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THE ORIGIN, TURNOVER AND REMOVAL OF NORMAL GLOMERULAR BASEMENT MEMBRANE

by

FREDERICK WALKER

SUMMARY

A comprehensive account of the natural history of normal glomerular basement membrane is prerequisite to elucidating the pathogenesis of numerous renal diseases.

The experimental argyric technique was investigated, adapted and applied in a long term sequential, electron microscopic study of normal glomerular basement membrane in the rat.

The results demonstrate that a major component of glomerular basement membrane is secreted by the visceral epithelial cells. This component is laid down on the epithelial side and slowly moves towards the endothelial side of the basement membrane as new basement membrane material continues to be secreted. The old basement membrane material is removed from the endothelial aspect of the membrane and passes by way of the lamina rara interna to the mesangial matrix for subsequent ingestion by the mesangial cells. This process is continuous and slow: the time for complete renewal of the glomerular basement membrane in the rat is of the order of twelve months. Secretion of this component, by the epithelial cells, is effected by a vascular-coated pit mechanism and removal, by the mesangial cells, is effected by a phagocytic mechanism.

The results further indicate the presence of a second component in glomerular basement membrane. This second component is probably of endothelial origin and has a much faster turnover rate than the main, or epithelial derived, component.

Study was also made of glomeruli from two cases of human argyria and though the observations perforce are limited, the results show that human glomerular basement membrane has a natural history essentially similar to rat glomerular basement membrane.

On the basis of these experimental observations, correlated with the results of previous investigations, a model of the functional morphology of glomerular basement membrane is proposed. The potential applications of this model are briefly indicated

The Origin, Turnover and Removal of Normal
Glomerular Basement Membrane

Thesis Submitted for the Degree of
Doctor of Medicine
of the University of Glasgow

by

Frederick Walker, M.B., Ch.B. (Commendation), Ph.D.

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PREFACE

This thesis is a study of the origin, turnover and removal of normal glomerular basement membrane.

The definite recognition of extracellular matrices, not un-naturally, awaited the definitive recognition of cells in the early nineteenth century. For some decades thereafter controversy raged over whether the cells produced, or were themselves derived from, the extracellular matrix. The domination of the cell and the complete acceptance of the cell theory did not occur until Virchow's (1855) classical pronouncement "Omnis cellula a cellula". This devastating truth, coupled with the lack of suit-able investigational techniques, largely restrained interest in the extracellular matrices until well into the twentieth century.

The study of basement membranes, which comprise part of the extracellular matrices, properly began with the introduction of the term and the concept by Bowman (1842; 1847) but has only resurged within the past fifteen years largely due to the develop-ment and application of electron microscopic techniques. In consequence the role of these lifeless but far from inert membranes in physiological and pathological processes is now better understood. It is hoped that this thesis will further contribute to this understanding.

The importance of basement membranes may be appreciated in a physiological context by the fact that any substance be it ingested, inhaled or injected into the mammalian body, has to cross an absolute minimum of three basement membranes in the course of its entry, metabolism and excretion. The importance of such membranes may also be appreciated in a pathological context in that carcinoma-in-situ has yet to be the cause of death in one patient while carcinoma causes the death of many and the difference, in the simplest possible terms, is transgression of basement membrane. It has also been suggested that altered permeability of basement membrane particularly in the vasa vasorum, may contribute to the formation of atheroma. It is therefore evident that basement membranes are involved significantly in the pathogenesis of many, common, important, human diseases.

The most studied basement membrane to date is that in the renal glomerulus which physiologically plays an important role in the initial urinary ultrafiltration process and which, pathologically, features prominently in a variety of disease processes both of renal and extra-renal origin. Because of these previous studies there are considerable data and reasonable general agreement about ultra-

structural features, chemical composition and functional capabilities of the glomerular capillary wall, including the basement membrane. There is, however, some dispute about the manner in which the glomerular wall functions. It is a rather remarkable membrane which will filter over one hundred and fifty litres of fluid per day for a lifetime without, in most instances, either becoming clogged or showing signs of wear and tear.

Such a membrane excites the curiosity. It was this, allied with a lack of comprehension of the genesis of certain glomerular basement membrane lesions, and the inability to retrieve the appropriate information from previously published investigations, which prompted the choice of the subject matter of this thesis. Other considerations influenced the form of this study. There is a great deal to be learned about basement membranes but expansive ideas, however stimulating, must be tempered by considerations of feasibility with the object of designing a practical, informative series of experiments which will elucidate the biological processes under consideration.

Essential to an understanding of any pathological process is an understanding of the corresponding normal process. As it has been aptly put "pathology is nothing but physiology with obstacles"

(Virchow 1855 translated by Rather, 1958). It was therefore decided, for this particular project, to dispose of at least some of the problems and study the physiology of the basement membrane.

Having decided to study normal glomerular basement membrane in an effort to achieve some general insight into its modus operandi the specific questions in mind initially were: Where does the basement membrane come from? How does it get there? Is it stable or does it turn over? If it turns over where does it go? How does it work? Why does it continue to work for a lifetime? Where can it go wrong?

Considering these questions, the already published data and available techniques it seemed that the answers might be forthcoming from a long term, sequential, electron microscopic examination of glomerular basement membrane which had been labelled in vivo, at appropriate times, with an electron dense marker. The progress of the marker in the basement membrane could then be studied as a function of time.

A more personal reason for selecting this approach is that the solutions to many important pathological problems appear to lie, for the present, in that limbo between ultrastructure and chemical

composition.   Here the electron microscopist expects the bio-
chemist to provide the answer and the biochemist has the same
expectation of the microscopist!   Having, in a previous thesis
("A Study of the Mucopolysaccharides in Normal and Diabetic
Vitreous Humour"), utilised a biochemical approach, it seemed
that if on this occasion an ultrastructural approach was used con-
siderable additional insight and experience would be gained which
would prove of value in tackling future problems.

The simplest appropriate electron-dense marker was silver.
The main advantage offered by this experimental model - the
argyric glomerulus - apart from simplicity, was the singular
lack of reported toxicity after ingestion of small amounts of silver
salts.   Silver offered one other major advantage in that it is the
only marker which enables direct comparison between the experi-
mental animal and the human.   Examples of human argyria are
nowadays of great rarity but material suitable for examination was
potentially retrievable from the files of pathology departments.
This proved to be the case.   The main disadvantage was that this
experimental model had been little used previously so lack of
essential background information necessitated considerable pre-
liminary and ancillary investigations in addition to the experiments

proper.

The thesis is presented in five parts.

Part I is an introduction outlining present information and current
ideas which provide a background to this particular invest-
igation.

Part II is concerned with preliminary and ancillary studies of
experimental argyria.

Part III describes the definitive experiments on glomerular base-
ment membrane in the rat.

Part IV records the findings in the glomerular basement membrane
in two cases of human argyria and correlates these with the
findings in the experimental animal.

Part V is a general conclusion which places the experimental
results in context with other data and a model of the functional
morphology of glomerular basement membrane is proposed.

Contents

Figure 1. The mammalian renal corpuscle showing the capsule (c), the glomerulus (m), the arterioles (af and ef) and the tubule (t). Reproduced from Bowman (1842).

Part I

INTRODUCTION

The Normal Mammalian Glomerulus in General
and its Basement Membrane in Particular

The functional and structural unit of the kidney is the nephron and
in the beginning of the nephron is the glomerulus.

Structure

The normal mammalian renal corpuscle (Malpighii, 1666) is

a hollow spheroid about 150 to 250 microns in overall diameter.

It is bounded by the capsular basement membrane which on its

outer aspect blends with the interstitial tissue of the renal cortex

and on its inner aspect is lined with the parietal epithelial cells.

Within the sphere is a large tuft of capillaries, the glomerulus

(Bowman, 1842), (Figure 1).

The glomerulus is supplied by a single afferent arteriole which

arises directly from an interlobular artery and enters the renal

corpuscle at the vascular pole or hilum.   The glomerulus is

drained by a single efferent arteriole which leaves the renal cor-

puscle at the vascular pole and eventually drains into an interlobular

vein.   The region between the arterioles at the vascular pole is

occupied by the lacis cells.   This region is closely related to the

macula densa of the distal convoluted tubule.

the

Normal glomeruli are remarkably uniform in appearance, not only in the individual and in the species but also in different mammalian species (Vernier, 1961). The preceding and following comments, except where specified, may be considered to apply to both human and rat glomeruli (Figures 2 and 3).

The glomeruli are distributed throughout the renal cortex and number well over one million in each adult human kidney: there are correspondingly fewer in the rat kidney. The capillaries of a single human glomerulus have a surface area of about 0·38 square millimetre (Book, 1937): the corresponding value in the rat is 0·19 square millimetre (Kirkman and Stowell, 1942). Glomeruli in a juxtamedullary situation are slightly larger than those elsewhere in the cortex.

Ultrastructure

(a) Glomerular basement membrane

The glomerular basement membrane completely surrounds the capillaries and no discontinuities have been demonstrated (Figure 4). The membrane is a trilaminar structure having thin outer and inner relatively electron-lucent layers, respectively called the lamina rara externa and the lamina rara interna, which sandwich a middle, thick, relatively electron-opaque layer, the lamina densa (Figure 5).
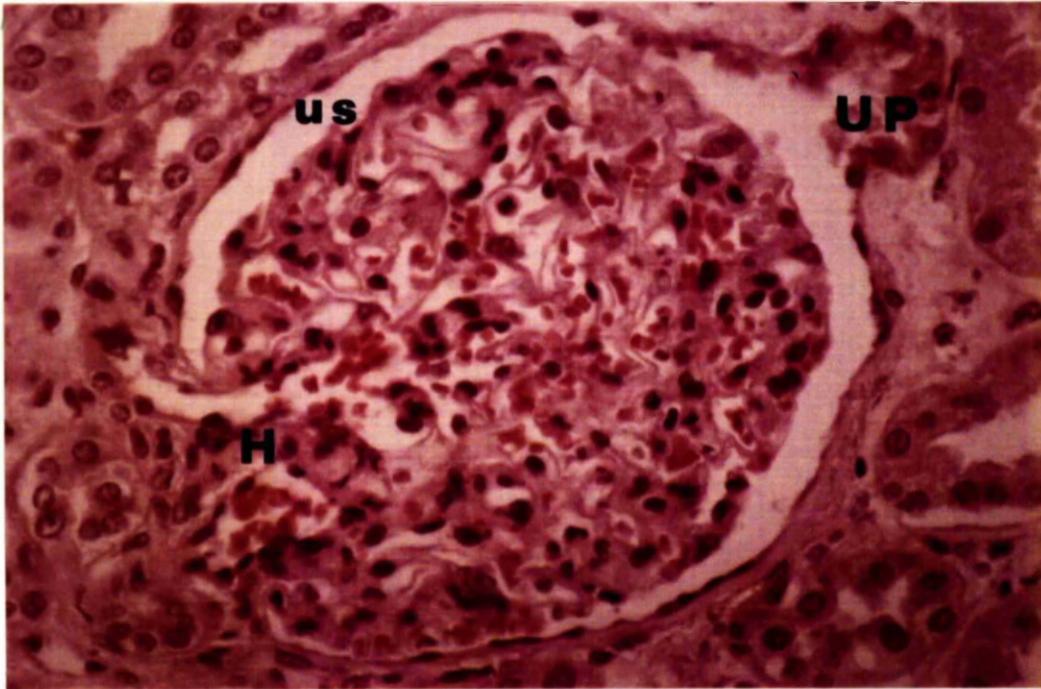
Figure 2.   Normal human glomerulus.   Haematoxylin and eosin (H & E). X 340
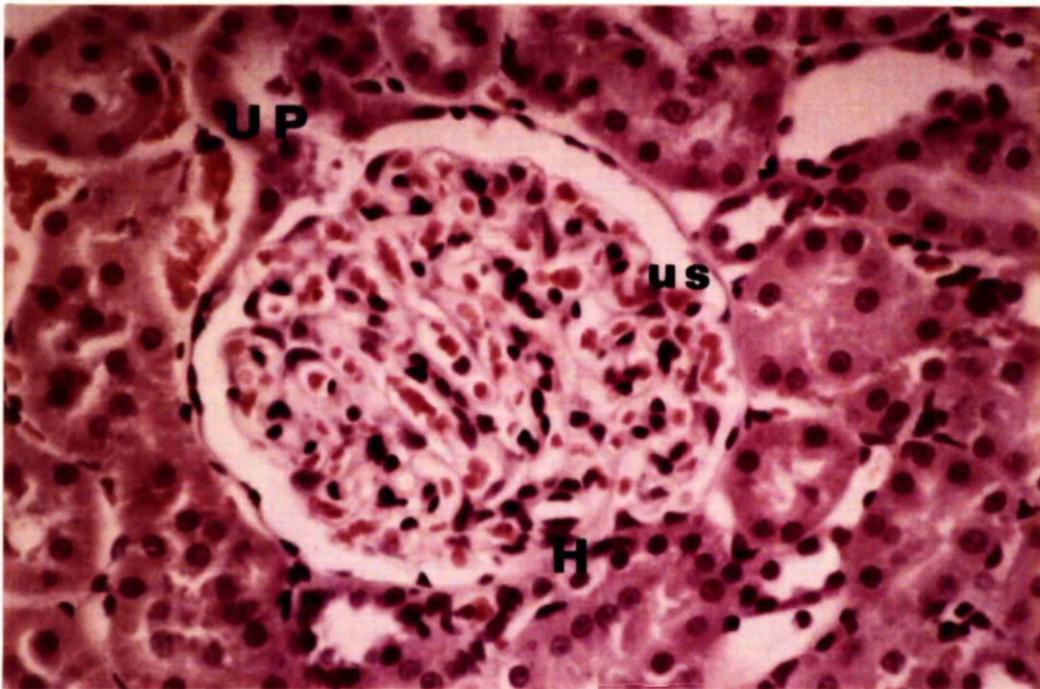
Figure 3.   Normal rat glomerulus.   H & E. X 340
H = hilus;   US = urinary space;   UP = urinary pole

Figure 4. Portion of a glomerulus demonstrating continuity
of the glomerular basement membrane and the relative positions
of epithelial, endothelial and mesangial cells.   X5,000

All the electron micrographs, unless otherwise indicated, are
of rat tissue stained with uranyl acetate and lead citrate.   All
magnifications refer to the finished print and are corrected to
the nearest thousand diameters.

BM	=	basement membrane
CBM	=	capsular basement membrane
CP	=	coated pit
DB	=	dense body
EC	=	visceral epithelial cell
EN	=	endothelial cell
F	=	fuzz
FP	=	foot process
G	=	Golgi apparatus
LD	=	lamina densa
LRE	=	lamina rara externa
LRI	=	lamina rara interna
M	=	mitochondrion
MC	=	mesangial cell
MM	=	mesangial matrix
N	=	nucleus
PC	=	parietal epithelial cell
R	=	ribosomes
RBC	=	red blood cell
SP	=	slit plate
V	=	vacuole

Figure 4

Figure 5a. Glomerular basement membrane with the foot processes of the visceral epithelial cells embedded in its outer aspect. The inner aspect is lined by attenuated endothelium with fenestrae. The three laminae of the basement membrane are clearly distinguishable. X 76,000

Figure 5b. Foot process showing thick plasma membrane and the filtration slit plates. X 148,000
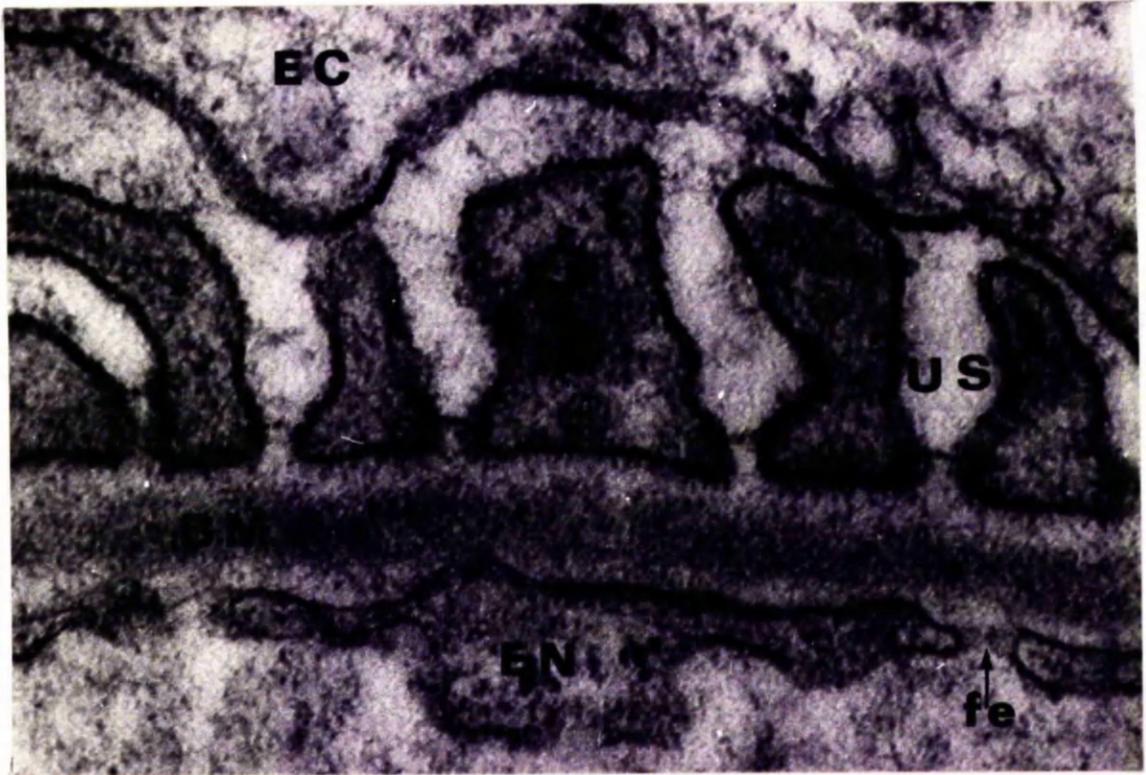
Figure 5 a
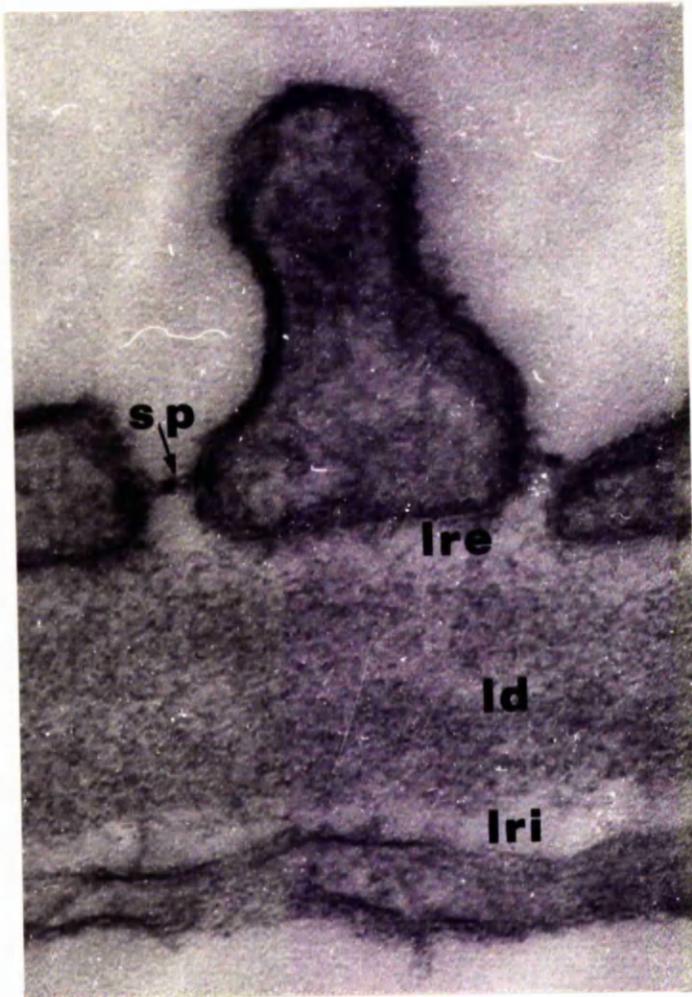
Figure 5 b

The existence of these three laminae is universally recognised
though it is not established whether they are entities in their own
right or whether they are preparative artefacts.   In this thesis
the term glomerular basement membrane refers to the entire
membrane, i.e. all three laminae, and the names of the individ-
ual laminae are used to refer to the corresponding topographical
locations.

Outwardly the glomerular basement membrane is discontin-
uously but regularly covered by the foot processes of the visceral
epithelial cells (Gautier, Bernhard and Oberling, 1950).   These
processes are embedded in the lamina rara externa and are very
firmly secured.   Foot process detachment has not been described.
Between adjacent foot processes lie the filtration slits in which
region the outer limit of the basement membrane is defined by a
single layered membrane, 50-70 $\overset{o}{A}$ thick, which joins the adjacent
foot processes (Yamada, 1955).   This, the filtration slit mem-
brane, is of such a width and electron density that it is usually
only visible when the plane of the microscope section is normal
with respect to the surface of the basement membrane (Farquhar,
Wissig and Palade, 1961).   Since it is apparent that the basement
membrane, as well as the adjacent structures, can be cut in an

infinite variety of planes, the filtration slit membrane is very useful in orientating the section and developing a more accurate three dimensional concept of the glomerulus.

Inwardly the glomerular basement membrane is limited largely by the plasma and fenestral membranes of the endothelial cells and to a lesser extent by the plasma membranes of the mesangial cells.

In the peripheral portions of the capillaries the glomerular basement membrane is regular in outline and fairly uniform in thickness. In the vicinity of mesangial cells the basement membrane is considerably and irregularly thickened on its internal aspect. This localised excess of basement membrane-like material is called the mesangial matrix.

It has already been mentioned that the glomerular basement membrane and the capsular basement membrane are continuous at the vascular pole. In this region there are also large, irregular deposits of basement membrane-like material which are in continuity with the glomerular basement membrane and with the basement membrane of the arterioles (Latta and Maunsbach, 1962; Barajas and Latta, 1963; Jacobsen, Jorgensen and Thomsen, 1966). The precise relationships of this basement membrane material, and

whether it is true basement membrane or mesangial matrix, are not yet determined.

The laminae rarae are almost devoid of ultrastructural features. They contain a few very fine fibrils which run almost at right angles from the vicinity of, or directly from, the neighbouring plasma membranes into the lamina densa (Jorgensen, 1967b). In current preparations the lamina densa appears as a network or feltwork of partially oriented, fine, aperiodic fibrils about 40 $\overset{o}{A}$ in diameter, set in an amorphous matrix of slightly lower electron density (Yamada, 1955; Kurtz and McManus, 1960; Farquhar, Wissig and Palade, 1961; Vernier, 1961, 1964; Kurtz, 1961; Farquhar, 1964a). About these observations there is good general agreement; about the interpretation of these observations there are disparate views. One view is that the fibrils exist in their own right and are an integral part of the lamina densa. The other is that these fibrils are the ultrastructural manifestation of micelles (Frey-Wyssling, 1948) whose appearance is exaggerated during the fixation of what is essentially a thixotropic gel (Menefee, Mueller, Bell and Myers, 1964; Menefee and Mueller, 1967). At the dimensional level involved in the foregoing interpretations preparative artefacts can seriously affect the organisation of biological

materials. Independent evidence obtained by other preparative procedures or by entirely different means is therefore highly desirable (Palade and Bruns, 1964).

The mean width of the glomerular basement membrane in the peripheral portions of the capillary loops in the adult human is about 3,200 $\overset{o}{A}$ though considerable variation occurs notably between different, rather than within the same, capillaries (Osawa, Kimmelstiel and Seiling, 1966; Jorgensen and Bentzon, 1968). Some of this variation appears to be related to the degree of dilation or contraction of the capillaries (Farquhar, Wissig and Palade, 1961; Shirai, Takasugi and Kitamura, 1969). The relative thickness of the laminae rarae vary from report to report and is apparently partly related to the amount of extraction occurring in different fixation and embedding procedures. A typical figure however, for present techniques, is 200 to 400 $\overset{o}{A}$ (Jorgensen, 1967b).

In the rat the glomerular basement membrane is thinner than the human and has been variously reported as having a mean width of about 1,600 $\overset{o}{A}$ (Latta, Maunsbach and Madden, 1960) to 1,900 $\overset{o}{A}$ (Shirai, Takasugi and Kitamura, 1969).

(b) Mesangial matrix

The mesangial matrix is smoothly limited on its outer aspect

by the inner surface of the lamina densa from which it is fre-
quently, but not invariably, distinguishable by being slightly
less electron dense. Inwardly its contours are tortuously
demarcated largely by the plasma membrane of mesangial cells
and to a lesser extent by the plasma membrane of endothelial
cells. At its lateral margins the matrix blends into the lamina
rara interna.

Mesangial matrix is looser in texture and less homogeneous
than the lamina densa. Its electron density varies irregularly
from that of the lamina densa to that of the lamina rara. Extens-
ive areas of the matrix appear structureless or faintly granular,
at least with the microscopic resolution used to date. In other
areas a variety of not readily characterised fibrils are present.
In addition to the fine 40 $\overset{o}{A}$ fibrils similar to those in the lamina
densa there are aperiodic fibrils about 100 $\overset{o}{A}$ in diameter (Farquhar
and Palade, 1962). The other main fibril occurs in lesser numbers
but is considerably larger, 400 to 500 $\overset{o}{A}$, in diameter. These fibrils
are generally thought to be collagen though they are considerably
shorter and have a less constant periodicity than typical collagen
(Michielson and Creemers, 1967). Such atypical collagen fibrils
are regularly found in the normal rat mesangial matrix (Latta, 1961)

but have not been described in the normal human glomerulus.
Typical collagen fibrils however have been widely reported in
a variety of human and rat pathological glomeruli (Spiro, 1959;
Bencosme, Stone, Latta and Madden, 1959; Hinglais-Guillaud
and Galle, 1961; Kimmelstiel, Kim and Beres, 1962; Suzuki,
Churg, Grishman, Mautner and Dachs, 1963).

(c) Visceral epithelial cells

The visceral epithelial cells are large and irregular in outline.
They are characterised by having many foot processes which inter-
digitate regularly and are firmly attached to the outer aspect of
the glomerular basement membrane (Yamada, 1955). The cells
are bounded by a plasma membrane which has a triple-layered unit
membrane structure differing from most other cells only in that the
outer layer of the membrane appears slightly thicker than usual
(Latta, 1962). This plasma membrane is completely enveloped
by a thick cell coat or "fuzz" (Rambourg and Leblond, 1967;
Mohos and Skoza, 1969; Jones, 1969; Groniowski, Biczyskowa
and Walski, 1969; Behnke and Zelander, 1970) which is considered
in detail later in this thesis.

The main body of the cell contains the nucleus and well developed
mitochondria, free ribosomes, rough surfaced endoplasmic reticulum

which in places is in the form of very large distended cisternae
(Farquhar, Wissig and Palade, 1961; Thoenes, 1967), round
vesicles up to 1,000 Å in diameter many of which surround the
prominent Golgi apparatus, and some large vacuoles up to 9,000 Å
in diameter. The vesicles and the vacuoles are surrounded by
a unit membrane of the same dimensions as the plasma membrane.
Multivesicular and membrane-bound dense bodies are commonly
present. The cytoplasm of the foot processes is frequently much
more dense than that elsewhere in the cell and contains a limited
range of organelles notably vesicles and vacuoles; ribosomes and
mitochondria are occasionally present but nucleus, Golgi apparatus
and rough surfaced endoplasmic reticulum have not been observed
(Trump and Benditt, 1962; Jorgensen, 1967a). Attachment zones
of the usual variety between adjacent epithelial cells are absent and
coalescence between visceral and parietal epithelial cells has not
been observed in the normal glomerulus though these cell types are
contiguous at the vascular pole.

The width of the foot processes is exceedingly variable but is
always greatest at the insertion of the basement membrane. In
humans the width varies from 1,000 to 15,000 Å though most pro-
cesses are in the lower reaches of this range, and the intervening

filtration slits range from 250 to 600 $\overset{o}{A}$ in width (Jorgensen, 1967a).
In rats the foot process width is equally variable and the filtration
slit width lies between 200 and 500 $\overset{o}{A}$ (Latta, Maunsbach and
Madden, 1960). It is interesting to note that foot processes,
though generally recognised only with the advent of electron micro-
scopy, were first described and accurately drawn by Zimmermann
in 1915 in the course of an optical microscopy study of armadillo
glomeruli. It is probable that this species has particularly large
foot processes but this does not detract from Zimmermann's
meticulous techniques and remarkable acuity (Majno, 1965).

(d) Endothelial cells

Endothelial cells completely line the glomerular capillaries.
The outer surface of these cells is related mainly to the lamina rara
interna and to a lesser extent to mesangial matrix. The inner sur-
face forms the boundary of the capillary lumen. Adjacent cells
are joined by desmosomes to form tight junctions and at the vascular
pole the endothelial cells lining the capillaries are continuous with
those lining the arterioles.

The main mass of the cytoplasm, containing the nucleus, Golgi
apparatus, free ribosomes, scanty mitochondria and occasional
vacuoles or vesicles, is usually, but not invariably, situated over

mesangial matrix. From this perikaryon the remainder of the cytoplasm extends in a lamina about 1,000 $\overset{o}{A}$ thick to invest the entire inner surface of the glomerular basement membrane. This attenuated portion of the cytoplasm is interrupted regularly by circular openings, the fenestrae, about 1,000 $\overset{o}{A}$ in diameter (Gautier, Bernhard and Oberling, 1950; Pease, 1955). The glomerular fenestrae may or may not be closed by a thin diaphragm (Majno, 1965) though in certain other sites in the body these diaphragms appear to be more constant (Rhodin, 1962). It has been suggested that the fenestral diaphragm consists of the external leaflets of two adjacent plasma membranes (Luft, 1964) and morphologically is very similar to the filtration slit plate (Luft, 1965). "This ingenious synthesis has little evidence to support it, however" (Karnovsky, 1968). The only organelles usually present in the attenuated portions of the cytoplasm are small vesicles. The endothelial cells also have a covering of cell coat or "fuzz" (Luft, 1966) but this is thinner than that on the visceral epithelial cells (Jones, 1969).

(e) Mesangial cells

The presence of a third cell type, that is apart from the endothelium and the visceral epithelium, was originally noted by Zimmermann (1933). His observations, though strictly correct,

were misinterpreted in the light of the then held view of glomerular development, namely invagination of the glomerular capsule by a tuft of capillaries. Such a process meant that the capillaries would be invested with a layer of the invaginated capsule rather in the way the gut is invested with a layer of peritoneum as it lies in the coelombic cavity. By analogy with the mesenterium Zimmermann introduced the term mesangium and thought that this third cell type was a fibroblast pulled in between the layers from the adjacent mesenchyme.

Mesangial cells are invariably situated between glomerular basement membrane and endothelial cells, surrounded by mesangial matrix. Their precise locus in the capillary loop is less certain. One view is that the cells are intercapillary in location, i.e. where capillary loops branch or anastomose (Latta, Maunsbach and Madden, 1960). The other view is that the cells are intracapillary in location, i.e. parietal (Farquhar and Palade, 1962). This problem is heatedly disputed (Zamboni and Martino, 1968b) but is not yet resolved and is a reflection of the difficulties of three dimensional reconstruction from two dimensional micrographs in a structure as complex as the glomerulus.* In the centrolobular region, close to the hilum, the mesangial cells are

continuous with, and similar to, lacis cells; they also bear a resemblance to the muscle cells of the afferent arteriole (Latta and Maunsbach, 1962; Zamboni and Martino, 1968b).

The mesangial cell has a remarkably irregular outline with numerous, small, projecting spines or pseudopodia, and numerous invaginating pockets or pits. The cytoplasm, apart from the nucleus, has a varied content of organelles; Golgi apparatus, smooth and rough surfaced endoplasmic reticulum, free ribosomes, multivesicular and dense bodies, and centrioles. There are focal areas of increased cytoplasmic density situated peripherally from which radiate bands of cytoplasmic filaments. Particularly noteworthy is an apparent continuity in sequence from the invaginating pockets through intracytoplasmic vacuoles to dense or residual bodies. Occasionally bulbous pseudopodia are observed displacing or penetrating the overlying endothelium to lie within the capillary

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* The original paper by Latta and his associates contains an explicit drawing of mesangial cells in an intercapillary location. This beautiful, but two dimensional, representation has been reproduced subsequently in numerous papers and books. This diagram and the ensuing controversy form a particularly good example of the point, made by Sidman, Sidman and Touchette (1964) in a study of programmed self-instruction, that showing anatomical features in only one view may lead to stereotyped concepts not usable in different contexts.

lumen.   Those pseudopodia are, as a rule, devoid of organelles
(Yamada, 1955; Farquhar and Palade, 1962; Jones, Mueller and
Menefee, 1962; Suzuki, Churg, Grishman, Mautner and Dachs,
1963; Farquhar, 1964a; Michielsen and Creemers, 1967).

(f) Capsular basement membrane and parietal epithelial cells

The capsular basement membrane and the parietal epithelium
are continuous respectively with the glomerular basement mem-
brane and the visceral epithelium at the vascular pole, and with
the tubular basement membrane and epithelium at the urinary pole.

The capsular basement membrane is substantial, over 5,000 $\overset{o}{A}$
in width, and is irregularly and inconstantly divided into laminae
of varying electron density.   Its inner limit is smoothly demarcated
by the plasma membrane of the parietal epithelial cells.   Its outer
limit is less well defined and contains small numbers of collagen
fibrils.   At the vascular pole the transition from capsular to
glomerular basement membrane is comparatively abrupt.

The parietal epithelial cell is flattened and contains the usual
complement of organelles.   It differs particularly from the visceral
epithelial cell in having no foot processes, fewer vesicles, a plasma
membrane of normal dimensions (Latta, 1962) and a thinner cell coat
(Jones, 1969).

(g) Variations

There are certain, minor, ultrastructural variations which occur inconstantly, yet sufficiently frequently, in the normal glomerulus to necessitate their recognition lest they be mistaken for pathological features.

Foot processes are not quite as regular as most descriptions would have them. In almost every section of a glomerulus occasional processes which are much larger than average are found, and slight degrees of foot process fusion can be observed especially in the vicinity of capillary branchings (Jorgensen, 1967a). Another variant is the presence of several slit plates in a single filtration slit (Ericsson, 1966).

Focal hemispherical thickening of the basement membrane occurs rarely (Jorgensen, 1967a).

Endothelial fenestrae vary in number and size in numerous published illustrations of what are undoubtedly normal glomeruli. This aspect of quantitative electron microscopy appears to have been imperfectly studied to date.

Mesangial cells form a heterogeneous population and the density of the cytoplasmic matrix and the number of organelles present is highly variable (Farquhar and Palade, 1962).

All of the above mentioned variations were encountered in the course of the present study.

Development and Maturation of the Glomerulus

The mammalian glomerulus develops in the definitive kidney, or metanephros, from the lower limb of the S-shaped metanephric vesicle and from the mesenchyme in the cleft between the middle and lower limbs. The cells in the lower surface of the lower limb flatten and form the parietal epithelial cells of the renal corpuscle. The cells in the upper surface of the lower limb are columnar and form the visceral epithelial cells of the glomerulus. The cavity of this lower limb becomes the urinary, or Bowman's, space. The cleft between the middle and lower limbs contains mesenchyme and a capillary which at all stages of development communicates with the surrounding blood vessels. This capillary eventually becomes the afferent and efferent arterioles: the mesenchyme in the cleft eventually becomes the mesangial, lacis and arteriolar wall cells.

By a process of differential growth the lower, or parietal surface almost completely envelops the upper or visceral, surface. At the same time the capillary in the cleft proliferates and occupies the developing concavity in the visceral surface, becoming intimately related to the visceral epithelial cells and forming the glomerulus.

The cells in the lower surface of the middle limb of the S-shaped vesicle, which are also related to the mesenchyme in the cleft, eventually become the macula densa of the distal tubule (Huber, 1905; Jokelainen, 1963; Potter, 1965; Potter and Osathanondh, 1966; Zamboni and Martino, 1968a).*

The oldest, most mature glomeruli are those in the juxtamedullary region. Those situated in the sub-capsular region are the youngest and least mature. Nephron induction in the human ordinarily ceases between the 32nd and 36th week of gestation after which time no new glomeruli are formed in any circumstances (Potter and Thierstein, 1943). Post natally in the rat it has been reported that new glomeruli continue to develop for up to three months though the vast majority

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* The sequence of events outlined above is that which fits most of the present known facts. However glomerular embryogenesis has been a particularly controversial topic during the past decade and other views are still held. The long held concept of invagination of a hollow sphere by a tuft of capillaries is well described and clearly illustrated by Ham (1966) though he concedes several pages later in his text that "This theory has the advantage of being easily understandable, but ......". The paper by Potter is particularly commendable for its clear account, solid appraisal and criticism of other theories, and excellent sequence of photomicrographs. Even this paper shows traces of the controversy in that emotive phrases appear in the text: instead of the more usual, objective "previous studies" or impersonal "other investigators" there appears the phrase "these men"! In a later paragraph another example is "Hall especially, has been vehement in denying that ......". The other papers cited are less enjoyable.

develop within the first month (Kittelson, 1917; Arataki, 1926).
After birth the most prominent feature of glomerular maturation
is an increase in the degree of capillary lobulation and the glomeruli
continue to increase in size until adulthood (Eckardt, 1888;
MacDonald and Emery, 1959; Bauer and Rosenberg, 1960).

As the glomerulus develops and matures so does its basement
membrane. Initially the plasma membranes of the foetal visceral
epithelial and the endothelial cells are apposed but they soon separate
slightly. The intervening space is almost devoid of ultrastructural
detail then either a single, or a double which soon coalesces into a
single, band of thin fibrillar material appears which eventually con-
solidates and progressively thickens into the definitive basement
membrane (Kurtz, 1958; Vernier, 1961; Vernier and Birch-
Andersen, 1962). It is presumed that both epithelial and endothel-
ial cells contribute to the formation of the basement membrane but
their respective role, and the part played by the mesangial cells
in the process, is not known (Rhodin, 1964).

At birth the glomerular basement membrane in the human has
a mean width of about 1,000 $\overset{o}{A}$. It progressively thickens until at
the age of three years it has a mean width of 2,800 $\overset{o}{A}$ (Bloom,
Hartmann and Vernier, 1959; Vernier, 1961). This was an early

study of basement membrane measurements and 2,800 Å was also given as the width of adult membrane. This is slightly less than the currently accepted adult figure of 3,200 Å (Osawa, Kimmelstiel and Seiling, 1964; Jorgensen and Bentzon, 1968) and while the difference is probably due to difference in measuring techniques, it might possibly be observational. In summary present information indicates that human glomerular basement membrane increases in width from birth to adulthood but by far the largest increment occurs before the age of three years.

In the rat glomerular basement membrane also increases in width with age (Ashworth and Erdmann, 1959; Ashworth, Erdmann and Arnold, 1960; Takebayashi, 1969) and it has been demonstrated that this is due to the laying down of new basement membrane on the epithelial aspect (Kurtz and Feldman, 1962). Though the new basement membrane appeared on the epithelial aspect it was only by inference that the epithelial cells were responsible.

This topic of the source of basement membrane material in the post natal development, maintenance and repair of the glomerulus is considered in detail later in this thesis.

Composition of Glomerular Basement Membrane

It is well established that the main constituent of mammalian

glomerular basement membrane is a protein of the collagen type (Goodman, Greenspon and Krakower, 1955; Rothbard and Watson, 1961; Lazarow and Speidel, 1964; Dische, Pappas, Grauer and Dische, 1965; Kefalides and Winzler, 1966; Misra and Berman, 1966; Lidsky, Sharp and Rudee, 1967; Spiro, 1967a). This collagen differs chemically from the general form of mammalian fibrillar collagen in several respects. Particularly significant differences are that basement membrane collagen contains more hydroxylysine and is associated with more carbohydrate than fibrillar collagen. The carbohydrate occurs in two different units, a disaccharide and a heteropolysaccharide (Dische, Pappas, Grauer and Dische, 1965; Spiro, 1967b). The disaccharide consists of glucose and galactose and is linked to hydroxylysine, the structure is 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine (Spiro, 1967c; Kefalides, 1969a). The heteropolysaccharide contains a total of sixteen monosaccharides made up of galactose, mannose, N-acetylglucosamine, sialic acids and fucose. The structure of this heteropolysaccharide is not yet determined but its linkage is probably to asparagine.

Concerning this heteropolysaccharide there are at present inadequate data to reach a definite conclusion but there are three

main suggestions. The first is that this carbohydrate is attached to a non-collagenous peptide which may be part of a separate glyco-protein (Dische, Pappas, Grauer and Dische, 1965; Kefalides, 1968). This suggestion implies that, in the main, glomerular basement membrane is a two component system. The second is that this carbohydrate is attached to the basement membrane collagen itself (Spiro, 1967b, 1969). This suggestion implies that, in the main, glomerular basement membrane is a one component system. The third suggestion is that much of this sialic acid containing poly-saccharide is derived from cell coat and is a contaminant of the glomerular basement membrane preparations (Mohos and Skoza, 1970).

These viewpoints may not be so disparate as they appear for immunochemical studies raise the possibility that the non collagenous peptide to which the heteropolysaccharide is attached is associated with the collagen molecule as a telopeptide (Sharp, Anderson and Lidsky, 1967; Kefalides, 1969b, 1969c). Moreover experiments described later in this thesis indicate that a component of glomerular basement membrane has certain features in common with visceral epithelial cell coat. It is likely that this particular problem will be solved in the next few years probably as a result of correlative biochemical-immunochemical investigations.

Numerous other minor components have been reported in studies of both normal and abnormal glomerular basement membrane. These include phospholipids, neutral lipids, globulin, fibrinogen and ribonucleic acid. Their exact relationships and distribution are obscure but they are generally regarded as being either filtration residues or cytoplasmic contaminants of basement membrane preparations.

It is of interest to note that the collagenous nature of basement membrane was not recognised electron microscopically. Mammalian fibrillar collagen has a characteristic ultrastructure consisting of striations with regular major and minor periodicities. Basement membrane collagen lacks this periodicity. This ultrastructural difference is attributable to differences in the degree of cross linking of the respective collagens. Fibrillar collagen shows a high degree of orientation and is extensively cross linked. Basement membrane collagen is considerably less so (Dische, 1964). Lysine and hydroxylysine have been shown to be the amino acids responsible for forming the cross links of the collagens (Piez, 1968; Bailey and Peach, 1968; Bailey, Peach and Fowler, 1970). It has therefore been proposed that the hydroxylysine-linked disaccharide may serve a regulatory function by substituting the hydroxylysine and removing it as a potential

partner in the cross link formation (Spiro, 1969).

Function of Glomerulus

The glomeruli produce an ultrafiltrate from the plasma which is subsequently modified in the renal tubules and eventually excreted as urine. In man this ultrafiltrate normally amounts to about 180 litres per 24 hours and consists of plasma constituents in varying dilutions. The main factors involved in production of the ultrafiltrate are the constituents of the plasma, the hydrodynamic pressure within the glomerular capillary and the nature of the glomerular capillary wall. The first two factors are normally assumed to be determined as a result of which most investigation and speculation concerning glomerular function has centred on the nature of the capillary wall. It is frequently assumed, though supported by remarkably little direct evidence, that ultrafiltration takes place by physical processes which involve little or no energy expenditure on the part of the cells in the wall of the capillary. It is further generally assumed and supported by considerable experimental evidence, that the capillary wall is a semi-permeable membrane though permeability in this context can imply flow and/or diffusion.

In terms of performance or 'what it can do', there is good general agreement about the capillary wall (Smith, 1951; Wallenius, 1954;

Hardwicke and Soothill, 1961). Small molecules up to about the size of inulin, which has a molecular weight (MW) of 5,500 and an Einstein-Stokes diffusion radius (RES) of 15 $\overset{o}{A}$, pass freely through the capillary wall and have a clearance about the same as creatinine. Progressively larger molecules pass through the capillary wall less freely; for instance serum albumen (MW = 69,000, RES = 32 $\overset{o}{A}$) has a clearance which is less than one hundredth that of creatinine. Although glomerular capillaries are relatively impermeable to proteins there is no upper limit to the permeability, only a pronounced falling off of the speed or amount of penetration with increasing molecular weight.

There is no single hypothesis of the mechanism of glomerular capillary permeability which can account for all the known facts. "This may be due to defective or incomplete hypothesis, or that some of the facts are wrong, or misinterpreted, or both" (Luft, 1965). At present the various hypotheses show four trends of thought which developed in chronological order more or less parallel to the application of particular investigational techniques.

(A) The first hypothesis is that ultrafiltration occurs between the endothelial cells and is largely regulated by the intercellular cement substance. This view stems mainly from light microscope

observations and was extensively reviewed by Chambers and Zweifach (1947). This hypothesis, though useful in other sites, has been of limited value in interpreting glomerular permeability.

(B) The second and, to date, dominant hypothesis is the so called "Pore Theory" developed by Pappenheimer (Pappenheimer, Renkin and Borrero, 1951; Pappenheimer, 1953, 1955). This hypothesis, based on physicochemical data such as that outlined earlier in this section and on physiological clearance studies, proposes that ultrafiltration occurs through pores some 75 to 100 $\overset{o}{A}$ in diameter largely by hydrodynamic flow and to a much lesser extent by diffusion. Two points made by Pappenheimer are of particular importance with regard to later consideration of this hypothesis: the first is that steric effects may restrict diffusion at the entrance to the pore; the second is that reduction in glomerular filtration, due for example to interference with the blood flow, will result in relatively larger amounts of protein appearing in the ultra-filtrate due to molecular sieving. Pappenheimer's hypothesis has been severely criticised on thermodynamic grounds and an alternative working hypothesis of diffusion across the capillary wall, rather than flow through pores, has been proposed (Chinard, 1952; Chinard, Vosburgh and Enns, 1955). Other studies in general

support Pappenheimer's views and further suggest the existence of either stretchable pores or the existence of a second, numerically smaller but dimensionally larger, pore population (Grotte, 1956; Mayerson, Wolfram, Shirley and Wasserman, 1960).

It must be emphasized that the hypothesis outlined above concerns a mechanical and mathematical model of the glomerular wall as a whole. It is a satisfactory functional concept but makes no attempt to ascertain the actual pathway taken by the transported material.

(C) The third hypothesis is intrinsically concerned with the actual pathway taken by the transported material. This view is really a series of electron microscopic observations which, as yet, lack succinct formal expression in a unifying hypothesis. Briefly, different investigators using different experimental models have placed varying degrees of emphasis on the endothelium, the basement membrane, the filtration slit plate and the foot processes, as the barrier which regulates ultrafiltration. These investigations are tabulated on the following page.

