | 10  |
|                       | 3      | 100                                  | ± | 10    | 47                       | ± | 2   |
| 300                   | 1      | 56,490                               | ± | 1,990 | 58,740                   | ± | 687 |
|                       | 2      | 1,143                                | ± | 83    | 683                      | ± | 41  |
|                       | 3      | 130                                  | ± | 12    | 108                      | ± | 20  |
| 400                   | 1      | 79,723                               | ± | 667   | 79,700                   | ± | 886 |
|                       | 2      | 2,394                                | ± | 81    | 1,599                    | ± | 90  |
|                       | 3      | 414                                  | ± | 68    | 329                      | ± | 66  |

Mean  $\pm$  Standard Error of the Mean.

n = 4.

counted. Table 11 shows the rate of incorporation of  $^{14}\text{C}$ -amino acids into DEOC insoluble material. Sonication of the washed glomeruli in DEOC resulted in a lower rate of incorporation than did only vortexing in DEOC. However, both treatments show incorporation into a structure which is ultrastructurally and chemically identical to basement membrane. Sonication in detergents would be expected to remove noncovalently bound proteins and amino acids.

Earlier experiments had shown that when large quantities of tissue were treated with DEOC a viscous gel was formed. This gel interfered with further extraction and manipulation of the tissue. To determine if a similar phenomenon might also interfere with glomerular biosynthetic experiments, isolated glomeruli were incubated with  $^3\text{H}$ -proline plus MEM amino acids. After 330 minutes of incubation, aliquots of varying volumes were removed from the batch incubation suspension and placed in conical microfuge tubes. The glomerular basement membrane was obtained by treating the glomeruli with two 1 ml saline washes, two 1 ml DEOC washes and a 1 ml saline wash, respectively. Solubilization of the basement membrane protein by heating at  $60^\circ\text{C}$  overnight in 0.1 N sodium hydroxide permitted the quantification of the protein in each tube. The plot of radiolabel incorporation into DEOC insoluble material versus protein concentration is shown in Figure 35. It appears that there is a linear relationship between incorporation and protein concentration between protein values of 50 to 200  $\mu\text{gms}$ . Tissue manipulation was not hindered by this experimental regime.

Table 11. Radiolabel Remaining After Multiple Deoxycholate Extractions

| Incubation Time | <u>Deoxycholate Insoluble Material</u><br>(CPM) |               |
|-----------------|---|---------------|
|                 | Vortex  | Sonication    |
| 150             | 1829 $\pm$ 20                                   | 1551 $\pm$ 20 |
| 300             | 2516 $\pm$ 107                                  | 2190 $\pm$ 95 |
| 400             | 3436 $\pm$ 52                                   | 2932 $\pm$ 84 |

Mean  $\pm$  Standard Error of the Mean.

N = 4

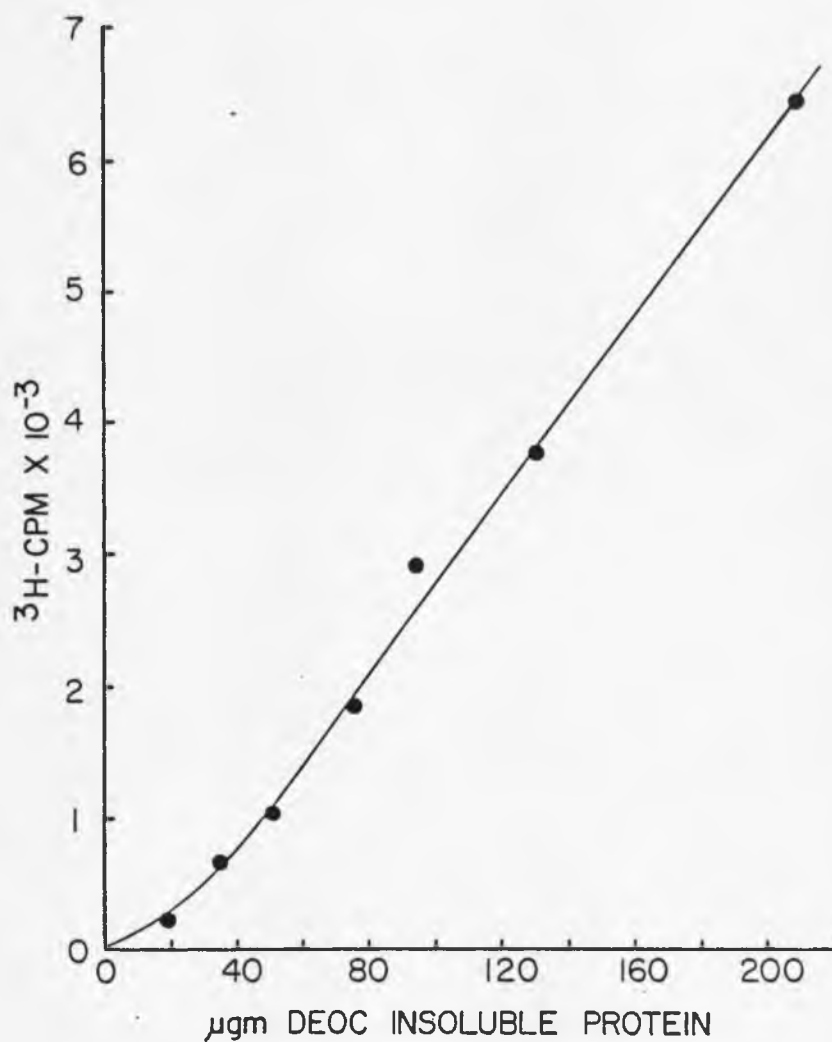


Figure 35. Incorporation of <sup>3</sup>H-Proline into DEOC Insoluble Material Plotted Against Protein Concentration

For comparison, isolated proximal tubules were incubated with a mixture of  $^{14}\text{C}$ -amino acids under similar conditions. The appearance of  $^{14}\text{C}$ -amino acids in the isolated tubule basement membrane obtained by DEOC treatment is shown in Figure 36. Using the isolated bovine retinal vessel system, White, Meezan, and Brendel (1975) have also observed radiolabel incorporation into isolated basement membrane.

Since it is generally agreed that basement membranes contain a collagenous component, we attempted to demonstrate the incorporation of radiolabeled amino acids known to be found in large amounts in collagens into DEOC insoluble material. Radiolabeled proline, glycine, lysine, glucosamine, and galactose have been observed to be taken up and incorporated into DEOC insoluble material. Figure 37 shows the incorporation of glucosamine into DEOC insoluble material. The incorporation of radiolabeled glucosamine, lysine, and glycine into the matrix was linear after the first hour of incubation. Krisko and Walker (1974) have reported the incorporation of radiolabeled glucosamine into glomerular protein to be linear over a 10 hour incubation period. Clark et al., (1975) using the embryonic rat parietal yolk sac have reported that the synthesis and laydown of  $^3\text{H}$ -glucosamine was linear throughout a two hour incubation. Glucosamine is a component of the heteropolysaccharide found in the non-collagen portions of the basement membrane proteins (Kefalides, 1973). The observation that glucosamine incorporation into basement membrane matrix bound proteins was linear through the incubation period suggests that non-collagenous proteins were being formed.

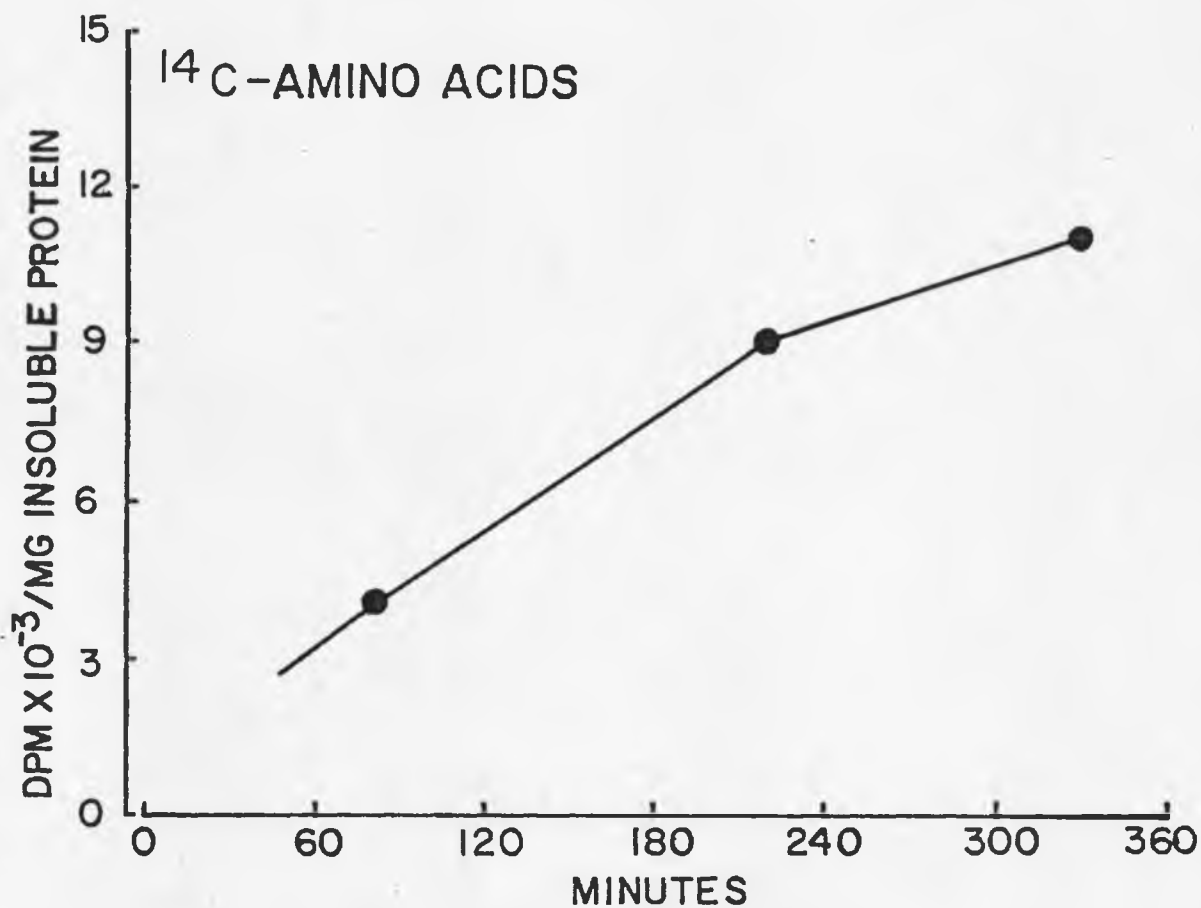


Figure 36. The Incorporation of <sup>14</sup>C-Amino Acids into Rabbit Tubule Basement Membrane Material

