THE RENAL GLOMERULUS OF THE DOG; ITS NORMAL STRUCTURE AND ITS REACTION TO IMMUNOLOGICAL INJURY

By

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DECLARATION

This is to declare that all work and techniques and photographs included in this study were carried out by myself, except photographs Figs. 11, 14, 16, 18, 19, 21 which were taken by Prof. N.G. Wright to whom I would like to record my thanks and gratitude.

SUMMARY

Familiarity with normal glomerular anatomy is an essential prerequisite to understanding the pathogenesis of glomerulonephritis and interpretation of glomerular abnormalities detected by light microscopy, immunofluorescence and electron microscopy. Glomerulonephritis is now recognised to be an important canine nephropathy and it is clear that it is the formation of immune complex deposits in the glomerular filter that is responsible for virtually all types of glomerulonephritis seen in man and animals.

In Chapter 3 of the present work, an attempt was made to highlight the most important gaps in our understanding of the normal dog glomerulus with particular reference to a number of important parameters such as methods of fixation and embedding, thickness of section and variation in glomerular size between inner and outer cortical levels.

Chapter 4 provided a detailed study of sequential autolytic changes in the dog glomerulus. Under controlled experimental conditions, glomerular changes in dogs killed from two minutes up to five days after death were recorded using combined light, transmission and scanning electron microscopy. This study showed that, while autolytic changes particularly when observed with the electron microscope, occurred very rapidly after death, nevertheless there was a significant amount of cytological preservation as late as three days.

In Chapters 5 and 6, a series of experiments were carried out to study the response of the dog glomerulus to various forms of immunological injury.

Chapter 5 provided an in depth study of experimentally-induced nephrotoxic (anti-glomerular basement membrane) nephritis in dogs with particular reference to the sequential changes occurring in the glomeruli from 30 minutes after receiving anti-GBM rabbit serum up to the termination of the experiment at 80 days. Using combined histologic, immunofluorescence and ultrastructural methods the present study revealed the remarkable ability of the canine glomerulus to recover from a severe immunologically-mediated attack.

During the last three decades, the increasing utilization of immunological techniques in the study of renal disease has clearly established that glomerulonephritis, the most important renal disease of man and his closest animal associates the dog and cat, are immunological in origin. In Chapter 6, a series of serum sickness experiments were undertaken, for the first time using the dog as an experimental animal, to induce a number of different forms of immune complex glomerulonephritis, namely "one-shot" serum sickness and accelerated serum sickness.

Furthermore, as membranous nephropathy is the most common form of spontaneous immune complex glomerulonephritis in the dog, a lesion which does not occur with the above two experimental methods, an attempt was made to make use of charged antigens (cationized bovine serum albumin) in a chronic serum sickness experiment. Overall, the immunofluorescence, histological and ultrastructural features encountered in this experiment resembled closely those described in spontaneous cases of canine membranous nephropathy.

KEY TO TABLES

KEY TO FIGURES

ABBREVIATIONS USED IN THE PRESENT WORK

GN	:	Glomerulonephritis.
TEM	:	Transmission Electron Microscope (or Microscopy).
SEM	:	Scanning Electron Microscope (or Microscopy).
BSA	:	Bovine Serum Albumin.
GBM	:	Glomerular Basement Membrane.
PBS	:	Phosphate Buffered Saline (pH, 7.3).
NTS	:	Nephrotoxic Serum.
NTabs	:	Nephrotoxic Antibodies.
NTN	:	Nephrotoxic Nephritis.
NBF	:	Neutral Buffered Formalin.
TBM	:	Tubular Basement Membrane.
PAP	:	Peroxidase-Anti Peroxidase technique.

EXPLANATORY TERMS

Focal	:	Only some glomeruli affected.
Diffuse	:	All glomeruli affected.
Segmental	:	Part of a glomerulus affected.
Global	:	All of a glomerulus affected.
Obsolescence	:	Shrunken scarred glomerular tuft with no patent capillaries.

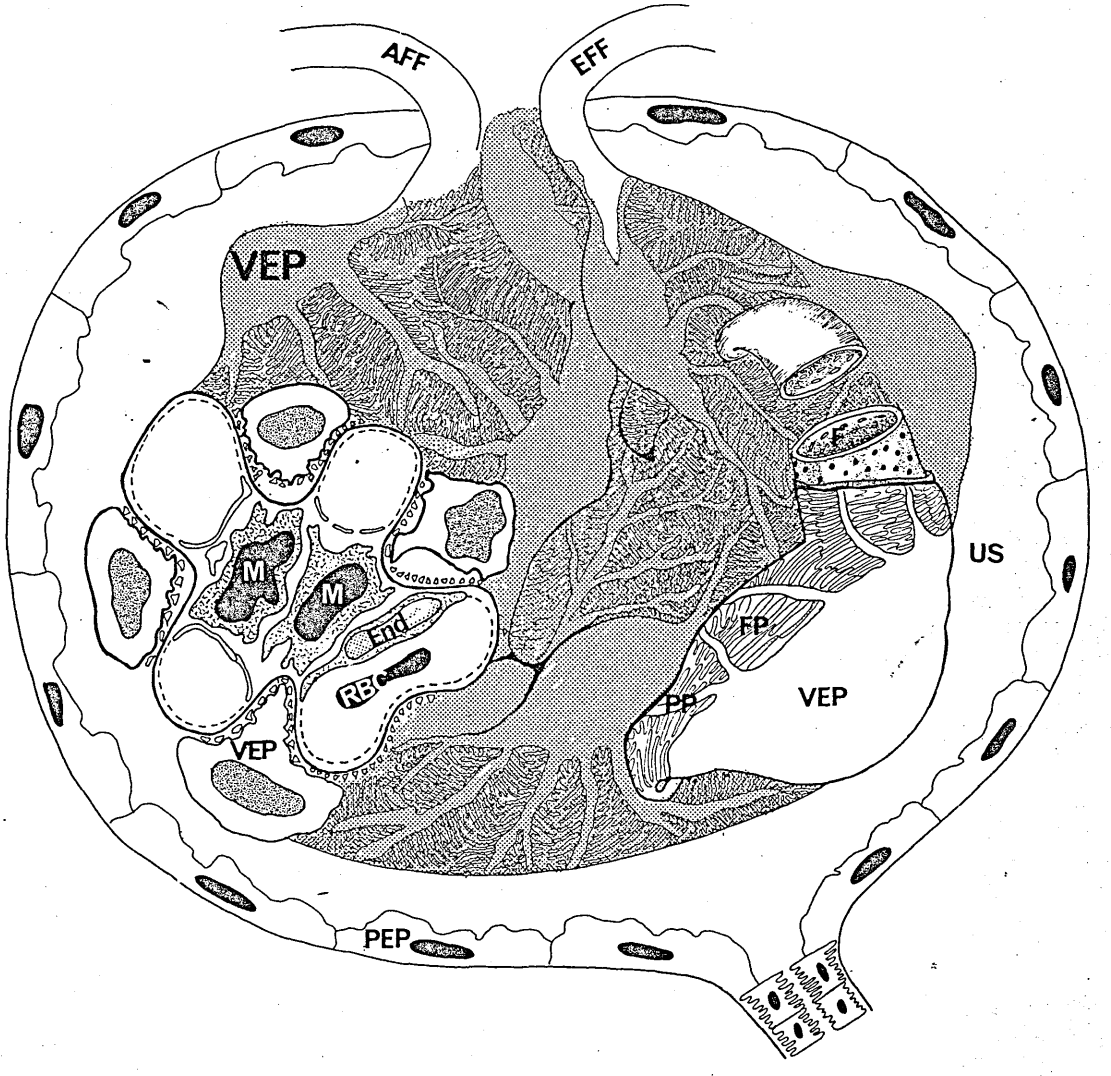
CHAPTER 1

GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

"It will never be possible for us to be completely ourselves until we have resolved the problems of the animal in relation to man...."

J. BOUTONNIER

Diagram of a normal dog renal corpuscle



AFF = Afferent arteriole
EFF = Efferent arteriole
End = Endothelial cell
FP = Foot processes
M = Mesangial cell
PEP = Parietal epithelium
PP = Primary process
RBC = Red blood cell
VEP = Visceral epithelium
US = Urinary space

The renal corpuscle of the mammal, with its functionally vital glomerulus, has fascinated anatomists, clinicians and pathologists for decades. Since the introduction of the modern light microscope, and more recently of the transmission and scanning electron microscopes, a vast literature dealing with many diverse aspects of glomerular structure has accumulated. These extensive morphological studies have allowed a general understanding of the functional role of glomerulus not only in the filtration of urine but also with respect to the mechanisms involved in renal failure.

It is not the intention of this dissertation to review all the histological and ultrastructural features of the mammalian glomerulus; the literature is now so extensive that this would not be possible. Only a brief summary of the major morphologic characteristics of the glomerulus are given in order to set the scene for the present work.

The renal glomerulus of the mammal:

In 1669 Marcello Malpighi (cited by Mueller and Syracuse, 1958) an Italian biologist who became the founder of microscopic anatomy, first described clearly the curious spherical tufted arrangement of capillaries in the renal cortex; these came to be known as "Malpighian" or "renal" corpuscles. It was not until 200 years later in 1842, when William Bowman showed that each corpuscle was enveloped by the expanded end of a renal tubule to form a capsule (Bowman's capsule) around the capillary tuft, that it became clear that it was into the space enclosed by the capsule that urine was produced from capillaries and subsequently carried away by the remaining tubular portion. Three years later in 1845, Gerlach found cells covering the outer surface of the capillaries and

concluded that they were derived from the capsule. Another early report was that by Vaughan (1879) who carried out an examination of sections of kidney with a hand lens and noted numerous minute red dots in the cortical portion, the Malpighian bodies.

Since these early steps in our understanding of glomerular morphology, there have been extensive investigations of the normal histology and ultrastructure of the mammalian glomerulus; these include those by McGregor (1929), McManus (1948), Pease and Baker (1950), Smith (1951), Trabucco and Marquez (1952), Mueller et al (1955), Pease (1955), Rhodin (1955) and Mueller and Syracuse (1958).

Most of the progress made in recent years in the study of the functional morphology of the glomerulus has been due to the technical possibilities offered by the transmission electron microscope (Rhodin, 1955; Yamada, 1955; Farquhar et al, 1961; Graham and Karnovsky, 1966; Jorgensen and Bentzon, 1968; Latta, 1970 and Venkatachalam et al, 1970). Despite the complicated arrangement of the glomerular capillaries, a general accepted plan of the renal corpuscle has been put forward by Boyer (1956), Elias et al (1960) Murakami et al (1971) and Murakami (1972). The major structural components that are organised to form the renal corpuscle are the capillary endothelial cells, mesangial cells and matrix, glomerular basement membrane (GBM) and visceral and parietal epithelial cells. A brief account of the major characteristics of these structures is now given:

a) Endothelial cells:

The thin cytoplasm of glomerular capillary endothelial cells intimately apposed to the GBM is characterized by large pores or

fenestrae which were first recognised by Hall (1954); these vary from 500A° to 1000A° in diameter (Farquhar et al, 1961). Although 60 A° thick diaphragms have been observed bridging the fenestrae of mouse endothelium (Rhodin, 1962), these have not been recognised in other species (Farquhar et al, 1961; Latta, 1970). The main body of the endothelial cell occupies the axial portion of the capillary loop and is directly contiguous with adjacent mesangial cells without an intervening basement membrane; the thin fenestrated portion of the cytoplasm forms a thin layer around the peripheral region of the capillary.

In the mouse, Yamada (1955) reported that the cytoplasm adjacent to the nuclear region contains mitochondria, Golgi bodies and a sparse endoplasmic reticulum and, in addition, a characteristic feature is the presence of numerous densely packed vesicles and caveolae.

b) Mesangium:

The mesangium of the glomerulus has been the object of considerable ultrastructural definition and controversy. The term "mesangium" was introduced by Zimmerman (1933) to designate the structure that he assumed served as a supporting "mesentery" for the renal glomerular capillary tuft. After years of dispute regarding the structural nature of the glomerular mesangium it is now appreciated that this supporting structure consists of an intercapillary population of distinctive glomerular cells. Yamada (1955) reported that the cytoplasm of mesangial "intercapillary" cells contains mitochondria, endoplasmic reticulum, minute vesicles, and numerous delicate filaments which arrange themselves along the axes of the cell processes and thus suggests a similarity with smooth

muscle cells.

Recently, Burkholder (1982) has reviewed the numerous functions attributed to mesangial cells; these include fibrocytic activity, smooth muscle contractility, phagocytosis, receptor activity for selected hormones and peptides, prostaglandin production, synthesis of mesangial matrix and proliferation in response to injury. Mesangial cells are bounded laterally by the GBM and are contiguous at the axial region of the capillary loops with the endothelial cells. In the normal animal, cytoplasmic projections often protrude between the endothelial cells into the capillary lumina (Crowell et al, 1974). Normally, mesangial matrix comprises a narrow band of amorphous fibrillar, basement membrane-like material of unknown origin and biochemical composition (Osborne et al, 1977). It has been suggested, however, that mesangial matrix is formed in part from deposition of old GBM in the mesangium following its turnover and renewal (Walker, 1973). The absence of reactivity of the mesangium with antibody to GBM antigens indicates that it is immunologically distinct from the GBM (Scheinman et al, 1974).

Mesangial cells are continuous with "Lacis" or pseudo-meissnerian cells of the juxtaglomerular apparatus; they also show many similarities in fine structure. Mesangial cells may supplement both "lacis" cells and granular epithelioid cells in controlling glomerular blood flow (Latta et al, 1962).

c) Glomerular basement membrane (GBM):

The GBM is sandwiched between endothelial cells and visceral epithelial cells in peripheral portions of capillaries and between

mesangial cells and visceral epithelial cells in the axial portions. It appears to be composed of three layers when examined by the electron microscope (Crowell et al, 1974; Churg and Grishman, 1975). Immediately adjacent to the endothelium there is an electron-lucent inner layer, the lamina rara interna. The bulk of the GBM, however, is composed of an electron-dense middle layer, the lamina densa; the outer layer, the lamina rara externa is electron - lucent and lies beneath the visceral epithelium.

The total thickness of the GBM varies from 700 to 1000 A^{o} in mice (Yamada, 1955); 1200 - 1500 A^{o} in rats (Farquhar et al, 1961), 1200 to 1900 A^{o} in rabbits (Latta, 1970), 1000 - 1300 A^{o} in the dog (Movat and Steiner, 1961) and 1000 - 1900 (Crowell et al, 1974), 2400 - 3200 A^{o} in the monkey (Latta, 1970) and 3288 A^{o} - 3500 A^{o} in man (Jorgensen and Bentzon, 1968).

The composition of the GBM from several species of animals has been extensively reviewed by Kefalides (1972). Chemical studies have established that the GBM has collagen and glycoprotein components. The collagen moiety differs from interstitial collagen. The carbohydrates of the glycoprotein make up 10 per cent of the GBM dry weight and are characterised by two molecular structures, disaccharides and heteropolysaccharides. The peptide portion is formed by an amino acid sequence similar to that of basement membranes in other organs (Albini et al, 1979).

d) Visceral epithelial cells:

Glomerular capillaries are invested with a layer of epithelial cells (podocytes) characterised by an elaborate layer of primary, secondary and tertiary cytoplasmic processes which extend to the GBM;

tertiary cytoplasmic processes are commonly called "foot processes". These abut on to the GBM and between each process there is a thin diaphragm 200 - 300 A^{o} wide "slit membrane" (Yamada, 1955).

Visceral epithelial cells are in direct continuation with the squamous, relatively simple, parietal epithelial cells which line Bowman's space (Osborne et al, 1977). In addition to their hypothesised function in the synthesis of one or more components of the GBM and regulation of permeability, the presence of microfilaments in the cytoplasm of visceral epithelial cells has suggested that they also have a contractile function (Karnovsky and Ainsworth, 1972). Among the cytoplasmic organelles are the Golgi apparatus, centrioles and a few mitochondria all of which are located near the nucleus. Farquhar et al (1961) has described the endoplasmic reticulum as having large distended cisternae containing basement membrane-like material.

In recent years, the introduction of scanning electron microscopy (SEM) has helped to illuminate the topography of the renal glomerulus particularly with regard to the peculiar interdigitating arrangement of the foot processes of visceral epithelial cells. The primary, secondary and tertiary arrangement of the cytoplasmic processes of visceral epithelium has been described in various mammalian species (Arakawa, 1970; Fujita et al, 1970; Arakawa, 1971; Andrews and Porter, 1974; Andrews, 1975; Spinelli, 1976). Furthermore, studies of vascular casts of glomerular capillaries with the scanning electron microscope take advantage of the exceptional depth of field of scanning electron micrographs to illuminate the spatial orientation of the glomerular capillaries (Murakami, 1972; Spinelli, 1976; Anderson and Anderson, 1976).

e) Parietal epithelium:

The epithelium of Bowman's capsule is a layer of thin, flattened cells intimately apposed to the capsular basement membrane. The cell junctions are distinct (McGregor, 1929) and this layer of cells is continuous with the epithelium of the proximal tubule and with the visceral epithelial cells covering the capillaries.

Electron microscopic studies of these cells have revealed that they have few cytoplasmic organelles and, apart from a few mitochondria scattered throughout the cytoplasm, they do not have the intricate organisation of internal structure which characterises the cells of proximal tubules (Mueller et al, 1955; Yamada, 1955).

Peripolar cells have recently been described by Ryan et al (1979) as distinctive granular parietal cells encircling the orifice of the proximal tubule. The function of these cells is still in dispute but they may be involved in juxtaglomerular complex activity or they may have some regulatory role on the glomerular filtrate which flows past these cells on its way to the proximal tubule.

Regarding the relationship of glomerular number and diameter to body size, Kunkel (1930) noted that interspecies differences exist in the number of glomeruli in the kidney of mammals. On the other hand, Smith (1951) concluded that the number of glomeruli in the kidney of adults within a species was relatively constant and that the correlation did not exist between numbers of glomeruli and body weight. This is in contrast to the earlier work of Rytand (1938) who stated that the number and size of mammalian glomeruli are related to kidney and body weight.

The renal glomerulus of the dog:

In terms of investigation of renal function, the dog has been and still is used probably more than any other mammalian species. Yet the majority of morphologic studies of the renal glomerulus have been carried out in the rat, mouse and man (Arakawa, 1970; Spinelli, 1976; Osborne et al, 1977).

Furthermore, the recent surge of interest into spontaneous glomerular disease of the dog (Murray and Wright, 1974; Mueller-Peddinghaus and Trautwein, 1977; Wright et al, 1981) has highlighted the importance of a real understanding of normal glomerular structure in this species, not only at the light microscopic level but also in view of the increasing use of the transmission and scanning electron microscopes.

Some aspects of canine glomerular morphology such as size and overall number have been studied and there are reports, mostly brief and incomplete, of the normal histological and ultrastructural features. The number of glomeruli in the kidney of a dog for example has been calculated by various workers to be 125,000 - 142,000 (Brodie, 1914), 500,000 (Vimtrup, 1928; Kunkel, 1930), 408,100 (Rytand, 1938), 415,000 (Smith, 1951) and 630,000 (Sellwood and Verney, 1955).

There is also some dispute as to whether or not the number of glomeruli is related to body weight. Thus, Kunkel (1930) reported that the number and size of glomeruli varied in dogs of different weight but there is no constant relationship between the number of glomeruli and kidney weight or body weight. This view was challenged by Smith (1951); he concluded that both kidney size and

number of glomeruli are related to body size. However, Finco and Duncan (1972) found a significant correlation did not exist between glomerular number and body weight or body surface. It appears that the mean diameter of juxtamedullary glomeruli of the dog is slightly greater than that of cortical glomeruli (Horster et al, 1971).

On the whole, however, there is relatively little published information on the histological and ultrastructural features of the normal dog glomerulus; there are only a few brief ultrastructural reports in the recent literature (Wright et al, 1973[b]; Osborne et al, 1977). Mueller et al (1955) in an early ultrastructural study, described the interdigitation of the foot processes of visceral epithelial cells which overlie and insinuate between the loops of each capillary. They also reported that the basement membrane of the dog glomerulus was a single homogeneous membrane 2,000 to 2400 A^{o} thick. They claimed that the glomerular capillaries divide at the vascular pole into four, six or eight primary branches which enter the glomerular space and emerge without any anastomosis between the individual primary branches. Each primary branch divides several times and subsequently re-unites and returns to the vascular pole where it unites with other primary branches to exit as the efferent arteriole. They claimed that there are no differences between the dog and human glomerulus.

Movat and Steiner (1961), in a more detailed ultrastructural study, attempted to establish some baseline information on the normal dog glomerulus. They stated that visceral epithelial cells consist of a main cytoplasmic mass which contains the nucleus and the majority of organelles. Large cytoplasmic extensions arose from the cell body and gave rise to numerous foot processes. They described

the endothelial cells lining the capillary lumina and noted that the main cytoplasmic mass of these cells contained the nucleus. In contrast to the visceral epithelial cells, smooth surfaced (pinocytic) vesicles were seen in large numbers. The attenuated portion of the endothelial cytoplasm arises from the main cytoplasmic mass and contains numerous perforations or fenestrae. With regard to the relation of endothelial cells to the GBM, this was found to be constant in the region of attenuated (peripheral) portion of the cytoplasm. The relation of the main cytoplasmic mass to the basement membrane, however, was less constant. Here the lamina rara interna was often focally widened indenting the endothelial cytoplasm. This was considered to be of importance in the interpretation of pathologic changes in this area. Three layers of the dog glomerular basement membrane were described. A lamina densa, measuring between 770 A^{o} and 830 A^{o} in width and lamina rara externa 200 - 250 A^{o} in width and lamina rara interna only a few A^{o} units thinner than that of the lamina externa.

From the available literature it is apparent that much has still to be established with regard to the normal histological and ultrastructural features of the normal dog glomerulus and that what may be true of the glomerulus of the rat or man might not necessarily apply to the dog. Furthermore, the increase in interest in primary immunological glomerular disease in the dog makes it necessary to study, under controlled experimental conditions, how the dog glomerulus reacts to such immunologic injury.

The purpose of Chapter 3 of the present work was not only to provide a brief description of the general morphologic features of the canine glomerulus in the adult and neonatal dog but also to compare

the importance of preparative techniques such as perfusion as distinct to immersion fixation.

Chapter 4 was designed to study the sequential changes occurring in the dog glomerulus due to autolysis, using combined light, transmission and scanning electron microscopy.

In Chapters 5 and 6, the reaction of the dog glomerulus to experimentally-induced immunologic injury is described. Chapter 5 deals with anti-GBM (nephrotoxic serum) nephritis while, in Chapter 6 various models of experimentally-induced immune complex glomerulonephritis are compared.

CHAPTER 2

GENERAL MATERIALS AND METHODS

1. Source of animals:

Litters of Collie-cross puppies aged 14 - 20 weeks were obtained from commercial sources. On arrival, they were immunised against canine parvovirus, distemper virus, leptospira canicola, leptospira icterohaemorrhagiae and adenovirus subtype I, using standard commercial vaccines. They were observed for at least three weeks before being used for experimental purposes.

2. Method of euthanasia:

Dogs were given a neuroleptanalgesic "Immobilon SA" (Reckitt and Colman Pharmaceutical Division, Hull, England) and subsequently euthanized by an intravenous injection of sodium pentobarbitone (Euthatal, May and Baker, Dagenham, England). As soon as deep anaesthesia was achieved, exsanguination was performed by severing the axillary artery. The abdominal wall was then incised, the kidneys exposed and portions removed for further study.

3. Light microscopic studies:

Small blocks of cortex were fixed in 10% neutral buffered formalin (NBF) for four days and subsequently post fixed for at least two days in mercuric chloride formol. The kidney tissue was then dehydrated, cleared and impregnated with paraffin wax. Sections two to three μm thick were cut and stained routinely with haematoxylin and eosin (H&E). Other stains used on occasion were Martius scarlet blue (collagen and fibrin) and periodic acid-Schiff (basement membranes).

4. Scanning electron microscopic studies:

Slices of kidney cortex less than two mm in thickness were gently washed in 0.1 M cacodylate buffer in order to remove surface blood and then immersed in Karnovsky's fixative (paraformaldehyde-glutaraldehyde) for 24 hours, the specimens were then washed in 0.1 M cacodylate buffer for four hours before dehydration in a series of acetones (70%, 90%, 100%). The specimens were then dried in a Polaron critical point dryer, mounted on stubs, and coated with gold in an Emscope sputter coater for four minutes. Specimens were then viewed in a Philips 501B scanning electron microscope.

5. Transmission electron microscope studies:

Small portions of cortex were diced into small pieces, fixed in paraformaldehyde-glutaraldehyde, washed with 0.1 M cacodylate buffer and post-fixed in 1% osmic acid. Specimens were then washed with distilled water, dehydrated in a series of acetones (70%, 90%, 100%) and subsequently placed in two changes of propylene oxide for 20 minutes each. The specimens were then placed in propylene oxide/ Emix resin (50:50) for one hour, Emix resin for four hours, and the specimens embedded in resin and polymerised at 60° overnight. Sections one µm thick were cut in an LKB pyramitome and stained with toluidine blue. Once glomeruli had been identified, ultrathin sections were cut in an LKB mark III ultramicrotome, stained with uranyl acetate and lead citrate and examined with an Hitachi HS8 electron microscope.

6. Kidney perfusion method:

Immediately following exsanguination the renal vein was clamped by means of an artery forceps and a cannula (No. 12 guage) was inserted into the renal artery. 40 ml of Karnovsky's fixative was

slowly injected manually through the cannula at a flow rate of 2 ml per minute. As soon as the fixative was observed emerging from the renal vein, the vein was severed and perfusion continued for five minutes. During perfusion the kidney rapidly became blanched and firm. Following perfusion small blocks of cortex were transferred to 10% NBF for histological studies and slices placed in Karnovsky's fixative for SEM. Small portions of cortex were diced into small pieces and immersed in Karnovsky's fixative for TEM.

7. Preparation of arterial casts:

Corrosion casts of kidney were prepared by inserting a cannula (gauge No. 12) into the renal artery and flushing out the renal circulation with PBS (approximately 40 ml at room temperature at a flow rate of 2ml/min). 15 - 20ml of a mixture of Tensol cement and No. 7 component A and B (ICI Plastic Division, Welwyn Garden City, Herts, England) in a ratio 25/1 was then injected slowly through the cannula. When filling was complete, the kidney was transferred to an incubator at 37°C for four hours and then placed in a 30% solution of potassium hydroxide for three days. Once the digestion process was completed, the kidney was placed in a bath of running water in order to remove any crystalized KOH. After drying at 37°C overnight, small pieces of cortex were carefully removed for examination with the SEM as described above.

8. Immunofluorescence methods:

Cryostat sections of kidney four µm thick were washed for five minutes in PBS and subsequently fixed in acetone for a further five minutes. They were then stained for 30 minutes in a moist chamber with rabbit anti-dog IgG, sheep anti-rabbit globulin, goat anti-dog

complement (C_3) or goat anti-dog fibrinogen all labelled with fluorescein isothiocyanate. After washing with PBS for 10 minutes the stained sections were examined using a Leitz Orthoplan microscope equipped for incident light fluorescence.

CHAPTER 3

A HISTOLOGICAL AND ULTRASTRUCTURAL STUDY OF THE NORMAL DOG GLOMERULUS

Introduction:

The purpose of this part of the work was to carry out for the first time a combined light microscopical and ultrastructural investigation of the normal dog glomerulus taking particular account of a number of important parameters such as method of fixation and embedding, thickness of section and variations between inner and outer cortical levels. In addition the main features of the normal glomerulus were studied with the transmission and scanning electron microscopes. With respect to the latter, a number of arterial casts were prepared in order to study the overall organisation of the glomerular capillaries. Also included in this section is a study of the normal post-natal development of the dog glomerulus.

Despite the extensive literature on the development of the neonatal mammalian glomerulus (Hall and Roth, 1956; Kurtz, 1958; Potter, 1965) little work has been done on nephrogensis in the dog. Eisenbrandt and Phemister (1977) described the histological features of nephrogensis in dogs from two to 200 days after birth and claimed that nephrogensis had diminished by eight days, and was absent by 14 days. They mentioned that the histologic appearance of kidneys at 70 and 200 days of age was similar to that of normal adult kidneys. Apart from this report, there is no sequential study of the post-natal development of the canine glomerulus.

Materials and methods:

These are given in detail in the general section on materials and methods in Chapter 2.

The kidneys from 15 five month old dogs and 10 newborn puppies

formed the basis of this study. In every case, the kidneys were shown to be histologically normal and immunofluorescence tests for the presence of canine IgG and C_3 proved negative. The number of kidney specimens used in the investigation of each morphologic parameter is given below.

In the five month old dogs one kidney from each animal was used for light microscopy either using immersion or perfusion fixation. The contralateral kidney was used for ultrastructural studies or for producing arterial casts. An opportunity arose to study two litters of newborn Collie cross puppies. In all, 10 puppies were killed by intracardiac injection of sodium pentobarbitone at four hours (2), 36 hours (2), three days (2), seven days (2) and 15 days (2) after birth. Only light microscopy (immersion fixation) was carried out in these animals.

	No of kidney specimens
Immersion Fixation (Light Microscopy)	10
Perfusion Fixation (Light Microscopy)	10
Immersion Fixation (Electron Microscopy, TEM and SEM)	5
Perfusion Fixation (Electron Microscopy, TEM and SEM)	5
Plastic Embedding (Light Microscopy)	5
Arterial Casts (SEM only)	10
Neonatal Puppies (Light Microscopy only)	10

Results:

Light microscopy:

1. Light microscopy (immersion fixation):

Fig. 1 shows a typical renal corpuscle from an immersion fixed kidney section three μm thick. This is a deep cortical glomerulus. Note the patent capillaries, and easily differentiated visceral epithelial, endothelial and mesangial cells. In contrast a six μm thick kidney section (Fig. 2) shows a glomerulus with closed capillaries and the glomerular tuft seems to be hypercellular with darkly stained nuclei which are difficult to identify.

In the outer cortex, the glomerular capillaries are often closed with resultant difficulty in identifying the various glomerular cell types (Fig. 3). In the mid cortex, some glomeruli show patent loops while in others some capillaries are open and some are closed (Fig. 4).