Tracer	Molecular weight or size	Reference
Horseradish peroxidase	40,000	Graham and Karnovsky, 1966
Haemoglobin	68,000	Ericsson, 1965
Myeloperoxidase	160,000–180,000	Graham and Karnovsky, 1966
Catalase	240,000	Venkatachalam, Karnovsky and Cotran, 1969
Ferritin	462,000 Total 100 Å. Visible 55 Å	Farquhar, Wissig and Palade, 1961; Vernier and Birch-Andersen, 1963
Globin	Aggregates	Menefee, Mueller, Bell and Myers, 1964
Thorotrast	up to several hundred Å	Latta, Maunsbach and Madden, 1960
Colloidal gold	40–250 Å	Farquhar and Palade, 1959
Colloidal carbon	200–400 Å	Vernier and Birch-Andersen, 1963

Summarizing these results it is clear that cells and very large colloidal particles normally do not cross the endothelium. All other tracers cross the endothelium readily, principally by way of the fenestrae, and enter the lamina rara interna. All of these molecules can subsequently transverse the lamina densa but many are retarded at the inner border of this layer and eventually finish up in the mesangial matrix then the mesangial cells. In general the larger the molecular size of the tracer the more of it goes by this route. Those molecules which cross the lamina densa lie in the lamina rara externa and are more or less retarded by the filtration slit plates and by the plasma membranes of the foot processes. Small molecules of the size of catalase readily pass through the slit plate but larger molecules pass through the foot processes by pinocytosis and so reach the urinary space.

These ultrastructural studies using electron-dense tracers have failed to demonstrate a pore system in the basement membrane. The tracers cross the membrane everywhere or at random, and no deformation of the membrane by the tracers has been observed. All the tracers investigated were found also in the mesangial matrix and cells to a greater or lesser extent.

From these studies it is established that the glomerular capillary

wall is stratified: this implies division of labour and functional specialization of each layer (Palade and Bruns, 1964). The endothelium is a coarse filter, the lamina densa is a medium filter and the slit plate is a fine filter. It has been suggested, and supported with sound experimental evidence, that filtration residues are removed to the mesangial matrix and incorporated into the mesangial cells (Farquhar and Palade, 1962; Graham and Karnovsky, 1966). It has further been suggested that the visceral epithelium functions as a monitor of the glomerular filtrate (Farquhar, 1964a). The results of studies using peroxidase as a tracer are in accord with this latter suggestion (Graham and Karnovsky, 1966; Venkatchalam, Karnovsky and Cotran, 1969).

The tracers used in these investigations are unusual molecules and, with the exception of haemoglobin, clearance data for them are either not available or incomplete. In consequence it is difficult to correlate the ultrastructural features with the physiological concepts outlined previously. Again there are two main views. One is that the filtration slits may function as 'pores' or at least be rate limiting (Hall, 1957; Landis and Pappenheimer, 1963). The other is that the basement membrane is a gel and the rate limiting factor is diffusion (Chinard, Vosburgh and Enns, 1955; Menefee and

Mueller, 1967; Ericsson, 1968).

Apart from these general deficiences all of the tracer models
are subject to particular criticisms.   In some the intraluminal
concentration of tracer molecules was too low to permit a system-
atic study (Palade and Bruns, 1964).   This applies particularly
to colloidal gold.   In certain of the early ferritin studies cadmium
was also present and the injected tracer was therefore toxic.
Ferritin is also produced endogenously and cannot be distinguished
from the exogenous tracer after a lapse of a few hours (Farquhar,
1964b).   Globin occurs in both a soluble and an aggregated form:
only the latter is electron-dense and locatable (Graham and Karnovsky,
1966).   Haemoglobin is extremely difficult to demonstrate electron
microscopically and often its location is more by inference than
recognition.

Despite these deficiences and criticisms the electron micro-
scopic studies of glomerular permeability have significantly contri-
buted to the elucidation of glomerular function.

(D)   The fourth and last main hypothesis concerning glomerular
filtration stems from the advances in biochemical studies of glomerular
basement membrane in the past few years.   These data are as yet
incomplete and so far no well developed hypothesis has emerged.

only random ideas. One such idea is that differential permeability is the result of differential cross-linking and aggregation of the basement membrane collagen (Kefalides, 1969c). This is a reason-able suggestion but, as yet, is in too general a form to be of value in particular situations. Another idea is that the physiological 'pores' are helical structures because the major structural compon-ent of glomerular basement membrane is a helical glycoprotein (Misra and Berman, 1969). This is an interesting but highly specul-ative suggestion for while the glycoprotein is helical there is little evidence that pores exist, far less helical pores. Primitive though these suggestions are, the molecular anatomy of the basement mem-brane promises formative ideas within the next few years.

(E) Apart from the main hypotheses two further ideas merit con-sideration, one because of its originality the other because it is a good compromise.

Gautier, Bernhard and Oberling (1950) who were the first to visualize and appreciate the nature of foot processes were consider-ably impressed by their contours and interdigitations. They there-fore surmised that the calibre of the glomerular capillary and the volume of the peri-capillary, i.e. urinary, space could actively vary and thereby act like a suction pump. Oddly enough it has subsequently

been shown that the calibre of the glomerular capillaries varies
(Shirai, Takasugi and Kitamura, 1969), that the epithelial cells
have myoid features (Pease, 1968) and that the glomerulus con-
tracts rhythmically and synchronously in tissue cultures (Bernik,
1969).

Bennett (1963), taking the morphological data especially con-
cerning the feltwork of filaments in the basement membrane and
the physiological data especially concerning the 'stretched pore'
proposal, put forward the idea that the filaments are not extensively
cross linked so filtration channels are present everywhere in the
basement membrane but are constantly capable of changing in res-
ponse to hydrodynamic and, by inference, other physical conditions.
He further suggested that the basement membrane of the capillary
is analogous to the glycocalyx of many cells.   These ideas antedate
but are in accord with the biochemical evidence concerning the
meagre cross linking in the basement membrane collagen.   They
also antedate experimental evidence supporting the analogy between
glycocalyx and basement membrane which is presented later in this
thesis.

Optical microscopy, physiology, electron microscopy and biochemistry have all contributed considerable data on the glomerulus. While within each of these fields of investigation there are conflicting views - Whether the ultrafiltrate is formed by diffusion or filtration; whether the basement membrane is fibrillar or a gel; whether the basement membrane is a single or a multicomponent system - it is probable that these views are less irreconcilable than they appear. Between these fields of investigation there is a lack of correlation - do the filtration slits correspond to the pores, are the fibrils the ultrastructural manifestation of weakly cross-linked collagen, is the heteropolysaccharide responsible for molecular sieving effects - but the lack is due to the absence of ill-defined though essentially small details. The gaps to be bridged between the individual and the molecule, between function and structure, between hypothesis and reality, are smaller in the glomerulus than in most other sites in the body.

Having considered this background information with respect to the subsequent part of this thesis, one basic, self-evident but none-the-less worth emphasizing fact is that glomerular basement membrane cannot be regarded properly in isolation. It must be considered with the visceral epithelial, the endothelial and the mesangial cells as a

functional and structural unit whose several components normally

maintain a delicately adjusted balance.

Part II

Experimental Argyria

Preliminary and Ancillary Experiments Directed to
the Development of a Suitable Experimental Model
for Basement Membrane Studies

Numerous experimental studies on argyria were performed
in the latter half of the 19th and the early years of the 20th cen-
turies.   Most of these studies were directed towards the thera-
peutic uses of silver compounds and the prevention of argyria.
One of the earliest, and most systematic, of such investigations
was that of Huet (1873) who fed silver salts in doses of up to 6 mg.
per day to 4 rats for periods ranging to more than a year.

The application of argyria as an experimental model for struc-
tural and ultrastructural studies emerged only in recent years and
commenced with the study of Gatz (1949).   This paper, which was
presented at a meeting and published only as an abstract, attracted
considerable attention as a potentially useful technique.   The pro-
cedure was subsequently used in electron microscopic studies first
by Dempsey and Wislocki (1955a, b, c, d) and since then by several
other workers (Wislocki and Ladman, 1955;   Deane, Hofmann,
Solomon and Wislocki, 1955;   van Breemen and Clemente, 1955a, b;
van Breemen, Reger and Cooper, 1956;   Olcott and Richter, 1958;

Kurtz and Feldman, 1962; Vernier, 1964; Oshima, Hatano,

Maeyama, Sugino and Takeuchi, 1967; Striker and Smuckler,

1970).

In theory experimental argyria is simple: rats are given silver

nitrate in their drinking water and after appropriate intervals the

animals are killed and the silver is located by light or electron

microscopy.   The very theoretical simplicity has marred method-

ological detail in the previously cited studies.   The concentration

of silver nitrate has ranged from 1·0 g per litre (Dempsey and

Wislocki, 1955c) to 5·0 g per litre (van Breemen and Clemente,

1955b) or is referred to as "dilute" (Vernier, 1964; Oshima,

Hatano, Maeyama, Sugino and Takeuchi, 1967).   The period of

administration has ranged from one (Oshima, Hatano, Maeyama,

Sugino and Takeuchi, 1967) to nineteen (Gatz, 1949) months.

The varied nature, or lack, of detailed information necessitated

extensive preliminary and ancillary experiments which are described

in this part of the thesis.   These particular observations were made

in different animal houses and laboratories (Glasgow, Minneapolis

and Aberdeen).   The definitive experiments described in part III

of this thesis were all carried out under standardised conditions in

Aberdeen.

Methodological Studies

(a) Method of administering silver nitrate

Silver nitrate is very soluble in water and is most conveniently administered orally. It deteriorates on exposure to light and precipitates readily in the presence of certain ions, particularly chloride. At different times precipitation problems arose with drinking containers, washers and mouthpieces. These were all tested, individually and assembled, by placing in 0.5 per cent aqueous silver nitrate for at least 24 hours to see if any silver was precipitated. Silver administration was eventually standardised to insulation tape covered glass bottles with plastic mouthpieces and grey, synthetic rubber washers devoid of identifying marks, source unknown (Figure 6). This particular drinking apparatus presented no precipitation problems: its only inconveniences in use were that inspection of the amount of fluid consumed took longer than with a transparent container, and that the mouthpieces were breakable.

En route to this almost obvious solution it was noted that the opaque, unbreakable, plastic water containers widely used in animal houses are made from a polymer which contains chloride. These containers slowly precipitate silver. One unexpected complication

Figure 6a.   Drinking bottle suitable for administering silver
nitrate solutions, on the left;   unsuitable type of mouthpiece on
the right.

Figure 6b.   Detail of corrosion produced, by steel mouth-
piece resting on zinc coated metal cage in the presence of
silver salts, within a period of 4 weeks.

Figure 6 a

Figure 6 b

occurred while using plastic coated metal mouthpieces. These do not precipitate silver but the plastic wears off at the point where the mouthpiece rests on the metal of the cage. The combination of galvanised metal cage, steel mouthpiece and silver nitrate solution is particularly corrosive and the mouthpiece erodes through in about 4 weeks (Figure 6).

(b) Concentration of silver nitrate in drinking water

The amount of water drunk by young adult rats varies from animal to animal, from day to day, and from animal house to animal house but a typical range is about 15 to 25 ml per animal per 24 hours.

Four groups of rats were given respectively ordinary tap drinking water, and 1·0, 2·5 or 5·0 g of silver nitrate per litre of distilled water. Analytical grade silver nitrate was used to eliminate possible effects from other heavy metals.

When given 1·0 g silver nitrate per litre of drinking water there is no obvious change in the drinking habits or water consumption of rats. When given 2·5 g silver nitrate per litre the rats' water consumption drops markedly on the first day then progressively builds up to a normal consumption in about 5 days. When given 5·0 g silver nitrate per litre the rats' water consumption drops precipitously then slowly builds up to about half the previously normal level in about

10 days. Three of 12 rats on this high concentration died on days 4 and 5 after commencing ingestion. The remaining rats survived but because of their rather miserable condition this particular experiment was discontinued after 2 weeks when ordinary tap water was substituted for the silver nitrate drinking fluid and the animals rapidly returned to normal.

Dark field microscopy of the kidneys of rats drinking 1·0 g silver nitrate per litre failed to show good glomerular basement membrane labelling within 3 months. The glomeruli of rats drinking 2·5 g silver nitrate per litre showed good labelling after 10 weeks. Five grammes of silver nitrate per litre had adverse effects on the animals. The concentration of silver nitrate was therefore standardised at 2·5 g per litre of water for all subsequent experiments. Apart from the first 5 days on this drinking fluid those rats were clinically distinguished from control rats on ordinary tap drinking water only by having a brown muzzle and discoloured teeth.

This concentration of silver nitrate has previously been used effectively (Kurtz and Feldman, 1962). Two papers on experimental argyria mention toxic concentrations of silver (Olcott, 1948; van Breemen, Reger and Cooper, 1956). The latter investigators

used a concentration of 5.0 g silver nitrate per litre for 6 to

8 months and clearly state that "silver appears in noticable

quantity in the rat kidneys only after several months of vital

administration".   In view of the findings recorded in the pre-

vious two paragraphs it seems that either most of van Breemen's

rats were atypical (such animals do occur and are considered

later in this chapter) or, more probably, their drinking vessels

were slowly precipitating out the silver nitrate.

Olcott (1948)* records that rats given 1 per cent silver salts

to drink "did not survive" and that 2 rats "were kept alive for over

500 days with no other fluid intake than 0.4 per cent solutions of

silver chloride in sodium thiosulfate but this dosage usually was

found to be excessive".   He gives no data on the onset of pigment-

ation but his illustrations are all from rats exposed to silver for

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*   This paper by Olcott contains much useful information and its
histological content is graced with descriptive precision.   However,
apart from some anachronistic references the paper might well have
been written in 1848 and not 1948.   Bright field and not dark field
microscopy is the basic technique employed.   Large segments are
written in the first person singular e.g. "I have given silver sol-
utions to ......".   There are personal references in the text
e.g. "Dr. N. C. Foot has examined some of my slides and believes
that ......".   This style contrasts markedly with the objectivity of
the results.   These are well correlated and spiced with a modicum
of tangential speculation but there is a remarkable absence of deduct-
ion.   It would appear that the results are an end in themselves.

more than 511 days.

(c) <u>Long term toxicity</u>

It is well established that continuous administration of silver solutions results in the progressive accumulation of silver in rats (Olcott, 1948; Wislocki and Leduc, 1952).   To determine when the standard 2·5 g per litre silver nitrate drinking fluid pro-duced long term toxic effects a group of six, male, Sprague-Dawley rats was given this fluid continuously from the age of six weeks.   The animals were observed and handled every week and compared with a control group of rats receiving ordinary, tap, drinking water.

From about the 25th week of silver ingestion onwards these argyric rats were distinguishable from the controls not only by discoloured muzzle and teeth but also by being slightly less in weight.   Their condition otherwise was unremarkable until between the 76th and 81st week of silver ingestion the condition of all six rats deteriorated markedly.   They became listless and poorly groomed and their fluid intake dropped considerably.   Each rat did this successively and five recovered when the silver nitrate solution was replaced with ordinary drinking water.   The sixth rat was killed and examined.   Apart from intense pigmentation,

which is described later, and weight loss it showed no pathological features.

A striking feature of human argyria is the absence of any evidence that the silver deposits produce any significant physiological disturbance of the involved organs or tissues, apart from pigmentation (Hill and Pillsbury, 1939). In rats, apart from those given very high concentrations of silver nitrate, or given lower concentrations for a very considerable time as described earlier, the only known circumstances in which silver salts are markedly toxic is in association with vitamin E deficiency (Shaver and Mason, 1951; Grasso, Abraham, Hendy, Diplock, Golberg and Green, 1969). The rats used in the experiments described in this thesis were fed on proprietary brands of rat cake, largely based on wheat and dried milk, which have an adequate content of vitamin E.

(d) Detection of silver - light microscopy

Silver deposited in biological tissues has constant and reasonably characteristic features when examined by bright field microscopy. The silver appears as a dark brown or black deposit depending on the intensity of the argyria. Slight degrees of argyria are easily overlooked in haematoxylin and eosin stained

sections. For practical purposes the only exogenous pigment which can cause confusion is carbon, and the only endogenous ones are melanin and haemosiderin. In theory the preparative artefactual pigments formalin and mercury could pose problems but were not encountered in the course of this study.

Silver deposited in biological tissues has constant and reasonably characteristic features when examined by dark field microscopy. The silver appears as a brilliant sheen which varies from silver to pale gold in colour depending on the intensity of the argyria. This is the technique of choice for detecting argyria with the light microscope and can be used both with stained and unstained slides. For reasons not made entirely clear, but possibly due to established custom, most previous studies with dark field microscopy have utilized unstained slides and localization, rather than detection, has been a problem (Hill and Montgomery, 1941; Boersma and Baker, 1948).

In order to gain familiarity with the light microscopic appearances of silver the kidneys from a series of 6 rats which had been given 0·25 per cent silver nitrate for 0, 2, 4, 6, 8 and 10 weeks were examined respectively unstained, eosin stained, and haematoxylin and eosin stained using both bright and dark field microscopy. Silver

was detectable in the 8 week specimen by the dark field technique
and was barely detectable in the 10 week specimen by the bright
field technique. The findings were in accord with the conclusion
of Wislocki and Leduc (1952) that the dark field brings out "much
more silver and finer particles of it than the eye was able to
detect with direct light".

Unstained sections were very difficult to locate under the
objective and by bright field are exceedingly trying on the eyes
(Figure 7). Eosin stained sections give particularly good dark
field appearances but are of limited value for bright field examin-
ation (Figure 8). Haematoxylin and eosin stained sections give
good bright field images, and dark field images only marginally
less satisfactory than eosin alone (Figure 9). As a routine all
subsequent specimens were stained with eosin alone and haematoxylin
and eosin, and were examined using both bright and dark fields.

Localization of the silver was not found to be a particular
problem as the same field can be readily examined by bright or
dark techniques. With rotating sub-stage bright and dark field
condensers (Zeiss Standard Universal Microscope with achromatic-
aplanatic condenser VZ with N.A. 1·4 front lens) this is no more
difficult or time consuming than rotating the objective turret.

Figure 7. Argyric glomerulus, unstained, (a) bright field, (b) dark field. X 340

Figure 8. Argyric glomerulus, stained with eosin alone, (a) bright field, (b) dark field. X 340

Figure 9. Argyric glomerulus, stained with haematoxylin and eosin, (a) bright field, (b) dark field. X 340

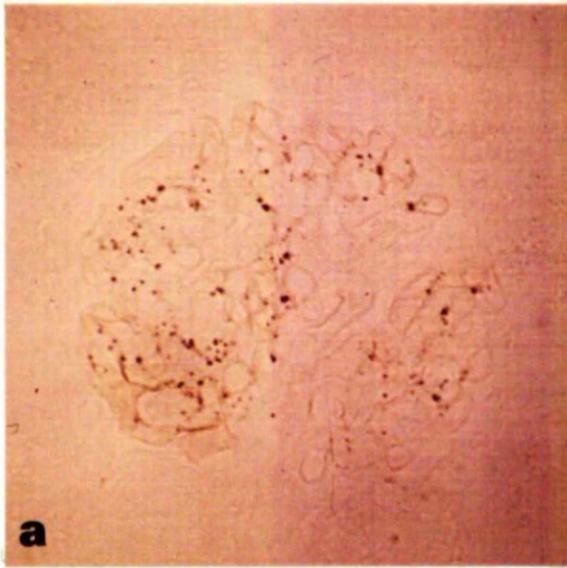These glomeruli are all from a rat exposed to silver nitrate drinking solution for 10 weeks.

Fig.
7
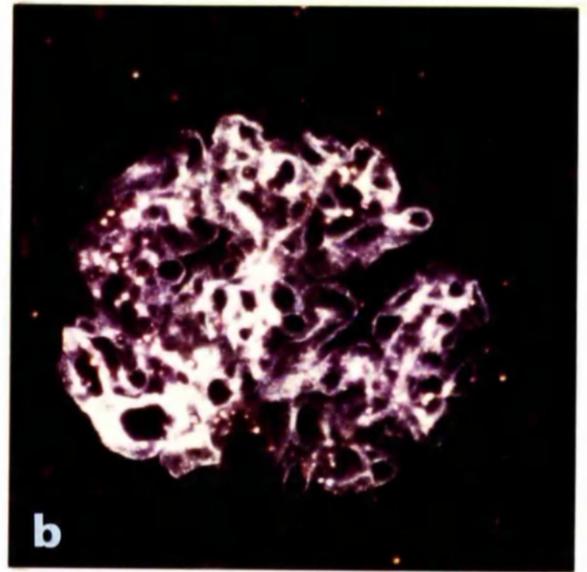
Fig.
8
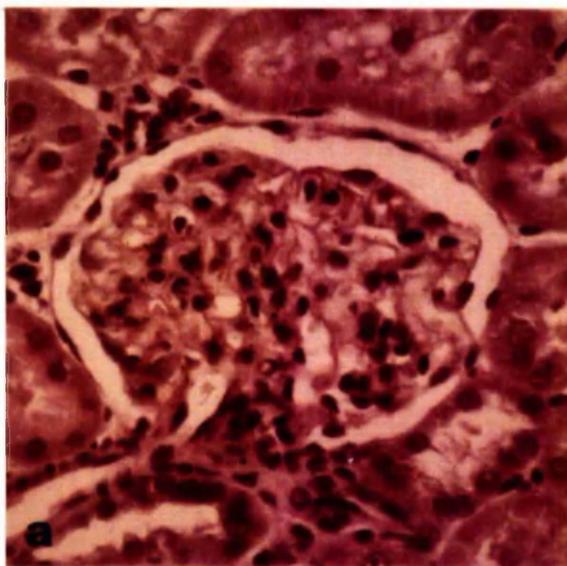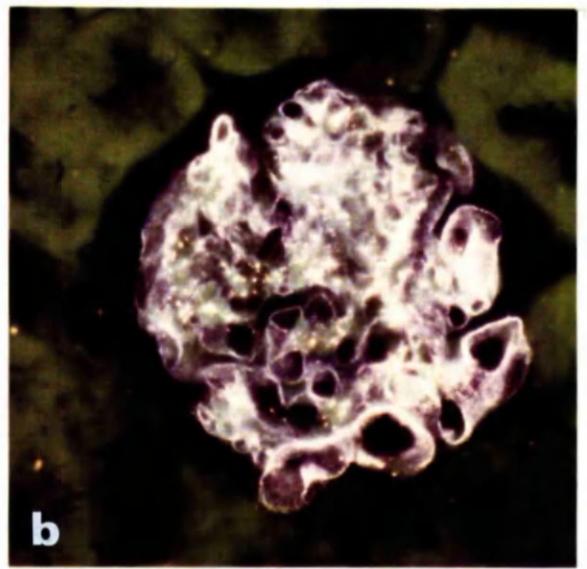
Fig.
9
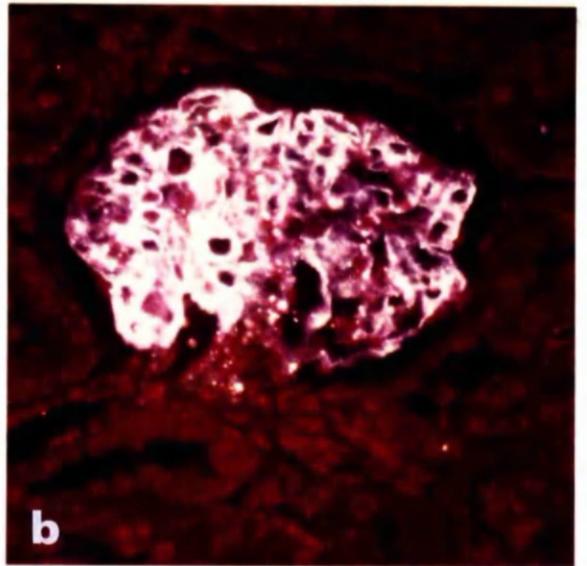
Such a facility was available to neither Hill and Montgomery nor Boersma and Baker.

Sections from the argyric rats were subjected to the standard chemical procedures for the detection of silver, as adapted for histochemistry (Lillie, 1965). The pigment was not removed by iodine alone, or by sodium thiosulphate alone, but was removed when treatment with iodine was followed by treatment with sodium thiosulphate. Concerning this latter reaction Olcott (1948) clearly states "The color can be made to return by the use of a photographic developer". This was attempted using a variety of proprietary, modern developers, e.g. "Unitol", without success. Olcott unfortunately did not specify which developer but, later, on reflection it seemed he might well have used one of the older developers which gave physical as well as chemical development. The experiment was therefore repeated using a variety of older developers, e.g. D-76 and paraphenylenediamine, and the colour did return to the tissues. The silver could be completely removed by 5 per cent sodium cyanide in which case the colour did not return with either group of developers.

(c) Detection of silver - electron microscopy

Vital staining with silver lends itself well to electron microscopic

techniques. Silver scatters electrons producing an easily identified, readily located, image of the metallic deposit. The silver appears in the form of single or aggregated particles with rounded, slightly angular or occasionally comma-shaped profiles which are characterised by a uniform and very intense electron density (Figure 10). The particles vary in size from less than 30 to several hundred $\overset{o}{A}$ in diameter. Such particles are not seen in non-argyric control animals. There is unanimous agreement on these features in all previous electron microscopic studies on experimental argyria.

These dense deposits can be removed by exposure to 5 per cent sodium cyanide for 10 minutes (Figure 11). The form of the deposit changes when the sections are exposed to iodine (Figure 12). Subsequent exposure of these grids to sodium thiosulphate gave micrographs of dubious validity due to severe contamination problems. The original deposit gives no electron diffraction pattern, which confirms the previous finding of Olcott and Richter (1958), but a diffraction pattern was obtained from the iodine treated material (Figure 13). This diffraction pattern altered during the course of examination from a spot to a ring pattern. Both the transmitted and diffracted images of the iodine treated material were comparable

Figure 10. Typical appearances of silver deposits in glomerular basement membrane. From a rat which ingested silver nitrate for 25 weeks. X 22,000

Figure 11. Sodium cyanide treated section of argyric glomerular basement membrane. The silver granules, which were deposited in the inner half of the basement membrane in this specimen, have dissolved. The outer half of the basement membrane contained no silver and is unaffected by this treatment. X 22,000

Figure 12. Iodide treated section of argyric glomerular basement membrane. The silver granules, which were deposited in the inner half of the basement membrane, have altered their morphology. The outer half of the basement membrane contained no silver and is unaffected by this treatment. X 22,000

Figure 13. Electron diffraction pattern of the deposit illustrated in figure 12.

Figure 11

Figure 12
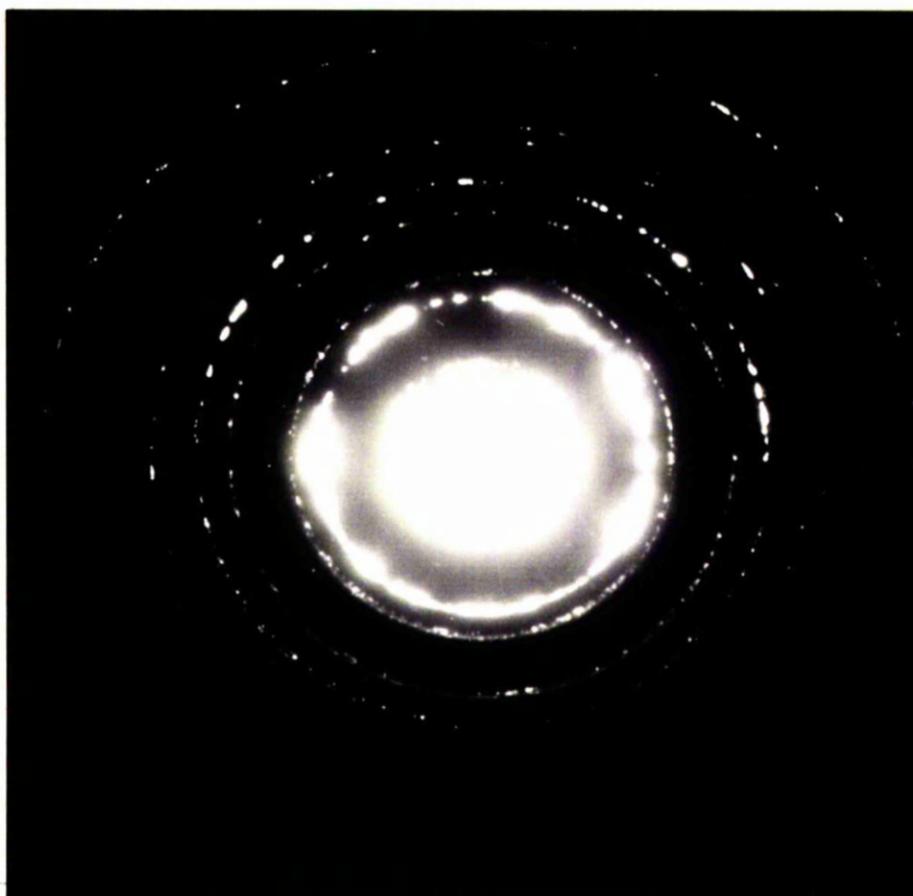
Figure 13

to 'Formvar' coated grids with silver iodide precipitates but this does not necessarily prove identity. Silver iodide does not have a sufficiently characteristic crystal shape to be positively identified, and certain silver salts change in structure when exposed to electron beams (Reimer, 1965; Weisenberger, 1965). Both the chemical and crystalline data strongly support the identity of these electron dense basement membrane deposits as silver.

There are, unfortunately, several other kinds of electron dense particles which can potentially complicate the interpreation of argyric electronmicrographs. There is no mention of these in any of the previous studies though both from textual descriptions and in illustrations it is evident that minor, unrecognised, confusions have arisen. A systematic search for such non-argyric, electron dense particles was therefore made in a series of argyric and non-argyric tissues, mainly kidney. Sections were examined unstained, stained with uranyl acetate, with lead citrate, and with combined uranyl acetate and lead citrate. Some of the sections were on 'Formvar' coated grids, most were on uncoated grids. Some of the specimens were coated with carbon but most were uncoated.

The electron dense particles observed fell into two distinct categories which are considered in turn below.

A. Artefactual densities.

Overstaining with lead citrate, overexposure of the film, or printing the negative on too high a grade of paper, all exaggerate the natural electron densities of the section so that even ribosomes can appear as dense as silver granules in the finished photograph. Such an artefact can be suspected by the lack of low contrast detail in the finished print and confirmed by examination of an unstained section from the same block in which case only the silver is electron dense, by examination of the negative in which case it is excessively dark, or by changing the grade of the photographic paper.

Microscope contamination due to deposition of hydrocarbons (Ennos, 1953, 1954; Heide, 1965) can produce particles almost as dense as silver. These particles are distinguished by appearing and increasing both in intensity and number, often with dramatic rapidity, while the section is being examined (Figure 14).

Stain precipitation has been observed on sections inadequately rinsed and dried. This tends to occur in tidemark regions near the periphery of grid squares. A more generalised distribution was observed if the lead citrate was breathed on during staining procedures and is presumably due to insoluble lead carbonate.

Figure 14.   Beam contamination.   The contaminant spots
are larger, less homogeneous and less electron-opaque than
silver deposits (arrow).   They are randomly distributed over
the field of view without regard to topography.   X120,000

Figure 15.   Lead artefact.   Small, electron-opaque globules
are distributed throughout the field of view and are especially
obvious in the vicinity of the plasma membrane.   Silver, for
comparison, is present in the glomerular basement membrane
in the lower half of the illustration.   X51,000

Figure 14
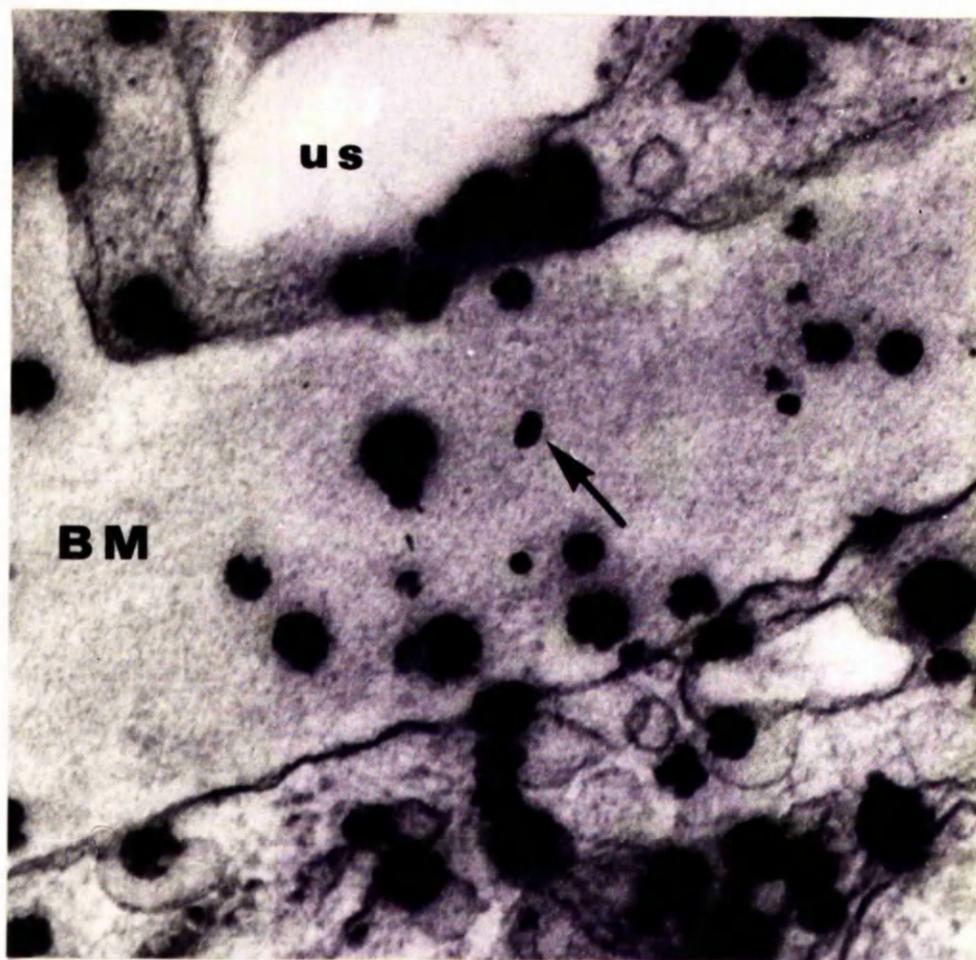
Figure 15
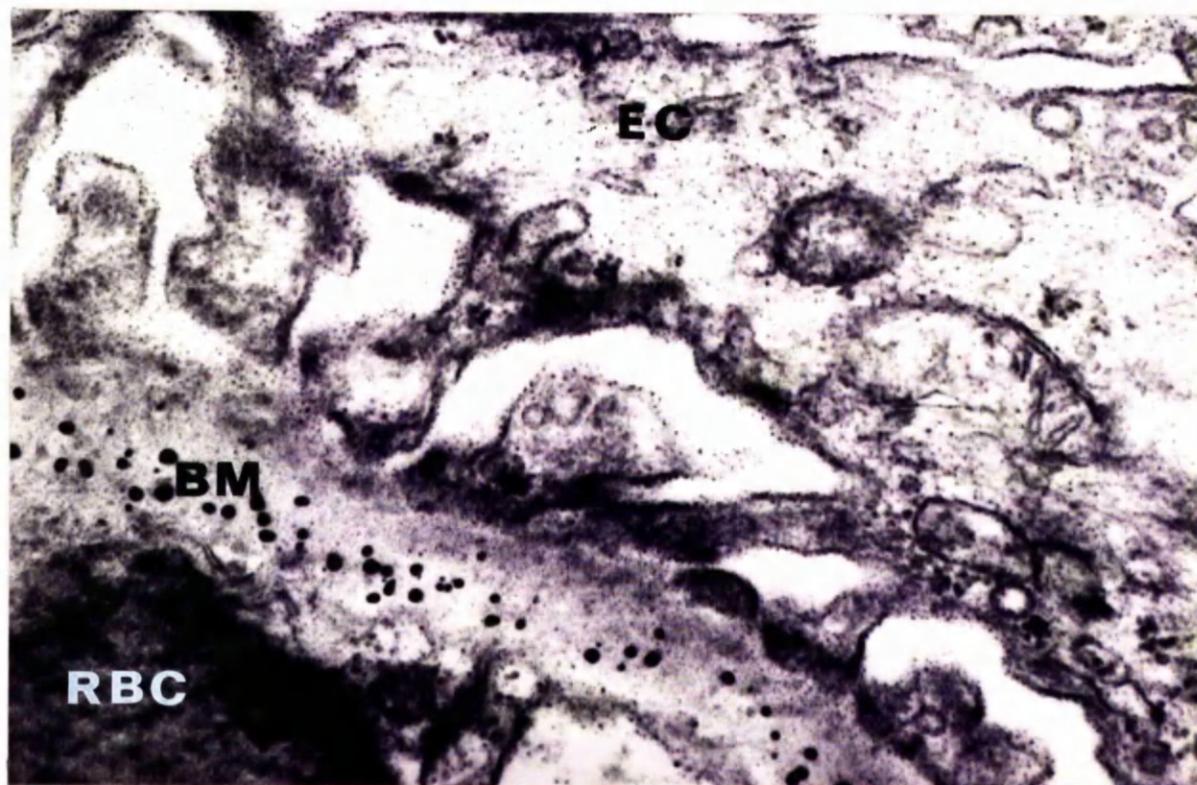
These deposits are not present in unstained sections from the same block.

An unusual artefact which occurs in overstained, over-irradiated sections is illustrated in figure 15. This produces globules as electron dense as silver and they have a vague relationship to membranous structures. The image on the screen quite suddenly loses contrast and when the beam is turned down an image such as that illustrated appears. It is probable that this is due to melting and subsequent resolidification of the lead stain.

Dirt occurs on sections in a variety of shapes, densities and distributions (Figure 16). In general because of their inconstant shape, variable electron density, non-topographical distribution and tendency to occur on sections which are recognisably 'dirty', the distinction of dirt particles from silver deposits poses few problems. Scrupulous care was taken at all times to avoid extraneous contamination of specimens (Pease, 1960) and the occasional grid which was obviously dirty, was discarded.*

There remains the problem of the solitary, small, uniformly dense particle of dirt which is indistinguishable from silver. Such a problem is illustrated in figure 17. In the light of experience, in that it has been observed that silver is not deposited in this part of

Figure 16. Dirt. Multiple, very large, irregular, electron-opaque materials are deposited on the surface of this section. X 21,000

Figure 17. Solitary, small, electron-opaque body in the inner half of the capsular basement membrane. Silver is not usually deposited in this site. The electron-opaque body is presumed to be extraneous material. X 55,000

Figure 16
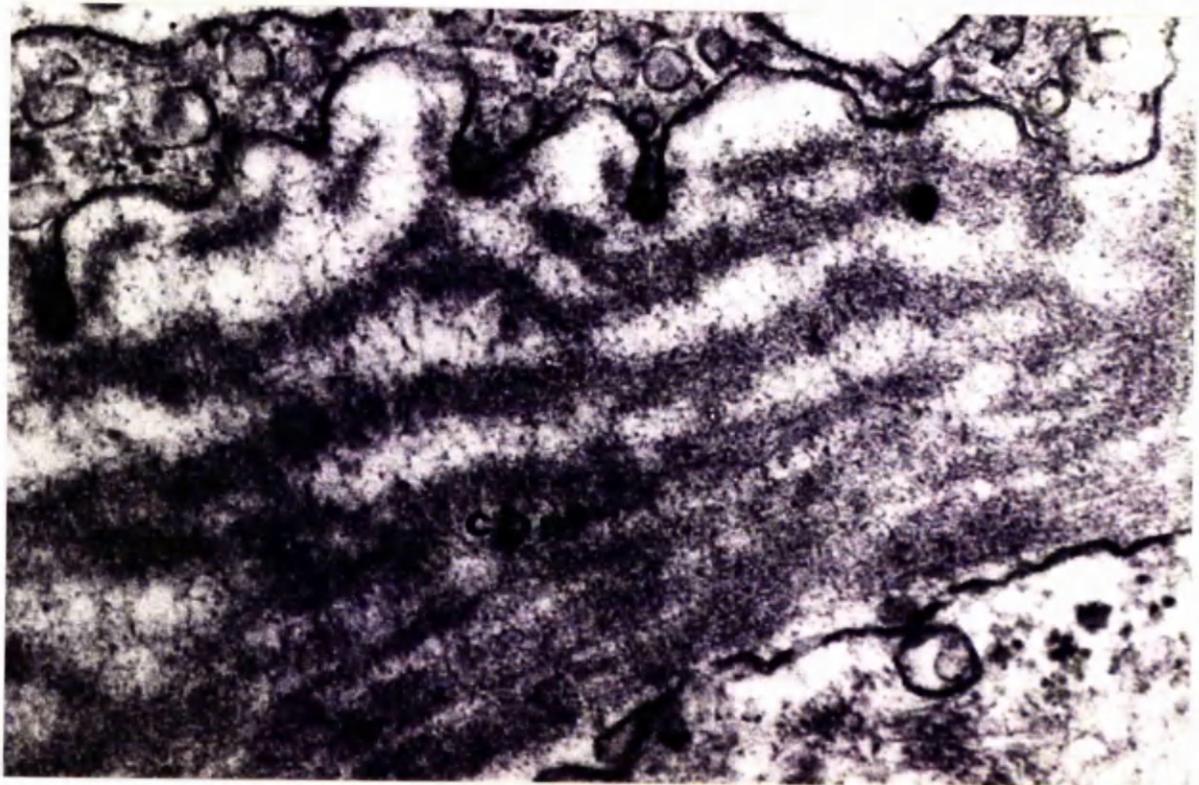
Figure 17

the capsular basement membrane, this particle is almost certainly
dirt.   On this particular occasion the problem is recognisable.
If such a particle occurred in a region where silver was deposited
the problem would not be recognised but it would not affect the
interpretation of the photomicrograph.   The problem in this case
is of more theoretical than practical importance.   In regions where
silver is deposited as scanty, solitary granules such as occurs in
visceral epithelial cell coat, the problem is real.   In such instances
no single particle can be interpreted with reliability, it is necessary
to take into account the pattern of distribution of several granules:
it is very unlikely that several isolated dirt particles would all have
the same topographical disposition.

B.   Natural densities.

Mitochondrial granules are fairly constant in size, shape, density

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*   It is only when specifically looking for electron dense particles
that the true amount of extraneous material on a specimen becomes
apparent.   Minor degrees are frequently overlooked even in pub-
lished photographs which are presumably carefully selected.   Out
of curiosity 50 photomicrographs from 5 different sources all published
in a single monograph (Dalton and Haguenau, 1967) were examined
carefully and the number of dirt particles visible in each was noted.
The total was 76 and the range was from 0 to 7.   In none however
did the dirt particles interfere with the information the illustration
was intended to convey.   The illustrations of the case of human
localized argyria reported by Buckley, Oster and Fassett (1965) are
particularly heavily covered with dirt particles: the illustrations
of Kurtz and Feldman (1962) are particularly free of dirt.

and above all, position. They are not a feature of glomerular cells but are particularly prominent in the tubular cells (Figure 18). It has been shown that divalent cations accumulate in these granules (Peachey, 1964) but silver was not used in this particular investigation. These granules have in certain previous investigations (Dempsey and Wislocki, 1955c; van Breemen, Reger and Cooper, 1956) been said to represent silver granules. At the time of these particular observations mitochondrial granules had not been clearly identified in normal cells, nor were residual bodies always clearly distinguished from mitochondria. The presence of silver in mitochondrial granules is therefore not proven. Certainly treatment with sodium cyanide does not noticably reduce the electron density of mitochondrial granules.

Ferritin is a common, widely distributed electron-dense particle but it appears to be infrequently present in the normal rat glomerulus though readily found in the spleen (Figure 19). It is well characterised electron-microscopically (Farrant, 1954; Richter, 1957, 1960; Richter and Bessis, 1965) both as individual molecules and as various aggregates. The ferritin molecule, with a visible diameter of 55 Å, is smaller than most silver particles and the degree of uniformity of size of ferritin molecules is usually a striking feature. Moreover in

Figure 18. Tubular cell cell showing mitochondrial granules. For comparison silver is present in the basement membrane in the lower left corner.
X 21,000
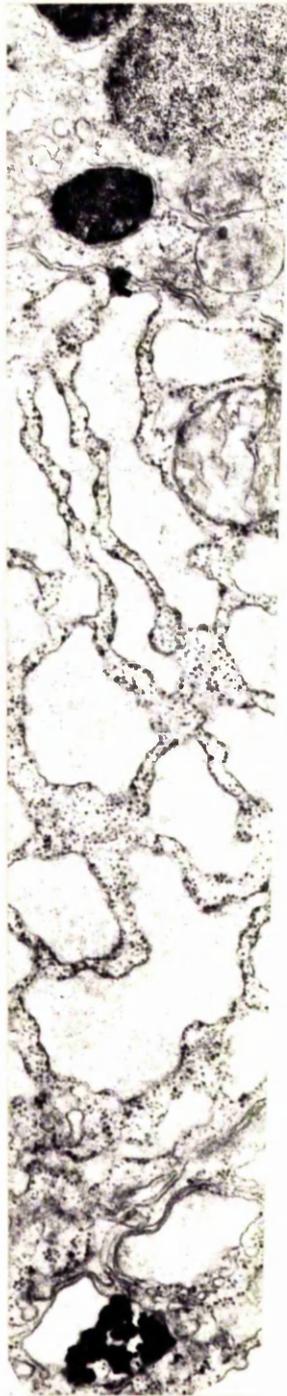
Figure 19. Spleen showing ferritin, at top, for comparison with silver, at foot. The ferritin particles are smaller, more uniform and slightly less electron dense than the silver. X 28,000

particularly well exposed and printed micrographs ferritin is not quite as dense as silver and at high resolution its four sub-units can be discerned.

Organelles of the lysosome, dense body and residual body type occur in a variety of sizes and shapes with a variable, usually inhomogeneous electron density. Their density is usually, but not invariably, less than that of silver. Silver particles are, in circumstances to be described later, incorporated into these organelles but are distinguishable by their large size, homogeneous electron density and tendency to be about the same size within any single organelle. In the few instances where such a distinction could not be made readily another grid from the same block was treated with 5 per cent sodium cyanide solution. Silver and non-silver containing bodies could be readily distinguished using this technique (Figure 20).

Lysosomes and related bodies were not definitively identified at the time many of the early electron microscopic studies on experimental argyria were performed. In consequence in the studies from the middle fifties there are minor degrees of confusion between these organelles and mitochondria. The presence of non-silver dense particles in these organelles was also poorly recognised at that time.