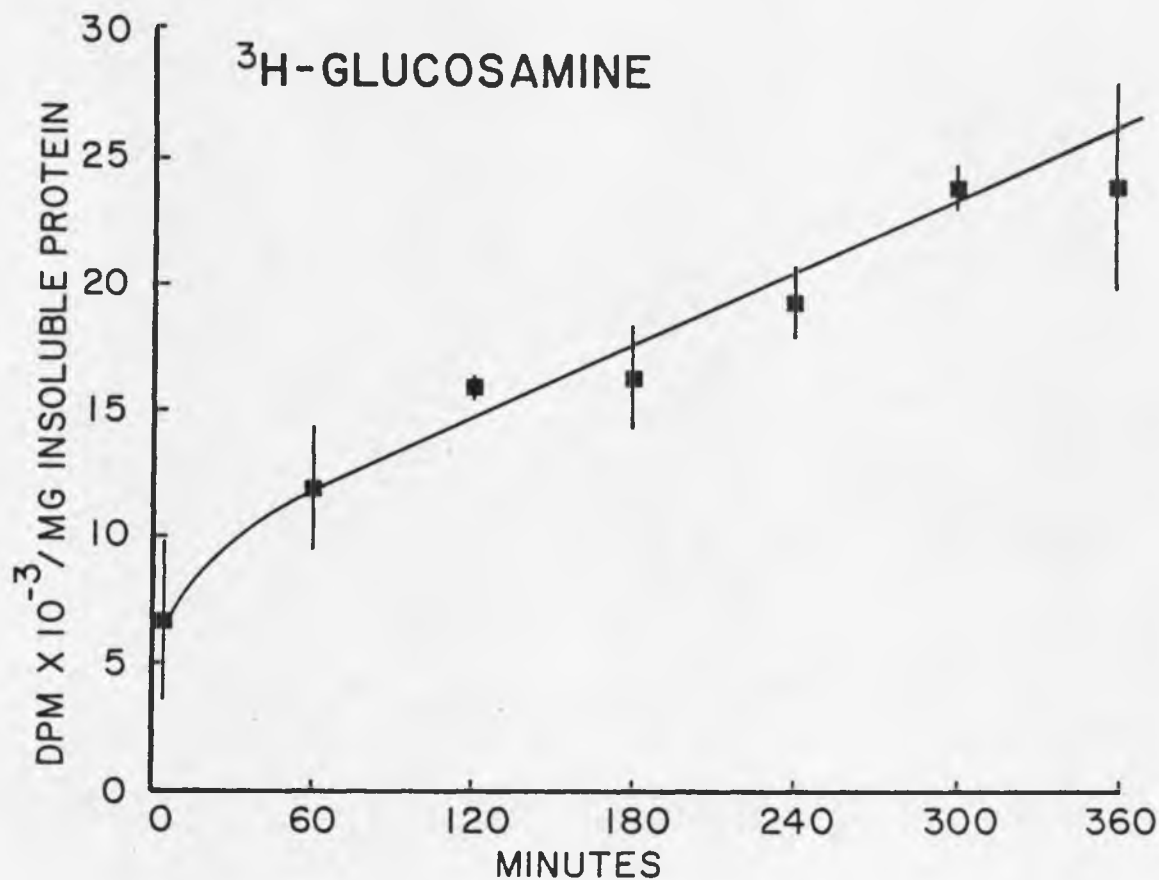


Figure 37. The Incorporation of <sup>3</sup>H-Glucosamine into Isolated Glomerular Basement Membrane

The isolated rat glomeruli were incubated with 100  $\mu$ Ci of <sup>3</sup>H-glucosamine in incubation buffer.

Glucosamine is not converted to anything but galactosamine and sialic acid to any appreciable extent.

The incorporation of radiolabeled lysine is also linear throughout the six hour incubation period (Figure 38). The incorporation of  $^{14}\text{C}$ -lysine into glomerular protein and the formation of  $^{14}\text{C}$ -hydroxylysine was reported by Cohen and Vogt (1975) to be approximately linear for the first 2 hours of incubation. The incorporation of lysine observed by Cohen and Vogt (1975) is similar to that observed in this study except that they measured soluble protein whereas matrix bound protein was measured in this study.

The incorporation of glycine into the basement membrane is shown in Figure 39. Again, the rate of radiolabel incorporation is linear throughout the six hour incubation. Since glycine constitutes approximately one third of the total number of residues found in collagen, glycine incorporation should be substantial in collagen forming tissues. Glycine incorporation itself, however, is not diagnostic for collagen synthesis.

$^3\text{H}$ -galactose incorporation into glomerular basement membrane was linear after the first hour of incubation. This is shown in Figure 40. Galactose is found in both the heteropolysaccharide and the disaccharide units of basement membrane. Krisko and Walker (1974) have also reported the incorporation of radiolabeled galactose into glomerular protein to be linear over a 10 hour incubation period.

When the incorporation of radiolabeled proline into the basement membrane was monitored, an increase in the rate of incorporation

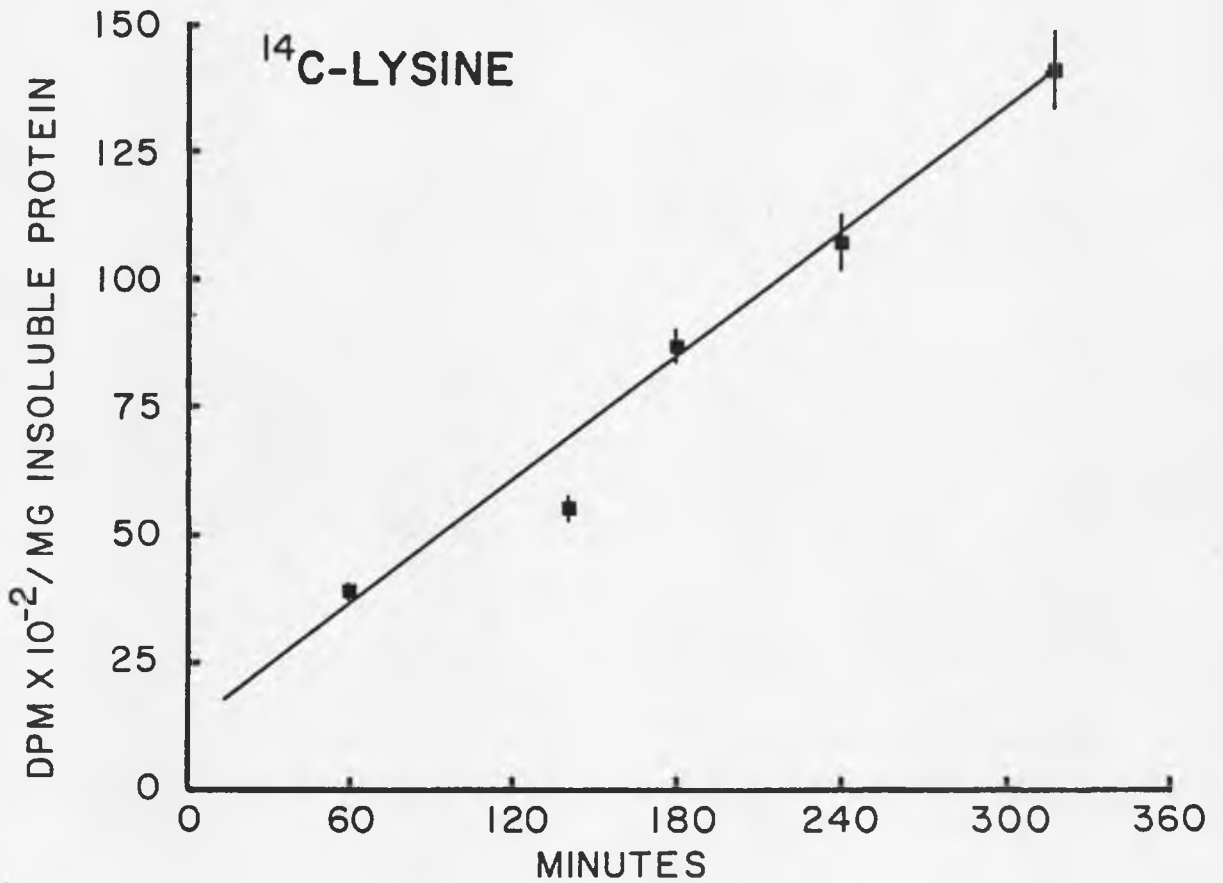


Figure 38. The Incorporation of <sup>14</sup>C-Lysine into Isolated Glomerular Basement Membrane

The isolated rat glomeruli were incubated with 10  $\mu$ Ci of <sup>14</sup>C-lysine in incubation buffer.

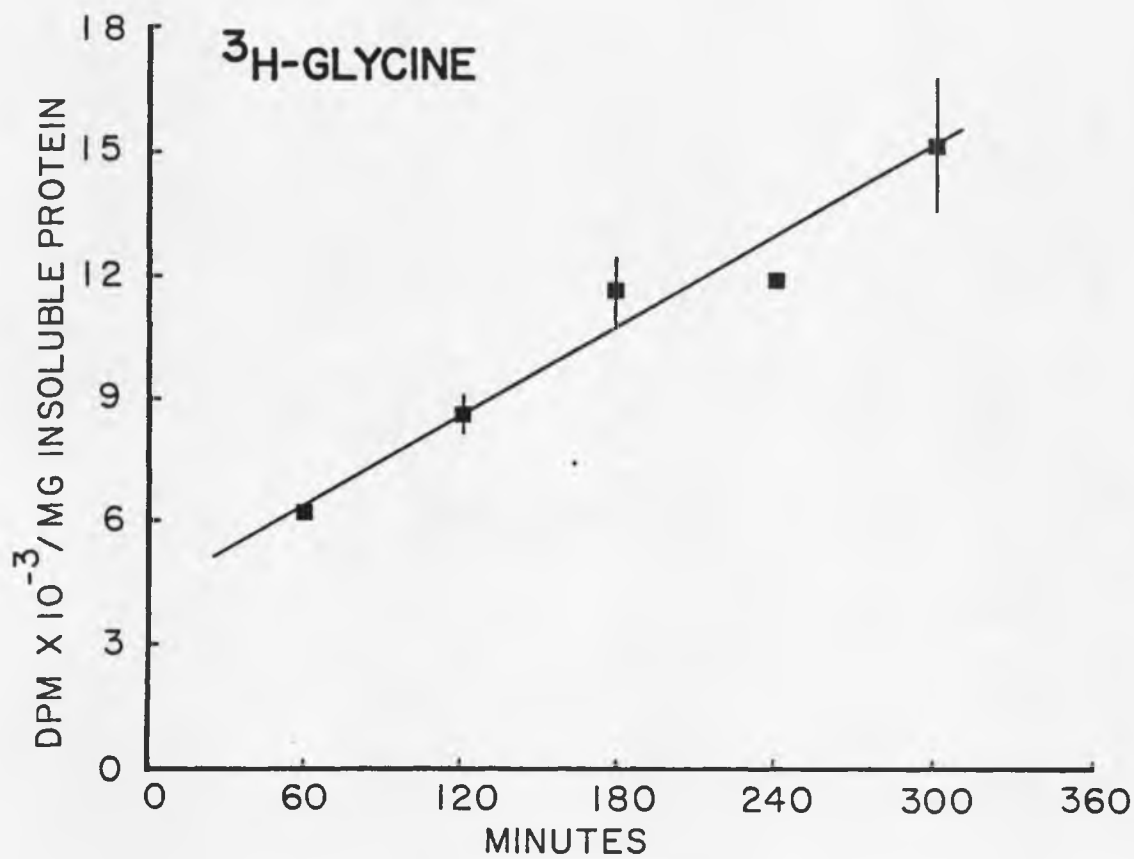


Figure 39. The Incorporation of  $^3\text{H}$ -Glycine into Isolated Glomerular Basement Membrane