2. Light microscopy (perfusion fixation):

In perfused kidney sections, the renal corpuscle often has a wide urinary space. The lumina of capillaries are usually patent and largely empty, allowing good differentiation between endothelial, visceral epithelial and mesangial cell types (Fig. 5). In the present study not all glomeruli are, however, perfused evenly, particularly the outer cortical glomeruli (Fig. 6).

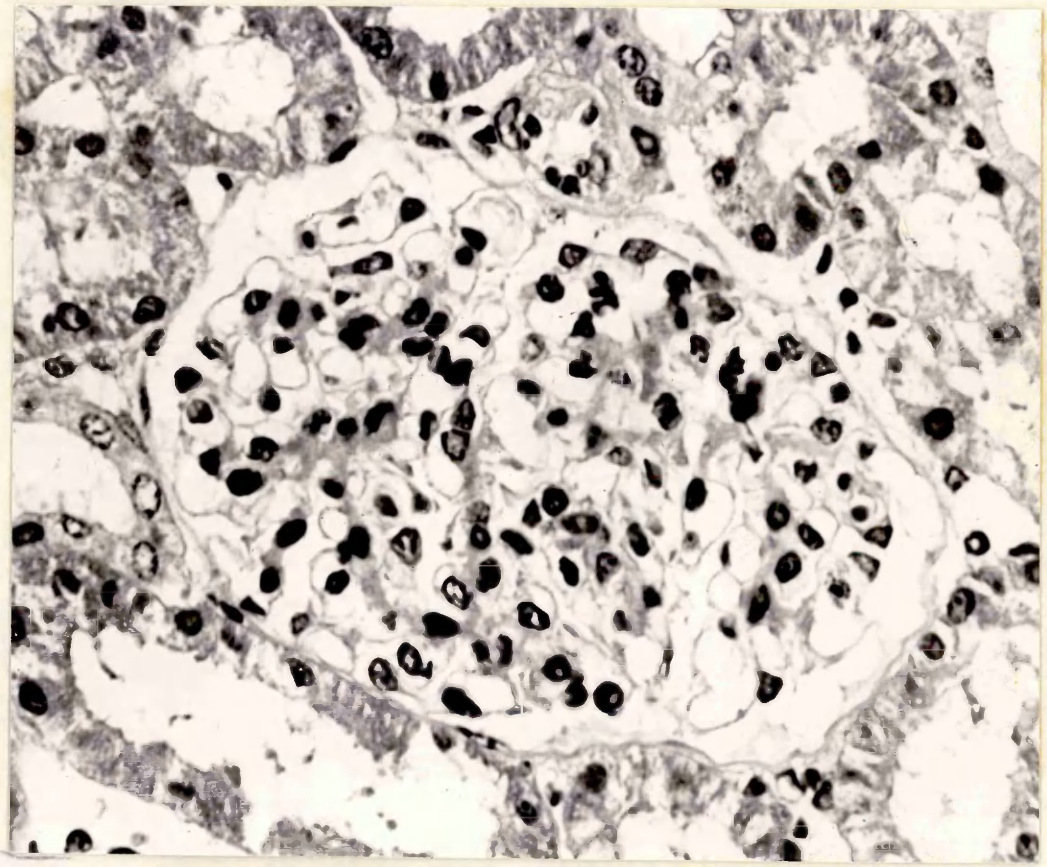
3. One μm plastic sections (non-perfused):

The renal corpuscle in plastic sections often shows diminished or obliterated urinary space. The capillary lumina are

usually patent and it is relatively easy to identify the glomerular cell types (Fig. 7). Fig. 8 depicts an outer cortical glomerulus with prominent visceral epithelial nuclei.

Fig. 1. A normal dog glomerulus: deep cortex, immersion fixation, three μm thick, showing patent capillaries; visceral epithelial cells, endothelial cells and mesangial cells can easily be identified. H & E x 300.

Fig. 2. A normal dog glomerulus: immersion fixation, six μm thick, showing closed capillaries and the glomerular tuft seems to be hypercellular with darkly stained nuclei which are difficult to identify. H & E x 300.



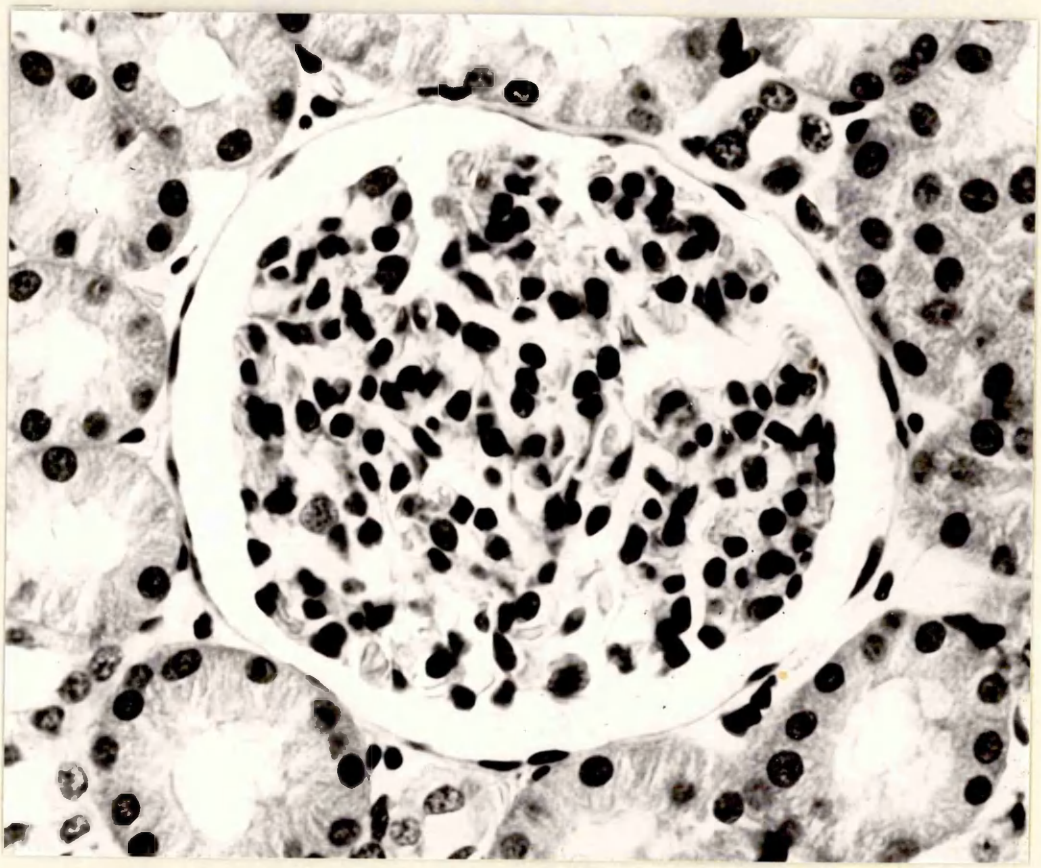
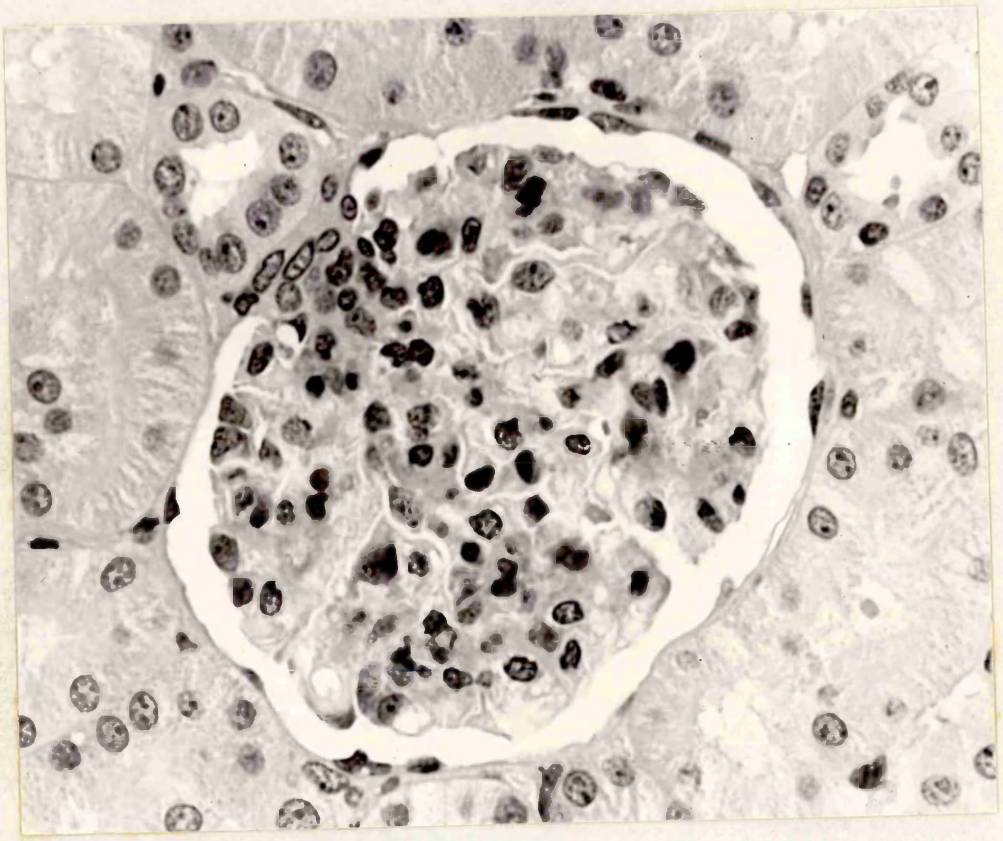


Fig. 3. Normal dog glomerulus: outer cortex, the glomerular capillaries are closed and it is difficult to identify the various glomerular cells. H & E x 300.

Fig. 4. Normal dog glomerulus: mid cortex showing some patent loops. H & E x 300.



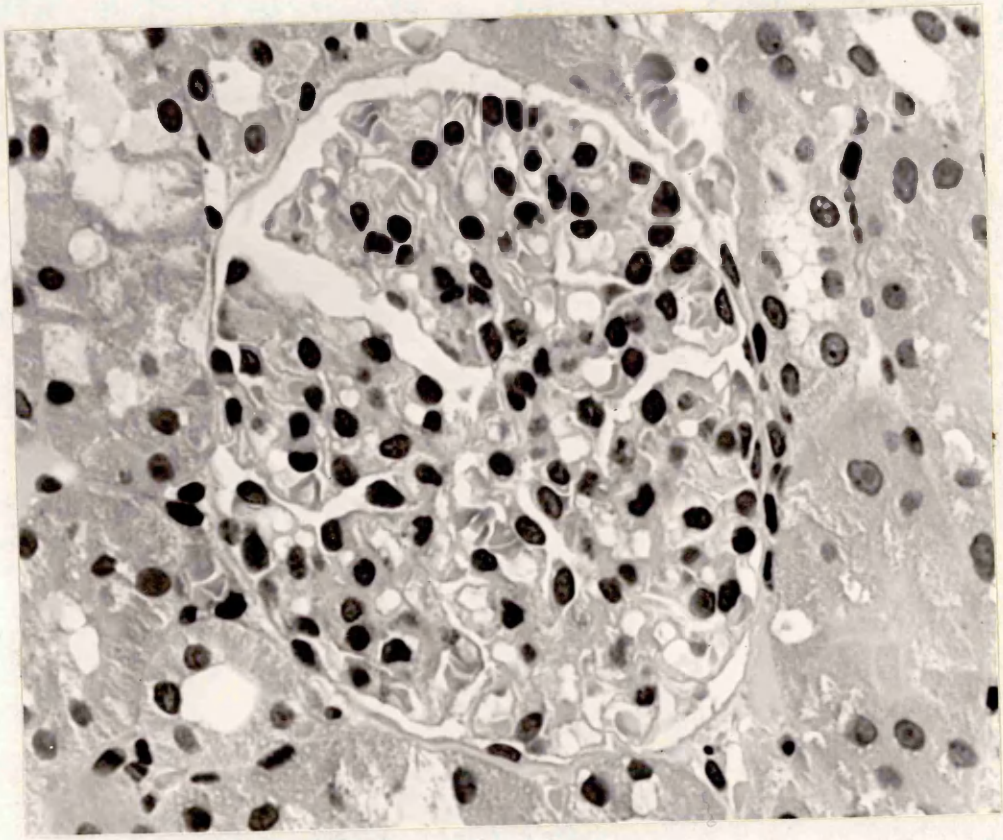
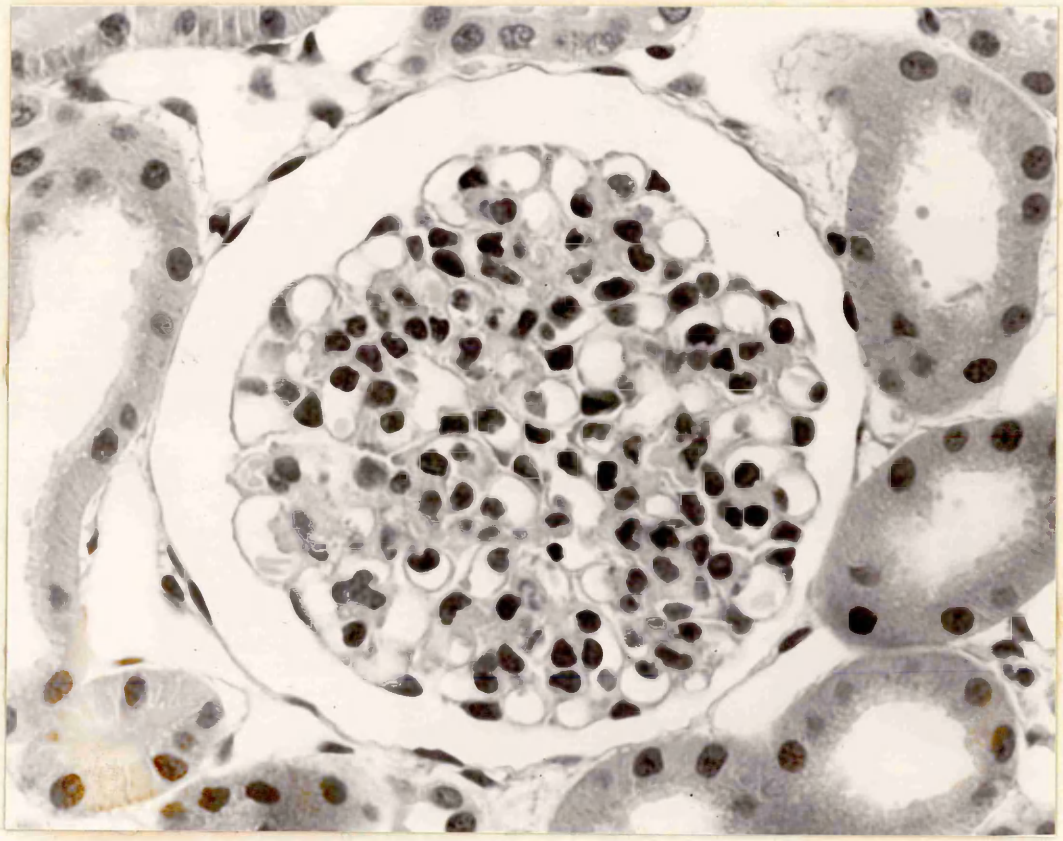


Fig. 5. Normal dog glomerulus: from a perfusion fixed kidney section three μm thick. The lumina of capillaries are patent and largely empty; the endothelial cells epithelial cells and mesangial cells are easily differentiated. H & E x 300.

Fig. 6. Normal dog glomeruli: from a perfusion fixed kidney section, showing three outer cortical glomeruli, one perfused evenly, a second partially perfused and the third not perfused. H & E x 180.



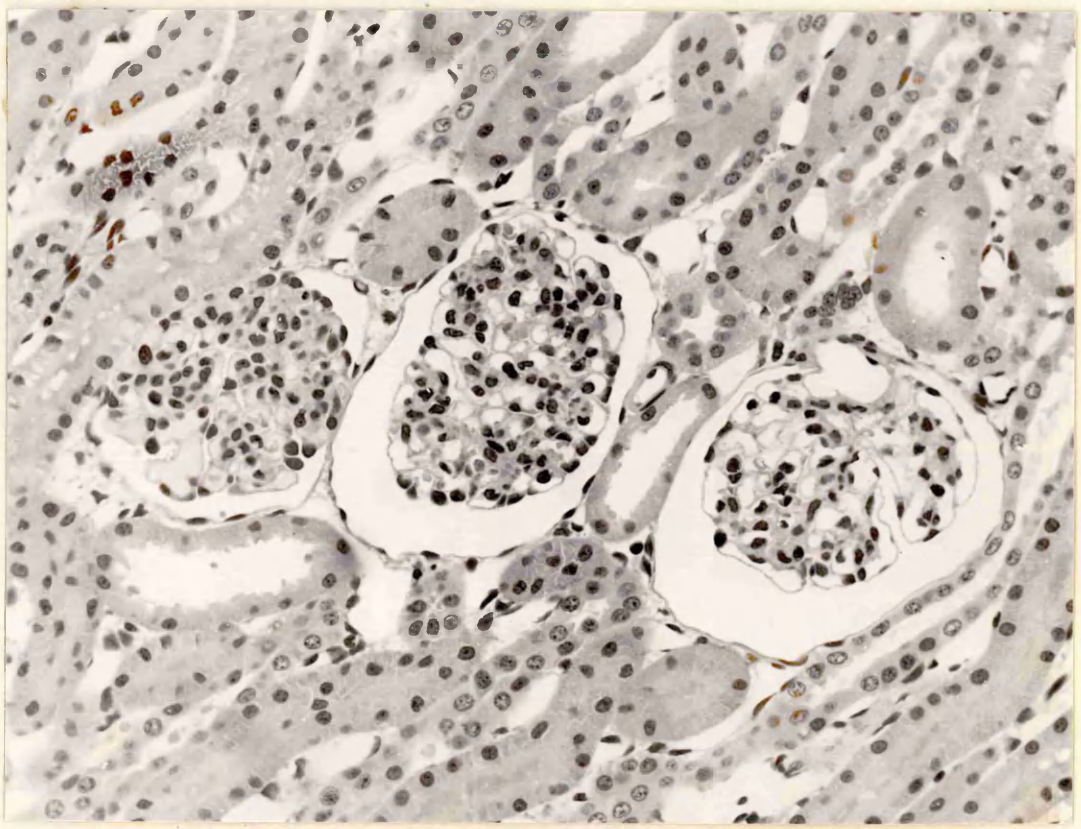
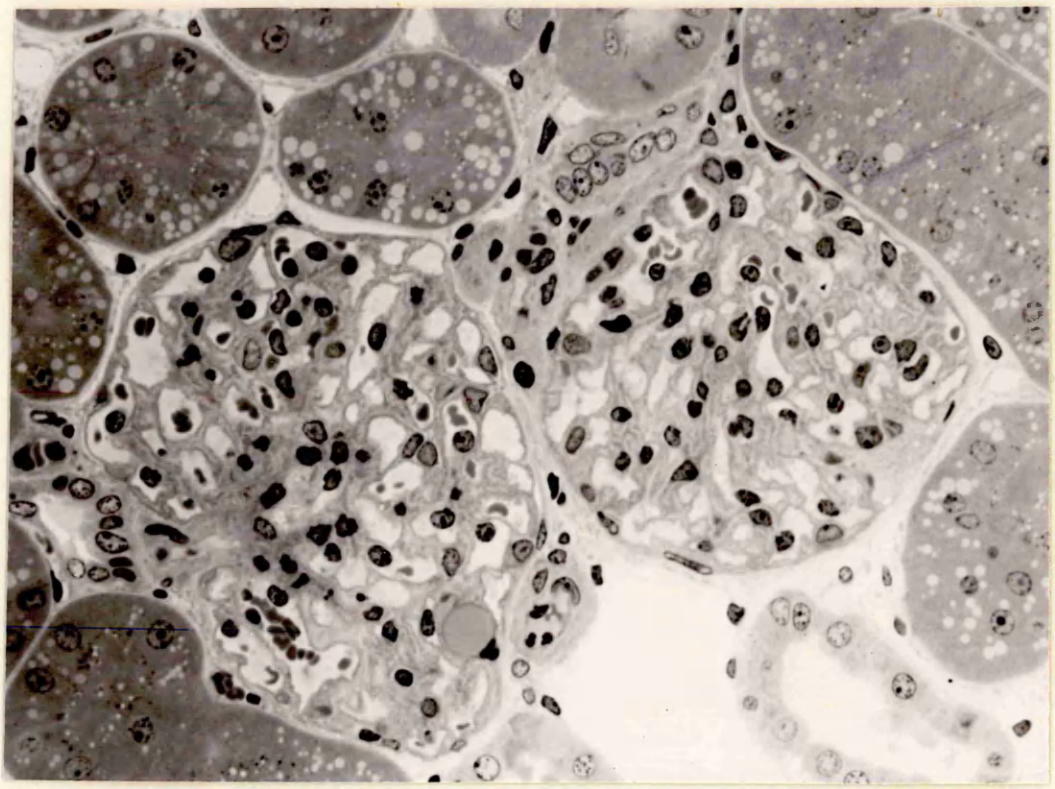
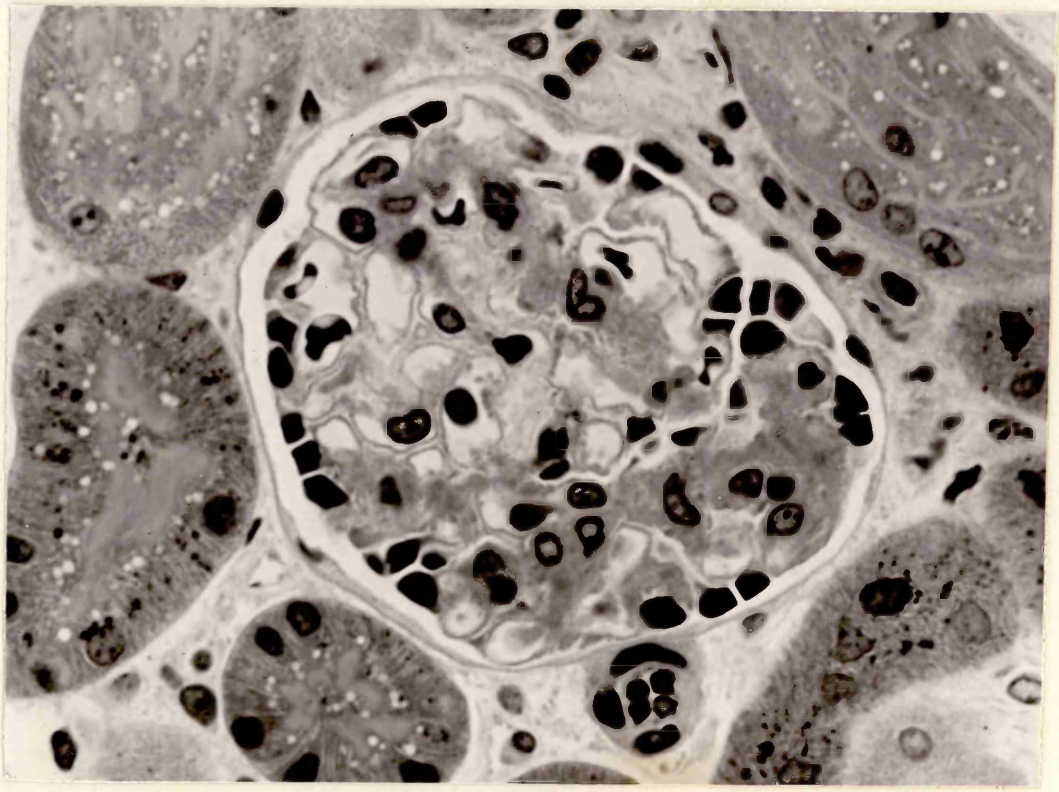


Fig. 7. Normal dog glomeruli: non perfused, showing patent capillary lumina, easily identified epithelial cells, endothelial cells and mesangial cells, one μm plastic section, Toluidine blue x 250.

Fig. 8. Normal dog glomerulus: non perfused, showing prominent nuclei of epithelial cells, one μm plastic section, Toluidine blue x 300.





Transmission electron microscopy:

Particular attention was paid to the four major components of the dog glomerulus namely (a) visceral epithelial cells (b) endothelial cells (c) the mesangium and (d) the glomerular basement membrane.

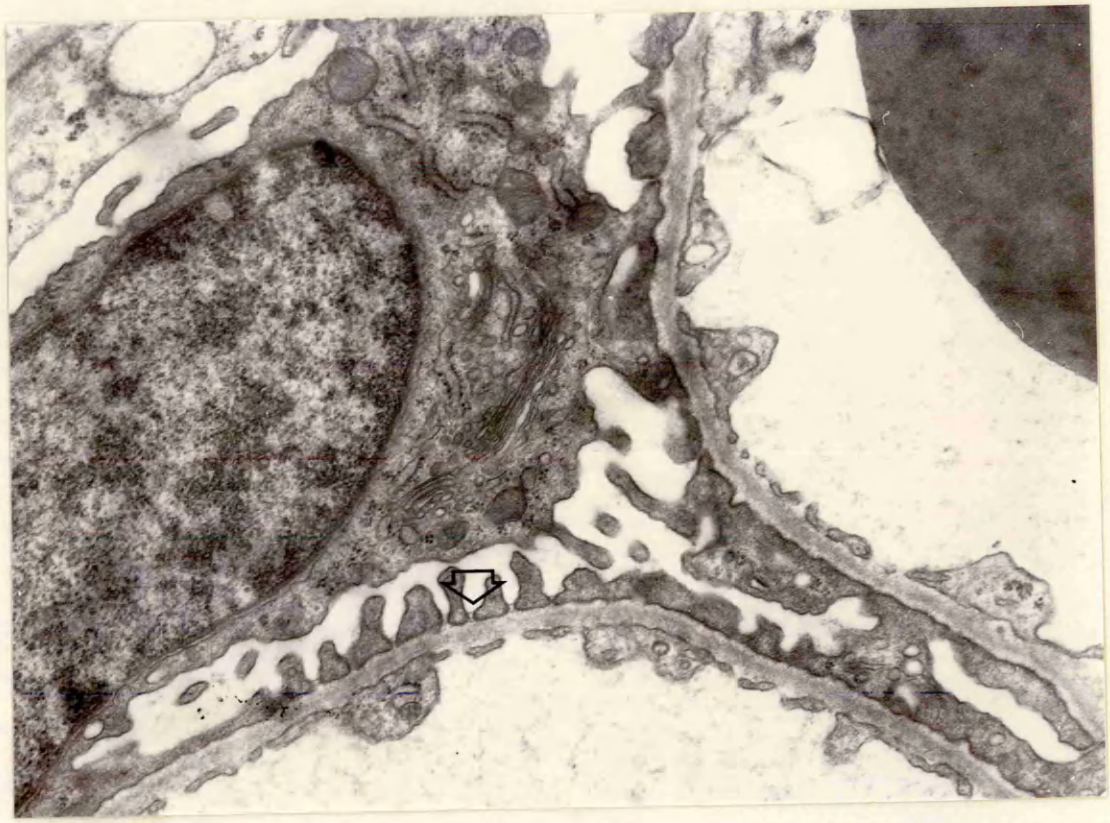
(a) Visceral epithelial cell:

From the main mass of the epithelial cell body in which the nucleus and the majority of organelles are located, a number of large primary cytoplasmic processes give rise to numerous secondary branches. The tertiary or foot processes are regularly arranged along the basement membrane. The single unit membrane, namely the slit membrane, is often clearly noticeable between neighbouring processes (Figs. 9 and 11). Cytoplasmic organelles are largely confined to the perinuclear region and to primary and to lesser extent secondary processes. Mitochondria are not conspicuous whereas a prominent Golgi apparatus is often present. The latter consists of flattened smooth surfaced sacs appearing as tubes in longitudinal and as vesicles in transverse sections.

The endoplasmic reticulum is sparse and consists of occasional flattened rough and smooth surfaced vesicles, the former having an outer coating of ribosomes. Fragments of endoplasmic reticulum are often found as far distant from the main cytoplasmic mass as the foot processes. In addition aggregates of ribosomes are commonly observed throughout the cytoplasmic processes of the cell. Clusters of fine microfilaments and occasional microtubules are also seen throughout the cell and extending as far as the foot processes.

Fig. 9. A section of normal dog glomerulus: reveals the relationship of epithelial cells to the basement membrane. Foot processes are implanted on the lamina rara externa. Epithelial cell cytoplasm shows sparse rough endoplasmic reticulum and a prominent Golgi body. Note the slit membranes between neighbouring processes (arrow). TEM x 11,000.

Fig. 10. Endothelial cell: the cytoplasm shows rough-surfaced endoplasmic reticulum. Note the fenestrated attenuated cytoplasm closely adherent to the GBM. TEM x 11,000.