Bizarre, isolated, electron dense bodies were observed on two

Figure 20.   Cyanide treated section of visceral epithelial cell and glomerular basement membrane from argyric rat. The silver granules in the basement membrane have dissolved. The electron-opaque material in the membrane-bound body in the epithelial cell is unaffected by the cyanide.   X 23,000

Figure 21.   Bizarre, electron-opaque body found in an argyric rat.   X 50,000
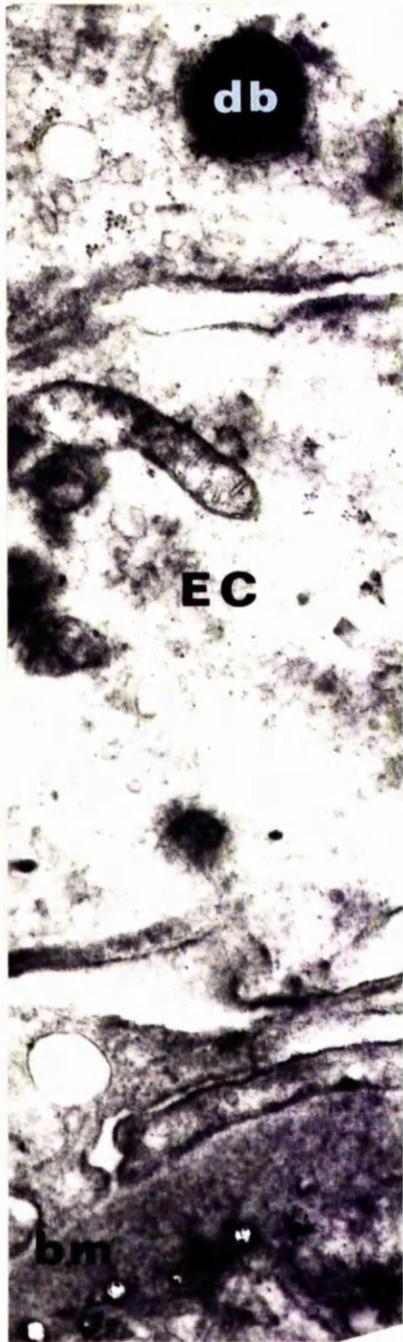
Figure 20

Figure 21
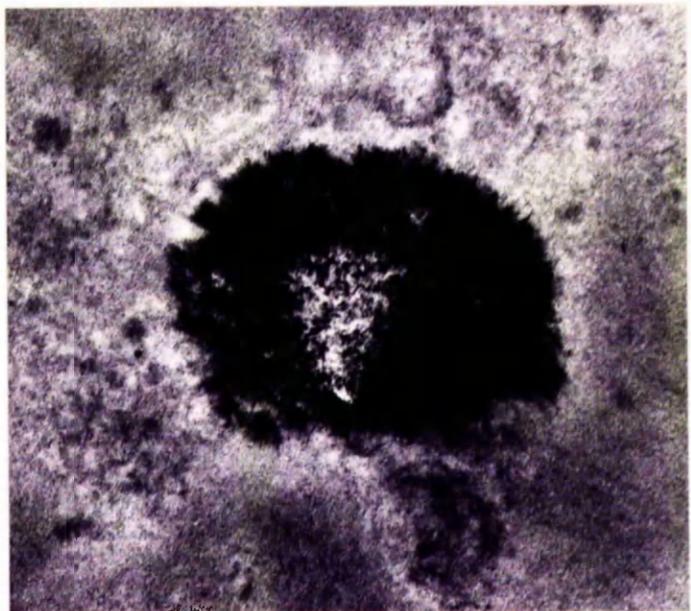
occasions in the course of this entire study on argyric animals
(Figure 21). They appeared to be non-artefactual and were
regarded as unexplained natural oddities perhaps due to some
crystalline deposition. However a not dissimilar body is illus-
trated in the single human argyric glomerulus so far examined
in the electron microscope (Prose, 1963) and appears to have been
accepted without question by that author as a silver deposit.
Further sections from the same block containing these bizarre
bodies were therefore examined but no other such bodies were
found. Attempts to treat the original section with sodium cyanide
were unsuccessful as the section was on an uncoated copper grid
which was severely etched and contaminated by the cyanide so that
the two original bodies could not be located. If these particular
deposits are silver, they are an excessively rare form.

(f) Artefacts attributable to silver deposition

Most electron microscopic techniques have their particular arte-
facts and experimental argyria is no exception. Two were recog-
nised which are directly attributable to deposited silver.

The first is observed in sections in which there are large, or
to a lesser extent very numerous, silver particles. Minute, ir-
regular fragments of silver appear to stream from the large particles

in the direction in which the section was cut by the knife (Figure 22).
This is commonly observed in heavily argyric animals but is un-
common in rats which have only ingested silver for up to 10 weeks.
The artefact is readily recognised because the daughter fragments
are smaller and more irregular in profile than the parent particles
and are situated only on one side of the parent. It is important to
take this into consideration when describing the location of silver in
certain specimens. This artefact appears in previous illustrations
(Dempsey and Wislocki, 1955c; Olcott and Richter, 1958) without
comment and therefore probably unrecognised.

The second, and rare, artefact is observed in areas with numer-
ous silver particles. If these areas are subjected to a particularly
intense electron beam the silver particles disappear from the section
within a period of about 1 to 2 minutes (Figure 23). Whether the
particles dropped out of the section or evaporated, could not be
decided. Sawkill (1955) records that he managed to evaporate a
solid silver crystal in a Vickers EM3 at 75 kV with the beam ad-
justed to maximum intensity. This artefact can be easily avoided.

Informational Studies

(a) Distribution of silver in the argyric rat

With a view to the potential application of experimental argyria

Figure 22. Streaming or 'screeving' artefact. The score lines indicate the direction in which the section was cut (large arrow). Minute, irregular, silver particles are present on the lee side of the main silver deposits (small arrows). The specimen is of a heavily argyric, partially cleared, glomerular capillary. X 8,000
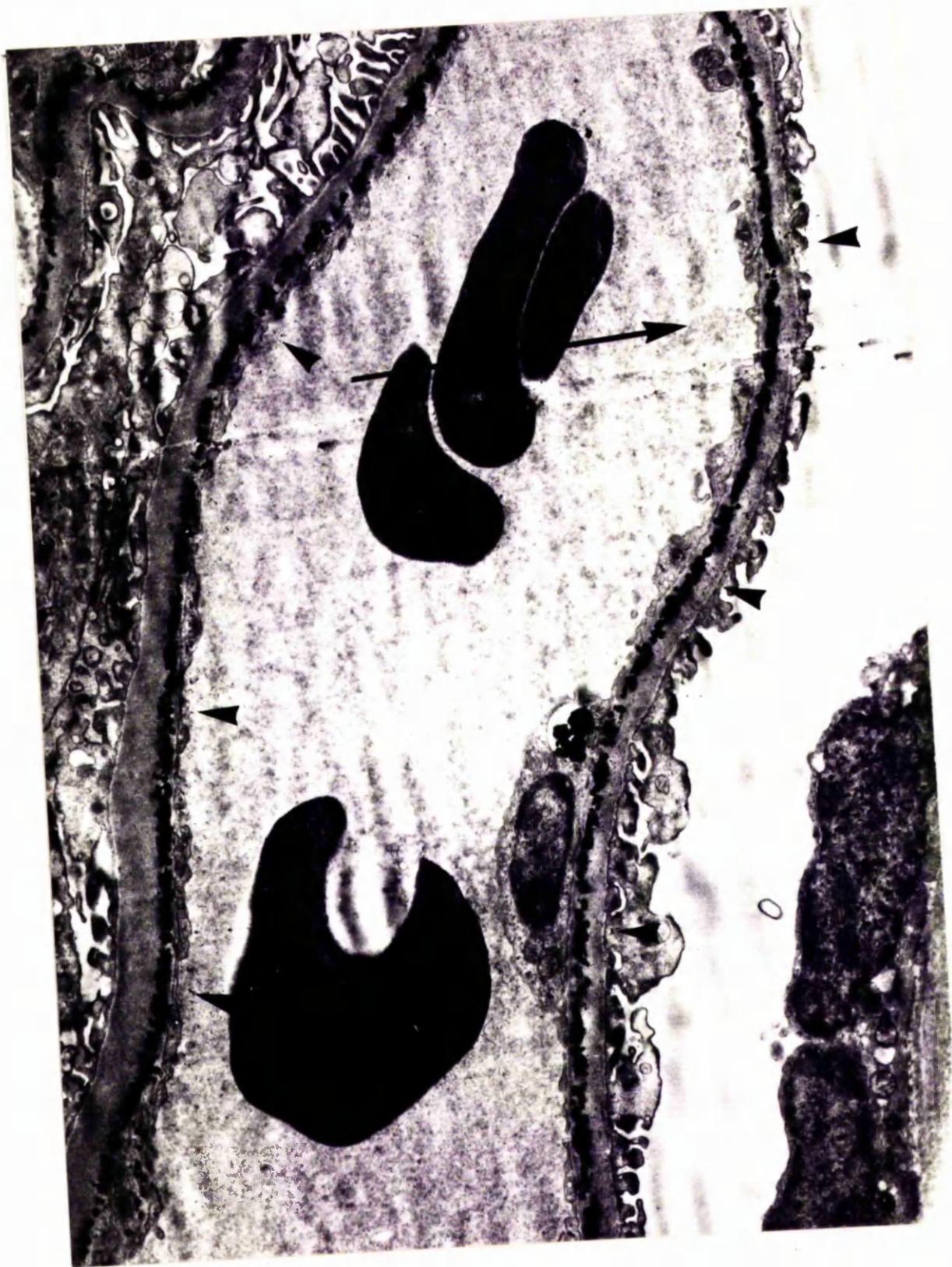
Figure 22

Figure 23. Beam artefact. Silver deposits exposed to a particularly intense electron beam. The time interval between (a) and (c) is approximately 2 minutes. X 46,000

Figure 23
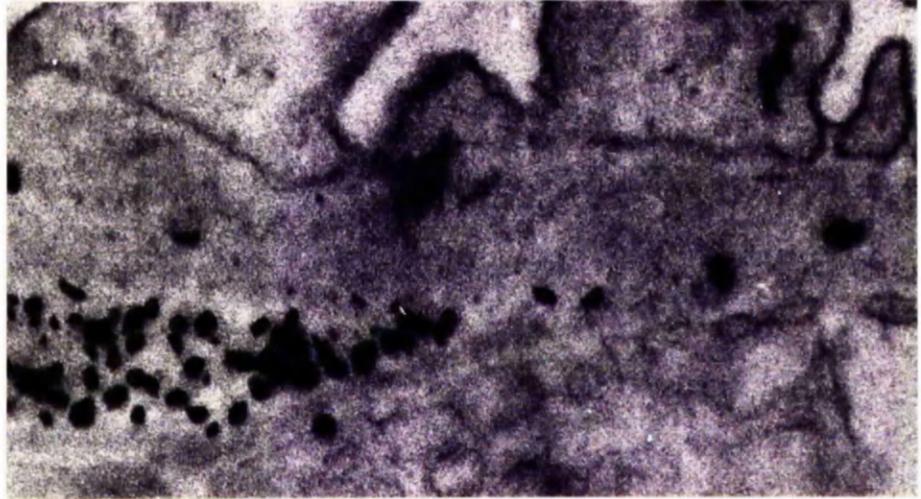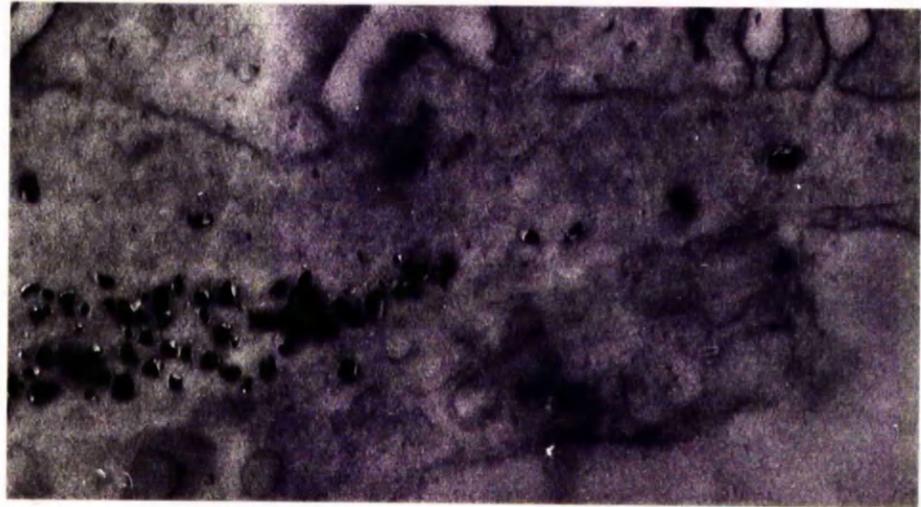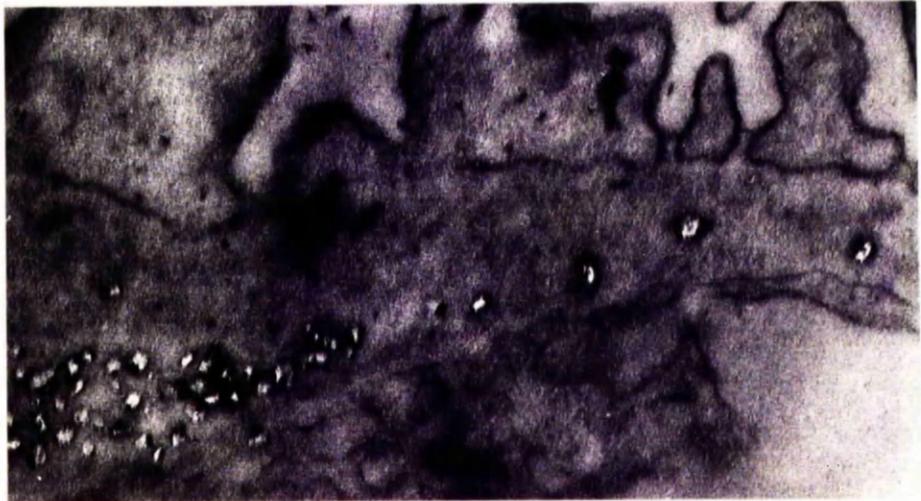
in sites other than the glomerulus, and to facilitate interpretation of glomerular observations by reference to more general situations, it was deemed advisable to determine the overall distribution of silver in the argyric rat. There are several published descriptions of the silver distribution in argyric animals but most are based on transmitted light rather than dark field observations which, as indicated previously, are more sensitive in detecting small amounts of silver. Moreover these observations were made with varying doses of silver administered for varying periods in a variety of species.

This survey was carried out using bright and dark field optical microscopy supplemented where necessary with electron microscopy.

Materials and methods

A series of male, Sprague-Dawley rats aged 6 weeks at the commencement of the experiment were given standard silver nitrate drinking fluid. Animals were killed after 0, 2, 4, 6, 8, 10, 12, 25 and 60 weeks' continuous exposure to the silver nitrate. These animals were all clinically healthy. A further animal showing signs of toxicity after 81 weeks on silver nitrate, as indicated previously, was also examined. A comprehensive, but not exhaustive, series of blocks was taken from head to tail of each animal as follows: (1) Cerebrum, (2) Eye, (3) Lachrymal gland, (4) Tongue, (5) Salivary gland,

(6) Trachea, oesophagus and thyroid, (7) Heart and lung, (8) Ventral skin, (9) Xiphoid process, (10) Rectus abdominis muscle, (11) Liver, (12) Pancreas, (13) Spleen, (14) Mesenteric lymph node, (15) Stomach, (16) Ileum, (17) Colon, (18) Kidney, (19) Bladder and prostate, (20) Testis and epididymis, (21) Tail.   The blocks were fixed in neutral, phosphate buffered formalin, embedded in paraffin, sectioned at a nominal 6μ, stained with haematoxylin and eosin and with eosin alone, and examined by both bright field and dark field microscopy. A few selected tissues were fixed in buffered osmium tetroxide (Palade, 1952), embedded in 'Epon', sectioned at a nominal 600-800 $\overset{o}{A}$, stained with uranyl acetate and lead citrate and examined by electron microscopy.

Results

The keratin layers of the tongue, oesophagus (Figure 24) and squamous portion of the stomach contained very heavy silver deposits. No silver was observed in the underlying epithelium.

Elsewhere in the body, with a few notable exceptions, silver was deposited in homologous locations in all the tissues examined namely extracellularly in basement membranes both epithelial and vascular, and intracellularly in fixed and wandering phagocytes of the reticulo-endothelial system.

Figure 24. Oesophagus of a rat which had ingested silver nitrate. The superficial portions of the keratin layer are impregnated with silver deposits. Bright field, H & E, X 140. Inset is the corresponding dark field appearance.

Figure 25. Choroid plexus of argyric rat. Dense silver deposits are present in the basement membrane. Dark field, E, X 170
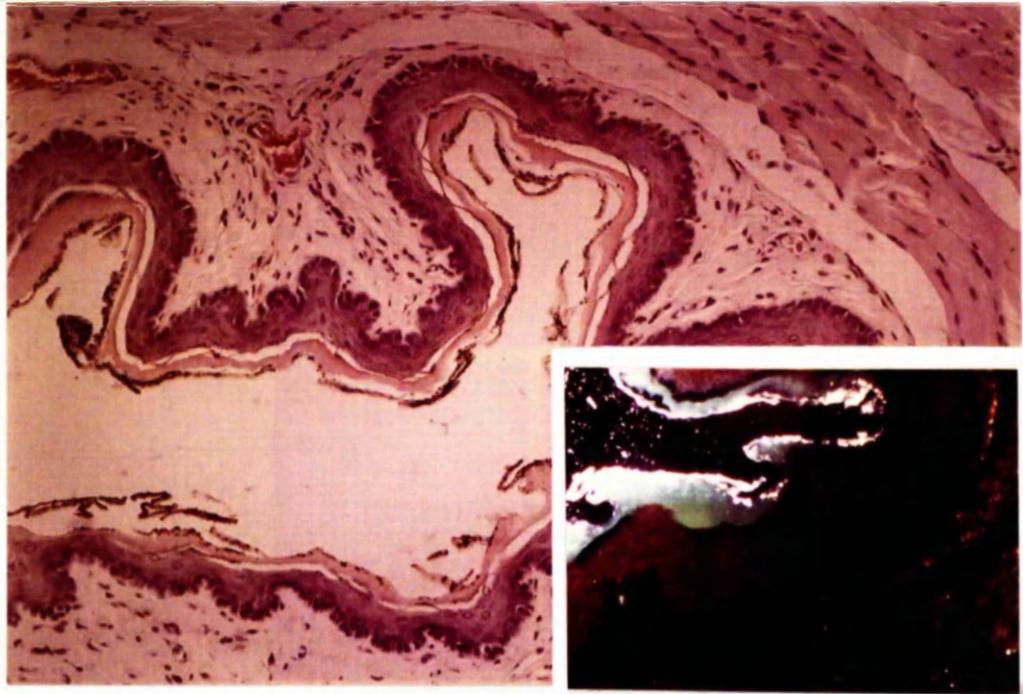
Figure 24
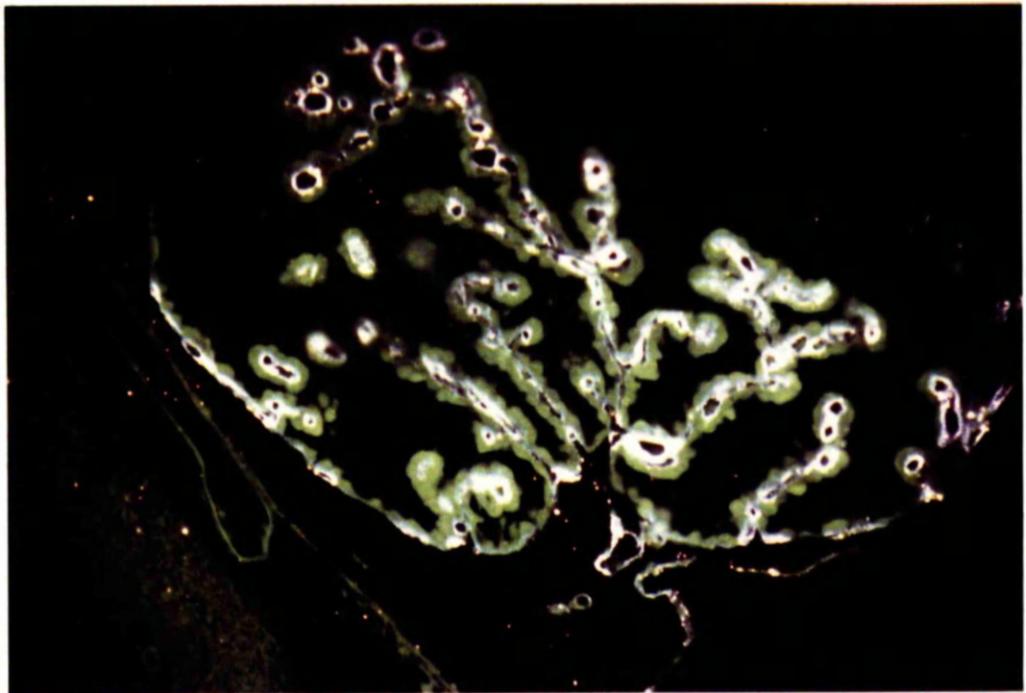
Figure 25

These features are well seen in choroid plexus (Figure 25), lachrymal gland (Figure 26), Bruch's membrane behind the retina (Figure 27), ciliary processes of the eye, salivary gland, thyroid (Figure 28), pancreas, glomerulus (Figure 8), prostate, bladder, epididymis and skin (Figure 29).

Silver deposits are notably absent from Bowman's and Descemet's membranes in the cornea, the lens capsule, tracheal and bronchial basement membranes and the basement membrane of seminiferous tubules. Silver is not detected in the capillaries or perimiseum of the rectus abdominis, or in Bowman's capsule (Figure 8), using dark field microscopy but is detected in these three sites by electron microscopy (Figure 30).

Throughout the gut silver is deposited in mucosal basement membranes especially at the apices of villi and the necks of glands. The macrophages in the lamina propria contain large deposits of silver. The histiocytes in the abdominal lymph nodes also contain large amounts of silver. The histiocytes in the red pulp of the spleen also contain considerable amounts of silver but this organ is principally distinguished by the deposition of silver outlining the sinusoids of the red pulp (Figure 31).

The pattern of silver deposition in the liver is complex (Figure 32).

Figure 26. Lachrymal gland of argyric rat. Dense silver deposits are present in the basement membranes. The intra-luminal, red deposits are small calculi which are commonly present in rat lachrymal glands. Dark field, E, X 140

Figure 27. Eye of argyric rat. The retina is on the left, the sclera is on the right. The brilliant intervening line is Bruch's membrane. Dark field, E, X 340
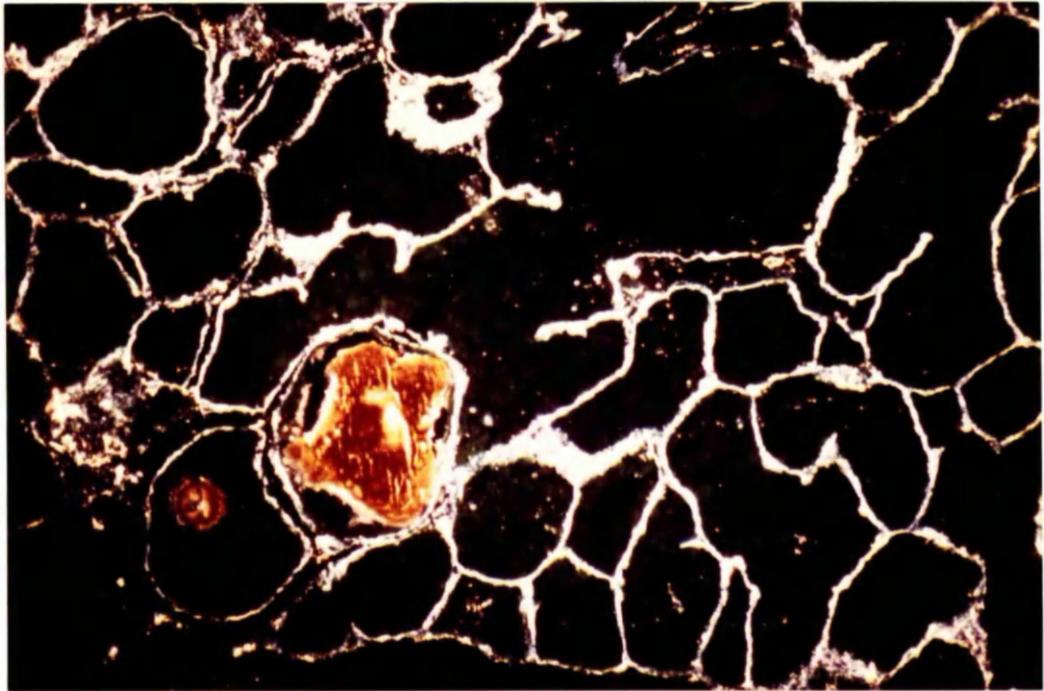
Figure 26
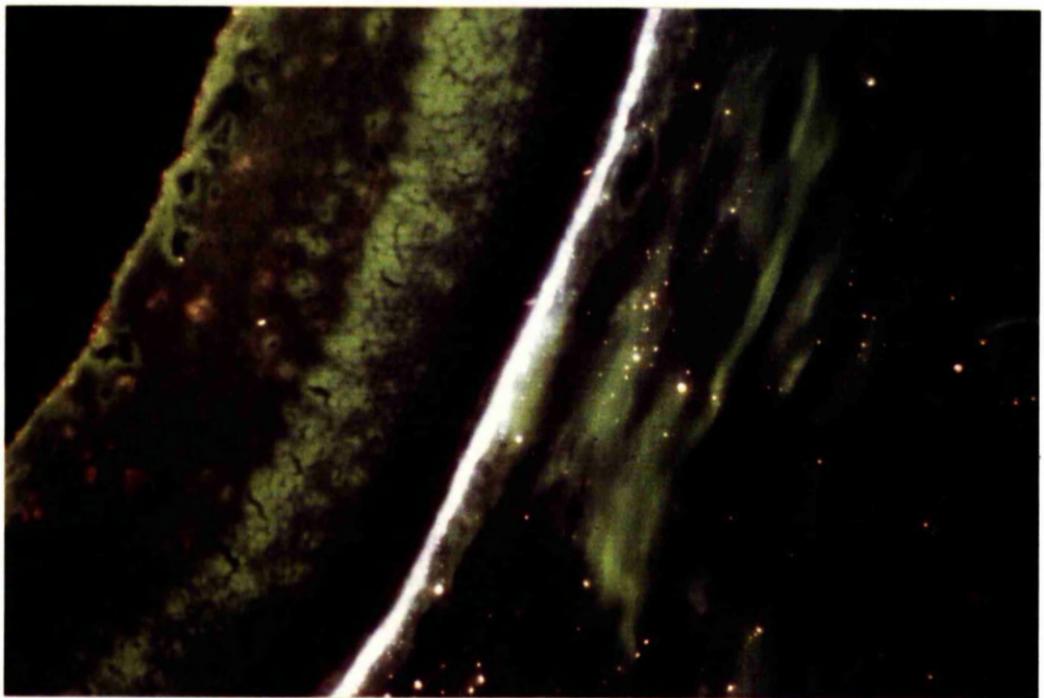
Figure 27

Figure 28. Thyroid gland of argyric rat. There is a dense silver deposit in the basement membrane surrounding each acinus. Dark field, E, X140

Figure 29. Skin of argyric rat. There is a dense silver deposit in the basement membrane beneath the surface epithelium and around the appendages. The surface basement membrane on the right side of the photograph appears more prominent and thicker because in this region the plane of the section is slightly tangential. Dark field, E, X340
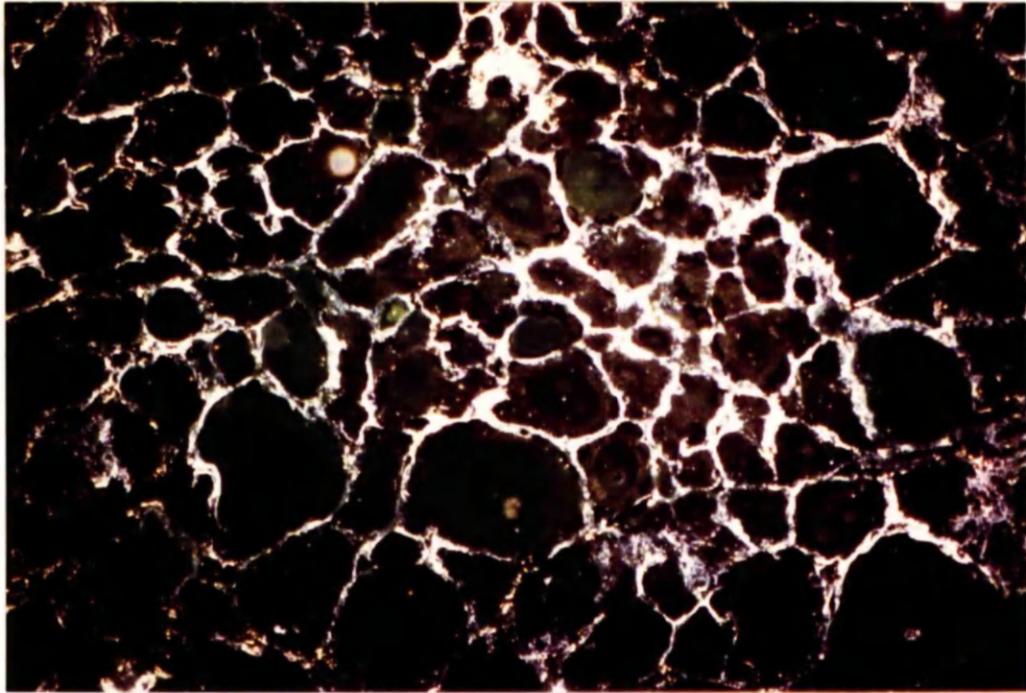
Figure 28
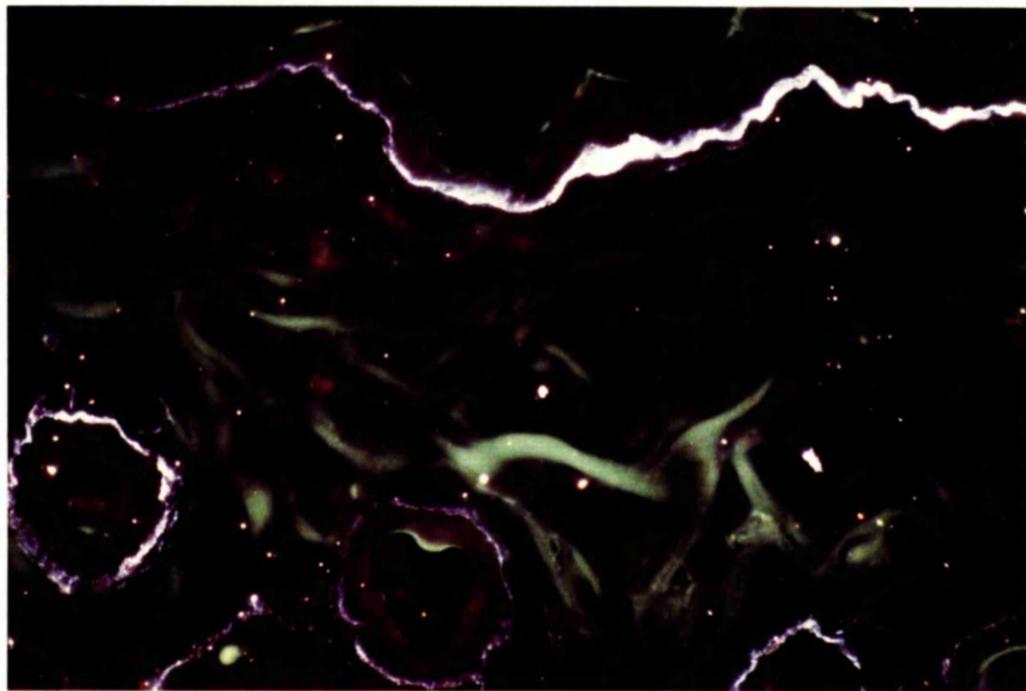
Figure 29

Figure 30. Capillary from rectus abdominis muscle. A very light deposit of silver is present in the capillary basement membrane. X18,000

Figure 31. Spleen of argyric rat. There are dense silver deposits in a reticulin-like pattern outlining sinusoids in the red pulp. Apart from silver within phagocytes the white pulp is free of silver. Dark field, E, X140

Figure 32. Liver of argyric rat. There are dense silver deposits in the portal tract in the centre of the photomicrograph. Lesser amounts of silver are present in the hepatic venules at the periphery of the illustration. The Kupffer cells are heavily laden with silver. Dark field, E, X140
Compare with figure 33.
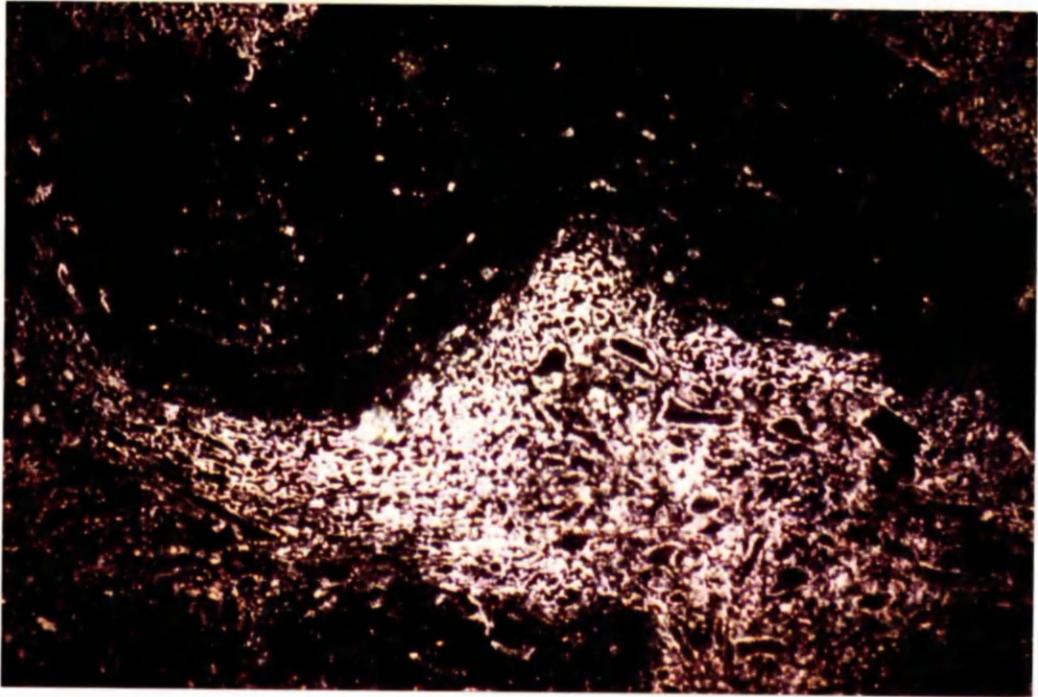
Figure 31
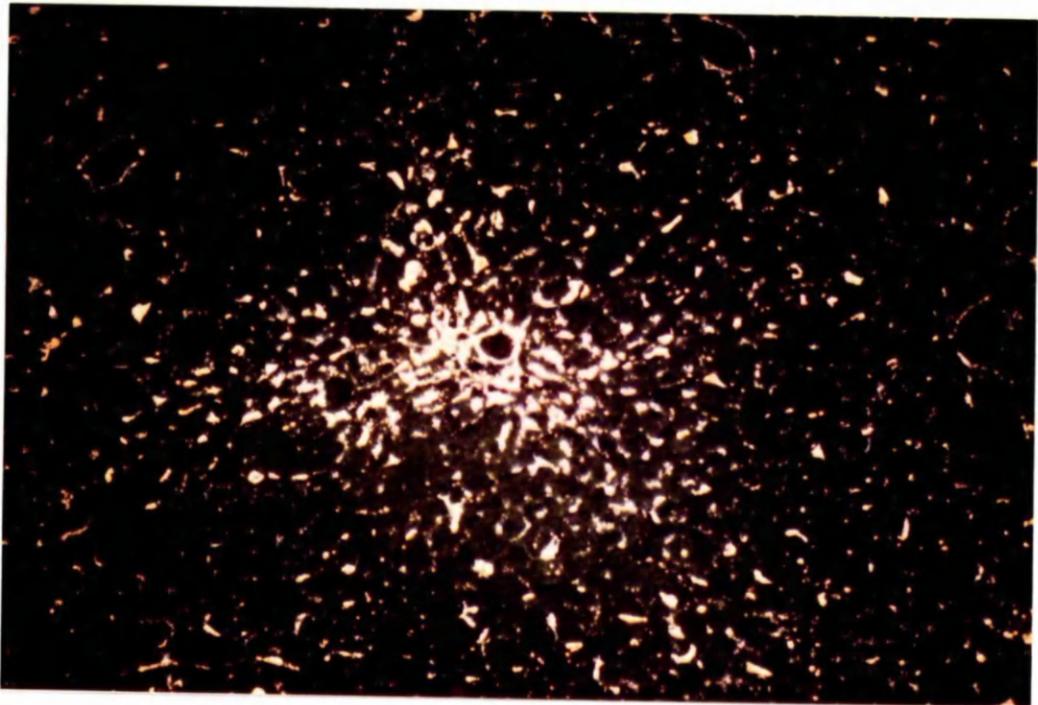
Figure 32

In the portal tracts the entire wall of the portal veins contains silver: the hepatic artery contains some silver in a sub-endothelial position and the bile ducts are outlined with silver. Deposition of silver in the hepatic venules is less intense than that in the portal venules. Macrophages in the portal tracts and Kupffer cells in the sinusoids are heavily laden with silver. Much smaller amounts of silver are present in some hepatic cells. Fine silver lines joining Kupffer cells are also present (Figure 33). These could not be positively located by dark field microscopy but electron microscopy shows the silver to be in the space of Disse (Figure 34).

Silver is present in systemic blood vessels mainly sub-endothelially, related to elastic laminae and around muscle cells (Figure 35). Electron microscopy shows that the silver is not deposited in the elastic proper but in the peripheral more electron dense regions of basement membrane-like material which surrounds not only the elastic lamina but also the muscle cells (Figure 36).

Within any single organ or tissue the deposition of silver is remarkably uniform. Between different organs or tissues there is some variation in silver deposition: choroid plexus, liver and glomerulus contain relatively dense silver deposits: skin, bladder

Figure 33. Liver of argyric rat, same specimen as figure 32. The Kupffer cells are laden with silver and these cells are joined by fine silver deposits whose precise histological location is not apparent at this magnification. Dark field, E, X 435

Figure 34. Electronmicrograph of liver shown in figure 33 demonstrating a light silver deposit in the space of Disse. This accounts for the fine silver lines seen in the dark field illustration. X 24,000
S = sinusoid; SD = space of Disse; H = hepatocyte.
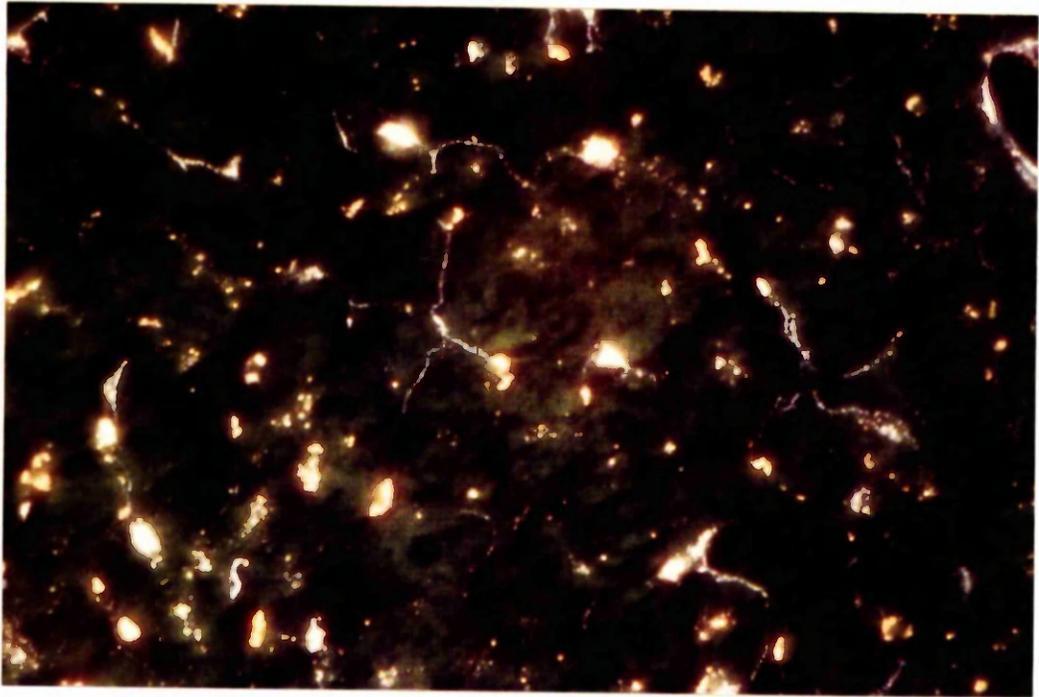
Figure 33
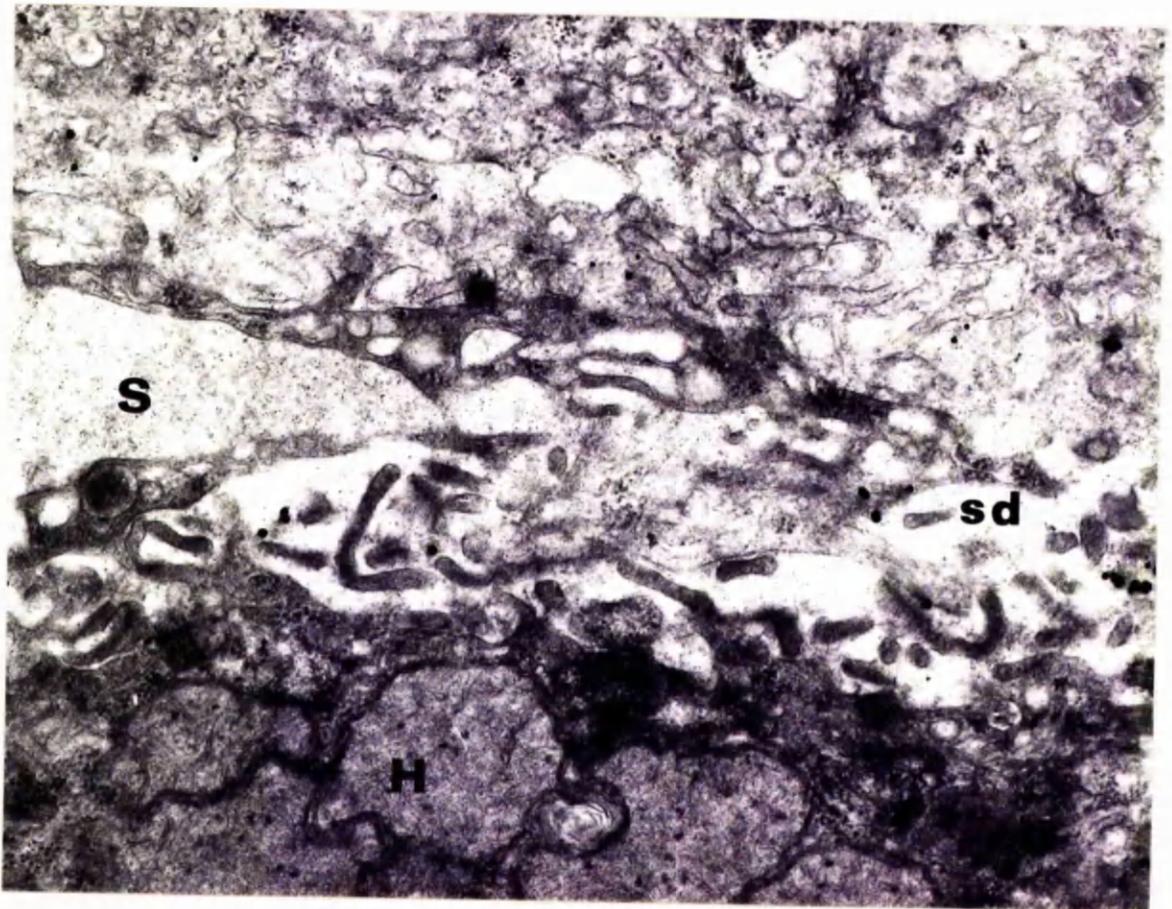
Figure 34

Figure 35. Small artery of argyric rat. Silver is deposited in the vicinity of the elastic lamina and around individual muscle cells. Silver aggregates are present in phagocytes in the surrounding connective tissue. Dark field, E, X 435

Figure 36. Electronmicrograph of small artery of argyric rat. Silver is deposited in the basement membrane-like material surrounding the elastic lamina (EL) and in similar material between adjacent muscle cells (M). X 25,000

Figures 35 and 36

and prostate contain relatively light silver deposits: muscle capillaries, perimiseum and Bowman's capsule contain such slight deposits that the silver can only be detected with the electron microscope.

Discussion

It has been recognised for over 70 years that exogenously administered silver becomes deposited in mammalian tissues in two main locations, phagocytic cells and connective tissue. There is good general agreement about the former location but concerning the latter location there have been divergent opinions as to which connective tissue components are the particular sites of silver deposition.

Elastic tissue was particularly favoured for many years (Dohi, 1908; Boersma and Baker, 1948) and was largely supported by histological studies of skin biopsies from cases of human argyria. Numerically these studies weigh heavily but the evidence presented is not very substantial for elastic tissue was not clearly distinguished from basement membrane either by name or description by many of the investigators. Furthermore many of the histochemical techniques used to demonstrate elastic tissue are now known to be of dubious specificity. The situation in skin biopsies was further

complicated by the locus, for silver has a predilection for regions
of senile elastosis (Schröpl, Oehlschlaegel and Drabner, 1968) and
there is little evidence that this condition, in spite of its name,
involves elastic tissue proper.

While the observations on this point in the present study are
limited, it is evident that silver is deposited not in, but around,
elastic tissue, an observation also made by Striker and Smuckler
(1970). The silver granules are clearly in a layer of what elec-
tron microscopically is basement membrane-like material. Such
a layer has previously been demonstrated histochemically (McManus,
1948a) and electron microscopically (Gottlob and Hoff, 1967) though
neither have described it as basement membrane-like, preferring
respectively to call it glycoprotein or ground substance. Though
silver is deposited in the immediate vicinity of elastic tissue it occurs
in many other locations and a unifying hypothesis for extra-cellular
deposition based solely on an elastic, or even a peri-elastic, distri-
bution, is no longer tenable.