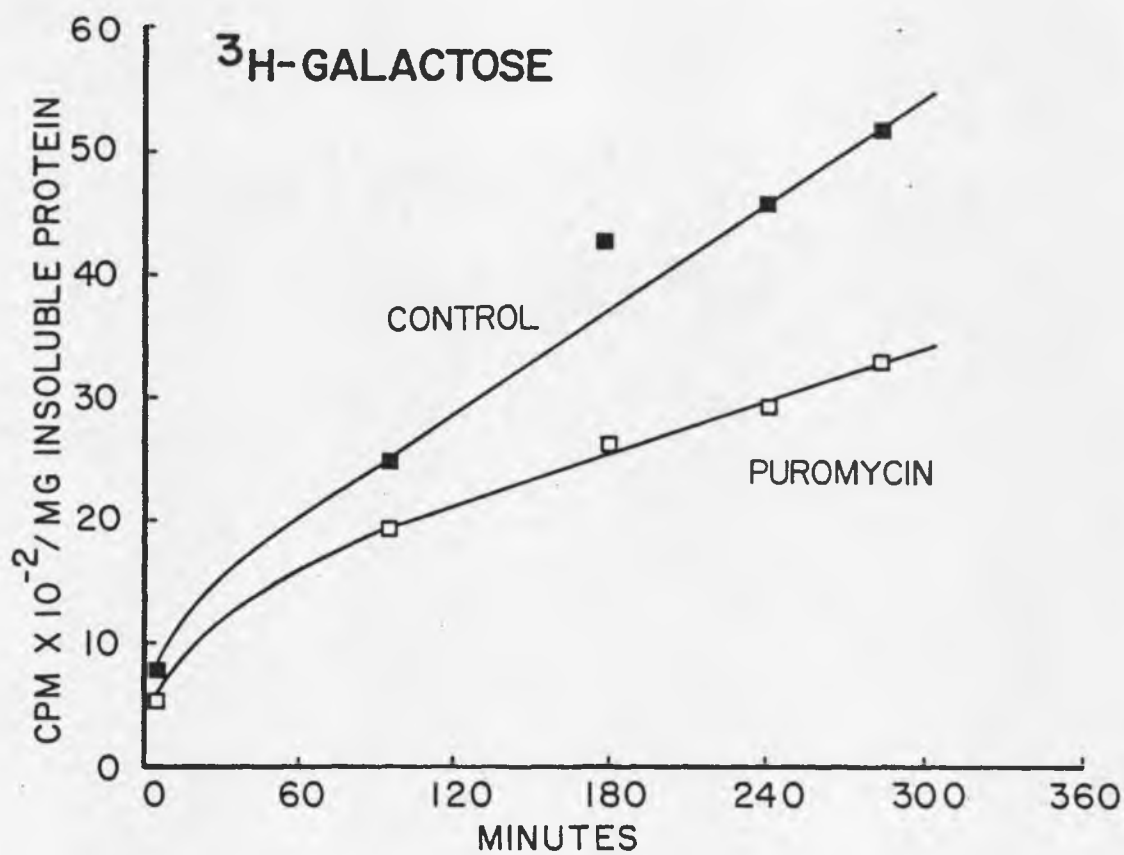


Figure 40. The Incorporation of  $^3\text{H}$ -Galactose into Isolated Glomerular Basement Membrane

was seen after three hours of incubation (Figure 41). This is in contrast to the incorporation rates of glycine, lysine, galactose, and glucosamine which were approximately linear throughout the incubation period. Since it is known that collagen secretion is slower than collagen synthesis (Grant, Kefalides, and Prockop, 1972a), the increase in the rate of radiolabel appearance in the isolated basement membrane matrix may be due to collagen synthesis and secretion processes.

Since collagen also contains hydroxyproline the synthesis and laydown of basement membrane proteins may be monitored by the appearance of radiolabeled hydroxyproline after incubation with radiolabeled proline. However, this procedure must be used cautiously if erroneous results are to be avoided. Collagen fibrils have been observed in the mesangial region of the glomerular tuft (Latta, 1973). Since basement membranes do not exhibit the classic striations of fibrillar collagen, this material is probably not basement membrane protein. Thus, the appearance of hydroxyproline in isolated glomerular preparations may be due to fibrillar synthesis in addition to glomerular basement membrane protein formation. By isolating the glomerular basement membrane by the DEOC method, mesangial fibrillar collagen is removed and the laydown of basement membrane proteins can be monitored by the appearance of radiolabeled hydroxyproline. To measure the formation of  $^{14}\text{C}$ -hydroxyproline from  $^{14}\text{C}$ -proline, glomeruli and proximal tubules were incubated with  $^{14}\text{C}$ -proline which had been purified by ion exchange chromatography. The time courses of hydroxylation expressed as the hydroxyproline/proline ratio times 100 is shown in Figures 42 and 43 for glomerular and

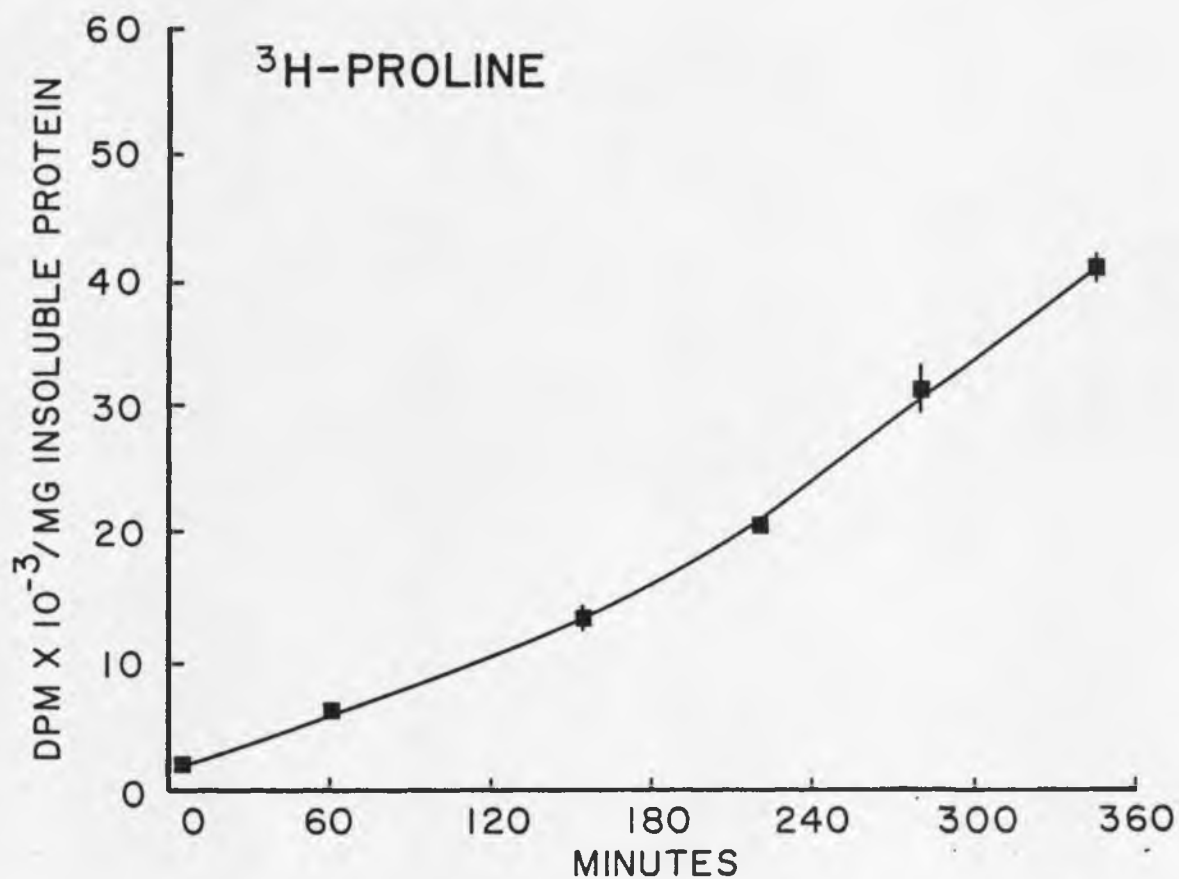


Figure 41. The Incorporation of <sup>3</sup>H-Proline into Isolated Glomerular Basement Membrane

The isolated rat glomeruli were incubated with 50  $\mu$ Ci of <sup>3</sup>H-proline in a volume of 4.5 ml of incubation buffer fortified with MEM amino acids.

# $^{14}\text{C}$ -PROLINE HYDROXYLATION

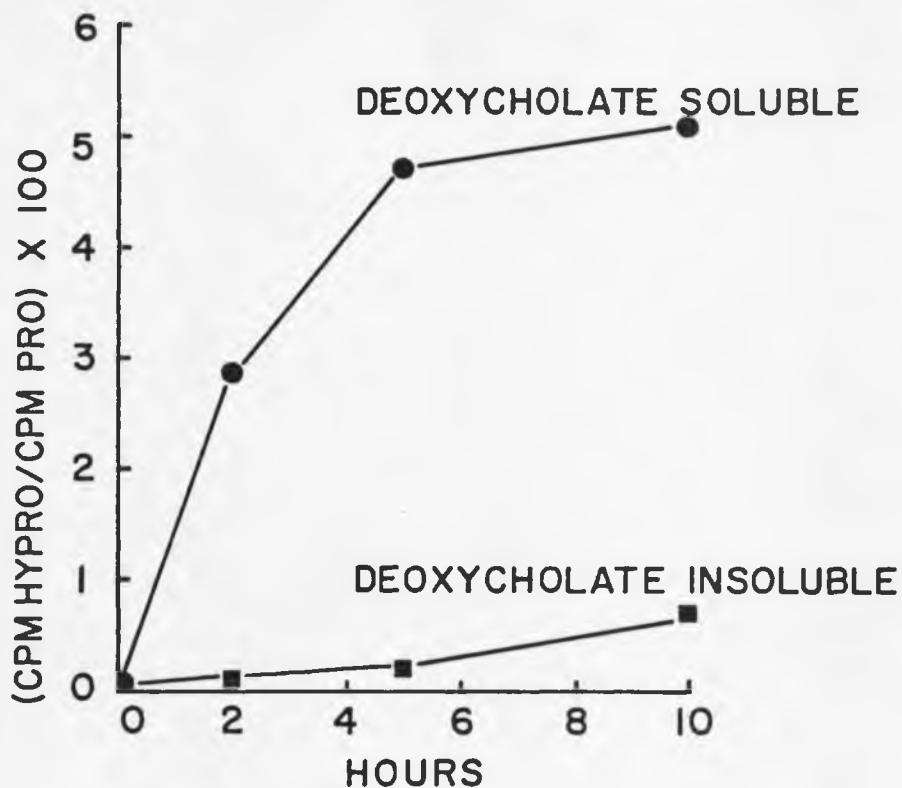


Figure 42. Appearance of  $^{14}\text{C}$ -Hydroxyproline in the Soluble and Basement Membrane Fractions Obtained from Isolated Rat Glomeruli

# $^{14}\text{C}$ -PROLINE HYDROXYLATION

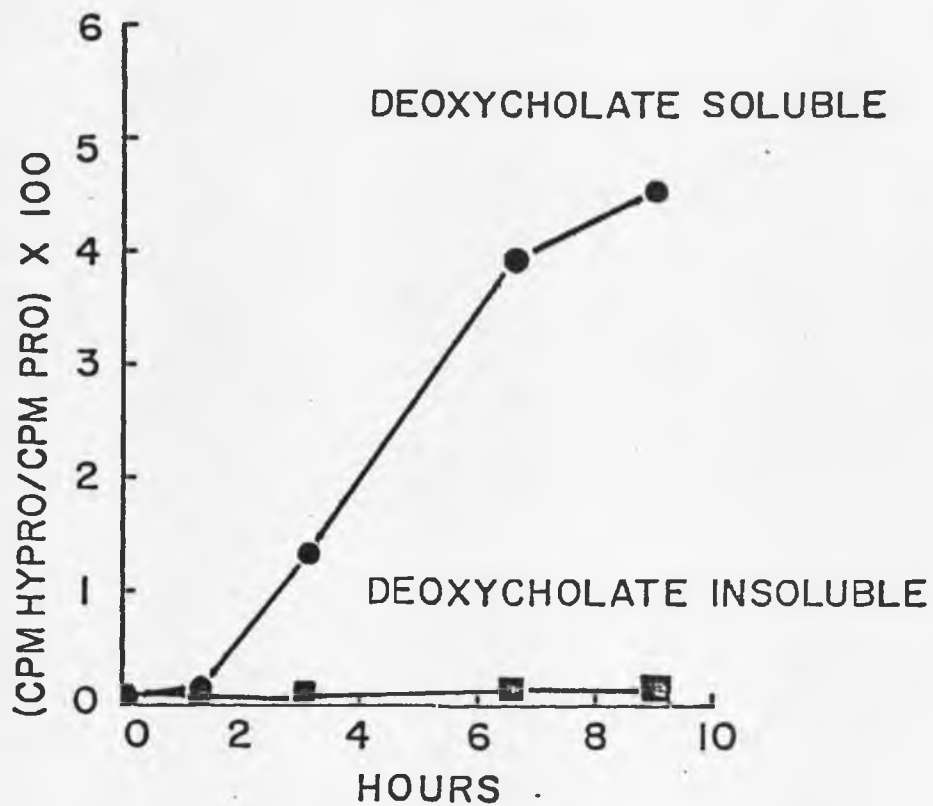


Figure 43. Appearance of  $^{14}\text{C}$ -Hydroxyproline in the Soluble and Basement Membrane Fractions Obtained from Isolated Rabbit Tubules