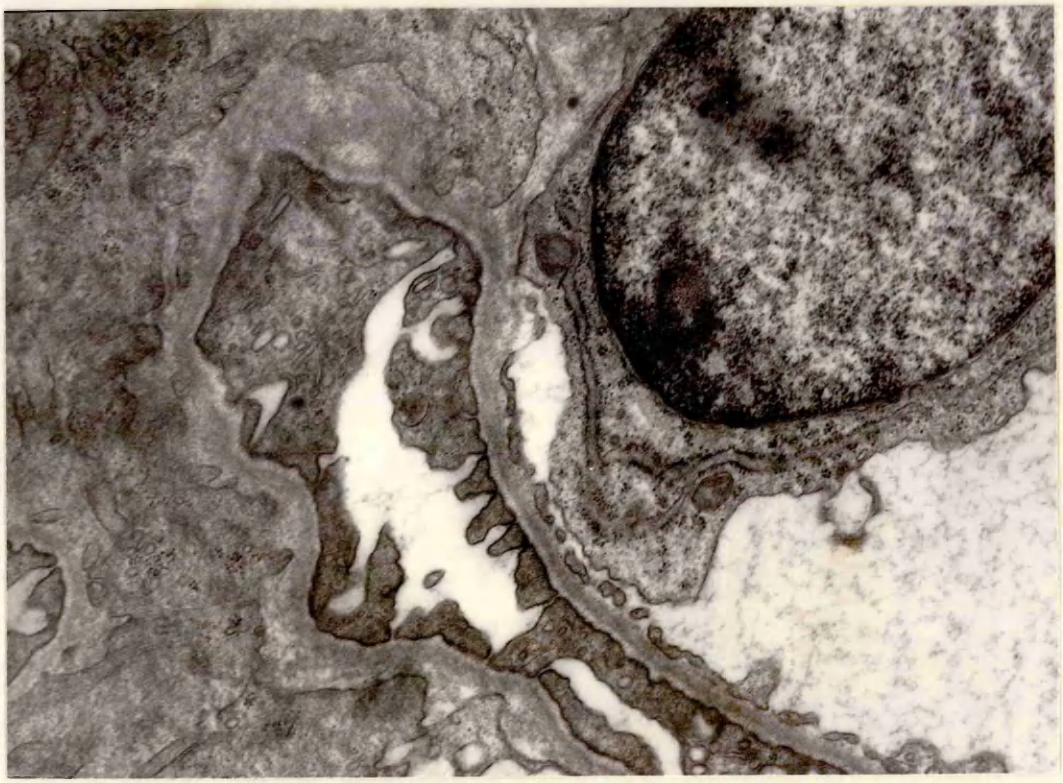
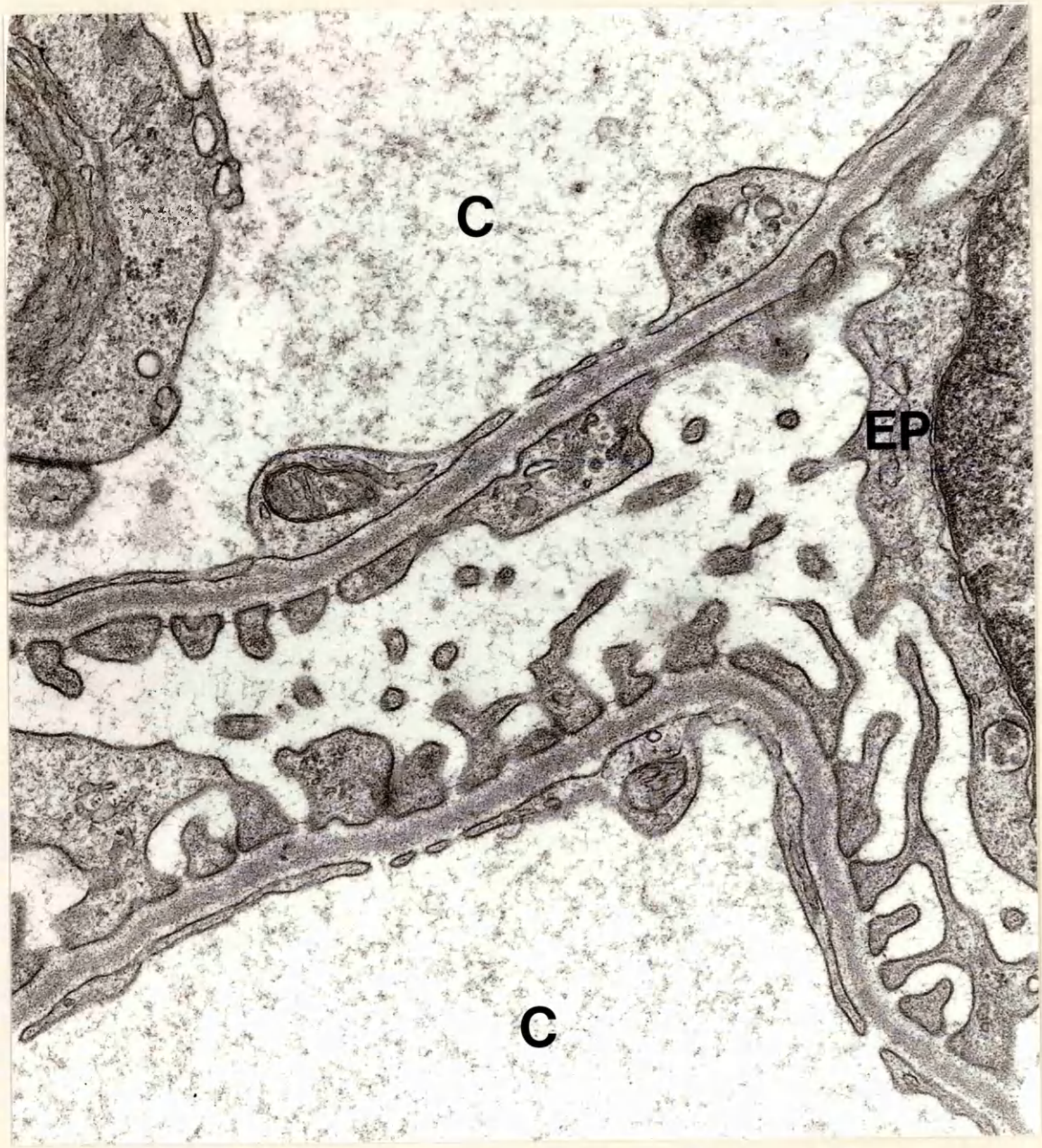


Fig. 11. This micrograph reveals the prominent basement membrane with its lamina rara externa, lamina densa and lamina rara interna. The fenestrated cytoplasm of the endothelial cell, the foot processes with their slit membranes can be seen. C = capillary lumina, EP = epithelial cell. TEM x 16,000.


C
EP
C

(b) Endothelial cell:

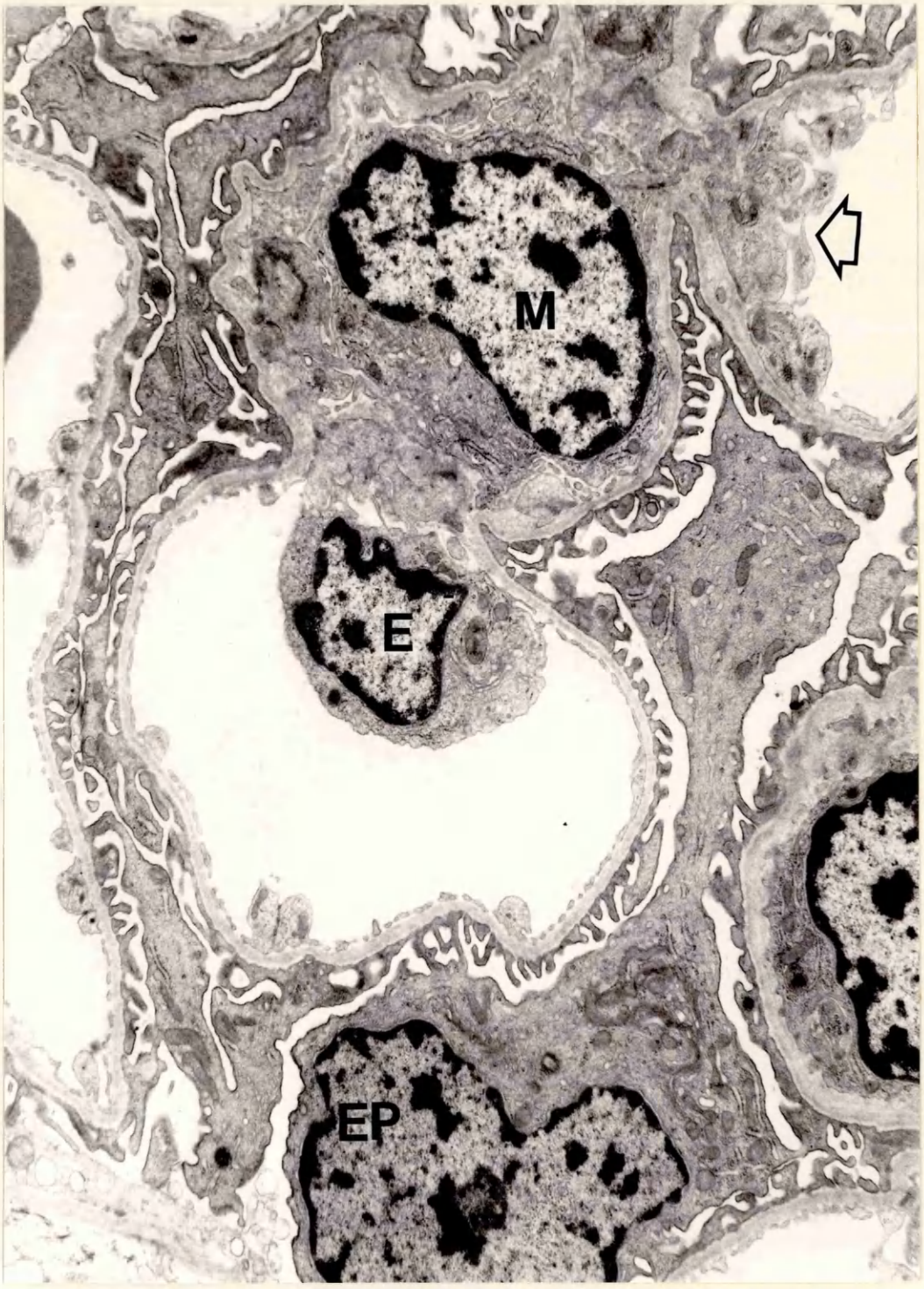
Endothelial cells lining the capillary lumen consist of a main cytoplasmic mass containing the nucleus and most of the organelles bulging into the axial portion of the loop and a very thin attenuated portion of cytoplasm which arises abruptly from the main cytoplasmic mass and extends out to cover the peripheral part of the capillary luminal surface (Fig. 10). Numerous gaps or fenestrae are present in the attenuated portion of the endothelial cytoplasm. The fenestrae are not bridged by a membrane and the plasma of the capillary loop appeared to have direct access to the lamina rara interna of the GBM (Fig. 11). The mitochondria appeared to be smaller than those of the epithelial cells and are found in small numbers throughout the cell, even in the fenestrated portion.

The rough surfaced endoplasmic reticulum with its associated clusters of ribosomes is sparse and mainly confined to the main cell mass. Occasional membrane-bound dense bodies are found and many small pinocytotic vesicles could always be seen. Golgi bodies are only rarely observed.

(c) The mesangium:

Between each capillary loop usually only one or two (rarely three) contiguous mesangial cells are found (Fig. 12); the cells are bounded laterally by the GBM and, at the axial portion of the loops, by the cell body of endothelial cells. Frequently mesangial cytoplasmic processes projected through the endothelial cell to bulge out into the capillary lumen (Fig. 12). Mesangial cells show a few mitochondria, scattered islands of endoplasmic reticulum mostly

Fig. 12. A micrograph of a normal dog glomerulus: note the endothelial cell (E), mesangial cell (M) and epithelial cell (EP). Some mesangial cytoplasmic processes (arrow) bulge out into the capillary lumen. TEM x 6,000.


M
E
EP

bounded with ribosomes and occasional round or irregular electron dense membrane-bound granules. Bundles of thin filaments are also to be seen in the irregular processes of mesangial cells while the surrounding mesangial matrix contains fluffy amorphous basement membrane-like material as well as fibrillar material (Fig. 13).

(d) The glomerular basement membrane:

The prominent GBM with its three layers namely, a central thick electron dense lamina densa, an outer electron lucent lamina rara externa on which the foot processes are implanted and an inner electron lucent lamina rara interna on which the attenuated part of the endothelial cells are lying is easily identifiable (Fig. 11). Towards the axial region of the loops the lamina rara interna often shows irregular expansions.

Scanning electron microscopy:

At low magnification, the cut surface of the renal cortex reveals numerous renal corpuscles as well as empty corpuscles (Fig. 14). For the most part the renal corpuscles are intact whereas sectioned glomeruli are less obvious. Fig. 15 shows an intact renal corpuscle with its glomerular capillary tuft.

At higher magnification, the detailed arrangement of the visceral epithelial cells becomes evident (Fig. 16). The podocytes with their interdigitating processes cover all the outer surface of the capillary loops. The cell bodies or nuclear portions of the podocytes are identified as more or less smooth-surfaced bodies attached to the wall of the capillaries. Most of the visceral epithelial cells lie between adjacent capillary loops extending their

processes to surround and encompass the surface of the capillaries. The surface of the main cell body containing the nuclear portion is often rather uneven, showing occasional microvillous projections (Fig. 17).

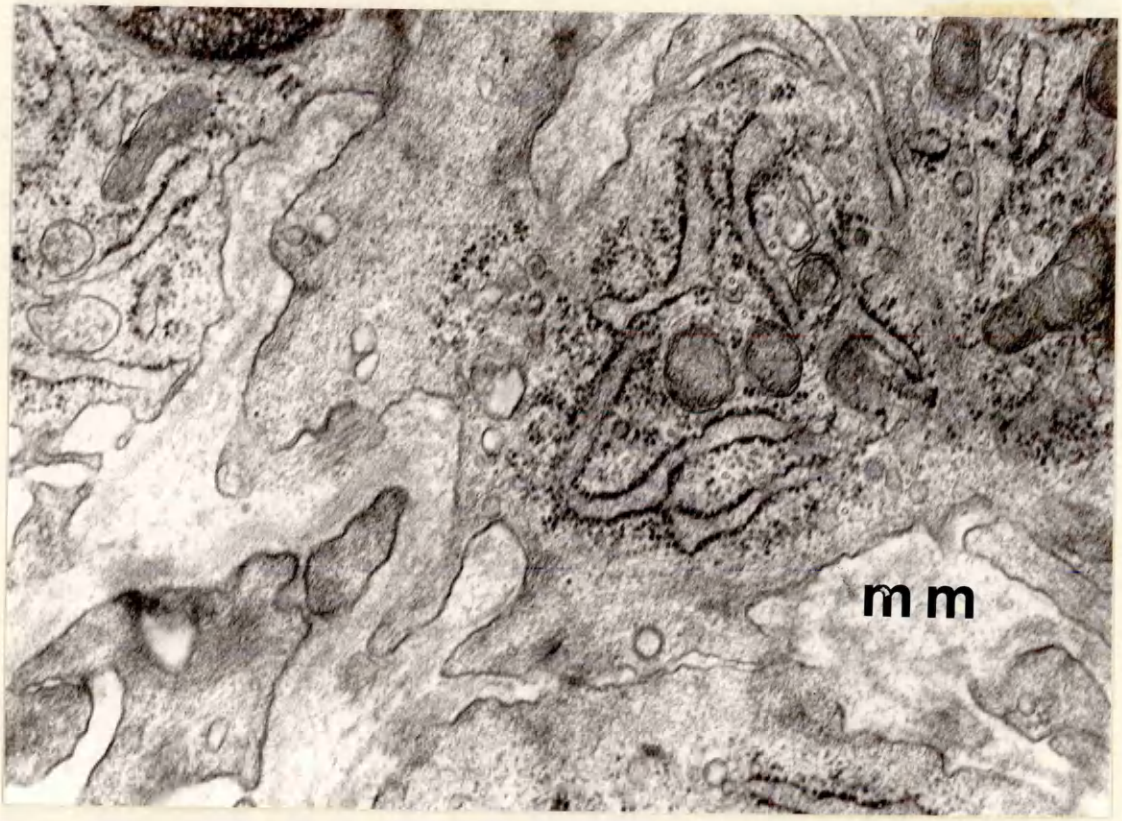
From each cell body several primary cytoplasmic processes radiate around the capillary loops. At right angles from its lateral side each primary process gives rise to thinner secondary branches. Both primary and secondary processes give rise to tertiary processes (foot processes) (Fig. 18). The foot processes are anchored to the GBM and correspond to the triangular profiles of foot processes seen with the TEM. They are very irregular in length, width and shape, are occasionally branched and issue at random angles. The spaces between each process, however, remain constant. In some specimens, the surface of the capillary endothelium is exposed to reveal the interior of the capillary (Fig. 19). The inner surface of the capillary wall is covered with flat fenestrated endothelium (Fig. 20). Bands of non-fenestrated endothelium are often seen running transversely across each capillary loop, rather akin to the hoops of a barrel.

Tensol casts of renal glomeruli successfully indicate the afferent arteriole arising from the interlobular artery and dividing into tightly knit capillary branches (Fig. 21) usually three - four in number; numerous interlobular anastomoses are observed. Extensive branching of these primary capillaries is observed (Fig. 22). The smaller efferent vessel emerges from the vascular pole of the capillary tuft to divide into a network of peritubular capillaries (Fig. 23).

In the present study no direct shunts between afferent and efferent arterioles were demonstrated. The diameter of the afferent arteriole is approximately twice that of the efferent vessel.

Fig. 13. Mesangial cytoplasm showing a few mitochondria and rough-surfaced reticulum. Note the fluffy amorphous basement membrane-like material of the mesangial matrix (mm). TEM x 16,000.

Fig. 14. A low-power scanning electron micrograph of the cut surface of a normal dog kidney. Bowl-like depressions (asterisks) are empty Bowman's capsules. SEM x 120.


m m

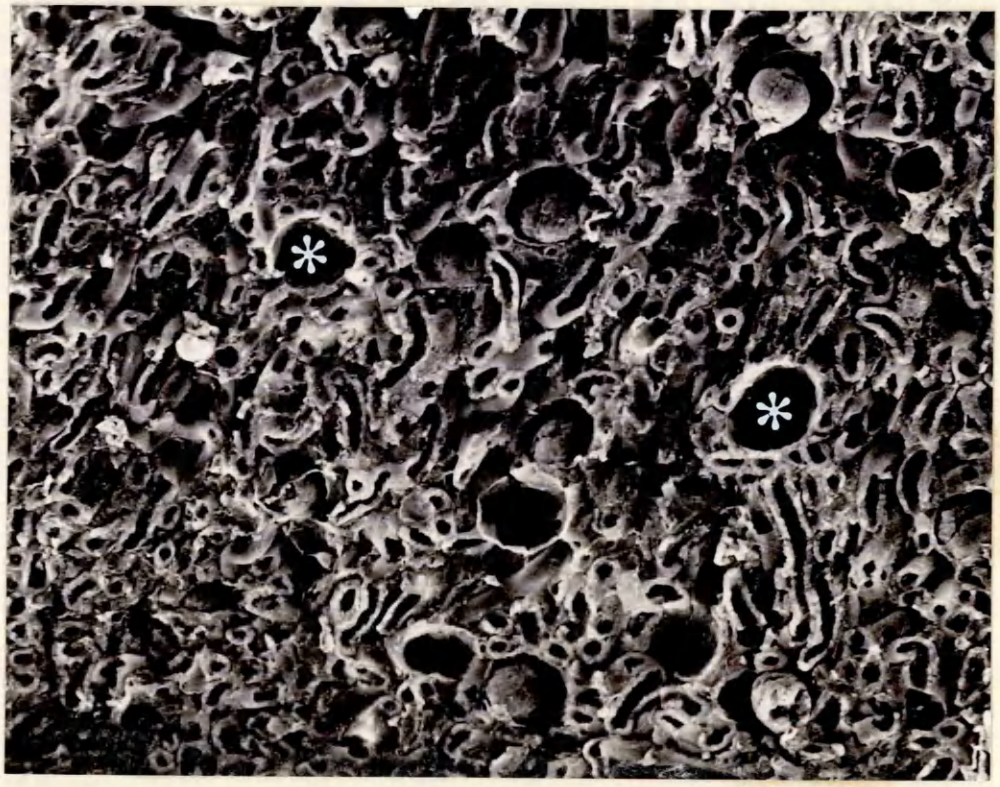
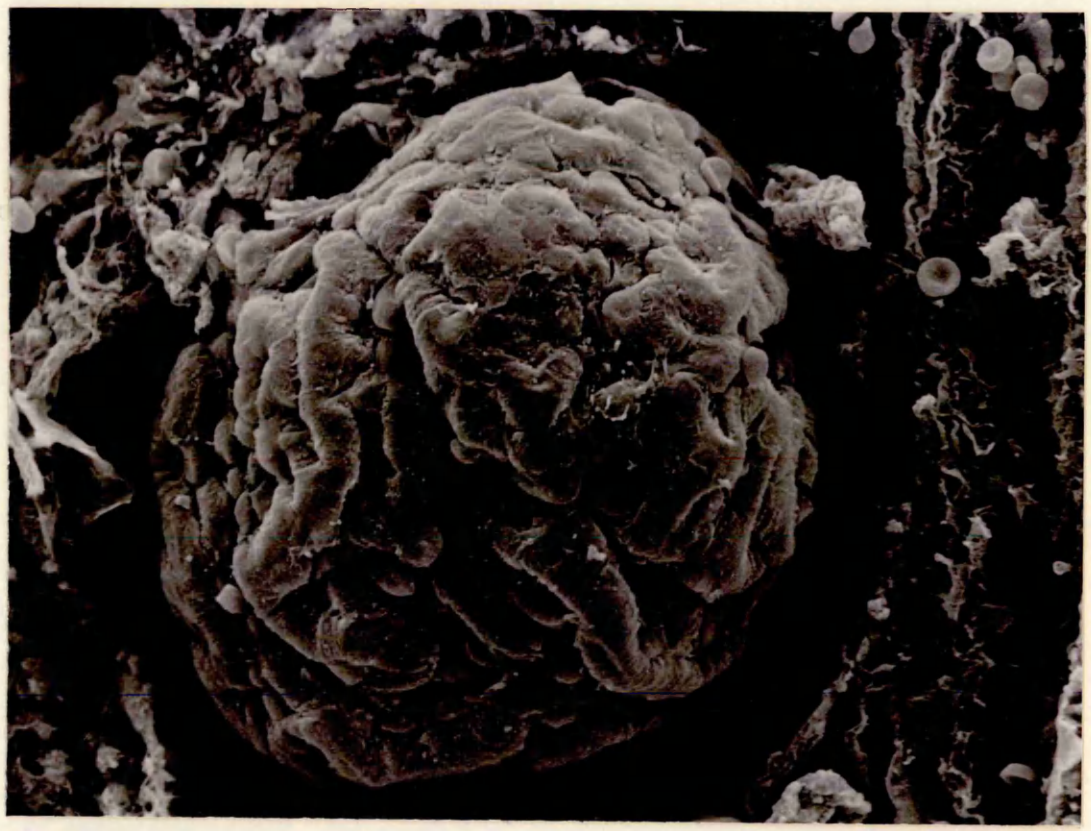


Fig. 15. A closer view of a renal corpuscle. The capsule of Bowman encircles the glomerulus leaving a space (urinary space). SEM x 1200.

Fig. 16. A higher magnification of a glomerulus showing the detailed arrangement of the visceral epithelium. A section through a capillary wall reveals the lumen and its endothelial lining. SEM x 5000.



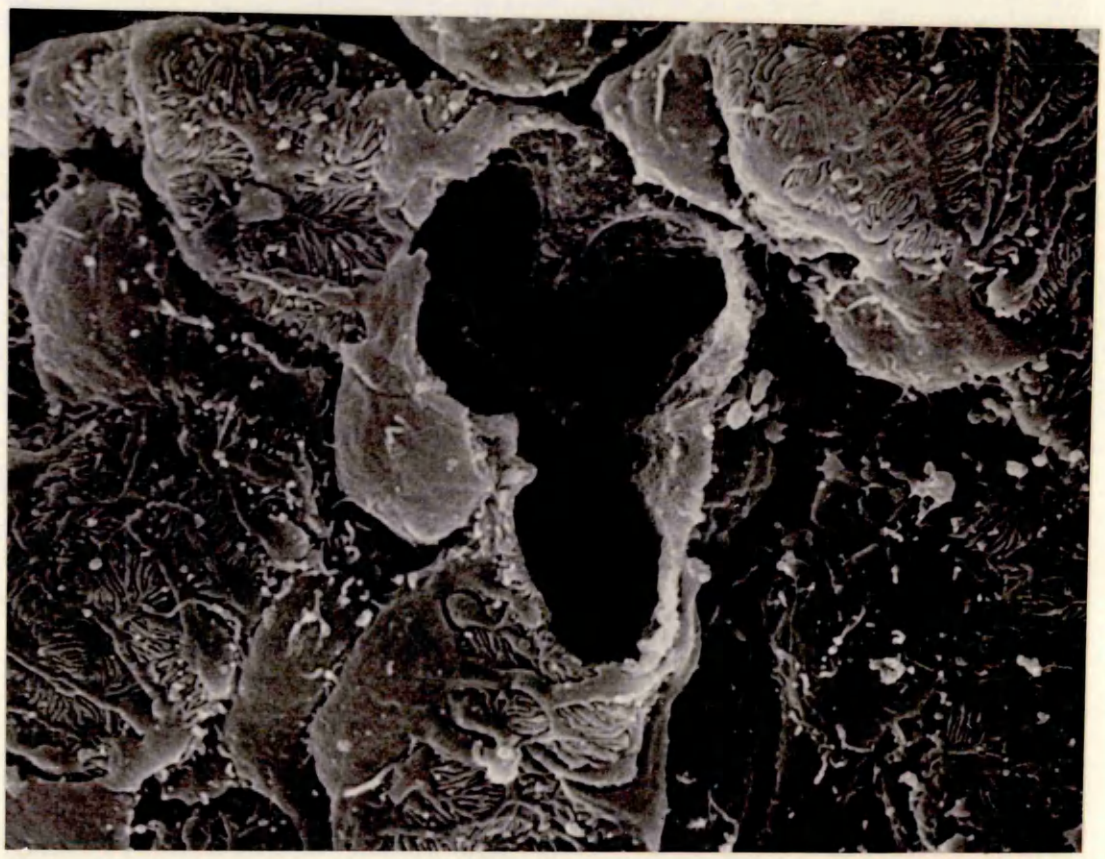


Fig. 17. A closer view of the capillary surface with its visceral epithelial cell body, note primary cytoplasmic processes, secondary cytoplasmic processes and foot processes; the latter may arise directly from either the primary or secondary processes. Note: occasional microvillous projections on the epithelial cell body (arrow). SEM x 5000.

Fig. 18. Surface view of a normal dog glomerulus. Note the podocyte cell body (CB) with its interdigitating foot processes. SEM x 10,000.

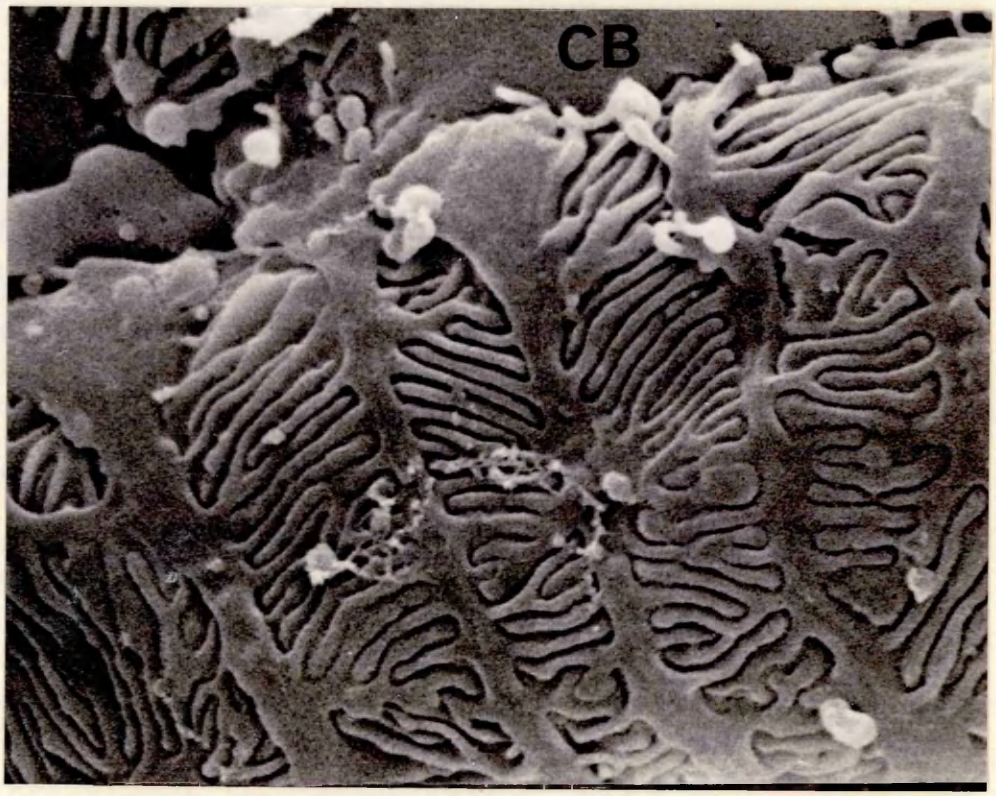
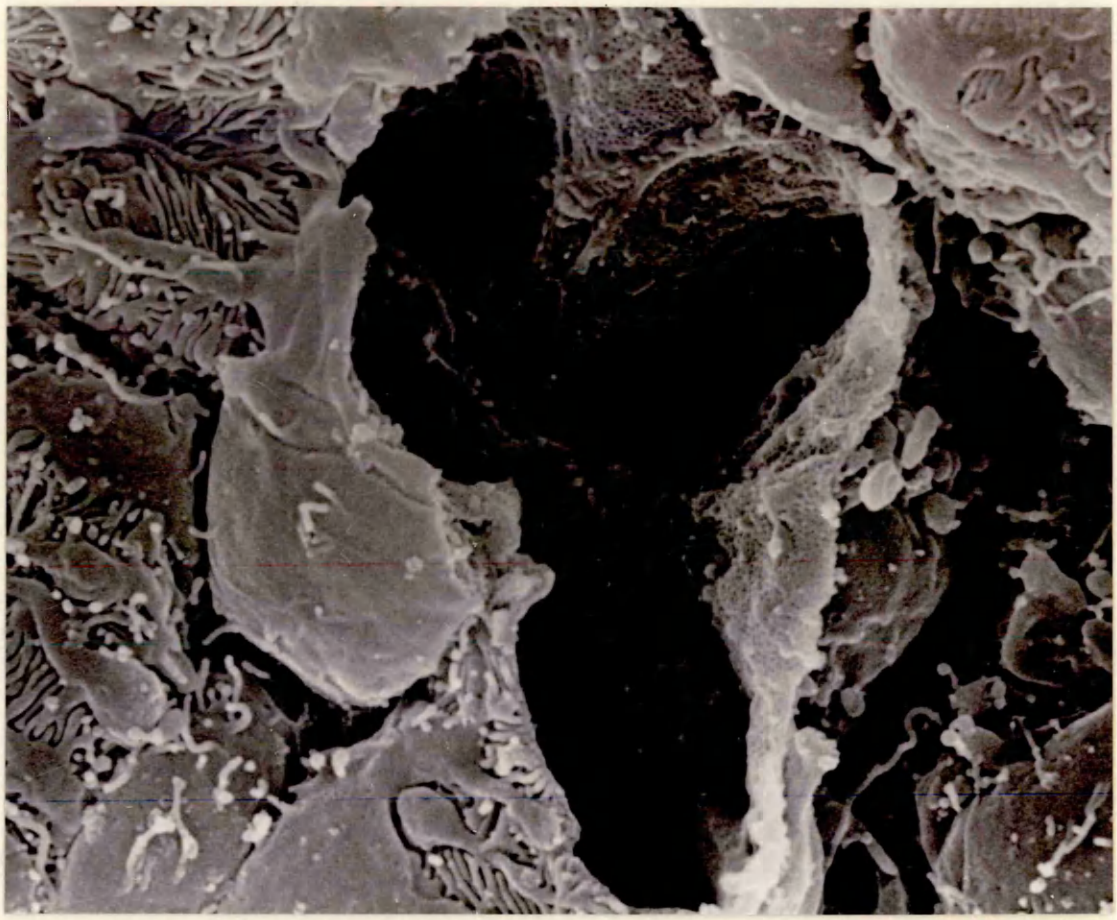

CB

Fig. 19. Normal dog glomerulus. The surface of the capillary endothelium is exposed to reveal the fenestrae of the attenuated part of the endothelial cytoplasm. Note the few microvilli on the epithelial cell body and on some primary processes. SEM x 10,000. (Higher power of Fig. 16).