Silver is widely distributed in blood vessels, an observation
emphasized by Gaul and Staud (1934, 1935), and confirmed in the
present study. These authors regarded this as the classical picture
of metallic retention and proposed that silver was deposited in a

direct complementary fashion to the capillary system.   This,
like the elastic tissue hypothesis, is partly correct but takes
little cognisance of the wide extra-vascular distribution of silver.

On the basis of the present observations one unifying feature
of all the extra-cellular deposits of silver is that they occur in
the homogeneous ground substances, or, more specifically, in
basement membranes and basement membrane-like material.
It is tempting to make this correlated evidence into the germ of
a unifying hypothesis.   This would be an improvement on the
two previous hypotheses in that it conveniently embraces both.
However 'unifying hypothesis' is slightly more emphatic than the
evidence truly permits and 'reasonable working hypothesis' is
more correct.

The first main stumbling block to a more definitive conclusion
is the absence of silver deposits in certain basement membranes.
This may merely reflect the relative insensitivity of dark field
techniques and could be solved by the more general application of
electron microscopy.   Alternatively it may reflect the failure
of silver to gain access to these sites – this almost certainly
accounts for the lack of silver in the cornea which becomes pig-
mented when silver is applied topically (Gutman and Crosswell,

1968) but not when silver is given systemically (Olcott, 1947; Wislocki and Ladman, 1955). Lastly the absence of silver in certain sites may reflect chemical differences in the membranes. All of these uncertainties could be resolved with presently available techniques but would be very costly in terms of time. Eventually such a general solution will be required but it is not essential to the main point of this thesis, glomerular basement membrane.

The second main stumbling block is the absence of data on the relationship between true basement membrane and basement membrane-like material, and between basement membrane and cell coat. This problem is considered later in this part of the thesis.

The various physiological and chemical mechanisms whereby silver is distributed and deposited in generalised argyria are not well established but on the basis of the meagre published evidence the sequence of events is probably as follows.

Only a very small amount, less than 0·1 per cent, of the total dose of silver nitrate ingested is absorbed from the gastro-intestinal tract (Scott and Hamilton, 1948). The chemical changes which the silver undergoes prior to absorption are not clear but the initial form of administration is of little importance as regards the final

compounds which are carried in the blood (Gager and Ellison, 1935). From the gastro-intestinal tract the silver enters the portal circulation to the liver where some is deposited and some is secreted in the bile (Scott and Hamilton, 1950) and some passes into the systemic circulation by which route generalised argyria occurs (Hill and Pillsbury, 1939). It is probable that the systemic concentration of silver may eventually build up to as high as 100µg per 100 ml of blood (Blumberg and Carey, 1934). The silver is carried in the blood mainly in association with the plasma proteins and the evidence indicates that it is the globulins which are principally involved (Scott and Hamilton, 1950; Polachek, Cope, Williard and Enns, 1960). This silver/protein reaction is an equilibrium and small amounts of ionic silver, almost certainly as the chloride, are available. These small molecules diffuse into the tissues, are reduced and become deposited.

The form in which the silver is deposited is not well established: metallic silver, silver oxide and many silver salts have been suggested but the only form which has much supporting experimental evidence at all is silver sulphide (van Breemen, Reger and Cooper, 1956; Buckley, Osler and Fassett, 1965). The optical and electron histochemistry described under methodological studies (d) and (e)

are consistent with the original deposit being, but do not prove that it is, silver sulphide. Because of this chemical uncertainty the terms 'silver granule', 'silver deposit' or 'silver particle' are used in this thesis in the sense that they indicate only the presence of silver which can be detected by microscopical techniques, but are non-committal as to the physical or chemical state of the silver (Dempsey and Wislocki, 1955d).

(b) Time of appearance of silver deposits in the argyric rat

It is well established that silver deposition in animal tissues is cumulative (Wislocki and Leduc, 1952) and that the amount of silver deposited varies from organ to organ (Gettler, Rhoads and Weiss, 1927; Bader, 1966; Constable, Morris and Burke, 1967). Both of these observations were confirmed in the previous experiment. What is not established is when silver first appears, or can be first detected, in different organs. This information is necessary if the experimental argyric technique is to be applied subsequently to basement membranes other than glomerular, and also to interpret certain human data for which information concerning the period of exposure to silver is not available.

Materials and methods

The specimens used in the previous experiment were examined

by dark field microscopy to determine in which particular animal, and therefore at which particular stage of silver ingestion, silver deposits were first detectable in a given site.

Results

The results are tabulated below, + indicates silver deposits present, - indicates silver deposits absent, b.m. indicates basement membrane.

Weeks on Ag NO3	0	2	4	6	8	10	12	25	60	81
Oesophageal keratin	-	+	+	+	+	+	+	+	+	+
Portal vein	-	-	+	+	+	+	+	+	+	+
Space of Disse	-	-	-	+	+	+	+	+	+	+
Glomerular b.m.	-	-	-	-	+	+	+	+	+	+
Choroid plexus b.m.	-	-	-	-	-	-	+	+	+	+
Thyroid acinar b.m.	-	-	-	-	-	-	+	+	+	+
Skin appendage b.m.	-	-	-	-	-	-	+	+	+	+
Skin surface b.m.	-	-	-	-	-	-	-	-	+	+
Urinary bladder b.m.	-	-	-	-	-	-	-	-	+	+
Prostate acinar b.m.	-	-	-	-	-	-	-	-	+	+
Seminiferous tubule b.m.	-	-	-	-	-	-	-	-	-	-

Discussion

From these results it is evident that in the argyric rat silver is not first detectable in all sites of deposition at the same time. The very early appearance of silver in the oesophageal keratin merely reflects the topical application of silver nitrate at this site. The early and intense deposits of silver in the portal vein reflect

one route of absorption of silver from the gut and it is probable that the silver content of portal blood is higher than that of systemic blood. The slightly later detection of silver in the space of Disse may be due to the extremely tenuous nature of this region as a result of which it is difficult to detect in lightly argyric animals.

In all other sites silver is presumably deposited after being transported via the systemic circulation and tissue fluid. It seems to be detectable earlier in sites where there is a high net transfer of fluid as indicated by the presence of fenestrated capillaries e.g. glomerulus, choroid plexus and thyroid. The converse holds in sites such as skin epidermal and urinary bladder basement membranes. This suggests the tentative, but not unreasonable, conclusion that silver deposition is proportional to the total cumulative exposure of a given structure to transported silver ions.

This phenomenon of sequential silver detection has not been recorded in previous studies of experimental argyria. This is undoubtedly partly because all previous multi-organ studies of experimental argyria have used long periods of exposure to silver, usually well over 25 weeks (Olcott, 1948; Gatz, 1949; Dempsey and Wislocki, 1955b, c) and partly because all previous shorter term studies have been on single organs (Kurtz and Feldman, 1962; Vernier, 1964;

Striker and Smuckler, 1970).

It is very likely that a similar sequential deposition of silver occurs in human argyria. This however has not been recognised for all the reported cases of generalised argyria (for reviews see Gettler, Rhoads and Weiss, 1927; Hill and Pillsbury, 1939) in which a suitably large number of organs and tissues were examined post mortem were initially recognised by skin changes. This, as indicated in the table, is a late site for silver deposition. No reports of cases of argyria prior to skin changes, and therefore recognised incidentally and retrospectively at post mortem examination could be traced. While such reports must exist it would appear that their retrieval is dependent on serendipity.

(c) <u>Clearance of silver deposits in the argyric rat</u>

Silver is a singularly effective intra-vital stain which becomes firmly deposited in the tissues. Because of the selective localization of the silver deposits experimental argyria is a particularly convenient and elegant model for demonstrating basement membranes. It is not, however, the mere demonstration of basement membranes that makes this model of informational value; rather it is because the silver is firmly bound to the membrane so that if the membrane moves the silver moves and this movement of silver can be detected.

Experimental argyria is therefore a convenient model for studying the origin, turnover and fate of certain basement membranes. This salient feature was first clearly appreciated by Kurtz and Feldman (1962) though the clearance of silver from argyric basement membranes had been previously observed in a different context (Enders, 1956; Enders and Moench, 1956).

The experiment described in part III of this thesis shows that silver is cleared slowly and progressively from glomerular basement membrane. As an ancillary investigation to determine if this behaviour was peculiar to glomerular basement membrane or not, several other tissues were screened using dark field microscopy.

Materials and methods

A group of standard argyric rats, given 2·5 g of silver nitrate per litre of drinking water for 10 weeks, were restored to ordinary tap drinking water and selected tissues were examined by dark field microscopy approximately 1, 6, 12 and 24 months later.

Results

The results are tabulated below, + indicates silver deposits present, − indicates silver deposits absent, b.m. indicates basement membrane.

Weeks off Ag NO3	0	4	26	51	100
Oesophageal keratin	+	−	−	−	−
Space of Disse	+	+	−	−	−
Choroid plexus b.m.	−	+	−	−	−
Glomerular b.m.	+	+	+	−	−
Thyroid acinar b.m.	−	?	−	−	−
Skin appendage b.m.	−	+	+	+	+
Skin surface b.m.	−	−	−	−	−
Portal vein	+	+	+	+	+

Though silver disappeared from the space of Disse, heavy deposits remained in the Kupffer cells. Though silver disappeared from the choroid plexus basement membrane, silver remained in the stroma. Though silver was no longer detected in the glomerular basement membrane, it was still present in cells though whether these were endothelial or mesangial or both could not be decided. The presence or absence of silver in thyroid acinar basement membrane was so borderline no firm decision could be made but silver was certainly present in the blood vessel walls and in macrophages between the acini. No change was noted in the skin surface or skin appendage basement membrane, or in the portal vein walls during the period of observation.

Discussion

An interesting point which emerged early in this study was that silver continued to be deposited for some time after silver ingestion

stopped. There is no detectable silver in choroid plexus, thyroid acinar or skin appendage basement membranes in the standard 10 week argyric rat but after a further month on ordinary drinking water silver is detectable in these locations. It has been shown that when doses of silver large enough to cause argyria are administered, the circulating level of silver remains high for some time after ingestion of silver ceases (Blumberg and Carey, 1934). This almost certainly explains the differences in the first two rats of this series, silver continued to be deposited for some time after cessation of silver administration.

The results clearly indicate that silver is progressively cleared from certain of the membranes studied and the phenomenon is not peculiar to glomerular basement membrane. However dark field microscopy, though it is an invaluable screening procedure enabling large numbers of membranes to be examined and the general distribution of silver to be established, is relatively insensitive compared with electron microscopy for experiments of this type.

It must be emphasized that the conclusions from this clearance study apply to the standard argyric rat. By comparison with most previous studies on argyric animals and by comparison with the intensity of silver deposition in many reported cases of human

argyria, this standard experimental animal is not particularly

argyric. Its tissues contain only enough silver to be conveniently

detected by microscopic techniques. It is quite possible that so

much silver could be administered to a rat that silver deposition

would be so intense as to make clearance undetectable or at least

markedly distorted.*

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* Human argyria, once established, is a lifelong state. Large
amounts of silver are permanently retained in the body. This is
undisputed and has been recognised for several centuries. The
very persistence of the condition has led to the assumption that once
deposited silver remains in that particular site. This assumption
has indeed been questioned (Buchanan, 1831) though the occasion
was used more to heap unjustified criticism on the then somewhat
primitive physiological concepts than on the assumption itself.
There is considerable indirect evidence to seriously question that
all silver deposits in human argyria are permanent. This derives
mainly from minor differences in the observations of different cases
of argyria by investigators who were undoubtedly competent with a
microscope (Frommann, 1859; Jahn, 1894; Gettler, Rhoads and
Weiss, 1927). There appears to be a trend for silver to appear
initially in membranes, later in cells and much later as aggregated
extra-cellular, extra-membranous deposits, though remaining
largely in the same organ or tissue.

This may be best illustrated by a specific example. In the late
19th and early 20th centuries a linear deposition of silver just deep to
the epidermis in cases of argyria was so well recognised as to be
given the eponym "Unna's line" (Unna, 1896). In a recent electron
microscopic study of the skin from a case of argyria it is clearly
stated that "The older concept of subepidermal accumulation of
silver deposit is challenged. It is settled that silver can be intra-
cellular ......" (Mehta, Dawson-Butterworth and Woodhouse, 1965).
Unna's line was typically described in what may be called 'active
argyria' in that the patients had only just stopped taking silver.
The case described by Mehta and his associates may be called

'burned out' argyria in that their patient had stopped taking silver 25 years prior to the skin biopsy being taken. To this time factor they apparently attach no significance and prefer to question the techniques or observations of previous investigators. Their observations are correct but they have challenged the wrong concept.

(d) Electron microscopic studies of silver deposition in the argyric rat glomerulus

There is unanimity in all the previous electron microscopic studies on experimental argyria regarding the morphological appearances of the silver granules in basement membrane. However no attempt has been made to describe the manner or to account for the mechanism whereby the silver granules are deposited. This information is essential in order to understand the experimental argyric model.

All previous observers agree that the silver granules vary in size in any one specimen but from the published illustrations it appears that the average size of the silver granules in the experiment of Oshima and his associates (1967) are smaller than those in the experiments of Kurtz and Feldman (1962) which in turn are smaller than those in the experiments of Dempsey and Wislocki (1955c). These particular studies were all of glomerular basement membrane in the rat, but the animals in the various experiments were exposed to silver nitrate for 4, 10 and 26 weeks respect-

ively.

A sequential study of silver deposition in the glomeruli of rats exposed to silver nitrate was therefore performed.

Materials and methods

Two groups of male, Sprague-Dawley rats, aged 6 weeks at the commencement of the experiment, were given silver nitrate in a concentration of 2·5 g per litre of distilled water to drink instead of ordinary tap water.   Animals were killed after 0, 2, 4, 6, 8 and 10 weeks in the first group and after 0, 2, 4, 6, 8, 10, 25, 60 and 81 weeks in the second group.   A third group of rats aged one year at the commencement of the experiment were similarly treated and killed after 0, 2, 4, 6, 8 and 10 weeks.

The animals were anaesthetised with chloroform, the abdomen was opened, the renal artery and vein were clamped simultaneously and the left kidney was removed.   One mm^3 blocks of the peripheral cortex were cut immediately and placed in cold s-collidine buffered osmium tetroxide (Bennett and Luft, 1959) in the case of the first group, and in cold veronal buffered osmium tetroxide (Palade, 1952) in the case of the second and third groups.   After fixation the blocks were dehydrated in alcohol and embedded in 'Epon'.   Six blocks were taken from each animal.   Thick, 0·5μ, sections

of each block were examined by phase contrast microscopy and two blocks containing appropriately situated glomeruli were selected. These blocks were trimmed, sectioned at a nominal 600 to 800 $\overset{o}{A}$, stained with uranyl acetate and lead citrate and examined in an electron microscope. Most of the grids were examined in an RCA-3F or a Zeiss EM 9A. A few grids were also examined in a Philips 75 or a Siemens Elmiskop 1.

Results

The glomeruli of rats killed after 0 and 2 weeks (Figure 37) on silver nitrate solution were of normal appearance and conformed to the description given in the introduction to this thesis. In particular no silver deposits were observed in these glomeruli.

Silver was observed first in glomeruli of rats exposed to silver nitrate for 4 weeks. In these animals a very few, small, isolated, randomly distributed silver granules were present in the glomerular basement membrane (Figure 38). Similar granules were present in the mesangial matrix and a very occasional granule was present in the urinary space.

In the 6 week specimen the number of granules had increased but their size had not appreciably altered, and they were reasonably uniformly distributed throughout the basement membrane

Figure 37.   Glomerular basement membrane from a rat which had ingested silver nitrate for 2 weeks.   No silver deposits are present.   X 65,000

Figure 38.   Glomerular basement membrane from a rat which had ingested silver nitrate for 4 weeks.   A few, small silver deposits are randomly distributed in the basement membrane. X 65,000

Figure 39.   Glomerular basement membrane from a rat which had ingested silver nitrate for 6 weeks.   There are more granules and some of them are slightly larger than those in the previous specimen.   X 65,000

Fig. 37
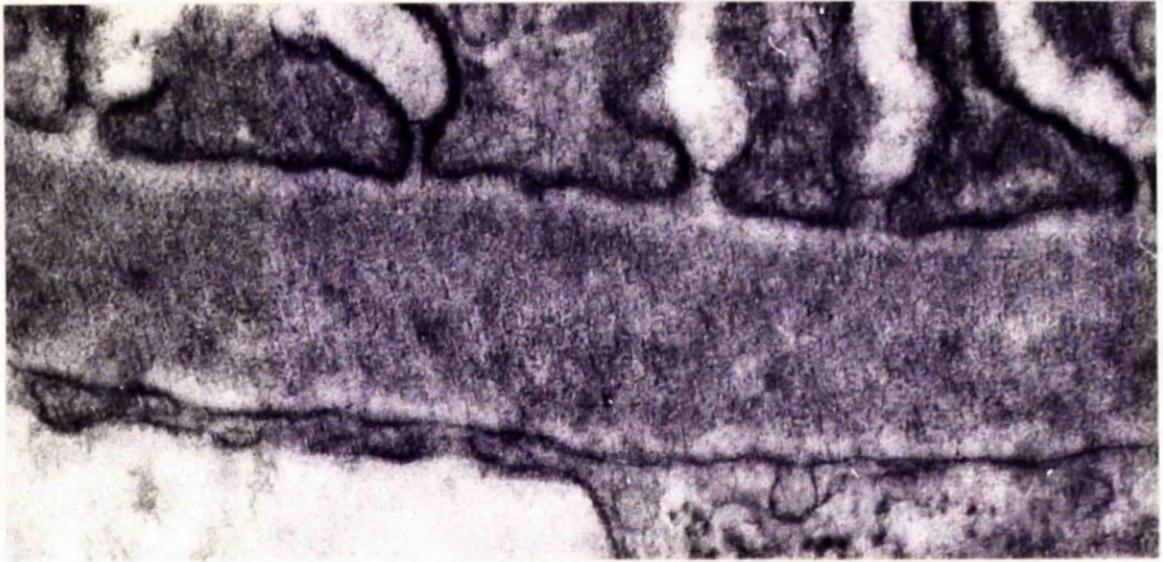
Fig. 38

Fig. 39
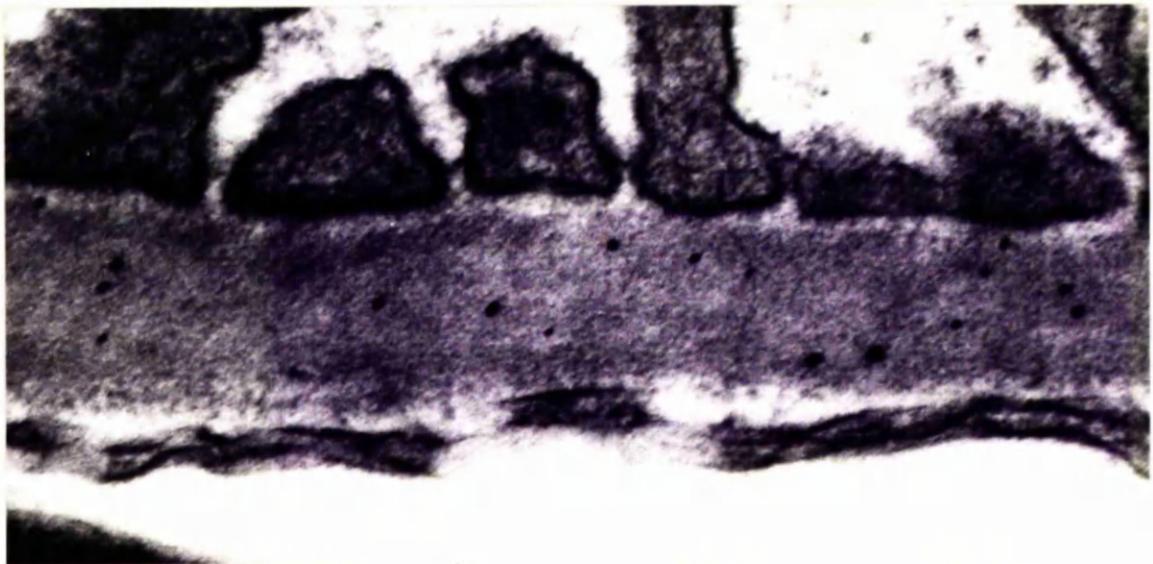
(Figure 39).   Granules were also present in mesangial matrix
and an occasional small group of granules was observed in mem-
brane bound bodies in mesangial cells.   A very few granules
were present in the urinary space.

In the 8 week specimen the number of granules had increased
slightly and there was a slight increase in size of some of the
granules (Figure 40).   Granules were also present in the sites
outwith the basement membrane noted in the previous specimen.

In two of the 10 week animals the number and distribution of
the silver granules was very like that in the previous specimen
but some of the granules were slightly larger (Figure 41).   Gran-
ules were present in the sites outwith the basement membrane noted
previously and some of the granules in the mesangial cells wore not
membrane bound.   The other 10 week animal was unusual and is
described later.

By 25 weeks the silver deposition in the basement membrane
is much more intense but the overall pattern resembles previous
specimens (Figure 42).   However the granules are larger in size
and some appear to have coalesced.   Extra-membranous silver
deposition is also more intense, particularly in mesangial cells.
In addition small amounts of silver are present in membrane-bound

Figure 40. Glomerular basement membrane from a rat which had ingested silver nitrate for 6 weeks. The granules of silver are slightly larger than the previous specimen. X 65,000

Figure 41. Glomerular basement membrane from a rat which had ingested silver nitrate for 10 weeks. Silver granules are randomly distributed throughout the entire thickness of the basement membrane. In one of the foot processes is a coated pit with a silver granule. X 65,000

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Figure 42. Glomerular basement membrane from a rat which had ingested silver nitrate for 25 weeks. The silver deposits are large and occupy a substantial portion of the basement membrane. X 65,000

Fig . 40
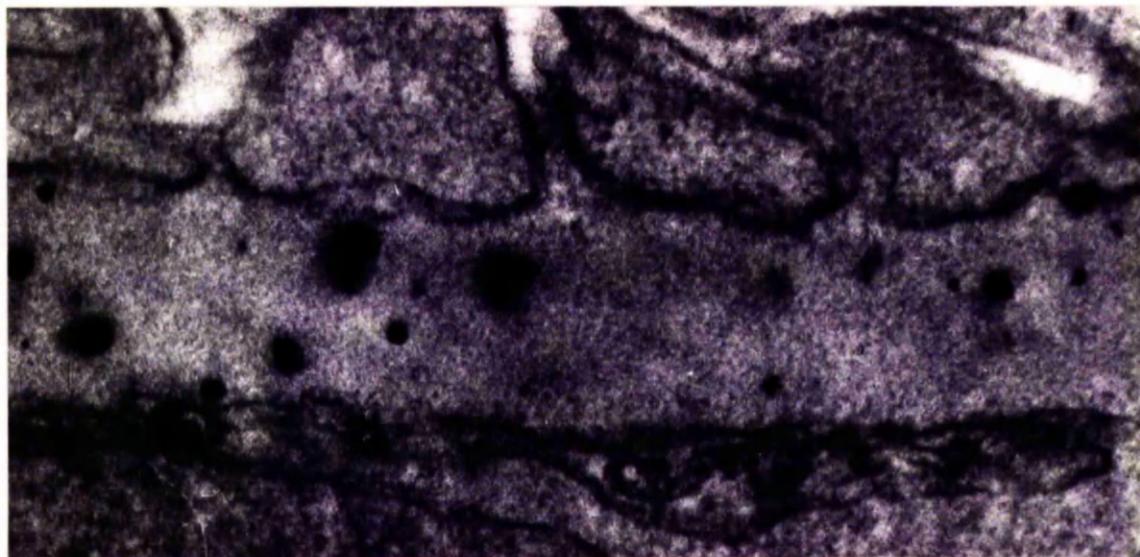
Fig . 41

Fig . 42
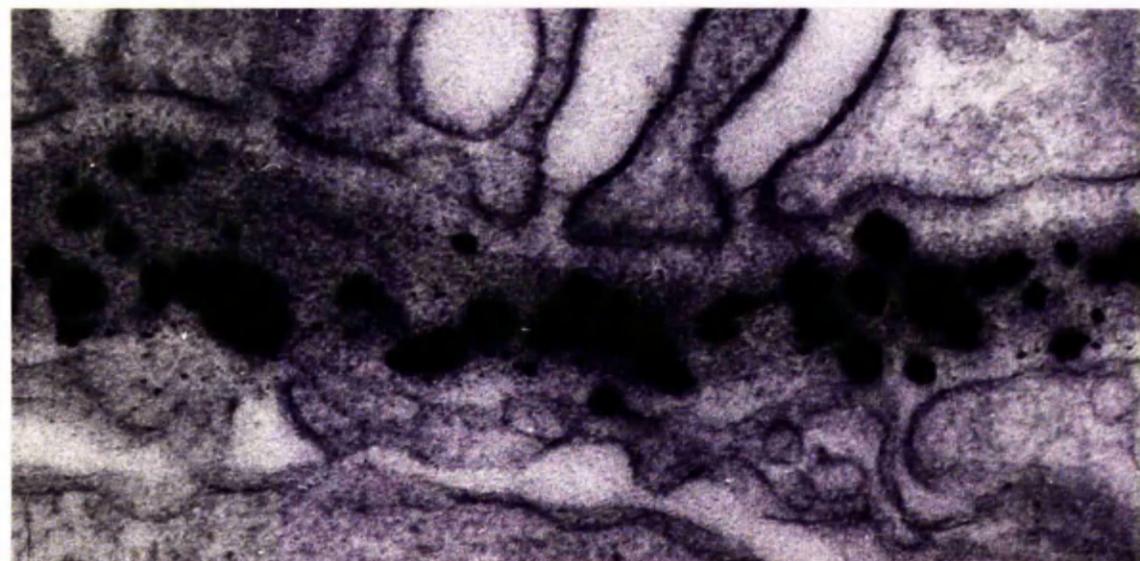
bodies in the perikaryon of endothelial cells but none is observed in the attenuated portion of the cytoplasm of these cells.

The typical appearances of the glomerular basement membrane after 60 weeks are illustrated in figure 43. Very large, aggregated, silver granules are concentrated along the inner aspect of the basement membrane, in places forming almost a solid barrier. Silver deposits are infrequent in the outer aspect of the basement membrane. No silver granules are observed in the urinary space otherwise the extra-membranous deposits are as previously described.

In the 81 week specimen the silver deposition pattern was not uniform (Figure 44). In some stretches of basement membrane large aggregates of silver similar to the 60 week specimen were present. In other stretches of basement membrane the pattern of deposition more closely resembled the 6 to 10 week specimens.

The following general observations were made. Up to and including the 25 week specimen the pattern of silver deposition was constant between glomeruli, within glomeruli and within individual capillary loops (Figure 45). In the 60 week specimen the pattern was relatively constant. In the 81 week specimen the pattern and density of silver deposition was inconstant (Figure 46).

Figure 43. Glomerular basement membrane from a rat which had ingested silver nitrate for 60 weeks. Very large silver deposits occupy most of the inner half of the basement membrane forming a continuous barrier in some areas. Practically no silver is deposited in the outer half of the basement membrane. X 35,000

Figure 44. Glomerular basement membrane from a rat which had ingested silver nitrate for 81 weeks. Very large silver deposits are present, focally in the inner half of the basement membrane. Small silver granules are randomly distributed throughout the entire thickness of the basement membrane. X 27,000
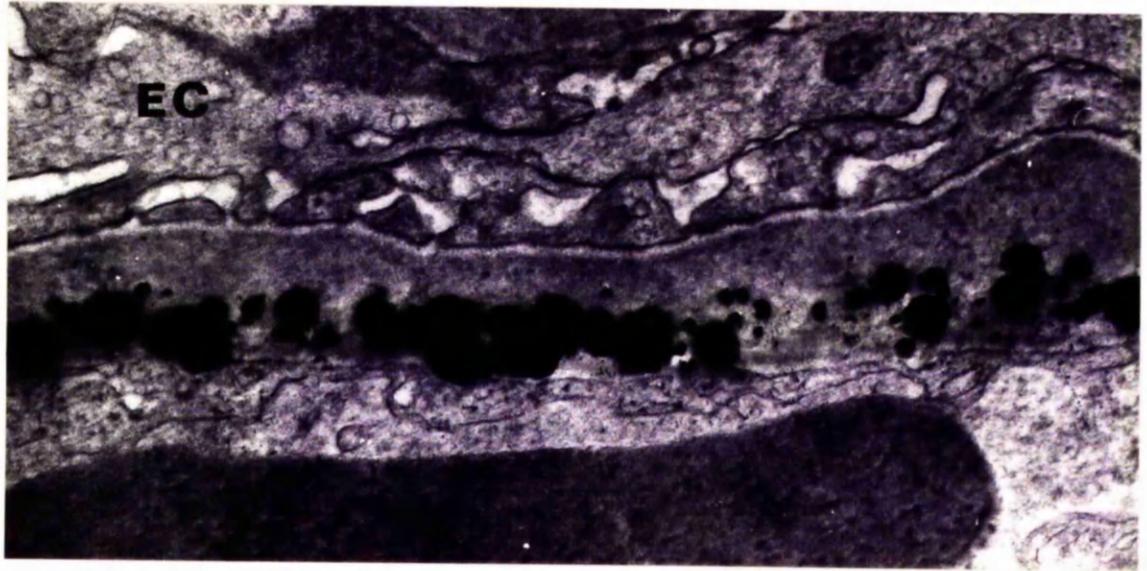
Figure 43
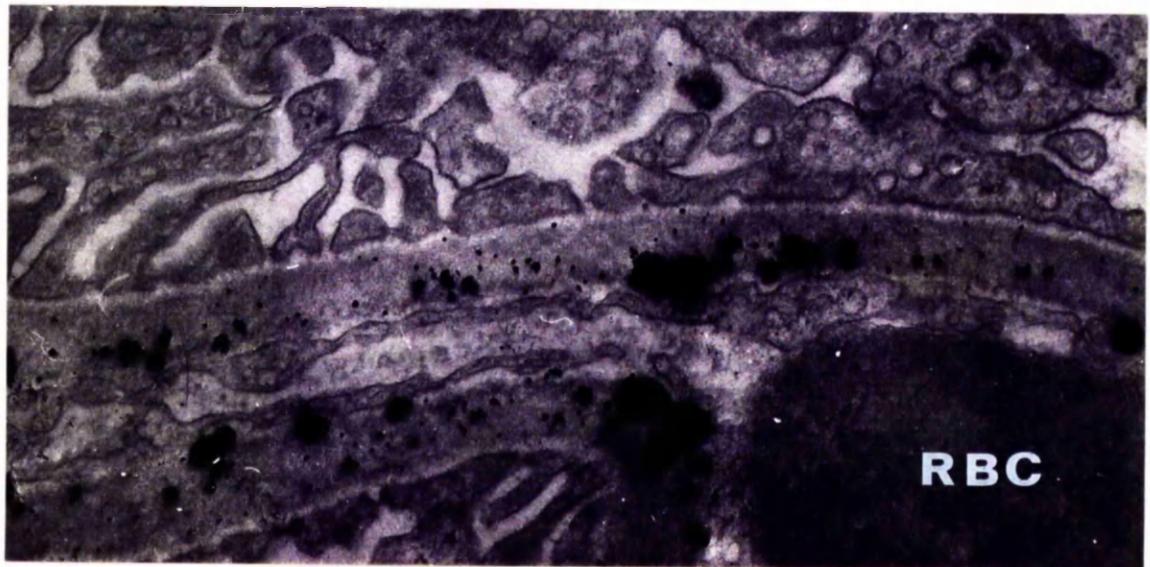
Figure 44

Figure 46. Glomerulus from a rat which had ingested silver nitrate for 25 weeks. The distribution of silver in the basement membrane is uniform. The arrows indicate where the membrane is cut tangentially. Compare with figure 46. X 7,000
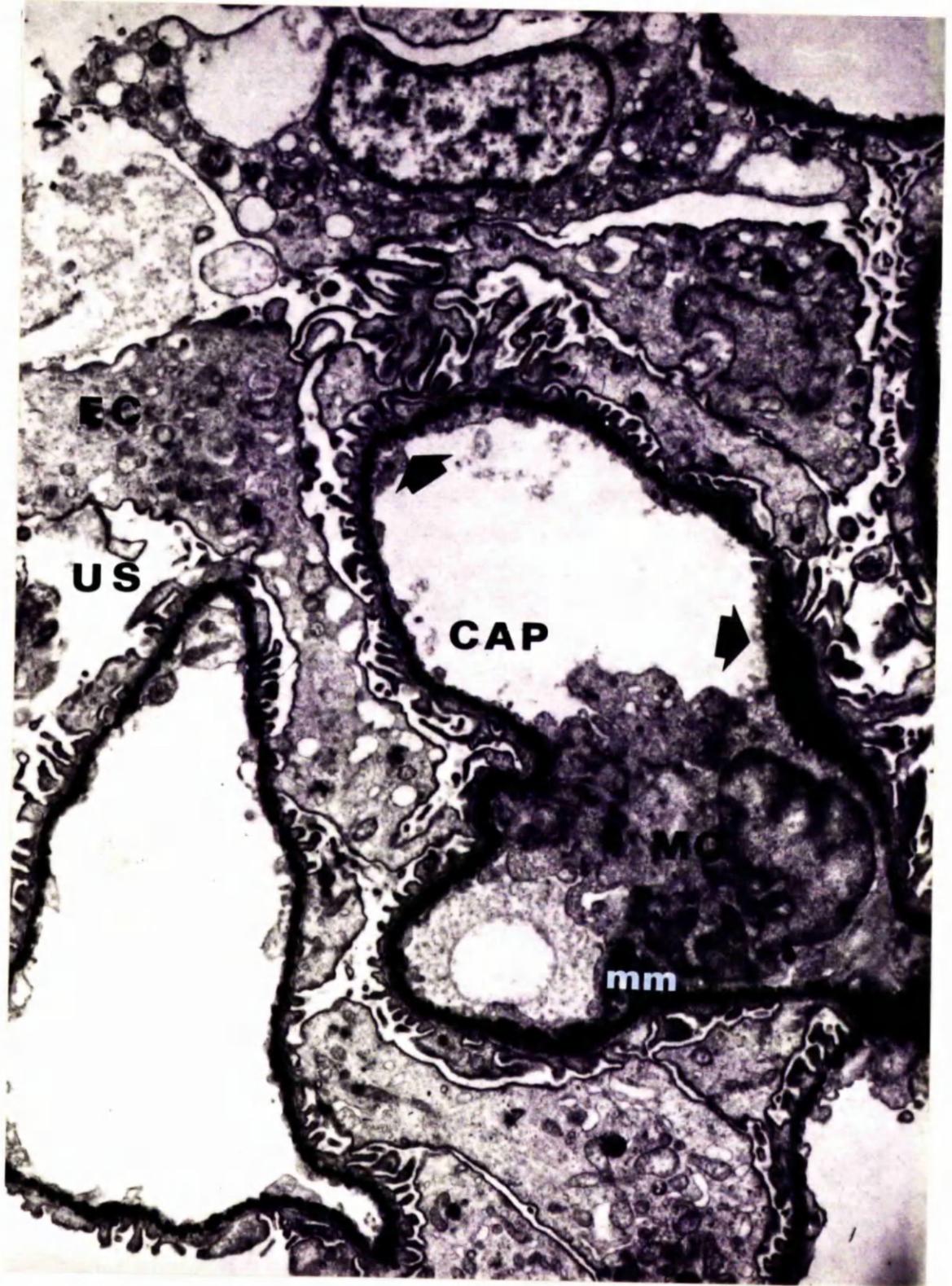
Figure 45

Figure 46. Glomerulus from a rat which had ingested silver nitrate for 81 weeks. The distribution of silver in the basement membrane is not uniform. The arrows indicate where silver granules are herniating through fenestrae. X 8,000

Figure 47. Very large silver granule herniating through an endothelial fenestra. X 76,000
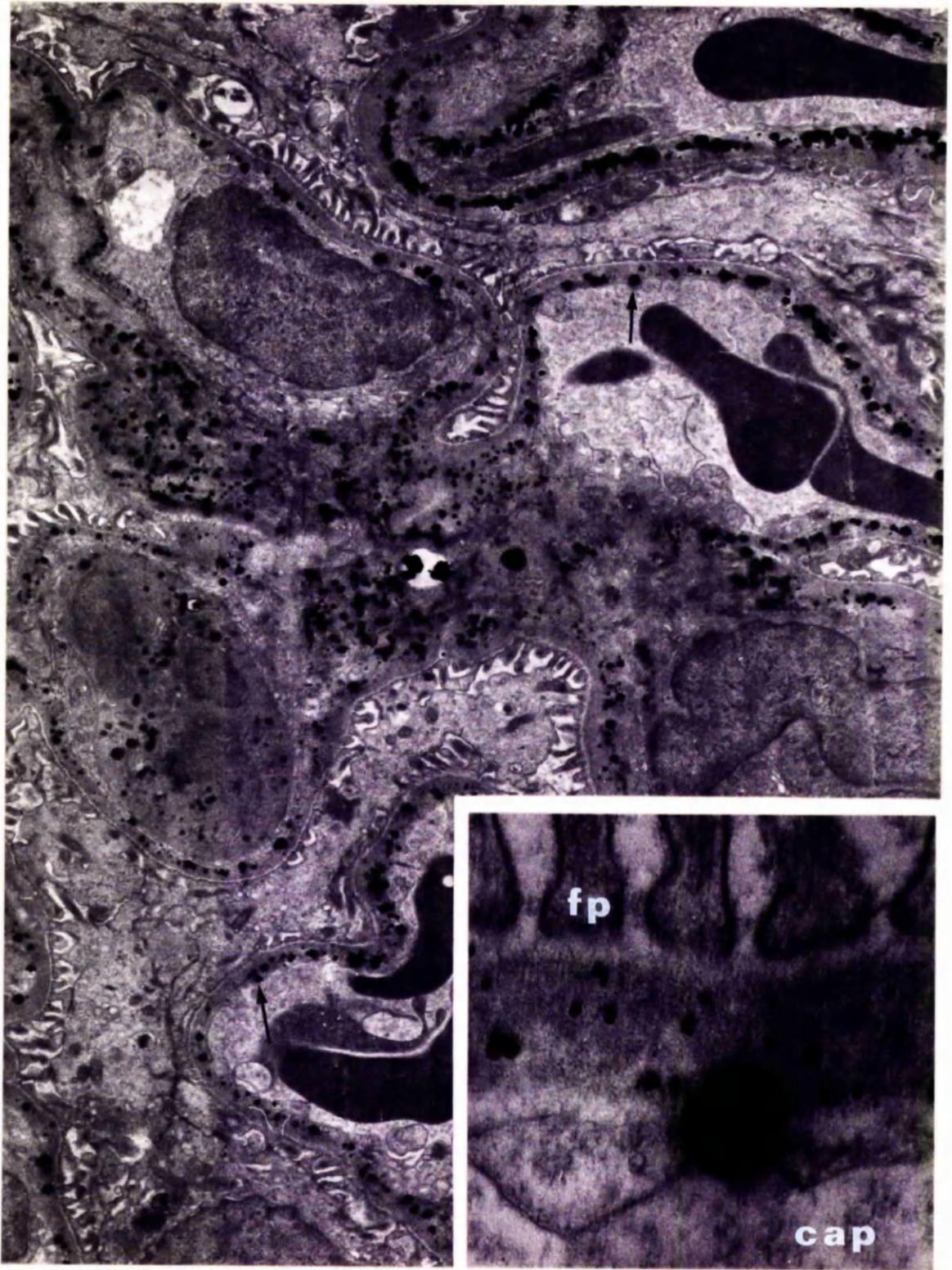
Figures 46 and 47

In no specimen was silver observed in visceral epithelial cells.
In no specimen was silver observed to distort the substance of the
basement membrane. In the 60 and 81 week specimens distort-
ion of endothelial cells and herniation of silver granules into endo-
thelial fenestrae were observed.

Discussion

Glomerular basement membrane is composed of glycoprotein
of the collagen type. Detailed molecular data for this particular
collagen are not, to date, available but by comparison with other,
characterised collagens it is almost certainly an elongated macro-
molecule of the order of 2,000 to 3,000 $\overset{o}{A}$ in length (Kefalides,
1969a). From the analytical data, presented in the introduction
to this thesis, it is evident that there can only be a finite, and
small, number of potential reducing groups in the basement mem-
brane collagen molecule. In contrast a silver molecule, or the
silver ion in a salt, is a small molecule. This great disparity
in size between collagen and silver, and the small number of
potential reducing groups, accounts for the silver being deposited
in a particulate, rather than a continuous pattern within the base-
ment membrane. As the silver halide passes through the base-
ment membrane it encounters a reducing group and is deposited at

that locus. Many reactive groups, in particular those that are reducing agents, are capable of producing a latent image with silver halides by direct chemical action. The process is that of chemography and is similar to the more familiar process of photography (Rogers, 1967).

However, not only is the silver molecule or ion smaller than a collagen molecule, it is also smaller than the best electron microscopic resolution obtained in any of these studies. The electron-dense particles observed are therefore not single molecules but aggregates. It is well established that once silver is deposited it catalyses the development and deposition of further silver salts with which it comes in contact. In this way the original silver particle grows in size until its presence is detectable. It is almost certainly a combination of the time taken to build up a suitable concentration of silver proteinate in the blood, plus the time for the original sub-microscopic or latent particle to grow to a detectable size which accounts for the failure to detect silver deposits until the end of the fourth week of silver nitrate ingestion.

The number of granules in a given length of basement membrane does not appreciably differ between the specimens examined after

8 and 10 weeks so presumably by this time all potential reducing sites are labelled. The granules are solitary, spherical or slightly comma-shaped and increase in size with time of silver ingestion. These features are typical of the physical development of silver (Caro and van Tubergen, 1962; Rogers, 1967; Gallyas, 1970). This process by which silver is deposited from solution on to minute particles of silver already present in the latent image (Lumiere, Lumiere and Seyewetz, 1911; James, 1966), accounts for the increase in size of the silver particles in the basement membrane. A collagenous basement membrane containing a sub-microscopic silver deposit which comes in contact with a mildly alkaline ultra-filtrate containing weak reducing agents and soluble silver salts is remarkably like a gelatin emulsion containing a latent silver image which comes in contact with a physical developer. The process in the basement membrane is many times slower than that in the photographic emulsion but is chemically similar. It is probable that the rate limiting feature is the amount of silver available. Such a mechanism also accounts for the early appear-ance of silver deposits in membranes through which there is a high net flow of ultrafiltrate and hence of silver salts, as tentatively suggested in the experiments described under Informational Studies (b).

From 25 weeks of drinking silver nitrate solution onwards
the pattern of silver deposition in glomerular basement mem-
brane becomes increasingly complex.   This is produced by a
combination of increasing granule size due to continuing develop-
ment and to granule aggregation due to their movement with the
basement membrane as it turns over.   These large silver deposits
continue to grow until by 60 weeks they form a definite physical
barrier in the inner lamina densa, in most regions effectively pre-
venting the silver in the ultrafiltrate from gaining access to the
outer portions of the basement membrane.   These silver deposits
are so large that some are still partly within the lamina densa,
bridge the lamina rara interna and distort the related endothelium.
They appear to be either impacted in the lamina rara interna or
herniating through the fenestrae.

Equally large granules are present in the 81 week specimen
but are fewer in number.   Focally, small silver granules are
deposited throughout the entire thickness of the basement membrane.
Though no large silver granules were observed in the capillary
lumen in the sections examined it is reasonable to suppose that
some large granules were eventually extruded from the basement
membrane via distorted fenestrae.   That particular segment of

basement membrane then reverted to normal, ultrafiltrate once more had free access and silver deposition commenced ab initio.

With the exception of the very early and the last specimens, these stages of progressive silver deposition have all been described and illustrated previously but only as isolated events. The 10 week stage is well illustrated by Kurtz and Feldman (1962), a 6 month stage by Dempsey and Wislocki (1955c) and a later stage by Olcott and Richter (1958). None of these investigators explained the pattern of silver deposition they obtained, or correlated it with the observations of the others. Recently Striker and Smuckler (1970) have studied silver deposition in the glomerular basement membrane at several stages of experimental argyria in a series of experiments similar to those reported here. These authors examined the glomeruli of rats after ingestion of 15 mM. Ag NO3 (c.f. 12 mM. in present experiment) for periods of 10, 17, 20, 26 and 52 weeks. Taking into account the slightly more concentrated silver solutions their rats ingested their observations in general agree well with those presented here. Their interpretation of these results is however markedly different from that presented earlier in this discussion and therefore warrants some detailed consideration.

Striker and Smuckler interpret the pattern of silver deposition to mean that "(1) GBM (glomerular basement membrane), formed by the epithelial cell, varies with age, and the silver labels only the initial material or (2) continuous feeding of Ag NO3 results in formation of an altered GBM which no longer accumulates granules after in vivo silver administration or (3) changes occur in silver transport affecting its exposure to the GBM". They also consider, only to discard in the next sentence as "unlikely", that "the absence of uniform staining may be based on the fact that silver does not have access to the sub-epithelial granule free zone". Eventually they conclude in favour of their suggestion (2) in spite of stating that "aside from the altered metal distribution there is no direct evidence for altered membrane or membrane formation".

The discussion and the conclusions of Striker and Smuckler are unconvincing for the following main reasons. First, the experimental data on which their conclusions are based are incomplete: they did not study silver deposition before 10 or after 52 weeks' ingestion of silver nitrate and both of these periods are essential to the overall picture. Second, their discussion covers only a small portion of the questions raised by their results, namely the distribution of the silver: they do not discuss, and apparently

have not considered, why the silver is deposited in a particulate manner or why the particles increase in size with continued silver ingestion and both of these are pertinent to the points they have attempted to establish. Third, they have failed to appreciate the differences between the pattern of silver deposition, as described here, and the pattern of silver clearance due to basement membrane turnover, as described in part III of this thesis.

In consequence of these deficiencies Striker and Smuckler have failed to elucidate the complexities of the experimental argyric model and reach conclusions which are of dubious validity.

The glomerular basement membrane of rats which have ingested 0·25 per cent silver nitrate drinking fluid for 10 weeks is lightly and uniformly labelled with silver granules. These granules can be easily recognised, are present in all three laminae of the membrane and do not deform the membrane or the related cells. These features make such argyric glomeruli suitable for the proposed studies outlined in the preface to this thesis. Any period of silver ingestion between 8 and 10 weeks gives suitable labelling of glomerular basement membrane for electron microscopic studies but 10 weeks was selected as the standard period of administration as the total amount of silver deposited by this time was also suitable

for parallel dark field microscopic studies.