tubules, respectively. Other workers using in vitro systems to study hydroxylation of  $^{14}\text{C}$ -proline have observed an apparent saturation of hydroxylation (Jaffe et al., 1975). This is also observed in our system in the deoxycholate soluble fraction. The hydroxyproline to proline ratio in the mature basement membrane is slightly greater than one. In contrast, the ratio of radiolabeled hydroxyproline to proline is much smaller (0.01). There are several possible explanations for the lower than expected hydroxyproline to proline ratio in the isolated glomerular basement membrane. The first and foremost is that basement membrane collagen synthesis constitutes only a very small percentage of the total protein synthesized by the glomerulus (Brown and Michael, 1973; Grant, Harwood, and Williams, 1975). The ratio of radiolabeled hydroxyproline to proline was found to be 0.014 in the media of 24 hour incubated glomeruli (Brown and Michael, 1973). Similar findings have been reported by Grant, Kefalides, and Prockop (1972a) for chick lens basement membrane collagen. A second explanation for the lower hydroxylation levels may be damage to the hydroxylating system. However, the ratio of hydroxyproline to proline increases in the DEOC soluble fraction for at least the first 5 hours of incubation indicating at least partial preservation of hydroxylation function. The appearance of hydroxyproline in the basement membrane does not occur until 5 hours indicating a lag in the secretion or laydown of collagenous proteins. A delay in the appearance of hydroxyproline in the basement membrane matrix has been observed in both embryonic and mature systems. The appearance of  $^{14}\text{C}$ -hydroxyproline in the embryonic parietal yolk sac basement membrane was

delayed 1 - 2 hours while the incorporation of  $^{14}\text{C}$ -proline into protein was linear throughout the 6 hour experiment. Grant, Kefalides, and Prockop (1972a, 1972b) reported that lens capsule cells did not start to secrete radiolabeled hydroxyproline until one hour after the addition of radiolabeled proline. Using an isolated glomerular system, Williams et al. (1976) reported that radiolabeled hydroxyproline did not appear in the incubation medium until 4 - 6 hours after the incubation was begun. This 4 - 6 hour lag period was thought to be due to the time required for assembly of the hydroxylated and glycosylated basement membrane collagen chains into triple helical form (Grant, Harwood, and Williams, 1975; Williams et al., 1976). The appearance of  $^{14}\text{C}$ -hydroxyproline in the DEOC insoluble basement membrane corresponds well with this observation of a 4 - 6 hour lag in collagen secretion. This finding of a slow time course of appearance of hydroxyproline contrasts to the linear incorporation rates observed for glucosamine, galactose, lysine, glycine, and amino acid mixture. Since glucosamine, galactose, lysine, and glycine are all found in the non-collagen glycoprotein described by Kefalides (1973), it is possible that a non-collagenous protein is synthesized and deposited into the basement membrane matrix at a faster rate than a collagen component of the basement membrane. This might account for the lower than expected ratio of radiolabeled hydroxyproline to proline in the isolated basement membrane.

From glucosamine and hydroxyproline incorporation experiments in the rat parietal yolk sac system, Clark et al. (1975) have proposed that basement membrane synthesis is a two component system: a non-collagenous

glycoprotein and a collagenous protein. The significant difference in the rate of radiolabeled  $^{14}\text{C}$ -hydroxyproline appearance in the isolated basement membrane as compared to that of glucosamine, galactose, lysine, and glycine would support the concept of a two component system.

#### Inhibition Experiments

To show that the incorporation of radiolabeled amino acids into basement membrane protein was dependent upon an intact energy producing system, glomeruli were incubated with sodium azide for 40 minutes at  $37^{\circ}\text{C}$  before the addition of radiolabeled amino acids. A significant inhibition of radiolabeled uptake into both the DEOC soluble and insoluble fractions was observed (Figures 44 and 45). To further show that protein synthesis was required for the uptake of radiolabeled amino acids into DEOC soluble and insoluble material, isolated glomeruli were incubated with the protein synthesis inhibitor, cycloheximide, for 40 minutes prior to the addition of the radiolabel. The effect of cycloheximide and sodium azide on the rate of incorporation of radiolabel into DEOC insoluble material is shown in Figure 45. The inhibition of  $^3\text{H}$ -galactose into DEOC insoluble material is shown in Figure 40. The inhibition of carbohydrate incorporation into basement membrane by the protein synthesis inhibitor puromycin is in agreement with the requirement for protein synthesis before addition of carbohydrate occurs in a post-translational step.

When the competitive proline antagonist L-2-azetidine-2-carboxylic acid was added to the incubation medium, the uptake of  $^3\text{H}$ -amino

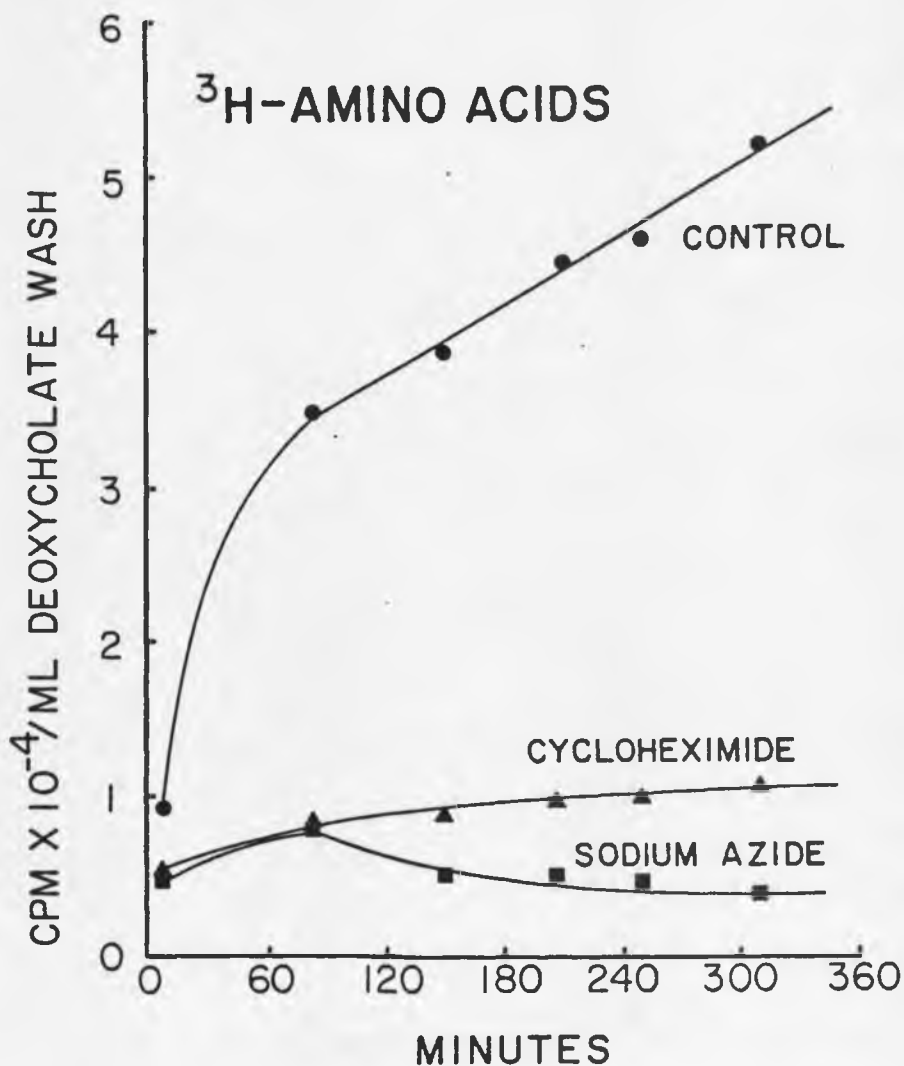


Figure 44. The Effect of Sodium Azide and Cycloheximide on the Appearance of Radiolabel in the Deoxycholate Soluble Fraction of Isolated Rat Glomeruli Incubated with a Mixture of  $^3\text{H}$ -Amino Acids

The isolated rat glomeruli were divided into three equal aliquots and suspended in 4.5 ml of incubation buffer alone or buffer containing either 46 mM  $\text{NaN}_3$  or 1.0 mM cycloheximide. After 40 minutes of preincubation, 50  $\mu\text{Ci}$  of  $^3\text{H}$ -amino acid mixture in buffer was added to each aliquot.

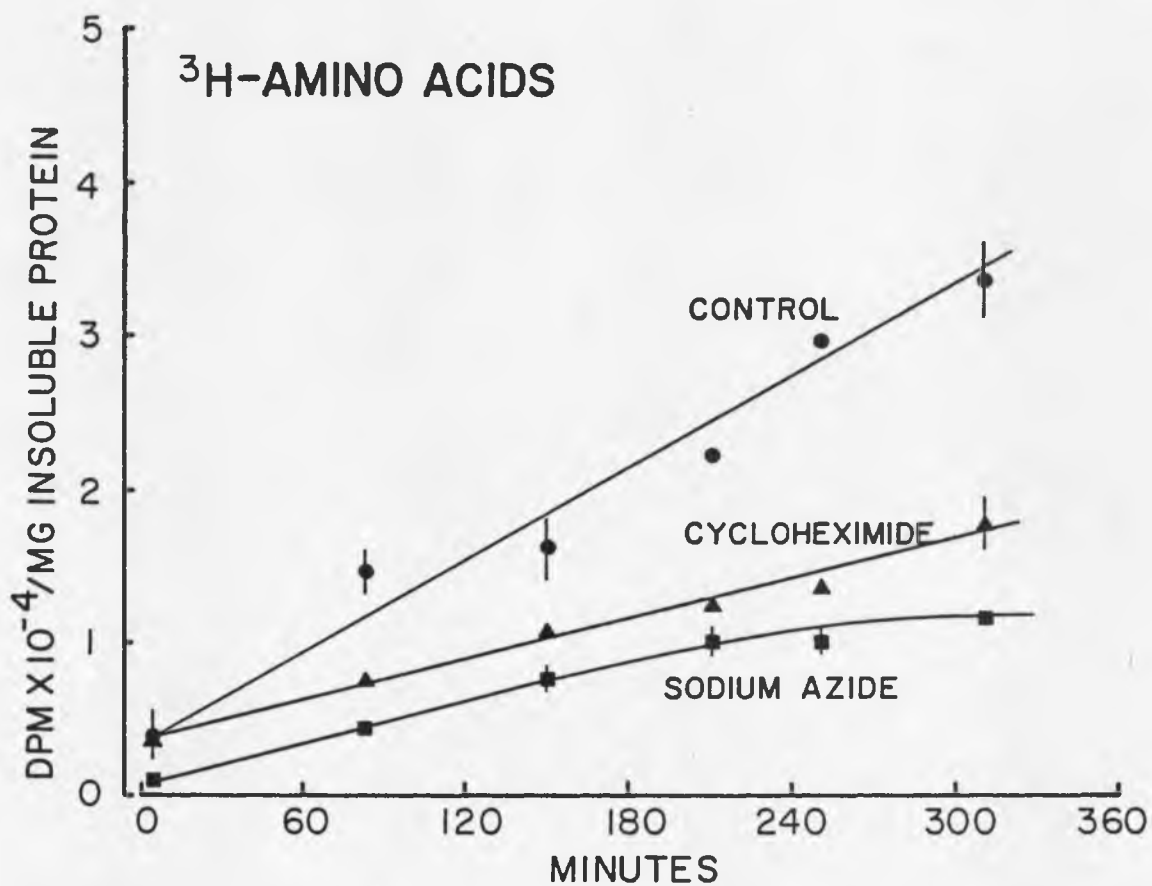


Figure 45. The Effect of Sodium Azide and Cycloheximide on the Appearance of Radiolabel in the Deoxycholate Insoluble Fraction Isolated from Rat Glomeruli Incubated with a Mixture of <sup>3</sup>H-Amino Acids

acids was inhibited by 40% while the inhibition of radiolabel appearance into the DEOC insoluble material was 15%. L-2-azetidine-2-carboxylic acid is thought to act by replacing proline during protein synthesis thus blocking further protein chain elongation. Although collagen is relatively rich in proline, the formation of any protein containing proline could be inhibited.