Fig. 20. A high-power view of the inner surface of a glomerulus or capillary of a dog uniform pores corresponding to fenestrae with unfenestrated ridges coursing over the endothelial surface. SEM x 40,000.



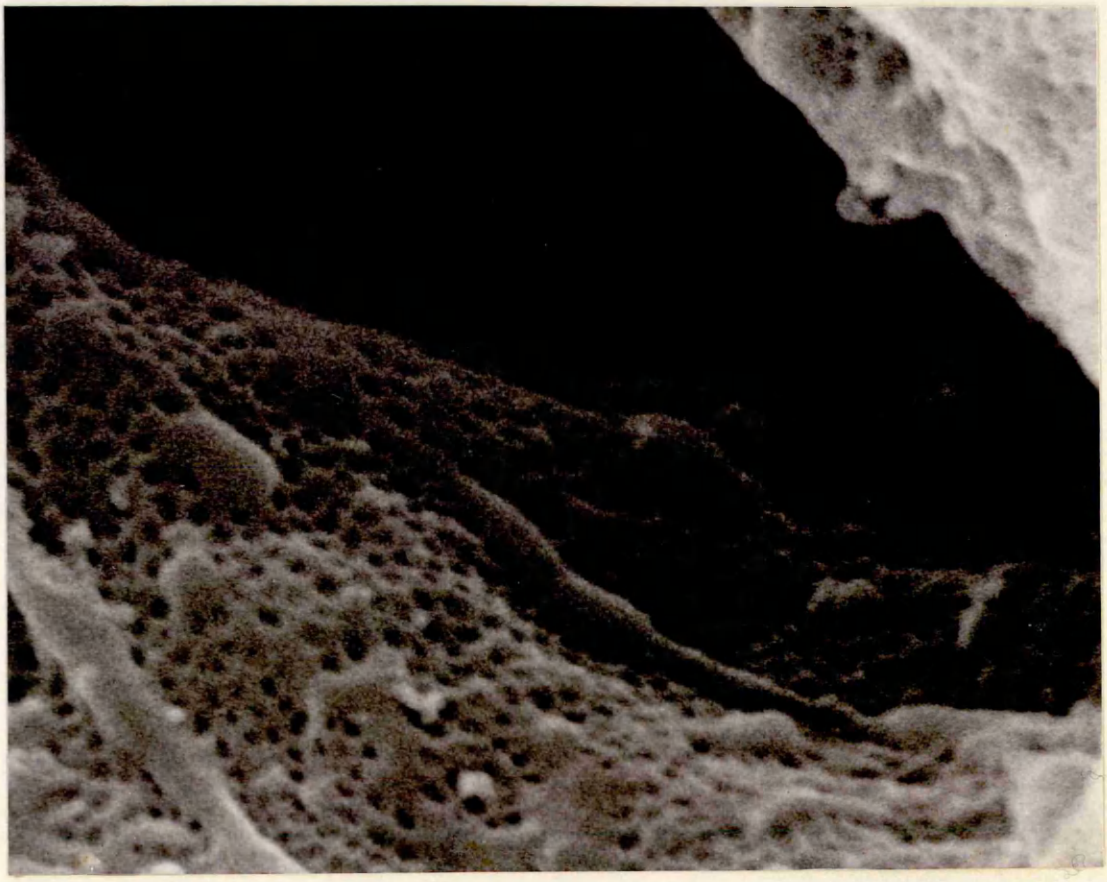
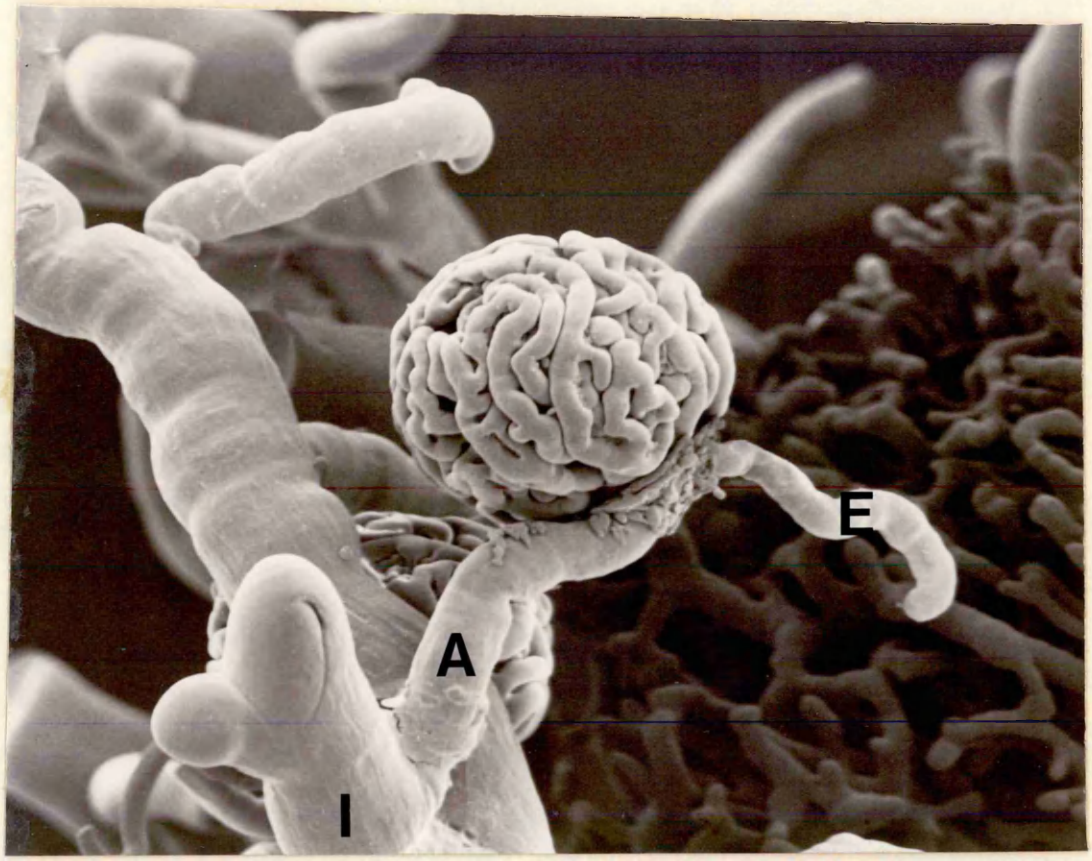


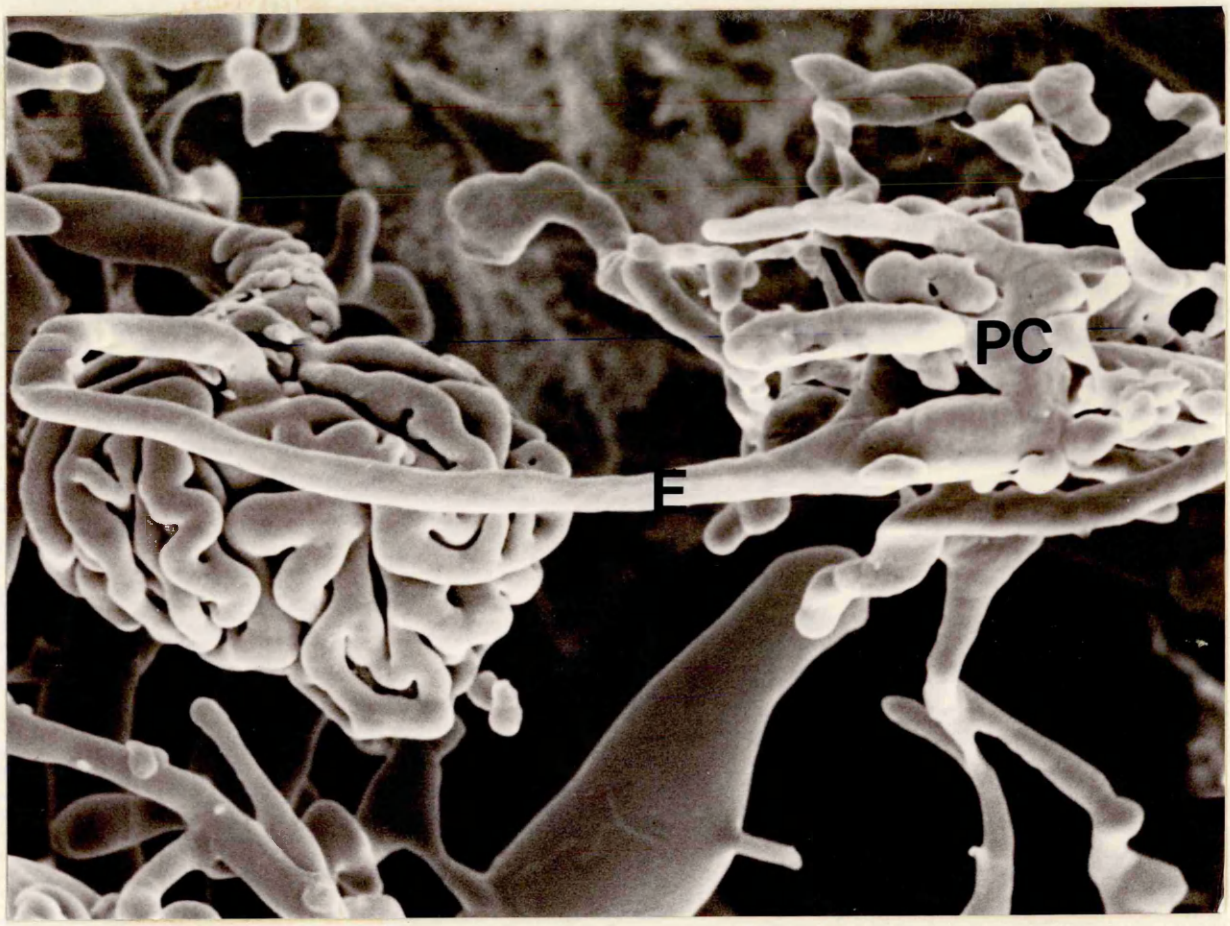
Fig. 21. Tensol cast. Low power view showing interlobular artery (I) afferent arteriole (A) and efferent arteriole (E). SEM x 120.

Fig. 22. A tensol cast of normal dog glomerulus. Note the afferent arteriole (A) and efferent arteriole (E). SEM x 320.


E
A
I


E
A

Fig. 23. Tensol cast of normal dog glomerulus. The efferent arteriole (E) can be seen to break up into peritubular capillaries (PC). SEM x 180.


PC
E

Neonatal puppies:

At four hours the nephrogenic zone is seen to be very wide extending to the mid-cortical region where some primitive glomeruli characterised by prominent cuboidal visceral epithelium and few patent capillaries can be observed. In the deep cortex, a few, well developed functioning glomeruli with patent capillaries are present (Fig. 24). In the outer cortex below the capsule numerous metanephric caps, vesicles and S-shaped developing glomeruli are seen.

At three days, the nephrogenic zone is still wide and extends to the mid cortical region. The outer cortex shows various stages of developing nephrons including caps of metanephric cells overlying the ampullae, oval masses of metanephric cells, vesicles, and S-shaped structures (Fig. 25). Scattered throughout the mid cortex and outer cortex, a number of functioning glomeruli can be detected, all characterised by basophilic cuboidal visceral epithelium. As these glomeruli contain few patent loops, they appear hypercellular. In the deep cortex more fully developed glomeruli are seen although in terms of the overall number of glomeruli in any one section these mature glomeruli are in the minority accounting for less than 25 per cent of the total.

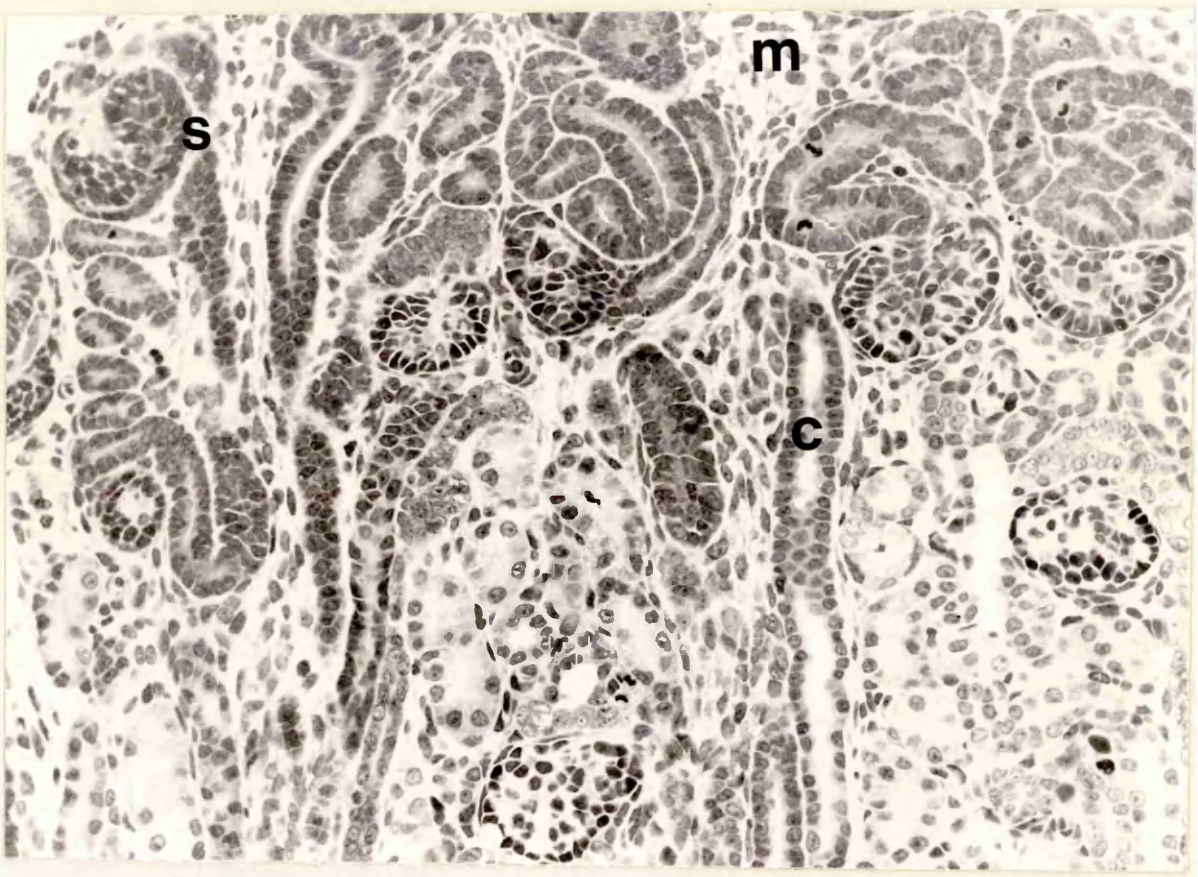
By seven days the nephrogenic zone is narrower and confined to the outer cortical region (Fig. 26). The mid and outer cortical region still contain some developing hypercellular glomeruli with few patent capillaries. However, many more mature glomeruli with patent capillaries can now be seen in the deep cortical region although some still appear hypercellular. These mature functioning glomeruli comprise approximately 50% of the total number of glomeruli.

By 15 days the nephrogenic zone has disappeared (Fig. 27) and the percentage of functioning glomeruli has increased to more than 50% of the total number of glomeruli. Many less well developed hypercellular glomeruli, however, are still to be found scattered throughout the outer cortex and mid cortex.

By 21 days, mature well developed functioning glomeruli predominate but even at this time approximately 10% of glomeruli still show prominent cuboidal visceral epithelium and with few patent capillaries (Fig. 28).

Fig. 24. Renal cortex from a four hour old puppy. The nephrogenic zone is subjacent to the capsule. Note the collecting tubules (C) with the terminal ampullae, the metanephric mesanchyme (M), and S-shaped developing glomerulus (S). A few functioning deep cortical glomeruli are also present. H & E x 120.

Fig. 25. Renal cortex from a three day old dog. Note the various stages of developing nephrons and collecting tubules (C), metanephric mesanchyme (M) and S-shaped structures. H & E x 120.


m
s
c

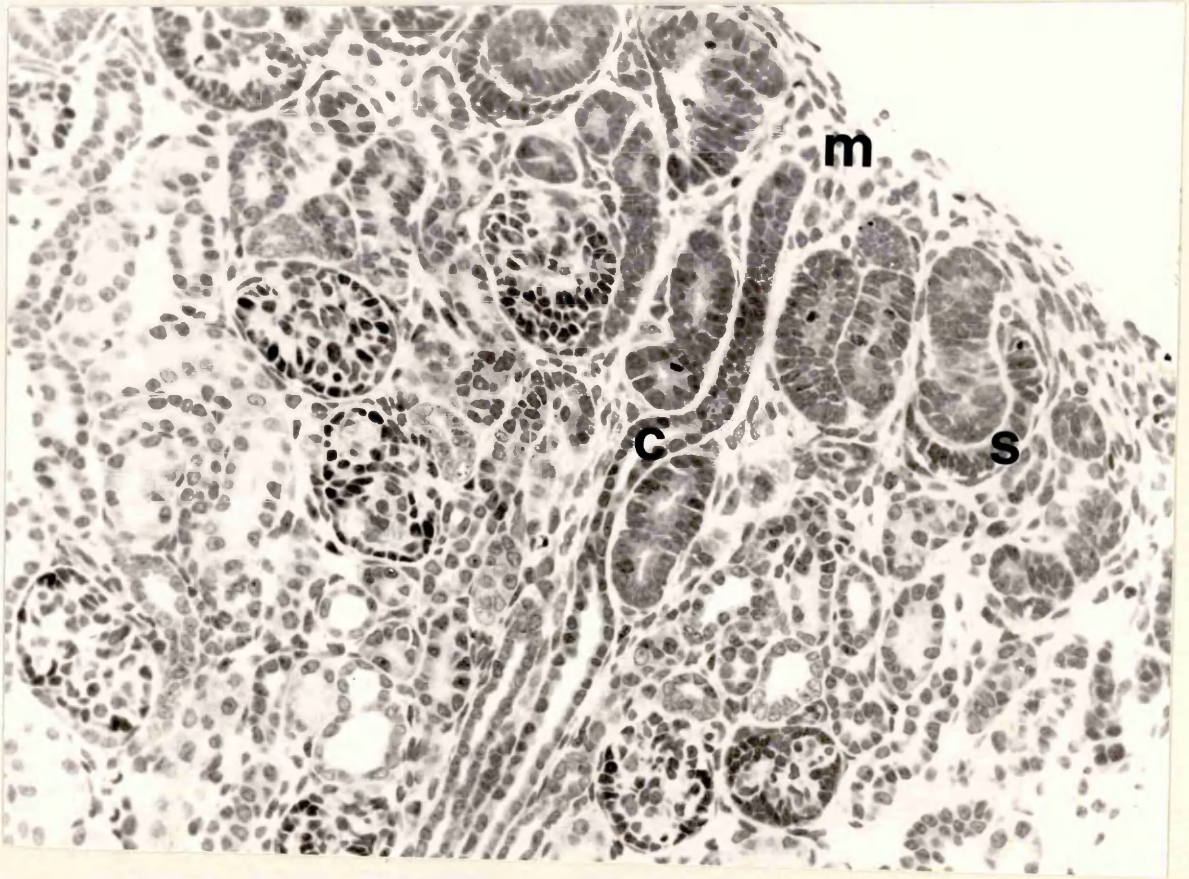

m
c
s

Fig. 26. Renal cortex of a seven day old dog.
Note that the narrow nephrogenic zone is
confined to the outer cortical region.
H & E x 120.

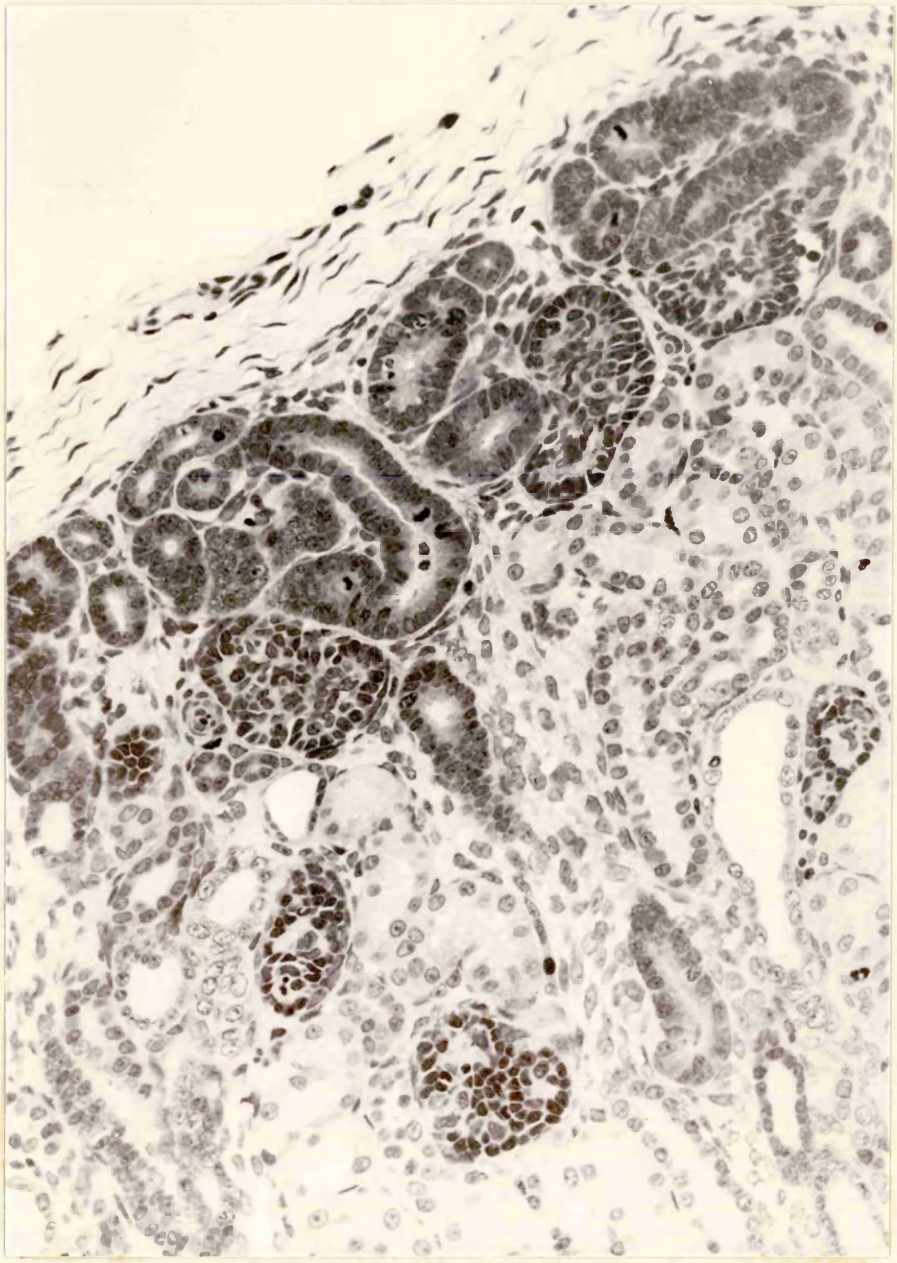
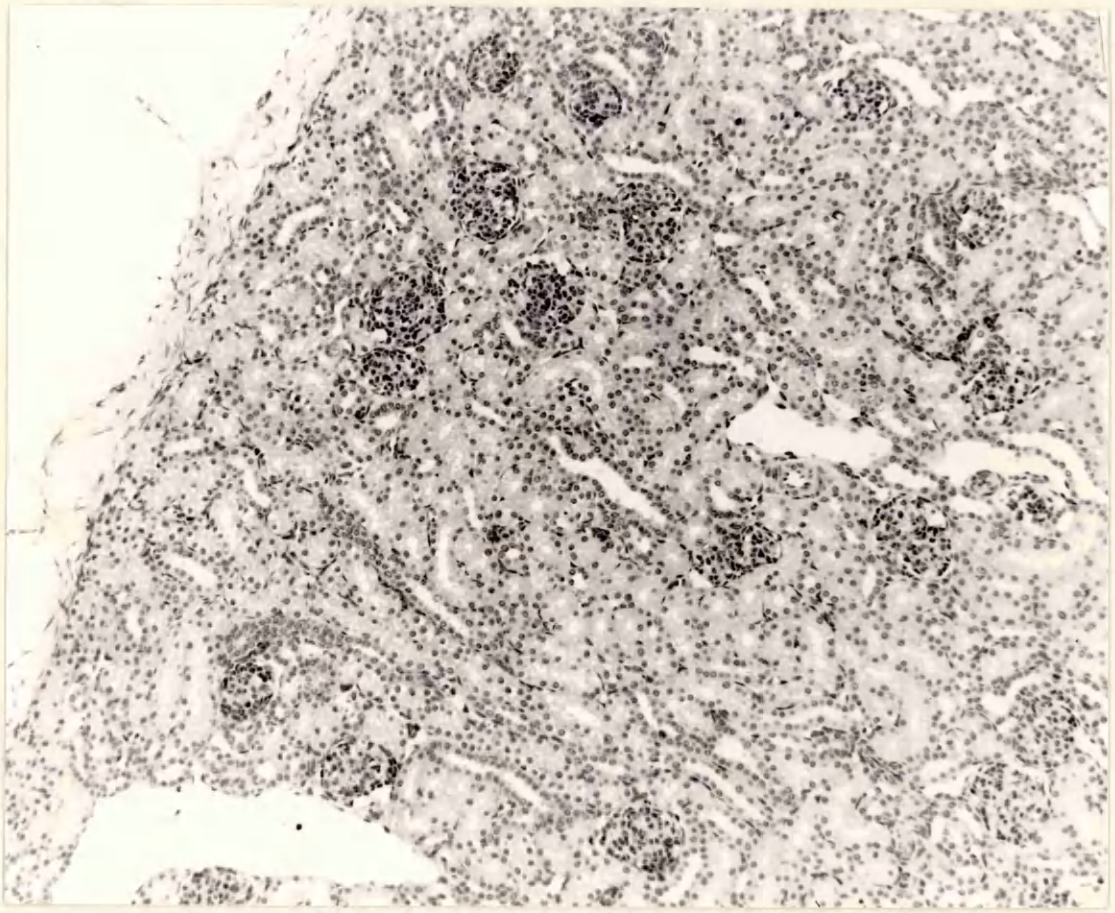
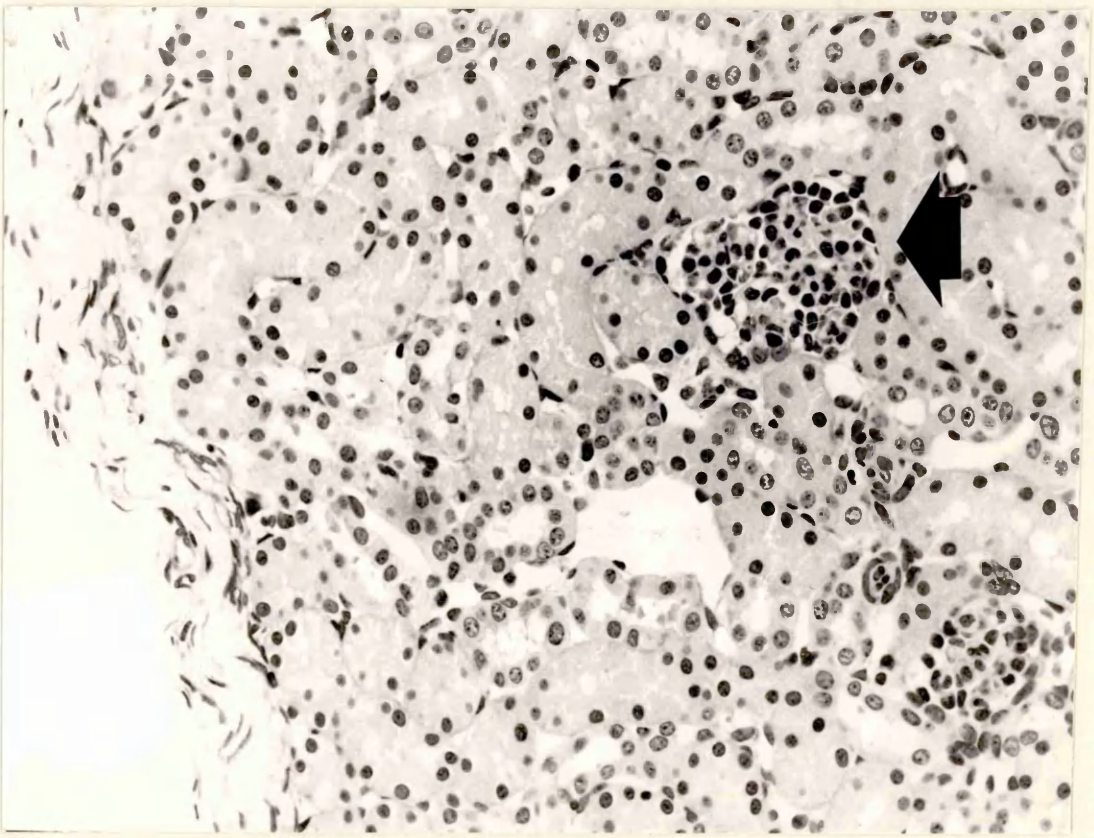


Fig. 27. Renal cortex of 15 day old dog. The outer cortical glomeruli are still hypercellular. H & E x 80.