(e) <u>Studies on the presence of silver outwith the glomerular basement membrane</u>

In the previous experiment it was noted that silver granules were occasionally observed outwith the glomerular basement membrane. Though these silver granules were usually isolated, examination of the photomicrographs indicated that their pattern of distribution was not random. They were consistently present in three well defined locations: (1) closely related to the outer aspect of the plasma membrane of foot processes (Figure 48); (2) overlying foot processes where these were cut obliquely (Figure 49); and (3) free in the urinary space but always in an ill-defined region of increased electron density (Figure 50). The constancy of these locations strongly suggests that the silver was deposited in an external cell coat or glycocalyx (Bennett, 1963; Rambourg and Leblond, 1967). This was investigated and found to be the case.

Materials and methods

A. The photomicrographs taken in the previous experiment and in the experiment described in part III of this thesis, were examined to determine where and when silver granules were present outwith the glomerular basement membrane.

Figure 48.   Silver granule outwith the basement membrane, closely related to the plasma membrane of a visceral epithelial cell.   X 64,000

Figure 49.   Silver granule outwith the basement membrane, overlying a tangentially cut foot process.   X 70,000

Figure 50.   Silver granule outwith the basement membrane lying in an ill-defined area of increased electron density in the urinary space.   X 59,000

Figure 48
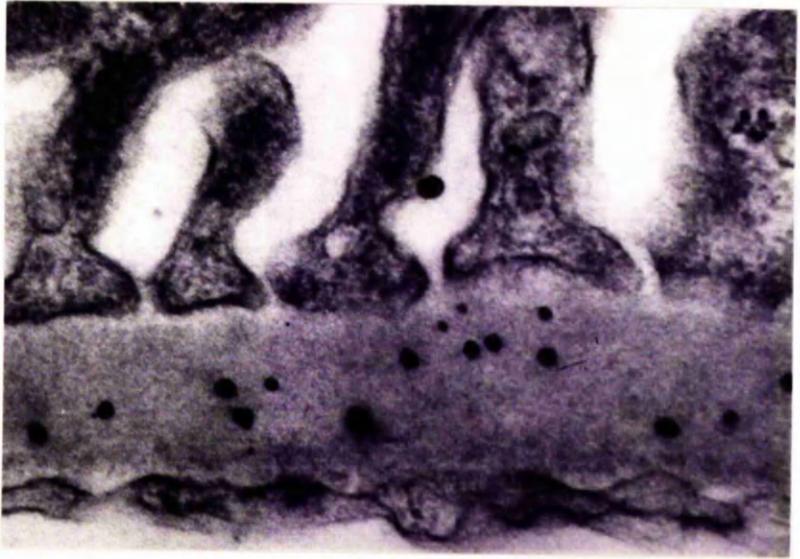
Figure 49
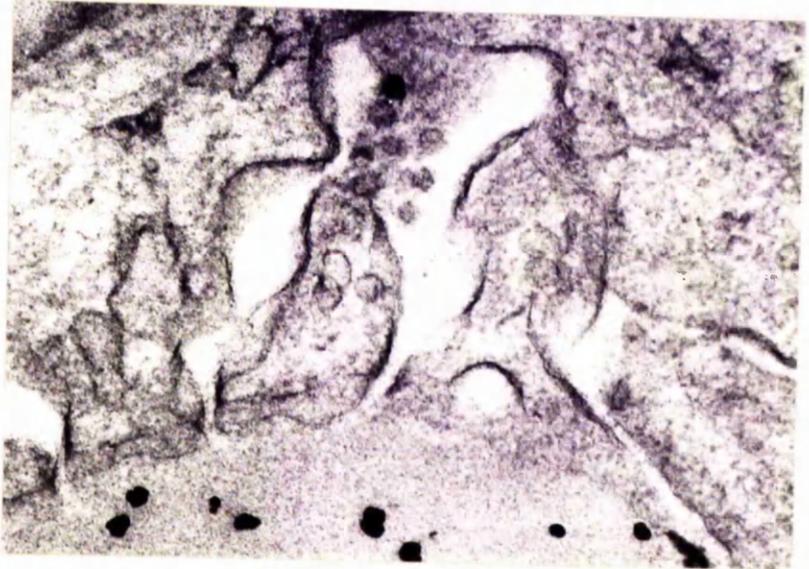
Figure 50

B.   Renal tissue from one standard 10 week argyric rat and
2 non-argyric control rats was prepared for electron microscopy
using the ruthenium red procedure (Luft, 1966).   Renal tissue
from the same animals was also prepared using standard osmium
fixation (Palade, 1952) followed by staining with uranyl acetate
and lead citrate.

Results

A.   Silver granules in the three locations illustrated (Figures
48, 49 and 50) were observed in the glomeruli of rats exposed to
0.25 per cent silver nitrate for 4, 6, 8, 10 and 80 weeks.   These
granules were also observed in the glomeruli of standard 10 week
argyric rats 2, 4 and 6 weeks after silver ingestion ceased.   Such
granules were not observed in the glomeruli of the other rats
examined.

B.   A thick, 200 $\overset{o}{A}$ or more, layer of ruthenium stained material
covered the entire free surface of the visceral epithelial cells and
the filtration slit plates.   Similar material lay free in the urinary
space (Figure 51).   This cell coat was clearly attached to the outer
leaflet of the plasma membrane (Figure 52).   No silver granules
could be discerned in the ruthenium red stained layer in the argyric
animal though such granules were observed in glomeruli from the
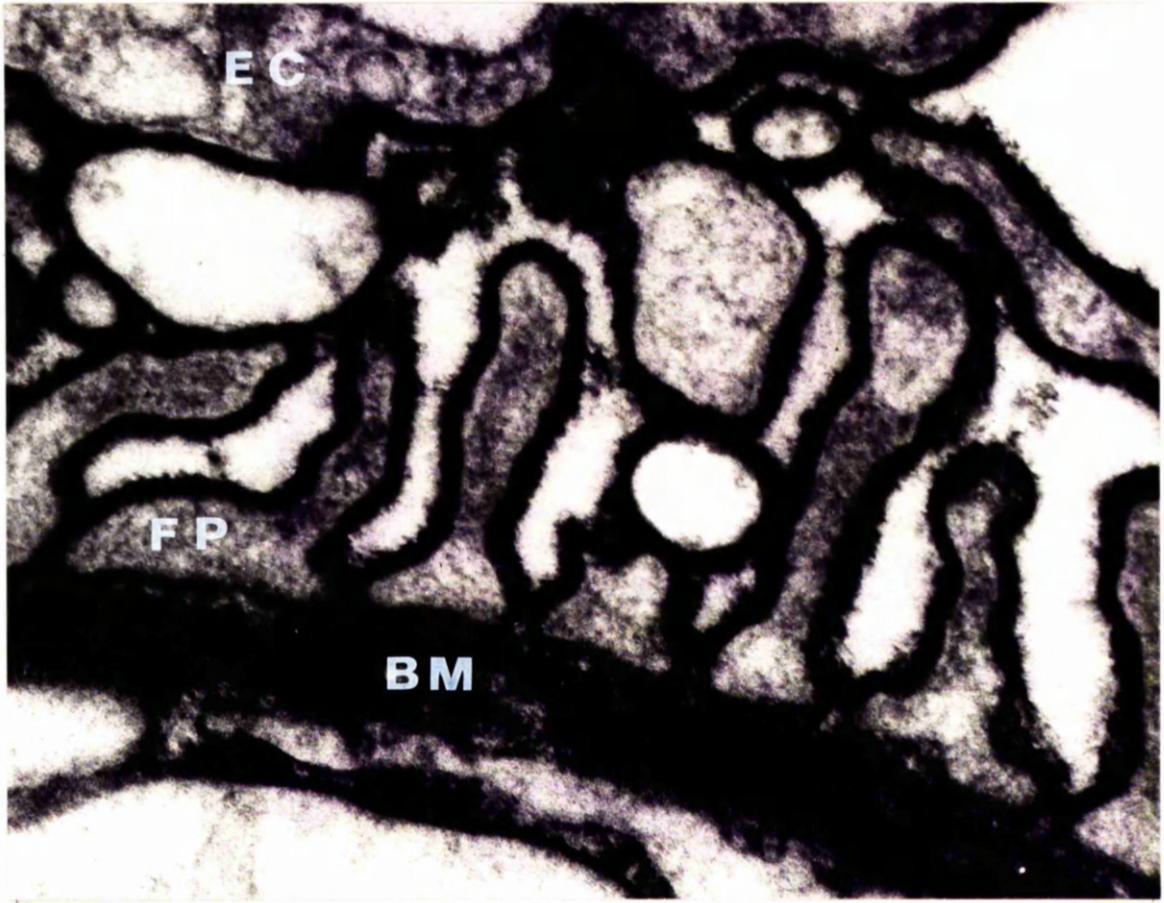
Figure 51. Glomerulus stained with ruthenium red. A thick layer of cell coat or "fuzz" invests the epithelial cell and some of this material is lying free in the urinary space. X 67,000
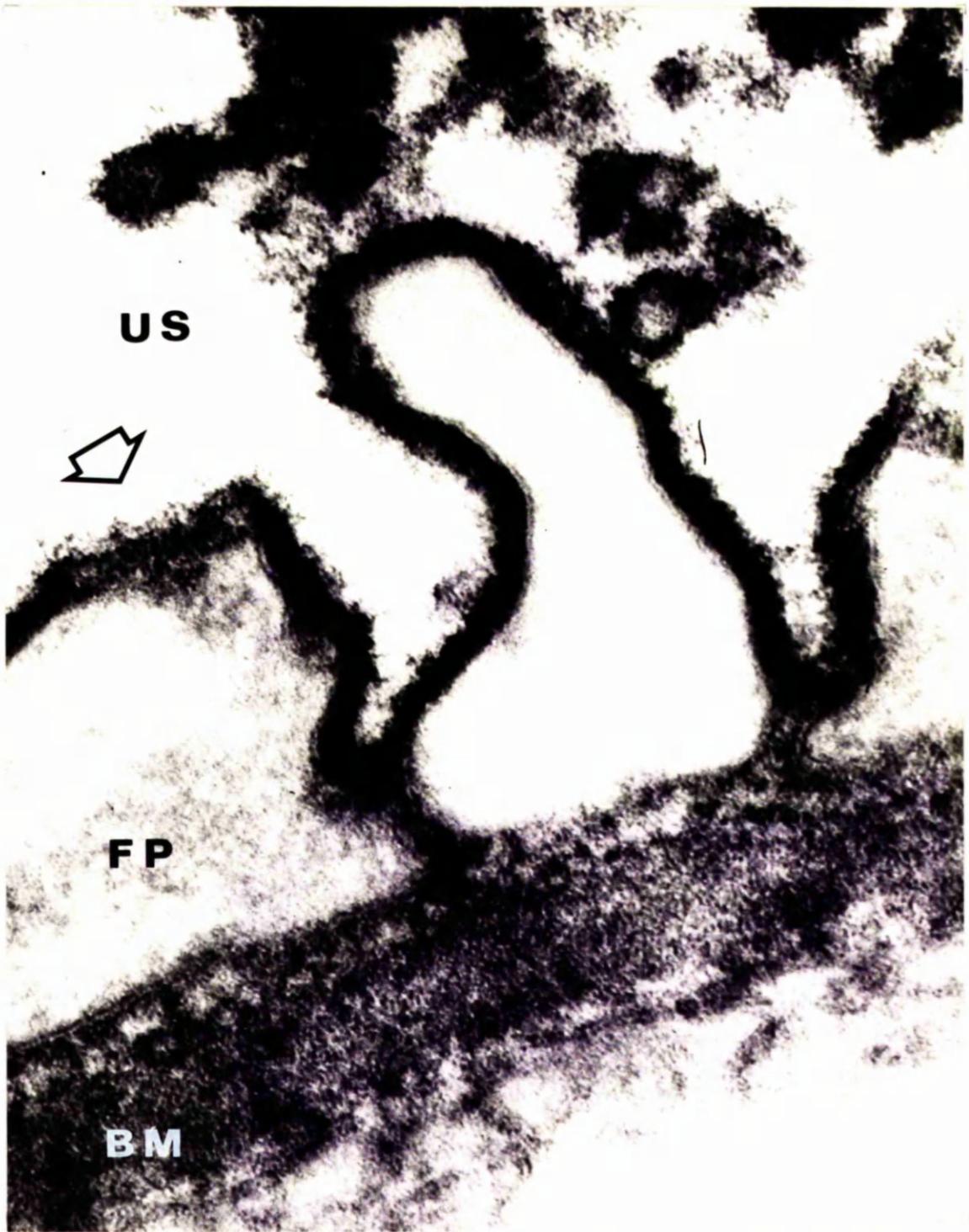
Figure 52. High magnification of foot processes and glomerular basement membrane stained with ruthenium red. The "fuzz" is clearly attached to the outer leaflet of the plasma membrane and also covers the slit plates. The basement membrane stains slightly less intensely than the "fuzz". The arrow indicates tangentially cut cell wall. X166,000

same animal which were examined after osmium fixation alone. The glomerular basement membrane was stained with ruthenium red, slightly less so than the cell coat but still sufficiently intensely to prevent positive identification of silver granules.

Silver granules were also observed in the 'coated pits' which open on to the free surface of the epithelial cell (Figure 53). These 'coated pits' are identical to those which open from the base of the foot processes into the basement membrane (Figure 54). These 'coated pits' are derived from a vacuolar system within the cell and are labelled with silver only when a communication to the outside of the cell is present. A well developed vacuolar/'coated pit' system is illustrated in figure 55. Not all the 'coated pits' contain silver but a substantial minority do. The visceral epithelial cells were particularly scrutinised for intracellular silver granules but none were observed.

Attempts to trace the vacuoles to their origin within the epithelial cell on morphological grounds alone were inconclusive. Though there was a suggestion of a relationship with the Golgi apparatus actual budding of a recognisable vacuole of this type from the Golgi apparatus was not observed.

These vacuoles are bounded by a thick unit membrane which

Figure 53. Silver granules in coated pits opening into the urinary space (a) "en face" view, X 44,000 (b) side view, X 42,000

Figure 54. Silver granules in coated pits opening into the glomerular basement membrane (a) "en face" view, X 68,000 (b) side view, X 66,000
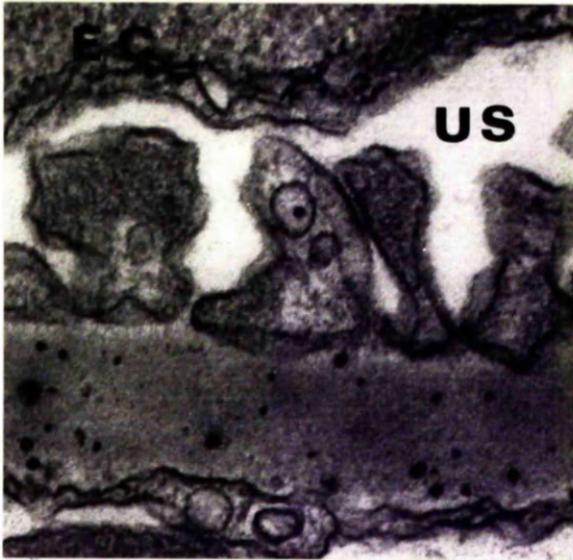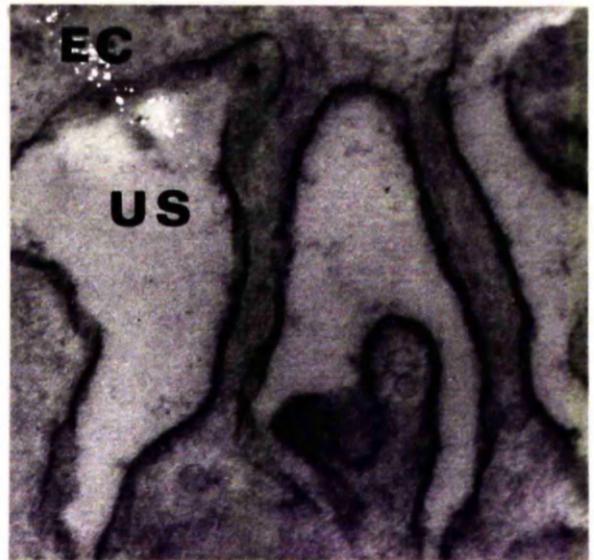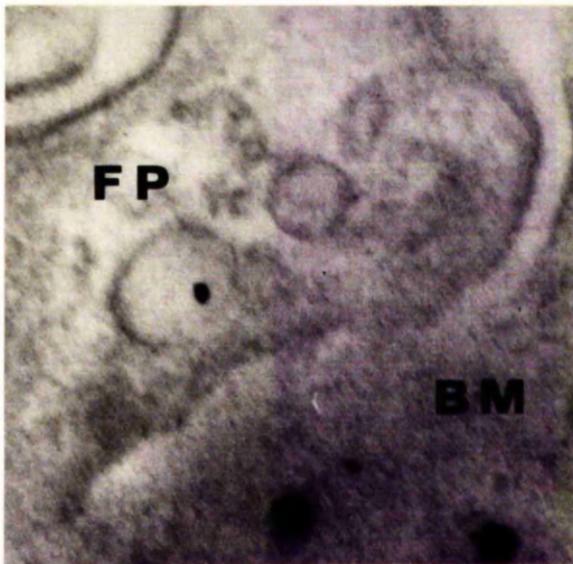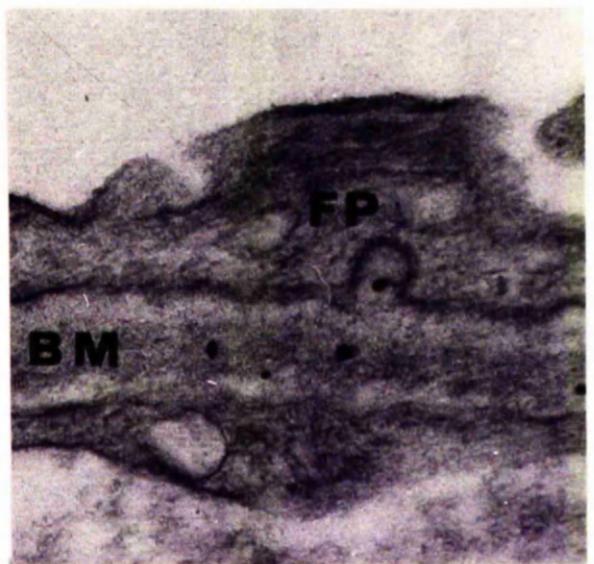
Figure 53

Figure 54

Figure 55. Visceral epithelial cell containing numerous vacuoles and with two coated pits opening into the urinary space. X 67,000

is slightly "fuzzy" on both the internal and external aspects but positive recognition of such a secretory system is, at present, only possible at the 'coated pit', silver labelled stage.

Discussion

On general principles, and from derived evidence, the presence of a cell coat, glycocalyx or "fuzz" around mammalian cells was first clearly proposed by Bennett (1963). Such a cell coat was subsequently demonstrated around glomerular visceral epithelial cells using thorium dioxide (Rambourg and Leblond, 1967), colloidal iron (Jones, 1969) and ruthenium red (Groniowski, Biczyskowa and Walski, 1969). Using the colloidal iron technique allied with neuraminidase digestion it has been shown that the visceral epithelial cell coat contains sialic acid and that small amounts of this material are present related to the anchored portion of the foot processes (Mohos and Skoza, 1969). The ruthenium red procedure, originally introduced to demonstrate the cell coat on endothelial cells, in general indicates the presence of an acid substituted large polymer, probably a polysaccharide or a glycoprotein (Luft, 1966).

There is general agreement that glomerular basement membrane contains significant amounts of carbohydrate. Two distinct

carbohydrate components are present (Dische, Pappas, Grauer and Dische, 1965; Spiro, 1967b; Kefalides, 1968) one of which contains galactose, mannose, hexosamine, fucose and sialic acid. The composition of very few cell coats has been investigated but of those, admittedly specialized ones, which have, sialoglyco-peptide has been regularly identified (Wallach and Esandi, 1964; Langley and Ambrose, 1967; Walborg, Lantz and Wray, 1969). The available evidence makes it not unlikely that such sialoglyco-peptides could be components of both the epithelial cell coat and the basement membrane. Dilution of this mucosubstance with the considerable amounts of collagen in the basement membrane could account for the basement membrane staining slightly less intensely than the cell coat in the ruthenium red procedure.

The vacuoles and the 'coated pits' have been described and well illustrated in previous studies (Farquhar, Wissig and Palade, 1961; Fawcett, 1965, 1966; Ericsson, 1968) under various names, "fuzzy vesicles", "coated vesicles", "alveolate vesicles", "acantho-somes", and "coated pits". These 'coated pits' open either on to the free surface of the visceral epithelial cell or into the glomerular basement membrane, are ultrastructurally indistinguishable, and differ only in the locus of their discharge - one outwith, the other

inside the filtration slit plate. No silver was observed within the visceral epithelial cell proper and the vacuoles only become labelled with silver at the coated pit stage when they communicate with the exterior of the cell. The vacuoles therefore probably contain reducing material in which silver is deposited when the vacuolar contents are exposed to silver halide in the extra-cellular fluid.

Ericsson (1965) observed these pits opening into the urinary space though he does not mention them opening into the basement membrane. Farquhar and her associates (1961) observed the pits opening into the basement membrane and into the urinary space and on the basis of ferritin tracer studies ascribed to them an absorption, transportation and secretion function from basement membrane to the urinary space. Not all the pits in the present study were labelled with silver. It is therefore probable that these vacuoles and pits, though not at present distinguishable on morphological grounds, are functionally two separate entities: one devoted to the synthesis and secretion of cell coat and basement membrane, the other devoted to the transport of ultrafiltrate material from the basement membrane to the urinary space by an intracellular route.

The visceral epithelial cell coat and the glomerular basement membrane are both labelled in vivo with silver and are both stained in vitro with ruthenium red. They are also both secreted by a vacuolar-coated pit system. Furthermore the experiment described in part III shows that the cell coat and the basement membrane are secreted at the same time. There is therefore sufficient experimental evidence to conclude that visceral epithelial cell coat and glomerular basement membrane, or a component thereof, are similar.

No evidence has been presented however to indicate that the cell coat and the basement membrane are identical. Indeed because the synthesis of the carbohydrate moiety of glycoprotein involves post-ribosomal, non-coded stages (Priestley, Pruyn and Malt, 1969; Spiro, 1969) and because of the potential multiplicity of membrane related glycosyl transferases (Hagopian, Bosmann and Eylar, 1968; Bosmann, 1969) the probability of absolute identity between visceral epithelial cell coat and basement membrane component is not particularly high. Nevertheless the similarity between the two substances is probably sufficient to cause considerable cross reaction immunologically. Evidence strongly suggestive of this phenomenon has recently been found by Hoedemaeker

(1970a, b) who in a series of nephrotoxic experiments with antibody to glomerular basement membrane has observed localization of the antibody not only in the basement membrane but also on the surface of visceral epithelial cells. In the present study considerable amounts of cell coat were observed lying in the urinary space. It is possible that this material could eventually be excreted in the urine. This shed cell coat might be the answer to Dixon's (1968) intriguing question of whether glomerular basement membrane antigens found in normal urine do in fact come from glomerular basement membrane.

In summary, on the basis of electron microscopic studies on rat glomeruli labelled in vivo with silver and stained in vitro with ruthenium red it has been shown that the visceral epithelial cell coat and a component of glomerular basement membrane are secreted in the same manner, by the same cell, at the same time, and share certain staining affinities with each other.

(f) Anomalous features of certain argyric rats

In the course of examining all the argyric rats utilized in the experiments described in this thesis one anomalous, and initially concerning, feature emerged. Very occasionally one of a group or series of rats was found to contain much less silver than expected

from comparison with other rats in the same group or series.
This was first noted in a rat which had ingested 0·25 per cent
silver nitrate for 10 weeks prior to being killed and was in the
first group of animals described in Information Studies (d), hence
the reason for the repeat series of rats in that particular experi-
ment.

The glomerular basement membrane of this rat was more like
that of an animal on silver nitrate for only 4 weeks instead of
10 weeks. The pattern was uniform in the 4 glomeruli examined
electron microscopically. Silver could not be detected, using
dark field microscopy, in any of the glomeruli in this animal and
on examination of other tissues only the liver contained detectable
but small amounts. The oesophageal keratin was however stained
normally with silver so the animal had been ingesting the silver
solution. This rat had been ingesting a standard solution of silver
nitrate under standard conditions for a standard time and yet ap-
parently had absorbed less than the standard amount of silver.

One rat, killed in the 20th week of the experiment described
in part III, had relatively little in the way of silver deposits and
was replaced in the series. A further rat from a group not des-
cribed in this thesis showed a similar picture. Out of a total of

112 argyric rats this phenomenon of markedly diminished silver deposition was observed in three. It is attributed to a lower than usual absorption of silver. The converse, namely excessive silver deposition in certain animals, was not observed.

It is disquieting to find an anomalous occurrence of this type appearing at random under standard experimental circumstances. Fortunately such animals can be recognised, and steps taken to replace them in the experiments proper.

This phenomenon of diminished silver absorption by certain individual rats has not previously been commented on in studies involving silver administration but in several of these studies the results are recorded in sufficient detail to make it possible to pick out, retrospectively, animals which had probably absorbed less silver than others in the same experiment. For instance Shaver and Mason (1951) gave 0·15 per cent silver nitrate to 23 vitamin E deficient rats and they died within 18 to 40 days "except for one rat which survived 7 months". Olcott (1948) records that "two rats were kept alive for over 500 days with no other fluid intake than 0·4 per cent solutions of silver chloride in sodium thiosulfate, but this dosage usually was found to be excessive". Striker and Smuckler (1970) note that "with one exception, intact animals on

15 mM. AgNO3 drinking water during any part of the experiment had lower body weights than the controls". In a somewhat specialised study on the behaviour of intravaginally applied radio-active silver Nyberg (1967) graphically records the uptake in liver, kidney and spleen, and one of 41 anoestric rats shows a markedly lower uptake in each of these organs.

It is therefore evident that there are occasional individual rats, probably the incidence is between 2 and 3 per 100 animals, which absorb considerably less silver than usual when exposed to silver solutions. The reasons for this anomaly are not known.

Experimental Argyria in Principle, Theory and Practice

The use of silver as an in vivo label for structural and ultra-structural studies on basement membranes is simple in principle and reasonable in theory. Silver nitrate administered orally to a rat is absorbed, distributed systemically via the blood and deposited in basement membranes as a firmly attached label which does not disturb the physiological situation at the site of deposition and can be readily detected and positively identified using optical and electron microscopic techniques.

In practice each of these stages can present problems which must be recognised and understood. The silver nitrate must be

delivered from containers which do not cause precipitation of silver.
Such containers have been described.   Silver nitrate must be ad-
ministered in a concentration which the rats will readily ingest
without signs of physiological upset for a period which is sufficient
to cause uniform and detectable deposition of silver in the membrane(s)
under study.   Two point five grammes of silver nitrate per litre
of distilled water (0·25 per cent w/v or 12 mM) administered con-
tinuously for 10 weeks is suitable for studying glomerular basement
membrane both by dark field and electron microscopy.   The argyric
rats must be examined in groups or series so that the occasional
rat which, for reasons unknown, absorbs less silver than normal
can be recognised.

The mechanism of silver deposition must be understood in order
that the pattern of silver deposition can be interpreted.   The prob-
able sequence of events is that silver is transported in the blood
reversibly bound to plasma proteins from which it dissociates and
diffuses or flows into the tissues where it is deposited.   Deposition
is most conveniently explained as a process of chemography by
reducing, or other active groups, resulting in the formation of
a sub-microscopic, or latent, deposit at the site of these groups.
This accounts for the widespread distribution of silver in particulate

form.   Subsequently these latent deposits grow to detectable
size by a process of physical development due to continued expos-
ure to silver ions.   The rate of growth of these particles, and
therefore the time they are first detectable, depends on the total
amount of silver which has access to the latent deposits: this is
greatest in membranes subjected to a high gross transfer of fluid
containing silver ions such as occurs in discontinuous or fene-
strated capillaries (Bennett, Luft and Hampton, 1957;   Majno,
1965).   This accounts for the early detection and subsequent
heavy deposits of silver in glomerular basement membrane.
Chemical differences in the basement membranes are also undoubt-
edly major factors especially with respect to the number of the
silver grains which is dependent on the number of groups with
chemographic potential, but there is as yet only limited data on
this aspect (Kefalides and Denduchis, 1969) so though this factor
can be recognised it is not yet adequately understood.

Silver can be readily detected with the optical microscope
using dark field techniques.   While its appearances are reason-
ably characteristic, further proof of its identity can be obtained
by using standard and simple histochemical tests for silver.
The only cells in which silver is regularly deposited are those of

the reticulo-endothelial system and, to a much lesser extent, hepatic parenchymal cells. Haemosiderin occurs in these sites but is distinguished not only by the tests mentioned above but also by being stainable in the Prussian blue reaction. Though this latter reaction involves cyanide solutions these are of insufficient strength to significantly alter deposited silver.

Silver can be readily detected with the electron microscope. Its appearances are highly characteristic but not absolutely specific. Isolated particles of dirt can exactly mimic silver granules so a scrupulously clean technique is essential and no significance can be attached to a single particle unless it forms part of a more general, topographically related, pattern of deposition. The other main particles from which silver must be distinguished are the electron dense particles found intracellularly in organelles of the lysosome, dense and residual body type. In most cases the distinction can be readily made on morphological grounds but in some instances histochemical procedures, as adapted for electron microscopy, are required.

Of the artefacts caused by silver, 'screeving' is the most important and must be recognised in order to define precisely where particular silver deposits are situated. In the standard

10 week argyric animal this artefact is not particularly prominent.

The experiments, and the trend in the discussions, in this chapter have largely been oriented towards the intended study of glomerular basement membrane which forms the principal part of this thesis. The experimental argyric technique is, however, adaptable to the study of other basement membranes provided the time of exposure to silver nitrate is selected to give a uniform, detectable, and not excessive deposition of silver in the membrane to be studied. The only membranes which, theoretically, are not amenable to such an approach are those which (1) contain no potentially chemographic groups; (2) have such a high turnover rate that the latent image is not exposed to silver solutions for a sufficient time to grow into a detectable deposit; (3) do not have a sufficient gross transfer of silver containing tissue fluid to produce, or result in, a detectable deposit.

There are several other techniques presently or potentially available whose application would yield the same sort of data as the experimental argyric technique. The foremost of these alternatives is autoradiography (Neutra and Leblond, 1969) which is much more versatile, more physiological and potentially more informative than experimental argyria. However ultrastructural

autoradiography poses certain technical problems not yet adequately solved and it is time consuming. A series of animals as large as that presented in this thesis could not be studied by a single investigator within a period of time deemed reasonable, using autoradiographic techniques alone. With the information from the experimental argyric experiments it will however be possible to design feasible autoradiographic experiments which are more refined, in the temporal sense, and will require fewer time points. Such experiments are essential, not least to determine where the vacuoles that become 'coated pits' arise, for the experimental argyric technique is not suitable for such intra-cellular studies. In two major respects however argyria is likely to remain better than autoradiography and that is in resolution and comparative human studies. The best resolution that can be obtained auto-radiographically will probably be about 300 $\overset{o}{A}$ (Bachmann and Salpeter, 1965) whereas with experimental argyria a resolution of 30 $\overset{o}{A}$ could be obtained. Finally, rare though argyric human glomeruli are, they are still more common than suitably labelled radio-active human glomeruli.

Part III

The Origin, Turnover and Removal of
Glomerular Basement Membrane

The origin of glomerular basement membrane, whether the membrane turns over and, if it does, the site of its removal, are not well established. This chapter describes the application of the experimental argyric technique in a long term investigation designed to yield an overall account of the natural history of normal glomerular basement membrane.

Concerning the origin various studies have implicated the visceral epithelial cells (Farquhar, Wissig and Palade, 1961; Andres, Morgan, Hsu, Rifkind and Seegal, 1962; Kurtz and Feldman, 1962; Vernier and Birch-Andersen, 1962; Thoenes, 1967), the endothelial cells (Farquhar, Vernier and Good, 1957; Movat, McGregor and Steiner, 1961; Jones, 1963) and the mesangial cells (Vernier, 1964), either individually or in various combinations. LeCompte (1964) admirably summed up the situation prior to 1964 by saying "I don't know whether basement membrane or basement membrane material comes from the epithelial or the endothelial or mesangial cells or from all three ......". Menefce and Mueller (1967) summed up the situation at that time as "From

these diverse reports it seems that epithelial cells, stalk (mesangial)cells, and endothelial cells may all be involved in the elaboration of basement membrane material and that different conditions may result in a selective increase of activity in one of the possible types ......".

Concerning turnover of glomerular basement membrane, this has been infrequently considered (Farquhar, 1964a; Vernier, 1964a) and even less frequently studied as it has been widely supposed that new basement membrane synthesis is for purposes of increasing the thickness or repairing a more or less permanent structure. Two investigations specifically directed to this problem do however indicate that the basement membrane turns over (Lazarow and Speidel, 1964; Chow and Drummond, 1969).

Since glomerular basement membrane turnover has not been widely considered, the site of removal of the basement membrane has been given scant attention. Farquhar and Palade (1962) demonstrated that mesangial cells phagocytosed ferritin tracer particles which did not penetrate the lamina dense. These investigators concluded that a function of the mesangial cells is to remove filtration residues and they also suggested the possibility that these cells "normally operate at the removal end of a turn-over process

by which the glomerular filter is continually renewed". This suggestion was reiterated by Farquhar (1964a) with the proviso "For the moment, however, these comments on basement membrane turnover should be recognised largely as speculations which clearly need to be tested by further experimentation.".

The various studies cited record, either as experimental fact or as reasonable supposition, the natural history of glomerular basement membrane at different times and in isolated stages whose interrelationships were not always apparent except with hindsight. The experiments to be described show that a major component of glomerular basement membrane is secreted by the visceral epithelial cells, turns over very slowly but continually and is removed by the mesangial cells.

Experiment 1.

Materials and Methods

A group of male Sprague-Dawley rats, bred and reared in the Foresterhill Animal House of the University of Aberdeen were given standard (2.5 g per litre) silver nitrate drinking fluid ad lib from the age of 6 weeks for a period of 10 weeks. One animal was examined at this time and the others were returned to ordinary tap drinking water. One animal was examined at the end of every

second week for a period of 30 weeks and at the end of every tenth week for a further 60 weeks.

The animal examined 20 weeks after ceasing to ingest silver nitrate contained very little deposited silver as described in the previous chapter. Accordingly a further animal was killed at 21 weeks for inclusion in the series proper. The animal examined 80 weeks after ceasing to ingest silver nitrate had chronic nephritis (Snell, 1967). Accordingly a further animal was killed at 83 weeks for inclusion in the series proper. In all, 24 argyric rats were examined in this experiment. No animals died or had to be destroyed because of illness. Six control rats, never exposed to silver nitrate, were examined at various times during the course of the experiment.

Blocks for electron microscopy were taken as previously described. All were fixed in cold veronal buffered osmium tetroxide (Palade, 1952). Most of the specimens were examined in a Zeiss EM 9A electron microscope: a few were examined in a Siemens Elmiskop I.

The sections were examined systematically in the microscope and the position of the silver granules was noted. The sections were then photographed. An average of 20 exposures were taken

per specimen mostly at primary magnifications of 1,750, 6,000 and 18,000 diameters and the negatives were photographically enlarged as required. The photographs were then systematically examined and the position of the silver granules was noted. There was excellent agreement on the distribution pattern of granules by direct examination of the image and by examination of the photographs but more granules were usually visible in the photographs than were detected on the screen. A minimum of 2 grids from each of 2 blocks from every specimen were examined.

The following sites were rigorously examined for the presence or absence of silver granules: visceral epithelial cells; visceral epithelial cell coat or "fuzz"; foot process coated pits; lamina rara externa; lamina densa; lamina rara interna; mesangial matrix; mesangial cells and endothelial cells (Figure 66). For recording purposes the lamina densa was sub-divided into an outer and an inner half. The observations on the location of silver granules in the basement membrane were made in regions where the plane of section could be accurately determined as indicated by the presence of filtration slit plates.

From each animal blocks of kidney, liver, pancreas, choroid plexus and spleen were taken for bright and dark field optical

Fuzz

Epithelial cell

Coated pit
Lamina rara externa

Lamina densa
Lamina rara interna
Mesangial matrix

Mesangial cell

Endothelial cell
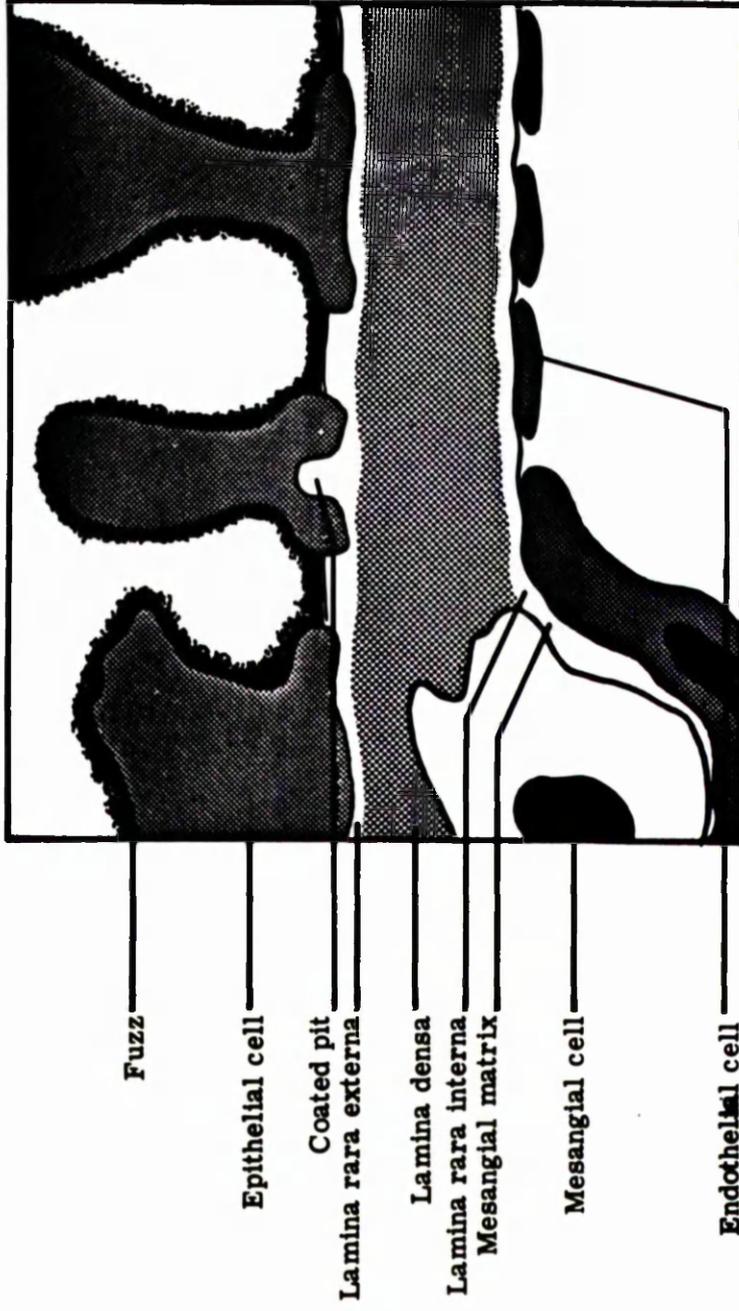
Figure 56

microscopy.

<p style="text-align:center">Results</p>

The distribution pattern of the silver granules in the individual specimens is summarized in the table: + indicates silver granules present, - indicates silver granules absent, ± indicates silver inconstantly present.

In no specimen was silver observed in the visceral epithelial cells, within the attenuated portion of the endothelial cytoplasm or within the capillary lumen.   Silver granules were never observed in that portion of the basement membrane which lies beneath the filtration slit plate between adjacent foot processes above the line of the 'sole' of the foot plate.

In the animal examined at the end of the 10 week period of silver nitrate ingestion silver granules were distributed as previously described namely in the fuzz, foot process pits, all the layers of the basement membrane, mesangial matrix and mesangial cells (Figure 57).   Silver granules were not observed in the perikaryon of the endothelial cells in this particular specimen.   This pattern was maintained in the rats examined 2 (Figure 58a), 4 and 6 weeks after ceasing to ingest silver nitrate with the exception that silver was present in the endothelial cells of these animals.
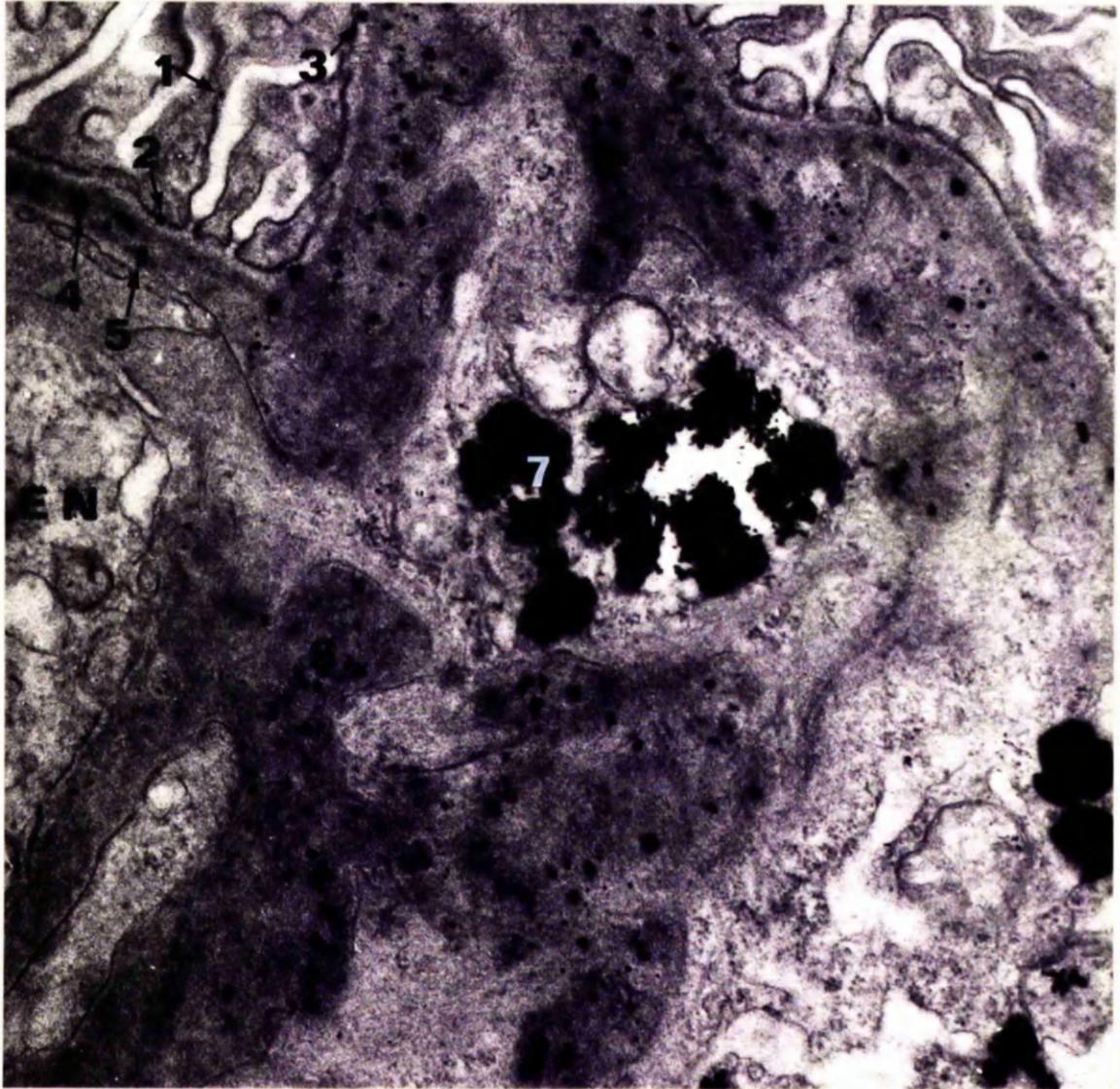
Figure 57. Mesangial region from standard argyric rat which had ingested silver nitrate for 10 weeks. All the regions in which silver was deposited are present in this single micrograph. 1 – fuzz; 2 – foot process pit; 3 – LRE; 4 – LD; 5 – LRI; 6 – mm; 7 – MC. X 26,000

Weeks on tap water	0	2	4	6	8	10	12	14	16	18	21	22	24	26	28	30	40	50	60	70	83	90
Visceral epithelial cells	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Visceral epithelial fuzz	+	+	+	+	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Foot process pits	+	+	+	+	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Lamina rara externa	+	+	+	+	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Lamina densa (outer)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•
Lamina densa (inner)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	•	+	•	•	•	•
Lamina rara interna	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	•	+	•	•	•	•
Mesangial matrix	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	•	+	++	+
Mesangial cells	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endothelial cells	•	+	+	+	+	+	+	+	+	+	•	+	+	+	•	•	+	+	•	•	•	•

Subsequent specimens (Figures 58b, c, d, e, f, 59 and 60) showed a slow and steady movement of silver granules towards the inner aspect of the basement membrane. By 8 weeks silver granules were not detected in the fuzz, the foot process pits or the lamina rara externa. By 24 weeks silver was not detected in the outer half of the lamina densa. By 40 weeks there was only a row of granules along the inner border of the lamina densa. From this time onwards granules were observed so infrequently and randomly in the lamina rara interna that they could not be said to constitute a definite pattern of deposition. From 50 weeks onwards the same applied to the mesangial matrix in that silver granules were detectable in the mesangial matrix in some grids of some glomeruli. Further grids and further glomeruli from these specimens were examined without improving the consistency of either the mesangial matrix or lamina rara interna observations.

In the 70, 83 and 90 week specimens only occasional, isolated granules were observed in the inner border of the lamina densa (Figure 60). These granules were insufficient to form a definite pattern.

Silver granules in the mesangial matrix appeared to become incorporated into the mesangial cells by a phagocytic process.

Figure 58. Glomerular basement membrane from peripheral portion of capillaries in a series of argyric rats at varying times after ceasing to ingest silver nitrate. The plane of the section is normal with respect to the glomerular basement membrane as indicated by the presence of slit plates (arrow). X 63,000

(a) 2 weeks. Silver granules are distributed throughout the entire thickness of the basement membrane.

(b) 12 weeks. Silver granules are no longer present in the foot process coated pits, the lamina rara externa and the outermost portion of the lamina densa.

(c) 24 weeks. The outer half of the lamina densa is now clear of silver.

(d) 40 weeks. Silver granules are aligned more or less along the inner border of the lamina densa.

(e) 60 weeks. The distribution of silver granules is similar to the previous specimen but the number of granules is considerably less.

(f) 90 weeks. Only a very occasional silver granule was observed in this specimen, usually near the inner border of the lamina densa.