Colchicine, an agent which inhibits collagen secretion, had no significant effect on radiolabel appearance in the isolated basement membrane during incubation with a mixture of  $^3\text{H}$ -amino acids (Figure 46). This would suggest that the extrusion of the deposited radiolabeled proteins does not require microtubule mediated processes. In addition, it suggests that the proteins being deposited are not collagenous. This is supported by the delayed appearance of radiolabeled hydroxyproline in the basement membrane and the extracellular medium (Williams et al., 1976).

Beta-aminopropionitrile (BAPN), an inhibitor of collagen cross-line formation, inhibited the increase in the rate of radiolabel deposition into the basement membrane matrix (Figure 47) that had been observed during incubations with radiolabeled proline (Figure 41). Unlike the earlier proline experiments (Figure 41), the glomeruli from both the control and BAPN groups were allowed to remain in deoxycholate for one week at room temperature before basement membrane isolation was completed. This delay in basement membrane isolation may be of sufficient duration to allow collagen crosslink formation. It is apparent from this study that only a portion of the radiolabel is susceptible to

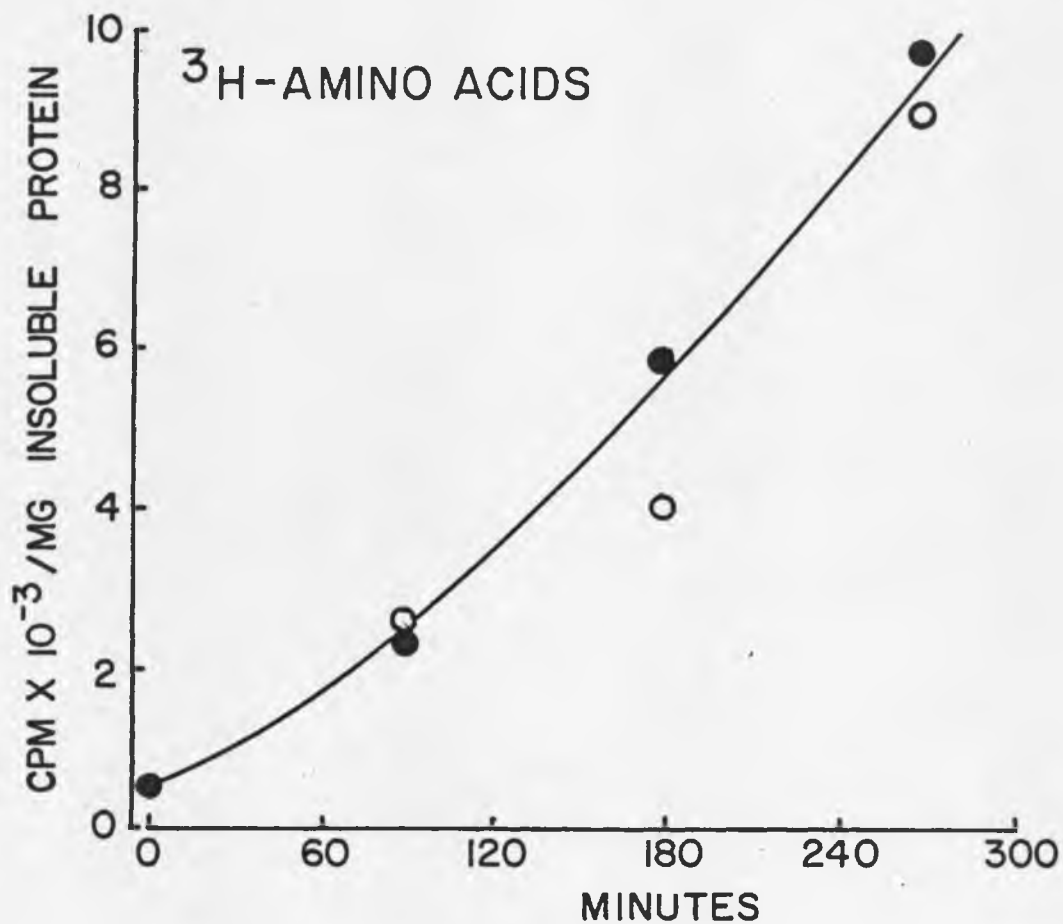


Figure 46. Effect of Colchicine on the Appearance of Radiolabel in the Isolated Basement Membrane Fraction Obtained from Rat Glomeruli

Control ● .

$1 \times 10^{-3}$  M colchicine for 30 minutes prior and during the incubation period ○ .

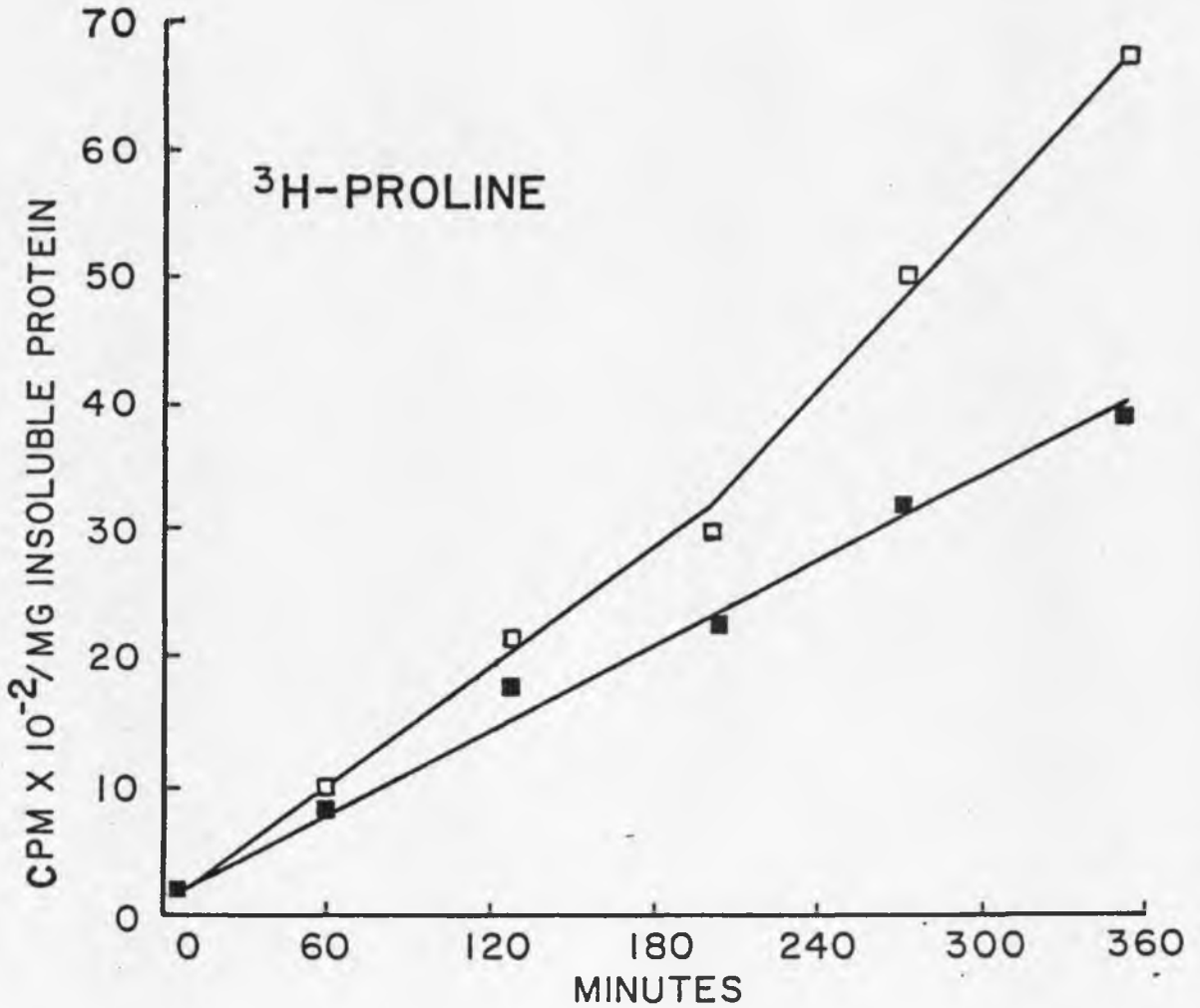


Figure 47. The Effect of  $\beta$ -Aminopropionitrile on the Appearance of Radiolabel in the Glomerular Basement Membrane Isolated from Rat Glomeruli Incubated with  $^3\text{H}$ -Proline

Control  $\square$  .

$7.1 \times 10^{-2}$  M  $\beta$ -aminopropionitrile for 10 minutes prior and during the incubation period  $\blacksquare$  .

deposition inhibition by BAPN suggesting that non-collagen crosslink interactions such as disulfide bonds are also involved in the deposition process. The possibility that a collagen component is attached to the basement membrane matrix by BAPN sensitive mechanisms while a non-collagen glycoprotein component is attached by BAPN resistant mechanisms is consistent with the linear incorporation rate observed for glucosamine (Figure 37). The inhibition by BAPN of radiolabel deposition may be due in part to cellular toxicity independent of lysyl oxidase inhibition.

It has been proposed that basement membrane protein synthetic activity might account for the high energy consumption rate thought to occur in the renal glomerulus (Cohen and Kamm, 1975). From the rate of basement membrane synthesis (Grant, Harwood and Williams, 1975) and deposition of radiolabeled proteins into the basement membrane matrix (Hjelle, Brendel and Meezan, 1976), it would appear that basement membrane protein synthesis does not account for the majority of protein synthetic activity associated with the glomerulus. The high energy consumption of the glomerulus may in part be accounted for by contractile processes associated with glomerular cells. An endogenous rhythmic contraction of glomerular capillaries has been observed by Bernik (1969). Hornyk, Beaufils and Richet (1972) have found that supracortical glomeruli exhibit a vasomotor tone and are susceptible to the pressor effects of epinephrine. Another energy dependent cellular activity which may affect glomerular energy utilization has been reported by Klebe (1975). He found that the attachment of cells to a layer of collagen was an energy requiring process. The maintenance of epithelial foot

process attachment to the basement membrane in the face of glomerular filtration pressures may also be an energy requiring process.

## CHAPTER 4

### CONCLUSIONS

In this study a method was developed for the isolation of morphologically and chemically intact basement membranes from a variety of suborgan fractions. This procedure has been demonstrated to be applicable to the study of basement membrane metabolism. Using this basement membrane isolation procedure, the deposition of newly formed proteins into the basement membrane matrix was studied. Evidence was accumulated which suggested that basement membrane formation might be a two component system. One component was deposited at a linear rate throughout most of the incubation period and was apparently not collagenous in nature. A second component was deposited after a lag period of 6 - 8 hours and was collagenous in nature.