Fig. 28. Renal cortex of 21 day old dog. An outer cortical glomerulus (arrow) shows prominent visceral epithelium. H & E x 120.





Discussion:

The criteria for judging the quality of kidney fixation are based on empirically determined principles which have become accepted as consistent with good tissue preservation.

There are two methods of fixation used to successfully preserve renal structures a) immersion fixation and b) perfusion fixation. The comparison made in the present study showed that although both methods produced good fixation for both conventional light microscopy and electron microscopy, perfusion fixation appeared to be superior to immersion fixation in respect to patency of the glomerular capillaries which allowed better differentiation of glomerular cells on light microscopy. The disadvantages of perfusion fixation was that the capillary lumina and urinary space were sometimes artificially widened and, while most glomeruli were evenly perfused, some particularly in the outer cortex did not seem to be perfused at all.

The crucial role of section thickness in the study of the renal glomerulus was underlined in the present study. In a thick kidney section, six μm thick the glomeruli appeared to be hypercellular and it was not always possible to identify the various glomerular cell types. On the other hand three μm paraffin sections allowed good visualisation of the glomerular capillaries and cellular differentiation was relatively easy.

The use of one μm plastic sections while offering optimal thin preparations for study had two major disadvantages. First, the overall surface area of the section was small and only a relatively few glomeruli were available for examination. Second, and although

not the subject of study in the present work the restriction to a relatively few staining procedures could in some instances be disadvantageous.

The present observation on the size of the outer and deep cortical (juxta-medullary) glomeruli concurred with those of Horster et al (1971) who noted that deep cortical glomeruli were larger in size than outer cortical glomeruli.

The ultrastructural findings confirmed and expanded those of Movat and Steiner (1961) and Crowell et al (1974) with regard to the general morphologic features of the renal glomerulus and provided useful baseline data for use in the study of pathologic glomerular alteration in the following sections of the work.

The present study also provided the first detailed scanning electron microscopic views of the normal dog glomerulus. As such, it provided a basis for future scanning electron microscopic (SEM) studies concerned with evaluating the morphologic changes which accompany various kidney disorders.

Most of the previous SEM studies on the renal glomerulus have been carried out on rat and rabbit kidney and there is some controversy as to the arrangement of the visceral epithelium and its processes. Thus, Buss and Kronert (1969) published a SEM study on the renal glomerulus of the rat and described the interdigitating foot-processes as arising either from different podocytes or from the same cell. However, Fujita et al (1970) in their study of the renal podocytes of the rat and rabbit clearly indicated that the neighbouring foot processes always arose from different cells. The present work concurs with the results of

Buss and Kronert (1969).

The presence of slender microvilli on the cell body as well as on the processes as described by Fujita et al (1970) in the rat and rabbit was also a feature of the dog glomerulus and the occurrence of stumpy bud-like projections on the podocyte cell body and processes as observed by Buss and Kronert (1969) and Fujita et al (1970) was also noted in the present work.

The present work reinforced the usefulness of corrosive casts in the study of glomerular capillaries although the reliability of such vascular casts has been questioned by some workers (Ljungqvist, 1963). The main argument against their use is the possibility that the vascular structures may be incompletely filled, owing to the high viscosity of the injected material. In the present investigation, all glomerular capillaries appeared to be evenly filled, there was no leakage of cast material from ruptured capillaries and even the delicate anastomosing channels between capillary loops of the same lobule were well filled and easily visible. The present study also confirmed the opinion of Spinelli et al (1972) in that no interarteriolar shunts are present in the dog glomerulus.

In the present study an opportunity arose to follow the distribution of mature and immature glomeruli in the dog renal cortex from four hours to 21 days after birth. It was only possible to do this with conventional light microscopy. Although, Eisenbrandt and Phemister (1977) have reported that nephrogenesis in the dog kidney diminishes by eight days of age and is absent by 14 days of age, in the present study a nephrogenic zone was still present at seven days and the outer cortical glomeruli were not fully

mature and functional until 15 - 21 days. Even at this time the capillaries of many outer cortical glomeruli were not fully patent and this together with the prominent cuboidal appearance of the visceral epithelial cells gave a hypercellular appearance in the tuft.

CHAPTER 4

A STUDY OF THE SEQUENTIAL AUTOLYTIC CHANGES IN THE DOG GLOMERULUS

Introduction:

The canine kidney, like that of man, cannot always be obtained under optimal conditions immediately after death; glomerular changes attributable to autolysis must therefore be differentiated from those due to disease processes. Despite this, only a few studies on the autolytic changes occurring in mammalian glomeruli have been carried out and, to date, no detailed sequential histological and ultrastructural investigation has been performed.

One of the first studies of the morphologic changes in the glomerulus due to autolysis was that by Osvaldo et al (1965) who used rat kidney as their model; kidney tissue was maintained at a constant temperature of 37° and samples taken for histological examination at intervals after death. It was found that a significant cellular swelling, sufficient to close the glomerular capillaries, had occurred as early as one hour. Pyknotic epithelial nuclei were recognised by four hours whereas the nuclei of endothelial and mesangial cells did not show pyknosis until 24 hours.

Cook et al (1965) have described some limited features of the electron microscopic changes in autolytic rat kidney and noted that Bowman's space was obliterated by 30 minutes after death and tubular epithelial reflux had occurred. Marked swelling of the processes of epithelial cells was observed by one hour while endothelial cytoplasmic projections into the capillary lumina were prominent by four hours and many capillaries were filled with swollen endothelial cytoplasm. Swelling of mesangial cell processes was noted as early as five minutes and was prominent by one hour.

More recently Langlinais (1981), using SEM, described the sequential post-mortem glomerular changes in rabbits, goats, mice and dogs. Dog kidney material was held at room temperature for the first four hours after death, then at 4 to 6°C until 24 hours. Surface blebs on the podocytes were observed as early as 15 minutes after death, while minimal focal fusion and obliteration of foot processes was noted by 90 minutes. At three hours, diffuse fusion of foot processes had occurred and, at 24 hours, more severe podocyte disruption was evident.

From these rather fragmentary reports it can be seen that much has still to be learned concerning the sequential histological and ultrastructural changes occurring in autolytic mammalian kidneys.

The purpose of this section of the work was to make a definitive study of the glomerular artefacts caused by autolytic changes in the canine kidney as detected by light microscopy as well as transmission and scanning electron microscopy.

Materials and methods:

Renal tissue from four healthy four month old dogs was used to study the sequential autolytic changes. The kidney material was held at room temperature (18°C) and samples taken at the following time intervals: immediately at death (0 mins - which served as control material), two, five, 10, 30 and 60 minutes, five and 24 hours and three and five days.

The histological, ultrastructural and scanning techniques used and the source of animals are described in the general section on materials and methods in Chapter 2.

Histological features:

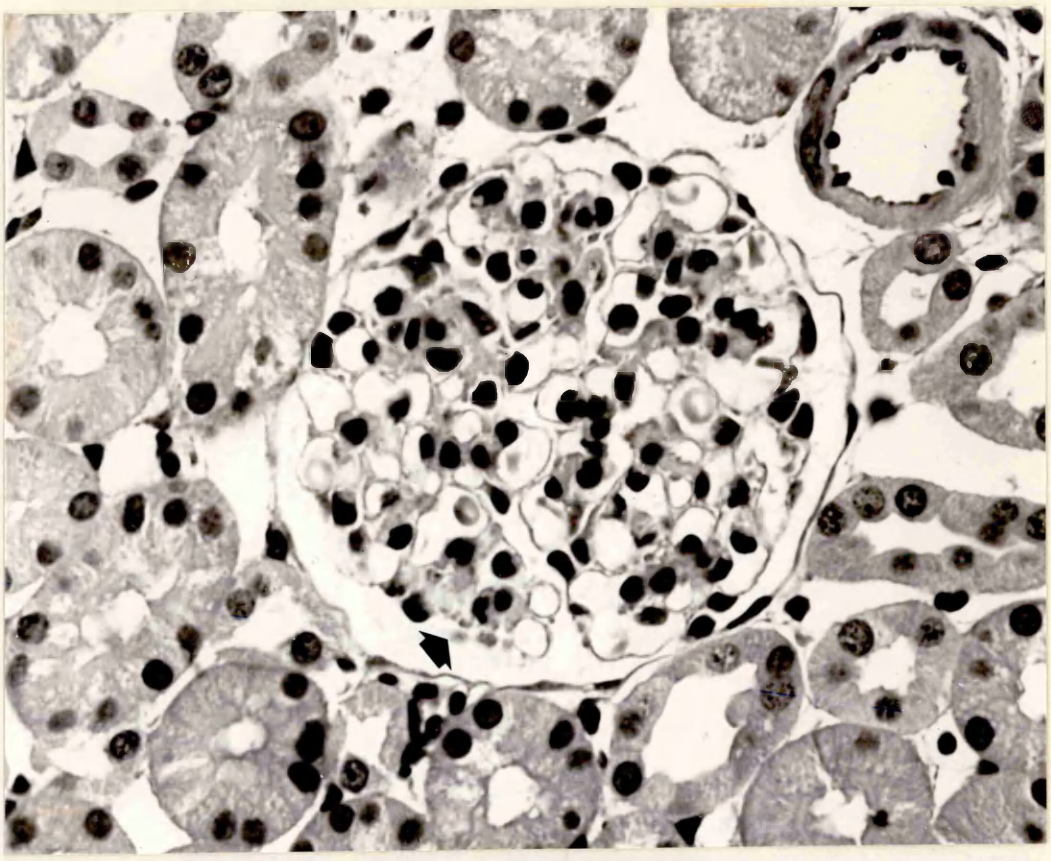
Although the nature and severity of autolytic changes often varied in different glomeruli in the same section, nevertheless the overall pattern of alterations was the same at each time interval in all four dogs. The main histological changes are summarised in Table 4.1.

At two minutes after death, the glomeruli were normal with the exception of reflux of small amounts of tubular epithelial cytoplasm into the urinary space which finding was observed in only a very few glomeruli (Fig. 29). Up to and including 30 minutes of death, tubular epithelial reflux could still be observed in a few glomeruli and, in addition, some capillaries appeared occluded with swollen endothelial cytoplasm (Fig. 30).

At one hour, in addition to an increase in tubular epithelial reflux, a few pyknotic endothelial, epithelial and mesangial nuclei had also appeared (Fig. 31); many capillary lumina were now closed. By five hours, the number of pyknotic nuclei had increased (Fig. 32) while at 24 hours tubular epithelial reflux was prominent in many glomeruli and all capillaries were now closed; nuclear pyknosis was marked (Fig. 33). At three days, there was complete closure of the capillaries, heavy tubular reflux into the urinary space and generalised nuclear pyknosis (Fig. 34). By five days, the normal histological configuration of the glomerulus was lost and the urinary space was almost completely filled with tubular epithelial debris (Fig. 35).

Fig. 29. Autolytic changes, two minutes after death: note the small amount of tubular epithelial cytoplasm in the urinary space (arrow).
H & E x 300.

Fig. 30. Autolytic changes, 30 minutes after death: note the occluded capillaries and tubular reflux in the urinary space (arrow).
H & E x 300.



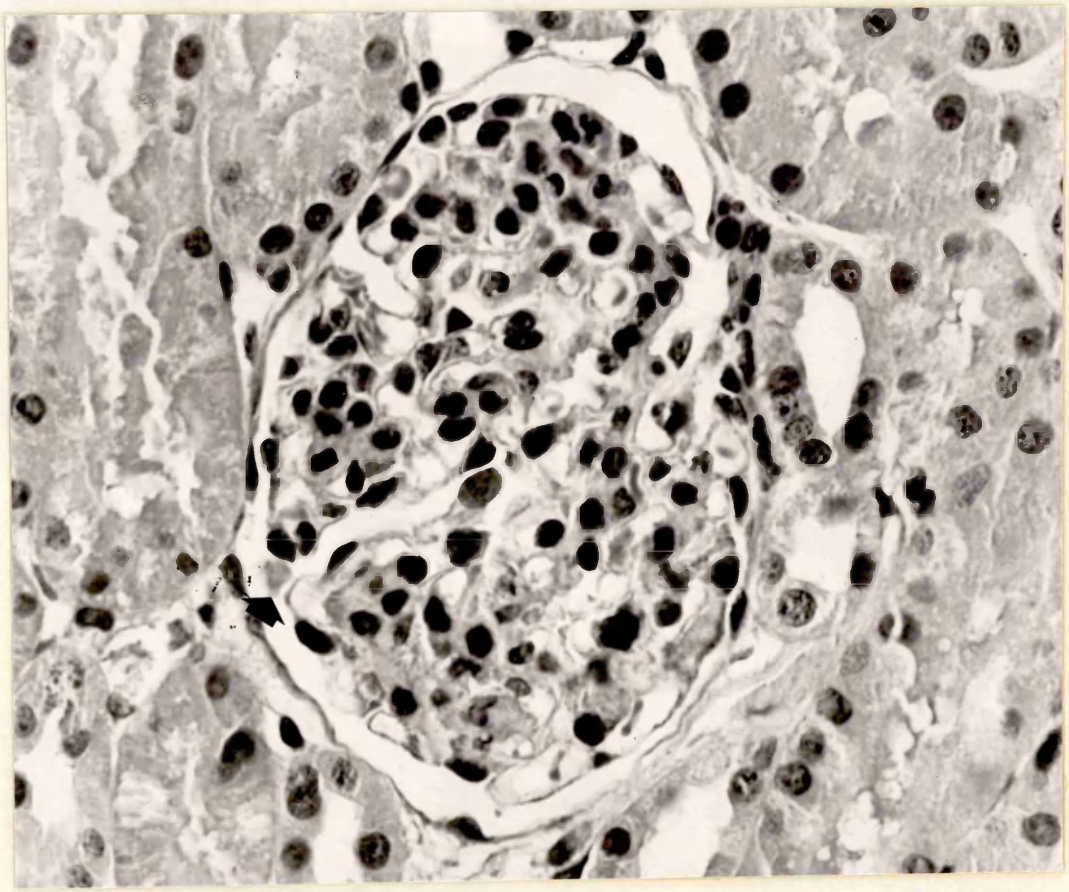
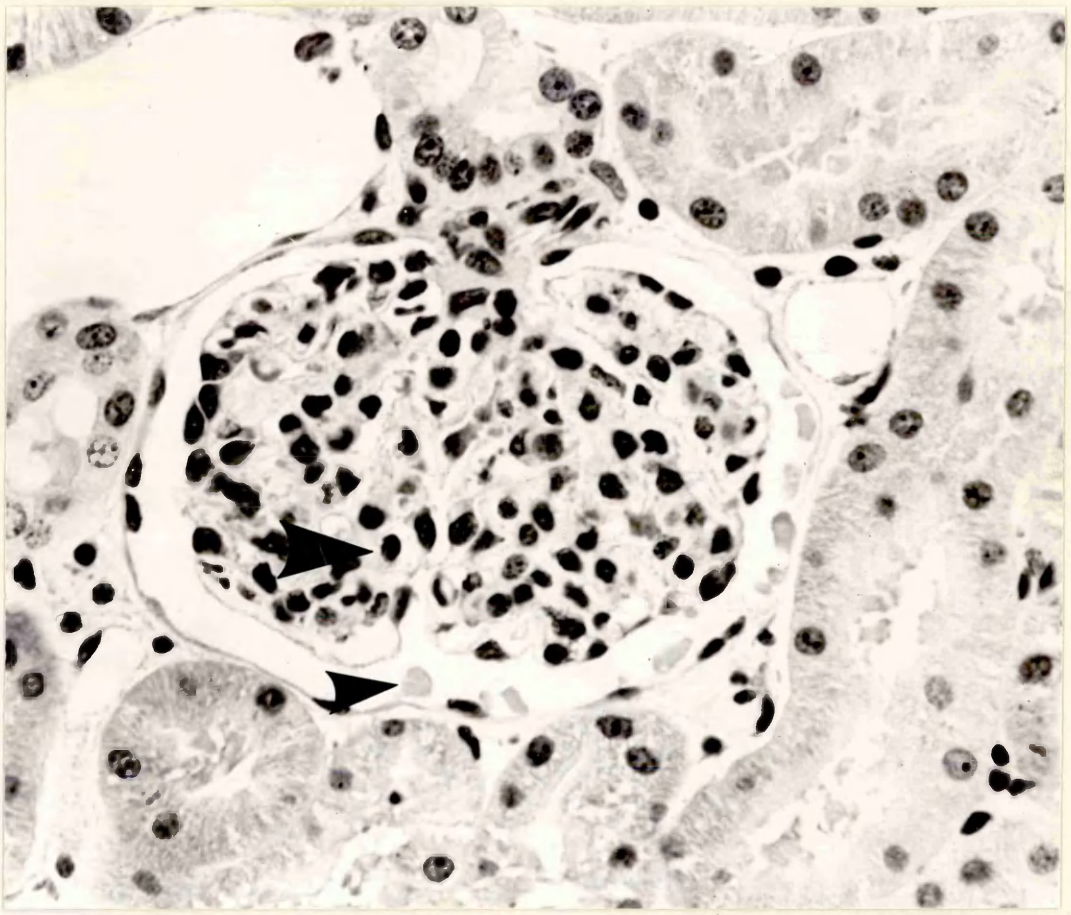


Fig. 31. Autolytic changes, one hour after death, showing tubular epithelial reflux in the urinary space (small arrow) and a few pyknotic cells (large arrow). H & E x 300.

Fig. 32. Autolytic changes, five hours after death, showing many pyknotic nuclei (arrow). H & E x 300.



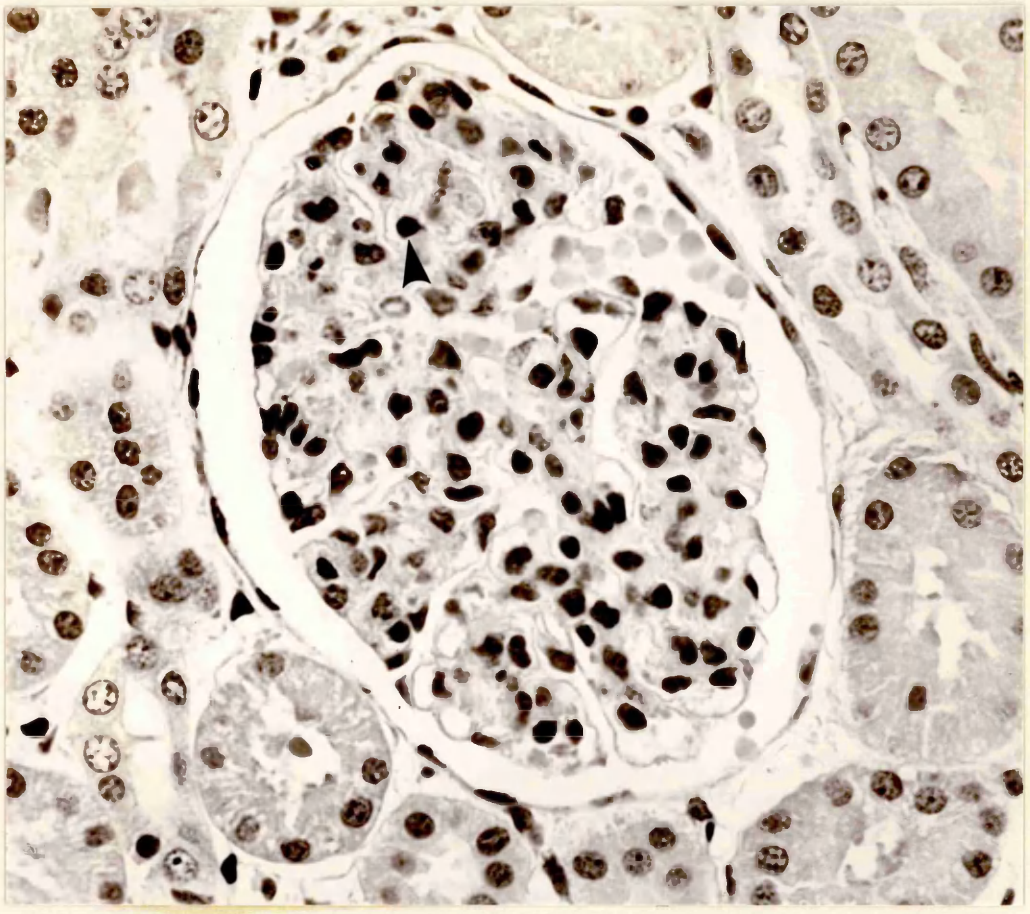
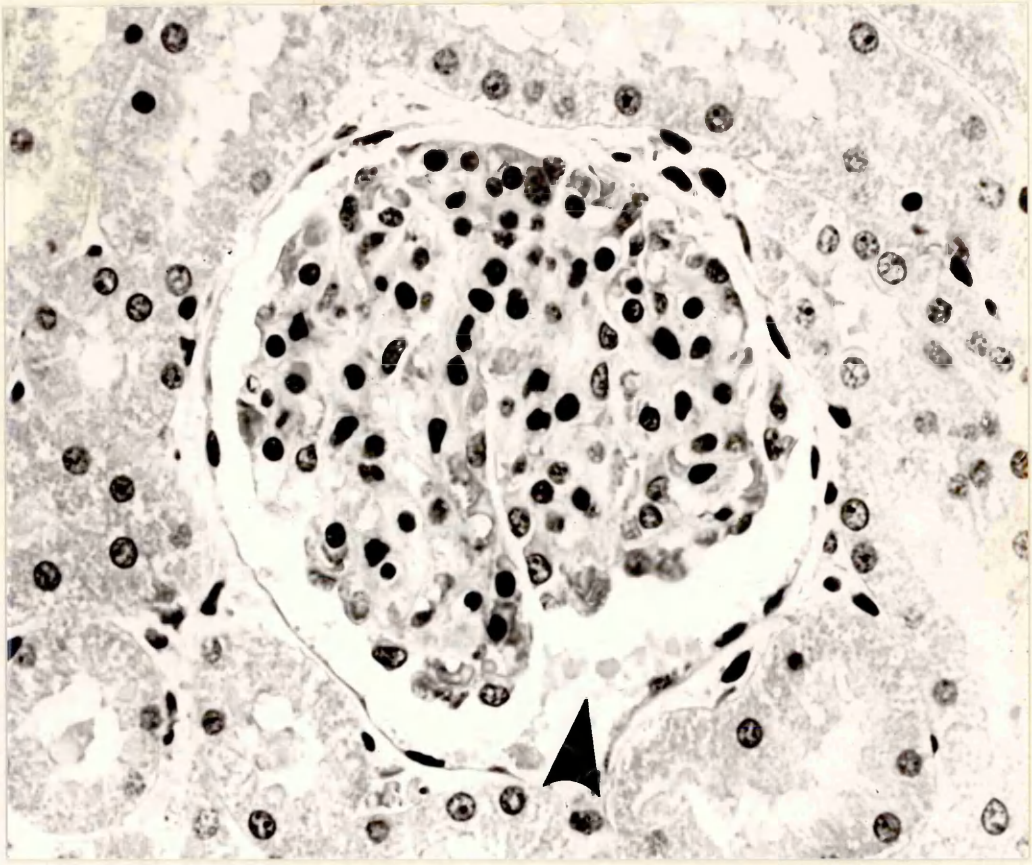


Fig. 33. Autolytic changes, 24 hours after death: note the prominent tubular epithelial reflux (arrow). All capillaries are now closed and there is pyknosis of nuclei. H & E x 300.

Fig. 34. Autolytic changes, three days after death. Note the heavy tubular reflux in to the urinary space (arrow) and generalized nuclear pyknosis. H & E x 300.



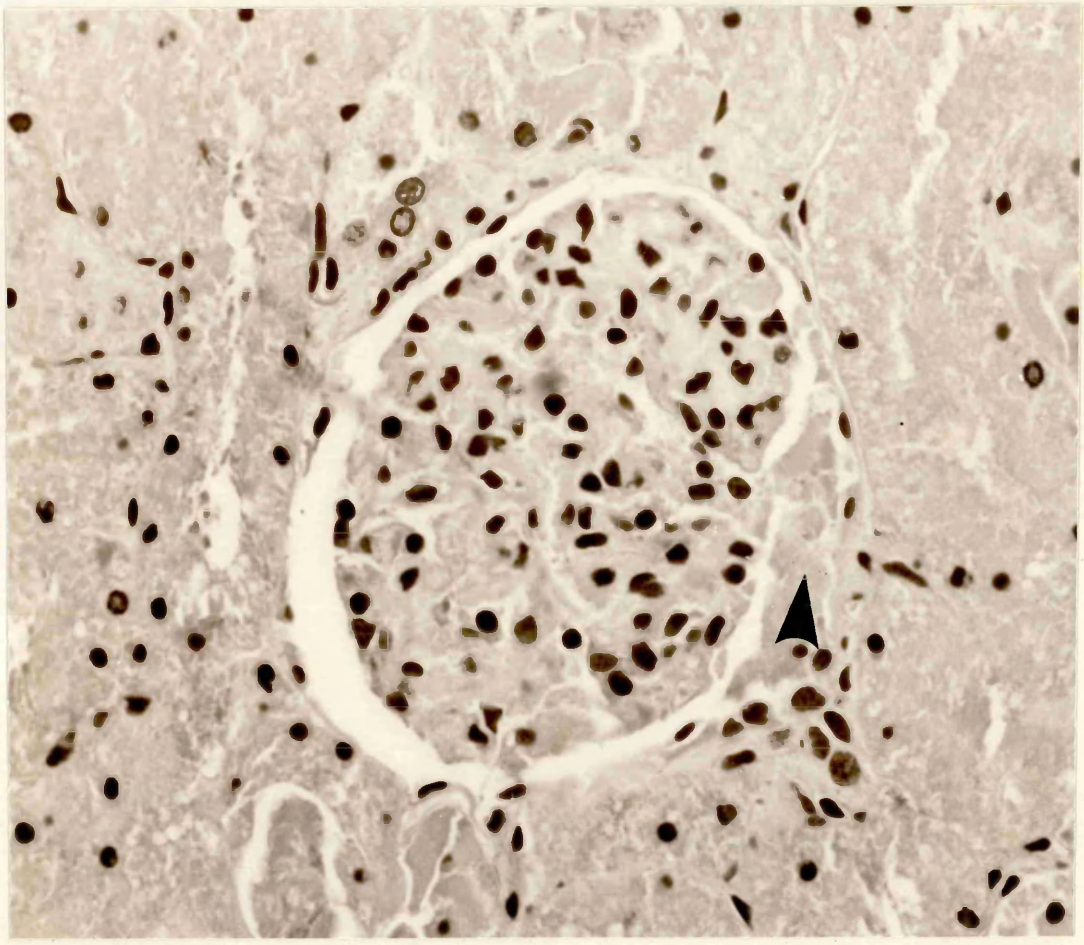


TABLE 4.1

AUTOLYTIC CHANGES IN THE DOG GLOMERULUS: HISTOLOGICAL FEATURES

Time	Tubular Reflux	Capillary Lumina	Nuclear Pyknosis
0 min.	-	open	-
2 min.	1+	open (very few closed)	-
5 min.	1+	few closed	-
10 min.	1+	few closed	-
30 min.	1+	few closed	-
60 min.	2+	many closed	1+
5 hours	3+	many closed	2+
24 hours	3+	many closed	3+
3 days	3+	all closed	3+
5 days	4+	all closed	4+

Lesions graded 1+ to 4+ according to severity.

Ultrastructural findings (TEM):

Localised swelling of the endothelial lining and focal subendothelial expansions was observed as early as two minutes after death (Fig. 36) but no tubular epithelial debris was seen at this time. By five minutes, however, reflux of tubular epithelial cytoplasm into the urinary space was observed (Fig. 37), this had increased considerably by 60 minutes (Fig. 38) and it was particularly noticeable at 24 hours and onwards.

Distinct epithelial changes were observed at 10 minutes when slight swelling of the epithelial foot processes had occurred. By 60 minutes, in addition to swelling, there was focal fusion of the foot processes and, by five hours, portions of necrotic foot processes were lying free in the urinary space. Even on days three and five after death, however, swollen foot processes could still be identified on intact glomerular basement membrane (Fig. 39).