Figure 58

Figure 58

Figure 59. Low power photomicrograph showing typical appearances in the glomeruli of an argyric rat 22 weeks after ceasing to ingest silver nitrate. Silver is deposited predominantly in the inner half of the basement membrane and in mesangial cells. Small amounts of silver are present in the mesangial matrix (arrow 1), and in endothelial cells (arrow 2). The appearances are remarkably uniform throughout the glomerulus. X 9,000
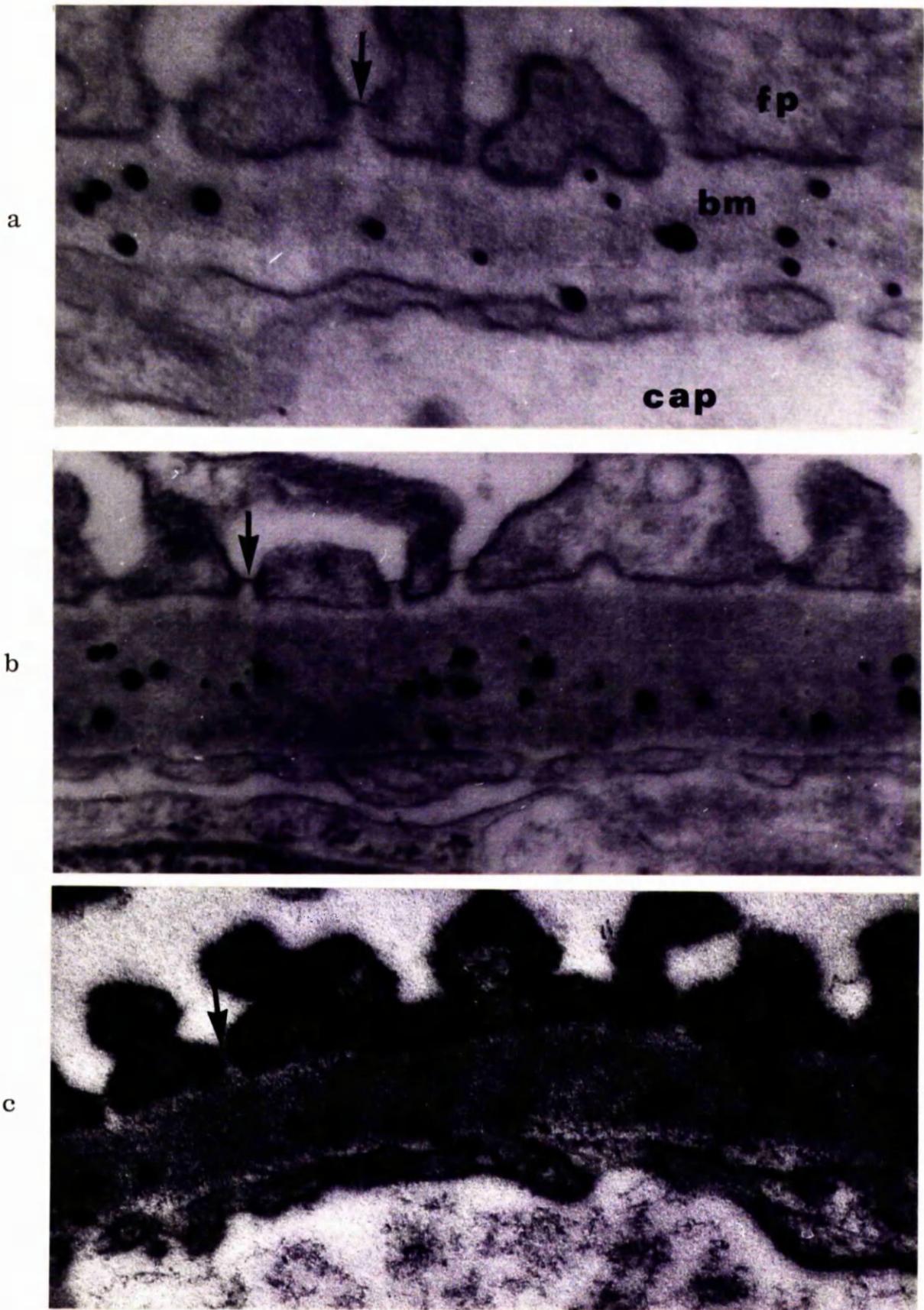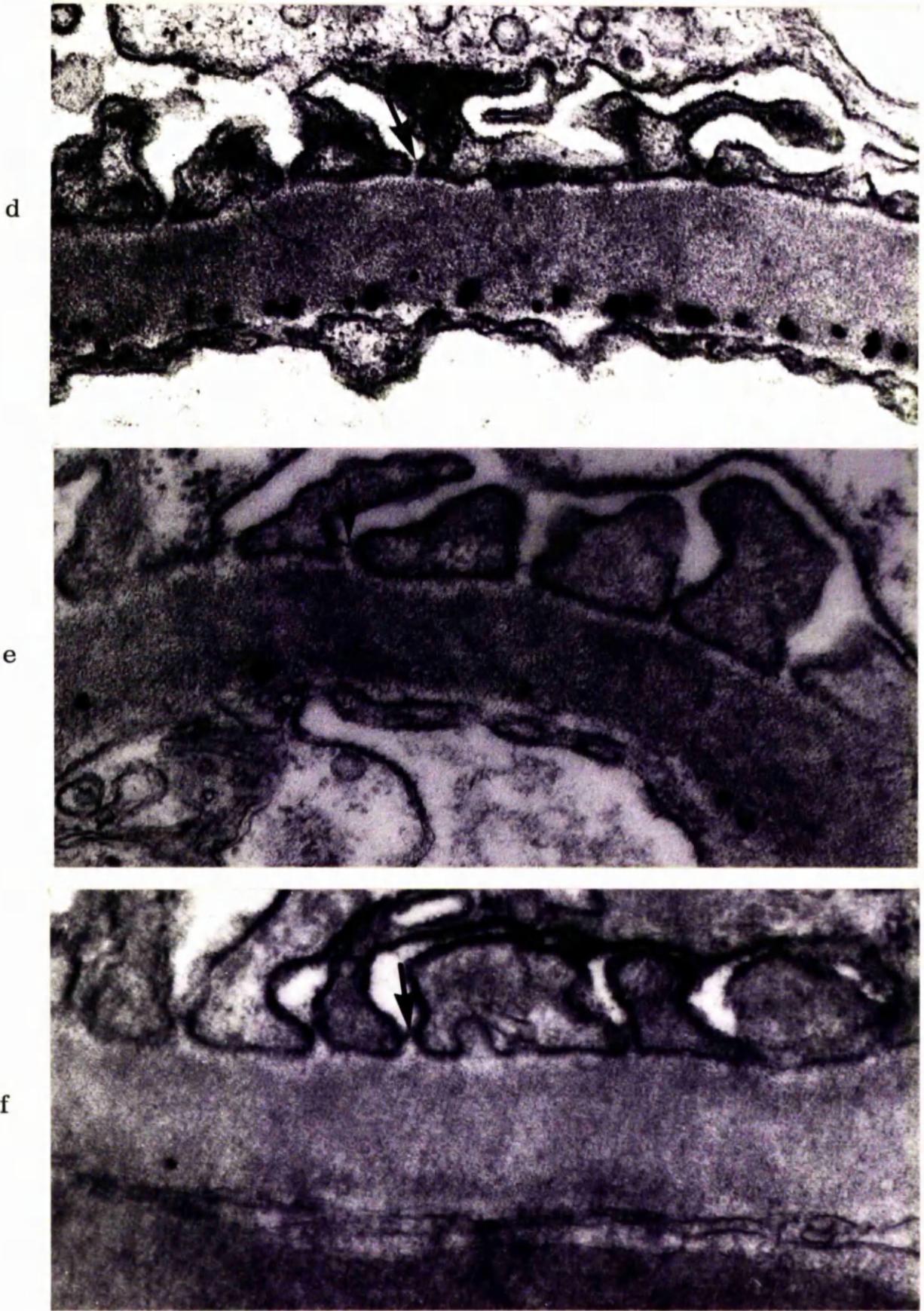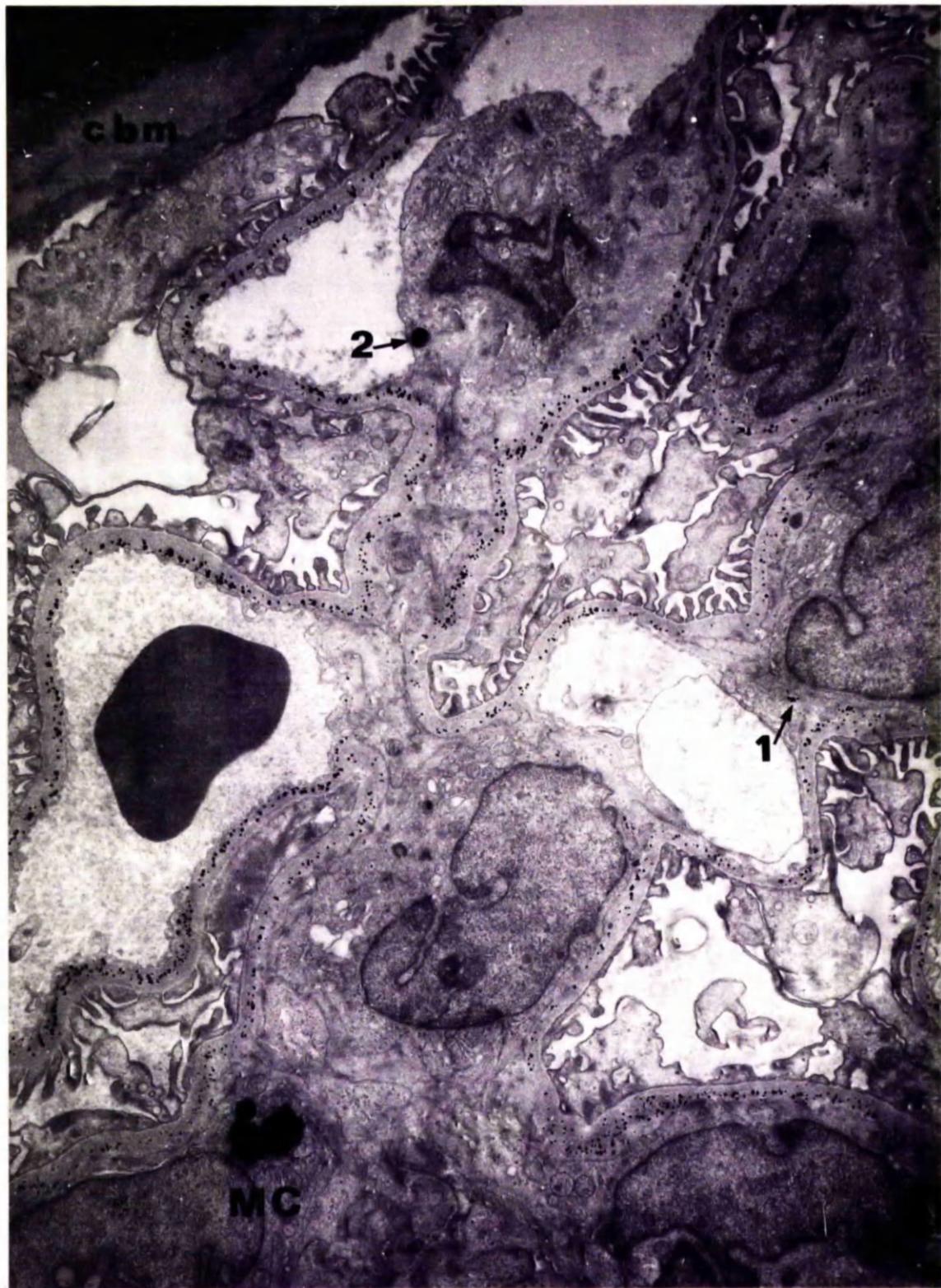
Figure 59

Figure 60. Low power photomicrograph showing typical appearances in the glomeruli of an argyric rat 83 weeks after ceasing to ingest silver nitrate. Silver is visible in the mesangial cell in the lower left corner. Elsewhere silver granules are very few and far between and are situated near the inner border of the lamina densa (arrows). X 8,000
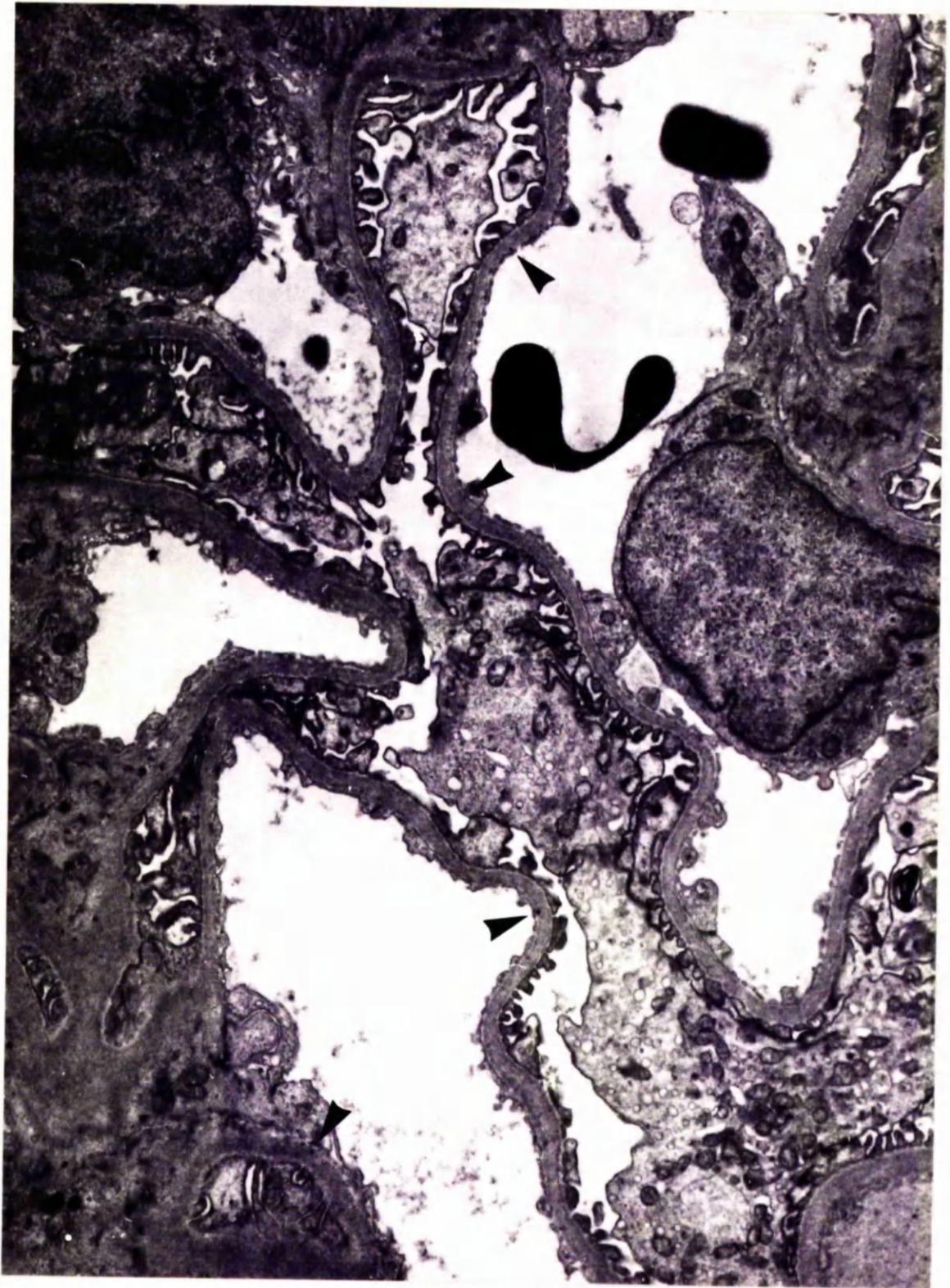
Figure 60

Mesangial matrix with silver granules was initially present in pockets or invaginations of the plasma membrane of the mesangial cell. These invaginations appeared to become vesicles containing matrix and silver granules. The matrix disappeared and concentrated aggregates of silver granules remained in the membrane bound vesicles. The membrane bound structures usually contained only silver but some contained, in addition, other electron dense material. On morphological grounds alone these heterogeneous organelles conform to the general pattern of lysosomes and residual bodies (Figure 61). In a few of the later specimens aggregates of silver granules were observed free in the cytoplasm of the mesangial cells.

In so far as quantitative estimates can be made of such specimens, as the amount of silver in the basement membrane decreased so the amount of silver in the mesangial cells increased up to the 20th to 30th week of observation. Thereafter the amount of intracellular silver slowly decreased but even in the 90 week specimen considerable deposits were still present within the mesangial cells. Attempts to find other sites of silver deposition in and around the glomerulus, both by electron and dark field optical microscopy, to account for this loss of silver from mesangial cells were un-

Figure 61. Incorporation of silver granules by mesangial cells. These illustrations are from different specimens and are presumed to show sequential stages of the ingestion process.

(a) Silver granules in mesangial matrix which is invaginated, or incorporated into a pocket, into the mesangial cell. X19,000

(b) Silver granules in a vacuole inside the mesangial cell. The other vacuolar contents are similar to mesangial matrix. X19,000

(c) Membrane-bound body partly filled with large silver aggregates. The other contents of the body appear to be breaking down. X22,000

(d) Concentrated aggregates of ingested silver in membrane-bound body (arrow 1) and apparently free in the cytoplasm (arrow 2). X22,000
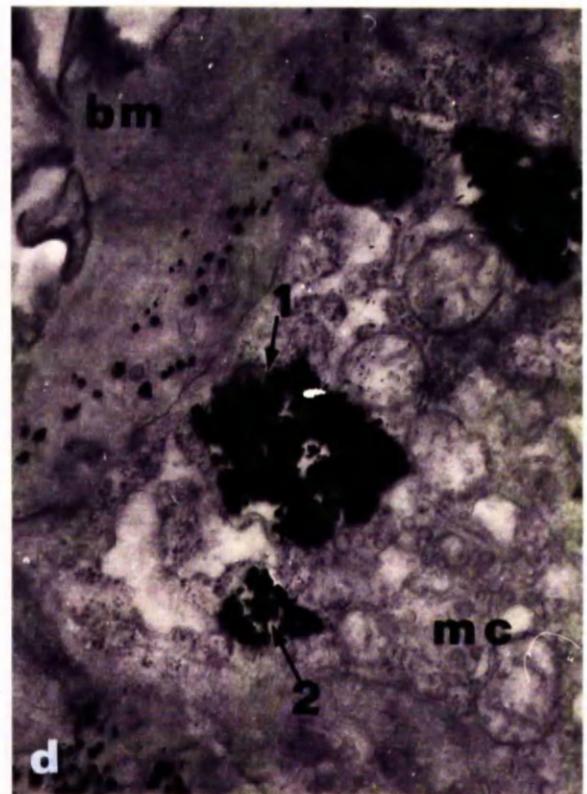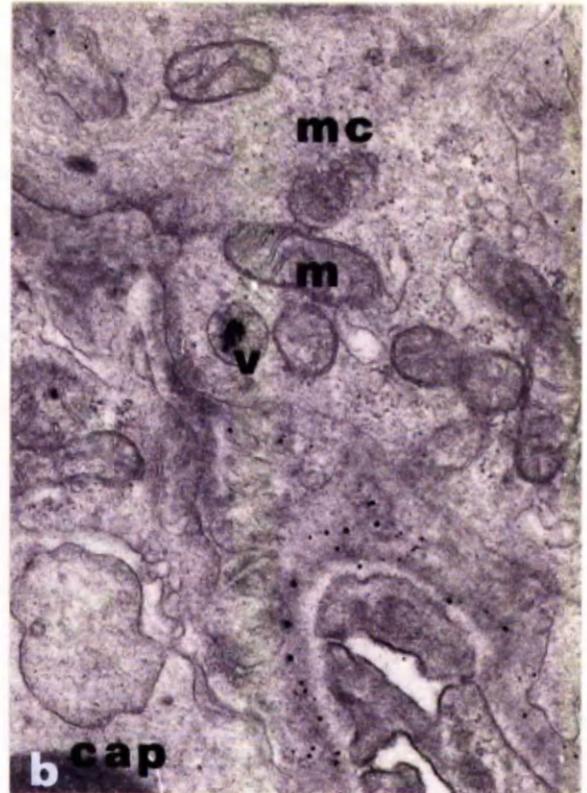
Figure 61

productive. The only point of possible note which emerged from this search was that intraluminal pseudopodia or "colliculi" from the mesangial cells (Figure 62) were much more readily observed in all of the argyric animals compared with non-argyric control animals. However silver was never observed in these pseudopodia.

Silver in the endothelial cells was always small in amount, membrane bound and in the perikaryon. The means by which the silver gained access to these cells was not observed.

In both the argyric and the non-argyric control animals the glomerular basement membrane was observed to thicken slightly with age and progressively more fibrils of a striated collagen pattern appear in the mesangial matrix.

Discussion

These results demonstrate that in the argyric rat silver granules are slowly and progressively cleared from the glomerular basement membrane, moreover this clearance is directional and proceeds from the outer, or epithelial aspect, to the inner, or endothelial aspect, of the basement membrane. This is the opposite direction to that taken by the glomerular ultrafiltrate as it passes from the capillary lumen to the urinary space. No
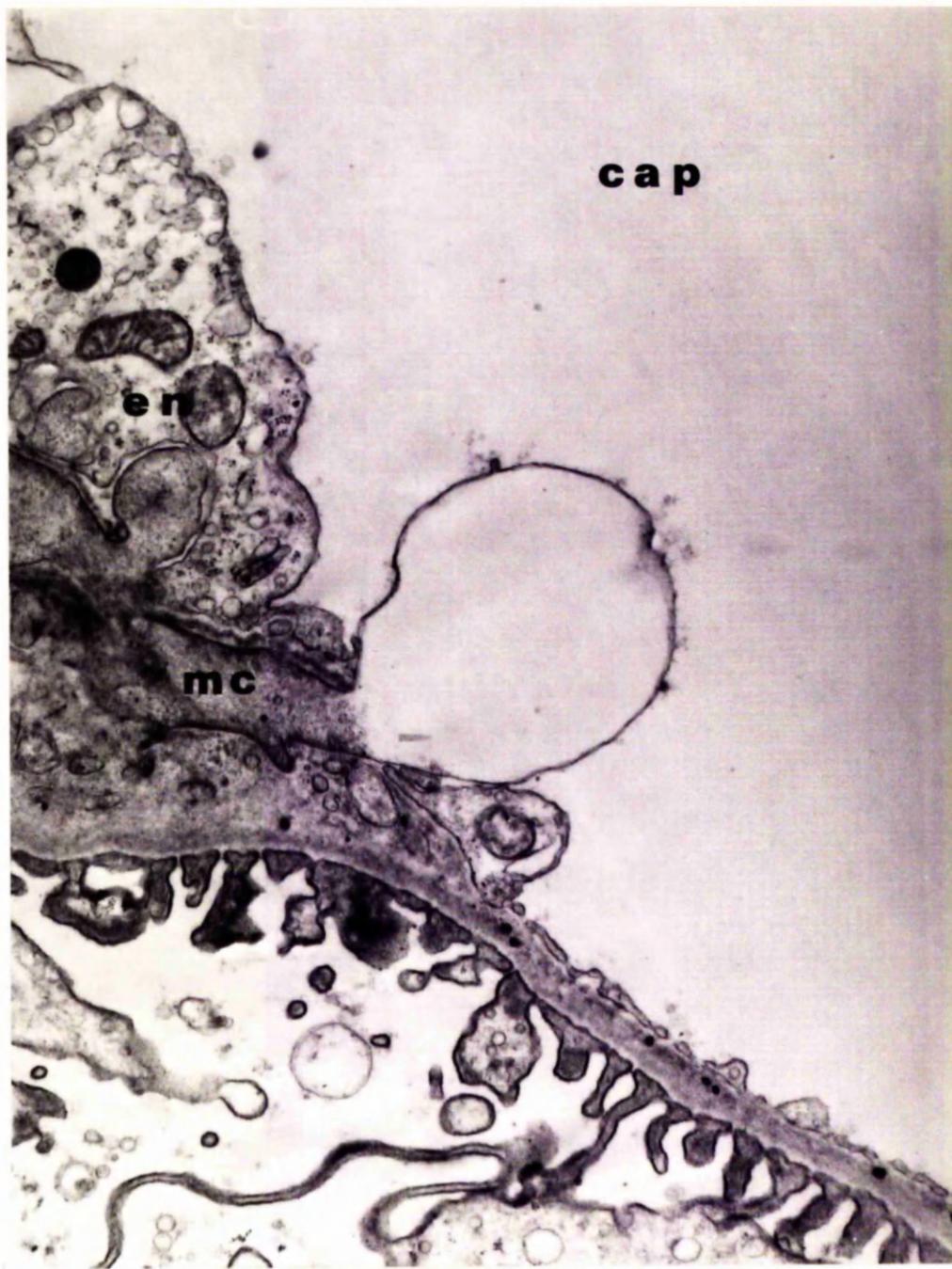
Figure 62. Intraluminal pseudopod or "colliculus" arising from mesangial cell. A silver granule is free in the mesangial cell cytoplasm but the pseudopod is devoid of contents. X16,000

reverse movement of silver granules, so that they come to rest against the filtration slit plate or within visceral epithelial cells, was observed. This is further evidence that the silver granules are firmly bound to basement membrane material.

As the silver is firmly bound to, and apparently an integral part of, the basement membrane, the movement of the granules indicates the movement of old, labelled, basement membrane and its replacement by new, unlabelled, basement membrane.

The observations clearly show that a component of glomerular basement membrane is secreted by the visceral epithelial cells. Secretion is effected by a coated pit mechanism which discharges into the basement membrane from the anchored 'sole' portion of the foot processes. This basement membrane material slowly migrates centripetally from the lamina rara externa to the lamina rara interna and is slightly retarded at the inner border of the lamina densa. Once past this region the basement membrane component passes, almost certainly by way of the lamina rara interna, to the mesangial matrix and is subsequently phagocytosed by the mesangial cells. This is a slow, continuous process without a sharp end-point and the time taken for the glomerular basement membrane to turn over completely is of the order of a year.

Previous studies using experimental argyric techniques have shown that new basement membrane material is laid down on the epithelial aspect (Kurtz and Feldman, 1962; Oshima, Hatano, Maeyama, Sugino and Takeuchi, 1967) but in neither of these studies was participation of the visceral epithelial cells, by way of the coated pits, observed. On the ultrastructural evidence that the visceral epithelial cells have a well developed endoplasmic reticulum and that the distended endoplasmic cisternae contain material with an electron density similar to basement membrane, it was suggested that the visceral epithelial cells secrete basement membrane material (Farquhar, Wissig and Palade, 1961; Thoenes, 1967). It has been demonstrated, using ferritin labelling techniques, that the contents of the endoplasmic cisternae and the glomerular basement membrane possess a common antigen (Andres, Morgan, Hsu, Rifkind and Seegal, 1962).

The experimental argyric technique because of the initial prolonged exposure to silver nitrate to cause adequate labelling and because of the lag phase when silver continues to be deposited after ingestion of silver nitrate ceases, is not suitable for accurate measurement of the time taken for the basement membrane to turn over. As an approximation, however, glomerular basement mem-

brane takes about a year to turn over completely. Such a turnover was not noted by Kurtz and Feldman (1962) who attributed the addition of new basement membrane on the epithelial aspect only to growth or to a pathologically reactive process, and did not consider turnover of the entire membrane. This conclusion was reasonable in their particular experimental circumstances - for the period of observation was very short, extending only from the eighth week of silver administration to the twelfth week after silver ingestion ceased - but incomplete considering the situation as a whole. Oshima and his associates (1967) did observe turnover of glomerular basement membrane and though their results are very briefly recorded they do note that "At 8 months, the granules had almost completely disappeared from the basement membrane.". This observation is in accord with those presented here.

The principle, though small, study on glomerular basement membrane turnover to date is that of Lazarow and Speidel (1964) who used an isotope labelling technique and found that normal rat glomerular basement membrane turned over and was completely replaced in less than 60 days. Though criticised (Spiro, 1964; Miller, 1964; Pressman, 1964) this study does not yet appear to

have been either extended or confirmed. Similar isotope techniques have been employed by Chow and Drummond (1969) and the results, though expressed in a different form, indicate that Lazarow and Speidel's figure of 60 days is conservative. The measurement of glomerular basement membrane synthesis and turnover using isotopic procedures is at present fraught with considerable technical and theoretical problems. This is reflected in the paucity of tracer and autoradiographic studies on the mammalian glomerulus though other extracellular matrices and other glycoproteins have been studied using these techniques (Hay and Revel, 1963; Neutra and Leblond, 1966).

Though direct evidence of glomerular basement membrane turnover is sparse, there is considerable indirect immunological evidence to indicate that replacement of the membrane takes a considerable time. Antibody to glomerular basement membrane once firmly attached has the long biological half-life of 20 days (Pressman and Yagi, 1964) and is still detectable in the glomerulus after a year or longer (Pressman, 1964). Similar studies have indicated the persistence of antibodies attached to the glomerular basement membrane for an observed period of 291 days and clearance was demonstrated in that by 8 months only about 10 per cent of the

amount originally present was found (Triedman, Metzger, Hsu, Rothenberg, Scegal and Urquhart, 1962; Scegal, Hsu, Rothenberg and Chapeau, 1962). These clearance figures for antibody firmly attached to glomerular basement membrane are very similar to the clearance estimates for silver attached to glomerular basement membrane observed in the present study.

For some considerable time after silver ingestion has ceased, silver granules continue to accumulate in the mesangial cells. This, more or less, is inversely proportional to the simultaneous clearance of silver from the basement membrane and is good evidence that the mesangial cells remove not only filtration residues but also old basement membrane as postulated by Farquhar (1964a). The incorporation of silver from the mesangial matrix into pockets which then become vacuoles which fuse with lysosomes and condense with the ultimate production of dense residual bodies is identical to that described and illustrated for filtration residues by Farquhar and Palade (1962). It has further been shown that the lysosomes contain enzymes capable of hydrolysing glycoprotein (Miller and Palade, 1964). However these organelles do not seem capable of breaking down the engulfed silver granules for these accumulate within the cell, occasionally free but usually in membrane bound

residual bodies, without evidence of degeneration of the cell.

The presence of silver deposits in mesangial cells has been variously recorded in other experimental argyric studies. Striker and Smuckler (1970) describe and illustrate silver in mesangial cells. Oshima and his associates (1967) do not mention silver in mesangial cells. Kurtz and Feldman (1962) say they found silver in the "interluminal or axial core cells". Olcott and Richter (1958) described silver in endothelial cells but illustrate it in a mesangial cell which is quite understandable as their study antedates the definite recognition of mesangial cells in electron microscopic specimens. Vernier (1964a) initially made no reference to silver in mesangial cells but subsequently (1964b) stated that silver was seen in 'intercapillary' cells.

Silver accumulation in mesangial cells was not observed to increase beyond the 20th to 30th weeks and thereafter steadily decreased. Mesangial cells therefore appear to have a mechanism for disposing of residues. There are several possible ways this could occur. The silver could be extruded into the capillary lumen via one of the numerous pseudopodia. The silver could be transferred to a neighbouring endothelial cell body and thence to the capillary lumen. The silver could be extruded into the

mesangial matrix and thence carried to the vascular pole and out-
with the glomerulus by the route proposed by Latta and Maunsbach
(1962). All these possible mechanisms for removing silver from
mesangial cells would probably take place within a short space of
time and would therefore be unlikely to be detected in an experi-
ment of the present design. Certainly neither electron nor dark
field microscopy of the present specimens yielded any evidence
on the ultimate fate of the silver granules. The conspicuousness
of the pseudopodia in these specimens, a feature not previously
noted in other renal tissue examined personally, slightly favours
this particular route for more detailed subsequent investigation.

The amount of silver in the endothelial cells was always small
and always in the perikaryon. It is well established that endothelial
cells can take up particulate matter (Florey, 1967) and it is further
recognised that this uptake can be enhanced in the glomerulus by
blockade of the reticulo-endothelial system for instance by thorium
dioxide (Benacerraf, McCluskey and Patras, 1959). It is there-
fore likely that the silver phagocytosed by the mesangial cells pro-
duced a mild degree of reticulo-endothelial blockade in consequence
of which some silver particles in the mesangial matrix were taken
up by the underlying endothelial, rather than by the overlying mes-

angial, cells.

Throughout the entire period of observation there was a slow steady movement of silver granules from the outer, towards the inner, surface of the basement membrane. Slight retardation occurred not at the surface of the endothelial cells but at the inner border of the lamina densa. Moreover accumulation of silver granules occurred in the mesangial matrix and cells and not in the lamina rara interna. This is most readily explained if the lamina rara interna is a functionally separate layer from the lamina densa. The existence of such a layer has been surmised from ultrafiltration tracer studies using ferritin, thorotrast and peroxidases (Farquhar, Wissig and Palade, 1961; Latta and Maunsbach, 1962; Graham and Karnovsky, 1966) to account for the sweeping of filtration residues to the axial region. It is reasonable to suppose that such a layer also sweeps old basement membrane, and contained silver granules, in the same way. To perform effectively this layer must move relatively rapidly, very much more rapidly - hours or days - than the time scale of synthesis and turnover of the basement membrane component demonstrated in the present experiment. This strongly suggests that a second component is present in glomerular basement membrane.

Such a component would have to be electron-lucent, or readily extractable by the standard electron microscopic preparative techniques, to account for the ultrastructural appearances of the lamina rara interna. Such a component would have to turn over fairly rapidly to clear residues from the inner surface of the lamina densa, and would be markedly diluted with serum both from the fenestrae and the discharged pinocytotic vesicles in the endothelium. Such a component would have little structural integrity compared with the major, silver labelled, epithelial derived component.

The presence of this second component, even with the present inadequate data, poses no serious conceptual problems as regards origin, function or even composition. Using immunological techniques such a component, a vascular basement membrane antigen, has been demonstrated (Pierce, Midgley and Sri Ram, 1963; Pierce and Nakane, 1967) and is of presumed, though not proven, endothelial origin. However, major conceptual difficulties do arise when attempts are made to visualize the structural relationship this second component has with the main, epithelial derived component (Kurtz, 1964). This particular problem demands considerable further experimental investigation but some tentative proposals concerning the relationship of the two components are

made in part V of this thesis.

Experiment 2.

The basement membrane turnover observed in the previous experiment might be a once and for all phenomenon which occurs during the growth phase of the rat. To investigate this possibility the experiment was repeated on an older group of rats.

Materials and Methods

A group of male, Sprague-Dawley rats, litter mates of those used in experiment 1, were allowed to age for one year before being given standard silver nitrate drinking fluid. The experiment was thereafter identical to that previously described except that a smaller number of animals was used and the animals were examined 0, 4, 8, 16, 32 and 52 weeks after ceasing to ingest silver nitrate.

Results

The distribution of the silver granules in the individual specimens is summarized in the table.

Weeks on tap water	0	4	8	16	32	52
Visceral epithelial cells	-	-	-	-	-	-
Visceral epithelial "fuzz"	+	+	-	-	-	-
Foot process pits	+	+	-	-	-	-
Lamina rara externa	+	+	-	-	-	-
Lamina densa (outer)	+	+	+	+	-	-
Lamina densa (inner)	+	+	+	+	+	+
Lamina rara interna	+	+	+	+	+	-
Mesangial matrix	+	+	+	+	+	±
Mesangial cells	+	+	+	+	+	+
Endothelial cells	+	+	+	+	+	-

The pattern of silver distribution and the sequence of silver

clearance from the basement membrane was the same as that in

the previously examined young animals.

Discussion

These results indicate that glomerular basement membrane

secretion, turnover and removal is a continuous process which

occurs not only in young, growing rats but also in mature, adult

rats.

Experiment 3.

The decision to use animals which had been ingesting silver

nitrate for 10 weeks as the standard argyric model was made because

of the essentially practical and experimentally proven reasons that

the silver deposits in such animals are reasonably uniform, are

distributed according to a standard pattern, do not distort neighbour-

ing structures and are readily detectable by optical and electron microscopic techniques. That such an experimental model is effective was demonstrated in the previous experiments. There remains the possibility, however remote, that the observed pattern of basement membrane behaviour was, in part, determined by the presence of the silver marker. This is merely a particular application of the general proposition that the experimental procedures necessary to detect certain biological phenomena may alter the phenomena which are being observed. Expressed in practical rather than philosophical form the question is, does the presence of the silver interfere with the normal behaviour of the glomerular basement membrane? In so far as such a question can be answered on internal experimental evidence alone, there is nothing to suggest that the silver deposits interfere with the behaviour of the glomerular basement membrane in the standard 10 week argyric rat.

From the studies on silver deposition described in part II of this thesis it is however evident that excessive amounts of silver, because of aggregation of granules and distortion of neighbouring structures, particularly endothelial cells, might well fail to reflect a normal basement membrane behaviour. From the same studies

it is also evident that lesser amounts of silver might still be an adequate tracer for electron microscopic though not for optical dark field observations. These assumptions were experimentally tested partly to determine the clearance pattern of different amounts of silver and partly to facilitate interpretation of the subsequent cases of human argyria for which no standards were available.

Materials and Methods

Two small groups of male, Sprague-Dawley rats aged 6 weeks at the commencement of the experiment were given 0.25 per cent silver nitrate drinking fluid: one group (a) for 5 weeks, the other group (b) for 25 weeks. At the end of these time periods the rats were returned to ordinary tap drinking water and glomeruli were examined at appropriate intervals over the following 16 months as described for the previous experiment.

Results

(a) Rats on 0.25 per cent silver nitrate for 5 weeks.

The amount of silver deposited and the size of the individual granules were less than in standard 10 week argyric rats, but the distribution and clearance patterns were otherwise identical to those described in experiments 1 and 2. One minor exception was that relatively very little silver was present in the endothelial

cells.

(b)   Rats on 0.25 per cent silver nitrate for 25 weeks.

The amount of silver deposited and the size of the individual granules were greater than in standard 10 week argyric rats. As time progressed there was considerable aggregation of individual granules into groups in the inner half of the lamina densa (Figure 22).   The diameter of these aggregates exceeded the width of the lamina rara interna and aggregates were observed impacted between the lamina densa and the endothelium, and herniating through the fenestrae (Figure 63).   Silver was constantly observed in mesangial and endothelial cells but the amount present in these locations was less than anticipated from the large amount of silver present in the basement membrane.

The overall deposition and clearance pattern was similar to that in the standard 10 week argyric animal only retardation in the inner border of the lamina densa was more pronounced. Furthermore the silver pattern was not as uniform as the standard argyric animal and different amounts of silver were observed in adjacent capillaries (Figure 64) in the intermediate clearance stages.   Complete clearance of silver from the basement membrane did occur and the last rat in this particular group, examined

Figure 63. Silver granule about to herniate an endothelial fenestra. X 22,000. See also figure 47.

Figure 64. Glomerulus from a heavily argyric rat showing non-uniform pattern of silver clearance. The distribution of silver in these adjacent capillary loops is essentially similar but there is a considerable difference in the density of the granule population. X 7,000
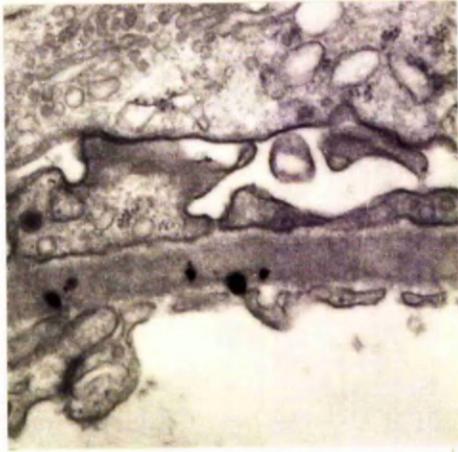
Figure 63
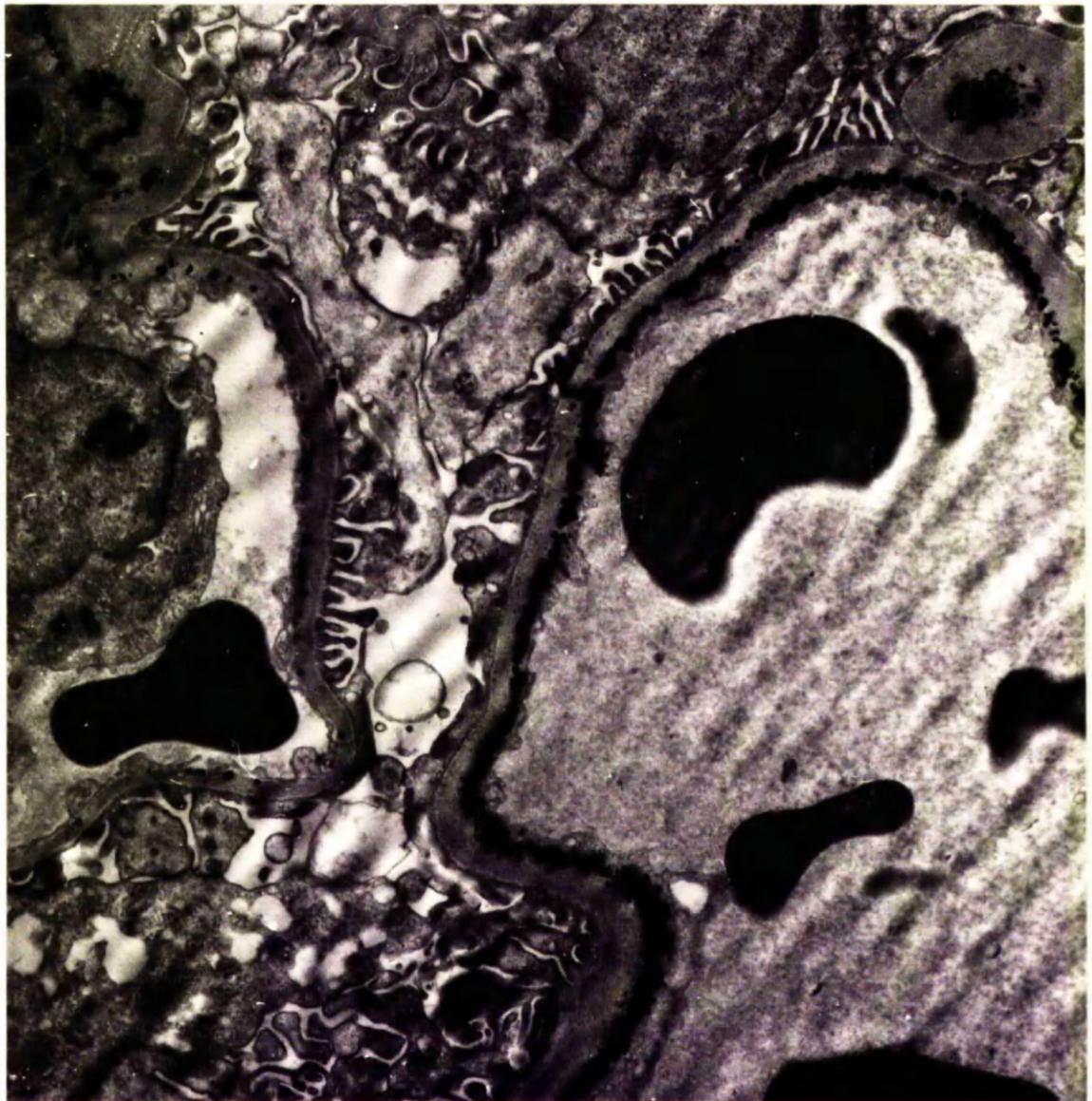
Figure 64

67 weeks after ceasing to ingest silver was similar to figure 60.

Discussion

The pattern of basement membrane synthesis, turnover and removal in argyric rats exposed to silver nitrate for 5 weeks is identical to that observed in standard argyric rats exposed to silver nitrate for 10 weeks.

The pattern of basement membrane synthesis and turnover in argyric rats exposed to silver nitrate for 25 weeks is similar to that observed in standard argyric rats exposed to silver nitrate for 10 weeks but the ultimate removal of silver granules from the basement membrane is different. The amount of silver in the mesangial and endothelial cells of these heavily argyric animals was less than expected relative to the amount of silver being cleared from the basement membrane. Furthermore the diameter of the large silver aggregates in these animals considerably exceeded the width of the lamina rara interna and were too large to be swept along this layer to the mesangial region. These large aggregates instead become impacted between the inner border of the lamina densa and the endothelial cells and appear to be eventually extruded through distorted fenestrae, into the capillary lumen.

It seems very unlikely that this particular route for removing

silver from glomerular basement membrane is of any physio-
logical importance. Conceivably it could operate in certain
pathological circumstances as a route for the removal of exception-
ally large filtration residues, or deposits such as fibrin and antigen-
antibody complexes.

From the results of these two small series of animals it is
concluded that experimental argyria as a model for studying normal
glomerular basement membrane synthesis and turnover, as dis-
tinct from removal, is valid for a wide time range of silver ingest-
ion. The experimental model is valid for studying normal glomer-
ular basement membrane removal only if the silver granules are
sufficiently small as not to physically impede the normal route
of removal. In practical terms, the size of the silver granules
must be small enough to pass along the lamina rara interna.
Five and 10 week argyric animals fulfil this criterion, 25 week
argyric animals do not.

Conclusions

In the rat a major component of renal glomerular basement
membrane is secreted by the visceral epithelial cells and is removed
by the mesangial cells. This is a slow, continuous process which
is not restricted to the period of growth of the animal and the time

for complete turnover of the membrane is about 12 months.

This basement membrane component is secreted by a vacuolar/coated pit mechanism, moves in a direction contrary to the flow of the glomerular ultrafiltrate, and is removed by a phagosome mechanism.

It is possible that in certain pathological circumstances some effete basement membrane material or large filtration residues may be extruded into the lumen of the capillary by way of the endothelial fenestrae.

The experimental data further indicate, but do not prove, the existence of a second, or minor, basement membrane component probably of endothelial origin which has a faster turnover rate than the major, epithelial cell derived, component.

Part IV

Human Glomerular Basement Membrane

While the existence of a turnover process for glomerular base-
ment membrane in the rat is of some biological interest, it is of
little medical significance unless there are reasonable grounds
for supposing that a similar process occurs in humans.   Since
silver is a label which occurs both in man and rat certain limited,
but nonetheless significant, direct comparisons can be made.

Since argyria does not cause any renal malfunction in humans
there is no justification for performing renal biopsies in such
patients.   Accordingly studies on the distribution of silver in
human glomerular basement membrane have to be performed on
tissue obtained post mortem.

The first reported case of pigmentation attributed to ingestion
of silver is probably that of Avicenna in 980, and the first undis-
puted case is that of Angelus Sala in 1647.   Several isolated case
reports appeared in the eighteenth century and the first small series
was described by Butini in 1814.   Shortly thereafter argyria was
positively identified and apparently widely recognised in many
European countries.   Early reports in English are those of Albers
(1816), Roget (1816) and Badeley (1818).   Of particular interest,

and not for parochial reasons, is the case described by Buchanan
(1831) in the Glasgow Royal Infirmary.   A variety of therapies
based on the then known physiological and chemical data were ap-
plied in an effort to remove the silver.   The patient was long
suffering and the attempts were unsuccessful.