The formation of the basement membrane matrix was observed to be a slow process. This suggested that the high energy consumption rate of the glomerulus was probably due to cellular processes other than basement membrane synthesis. It also suggested that in short term experiments changes in basement membrane synthetic rate would have little effect upon the gross structure of the basement membrane. This is of importance in the study of glomerular filtration when the question of the basement membrane as the primary barrier to filtration is discussed.

When glomerular preparations were examined for gluconeogenic activity, it was found that the activity resided in either the Bowman's

capsule or minute quantities of proximal tubule attached to Bowman's capsule. Protein synthetic activity was found in both glomeruli with attached Bowman's capsule and glomerular tufts. The indication of the non-gluconeogenic nature of the glomerular tuft may provide some further insight into glomerular function and metabolism and their alteration during disease.

## LIST OF REFERENCES

- Anderson, W. A. and Ellis, R. A.: Ultrastructure of Trypanosoma lewisi: flagellum, microtubules, and the kinetoplast. J. Protozool. 12: 483, 1965.
- Beisswenger, P. J. and Spiro, R. G.: Human glomerular basement membrane: chemical alteration in diabetes mellitus. Science 168: 596, 1970.
- Beisswenger, P. J. and Spiro, R. G.: Studies on the human glomerular basement membrane: composition, nature of the carbohydrate units and chemical changes in diabetes mellitus. Diabetes 22: 180, 1973.
- Berg, R. A. and Prockop, D. J.: The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. Biochem. Biophys. Res. Commun. 52: 115, 1973.
- Bernik, M. B.: Contractile activity of human glomeruli in culture. Nephron 6: 1, 1969.
- Blau, E. and Michael, A. F.: Rat glomerular basement membrane composition and metabolism in amino nucleoside nephrosis. J. Lab. Clin. Med. 77: 97, 1971.
- Bloodworth, J. M. B., Jr. and Engerman, R. L.: Diabetic microangiopathy in the experimentally-diabetic dog and its prevention by careful control with insulin. Diabetes 22: 290, (Abstract) 1973.
- Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S.: Fluorometric assay of proteins in the nanogram range. Arch. Biochem. Biophys. 155: 213, 1973.
- Bornstein, P., von der Mark, K., Wyke, A. W., Ehrlich, H. P., and Monson, J. M.: Characterization of the pro- $\alpha_1$  chain of procollagen. J. Biol. Chem. 247: 2808, 1972.

- Brendel, K. and Meezan, E.: Properties of a pure metabolically active glomerular preparation from rat kidneys. II. Metabolism. *J. Pharmacol. Exp. Ther.* 187: 342, 1973.
- Brendel, K. and Meezan, E.: A simple apparatus for the continuous monitoring of  $^{14}\text{CO}_2$  production from several small reaction mixtures. *Anal. Biochem.* 60: 88, 1974.
- Brendel, K. and Meezan, E.: Isolation and properties of a pure preparation of proximal kidney tubules obtained without collagenase treatment. *Fed. Proc.* 34: 803, 1975.
- Brendel, K., Meezan, E., and Carlson, E. C.: Isolated brain microvessels: a purified metabolically active preparation from bovine cerebral cortex. *Science* 185: 953, 1974.
- Brown, D. M. and Michael, A. F.: Glomerular basement membrane synthesis by isolated glomeruli. *Fed. Proc.* 32: 650, 1973.
- Burg, M. B. and Orloff, J.: Oxygen consumption and active transport in separated renal tubules. *Am. J. Physiol.* 203(2): 327, 1962.
- Carlson, E. C., Meezan, E., Brendel, K., and Hjelle, T.: The effect of enzymes on ultrastructurally pure basal lamina isolated from rabbit renal tubules. *Anat. Rec.* 84: 371, (Abstract) 1976.
- Chang, R. L. S., Deen, W. M., Robertson, C. R., and Brenner, B. M.: Permeability of the glomerular capillary wall: III. Restricted transport of polyanions. *Kidney International* 8: 212, 1975.
- Chow, A. Y. K. and Drummond, K. N.: Incorporation and hydroxylation of proline 3-4- $^3\text{H}$  as an index of glomerular basement membrane synthesis in normal and nephrotoxic nephrotic rats. *Lab. Invest.* 20: 213, 1969.
- Churg, Jacob and Grishman, Edith: Ultrastructure of glomerular disease: a review. *Kidney Int.* 7: 254, 1975.
- Clark, Charles C., Tomichuk, Emilie A., Koszalka, Thomas R., Minor, Ronald R., and Kefalides, Nicholas A.: The embryonic rat parietal yolk sac. *J. Biol. Chem.* 250: 5259, 1975.
- Cohen, J. J. and Kamm, D. E.: Renal metabolism: relation to renal function in the kidney. In: *The Kidney*, ed. by Brenner, B. M. and Rector, F. C., p. 126, Saunders, Philadelphia, 1975.
- Cohen, M. P. and Vogt, C. A.: Collagen synthesis and secretion by isolated rat renal glomeruli. *Biochem. Biophys. Acta* 393: 78, 1975.

- Cook, W. F. and Pickering, G. W.: A rapid method for separating glomeruli from rabbit kidney. *Nature* 182: 1103, 1958.
- Cruickshank, B. and Hill, A. G. S.: The histological identification of a connective tissue antigen in the rat. *J. Path. Bact.* 66: 283, 1953.
- Cunningham, L. W. and Ford, J. D.: A comparison of glycopeptides derived from soluble and insoluble collagens. *J. Biol. Chem.* 243: 2398, 1968.
- Dahlstrom, A.: Effect of colchicine on transport of amine storage granules in sympathetic nerves of rat. *European J. Pharmacol.* 5: 111, 1968.
- Daniels, J. R. and Chu, G. H.: Basement membrane collagen of renal glomerulus. *J. Biol. Chem.* 250: 3531, 1975.
- Davison, P. F. and Drake, M. P.: The physical characterization of monomeric tropocollagen. *Biochemistry* 5: 313, 1966.
- Dehm, P. and Prockop, D. J.: Synthesis and extrusion of collagen by freshly isolated cells from chick embryo tendon. *Biochim. Biophys. Acta* 240: 358, 1971.
- Dische, Z.: The glycans of the mammalian lens capsule -- a model of basement membranes. In: Small Blood Vessel Involvement in Diabetes Mellitus, ed. by Siperstein, M. D., Colwell, A. R., and Meyer, D., p. 201, Amer. Inst. Biol. Sci., Washington, 1964.
- Dische, Z., Zelmanis, G., and Rothchild, C.: The hexosaminohexuronide of the bovine lens capsule. *Arch. Biochem. Biophys.* 121: 685, 1967.
- Dohlman, C. H. and Balazs, E. A.: Chemical studies on Descemet's membrane of the bovine cornea. *Arch. Biochem.* 57: 445, 1955.
- Farquhar, M. G.: Glomerular permeability investigated by electron microscopy. In: Small Blood Vessel Involvement in Diabetes Mellitus, ed. by Siperstein, M. D., Colwell, A. R., and Meyer, K., p. 31, Amer. Inst. Biol. Sci., Washington, 1964.
- Farquhar, M. G. and Palade, G. E.: Glomerular permeability II. Ferritin transfer across the glomerular capillary wall in nephrotic rats. *J. Exp. Med.* 114: 699, 1961.
- Farquhar, M. G., Wissig, S. L., and Palade, G. E.: Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J. Exp. Med.* 113: 47, 1961.

- Fawcett, D. W.: Comparative observations on the fine structure of blood capillaries. In: The Peripheral Blood Vessels, ed. by Orbison, J. L. and Smith, D. S., p. 17, Williams & Wilkins, Baltimore, 1963.
- Fawcett, D. W.: An Atlas of Fine Structure. The Cell: Its Organelles and Inclusions, W. B. Saunders Co., Philadelphia and London, 1966.
- Ferwerda, W., Meijer, J. F. M., Eijnden, D. H., and van Dijk, W.: Epithelial basement membrane of bovine renal tubuli. Hoppe-Seyler's Z. Physiol. Chem. 355: 976, 1974.
- Fisher, E. R. and Klein, H. Z.: Ultrastructural observations concerning the effect of adenine on aminonucleoside mechanism of proteinuria. Lab. Invest. 12: 499, 1963.
- Friederici, H. H. R., Tucker, W. R., and Schwartz, T. B.: Observations on small blood vessels of skin in the normal and in diabetic patients. Diabetes 15: 233, 1966.
- Fukushi, S. and Spiro, R. G.: The lens capsule: sugar and amino acid composition. J. Biol. Chem. 244: 2041, 1969.
- Gallop, P. M. and Paz, M. A.: Posttranslational protein modifications, special attention to collagen and elastin. Physiol. Rev. 55: 418, 1975.
- Gehrke, C. W., Roach, D., Zumwalt, R. W., Stalling, D. L., and Wall, L. L.: Quantitative Gas-Liquid Chromatography of Amino Acids in Proteins and Biological Substances, Analytical Biochemistry Laboratories, Columbia, 1968.
- Goodman, M., Greenspon, S. A., and Krakower, C. A.: The antigenic composition of the various anatomic structures of the canine kidney. J. Immun. 75: 96, 1955.
- Grant, Michael E., Harwood, Richard, and Williams, Isabel F.: The biosynthesis of basement-membrane collagen by isolated rat glomeruli. Eur. J. Biochem. 54: 531, 1975.
- Grant, M. E., Kefalides, N. A., and Prockop, D. J.: The biosynthesis of basement membrane collagen in embryonic chick lens. I. Delay between the synthesis of polypeptide chains and the secretion of collagen by matrix-free cells. J. Biol. Chem. 247: 3539, 1972a.
- Grant, M. E., Kefalides, N. A., and Prockop, D. J.: The biosynthesis of basement membrane collagen in embryonic chick lens. II. Synthesis of a precursor form by matrix-free cells and a time-dependent conversion to  $\alpha$  chains in intact lens. J. Biol. Chem. 247: 3545, 1972b.

- Grant, M. E. and Prockop, D. J.: The biosynthesis of collagen. *New Engl. J. Med.* 286: 194, 1972.
- Grimes, W. J.: Biological and biochemical characterization of surface changes in normal, MSV- and SV40-transformed, and spontaneously transformed clones of Balb/c cells. In: Control of Proliferation in Animal Cells, ed. by Clarkson, B. and Baserga, R., p. 517, Cold Spring Harbor Laboratory, New York, 1974.
- Groniowski, J., Biczyskova, W., and Walski, M.: Electron microscope studies on the surface coat of the nephron. *J. Cell Biol.* 40: 585, 1969.
- Hardin, R. C., Jackson, R. L., Johnston, T. L., and Kelly, H. G.: The development of diabetic retinopathy. Effects of duration and control of diabetes. *Diabetes* 5: 397, 1956.
- Hay, E. D.: Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. In: Epithelial-Mesenchymal Interactions, ed. by Fleischmajer, R. and Billingham, R. E., 31, Williams & Wilkins, Baltimore, 1968.
- Hjelle, J. T., Brendel, K., and Meezan, E.: Basement membrane synthesis by isolated rat renal glomeruli. *Fed. Proc.* 35: 680, 1976.
- Hjelle, J. T., Meezan, E., and Brendel, K.: Localization of gluconeogenic activity in the Bowman's capsule of isolated kidney glomeruli. *The Pharmacol.* 17: 252, 1975.
- Hornych, H., Beaufils, M., and Richet, G.: The effect of exogenous angiotensin on superficial and deep glomeruli in the rat kidney. *Kidney Int.* 2: 336, 1972.
- Huang, F. and Kalant, N.: Isolation and characterization of antigenic components of rat glomerular basement membrane. *Canad. J. Biochem.* 46: 1523, 1968.
- Hudson, B. G. and Spiro, R. G.: Studies on the native and reduced alkylated renal glomerular basement membrane. Solubility, subunit size and reaction with cyanogen bromide. *J. Biol. Chem.* 247: 4229, 1972a.
- Hudson, B. G. and Spiro, R. G.: Fractionation of glycoprotein components of the reduced alkylated renal glomerular basement membrane. *J. Biol. Chem.* 247: 4239, 1972b.