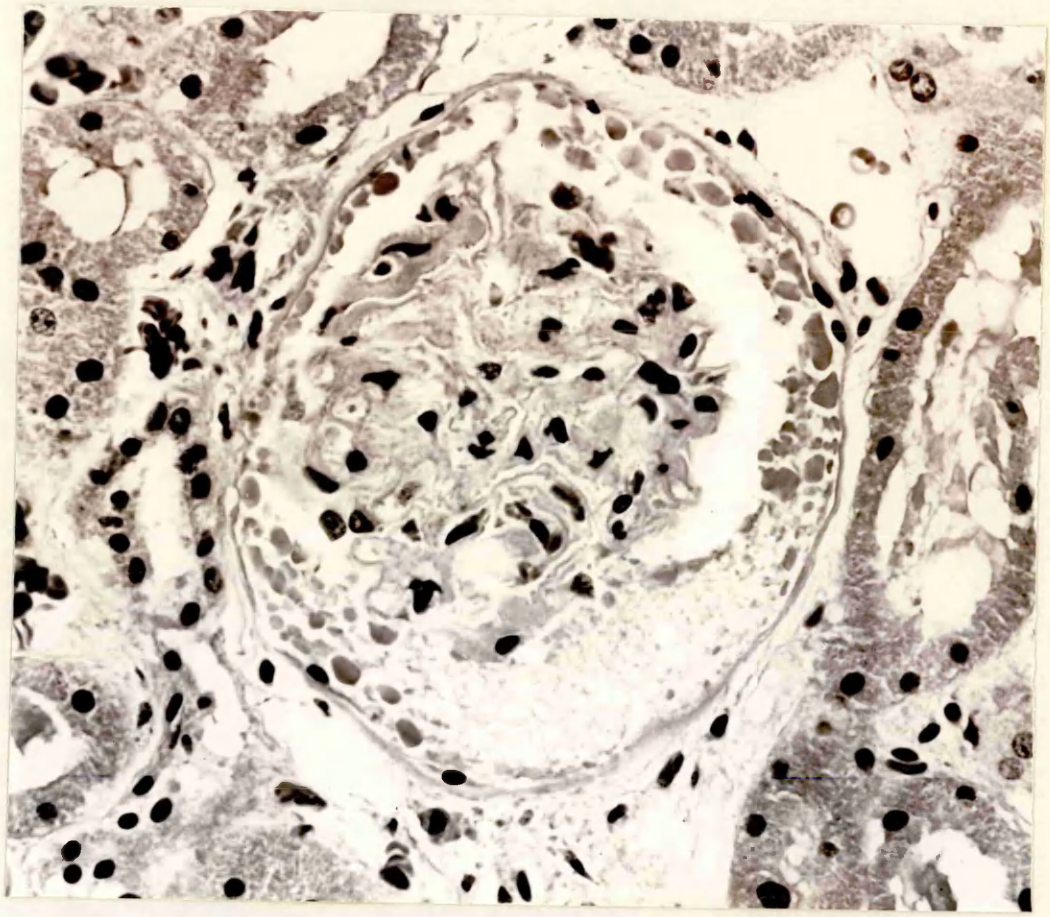
Mesangial cell changes were found from five minutes onwards when processes of these cells were noted extending into the axial portion of the loops, in so doing displacing the endothelial cells. This mesangial cell swelling together with the swelling and displacement of endothelial cells led to occlusion of the capillary lumina. At five hours there was almost total destruction of endothelial cells and the capillary lumina were filled with debris (Fig. 40).

Ultrastructural findings (SEM):

The visceral epithelial cells investing the capillaries often showed varying degrees of alteration, even in the same glomerulus.

Fig. 35. Autolytic changes, five days after death. Note that the normal histological configuration of the glomerulus is lost and the urinary space is filled with tubular epithelial debris. H & E x 300.

Fig. 36. Autolytic changes, two minutes after death, capillary wall shows focal subendothelial expansions (arrow). TEM x 8,000.



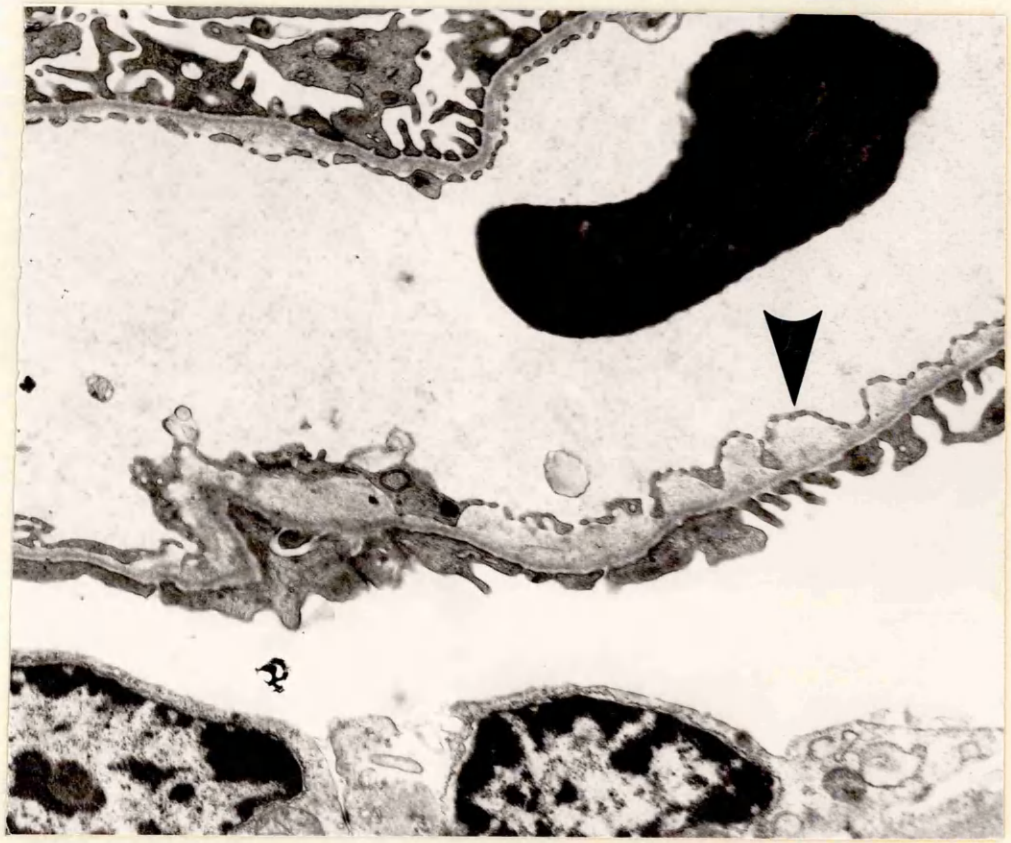
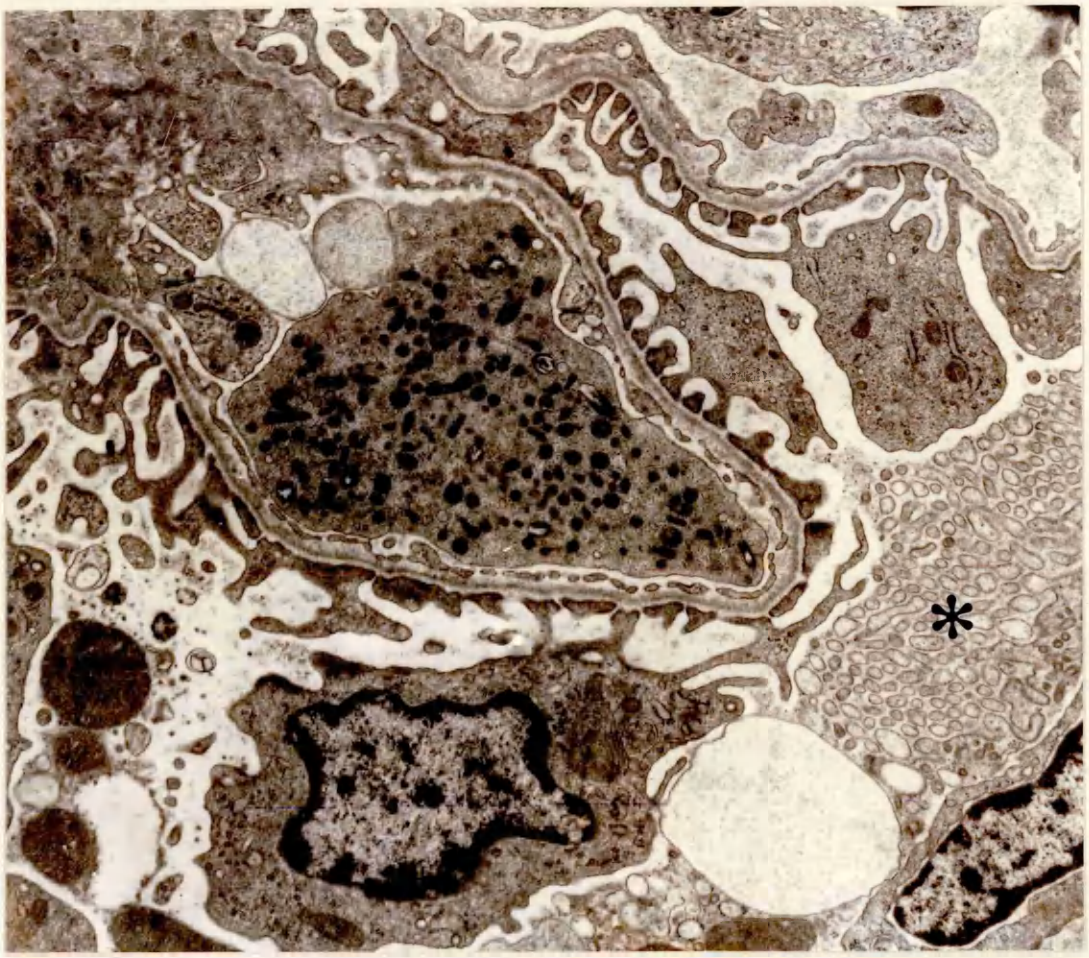


Fig. 37. Autolytic changes, five minutes after death. The urinary space shows reflux of tubular epithelial cytoplasm (asterisk). TEM x 8,000.

Fig. 38. Autolytic changes, 60 minutes after death. The urinary space shows tubular reflux (asterisk) and a visceral epithelial cell shows nuclear pyknosis. TEM x 8,000.



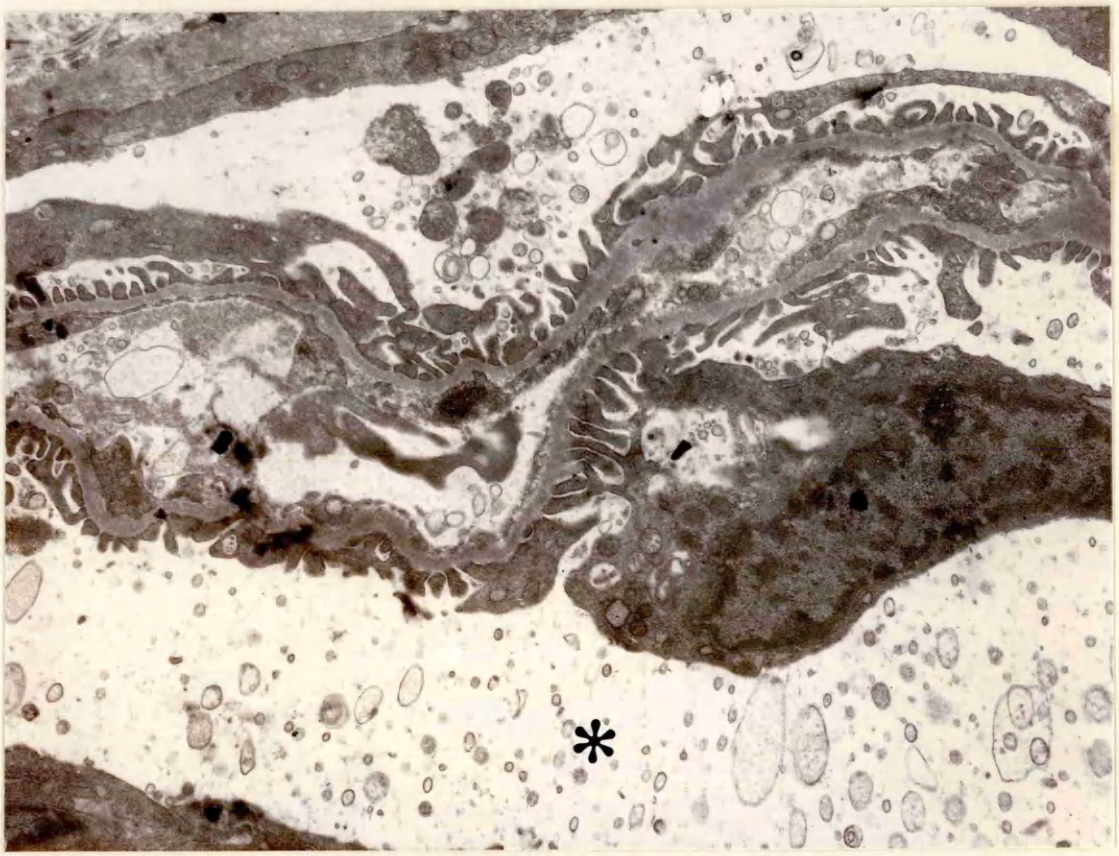
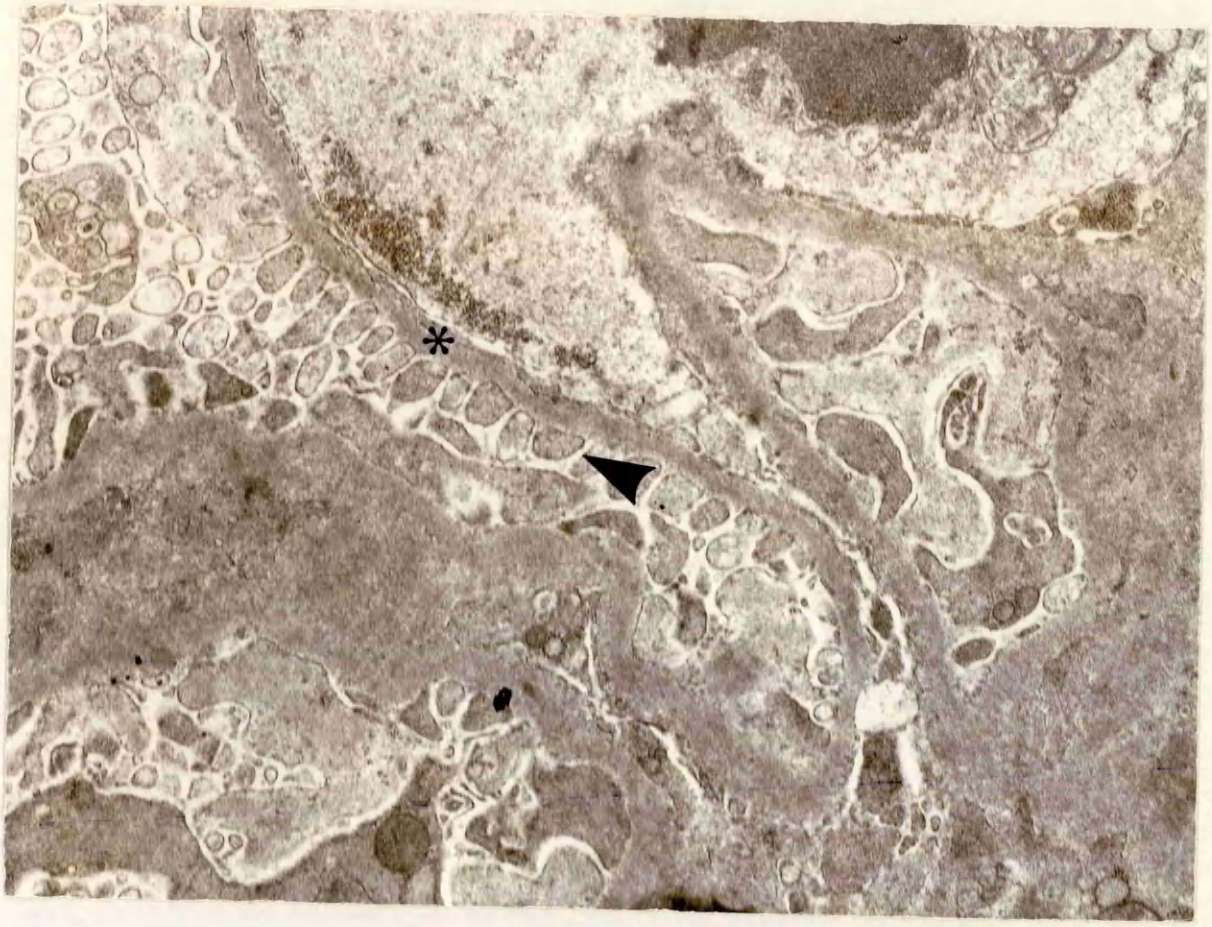


Fig. 39. Autolytic changes, three days after death. Note the swollen foot processes (arrow) on intact GBM (asterisk). TEM x 8,000.

Fig. 40. Autolytic changes, five hours after death. A glomerulus shows destruction of an endothelial cell (E) and capillary lumen full of debris (asterisk). TEM x 8,000.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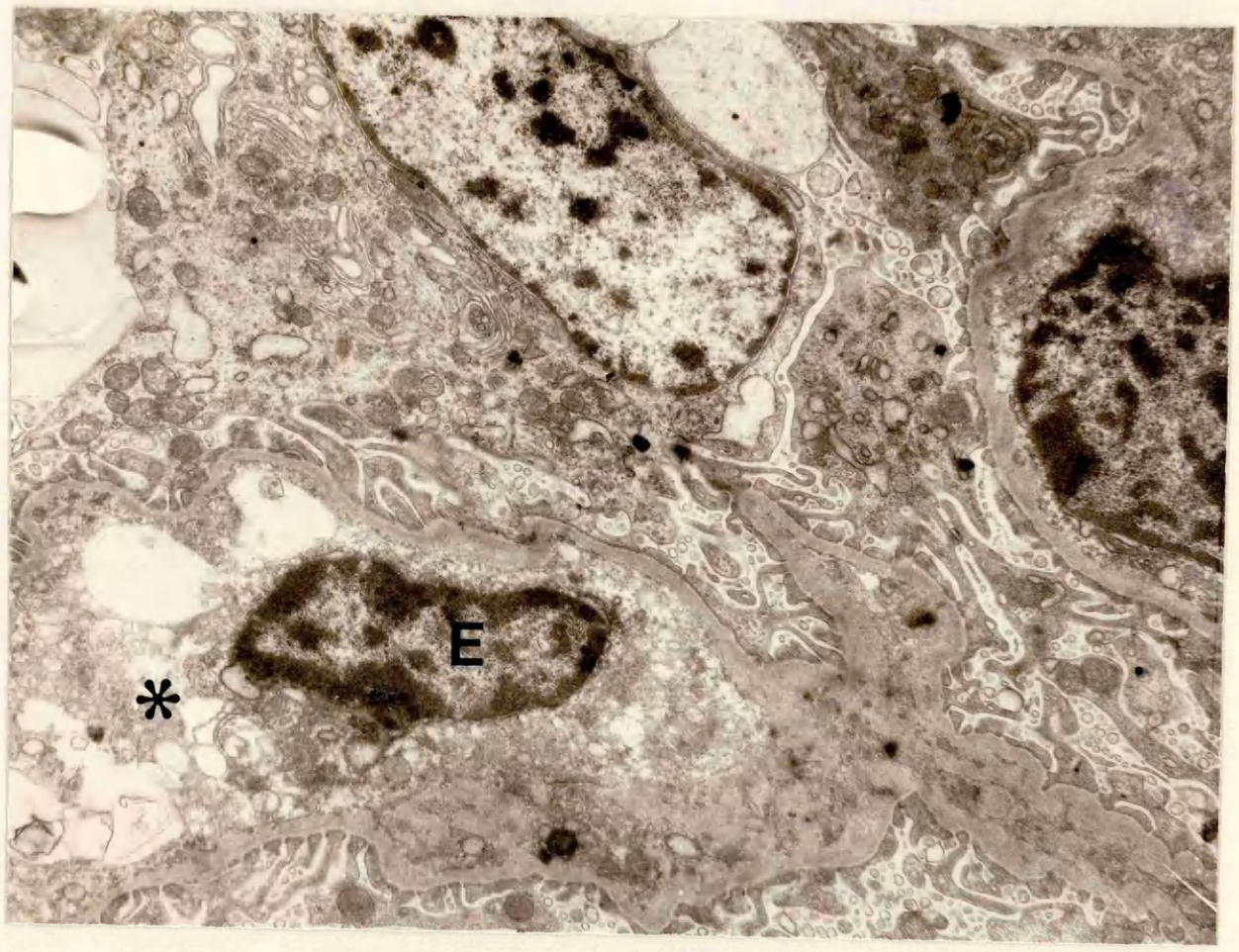

E
*

At two and five minutes after death, the epithelial cytoplasmic processes appeared thickened and the foot processes showed swelling with some localised irregularity in their interdigitation (Fig. 41). In some glomeruli only a few microvilli were seen at this time, mainly on the cell body and primary processes while in others, numerous microvilli were observed covering most of the cell bodies and processes. At this time, however, the epithelial investment of many capillary loops was within normal range (Fig. 42).

Between 10 and 60 minutes, the degree of irregularity and swelling of the foot processes had increased (Fig. 43). Again, in some glomeruli, only a few surface microvilli were found mainly on the epithelial cell body and major processes while, in others, many surface microvillous projections were evident (Fig. 44). In some glomeruli, obliteration of the most of foot processes with loss of the characteristic orderly structural morphology of the visceral epithelium was found (Fig. 45). Even in these severely altered loops, however, there were still areas showing the normal morphological pattern of the epithelium (Figs. 46 and 47).

At five and 24 hours after death, most glomerular capillaries showed loss of the basic morphological characteristics of the epithelial cells with disappearance of foot process interdigitations and marked swelling of epithelial cytoplasmic processes. The epithelial surface of most capillaries was transformed into a roughened sheet-like structure without any discernible foot processes (Fig. 48). Nevertheless, occasional segments of glomerular capillaries still showed normal features of visceral epithelium (Fig. 49).

Fig. 41. Dog glomerulus, two minutes after death. The epithelial cytoplasmic processes are thickened, the foot processes are swollen with some localized irregularity in their interdigitation; many surface microvilli can be seen (arrow). SEM x 5,000.

Fig. 42. Dog glomerulus, two minutes after death. The visceral epithelium is within normal range. SEM x 5,000.

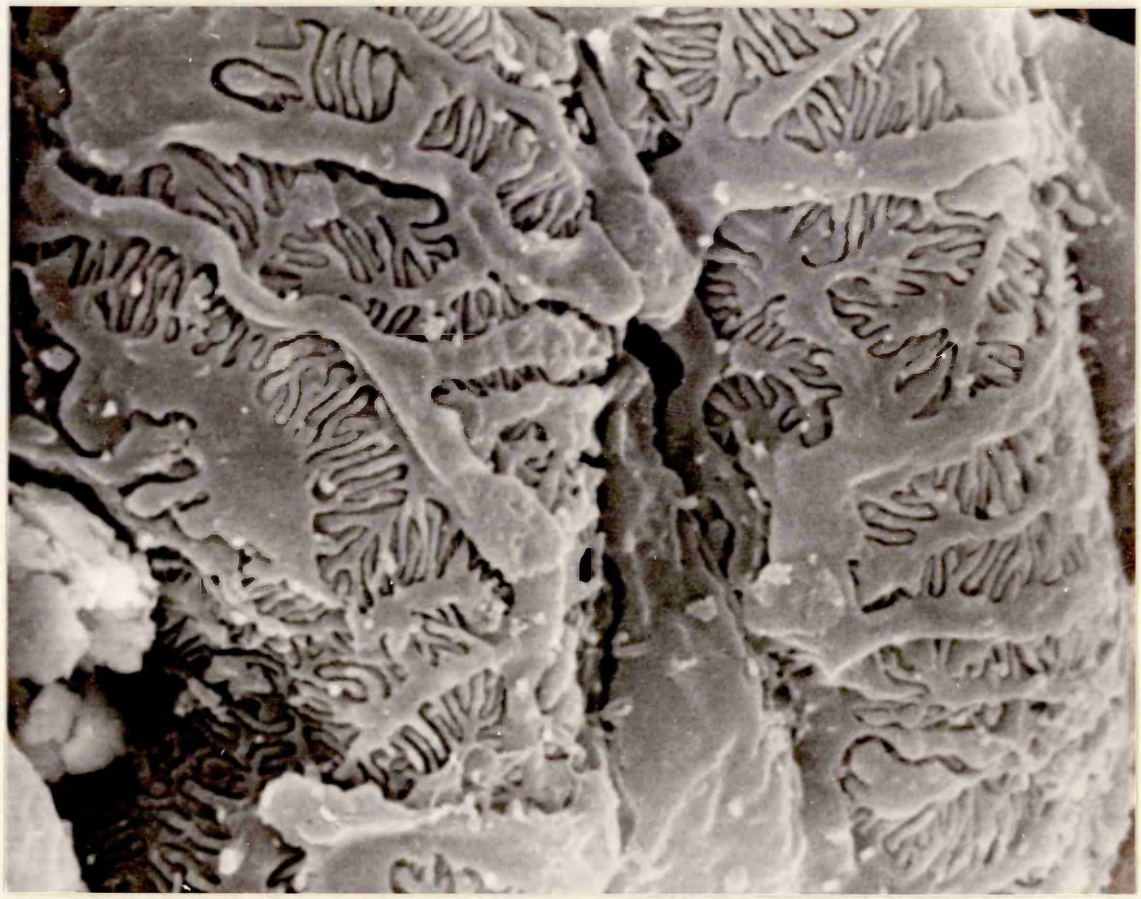
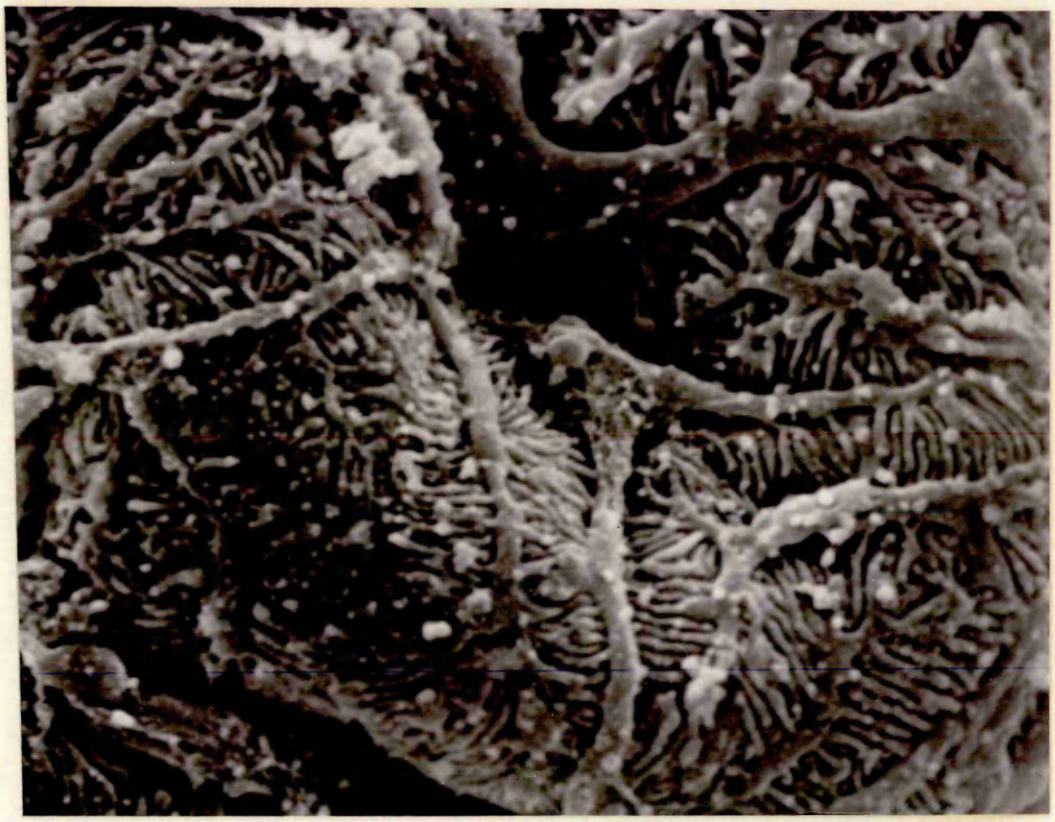


Fig. 43. Dog glomerulus, 10 minutes after death. This shows irregularity and swelling of the foot processes; some microvilli and surface blebs on the primary and secondary cytoplasmic processes can also be seen. SEM x 5,000.

Fig. 44. Dog glomerulus, 30 minutes after death. Note the microvilli on the epithelial cell body (arrow) and on the primary and secondary processes. SEM x 5,000.