The first post mortem on a case of argyria was reported by
Lelut (1830) but nearly 30 years elapsed before the microscopic
appearances of such a case were recorded (Frommann, 1859).
A particularly clear and well illustrated account of the gross and
histological appearances in a case of argyria is the paper by Gettler,
Rhoads and Weiss (1927).   These authors also review the pre-
viously published cases of argyria with post mortem examinations
but incorrectly attribute the first to Frommann and not to Lelut.*

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*   The early literature on human argyria contains numerous incid-
ental, but nonetheless informative, comments.   Albers tried to
trace an earlier English reference and records "Professor Reurs
of Gottingen, who is so eminent for literary erudition, likewise
hunted for it, but without any better success.".   Lelut's case had
been treated with silver nitrate because of epilepsy.   The illness
was attributed to the fatigues and terrors of war as the patient had
served in the Imperial Guard during the Napoleonic Wars.
Buchanan's case had also been treated with silver nitrate because
of epilepsy which developed after he fell from a horse and fractured
his skull.   The patient first noticed his unusual pigmentation only
on Sundays, this being the day on which he washed.   Buchanan was
sufficiently intrigued that he called in an artist and had the patient's
portrait done in oils.   Frommann's case was reported not from
Germany but from London.   Badeley, apart from describing a clear

case of argyria including dose and time of exposure to silver nitrate, digresses on more than one occasion to the subject of air pollution. The case described by Gettler and his associates was the famous "blue man" of the Barnum and Bailey circus.

Materials and Methods

Argyria is a rare condition and post mortems on such cases are even more rare so there was no reasonable probability of obtaining a series of specimens. It appeared that the best that could be hoped for was tissue from proven cases of argyria, free of renal disease (a) who died while still taking silver or shortly after ceasing to take silver and (b) who died a considerable time, preferably more than a year, after ceasing to take silver. On the basis of the rat experiments it was hoped that the first of these specimens would contain silver and the second would be largely clear of silver.

A search for suitable material was therefore instituted. Two unexpected problems complicated this search. Cases of argyria seem to have an intrinsic appeal to pathologists for though reports can be traced in the files, slides and blocks can not be traced in the stores. It appears that such tissues are the sort that lie around individual pathologists' rooms awaiting later detailed examination but suffering earlier unintentional loss. The second prob-

lem is that reliable information concerning details of silver in-
gestion is particularly difficult to obtain in many cases of argyria.
The psychological and communication reasons for this are des-
cribed and discussed by Brinton (1949).   Eventually, thanks to
the good memories and efforts of Dr. J. R. Dawson of Minneapolis
and Dr. J. G. Simpson of Aberdeen, two suitable specimens were
obtained.

Case 1.   This was a man in his late fifties who was admitted with
cardio-respiratory failure and was incidentally found to be argyric.
He had been ingesting a proprietary silver preparation for about a
year and his skin pigmentation was of recent onset.   He died 12
weeks after admission.   During this 12 week period he was con-
tinuously in hospital and did not ingest any silver.

A post mortem examination was performed 14 hours after death.
The glomeruli were visibly dark to the unaided eye but there was
no other gross or histological evidence of renal disease.

The specimen which was received consisted of the shavings of
tissue retained when the original block of kidney was trimmed prior
to embedding.   These shavings had been in unbuffered formalin
fixative for 23 years.   The specimen was thoroughly washed with
water, cut into 1 mm^3 blocks, post fixed in phosphate buffered

osmium tetroxide and embedded in Epon.

Case 2. This was an 86 year old man who died of broncho-
pneumonia. He had suffered from epilepsy for very many years
and had spent much of his working life in Australia where he had
been treated with a "metallic compound". Argyria was diag-
nosed 14 years prior to his death. During these 14 years he
was regularly medically supervised, had several hospital admis-
sions, was treated with phenobarbitone and anti-convulsant drugs
and was not exposed to silver.

A post mortem examination was performed 5 hours after death.
The glomeruli were not visible to the unaided eye and there was
no gross or histological evidence of renal disease.

The specimens received consisted of the neutral phosphate-
buffered formalin-fixed blocks taken post mortem and embedded
in paraffin. The tissue was deparaffinised, 1 mm^3 blocks were
cut, post fixed in veronal buffered osmium tetroxide (Palade,
1952) and embedded in Epon.

Review of the original skin biopsy and post mortem tissues by
bright and dark field microscopy (Figure 65) and treatment of these
tissues with iodine and sodium thiosulphate confirmed that this
was a genuine case of argyria.

Figure 66. (a) Original skin biopsy from case 2. There is a dense silver deposit especially in the basement membrane around sweat glands. Dark field, H & E, X 340

(b) Original post mortem liver section from case 2. There is a dense silver deposit in the wall of the portal vein and silver granules are present intra- and extra-cellularly elsewhere in the portal tract. Dark field, H & E, X 340. Note that different eosins give different dark field colours.
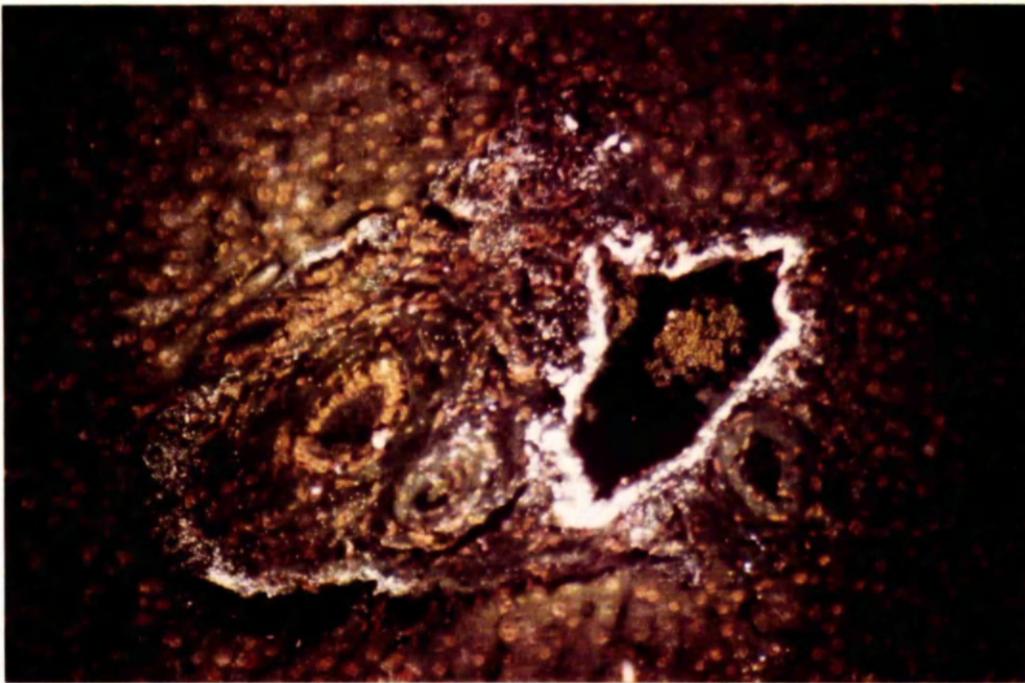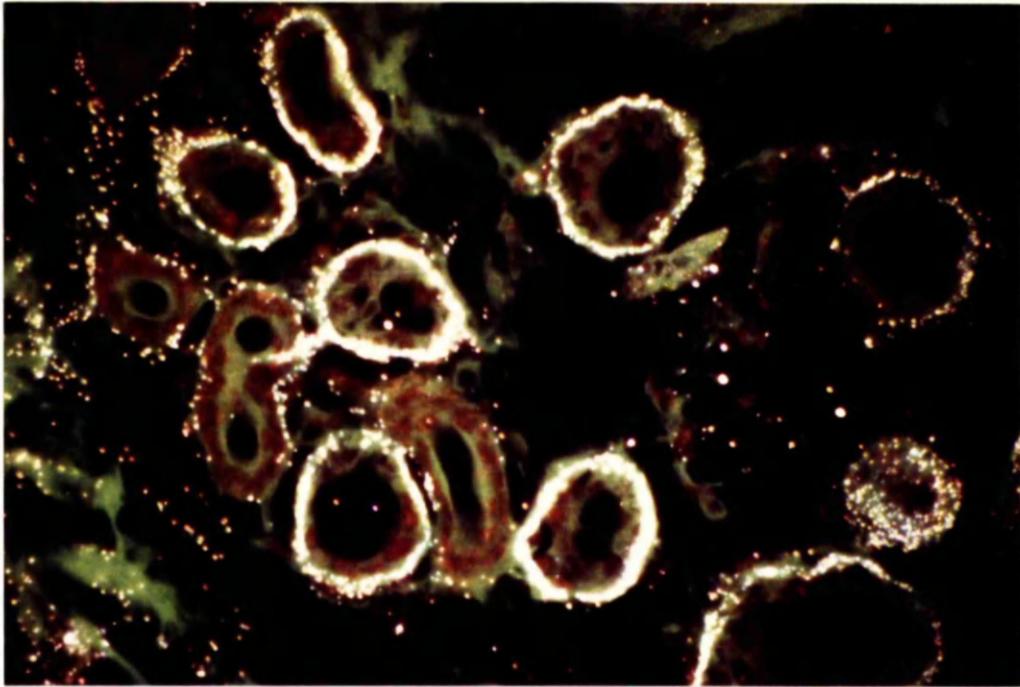
Figure 65

Results

Case 1.   Preservation of this tissue was very poor but glomer-
ular basement membrane was clearly identifiable and contained
numerous silver granules.   Silver granules were also present
intracellularly but it was not possible to identify the cell type
with any certainty (Figure 66).

Case 2.   Both bright and dark field optical microscopy of the
glomeruli failed to demonstrate any silver in the glomerular base-
ment membrane.   Very small deposits of silver were present
intracellularly, notably in the axial region (Figure 67).

Preservation of this tissue for electron microscopy was slightly
better than case 1, but still far from good.   Glomerular basement
membrane was clearly identifiable and contained no silver deposits.
Intracellular silver deposits were very sparse indeed and the cell
type could not be identified with certainty (Figure 68).

Discussion

The presence of silver deposits in the glomerular basement
membrane in human argyria is well established.   There are good
bright field illustrations of argyric glomeruli in the reports by
Gettler, Rhoads and Weiss (1927) and by Christensen, Jorgensen
and Ohlsen (1964).   The former authors further state that "in

Figure 66.   (a) Silver granules in glomerular basement membrane in human argyric case 1.   Tissue preservation is very poor but glomerular basement membrane is recognisable and the silver granules are similar to those previously observed in the rat.   X 27,000

(b) Silver granules in glomerular basement membrane and what is probably mesangial matrix.   X 20,000
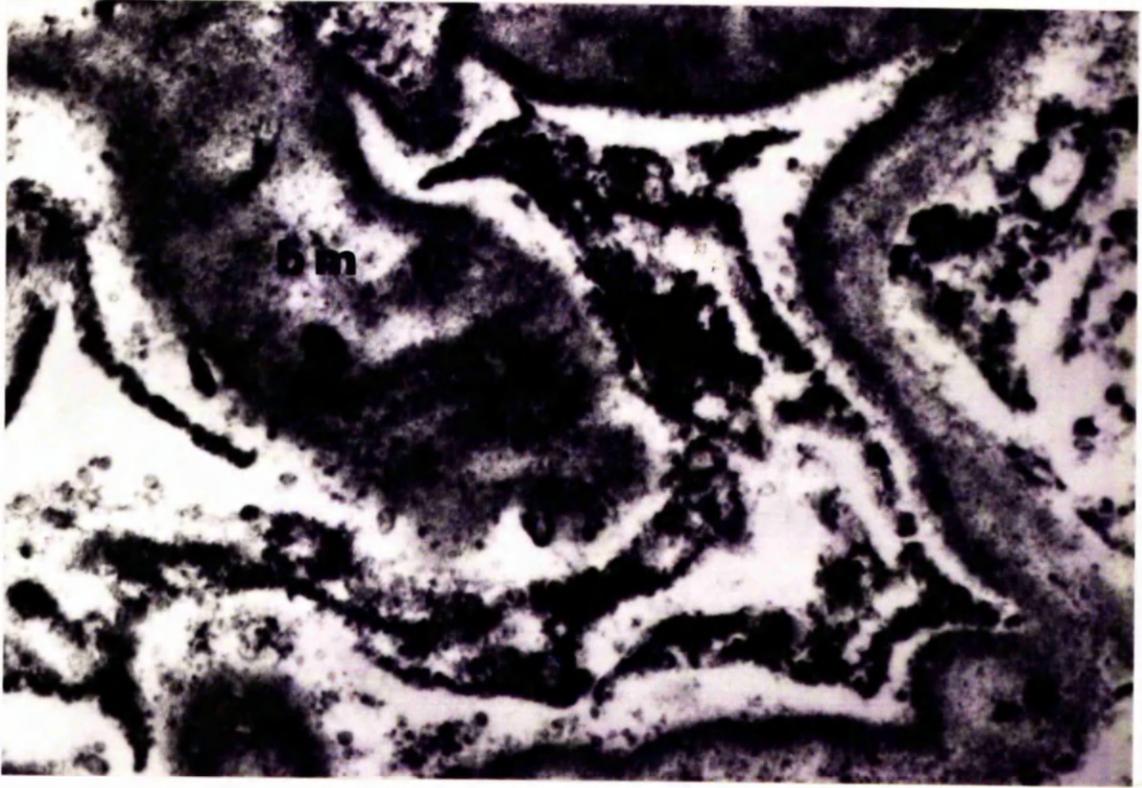
Figure 66 a
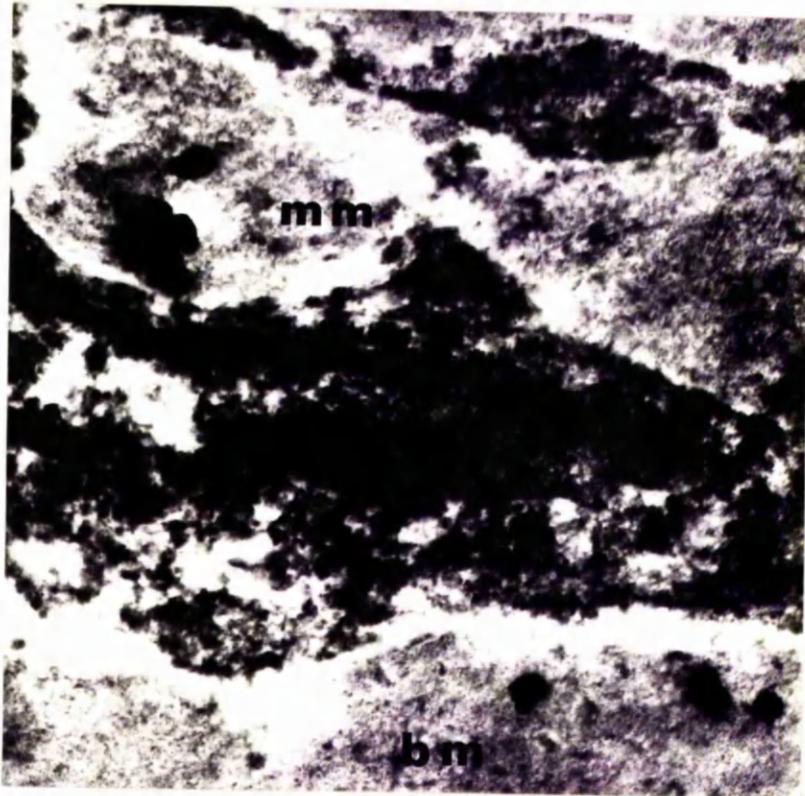
Figure 66 b

Figure 67. Glomerulus from human argyric case 2 showing absence of silver deposits from the basement membrane. A few, small, isolated deposits are present intracellularly, probably, but not definitely, in mesangial cells. (a) bright field, (b) dark field, X 340

a
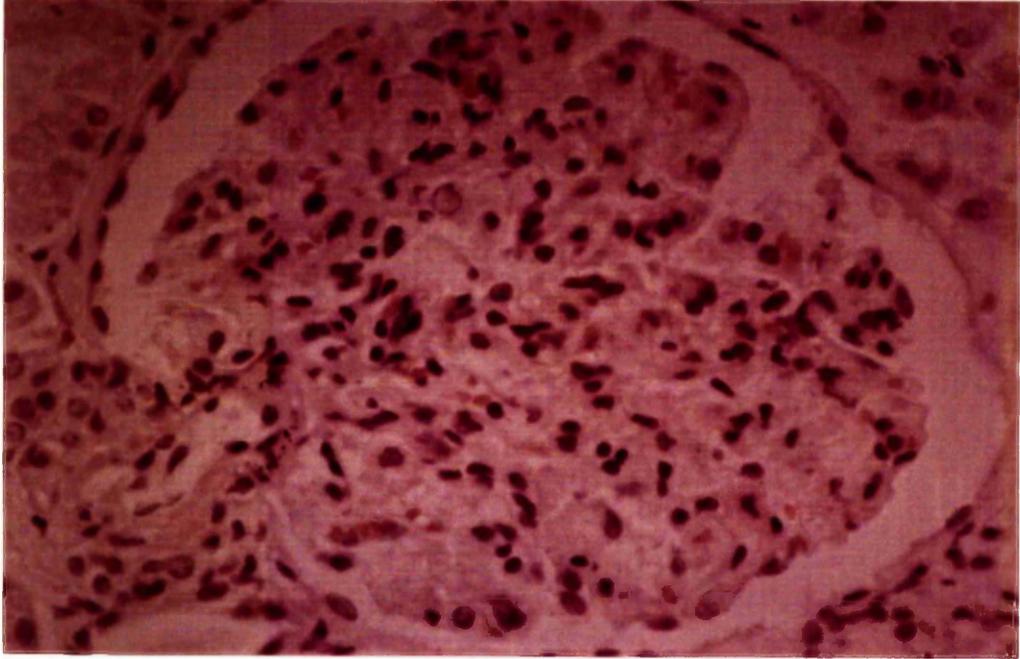
b
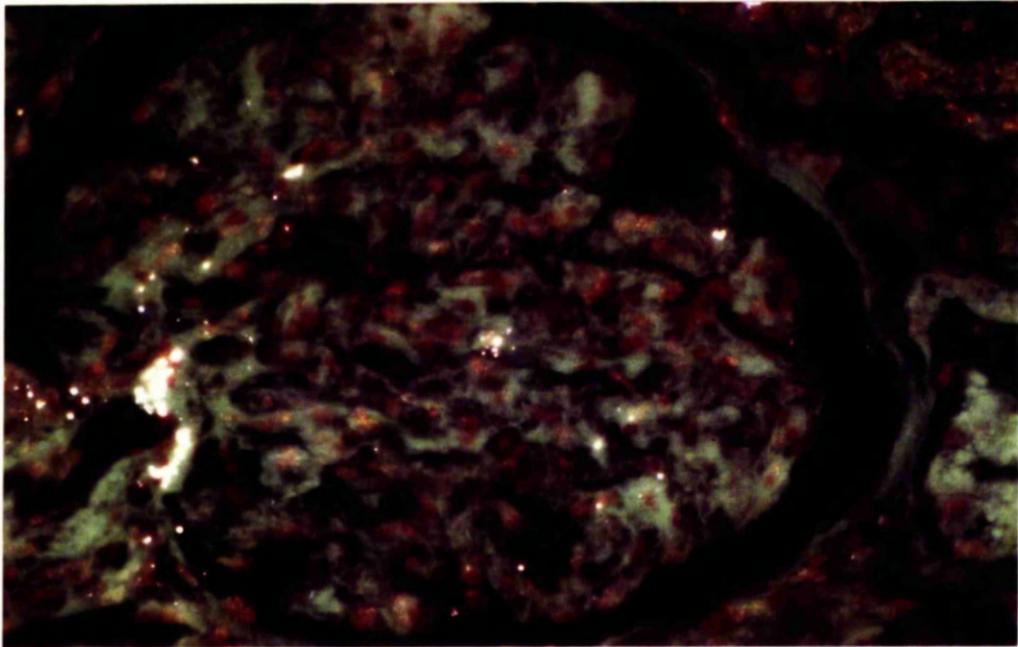
Figure 67

Figure 66. (a) Low power photomicrograph showing general features of glomerulus from case 2. Capsular basement membrane and glomerular basement membrane are readily identified. Red blood cells and a polymorph are present in the capillaries. The epithelial cells are markedly autolytic. Endothelial and mesangial cells cannot be positively identified but the two cells indicated are tentatively recognised as mesangial cells (MC). X 5,000

(b) Glomerular basement membrane. There are no silver granules. X 26,000

(c) Small, focal collection of silver granules found in intercapillary location. Tissue preservation is so poor the locus cannot be identified from ultrastructural features but the topography suggests this deposit is in mesangial cell or mesangial matrix. X 26,000

Figure 68 a
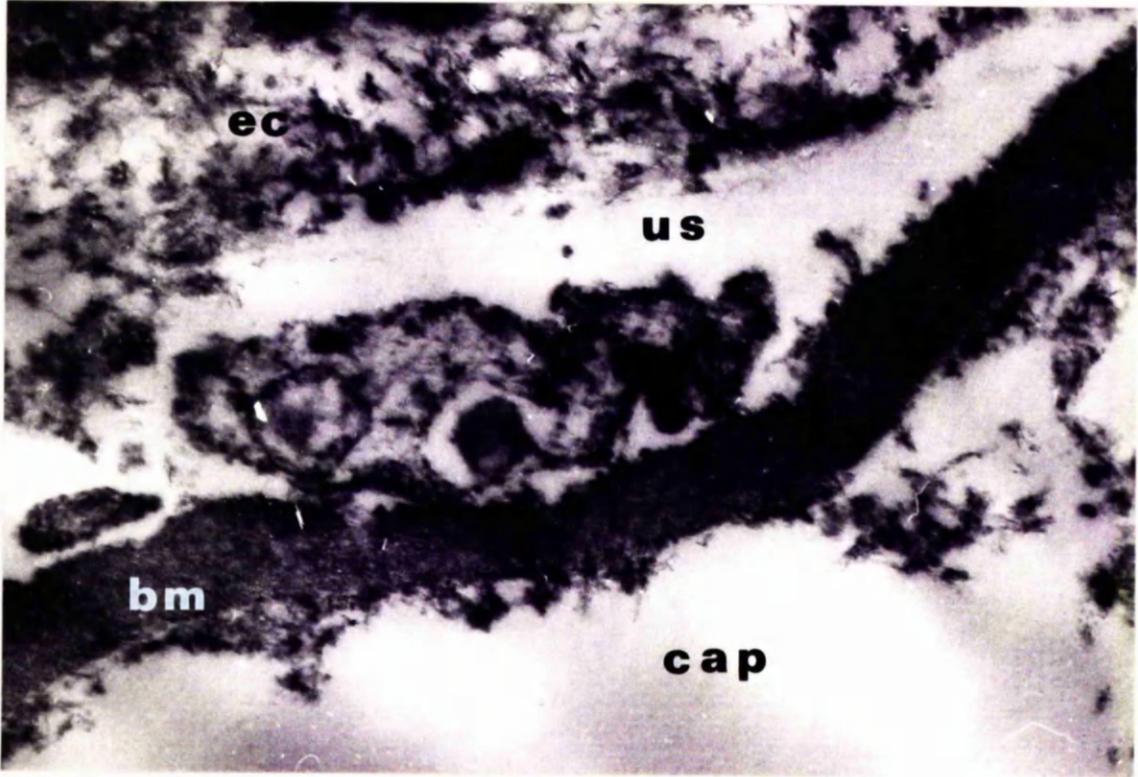
Figure 68 b
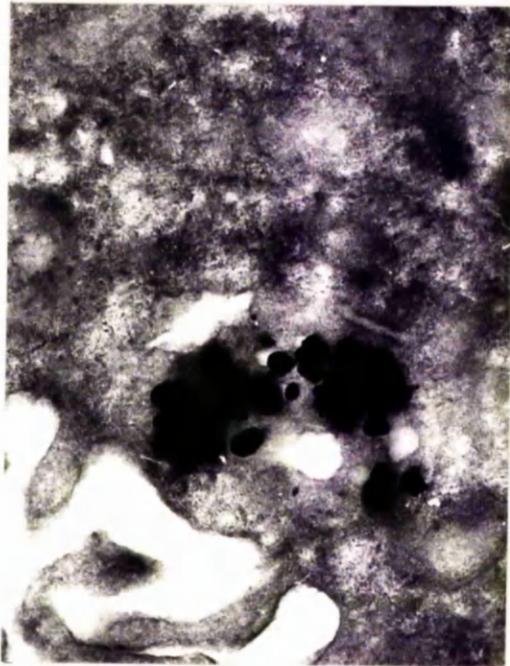
Figure 68 c

some places it is difficult to say that the metal is not actually in the endothelial cells". However the illustrations of Gettler and of Christensen, and the electronmicrographs depicted here, are dismal compared with the illustrations in the paper by Jahn (1894), reproduced in figure 69. These clearly show silver in the glomerular basement membrane and with hindsight, in the mesangial matrix and mesangial cells. Case 1 is in accord with these findings.

It is not well established that in some cases of human argyria the glomerular basement membrane is free of silver deposits. Several reports of post mortems on cases of argyria do not specifically mention the glomerulus but there appears to be only one tenuous reference to the effect that glomeruli in such cases can be free of silver. Hill and Pillsbury (1939) state that Moslener in his Inaugural Dissertation at Kiel in 1889 quoted Kahlden as having reported such a case. Dohi (1908) refers to this same case.

There is only a single report of the electron microscopic examination of the kidneys in a case of argyria. This is the paper entitled "An Electron Microscopic Study of Human Generalised Argyria" by Prose (1963). This paper has five illustrations, four of a skin biopsy and one of a renal glomerulus. This last

Figure 69. Human argyric glomerulus showing silver granules in basement membrane and in mesangial regions, a = epithelial cell, b = endothelial cell. Bright field microscopy, original magnifications not stated but appear to be of the order of X 300 and X 900 respectively. Reproduced from Jahn (1894).

Fig.3.

b

a

b

a

a

b

Fig.4.

b
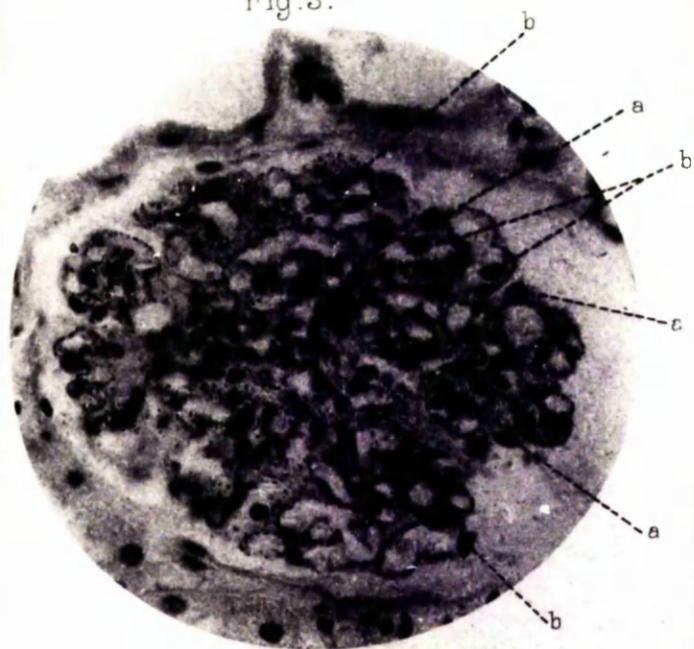
Figure 69

illustration consists of a portion of a glomerular capillary and a portion of Bowman's capsule. In the latter site is a solitary electron dense deposit said to be silver. As has been previously discussed (Part II, Methodological Studies (e)) it is far from certain that this particular electron dense deposit is indeed silver. Of more significance, however, is the absence of silver granules from the considerable segment of glomerular basement membrane in the same illustration.

The morphological description and the discussion in text of Prose's paper are far from clear and do not correspond to the illustration. He states "Infrequent silver deposits were seen in the connective tissue of the spleen and liver; in the focally thickened finely filamentous basement membranes surrounding renal glomerular capillaries and the glomerular capsule ......". Further on he records "As observed in the renal glomeruli in the rat Kurtz and Feldman, 1962 , silver granules were deposited in bandlike form in the basement membranes around the glandular portion of the sweat glands" (sic). While the evidence for the presence of silver in the skin of Prose's case is excellent, the evidence for the presence of silver in the glomerular basement membrane is insubstantial in the extreme. It seems possible

that in the expectancy of finding silver in the glomerulus he has alighted on a few isolated, electron-dense deposits of indeterminate nature. Significantly the interval between Prose's case ceasing to ingest silver and dying was thirty four years, more than long enough to clear silver deposits from glomerular basement membrane.

In case 2 reported here, the patient ceased taking silver at least fourteen years prior to death. Though he still had the clinical stigmata of argyria, his glomerular basement membrane contained no silver deposits. In the experiments on silver deposition in the rat described in part II of this thesis it was shown that silver deposition in the glomeruli is particularly intense and antedates silver deposition in the skin. Silver deposition in both these sites is well recognised in humans though the order of pigmentation is not known. There is therefore little reason to suppose that the glomerular basement membrane of case 2 was other than pigmented while the patient was taking silver and for an indeterminate time after he stopped taking silver. The small, intracellular traces of silver still present at death further support this view.

The amount of silver ingested by these two patients could not

be established but, by comparsion with other reported cases, neither was particularly heavily argyric. In experiment 3 on rats described in part III of this thesis it was shown that silver deposits were cleared from glomerular basement membrane over a five fold range of silver administration. The amount of silver ingested by the 2 human cases, in so far as it affects glomerular behaviour, does not therefore appear to be a critical factor.

Because of the well established time intervals between ceasing to ingest silver preparations and death it is possible to state from the two cases described, and to infer from previously described cases, that human glomerular basement membrane can be labelled with silver and this silver is subsequently cleared from the glomerular basement membrane.

Extrapolating results from experimental animals to humans is not always justifiable (Walker and Patrick, 1967, 1968, 1969) but the similarities between the observations on glomerular basement membrane in argyric rats and argyric humans are striking. It is therefore reasonable to conclude that the process of glomerular basement membrane synthesis, turnover and removal is at least similar in both species.

Conclusion

Human glomerular basement membrane turns over and is probably synthesized and removed in a manner similar to that previously demonstrated in the rat.

Part V

Ceneral Conclusions

The Functional Morphology of Glomerular Basement Membrane

The experiments recorded in this thesis demonstrate that a
major component of glomerular basement membrane is secreted
by the visceral epithelial cells. This component is laid down
on the epithelial side and slowly moves towards the endothelial
side of the basement membrane as new basement membrane mat-
erial is secreted. The old basement membrane material is re-
moved from the endothelial aspect of the membrane and passes by
way of the lamina rara interna and the mesangial matrix to the
mesangial cells. This entire process is continuous and slow:
the time for complete renewal of glomerular basement membrane
in the rat is of the order of 12 months: the time for complete re-
newal in the human is not established but is probably longer.
Secretion of this component by the epithelial cells is effected by
a vacuolar-coated pit system and removal by the mesangial cells
is effected by a phagocytic mechanism. The process is diagramat-
ically summarized in figure 70.

All the stages in this process have been described or surmised
previously as isolated events but the entire, interrelated sequence
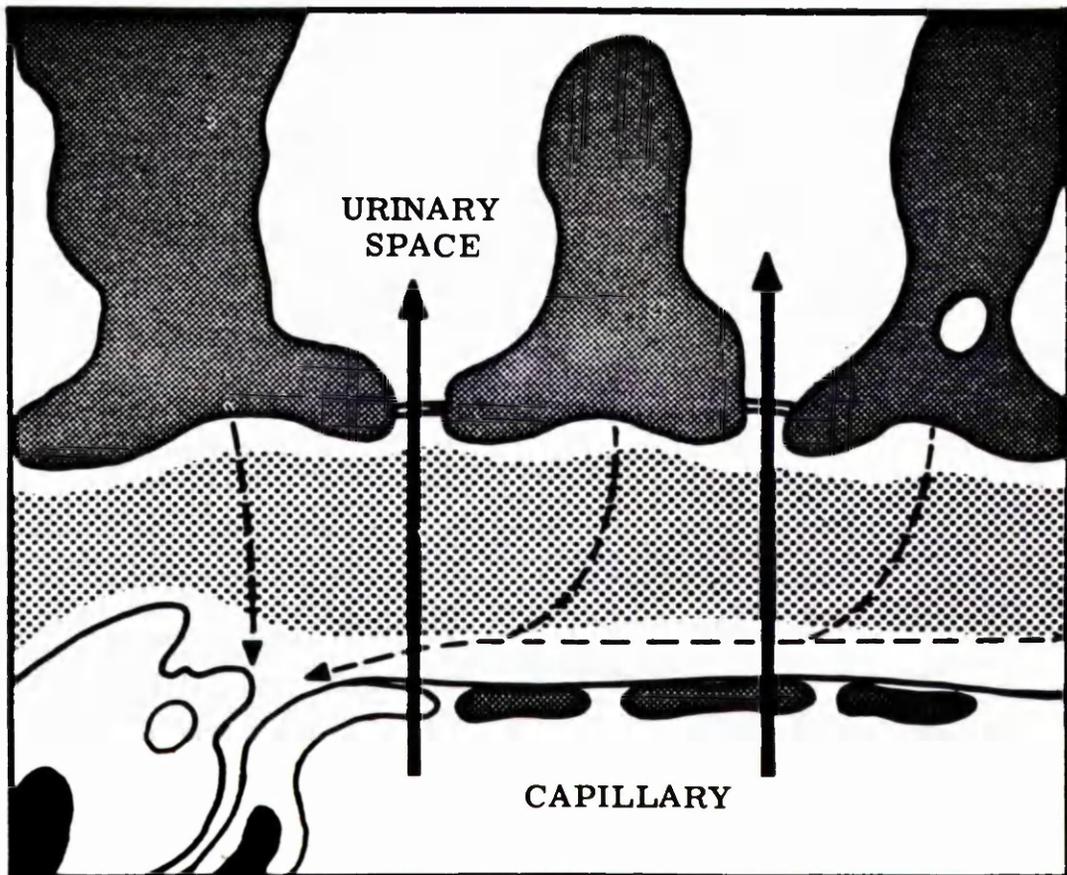
Figure 70. Diagram of glomerular basement membrane and related structures. The heavy black arrows indicate the path taken by the glomerular ultrafiltrate. The broken arrows indicate the path taken by the epithelial derived component of the glomerular basement membrane. Note that the basement membrane is renewed in a direction which is countercurrent to the flow of the ultrafiltrate.

of events, to the best of the author's knowledge, has not been demonstrated before, nor is there any previous direct evidence to show that this process occurs in the human and not just in the rat.

Apart from the main basement membrane component considered above, from the experimental observations -- in particular that the silver marker does not progress smoothly all the way through the basement membrane but is retarded at the inner border of the lamina densa, and that the lamina rara interna clears more rapidly than the lamina densa -- it is inferred that a second basement membrane component exists. The presence of such a second component has been suspected or demonstrated by several previous investigators in particular by Pierce and his associates. This second component is probably of endothelial origin.

The relationship of these two components in glomerular basement membrane is, at present, largely conjecture but Kurtz (1964) has made the reasonable suggestion that the second, or endothelial component could be the electron-lucent substance between the dense fibrils of the lamina densa.

From a consideration of all the available data there emerges a concept of glomerular basement membrane in terms of functional morphology. Glomerular basement membrane is a two component

structure. The major component is a loosely crosslinked, fibrillar meshwork of a collagenous protein (Spiro, 1967a; Kefalides, 1968) predominantly concentrated in the lamina densa but with occasional fibrils attached to the epithelial and endothelial cells (Trump and Benditt, 1962; Jorgensen, 1967b) thereby conferring structural integrity to the basement membrane. This component is secreted by the visceral epithelial cells, turns over slowly and becomes more crosslinked with time being maximally so at the inner border of the lamina densa thereby conferring a degree of coarse filtration selection at this level (Farquhar, Wissig and Palade, 1961; Latta and Maunsbach, 1962). At the inner border of the lamina densa this collagenous component is depolymerised and carried with the filtration residues by way of the lamina rara interna to the mesangial matrix to be subsequently ingested by the mesangial cells (Farquhar and Palade, 1962; Farquhar, 1964a).

The second component is a hydrated gel of endothelial cell origin which is intimately intermingled with and diluted by, the ultrafiltrate. This gel permeates the interstices of the fibrillar meshwork but is predominantly concentrated in the lamina rara interna and the lamina rara externa. It confers form, as opposed

to integrity, to the basement membrane. This is essentially an extension of the biomechanical concept of extracellular matrix structure developed by Fessler (1957; 1960) and expressed independently by Dische (1964). This second, or endothelial, component has a much faster turnover than the first, or epithelial, component. Much of this endothelial component flows circumferentially in the lamina rara interna carrying with it effete collagenous component and filtration residues to the mesangial matrix for subsequent ingestion by the mesangial cells.

Though at this juncture hypothetical, the proposed model of glomerular basement membrane accords with most of the established facts and many of the reasonable assumptions. It implies that glomerular basement membrane acts only as a coarse prefilter and assigns to the slit plates and foot processes the ultimate regulation of the glomerular ultrafiltrate which is in accord with the studies of Karnovsky and his associates on transcapillary exchanges and formation of the ultrafiltrate (Graham and Karnovsky, 1966; Venkatachalam, Karnovsky and Cotran, 1969). The model further implies that apart from regulating and monitoring the ultrafiltrate on a short term basis (Fisher and Hellstrom, 1962; Farquhar, 1964a), the visceral epithelial cells can, in the long term, alter the

nature of the pre-filter or basement membrane by synthesizing more or different basement membrane component. Much of the synthesis of the collagenous component involves post-ribosomal stages which are susceptible to environmental influences (Spiro, 1969).

Above all this hypothetical model is dynamic and offers possible explanations as to how certain glomerular basement membrane lesions develop and why, in many circumstances, these lesions resolve. In such a pathological context two subsidiary observations from the present series of experiments are probably of importance namely the ability of the mesangial cells to clear themselves of undigested residues and the ability of large filtration residues or basement membrane fragments to be herniated through the fenestrae into the capillary lumen.

At an intellectual level a hypothetical model is satisfactory if it explains and correlates most of, and preferably all, the present known facts pertaining to the situation under consideration. Apart from this there is little intrinsic value in such a model: its usefulness and validity rest in its application. There are two facets to this application, (a) the model should indicate deficiencies in

present knowledge which require further experimental investigation and (b) the model should assist in explaining the pathogenesis of certain naturally occurring glomerular lesions.

Application of the model in the first of these ways indicates several major points requiring further experimental elucidation. These are enumerated below.

(1) The role of fuzz in the glomerulus is far from clear. This lack of information is not peculiar to the the glomerulus but is common to cell coats generally. Ito (1969) has summarized the present situation as "It seems reasonable to speculate that the surface component may be characteristic for each species and for each cell type and furthermore that it may vary in its structure and function on the same cell under different physiological conditions. When the structure and function of the complete plasma membrane are understood and established, we may find that the glycocalyx or some of its components may be even more important than we now realize.".

(2) There are two vacuolar-coated pit systems, one concerned with absorption the other with secretion, in the visceral epithelial cell. The first of these has been recognised for some time (Fawcett, 1965, 1966), the second has only been recognised recently

(Haust, 1970).   No morphological criteria for distinguishing these two systems are presently available;   they can only be distinguished functionally.

(3) The chemical composition, physical nature, turnover and clearance of the second, or endothelial, component in glomerular basement membrane are not established.   It is suggested that this component may be related to endothelial cell coat, is probably a hydrated gel markedly diluted by the ultrafiltrate, and turns over more rapidly than the first, or epithelial, component.   Mohos and Skoza (1970) have demonstrated that the sialic acid content of glomerular basement membrane preparations varies directly with the centrifugal force employed in isolating these preparations and they suggest that this is due to contamination of the basement membrane preparations with cell coat.   Their data can equally well be interpreted as showing that increasing the preparative centrifugal force increases the yield of the second basement membrane component.   These investigators also note, but do not explain, that the sialic acid content of basement membrane preparations is higher in young animals than in old animals.   This is remarkably similar to the age changes which occur in the widths of the laminae rarae where the second component may well be present

in greatest amounts.

(4) The mechanism for the removal of the main, or collagen-ous, basement membrane component at the inner border of the lamina densa poses interesting problems. A possible way this removal could be effected is by a circulating collagenolytic mechan-ism in which the collagenase activity resides in molecules of such a size that ordinarily they are trapped as filtration residues at the inner border of the lamina densa. If the resultant products are of suitably small size they will be carried away by the ultrafiltrate and larger products will be disposed of as filtration residues in the mesangial region. Such collagenases have been identified and partially characterized (Jeffrey and Gross, 1970), but not, so far, in glomeruli.

(5) To maintain a glomerular basement membrane of more or less uniform appearance throughout life in the presence of so much turnover and other dynamic activities requires the presence of induction and control mechanisms between the epithelial, endo-thelial and mesangial cells. The study of such mechanisms is in its infancy and though their existence is well recognised (Kallman and Grobstein, 1965) and they have been demonstrated in the kidney (Grobstein, 1965), the manner in which they operate in mammalian

cells is unexplained.

(6) While it is clear that mesangial cells are phagocytic and remove effete basement membrane and filtration residues, other functions have also been ascribed to these cells. In particular it has been suggested that these cells may produce the collagen type fibres sometimes found in the mesangial matrix (Michielsen and Creemers, 1967). These fibres are not necessarily produced by the mesangial cells and may well be due to reaggregation of partially degraded basement membrane collagen transported to the mesangial matrix after being removed from the lamina densa (Rowlatt, 1970). Certainly not dissimilar fibres have been observed in other basement membranes (Palade and Farquhar, 1965; Rowlatt, 1969), reaggregation of collagenase digested collagen fibres has been demonstrated (Jeffrey and Gross, 1970), and it has been shown that under certain circumstances basement membrane collagen will aggregate into fibrils (Kefalides, 1969a). However all this evidence is circumstantial and further direct experimental information is required.

Application of the proposed model in the second way, namely to explain the pathogenesis of certain glomerular diseases in terms of structure and function, is outwith the scope of this thesis but a

few, brief, general considerations and specific examples drawn from human pathology may be given.

(1) The proposed model, like most biological systems, has to function satisfactorily within a relatively wide range of conditions the extremes of which may be regarded nicely as more pathological than physiological. Such an extreme condition may be said to occur if the glomerular filtration rate is maintained when the effective renal plasma flow is chronically reduced. In such conditions the glomerular filtration rate could only be maintained if an increased proportion of the plasma went to form the ultrafiltrate. This would increase the amount of filtration residues which would conceivably be reflected in the mesangial matrix and mesangial cells. Such a condition is exemplified by children with Fallot's tetrad and a high packed cell volume. Ultrastructural studies on glomeruli in such cases do not appear to have been reported but by light microscopy there is an increase in eosinophilic or hyaline intercellular substance which is thought to involve the mesangium (Spear, 1960; Bauer and Rosenberg, 1960).

(2) The proposed model is consonant with complete turnover of the basement membrane though this takes considerable time. If the glomerular basement membrane is damaged by a disease

process which is essentially extraglomerular in origin and the cause of the damage then ceases to operate, the damaged basement membrane should be slowly removed either via the mesangial cells, the endothelial cell perikaryon or by extrusion of fragments into the capillary lumen, and replaced by newly synthesized basement membrane. Such a situation occurs in acute glomerulonephritis during and after which there are conspicuous basement membrane abnormalities (Movat, Steiner and Huhn, 1962) yet these resolve in most cases for when death occurs many years later from some entirely unrelated disease and a post mortem examination is performed, the glomerular basement membrane is found to be essentially normal. Such a case has been followed by serial renal biopsy over a period of 560 days by which time the glomerular basement membrane had almost returned to normal (Strunk, Hammond and Benditt, 1964).

(3) Abnormalities of glomerular basement membrane must not be considered in isolation. Though glomerular basement membrane is eye-catching and much studied it is merely one part of a unit comprising in addition visceral epithelial cells, mesangial matrix, mesangial cells and endothelial cells. Subsidiary to this it should be appreciated that some conspicuous morphological features,

though of undoubted diagnostic value to the pathologist, may be of little functional and prognostic significance to the patient. This is exemplified in the focal, nodular, intercapillary glomerulo-sclerosis (Kimmelstiel and Wilson, 1936) which is to all intents and purposes pathognomonic of diabetes mellitus. There is remarkably little evidence that this particular feature is of any significance in terms of renal function (Gellman, 1964) and it is the less conspicuous diffuse membraneous lesion (Bell, 1953) which correlates better with glomerular malfunction. Recent investigations suggest that this may be due to the visceral epithelial cells secreting a basement membrane component which is slightly different from normal (Beisswenger and Spiro, 1970). It is possible that the mesangial cells ingest this component less avidly than normal effete basement membrane. However this, like so many other problems concerning glomerular structure and function awaits final elucidation.

Summary

A detailed and comprehensive account of the natural history of normal glomerular basement membrane is prerequisite to elucidating the pathogenesis of several renal diseases.

The experimental argyric technique was investigated, adapted and then applied in a long term, sequential, electron microscopic study of normal glomerular basement membrane in the rat.

The results demonstrate that a major component of glomerular basement membrane is secreted by the visceral epithelial cells. This component is laid down on the epithelial side and slowly moves towards the endothelial side of the basement membrane as new basement membrane material continues to be secreted. The old basement membrane material is removed from the endothelial aspect of the basement membrane and passes by way of the lamina rara interna to the mesangial matrix for subsequent ingestion by the mesangial cells. This process is continuous and slow: the time for complete renewal of the glomerular basement membrane in the rat is of the order of 12 months. Secretion of this component by the epithelial cells is effected by a vacuolar-coated pit mechanism and removal by the mesangial cells is effected by a phagocytic mechanism.

The results further indicate the presence of a second component in the basement membrane. This second component is probably of endothelial origin and has a much faster turnover rate than the main, or epithelial derived, component.

Study was also made of glomeruli from 2 cases of human argyria and though the observations perforce are limited the results show that human glomerular basement membrane has a natural history essentially similar to rat glomerular basement membrane.

On the basis of these experimental observations, correlated with the results of previous investigations, a model of the functional morphology of glomerular basement membrane is proposed. The potential applications of this model are briefly indicated.

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This thesis, though the techniques and tissues are quite different, embodies the principles and practices learned during the course of working for my previous thesis. For this sound basis I am much indebted to Dr R. S. Patrick, my former Ph.D. supervisor; and it is a pleasure to acknowledge his continued interest and encouragement.