- Jackson, R., Guthrie, R., Esterly, J., Bilginturan, N., James, R., Yeost, J., Saathoff, J., and Guthrie, D.: Muscle capillary basement membrane changes in normal and diabetic children. *Diabetes* 24: 400 (Abstract), 1975.
- Jaffe, E. A., Adelman, B., Minick, C. R., Becker, C. G., and Nachman, R. Synthesis of basement membrane by cultured human endothelial cells. *Circulation* 52: 235, 1975.
- Jakus, M. A.: Ocular Fine Structure, Little Brown & Co., Boston, 1964.
- Jimenez, S. A., Dehm, P., Olsen, B. R., and Prockop, D. J.: Intracellular collagen and protocollagen from embryonic tendon cells. *J. Biol. Chem.* 248: 720, 1973.
- Job, D., Eschwege, E., Guyot, C., and Tchobroutsky, G.: Effect of multiple daily insulin injections on the course of diabetic retinopathy. *Diabetes* 24: 397, (Abstract), 1975.
- Jones, D. B.: Mucosubstances of the glomerulus. *Lab. Invest.* 21: 119, 1969.
- Kaiser, F. E., Gehrke, C. W., Zumwalt, R. W., and Kuo, K. C.: Amino Acid Analysis: Hydrolysis, Ion-Exchange Cleanup, Derivatization, and Quantitation by Gas-Liquid Chromatography, Analytical Biochemistry Laboratories, Columbia, 1973.
- Karnovsky, M. J.: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* 27: 137a, (Abstract # 270), 1965.
- Karnovsky, M. J. and Ainsworth, S. K.: The structural basis of glomerular filtration. *Adv. Nephrol.* 2: 35, 1973.
- Karrer, H. E.: The ultrastructure of the mouse lung. *J. Biophys. Biochem. Cytol.* 2: 241, 1956.
- Kefalides, N. A.: A collagen of unusual composition and a glycoprotein isolated from canine glomerular basement membrane. *Biochem. Biophys. Res. Commun.* 22: 26, 1966.
- Kefalides, N. A.: Isolation and characterization of the collagen from glomerular basement membrane. *Biochemistry* 7: 3103, 1968.
- Kefalides, N. A.: Characterization of the collagen from lens capsule and glomerular basement membranes. In: Proceedings of the Sixth Congress of the International Diabetes Federation, ed. by Ostman, J., p. 307, Excerpta Medica Foundation, Amsterdam, 1969a.

- Kefalides, N. A.: The chemistry of the antigenic components of vascular and capsular basement membranes. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 28: 699, (Abstract), 1969b.
- Kefalides, N. A.: Comparative biochemistry of mammalian basement membranes. In: Chemistry and Molecular Biology of the Intercellular Matrix, ed. by Balazs, E. A., Vol. 1, p. 535, Academic Press, New York, 1970.
- Kefalides, N. A.: Isolation of collagen from basement membranes containing three identical  $\alpha$ -chains. *Biochem. Biophys. Res. Commun.* 45: 226, 1971.
- Kefalides, N. A.: The chemistry of antigenic components isolated from glomerular basement membrane. *Connect. Tiss. Res.* 1: 3, 1972.
- Kefalides, N. A.: Structure and biosynthesis of basement membrane. *Int. Rev. Connect. Tissue Res.* 6: 63, 1973.
- Kefalides, N. A.: Biochemical properties of human glomerular basement membrane in normal and diabetic kidneys. *J. Clin. Invest.* 53: 403, 1974.
- Kefalides, N. A. and Denduchis, B.: Structural components of epithelial and endothelial basement membranes. *Biochem.* 8: 4613, 1969.
- Kefalides, N. A. and Forsell-Knott, L.: Structural changes in the protein and carbohydrate components of glomerular basement membrane in aminonucleoside nephrosis. *Biochim. Biophys. Acta* 203: 62, 1970.
- Kefalides, N. A. and Winzler, R. J.: The chemistry of glomerular basement membrane and its relation to collagen. *Biochemistry* 5: 702, 1966.
- Keiding, N. R., Root, H. F., and Marble A.: Importance of control of diabetes in prevention of vascular complications. *J. Amer. Med. Ass.* 150: 964, 1952.
- Khalifa, A. and Cohen, M. P.: Glomerular protocollagen lysyl-hydroxylase activity in streptozotocin diabetes. *Biochim. Biophys. Acta.* 386: 332, 1975.
- Killen, P. D., Quadracci, L. J., and Striker, G. E.: Basal lamina synthesis in vitro by glomerular cells. *Fed. Proc.* 33: 617, 1974.

- Kilo, C., Vogler, N., and Williamson, J. R.: Muscle capillary basement membrane changes related to aging and to diabetes mellitus. *Diabetes* 21: 881, 1972.
- Klebe, Robert J.: Cell attachment to collagen: the requirement for energy. *J. Cell Physiol.* 86: 231, 1975.
- Krakower, C. A. and Greenspon, S. A.: Localization of the nephrotoxic antigen within the isolated renal glomerulus. *A. M. A. Arch. Path.* 51: 629, 1951.
- Krakower, C. A. and Greenspon, S. A.: The antigens of capillary and venular basement membranes elucidated by the use of lens capsule. In: Small Blood Vessel Involvement in Diabetes Mellitus, ed. by Siperstein, M. D., Colwell, A. R., and Meyer, K., p. 161, Amer. Inst. Biol. Sci., Washington, 1964.
- Krisko, I. and Walker, W. G.: Protein and glycoprotein synthesis by isolated kidney glomeruli. *Proc. Soc. Exper. Biol. Med.* 146: 942, 1974.
- Kurokawa, K. and Rasmussen, H.: Ionic control of renal gluconeogenesis: I. The interrelated effect of calcium and hydrogen ions. *Biochim. biophys. Acta* 313: 17, 1973.
- Kurtz, S. M.: The fine structure of the lamina densa. *Lab. Invest.* 6: 1189, 1961.
- Kurtz, S. M. and McManus, J. F. A.: A reconsideration of the development, structure, and disease of the human renal glomerulus. *Amer. Heart J.* 58: 357, 1959.
- Latta, H.: Ultrastructure of the glomerulus and juxtaglomerular apparatus. In: Handbook of Physiology: Renal Physiology, ed. by Orloff, J. and Berlinger, R. W., p. 22, The Williams and Wilkins Co., Baltimore, 1973.
- Latta, H., Johnston, W. H., and Stanley, T. M.: Sialoglycoproteins and filtration barriers in the glomerular capillary wall. *J. Ultrastruct. Res.* 51: 354, 1975.
- Latta, H., Maunsbach, A. B., and Osvaldo, L.: The fine structure of renal tubules in cortex and medulla. In: Ultrastructure of the Kidney, ed. by Dalton, A. J. and Haguenu, F., p. 1, Academic Press, New York, 1967.
- Lazarides, E. L., Lukens, L. N., and Infante, A. A.: Collagen polysomes: site of hydroxylation of proline residues. *J. Mol. Biol.* 53: 831, 1971.

- Lazarow, A. and Speidel, E.: The chemical composition of the glomerular basement membrane and its relationship to the production of diabetic complications. In: Small Blood Vessel Involvement in Diabetes Mellitus, ed. by Siperstein, M. D., Colwell, A. R., and Meyer, K., p. 127, Americal Institute of Biological Sciences, Washington, 1964.
- Leblond, C. P.: Distribution of periodic acid reactive carbohydrates in the adult rat. *Am. J. Anat.* 86: 1, 1950.
- Lehotay, D. C.: Studies of normal and nephritic rat glomerular basement membrane. *Biochemica et Biophysica Acta* 394: 193, 1975.
- Lidsky, M. D., Sharp, J. T., and Rudee, M. L.: Studies on acellular bovine glomeruli. Isolation, chemical composition and demonstration of collagen with an unusual hydroxylysine: hydroxyproline ratio. *Arch. Biochem. Biophys.* 121: 491, 1967.
- Lillie, R. D.: Reticulum staining with Schiff reagent after oxidation by acidified sodium periodate. *J. Lab. Clin. Med.* 32: 910, 1947.
- Lillie, R. D.: Histochemistry of connective tissue. *Lab. Inv.* 1: 30, 1952.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265, 1951.
- Luft, R. and Guillemin, R.: Growth hormone and diabetes in man. Old concepts -- new implications. *Diabetes* 23: 783, 1974.
- Lundbaek, K.: Growth hormone and diabetic angiopathy. In: Blood vessel Disease in Diabetes Mellitus. V Capri Conference, ed. by Lundbaek, K. and Keen, H., p. 344, The Publishing House "Il Ponte", Milan, 1971.
- McIntosh, R. M., Kihara, H., Kulvinskis, C., Kaufman, D. B., and Wong, S. R.: Chemical and immunological characterization of a basement membrane-like glycoprotein in the rat. *Ann. Rheu. Dis.* 30: 631, 1971.
- McKeehan, M. S.: Cytological aspects of embryonic lens induction in the chick. *J. Exp. Zool.* 117: 31, 1951.
- McManus, J. F. A.: Histological and histochemical uses of periodic acid. *Stain Technol.* 23: 99, 1948a.

- McManus, J. F. A.: The periodic routine applied to the kidney. *Am. J. Path.* 26: 643, 1948b.
- McManus, J. F. A.: Structure of the glomerulus of the human kidney. *Am. J. Path.* 24: 1259, 1948c.
- Mahieu, P. and Winand, R. J.: Chemical structure of tubular and glomerular basement membranes of human kidney. *Eur. J. Biochem.* 12: 410, 1970.
- Malawista, S. E.: On the action of colchicine. *J. Exptl. Med.* 122: 361, 1965.
- Markowitz, A. S. and Lange, C. F., Jr.: Streptococcal related glomerulonephritis. I. Isolation, immunochemistry and comparative chemistry of soluble fractions from type 12 nephritogenic streptococci and human glomeruli. *J. Immun.* 92: 565, 1964.
- Mauer, S. M., Steffes, M. W., Sutherland, D. E. R., Najarian, J. S., Michael, A. F., and Brown, D. M.: Studies of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. *Diabetes* 24: 280, 1975.
- Meezan, E., Brendel, K., and Carlson, E. C.: Isolation of a purified preparation of metabolically active retinal blood vessels. *Nature* 251: 65, 1974.
- Meezan, E., Brendel, K., Ulreich, J., and Carlson, E. C.: Properties of a pure, metabolically active glomerular preparation from rat kidneys. I. Isolation. *J. Pharmacol. Exp. Ther.* 187: 332, 1973.
- Meezan, Elias, Hjelle, J. Thomas, Brendel, Klaus, and Carlson, Edward C.: A simple, versatile, nondisruptive method for the isolation of morphologically and chemically pure basement membranes from several tissues. *Life Sci.* 17: 1721, 1975.
- Merimee, T. J., Siperstein, M. D., Hall, J. D., and Fineberg, S. W.: Capillary basement membrane structure: a comparative study of diabetics and sexual ateliotic dwarfs. *J. Clin. Invest.* 49: 2161, 1970.
- Michael, A. F., Blau, E., and Vernier, R. L.: Glomerular polyanion. Alteration in aminonucleoside nephrosis. *Lab. Invest.* 23: 649, 1970.
- Miki, E., Fukuda, M., Kuzuya, T., Kosaka, K., and Nakao, K.: Relationship of the course of retinopathy to control of diabetes, age, and therapeutic agents in diabetic Japanese patients. *Diabetes* 18: 773, 1969.