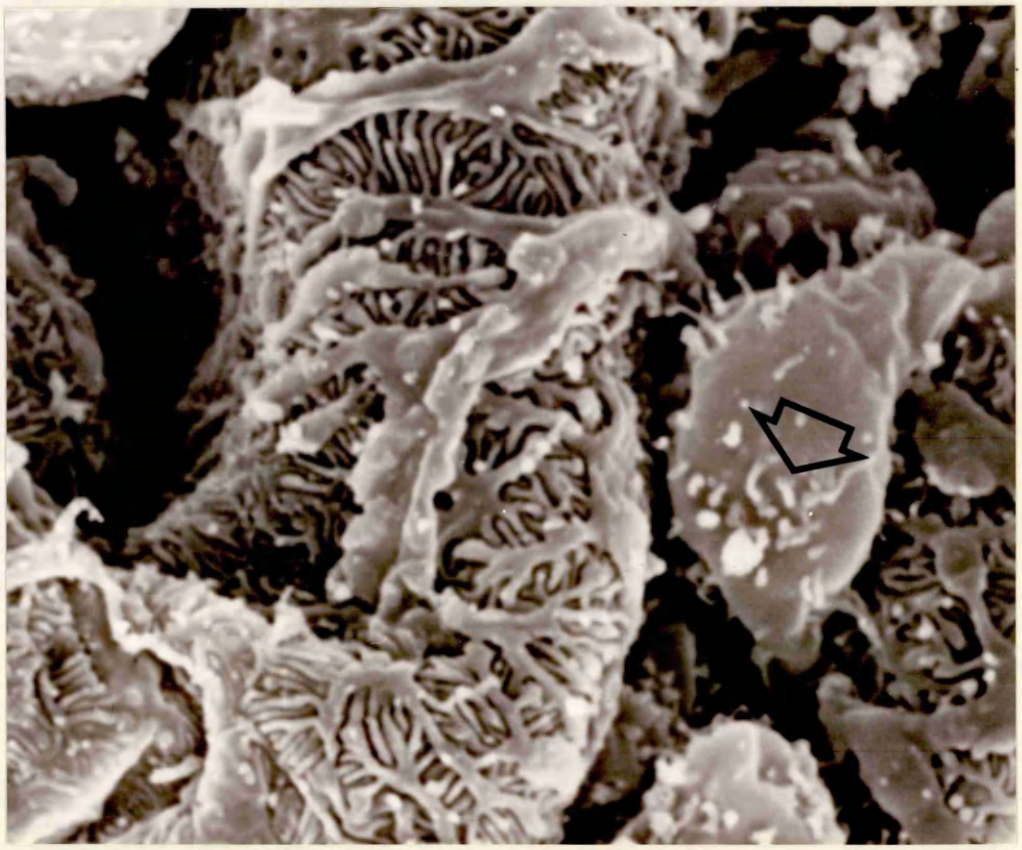
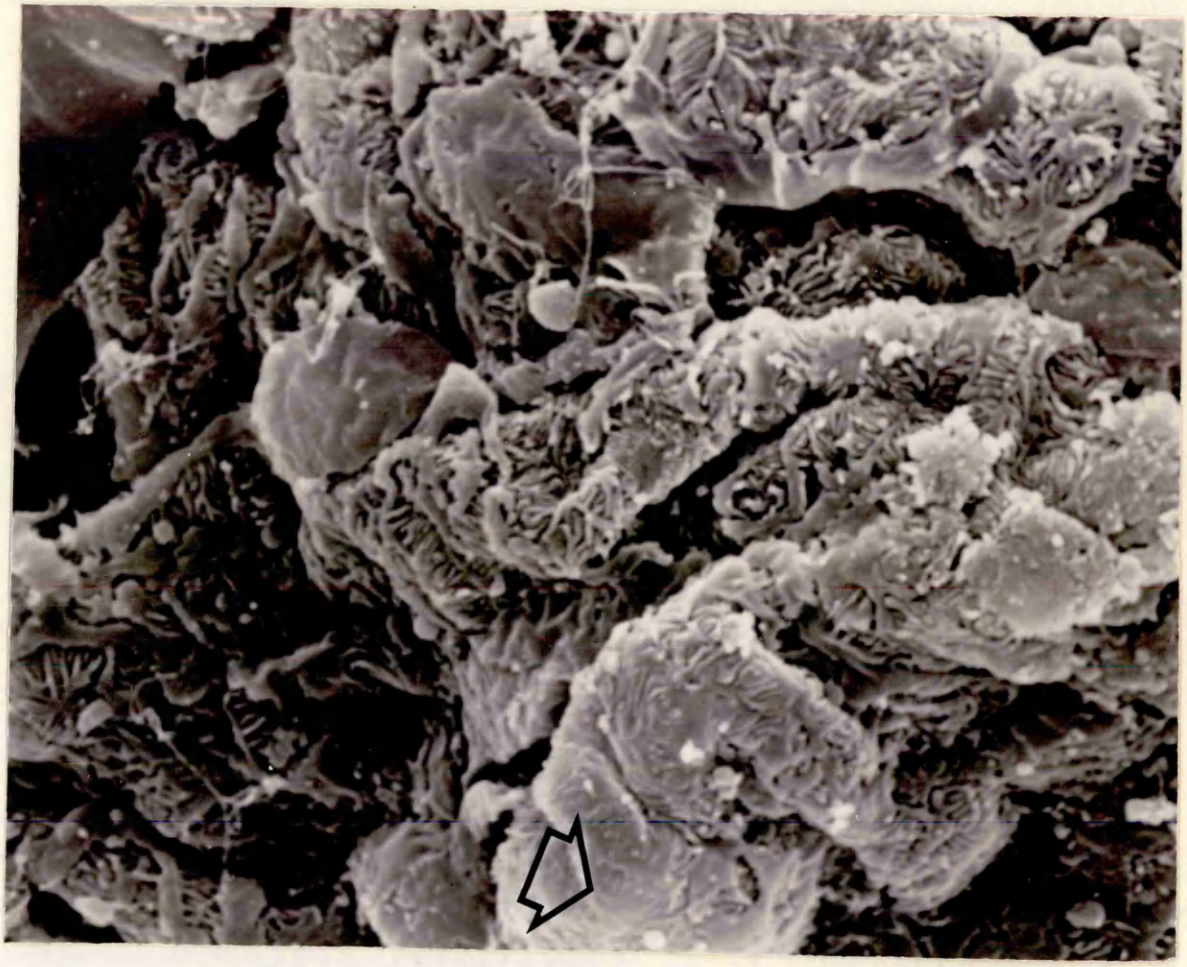


Fig. 45. Dog glomerulus, 60 minutes after death, showing obliteration of the most of foot processes with loss of the characteristic orderly structural morphology of the visceral epithelium (arrow). SEM x 2,500.

Fig. 46. Dog glomerulus, 30 minutes after death: it is within normal range. SEM x 5,000.



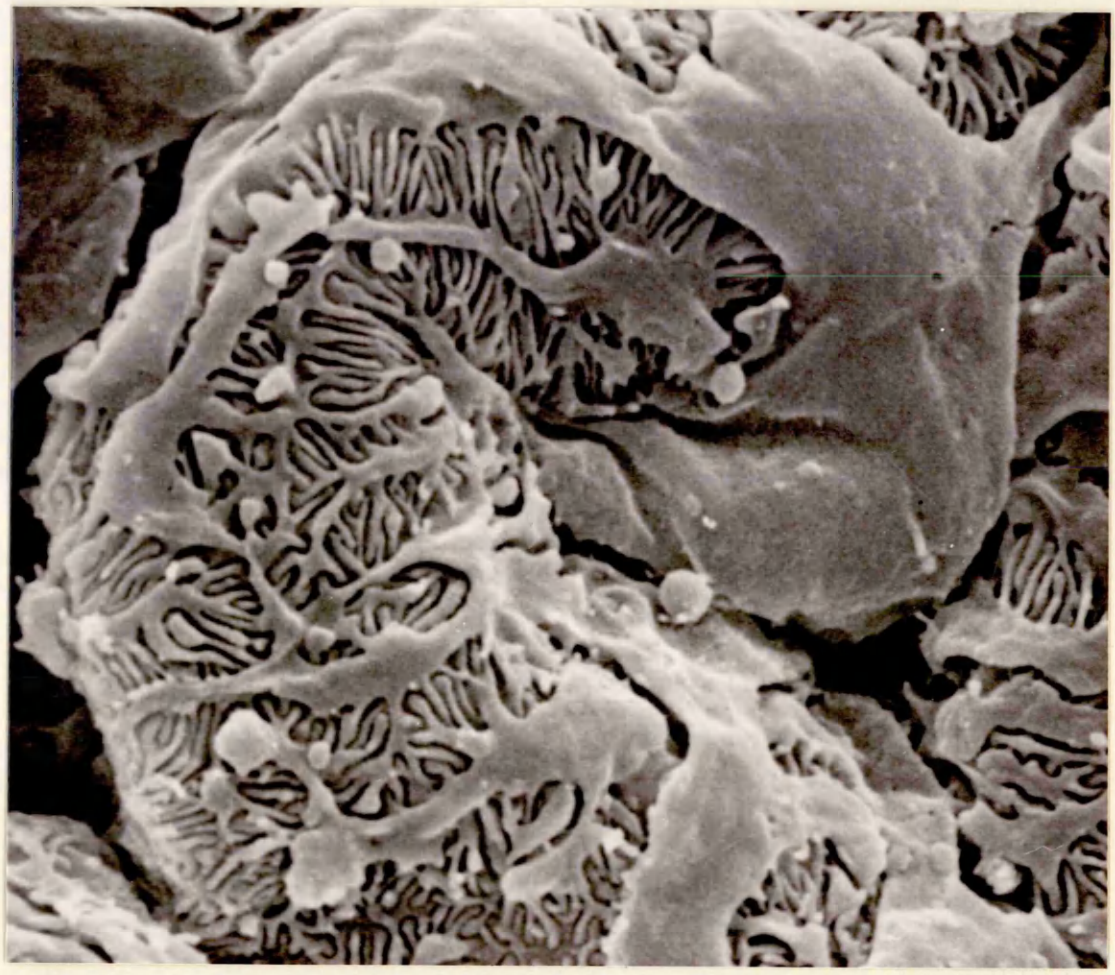


Fig. 47. Dog glomerulus, 60 minutes after death, showing normal interdigitation of the foot processes. SEM x 15,000.

Fig. 48. Dog glomerulus, 24 hours after death, most capillaries show loss of the basic morphological characteristics of the epithelial cells with disappearance of foot processes. SEM x 2,500.

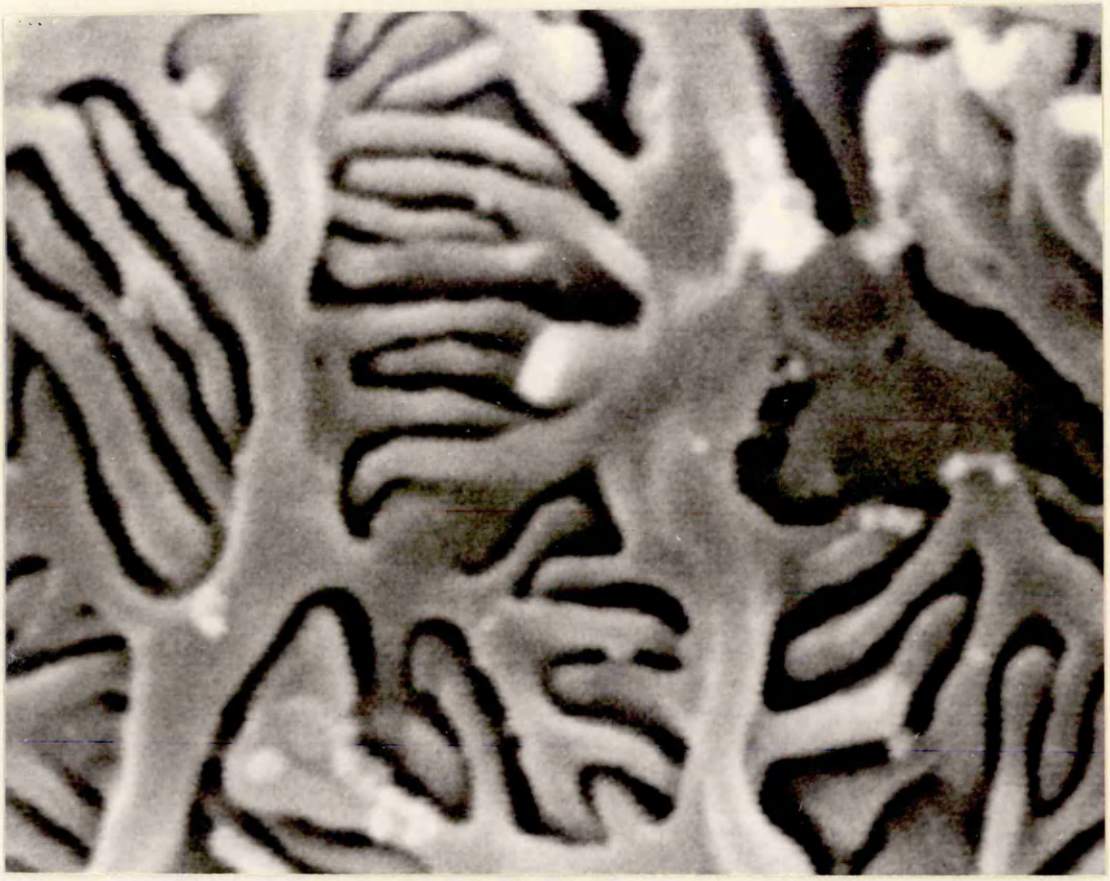


Fig. 49. Dog glomerulus 24 hours after death, showing occasional segments of glomerular capillaries still within normal range (arrow). SEM x 2,500.

TABLE 4.2

AUTOLYTIC CHANGES IN THE DOG GLOMERULUS: TEM FINDINGS

Time	Tubular Reflux	Epithelial Cells	Endothelial Cells	Capillary Lumina
0 min.	-	-	-	-
2 min.	-	-	focal subendothelial expansion	-
5 mins.	2+	-	cell body pushed by mesangial pseudopods	closed 1+
10 mins.	2+	slightly swollen F.P.	focal disapp. of cytoplasm	closed 2+
30 mins.	2+	F.P. swollen 1+	focal disapp. of cytoplasm	closed 2+
60 mins.	3+	focal fusion of F.P. swollen 2+	mostly disapp. of cytoplasm	closed 3+
5 hours	3+	focal fusion of F.P. and destruction	disapp. of cytoplasm	closed 3+
24 hours	4+	F.P. swollen 3+ and focal fusion and destruction	disapp. of cytoplasm	closed 3+
3 days	4+	still F.P. recognisable on GBM	disapp. of cytoplasm	closed 4+
5 days	4+	same as above	-	closed 4+

Lesions graded 1+ to 4+ according to severity.

TABLE 4.3

AUTOLYTIC CHANGES IN THE DOG GLOMERULUS: SEM FINDINGS

Time	Microvilli	Epithelial Cell				
		Foot Processes		Primary & Secondary Processes Thickening	Cell Body Swelling	Surface Blebs
		Swelling	Fusion			
2 mins.	1+	1+	-	1+	1+	-
5 mins.	1+	1+	-	1+	1+	-
10 mins.	2+	2+	2+	1+	1+	-
30 mins.	1+	2+	2+	1+	1+	-
60 mins.	1+	2+	2+	1+	1+	-
5 hours	1+	2+	3+	2+	2+	-
24 hours	-	2+	3+	2+	2+	2+
3 days	-	-	4+	1+	1+	3+
5 days	-	-	4+	-	-	4+

Lesions graded 1+ to 4+ according to severity.

On the third and fifth days after death, every glomerulus was converted into a homogeneous rounded mass (Fig. 50); all the morphological characteristics of the visceral epithelial cells had disappeared and identifyable foot processes could no longer be seen (Fig. 51).

Discussion:

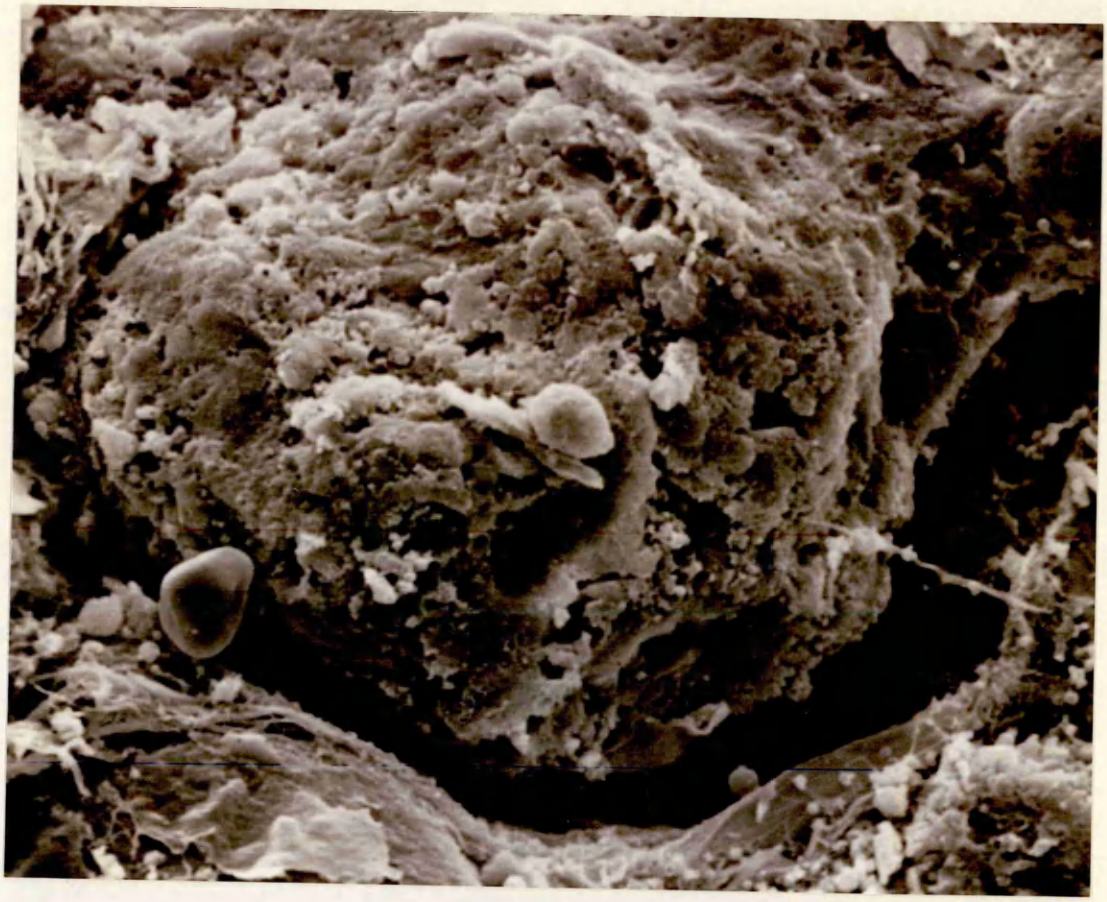
The earliest autolytic changes observed by light microscopy occurred as early as two minutes when a small amount of tubular epithelial debris was found in the urinary space in a very few glomeruli. This tubular epithelial reflux subsequently increased as the time of sampling after death lengthened. From five minutes onwards occlusion of the capillaries as a result of endothelial and mesangial swelling gradually became more and more pronounced and was virtually complete by 24 hours. These findings are in accord with the report by Osvaldo et al (1965) who noted similar progressive capillary obliteration in the rat kidney. The latter workers, however, made no mention of tubular epithelial reflux into the urinary space which, in the present study, was observed from two minutes and gradually became more prominent as the length of time after death increased.

The presence of tubular epithelial reflux in the kidneys of dogs and rats has, however, been shown to be influenced by a range of factors including antemortem conditions such as ischaemia, the method of fixation and even palpation of the kidneys at autopsy (Mullink and Feron, 1967).

The TEM findings in the present work were, in general, similar to those of Crowell et al (1974), although these workers did

Fig. 50. Dog glomerulus, three days after death. All morphological characteristics have disappeared. SEM x 1,200.

Fig. 51. Dog glomerulus, three days after death. At this higher magnification, no foot processes can be seen. SEM x 2,500.



not begin sampling their material until 30 minutes after death. The main feature of interest was the remarkable preservation of the GBM and attached foot processes despite the early disintegration of endothelial cells and the cell bodies of visceral epithelial cells. Some foot processes still remained morphologically recognisable even as late as three and five days after death.

It was evident with the SEM that glomeruli appeared to react at different rates to autolytic change. As early as two minutes after death, some visceral epithelial cells showed the development of surface microvilli and there was swelling and thickening of foot processes. These changes were more severe at 10 and 30 minutes with more widespread fusion of foot processes. Nevertheless, even at one - 24 hours after death, some capillaries were still showing areas of normal morphology. On days three and five, however, no trace of orderly interdigitation of foot processes was found.

It was not possible to make a direct comparison with the findings of Langlinais (1981), who studied the SEM changes in dog kidney material held at room temperature for the first four hours, and subsequently at 4 to 6°C for 24 hours. He did, however, describe surface blebs on visceral epithelial cells at 15 minutes, focal fusion and obliteration of foot processes at 90 minutes, and widespread effacement of foot processes at three hours.

In the light of these results a number of important considerations have arisen. Firstly, the rate at which autolytic changes occur in different glomeruli varies; thus although early changes were observed with the SEM at two minutes, areas of normal glomerular architecture were still maintained as late as 24 hours.

Secondly, although early changes were identified by TEM at two minutes and cellular disintegration, particularly of endothelial cells followed rapidly the GBM although swollen remained intact and foot processes could still be observed as late as three to five days after death.

CHAPTER 5

EXPERIMENTAL NEPHROTOXIC NEPHRITIS IN DOGS

Introduction:

Due to its apparent predisposition to damage by immunological mechanisms, the kidney has probably attracted more attention from investigators in the field of immunopathology than any other organ. A search for the role of immune mechanisms in the pathogenesis of kidney disease dates back as early as the foundation of immunology itself in the last decades of the 19th and the early part of the 20th centuries.

One of the first steps in our understanding of how immune mechanisms affect the kidney was the work of Weiss (1896) who showed that the injection of heterologous serum induces proteinuria. It is Lindemann (1900), however, who is considered to be the first real investigator of immunologically induced kidney injury when he injected rabbit kidney homogenates intraperitoneally into guinea pigs. The resultant anti-kidney serum when inoculated into rabbits caused proteinuria and death two to five days after injection. Pearce (1903) subsequently presented more detailed information on this so-called "nephrotoxic serum nephritis" (NTN). He described the glomerular lesions induced by nephrotoxic serum and pointed out that homogenates of cortex were much more efficient in inducing NTN than homogenates of renal medulla. More recently, a detailed morphological description of NTN in rats was given by Masugi (1934) and, since then, NTN has been produced in several animal species and has become generally known as "Masugi nephritis".

NTN can now be defined as an immunological injury to the renal glomerulus induced by injection of heterologous antibodies to the GBM. Thus, for example, antibodies to rat (or human or guinea pig) GBM raised in rabbits induces NTN when injected intravenously or

intraperitoneally back into rats (or human§or guinea pigs).

It has been clearly established that NTN develops in two often overlapping phases each based on somewhat different pathogenetic mechanisms (Hammer and Dixon, 1963; Fujimoto et al, 1964; Unanue and Dixon, 1964). The initial or "heterologous phase" takes place very soon after injection of nephrotoxic antibodies (NTabs) and is dependent upon their interaction with GBM antigens. The "autologous phase" develops later when the host mounts an immune response to the injected heterologous NTabs implanted on the GBM.

The heterologous phase:

The NTabs fix to antigens located in the GBM, the main antigenic component of the glomerulus (Krakower and Greenspon, 1951). Numerous studies on the isolation and purification of these GBM antigens have now been carried out (Krakower and Greenspon, 1951; Goodman et al, 1955; Steblay and Lepper, 1961; Terman et al, 1977). It was not until 1972, however, that Naruse and Shibata provided evidence that a water soluble extract of trypsin-digested GBM has the ability not only to absorb nephrotoxic activity from nephrotoxic antiserum but also to actively induce potent nephrotoxic serum in animals. It must be stated that antisera raised to other connective tissue stromal elements such as epithelial basement membrane, collagen or organ sediments rich in blood vessels, when injected into animals, will also fix to the GBM (Seegal, 1958) and in some instances cause NTN (Baxter and Goodman, 1956); thus it is concluded that the GBM shares a common antigen with other tissues (Goodman et al, 1955).

The immunological events of the heterologous phase occur rapidly after intravenous injection of NTabs. The majority of NTabs

leave the circulation during the first 30 minutes after injection and become fixed to the GBM (Blau et al, 1957); this is the crucial event responsible for glomerular injury (Kay, 1940; Hammer and Dixon, 1963). As the GBM, however, shares antigens with other connective tissue elements not unexpectedly some NTabs fix elsewhere, particularly in the liver, lung, spleen, muscle, adrenal gland, ovary and gastrointestinal tract (Pressman, 1951, Blau et al, 1957). Despite this widespread organ localisation of NTabs, significant injury occurs in the kidney; in other organs the concentration of the antibody appears to be too low and the fixation too transient to cause injury.

Unanue and Dixon (1965) considered the most important factor in the development of renal injury during the heterologous phase to be the amount of antibody which fixes to the GBM. Likewise, Dixon (1967) states that a correlation exists between the amount of antibody, the amount fixed to the GBM, and the degree of clinical and pathologic changes. As will become clear later, however, once the NTabs are fixed to the GBM a number of other factors such as complement activation and neutrophil infiltration influence subsequent events (Stavitsky et al, 1954; Dixon, 1967).

Descriptions of the main histological, immunofluorescence and ultrastructural alterations in NTN in rabbits have been given by Kondo et al (1972); Kondo et al (1976) and in rats by Shigematsu (1970) and Shigematsu and Kobayashi (1973). The major morphologic changes found in the glomeruli during the heterologous phase of NTN can be summarised as influx of neutrophils (and later) monocytes, capillary thrombosis with leakage of fibrin into urinary space, segmental or global tuft necrosis, expansion of the subendothelial

space and mesangial hypercellularity.

Shibata et al (1978) reported that exfoliation of endothelial cytoplasm with denudation of the GBM is the initial significant glomerular change in NTN; this alteration appeared as early as 10 - 15 minutes after inoculation of antiserum to soluble GBM but not until two to four hours in animals receiving antiserum against insoluble GBM preparations. This confirmed a previous report by Winemiller et al (1961) who claimed that the initial significant glomerular change at four hours after injection of potent NTabs is not damage to the GBM itself but focal exfoliations of endothelial cytoplasm, denuding the GBM. Shigematsu (1970) noted attachment of neutrophils to the denuded GBM within two to 12 hours after injection of NTabs. Unanue et al (1969) concluded that the actual nephritogenic action of these cells is not known but it has been suggested by Cochrane et al (1965) that they might damage the GBM by releasing hydrolytic enzymes. Albini et al (1979) reported that neutrophils are attracted to the GBM by cleavage products of complement (particularly C_3 and C_5 fragments); they can adhere to and strip off the endothelium and in this way reach the underlying GBM.

The role of complement in NTN has been the subject of many investigations. Cochrane et al (1965) showed that depletion of serum complement resulted not only in inhibition of infiltration of neutrophils but also the development of proteinuria. On the other hand, duck NTabs apparently do not fix mammalian complement yet can cause immediate renal injury when injected in appropriate doses (Hasson et al, 1957; Hammer and Dixon, 1963; Unanue and Dixon, 1964).

Extensive studies have also been carried out on the role of monocytes in the pathogenesis of NTN. Cotran (1981) concluded that blood monocytes, of bone marrow origin, infiltrate the glomerulus and play an important role in inducing glomerular injury. Glomerular infiltration by monocytes in NTN has also been studied by Kondo et al (1972), Thomson et al (1979) and Shigematsu (1981) who provided morphological evidence of imigration, transformation and phagocytosis in the inflamed glomerulus. They are found in greatest numbers seven to 11 days after administration of NTabs (Kondo and Shigematsu, 1972). In NTN and indeed in various other types of experimental glomerulonephritis they are seen to migrate into subendothelial areas, mesangial matrix and the urinary space; emigration of monocytes into the urinary space is often seen in close proximity to extravasated fibrin-like material. Schreiner et al (1981) reported that mononuclear cells are an important cellular component of immunological injury to the renal glomerulus, and correlated with increased glomerular cellularity and maximal proteinuria. They concluded that a predominant part of the glomerular hypercellularity is due to bone marrow derived mononuclear cells infiltrating the glomerular mesangium.

Kondo et al (1976), in their studies of NTN in rabbits, paid special attention to mesangial changes. They confirmed that the mesangium is not the primary site of antigen antibody interaction, and that nephrotoxic antibodies, host antibodies and complement are all found to be localised in a linear fashion along the GBM rather than in the mesangium. They did point out, however, that the severe mesangiolytic changes observed in some models of NTN were obscured by marked hypertrophy and proliferation of mesangial cells and large scale infiltration of monocytes into

the loosened mesangium.

Although it is the endothelial and mesangial cells that show the most changes in the heterologous phase of NTN, SEM and TEM studies have indicated that visceral epithelial cells are also affected. Buss and Lamberts (1975) carried out a detailed combined TEM and SEM study of the podocytes in rat NTN in the course of which the processes of these cells were shown to be generally preserved although proteinuria was high. There was, however, irregular swelling of the cell bodies and foot processes in some glomeruli and a most striking feature with SEM was the presence of microvillous formation.

The autologous phase:

The study of the second or autologous phase of NTN was initiated by Kay (1940) and recently reviewed by Albini et al (1979). It begins one or more weeks after injection of NTabs and is due to the production by the recipient animal of antibodies to the heterologous serum implanted in the GBM. This second immune reaction usually induces a proliferative, exudative and sometimes necrotizing glomerulonephritis. There is swelling of endothelial cells, mesangial cell proliferation and an increase in mesangial matrix. Occasional accumulation of collagen fibres and fibrin in the mesangium is sometimes observed and there is often formation of crescents. The latter are considered to be formed by proliferating visceral and parietal epithelial cells and infiltrating neutrophils and macrophages in response to extravasated fibrin (Albini et al, 1979). A marked variation in severity of these lesions from glomerulus to glomerulus often exists. Ultrastructurally, expansion of the subendothelial space is more evident than in the heterologous

phase and there is thickening and splitting of the GBM. The disease may have a mild or rapidly progressive course, ending with chronic renal failure; the outcome is dependent on the amount of antibody injected and on the animal model used.

The mediators involved during this phase have not been fully characterised. It is suggested that only partial fixation of complement might be necessary to induce further glomerular damage or that complement is not even essential (Dixon, 1967). Kondo et al, (1972) reported the occurrence of monocyte accumulation while in 1979, Albini et al suggested that components of the coagulation system also participate in the production of glomerular damage. Thus at least some of the mediators operative during the heterologous stage are also participating in the autologous phase.

The pathology of the autologous phase of NTN is characterised generally, however, by progressive alteration to the glomeruli. Mesangial zones enlarge and contain a greater than normal number of cells some of which are monocytic in origin. The GBM exhibits increased thickening, becomes wrinkled and often finally collapses. Some animals with NTN show subepithelial electron dense deposits which are normally associated with immune complex disease (Albini et al, 1979). Whether or not these deposits are associated with the release of kidney antigens by the nephrotoxic serum and subsequent anti-kidney antibody formation with deposition of circulating complexes in the GBM is still unclear.

NTN in the dog:

NTN has been the subject of intensive studies in laboratory animals (particularly the rat, mouse and rabbit). The dog model,

however, has only rarely been used. The earliest study on canine NTN was carried out by Wilson and Oliver (1920) who established the specificity of rabbit anti-dog kidney serum in producing nephritis. A more comprehensive histological and ultrastructural description of experimental NTN in the dog was reported by Movat et al (1961) who used whole kidney suspensions to prepare nephrotoxic serum. Since then, Dhar and Pathak (1970) have also given a brief histological description of NTN in dogs. Fouts et al (1941) reported observations on the clinical and functional course of NTN in dogs; they suggested that the increase of renal blood flow is due to inflammatory hyperemia, while the decrease in filtration is due to thickening of the GBM, and they also declared that clinical and functional alterations occurring during the course of the disease was similar to many phases of Bright's disease in human beings.