References

ALBERS, I.A. (1816) Observations on a change of colour in the skin produced by the internal use of the nitrate of silver.
Medico-Chirurgical Transactions, London, 7, 284-290.

ANDRES, G.A., MORGAN, C., HSU, K.C., RIFKIND, R.A. and SEEGAL, BEATRICE C. (1962) Electron microscopic studies of experimental nephritis with ferritin-conjugated antibody.
Journal of Experimental Medicine, 115, 929-936.

ARATAKI, M. (1926) On the postnatal growth of the kidney, with special reference to the number and size of the glomeruli (Albino rat).
American Journal of Anatomy, 36, 399-436.

ASHWORTH, C.T. and ERDMANN, R.R. (1959) Age changes in the renal basement membranes of rats.
American Journal of Pathology, 35, 670 only.

ASHWORTH, C.T., ERDMANN, R.R. and ARNOLD, N.J. (1960) Age changes in the renal basement membrane in rats.
American Journal of Pathology, 36, 165-179.

BACHMANN, L. and SALPETER, M.M. (1965) Autoradiography with the electron microscope. A quantitative evaluation.
Laboratory Investigation, 14, 1041-1053.

BADELEY (1818) On the effect of nitrate of silver on the complexion.
Medico-Chirurgical Transactions, London, 9, 234-239.

BADER, K.F. (1966) Organ deposition of silver following silver nitrate therapy of burns.
Plastic and Reconstructive Surgery, 37, 550-551.

BAILEY, A.J. and PEACH, CATHERINE M. (1968) Isolation and structural identification of a labile intermolecular cross-link in collagen.
Biochemical and Biophysical Research Communications, 33, 812-819.

BAILEY, A.J., PEACH, CATHERINE M. and FOWLER, L.J.
(1970) Chemistry of the collagen cross-links.
Biochemical Journal, 117, 819-831.

BARAJAS, L. and LATTA, H. (1963) A three-dimensional study
of the juxtaglomerular apparatus in the rat.
Laboratory Investigation, 12, 257-269.

BAUER, W.C. and ROSENBERG, BARBARA F. (1960) A quant-
itative study of glomerular enlargement in children with
tetralogy of Fallot.
American Journal of Pathology, 37, 695-712.

BEHNKE, O. and ZELANDER, T. (1970) Preservation of inter-
cellular substances by the cationic dye Alcian blue in prepar-
ative procedures for electron microscopy.
Journal of Ultrastructure Research, 31, 424-438.

BEISSWENGER, P.J. and SPIRO, R.G. (1970) Human glomerular
basement membrane: chemical alteration in diabetes mellitus.
Science, 168, 596-598.

BELL, E.T. (1953) Renal vascular disease in diabetes mellitus.
Diabetes, 2, 376-389.

BENACERRAF, B., McCLUSKEY, R.T. and PATRAS, DOROTHY
(1959) Localization of colloidal substances in vascular endo-
thelium. A mechanism of tissue damage.
American Journal of Pathology, 35, 75-91.

BENCOSME, S.A., STONE, R.S., LATTA, H. and MADDEN,
S.C. (1959) Acute alterations produced by uranyl nitrate in
glomeruli of rat kidneys: light and electron microscopic
studies.
American Journal of Pathology, 35, 670 only; also Journal
of Ultrastructure Research, 3, 171-185.

BENNETT, H.S. (1963) Morphological aspects of extracellular
polysaccharides.
Journal of Histochemistry and Cytochemistry, 11, 14-23.

BENNETT, H.S. and LUFT, J.H. (1959) S-collidine as a basis for buffering fixatives.
Journal of Biophysical and Biochemical Cytology, 6, 113-114.

BENNETT, H.S., LUFT, J.H. and HAMPTON, J.C. (1959) Morphological classification of vertebrate blood capillaries.
American Journal of Physiology, 196, 381-390.

BERNIK, MARIA B. (1969) Contractile activity of human glomeruli in culture.
Nephron, 6, 1-10.

BLOOM, P.M., HARTMANN, J.F. and VERNIER, R.L. (1959) An electron microscopic evaluation of the width of normal glomerular basement membrane in man at various ages.
Anatomical Record, 133, 251 only.

BLUMBERG, H. and CAREY, T.N. (1934) Argyremia.
Journal of the American Medical Association, 103, 1521-1524.

BOERSMA, D. and BAKER, B.L. (1948) Sites of deposition of silver in argyria.
Archives of Dermatology and Syphilology, 57, 1009-1012.

BOOK, M.H. (1937) The secreting area of the glomerulus.
Journal of Anatomy, 71, 91-97.

BOSMANN, H.B. (1969) Collagen-galactosyl transferase: subcellular localization and distribution in fibroblasts transformed by oncogenic viruses.
Life Sciences, 8, part II, 737-746.

BOWMAN, W. (1842) On the structure and use of the Malpighian
bodies of the kidney, with observations on the circulation
through that gland.
Philosophical Transactions of the Royal Society of London,
part I, 57-80.

BOWMAN, W. (1847) Mucous membrane. In "The Cyclopaedia
of Anatomy and Physiology" edited by R.B. Todd. Sherwood,
Gilbert and Piper, London. Volume 3, pp. 484-506.

BOYER, C.C. (1956) The vascular pattern of the renal glomerulus
as revealed by plastic reconstructions from serial sections.
Anatomical Record, 125, 433-441.

BRINTON, D.W. (1949) Argyria: report of a case.
Guy's Hospital Reports, 98, 88-94.

BUCHANAN, M.S. (1831) Report of a few of the surgical cases,
which occurred, in the Glasgow Royal Infirmary, during the
summer and autumn of 1830.
Glasgow Medical Journal, 4, 174-197.

BUCKLEY, W.R., OSTER, C.F. and FASSETT, D.W. (1965)
Localized argyria: II, chemical nature of the silver contain-
ing particles.
Archives of Dermatology, 92, 697-705.

CARO, L.G. and van TUBERGEN, R.P. (1962) High resolution
autoradiography. I Methods.
Journal of Cell Biology, 15, 173-188.

CHAMBERS, R. and ZWEIFACH, B.W. (1947) Intercellular cement and capillary permeability.
Physiological Reviews, 27, 436-463.

CHINARD, F.P. (1952) Derivation of an expression for the rate of formation of glomerular fluid (GFR). Applicability of certain physical and physico-chemical concepts.
American Journal of Physiology, 171, 578-586.

CHINARD, F.P., VOSBURGH, G.J. and ENNS, T. (1955) Transcapillary exchange of water and of other substances in certain organs of the dog.
American Journal of Physiology, 183, 221-234.

CHOW, A.Y.K. and DRUMMOND, K.N. (1969) Incorporation and hydroxylation of proline-3-4-H^3 as an index of glomerular basement membrane synthesis in normal and nephrotoxic nephritic rats.
Laboratory Investigation, 20, 213-218.

CHRISTENSEN, S., JORGENSEN, K. and OHLSEN, A.S. (1964) A case of generalized argyria diagnosed by X-ray fluorescence analysis.
Danish Medical Bulletin, 11, 227-229.

CONSTABLE, J.D., MORRIS, P.J. and BURKE, J.F. (1967) Absorption pattern of silver nitrate from open wounds.
Plastic and Reconstructive Surgery, 39, 342-348.

DALTON, A.J. and HAGUENAU, FRANCOISE (1967) Ultrastructure of the kidney.
Academic Press, New York. 240 p.

DEANE, HELEN W., HOFMANN, F.G., SOLOMON, A.K. and WISLOCKI, G.B. (1955) Selective uptake of silver by the adrenal zona glomerulosa.
Anatomical Record, 121, 283 only.

DEMPSEY, E.W. and WISLOCKI, G.B. (1955, a) An electron microscopic study of the blood-brain barrier in the rat, employing silver nitrate as a vital stain.
Anatomical Record, 121, 283-284.

DEMPSEY, E.W. and WISLOCKI, G.B. (1955, b) The use of silver nitrate as a vital stain, and its distribution in several mammalian tissues as studied with the electron microscope. Anatomical Record, 121, 392-393.

DEMPSEY, E.W. and WISLOCKI, G.B. (1955, c) The use of silver nitrate as a vital stain, and its distribution in several mammalian tissues as studied with the electron microscope. Journal of Biophysical and Biochemical Cytology, 1, 111-118.

DEMPSEY, E.W. and WISLOCKI, G.B. (1955, d) An electron microscopic study of the blood-brain barrier in the rat, employing silver nitrate as a vital stain. Journal of Biophysical and Biochemical Cytology, 1, 245-256.

DISCHE, Z. (1964) The glycans of the mammalian lens capsule – a model of basement membranes. In "Small blood vessel involvement in diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute of Biological Sciences, Washington, D.C., pp. 201-213.

DISCHE, R.M., PAPPAS, G.D., GRAUER, A. and DISCHE, Z. (1965) The carbohydrate of basement membranes of human kidney glomeruli. Biochemical and Biophysical Research Communications, 20, 63-70.

DIXON, F.J. (1968) The pathogenesis of glomerulonephritis. American Journal of Medicine, 44, 493-498.

DOHI, Sh. (1908) Über Argyrie. Virchows Archiv für pathologische Anatomie und Physiologie, 193, 148-164.

ECKARDT, C.Th. (1888) Ueber die compensatorische Hypertrophie und das physiologische Wachsthum der Niere. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 114, 217-245.

ENDERS, A. (1956) Silberelimination nach experimenteller Argyrose. Archiv für experimentelle Pathologie und Pharmakologie, 228, 206-207.

ENDERS, A. and MOENCH, A. (1956) Silberelimination im
Organismus bei experimenteller Argyrie.
Archiv für experimentelle Pathologie und Pharmakologie,
229, 16-25.

ENNOS, A.E. (1953) The origin of specimen contamination in
the electron microscope.
British Journal of Applied Physics, 4, 101-106.

ENNOS, A.E. (1954) The source of electron-induced contamin-
ation in kinetic vacuum systems.
British Journal of Applied Physics, 5, 27-31.

ERICSSON, J.L.E. (1968) Fine structural basis for hemoglobin
filtration by glomerular capillaries.
Nephron, 5, 7-23.

FARRANT, J.L. (1954) An electron microscopic study of ferritin.
Biochimica et Biophysica Acta, 13, 569-576.

FARQUHAR, MARILYN G. (1964) Glomerular permeability in-
vestigated by electron microscopy. In "Small blood vessel
involvement in diabetes mellitus" edited by M.D. Siperstein,
A.R. Colwell and K. Meyer. American Institute of Biological
Sciences, Washington, D.C., (a) pp. 31-38 (b) discussion
p. 52.

FARQUHAR, MARILYN G. and PALADE, G.E. (1959) Behaviour
of colloidal particles in the glomerulus.
Anatomical Record, 133, 378-379.

FARQUHAR, MARILYN G. and PALADE, G.E. (1962) Functional
evidence for the existence of a third cell type in the renal
glomerulus.
Journal of Cell Biology, 13, 55-87.

FARQUHAR, MARILYN G., VERNIER, R.L. and GOOD, R.A.
(1957) An electron microscope study of the glomerulus in
nephrosis, glomerulonephritis, and lupus erythematosus.
Journal of Experimental Medicine, 106, 649-660.

FARQUHAR, MARILYN G., WISSIG, S.L. and PALADE, G.E. (1961) Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. Journal of Experimental Medicine, 113, 47-66.

FAWCETT, D.W. (1965) Surface specializations of absorbing cells. Journal of Histochemistry and Cytochemistry, 13, 75-91.

FAWCETT, D.W. (1966) The cell. Its organelles and inclusions. W.B. Saunders, Philadelphia, 448 p.

FESSLER, J.H. (1957) Water and mucopolysaccharide as structural components of connective tissue. Nature, 179, 426-427.

FESSLER, J.H. (1960) A structural function of mucopolysaccharide in connective tissue. Biochemical Journal, 76, 124-132.

FISHER, E.R. and HELLSTROM, H.R. (1962) Mechanism of proteinuria: functional and structural correlation of effects of infusion of homologous and heterologous protein (bovine serum albumin) in the rat. Laboratory Investigation, 11, 617-637.

FLOREY, LORD (1967) The uptake of particulate matter by endothelial cells. Proceedings of the Royal Society, B, 166, 375-383.

FREY-WYSSLING, A. (1948) Submicroscopic morphology of protoplasm and its derivatives. Elsevier, Amsterdam, 255 p.

FROMMANN, C. (1859) Ein Fall von Argyria mit Silberabscheidungen im Darm, Leber, Nieren und Milz. Archiv für pathologische Anatomie und Physiologie, 17, 135-147.

GAGER, L.T. and ELLISON, E.M. (1935) Generalized (therapeutic) argyria. Case report with necropsy and notes on diagnosis and prevention. International Clinics, 4, 118-132.

GALLYAS, F. (1970) Silver staining of collagen and reticulin fibres and cerebral capillaries by means of physical development.
Journal of Microscopy, 91, 119-124.

GATZ, A.J. (1949) Experimental argyria in albino rats.
Anatomical Record, 103, 454-455.

GAUL, L.E. and STAUD, A.H. (1934) Clinical spectroscopy. A study of biopsy material from patients who had received intravenous injections of silver arsphenamine.
Archives of Dermatology and Syphilology, 30, 433-438.

GAUL, L.E. and STAUD, A.H. (1935) Clinical spectroscopy. Quantitative distribution of silver in the body or its physiopathologic retention as a reciprocal of the capillary system.
Archives of Dermatology and Syphilology, 32, 775-780.

GAUTIER, A., BERNHARD, W. and OBERLING, Ch. (1950) Sur l' existence d' un appareil lacunaire péri-capillaire du glomérule de Malpighi révelée par le microscope électronique.
Comptes Rendus de la Societe de Biologie, 144, 1605-1607.

GELLMAN, D.D. (1964) The correlation between the pathological and clinical manifestations of diabetic renal disease. In "Small blood vessel involvement in diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute for Biological Sciences, Washington, D.C., pp. 13-24 and discussion p. 25.

GETTLER, A.O., RHOADS, C.P. and WEISS, SOMA (1927) A contribution to the pathology of generalized argyria with a discussion of the fate of silver in the human body.
American Journal of Pathology, 3, 631-651.

GOODMAN, M., GREENSPON, S.A. and KRAKOWER, C.A. (1955) The antigenic composition of the various anatomic structures of the canine kidney.
Journal of Immunology, 75, 96-104.

GOTTLOB, R. and HOFF, H.F. (1967) A study of the relation between endothelial silver lines, medial transverse silver lines, and the ultrastructural morphology of blood vessels.
Vascular Surgery, 1, 92-100.

GRAHAM, R.C. and KARNOVSKY, M.J. (1966) Glomerular permeability. Ultrastructural cytochemical studies using peroxidases as protein tracers.
Journal of Experimental Medicine, 124, 1123-1134.

GRASSO, P., ABRAHAM, R., HENDY, R., DIPLOCK, A.T., GOLBERG, L. and GREEN, J. (1969) The role of dietary silver in the production of liver necrosis in vitamin E-deficient rats.
Experimental and Molecular Pathology, 11, 186-199.

GROBSTEIN, C. (1965) In report of meeting on extracellular matrices by K. Meyer, D.W. Richards and S.E. Bradley.
Science, 147, 760-761.

GRONIOWSKI, J., BICZYSKOWA, W. and WALSKI, M. (1969) Electron microscope studies on the surface coat of the nephron.
Journal of Cell Biology, 40, 585-601.

GROTTE, G. (1956) Passage of dextran molecules across the blood-lymph barrier.
Acta Chirurgica Scandinavica, Supplement 211, 84 p.

GUTMAN, F.A. and CROSSWELL, H.H. (1968) Argyrosis of the cornea without clinical conjunctival involvement.
American Journal of Ophthalmology, 65, 183-187.

HAGOPIAN, A., BOSMANN, H.B. and EYLAR, E.H. (1968) Glycoprotein biosynthesis: the localization of polypeptidyl: N-acetylgalactosaminyl, collagen: glucosyl, and glycoprotein galactosyl transferases in HeLa cell membrane fractions.
Archives of Biochemistry and Biophysics, 128, 387-396.

HALL, B.V. (1955) The organization of the renal glomerulus into independent lobular systems of intercommunicating anastomosing capillaries.
Anatomical Record, 121, 433 only.

HALL, V. (1957) The protoplasmic basis of glomerular ultra-
filtration.
American Heart Journal, 54, 1-9.

HAM, A.W. (1965) Histology. 5th edition. Pitman, London,
pp. 772-813.

HARDWICKE, J. and SOOTHILL, J.F. (1961) Glomerular damage
in terms of "pore size". In "Ciba Foundation Symposium on
Renal Biopsy" edited by G. Wolstenholme and Margaret P.
Cameron. Churchill, London, pp. 32-42.

HAUST, M.D. (1970) The fibroblast: its structure and function
in health.
American Journal of Pathology, 59, 18a-19a.

HAY, ELIZABETH D. and REVEL, J.P. (1963) Autoradiographic
studies of the origin of the basement lamella in Ambystoma.
Developmental Biology, 7, 152-168.

HEIDE, H.G. (1965) Contamination and irradiation effects and
their dependence on the composition of residual gases in the
electron microscope.
Laboratory Investigation, 14, 1134-1139.

HILL, W.R. and MONTGOMERY, H. (1941) Argyria: with special
reference to the cutaneous histopathology.
Archives of Dermatology and Syphilology, 44, 588-599.

HILL, W.R. and PILLSBURY, D.M. (1939) Argyria: The
pharmacology of silver. Bailliere, Tindall and Cox, London.

HINGLAIS-GUILLAUD, NICOLE and GALLE, P. (1961) Mise
en évidence au microscope électronique de fibres collagenes
dans les régions interluminaires de glomérules humains
pathologiques.
Comptes Rendus de l' Académie des Sciences, 253, 1627-1629.

HOEDEMAEKER, Ph.J. (1970, a) Localisation of tissue antigens
at the ultrastructural level using peroxidase-coupled antibodies.
Paper presented at 120th meeting of the Pathological Society
of Great Britain and Ireland.

HOEDEMAEKER, Ph.J. (1970, b) Personal communication.

HUBER, G.C. (1905) On the development and shape of uriniferous tubules of certain of the higher mammals.
American Journal of Anatomy, 4, supplement, 98 p.

HUET, M. (1873) Recherches sur l' Argyrie.
Journal de l' Anatomie et de la Physiologie, 9, 408-434.

ITO, S. (1969) Structure and function of the glycocalyx.
Federation Proceedings, 28, 12-25.

JACOBSEN, N.O., JØRGENSEN, F. and THOMSEN, A.C. (1966) An electron microscopic study of small arteries and arterioles in the normal human kidney.
Nephron, 3, 17-39.

JAHN, (1894) Ueber Argyrie.
Beiträge zur pathologischen Anatomie und zur allgemeinen Pathologie, 16, 218-239.

JAMES, T.H. (1966) The mechanism of development. In "The theory of the photographic process" edited by C.E.K. Mees and T.H. James, 3rd edition, Macmillan, New York, pp. 324-349.

JEFFREY, J.J. and GROSS, J. (1970) Collagenase from rat uterus. Isolation and partial characterization.
Biochemistry, 9, 268-273.

JOKELAINEN, P. (1963) An electron microscope study of the early development of the rat metanephric nephron.
Acta Anatomica, 52, supplement 47, 71 p.

JONES, D.B. (1963) The nature of scar tissue in glomerulonephritis.
American Journal of Pathology, 42, 185-199.

JONES, D.B. (1969) Mucosubstances of the glomerulus.
Laboratory Investigation, 21, 119-125.

JONES, D.B., MUELLER, C.B. and MENEFEE, M. (1962)
The cellular and extracellular morphology of the glomerular
stalk.
American Journal of Pathology, 41, 373-388.

JØRGENSEN, F. (1967, a) Electron microscopic studies of nor-
mal visceral epithelial cells.
Laboratory Investigation, 17, 225-242.

JØRGENSEN, F. (1967, b) Electron microscopic studies of nor-
mal glomerular basement membrane.
Laboratory Investigation, 17, 416-424.

JØRGENSEN, F. and BENTZON, M.W. (1968) The ultrastructure
of the normal human glomerulus. Thickness of glomerular
basement membrane.
Laboratory Investigation, 18, 42-48.

KALLMAN, F. and GROBSTEIN, C. (1965) Source of collagen
at epitheliomesenchymal interfaces during inductive interaction.
Developmental Biology, 11, 169-183.

KARNOVSKY, M.J. (1968) The ultrastructural basis of trans-
capillary exchanges.
Journal of General Physiology, 52, 64s-95s.

KEFALIDES, N.A. (1968) Isolation and characterization of the
collagen from glomerular basement membrane.
Biochemistry, 7, 3103-3112.

KEFALIDES, N.A. (1969, a) Characterization of the collagen from
lens capsule and glomerular basement membranes. Proceed-
ings of the 6th Congress of the International Diabetes Feder-
ation, Excerpta Medica, Amsterdam, pp. 307-322.

KEFALIDES, N.A. (1969, b) The chemistry of the antigenic
components of vascular and capsular basement membranes.
Federation Proceedings, 28, 699 only.

KEFALIDES, N.A. (1969, c) The chemical basis for the structure
and function of basement membranes. Proceedings of the 6th
Congress of the International Diabetes Federation, Excerpta
Medica, Amsterdam, pp. 610-611.

KEFALIDES, N.A. and DENDUCHIS, BERTA (1969) Structural components of epithelial and endothelial basement membranes. Biochemistry, 8, 4613-4621.

KEFALIDES, N.A. and WINZLER, R.J. (1966) The chemistry of glomerular basement membrane and its relation to collagen. Biochemistry, 5, 702-713.

KIMMELSTIEL, P., KIM, O.J. and BERES, J. (1962) Studies on renal biopsy specimens with the aid of the electron microscope. 1. Glomeruli in diabetes. American Journal of Clinical Pathology, 38, 270-279.

KIMMELSTIEL, P. and WILSON, C. (1936) Intercapillary lesions in the glomeruli of the kidney. American Journal of Pathology, 12, 83-97.

KIRKMAN, H. and STOWELL, R.E. (1942) Renal filtration surface in the albino rat. Anatomical Record, 82, 373-391.

KITTELSON, J.A. (1917) The postnatal growth of the kidney of the albino rat, with observations on an adult human kidney. Anatomical Record, 13, 385-408.

KURTZ, S.M. (1958) The electron microscopy of the developing human glomerulus. Experimental Cell Research, 14, 355-367.

KURTZ, S.M. (1961) The fine structure of the lamina densa. Laboratory Investigation, 10, 1189-1208.

KURTZ, S.M. (1964) The kidney. In "Electron Microscopic Anatomy" edited by S.M. Kurtz. Academic Press, New York, pp. 239-265.

KURTZ, S.M. and FELDMAN, J.D. (1962) Experimental studies on the formation of glomerular basement membrane. Journal of Ultrastructure Research, 6, 19-27.

KURTZ, S.M. and McMANUS, J.F.A. (1960) The fine structure of the human glomerular basement membrane. Journal of Ultrastructure Research, 4, 81-87.

LANDIS, E.M. and PAPPENHEIMER, J.R. (1963) Exchange of substances through the capillary walls. In "Handbook of Physiology" edited by W.F. Hamilton and P. Dow. Section 2. Volume II, pp. 961-1034.

LANGLEY, O.K. and AMBROSE, E.J. (1967) The linkage of sialic acid in the Ehrlich ascites-carcinoma cell surface membrane.
Biochemical Journal, 102, 367-372.

LATTA, H. (1961) Collagen in normal rat glomeruli.
Journal of Ultrastructure Research, 5, 364-373.

LATTA, H. (1962) The plasma membrane of glomerular epithelium.
Journal of Ultrastructure Research, 6, 407-412.

LATTA, H. and MAUNSBACH, A.B. (1962) Relations of the centrolobular region of the glomerulus to the juxtaglomerular apparatus.
Journal of Ultrastructure Research, 6, 562-578.

LATTA, H., MAUNSBACH, A.B. and MADDEN, S.C. (1960) The centrolobular region of the renal glomerulus studied by electron microscopy.
Journal of Ultrastructure Research, 4, 455-472.

LAZAROW, A. and SPEIDEL, EDNA (1964) 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" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute of Biological Sciences, Washington, D.C., pp. 127-150.

LeCOMPTE, P.M. (1964) Discussion in "Small blood vessel involvement in diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute of Biological Sciences, Washington, D.C., p. 8.

LÉLUT, H. (1830) Coloration bronzée des tégumens chez un épileptique, produite par l' usage intérieur du nitrate d' argent.
Journal Hebdomadaire de Médecine, 6, 305-310.

LEWIS, O.J. (1958, a) The development of the blood vessels of the metanephros.
Journal of Anatomy, 92, 84-97.

LEWIS, O.J. (1958, b) The vascular arrangement of the mammalian renal glomerulus as revealed by a study of its development.
Journal of Anatomy, 92, 433-440.

LIDSKY, M.D., SHARP, J.T. and RUDEE, M.L. (1967) Studies on acellular bovine glomeruli. Isolation, chemical composition, and demonstration of a collagen with an unusual hydroxylysine: hydroxyproline ratio.
Archives of Biochemistry and Biophysics, 121, 491-501.

LILLIE, R.D. (1965) Histopathologic technic and practical histochemistry. McGraw-Hill, New York, p. 441.

LUFT, J.H. (1964) Fine structure of the diaphragm across capillary "pores" in mouse intestine.
Anatomical Record, 148, 307-308.

LUFT, J.H. (1965) The ultrastructural basis of capillary permeability. In "The inflammatory process" edited by B.W. Zweifach, L. Grant and R. T. McCluskey. Academic Press, New York, pp. 121-159.

LUFT, J.H. (1966) Fine structure of capillary and endocapillary layer as revealed by ruthenium red.
Federation Proceedings, 25, 1773-1783.

LUMIÈRE, A., LUMIÈRE, L. and SEYEWETZ, A. (1911) Sur le développment des images photographiques après fixage.
Comptes Rendus de l' Académie des Sciences, 153, 102-104.

MACDONALD, MORAG S. and EMERY, J.L. (1959) The late intrauterine and postnatal development of human renal glomeruli.
Journal of Anatomy, 93, 331-340.

McMANUS, J.F.A. (1948, a) The periodic routine applied to the
kidney.
American Journal of Pathology, 24, 643-653.

McMANUS, J.F.A. (1948, b) Structure of the glomerulus of the
human kidney.
American Journal of Pathology, 24, 1259-1269.

MAJNO, G. (1965) Ultrastructure of the vascular membrane.
In "Handbook of Physiology" edited by W.F. Hamilton and
P. Dow. Section 2. Volume III, American Physiological
Society, Washington, D.C., pp. 2293-2375.

MALPIGHII, M. (1666) De viscerum structura exercitatio
anatomica. In "Opera Posthuma", Amstelodami MDCC,
pp. 48-58.

MAYERSON, H.S., WOLFRAM, C.G., SHIRLEY, H.H. and
WASSERMAN, K. (1960) Regional differences in capillary
permeability.
American Journal of Physiology, 198, 155-160.

MEHTA, A.C., DAWSON-BUTTERWORTH, K. and WOODHOUSE,
M.A. (1966) Argyria. Electron microscopic study of a case.
British Journal of Dermatology, 78, 175-179.

MENEFEE, M.G. and MUELLER, C.B. (1967) Some morpho-
logical considerations of transport in the glomerulus. In
"Ultrastructure of the kidney" edited by A.J. Dalton and
Francoise Haguenau. Academic Press, New York, pp. 73-
100.

MENEFEE, M.G., MUELLER, C.B., BELL, A.L. and
MYERS, J.K. (1964) Transport of globin by the renal
glomerulus.
Journal of Experimental Medicine, 120, 1129-1138.

MICHIELSEN, P. and CREEMERS, JULIA (1967) The structure
and function of the glomerular mesangium. In "Ultrastructure
of the kidney" edited by A.J. Dalton and Francoise Haguenau.
Academic Press, New York, pp. 57-72.

MILLER (1964) Discussion, p. 158. In "Small blood vessel involvement in diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute of Biological Sciences, Washington, D.C.

MILLER, F. and PALADE, G.E. (1964) Lytic activities in renal protein absorption droplets. An electron microscopical cyto-chemical study.
Journal of Cell Biology, 23, 519-552.

MISRA, R.P. and BERMAN, L.B. (1966) Studies on glomerular basement membrane 1. Isolation and chemical analysis of normal glomerular basement membrane.
Proceedings of Society for Experimental Biology and Medicine, 122, 705-710.

MISRA, R.P. and BERMAN, L.B. (1969) Glomerular basement membrane: insights from molecular models.
American Journal of Medicine, 47, 337-339.

MOHOS, S.C. and SKOZA, L. (1969) Glomerular sialoprotein.
Science, 164, 1519-1521.

MOHOS, S.C. and SKOZA, L. (1970) Variations in the sialic acid concentration of glomerular basement membrane prepar-ations obtained by ultrasonic treatment.
Journal of Cell Biology, 45, 450-455.

MOVAT, H.Z., McGREGOR, D.D. and STEINER, J.W. (1961) Studies of nephrotoxic nephritis II. The fine structure of the glomerulus in acute nephrotoxic nephritis of dogs.
American Journal of Clinical Pathology, 36, 306-321.

MOVAT, H.Z., STEINER, J.W. and HUHN, D. (1962) The fine structure of the glomerulus in acute glomerulonephritis.
Laboratory Investigation, 11, 117-135.

NEUTRA, MARIAN and LEBLOND, C.P. (1966) Synthesis of the carbohydrate of mucus in the Golgi complex as shown by electron microscope radioautography of goblet cells from rats injected with glucose-H^3.
Journal of Cell Biology, 30, 119-136.

NEUTRA, MARIAN and LEBLOND, C.P. (1969) The Golgi
   apparatus.
   Scientific American, 220, number 2, 100-107.

NYBERG, R. (1967) The behaviour of intravaginally applied
   Ag^{110m}-labelled silver nitrate.
   Acta Obstetricia et Gynecologica Scandinavica, 46, supple-
   ment 3, 72 p.

OLCOTT, C.T. (1947) Experimental argyrosis III.   Pigment-
   ation of the eyes of rats following ingestion of silver during
   long periods of time.
   American Journal of Pathology, 23, 783-791.

OLCOTT, C.T. (1948) Experimental argyrosis IV.   Morphologic
   changes in the experimental animal.
   American Journal of Pathology, 24, 813-833.

OLCOTT, C.T. and RICHTER, G.W. (1958) Experimental
   argyrosis VI.   Electron microscopic study of ingested
   silver in the kidney of the rat.
   Laboratory Investigation, 7, 103-109.

OSAWA, G., KIMMELSTIEL, P. and SEILING, VIRGINIA (1966)
   Thickness of glomerular basement membranes.
   American Journal of Clinical Pathology, 45, 7-20.

OSHIMA, K., HATANO, M., MAEYAMA, Y., SUGINO, N.
   and TAKEUCHI, T. (1967) Electron microscopy of the
   glomerular basement membrane of the rat kidney.
   Proceedings of 3rd International Congress of Nephrology,
   Karger, Basel, pp. 45-53.

PALADE, G.E. (1952) A study of fixation for electron microscopy.
   Journal of Experimental Medicine, 95, 285-298.

PALADE, G.E. and BRUNS, R.R. (1964) Structure and function
   in normal muscle capillaries.   In "Small blood vessel involve-
   ment in diabetes mellitus" edited by M.D. Siperstein, A.R.
   Colwell and K. Meyer.   American Institute of Biological
   Sciences, Washington, D.C., pp. 39-49.

PALADE, G.E. and FARQUHAR, MARILYN G. (1965) A
special fibril of the dermis.
Journal of Cell Biology, 27, 215-224.

PAPPENHEIMER, J.R. (1953) Passage of molecules through
capillary walls.
Physiological Reviews, 33, 387-423.

PAPPENHEIMER, J.R. (1955) Über die Permeabilität der
Glomerulummembranen in der Niere.
Klinische Wochenschrift, 33, 362-365.

PAPPENHEIMER, J.R., RENKIN, E.M. and BORRERO, L.M.
(1951) Filtration, diffusion and molecular sieving through
peripheral capillary membranes. A contribution to the pore
theory of capillary permeability.
American Journal of Physiology, 167, 13-46.

PEACHEY, L.D. (1964) Electron microscopic observations on
the accumulation of divalent cations in intramitochondrial
granules.
Journal of Cell Biology, 20, 95-109.

PEASE, D.C. (1955) Electron microscopy of the vascular bed
of the kidney cortex.
Anatomical Record, 121, 701-721.

PEASE, D.C. (1960) Histological techniques for electron micro-
scopy. Academic Press, New York, pp. 137 and 172.

PEASE, D.C. (1968) Myoid features of renal corpuscles and
tubules.
Journal of Ultrastructure Research, 23, 304-320.

PIERCE, G.B., MIDGLEY, A.R. and SRI RAM, J. (1963)
The histogenesis of basement membranes.
Journal of Experimental Medicine, 117, 339-348.

PIERCE, G.B. and NAKANE, P.K. (1967) Antigens of epithelial
basement membranes of mouse, rat and man.
Laboratory Investigation, 17, 499-514.

PIEZ, K.A. (1968) Cross-linking of collagen and elastin.
Annual Review of Biochemistry, 37, 547-570.

POLACHEK, A.A., COPE, C.B., WILLIARD, R.F. and
ENNS, T. (1960) Metabolism of radioactive silver in a
patient with carcinoid.
Journal of Laboratory and Clinical Medicine, 56, 499-505.

POTTER, EDITH L. (1965) Development of the human glomerulus.
Archives of Pathology, 80, 241-255.

POTTER, EDITH L. and OSATHANONDH, V. (1966) Normal
and abnormal development of the kidney. In "The Kidney"
edited by F.K. Mostofi and D.E. Smith. Williams and
Wilkins, Baltimore, pp. 1-16.

POTTER, EDITH L. and THIERSTEIN, S.T. (1943) Glomerular
development in the kidney as an index of fetal maturity.
Journal of Pediatrics, 22, 695-706.

PRESSMAN, D. (1964) Discussion.
PRESSMAN, D. and YAGI, Y. (1964) "Chemical differences in
vascular beds". In "Small blood vessel involvement in
diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell
and K. Meyer. American Institute of Biological Sciences,
Washington, D.C., pp. 187-188 and pp. 177-183.

PRIESTLEY, G.C., PRUYN, M.L. and MALT, R.A. (1969)
Glycoprotein synthesis by membrane-bound ribosomes and
smooth membranes in kidney.
Biochimica et Biophysica Acta, 190, 154-160.

PROSE, P.H. (1963) An electron microscopic study of human
generalized argyria.
American Journal of Pathology, 42, 293-299.

RAMBOURG, A. and LEBLOND, C.P. (1967) Electron micro-
scope observations on the carbohydrate-rich cell coat present
at the surface of cells in the rat.
Journal of Cell Biology, 32, 27-53.

RATHER, L.J. (1958) Disease, life and man. Selected essays
    by Rudolph Virchow. Stanford University Press, Stanford.

REIMER, L. (1965) Irradiation changes in organic and inorganic
    objects.
    Laboratory Investigation, 14, 1082-1096.

RHODIN, J.A.G. (1962) The diaphragm of capillary endothelial
    fenestrations.
    Journal of Ultrastructure Research, 6, 171-185.

RHODIN, J.A.G. (1964) Discussion in "Small blood vessel involve-
    ment in diabetes mellitus" edited by M.D. Siperstein, A.R.
    Colwell and K. Meyer. American Institute of Biological
    Sciences, Washington, D.C., p. 74.

RICHTER, G.W. (1957) A study of hemosiderosis with the aid of
    electron microscopy; with observations on the relationship
    between hemosiderin and ferritin.
    Journal of Experimental Medicine, 106, 203-218.

RICHTER, G.W. (1960) The nature of storage iron in idiopathic
    hemochromatosis and in hemosiderosis. Electron optical,
    chemical and serologic studies on isolated hemosiderin gran-
    ules.
    Journal of Experimental Medicine, 112, 551-570.

RICHTER, G.W. and BESSIS, M.C. (1965) Commentary on
    hemosiderin.
    Blood, 25, 370-374.

ROGERS, A.W. (1967) Techniques of autoradiography.
    Elsevier, Amsterdam, pp. 3, 10-32 and 89-92.

ROGET, P.M. (1816) Additional facts relative to the preceding
    paper. [that of ALBERS]
    Medico-Chirurgical Transactions, London, 7, 290-295.

ROTHBARD, S. and WATSON, R.F. (1961) Antigenicity of rat
    collagen. Demonstration of antibody to rat collagen in the
    renal glomeruli of rats by fluorescence microscopy.
    Journal of Experimental Medicine, 113, 1041-1052.

ROTHBARD, S. and WATSON, R.F. (1969) Comparison of re-
actions of antibodies to rat collagen and to rat kidney in the
basement membranes of rat renal glomeruli.
Journal of Experimental Medicine, 129, 1145-1161.

ROWLATT, C. (1969) Subepithelial fibrils associated with the
basal lamina under simple epithelia in mouse uterus: pos-
sible tropocollagen aggregates.
Journal of Ultrastructure Research, 26, 44-51.

ROWLATT, C. (1970) Personal communication.

SAWKILL, J. (1955) Nucleation in silver azide: an investigation
by electron microscopy and diffraction.
Proceedings of the Royal Society of London, A 229, 135-142.

SCHRÖPL, F., OEHLSCHLAEGEL, G. and DRABNER, J.
(1968) Schwermetallnachweis in der Haut bei Argyrose mittels
Neutronenaktivierungsanalyse.
Archiv für klinische und experimentelle Dermatologie, 231,
398-407.

SCOTT, K.G. and HAMILTON, J.G. (1948) The metabolism of
silver.
Journal of Clinical Investigation, 27, 555-556.

SCOTT, K.G. and HAMILTON, J.G. (1950) The metabolism
of silver in the rat with radio-silver used as an indicator.
University of California Publications in Pharmacology, vol-
ume 2 (1941-1955), University of California Press, Berkeley,
1955, pp. 241-262.

SEEGAL, BEATRICE C., HSU, K.C., ROTHENBERG, MILDRED S.
and CHAPEAU, MADELEINE L. (1962) Studies of the mech-
anism of experimental nephritis with fluorescein-labelled anti-
body II.
American Journal of Pathology, 41, 183-203.

SHARP, J.T., ANDERSON, M.S. and LIDSKY, M.D. (1967)
Studies on bovine glomeruli II. Localization of glomerular
collagen.
Journal of Immunology, 99, 1254-1263.

SHAVER, S.L. and MASON, K.E. (1951) Impaired tolerance to silver in vitamin E deficient rats.
Anatomical Record, 109, 382 only.

SHIRAI, T., TAKASUGI, M. and KITAMURA, A. (1969) Variation of thickness of glomerular basement membrane in various experimental circumstances.
Experientia, 25, 1071-1073.

SIDMAN, R.L., SIDMAN, M. and TOUCHETTE, P.E. (1964) Programmed self-instruction: an adjunct to the basic neuroanatomy course.
Anatomical Record, 148, 421 only.

SMITH, H.W. (1951) The Kidney. Structure and function in health and disease. Oxford University Press, New York, 1049 p.

SNELL, KATHARINE C. (1967) Renal disease of the rat. In "Pathology of laboratory rats and mice" edited by E. Cotchin and F.J.C. Roe. Blackwell, Oxford, pp. 105-145.

SPEAR, G.S. (1960) Glomerular alterations in cyanotic congenital heart disease.
Bulletin of the Johns Hopkins Hospital, 106, 347-367.

SPIRO, D. (1959) The structural basis of proteinuria in man. Electron microscope studies of renal biopsy specimens from patients with lipid nephrosis, amyloidosis, and subacute and chronic glomerulonephritis.
American Journal of Pathology, 35, 47-73.

SPIRO, R.G. (1964) Discussion in "Small blood vessel involvement in diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute of Biological Sciences, Washington, D.C., pp. 151-155.

SPIRO, R.G. (1967, a) Studies on the renal glomerular basement membrane. Preparation and chemical composition.
Journal of Biological Chemistry, 242, 1915-1922.

SPIRO, R.G. (1967, b) Studies on the renal glomerular basement membrane. Nature of the carbohydrate units and their attachment to the peptide portion.
Journal of Biological Chemistry, 242, 1923-1932.

SPIRO, R.G. (1967, c) The structure of the disaccharide unit of the renal glomerular basement membrane.
Journal of Biological Chemistry, 242, 4813-4823.

SPIRO, R.G. (1969) Glycoproteins: their biochemistry, biology and role in human disease.
New England Journal of Medicine, 281, 991-1001 and 1043-1056.

STRIKER, G.E. and SMUCKLER, E.A. (1970) An ultrastructural study of glomerular basement membrane synthesis.
American Journal of Pathology, 58, 531-555.

STRUNK, S.W., HAMMOND, W.S. and BENDITT, E.P. (1964) The resolution of acute glomerulonephritis. An electron microscopic study of four sequential biopsies.
Laboratory Investigation, 13, 401-429.

SUZUKI, Y., CHURG, J., GRISHMAN, EDITH, MAUTNER, W. and DACHS, S. (1963) The mesangium of the renal glomerulus. Electron microscopic studies of pathologic alterations.
American Journal of Pathology, 43, 555-578.

TAKEBAYASHI, S. (1969) Ultrastructural studies on glomerular lesions in experimental hypertension.
Acta Pathologica Japonica, 19, 179-200.

THOENES, W. (1967) Endoplasmatisches Reticulum und "Sekretkörper" im Glomerulum-Epithel der Säugerniere.
Zeitschrift für Zellforschung, 78, 561-582.

TRIEDMAN, RUTH S., METZGER, H., HSU, K.C., ROTHENBERG, MILDRED, SEEGAL, BEATRICE C. and URQUHART, A. (1962) Studies of the mechanism of experimental nephritis with fluorescein-labelled antibody I.
American Journal of Pathology, 41, 95-117.

TRUMP, B.F. and BENDITT, E.P. (1962) Electron microscopic studies of human renal disease. Observations of normal visceral glomerular epithelium and its modification in disease.
Laboratory Investigation, 11, 753-781.

UNNA, P.G. (1896) The histopathology of the diseases of the skin. Translated by N. Walker and P.G. Unna. Clay, Edinburgh, pp. 1189-1192.

van BREEMEN, V.L. and CLEMENTE, C.D. (1955, a) An electron microscopic study of silver deposition in the brain.
Anatomical Record, 121, 454 only.

van BREEMEN, V.L. and CLEMENTE, C.D. (1955, b) Silver deposition in the central nervous system and the hematoencephalic barrier studied with the electron microscope.
Journal of Biophysical and Biochemical Cytology, 1, 161-166.

van BREEMEN, V.L., REGER, J.F. and COOPER, W.G. (1956) Observations on the basement membranes in rat kidney.
Journal of Biophysical and Biochemical Cytology, 2, supplement, 283-286.

VENKATACHALAM, M.A., KARNOVSKY, M.J. and COTRAN, R.S. (1969) Glomerular permeability. Ultrastructural studies in experimental nephrosis using horseradish peroxidase as a tracer.
Journal of Experimental Medicine, 130, 381-399.

VERNIER, R.L. (1961) Ultrastructure of the glomerulus and changes in fine structure associated with increased permeability of the glomerulus to protein. In "Ciba Foundation Symposium on Renal Biopsy" edited by G.E.W. Wolstenholme and Margaret P. Cameron. Churchill, London, pp. 4-28.

VERNIER, R.L. (1964 a and b) Electron microscopic studies of the normal basement membrane. In "Small blood vessel involvement in diabetes mellitus" edited by M.D. Siperstein, A.R. Colwell and K. Meyer. American Institute of Biological Sciences, Washington, D.C., pp. (a) 57-63 and (b) 78.

VERNIER, R.L. and BIRCH-ANDERSEN, A. (1962) Studies of the human fetal kidney I. Development of the glomerulus. Journal of Pediatrics, 60, 754-768.

VERNIER, R.L. and BIRCH-ANDERSEN, A. (1963) Studies of the human fetal kidney II. Permeability characteristics of the developing glomerulus. Journal of Ultrastructure Research, 8, 66-88.

VIRCHOW, R. (1855) Cellular-Pathologie. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 8, 3-39.

WALBORG, E.F., LANTZ, ROBERTA S. and WRAY, VIRGINIA P. (1969) Isolation and chemical characterization of a cell-surface sialoglycopeptide fraction from Novikoff ascites cells. Cancer Research, 29, 2034-2038.

WALKER, F. and PATRICK, R.S. (1967) Constituent monosaccharides and hexosamine concentration of diabetic human vitreous humour. Experimental Eye Research, 6, 327-331.

WALKER, F. and PATRICK, R.S. (1968) Effect of insulin on the hexosamine content of alloxan diabetic rabbit vitreous humour. Diabetes, 17, 105-107.

WALKER, F. and PATRICK, R.S. (1969) Vitreous humour in diabetes mellitus. In "The William Mackenzie Centenary Symposium on the Ocular Circulation in Health and Disease" edited by J.S. Cant. Kimpton, London, pp. 216-218.

WALLACH, D.F.H. and ESANDI, M.V.P. (1964) Sialic acid and the electrophoretic mobility of three tumor cell types. Biochimica et Biophysica Acta, 83, 363-366.

WALLENIUS, G. (1954) Renal clearance of dextran as a measure of glomerular permeability. Acta Societatis Medicorum Upsaliensis. Supplement 4.

WIESENBERGER, E. (1965) The use of the electron microscope in analytical chemistry.
Laboratory Investigation, 14, 1026-1040.

WISLOCKI, G.B. and LADMAN, A.J. (1955) The demonstration of a blood-ocular barrier in the albino rat by means of the intravitam deposition of silver.
Journal of Biophysical and Biochemical Cytology, 1, 501-510.

WISLOCKI, G.B. and LEDUC, ELIZABETH H. (1952) Vital staining of the hematoencephalic barrier by silver nitrate and trypan blue.
Journal of Comparative Neurology, 96, 371-413.

YAMADA, E. (1955) The fine structure of the renal glomerulus of the mouse.
Journal of Biophysical and Biochemical Cytology, 1, 551-566.

ZAMBONI, L. and MARTINO, C.D. (1968, a) Embryogenesis of the human renal glomerulus.
Archives of Pathology, 86, 279-291.

ZAMBONI, L. and MARTINO, C.D. (1968, b) A re-evaluation of the mesangial cells of the renal glomerulus.
Zeitschrift für Zellforschung, 86, 364-383.

ZIMMERMANN, K.W. (1915) Über das Epithel des glomerularen Endkammerblattes der Säugerniere.
Anatomischer Anzeiger, 48, 335-341.

ZIMMERMANN, K.W. (1933) Über den Bau des Glomerulus der Säugerniere.
Zeitschrift für mikroskopische-anatomie Forschung, 32, 176-278.