- Miller, E. J.: Isolation and characterization of a collagen from chick cartilage containing three identical  $\alpha$  chains. *Biochemistry* 10: 1652, 1971.
- Miller, E. J. and Matukas, V. J.: Biosynthesis of collagen. The biochemist's view. *Fed. Proc.* 33: 1197, 1974.
- Miller, R. L. and Udenfriend, S.: Hydroxylation of proline residues in collagen nascent chains. *Arch. Biochem. Biophys.* 139: 104, 1970.
- Misra, R. P. and Berman, L. B.: Studies on glomerular basement membrane. I. Isolation and chemical analysis of normal glomerular basement membrane. *Proc. Soc. Expl. Biol. Med.* 122: 705, 1966.
- Mogensen, C. E. and Andersen, M. J. F.: Increased kidney size and glomerular filtration rate in untreated juvenile diabetes: normalization by insulin treatment. *Diabetologia* 11: 221, 1975.
- Mohos, S. C. and Skoza, L.: Glomerular sialoprotein. *Science* 164: 1519, 1969.
- Mohos, S. C. and Skoza, L.: Variations in the sialic acid concentration of glomerular basement membrane preparations obtained by ultrasonic treatment. *J. Cell Biol.* 45: 450, 1970.
- Moore, S. and Stein, W. H.: A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* 211: 907, 1954.
- Murphy, M. E. and Johnson, P. C.: Possible contribution of basement membrane to the structural rigidity of blood capillaries. *Microvas. Res.* 9: 242, 1975.
- Myers, C. and Bartlett, P.: Separation of glomerular basement membrane substances by sodium dodecylsulfate disc gel electrophoresis and gel filtration. *Biochim. Biophys. Acta* 290: 150, 1972.
- Nimni, M. E.: Metabolic pathways and control mechanisms involved in the biosynthesis and turnover of collagen in normal and pathological connective tissues. *J. Oral Path.* 2: 175, 1973.
- Ohno, M., Riquetti, P., and Hudson, B. G.: Bovine glomerular basement membrane. Isolation and characterization of a glycoprotein component. *J. Biol. Chem.* 250: 7780, 1975.
- Østerby, R.: Kidney structural abnormalities in early diabetes. In: *Vascular and neurological changes in early diabetes*, ed. by Camarini-Dávalos, R. A. and Cole, H. S., p. 323, Academic Press, New York, 1973.

- Østerby, H. R. and Lundbaek, K.: The basement membrane morphology in diabetes. In: Diabetes Mellitus: Theory and Practice, ed. by Ellenberg, M. and Rifkin, H., p. 178, McGraw-Hill Book Co., New York, 1970.
- Otto, H., Themann, H., and Wagner, H.: Qualitative and quantitative elektronenmikroskopische Untersuchungen an Hauptcapillaren jugendlicher Diabetiker. Klin. Wschr. 45: 299, 1967.
- Palade, G. E. and Bruns, R. R.: Structure and function of normal muscle capillaries. In: Small Blood Vessel Involvement in Diabetes Mellitus, ed. by Siperstein, M. D., Colwell, A. R., and Meyer, K., p. 39, American Institute of Biological Sciences, Washington, 1964.
- Peterson, D. T., Greene, W. C., and Reaven, G. M.: Effect of experimental diabetes mellitus on kidney ribosomal protein synthesis. Diabetes 20: 649, 1971.
- Pierce, G. B., Midgley, A. R., and Sri Ram, J.: The histogenesis of basement membranes. J. Exp. Med. 117: 339, 1963.
- Pirart, J., Lauvaux, J. P., and Eisendrath, C.: Diabetic retinopathy, nephropathy. Relation to duration and control. Diabetologia 11: 370, (Abstract), 1975.
- Pirie, A.: Composition of ox lens capsule. Biochem. J. 48: 368, 1951.
- Ramachandran, G. N., Sasisekharan, V., and Thathachari, Y. T.: Structure of collagen at the molecular level. In: Collagen, ed. by Ramanathan, N., p. 81, Interscience, New York, 1962.
- Rambourg, A. and Leblond, C. P.: Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. J. Cell Biol. 32: 27, 1967.
- Ray, B. S., Pazianos, A. G., Greenberg, E., Peretz, W. L., and McLean, J. M.: Pituitary ablation for diabetic retinopathy. I. Results of hypophysectomy (a ten-year evaluation). J. Amer. Med. Ass. 203: 79, 1968.
- Rhodin, J. A. G.: Electron microscopy of the glomerular capillary wall. Exp. Cell Res. 8: 572, 1955.
- Richterich, R. and Franz, H. E.: Isolation of glomeruli from rat kidney. Nature 188: 498, 1960.

- Rinehart, J. F.: Reticulum: its origin: the occurrence of reticulum in capillary endothelium: a new method of demonstration: II. the finer capillary bed. *Am. J. Path.* 6: 525, 1930.
- Roberts, D. St. C.: Studies on the antigenic structure of the eye using the fluorescent antibody technique. *Brit. J. Ophthal.* 41: 338, 1957.
- Rossle, R. and Yoshida, T.: Das Gitterfasergerüst der Lymphdrüsen unter normalen und pathologischen Verhältnissen. *Ziegler's Beitr. z. path. Anat. u. z. allg. Path.* 45: 472, 1909.
- Rothbard, S. and Watson, R. F.: Comparison of reactions of antibodies to rat collagen and to rat kidney in the basement membranes of rat renal glomeruli. *J. Exp. Med.* 129: 1145, 1969.
- Roy, L. P., Vernier, R. L., and Michael, A. F.: Effect of protein load proteinuria on glomerular polyanion. *Proc. Soc. Exp. Biol. Med.* 141: 870, 1972.
- Ryan, Graeme B., Hein, Stephen J., and Karnovsky, Morris J.: Glomerular permeability to proteins. *Lab. Invest.* 34: 415, 1976.
- Ryan, G. B. and Karnovsky, M. J.: An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis. *Kidney Int.* 8: 219, 1975.
- Sachot, N., Sternberg, M., Skwarlo, K., Rebeyrotte, P., and Lagrue, G.: Comparison of isolation and chemical composition of kidney glomerular basement membranes in rabbit, rat, and man. *Comp. Biochem. Physiol.* 50A: 575, 1975.
- Siperstein, M. D., Unger, R. H., and Madison, L. L.: Studies of muscle capillary basement membranes in normal subjects, diabetic and prediabetic patients. *J. Clin. Invest.* 47: 1973, 1968.
- Sjostrand, F. S. and Rhodin, J.: The ultrastructure of the proximal convoluted tubules of the mouse kidney as revealed by high resolution electron microscopy. *Exp. Cell Research* 4: 426, 1953.
- Spiro, M. J., and Spiro, R. G.: Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membrane and collagens. II. Kidney galactosyltransferase. *J. Biol. Chem.* 246: 4910, 1971.
- Spiro, R. G.: Studies on the renal glomerular basement membrane: preparation and chemical composition. *J. Biol. Chem.* 242: 1915, 1967a.

- Spiro, R. G.: Studies on the renal glomerular basement membrane. Nature of the carbohydrate units and their attachment to the peptide portion. *J. Biol. Chem.* 242: 1923, 1967b.
- Spiro, R. G.: The structure of the disaccharide unit of the renal glomerular basement membrane. *J. Biol. Chem.* 242: 4813, 1967c.
- Spiro, R. G.: Characterization and quantitative determination of the hydroxylysine-linked carbohydrate units of several collagens. *J. Biol. Chem.* 244: 602, 1969.
- Spiro, R. G.: Glycoproteins and diabetic microangiopathy. In: Joslin's Diabetes Mellitus, ed. by Marble, A., p. 146, Lea and Febiger, Philadelphia, 1971.
- Spiro, R. G.: Search for a biochemical basis of diabetic microangiopathy. *Diabetologia* 12: 1, 1976.
- Spiro, R. G. and Fukushi, S.: The lens capsule. Studies on the carbohydrate units. *J. Biol. Chem.* 244: 2049, 1969.
- Spiro, R. G. and Spiro, M. J.: Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membrane and collagens. I. Kidney glucosyltransferase. *J. Biol. Chem.* 246: 4899, 1971a.
- Spiro, R. G. and Spiro, M. J.: Effect of diabetes on the biosynthesis of renal glomerular basement membrane: studies on the glucosyltransferase. *Diabetes* 20: 641, 1971b.
- Spiro, R. G. and Spiro, M. J.: Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membrane and collagens. III. Tissue and subcellular distribution of glucosyltransferases and the effect of various conditions on the enzyme levels. *J. Biol. Chem.* 246: 4919, 1971c.
- Todd, R. B. and Bowman, W.: The Physiological Anatomy and Physiology of Man, pp. 129, 523, 660, Blanchard & Lea, Philadelphia, 1857.
- Törnblom, N.: Mechanical isolation of glomeruli from human kidneys and of hyaline substance from glomeruli with Kimmelstiel-Wilson changes. *Acta Med. Scand.* 159: 369, 1957.
- Udenfriend, S.: Biosynthesis of hydroxyproline in collagen. In: Chemistry and Molecular Biology of the Intercellular Matrix, ed. by Balazs, E. A., Vol. 1, p. 371, Academic, New York and London, 1970.

- Venable, J. H. and Coggeshall, R.: A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25: 407, 1965.
- Vernier, R. L.: Electron microscopic studies of normal basement membrane. In: Small Blood Vessel Involvement in Diabetes Mellitus, ed. by Siperstein, M. D., Colwell, A. R., and Meyer, K., p. 57, American Institute of Biological Sciences, Washington, 1964.
- Von Bruchhausen, F. and Merker, H. J.: Morphologischer und Chemischer Aufbau Isolierter Basal membranen aus der Nierenrinde der Ratte. *Histochemie* 8: 90, 1967.
- Vracko, R.: Basal lamina scaffold: anatomy and significance of maintenance of orderly tissue structure. *Amer. J. Pathol.* 77: 314, 1974a.
- Vracko, R.: Basal lamina layering in diabetes mellitus: evidence for accelerated rate of cell death and cell regeneration. *Diabetes* 23: 94, 1974b.
- Vracko, R. and Benditt, E. P.: Capillary basal lamina thickening: its relationship to endothelial cell death and replacement. *J. Cell Biol.* 47: 281, 1970.
- Vracko, R. and Benditt, E. P.: Basal lamina: the scaffold for orderly cell replacement. *J. Cell Biol.* 55: 406, 1972.
- Vracko, R. and Benditt, E. P.: Manifestations of diabetes mellitus: their possible relationships to an underlying cell defect. *Am. J. Pathol.* 75: 204, 1974.
- Wahl, P., Krezdorn, W., and Deppermann, D.: Untersuchungen zur Pathogenese der diabetischen Mikroangiopathie. *Klin. Wschr.* 48: 653, 1970.
- Walker, F.: The origin, turnover and removal of glomerular basement membrane. *J. Pathol.* 110: 233, 1973.
- Weibel, E. R.: Morphometry of the Human Lung, Academic Press, New York, 1963.
- Westburg, N. G. and Michael, A. F.: Human glomerular basement membrane preparation and composition. *Biochemistry* 9: 3837, 1970.
- Westberg, N. G. and Michael, A. F.: Human glomerular basement membrane: chemical composition in diabetes mellitus. *Acta Med. Scand.* 194: 39, 1973.

- White, R. H., Meezan, E., and Brendel, K.: Basement membrane synthesis by isolated bovine retinal vessels. *The Pharmacol.* 17: 215, 1975.
- Williams, I. F., Harwood, R., and Grant, M. E.: Triple helix formation and disulphide bonding during the biosynthesis of glomerular basement membrane collagen. *Biochem. Biophys. Res. Commun.* 70: 200, 1976.
- Williams, J. A. and J. Wolff: Possible role of microtubules in thyroid secretion. *Proc. Natl. Acad. Sci. U.S.* 67: 1901, 1970.
- Yamada, E.: The fine structure of the renal glomerulus of the mouse. *J. Biochem. Biophys. Cytol.* 1: 551, 1955.