More recently, the sequential pathology of canine NTN has been described by Wright et al (1973[a]) who induced NTN by intravenous administration of anti-GBM serum, and examined their experimental animals from four hours up to 60 days. In a similar fashion, Shirota and Fujiwara (1982) carried out a brief experiment on canine NTN using only three dogs which were sacrificed three, five, seven days after intravenous administration of NTabs. Since then, no further reports are available of NTN in dogs and much on how the dog glomerulus reacts to immunological injury has still to be defined. This is despite the fact that, although spontaneously occurring anti-GBM glomerulonephritis has not so far been described in dogs, other forms of immunological injury to the glomerulus are increasingly being diagnosed (Murray and Wright, 1974; Wright et al, 1981).

The purpose of this chapter of the work was to make an in depth study of experimentally induced NTN in dogs with particular reference to the sequential changes occurring in the damaged glomeruli. In order to study as many morphological parameters as possible a combined histologic, immunofluorescence and ultrastructural (transmission and scanning electron microscopy) was carried out.

Materials and Methods:

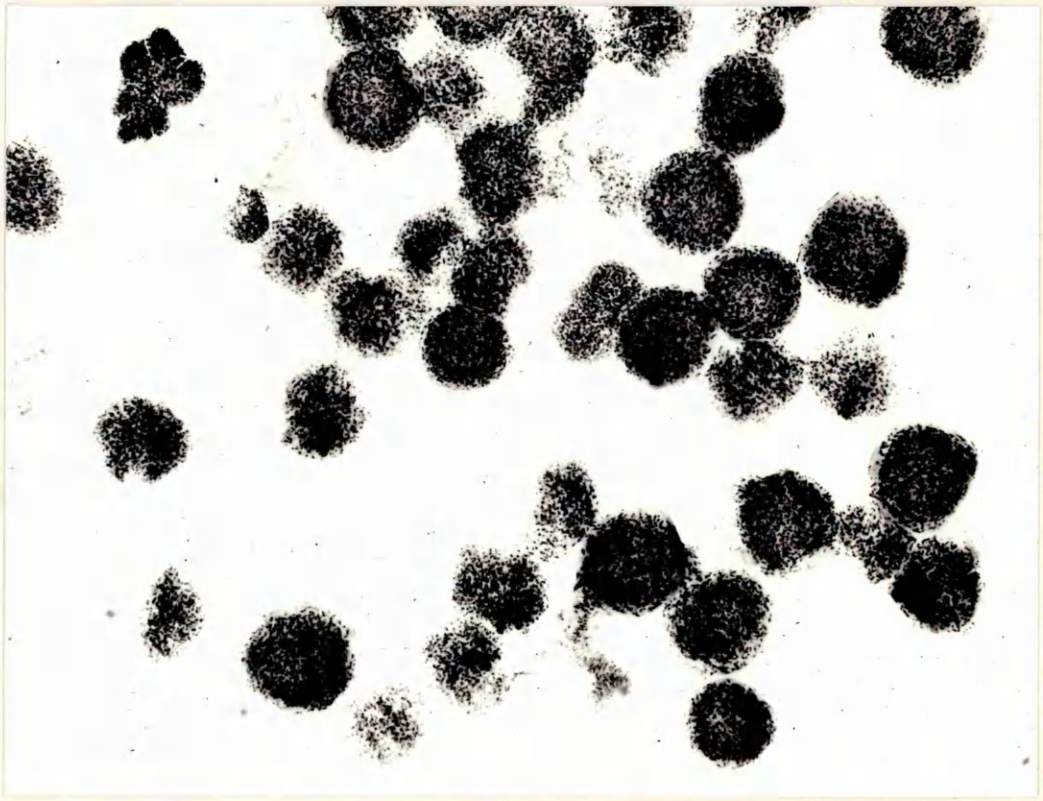
Preparation of GBM antigen:

The methodology used was that described by Wright et al (1973[a]). Glomerular basement membrane (GBM) was prepared from the kidneys of three sixteen week-old dogs. Histological examination of a small portion of each kidney showed no histological abnormalities of the glomeruli and immunofluorescence tests did not show deposition of IgG or complement. The kidneys were decapsulated, the medullary region removed by dissection and the cortices minced into fine pieces. After repeated washing in cold phosphate buffered saline (PBS) pH 7.3 to remove red blood cells, the diced kidney cortex was forced very gently by means of a small beaker through a 210 um stainless steel sieve, while flushing continuously with cold PBS. The washings were then poured through a 60 µm sieve which retained the glomeruli but allowed through the unwanted non-glomerular fragments; the latter was discarded or stored at -20° for future preparation of tubular epithelial antigen. The glomeruli were washed off the surface of the sieve with cold PBS and centifuged at 1000 x g at 4°C for five minutes. The glomerular rich sediment was resuspended in PBS and allowed to settle. This washing process was repeated three times until phase contrast microscopy demonstrated a rich suspension of glomeruli relatively free of tubular fragments.

Smears of glomerular rich sediment and non-glomerular supernatant were stained for five minutes with methylene blue in order to confirm the relative purity of the glomerular samples (Figs. 52, 53). The final glomerular rich sediment was suspended in equal volume of PBS, ultrasonicated for five minutes and stored at -70°C. Methylene blue staining confirmed the disruption of the

Fig. 52. A smear from supernatant, showing only cellular debris without any glomeruli. Methylene blue x 200.

Fig. 53. A smear from sediment stained with methylene blue showing large numbers of intact glomeruli. Methylene blue x 200.



glomeruli and no whole glomerular tufts were found.

Preparation of NTabs:

Twelve young adult rabbits were inoculated subcutaneously in the dorsal thoracic region with 2 ml of ultrasonicated (10 mg wet weight/ml) GBM antigen in PBS with a similar volume of Freunds complete adjuvant (Miles Research Products Division, Elkjart, Indiana). After a period of four weeks, a further injection of 20 mg GBM antigen was given subcutaneously without adjuvant; the rabbits were exsanguinated 10 - 14 days later. The sera were pooled and stored in sterile vials at -20°C until used. Prior to use the serum was decomplemented at 56°C for 30 minutes.

The specificity of the nephrotoxic serum for basement membranes was tested by an indirect immunofluorescence test. Cryostat sections of normal dog kidney were exposed to anti-GBM serum for 30 minutes at room temperature in a moist chamber. After washing in PBS, the sections were stained with sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate. The specific sharp linear fluorescence of the GBM and tubular basement membrane showed that the serum did not contain antibodies directed against other areas of renal tissue such as tubular epithelium.

Experimental protocol:

Thirty-one 16 week old Collie-cross dogs weighing six to 10 kg were inoculated intravenously with 2 ml of nephrotoxic serum per kg body weight. Animals were killed at each of the intervals summarized in Table 5.1. For purposes of control nine 16 week old dogs were given normal rabbit serum (2ml/kg) intravenously and killed at three (two dogs), six (two dogs), 10 (two dogs), 14 (two dogs) and 20 (one dog)

days after inoculation.

The procedures adopted for light microscopy, TEM and SEM are as described in the general section on materials and methods in Chapter 2.

One kidney from each sampled time interval was used for arterial casting, again as described in Chapter 2.

Results:

Histological observations : These are summarised in Table 5.1.

30 minutes:

No changes were observed in the glomeruli at this time.

One - six hours:

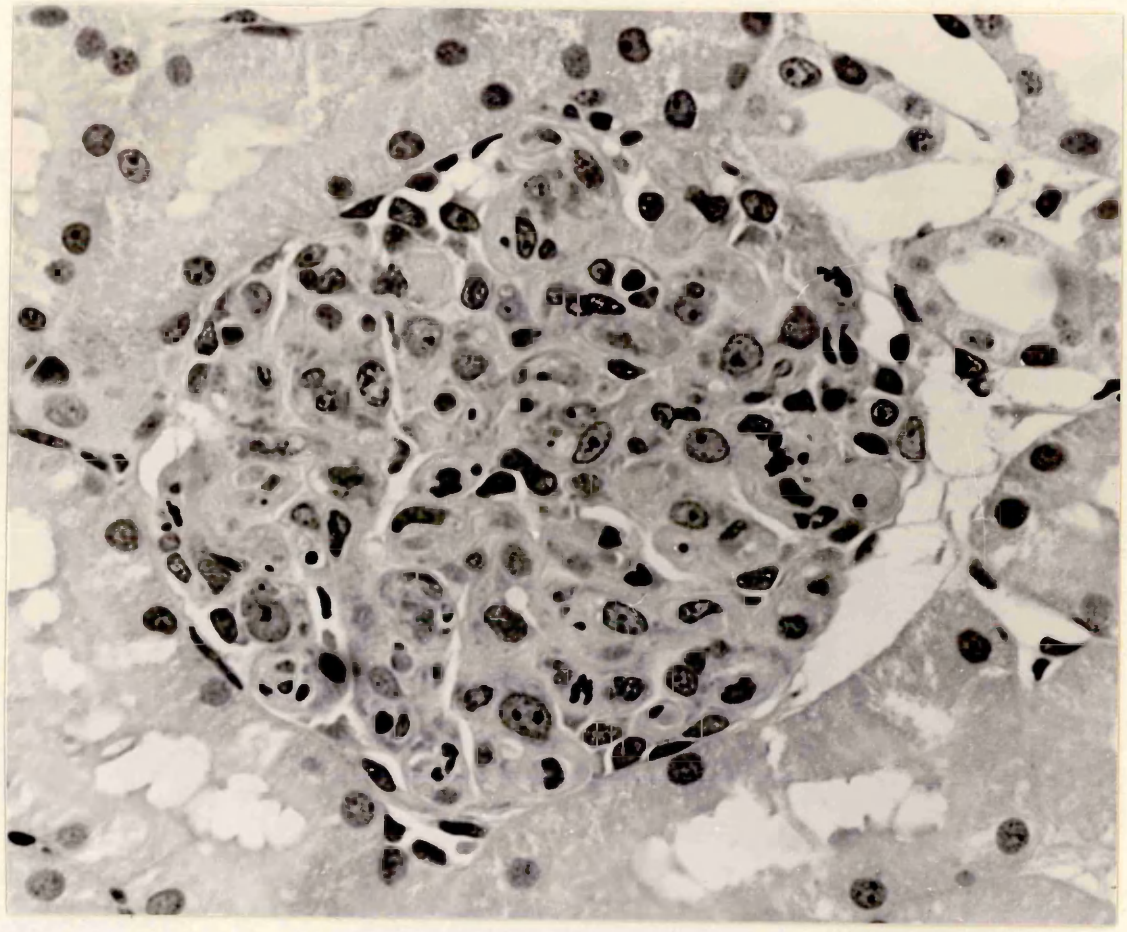
The first noticeable change at one hour after administration of NTabs was the appearance of small numbers of neutrophils in many of the glomerular capillaries. Although neutrophils are often seen in the glomeruli of normal dogs there are seldom more than two-three per glomerulus and, in any one histological section, most glomeruli are free of neutrophils. In addition, by four hours, there was distinct swelling of all three types of glomerular cells with occlusion of many of the capillaries (Fig. 54).

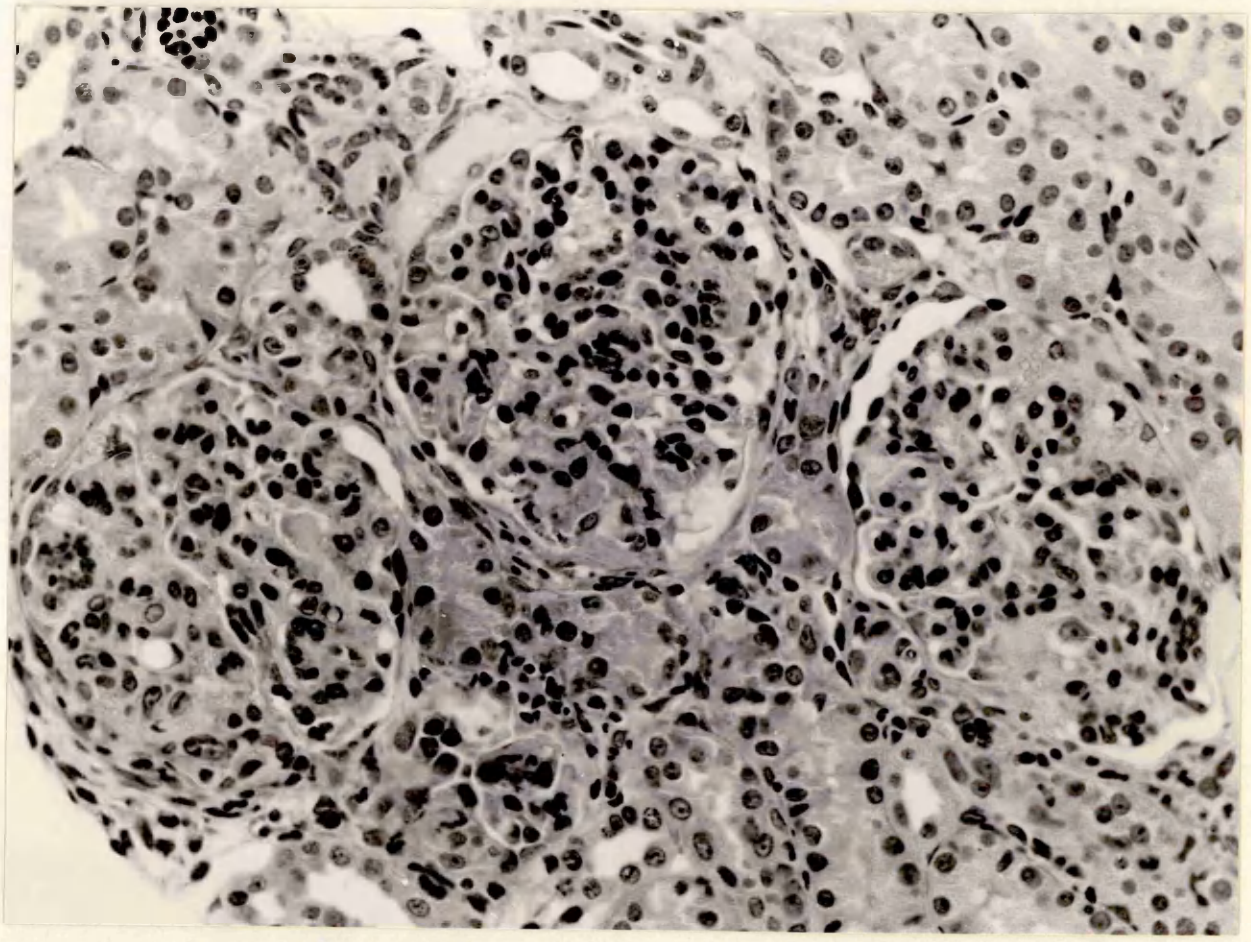
24 - 48 hours:

By 24 hours neutrophilic infiltration of glomeruli had increased and, in addition to swelling of the tuft and occlusion of capillaries, there was now evidence of mesangial hypercellularity (Fig. 55). One dog killed at 48 hours (Dog No. W943) showed segmental capillary thrombosis and necrosis involving many

Fig. 54. NTN: four hours. A glomerulus showing distinct swelling of epithelial, endothelial and mesangial cells, and occlusion of most of the capillaries. H & E x 300.

Fig. 55. NTN: 24 hours. Three glomeruli showing occlusion of capillaries and mesangial hypercellularity. H & E x 250.





glomeruli (Fig. 56) in this case, some glomeruli showed adhesions between the tuft and Bowman's capsule. In all five dogs killed at this time many proteinaceous casts could be seen in the tubules.

Three days:

The structural changes in the glomeruli had now intensified with striking tuft swelling, capillary thrombosis and mesangial hypercellularity. Deposits of fibrin in the capillaries and in the urinary space were commonly observed (Fig. 57) and protein casts in the tubules were plentiful.

Five - seven days:

At this stage, very severe tuft swelling with occlusion and thrombosis of the capillaries and diffuse mesangial hypercellularity were observed (Fig. 58). Segmental necrosis of glomeruli was a common feature and many capsular adhesions were found in association with fibrinous deposits in the urinary space. Many proteinaceous casts were present in the renal tubules.

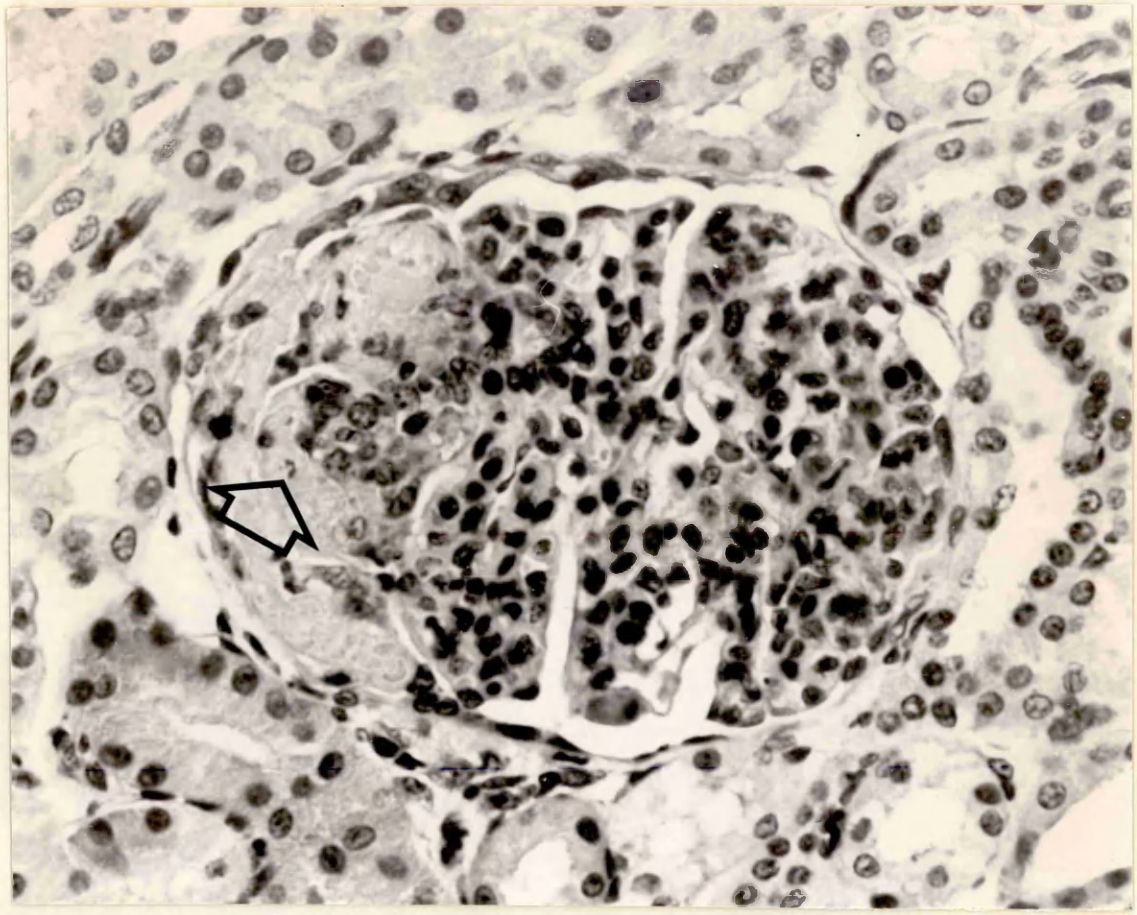
At day five, evidence of glomerular scarring was minimal in the two dogs examined at this time while, by day seven (Dog No. W928), 15% of glomeruli showed $<$ 50% scarring, a further 6% of glomeruli were $>$ 50% scarred and 3% of glomeruli were completely obsolescent.

14 - 21 days:

During this period, mesangial hypercellularity with many capsular adhesions were the main findings (Fig. 59). Apart from Dog No. W927, killed at 21 days, the amount of fibrin deposited in the loops and urinary space had, however, diminished. There was also a

Fig. 56. NTN: 48 hours. Glomerulus from Dog No. (W943) showing segmental capillary thrombosis and necrosis (arrow). H & E x 300.

Fig. 57. NTN: three days. The swollen glomerulus on the left shows fibrinous exudation into the urinary space, with segmental thrombosis and necrosis, the other glomerulus is relatively normal. H & E x 200.



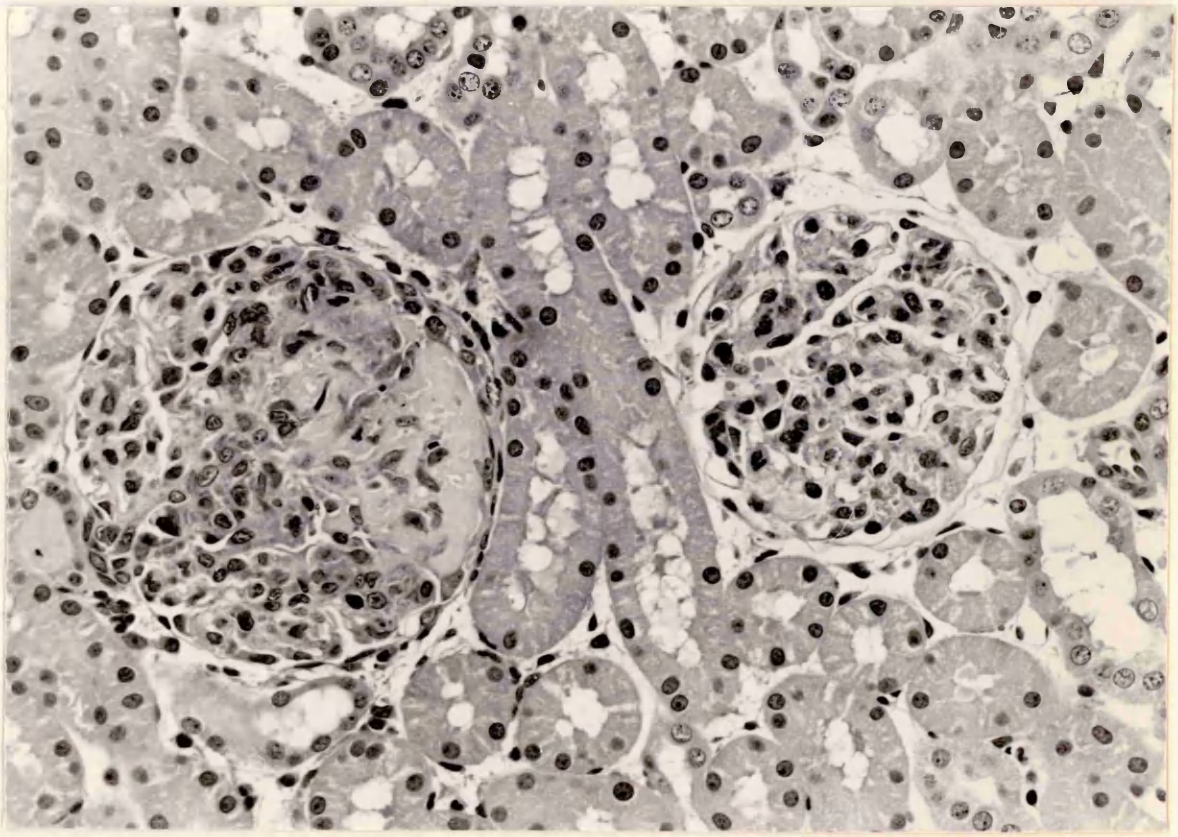
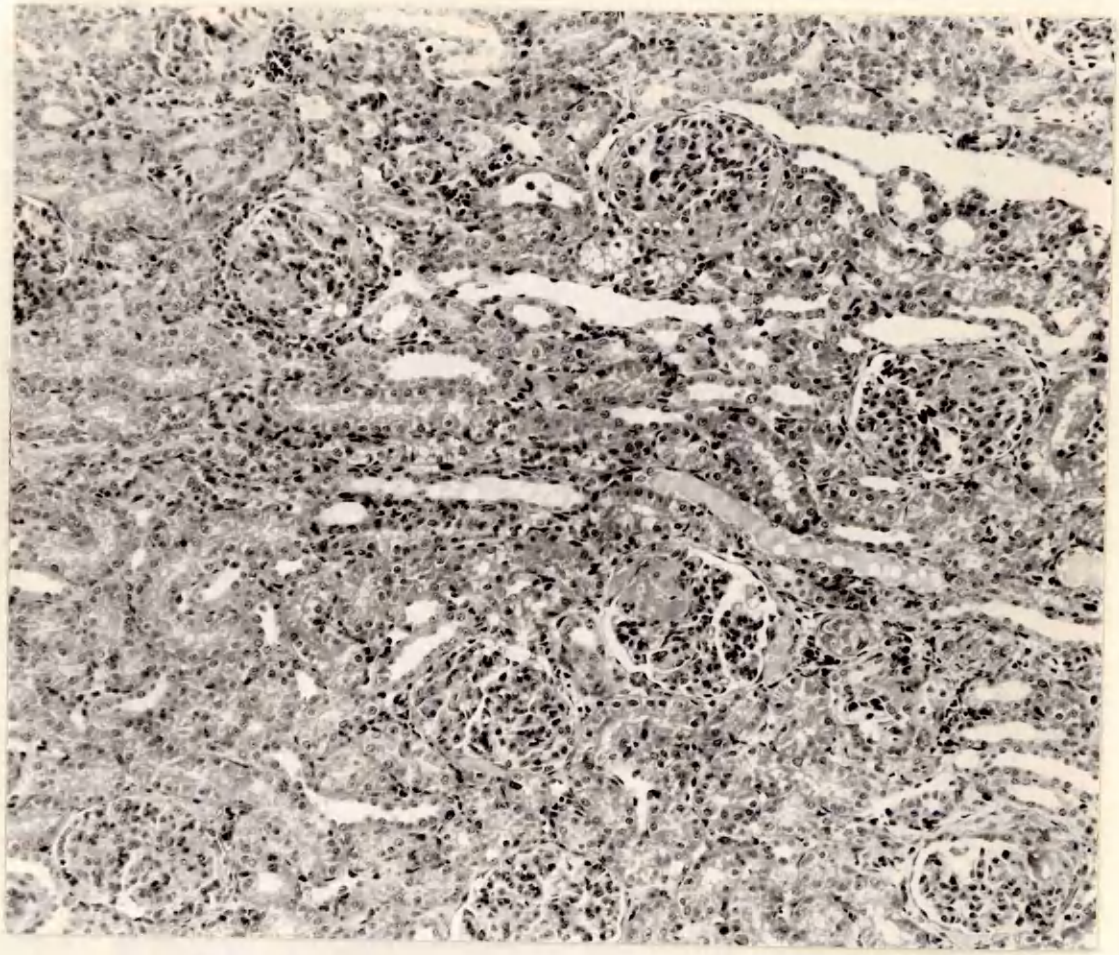
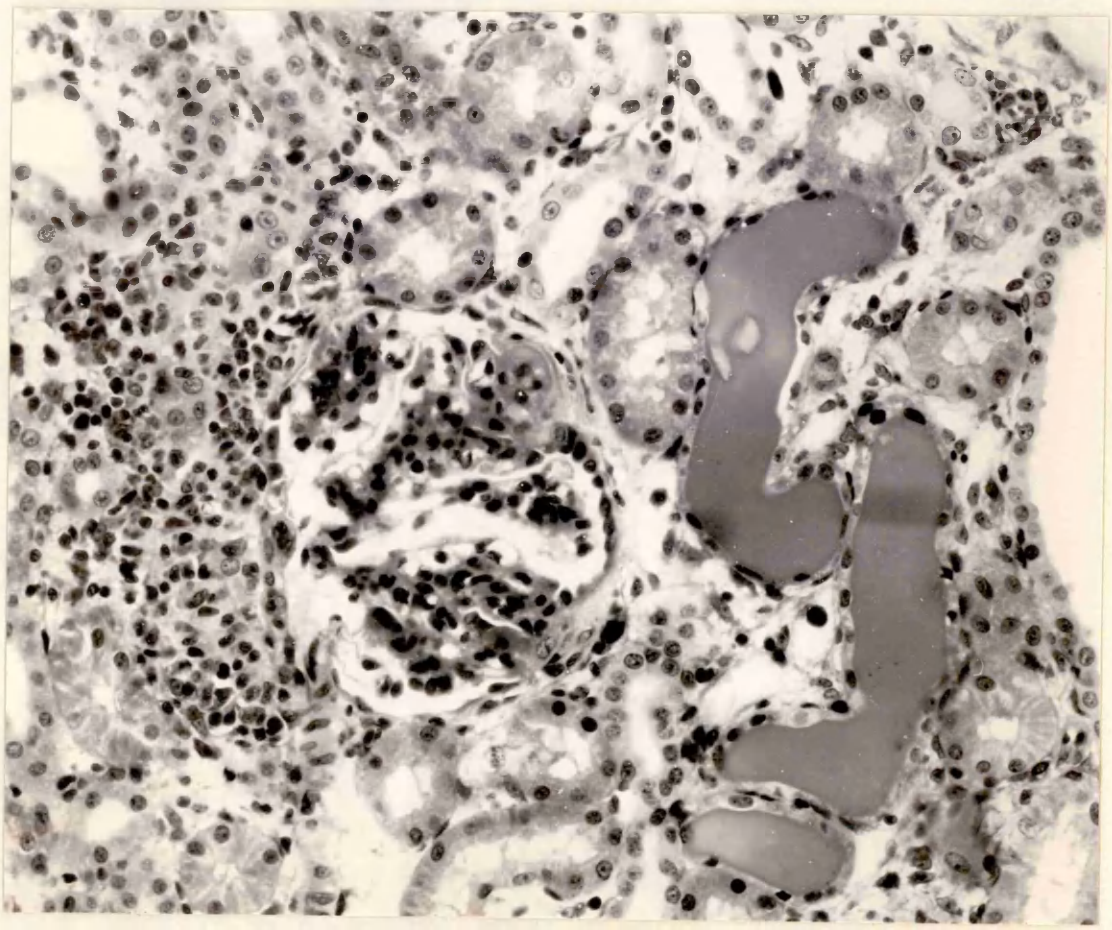


Fig. 58. NTN: five days. A section of cortical region showing severe tuft swelling with occlusion and thrombosis of the capillaries and diffuse mesangial hypercellularity. H & E x 80.

Fig. 59. NTN: 14 days. A glomerulus shows capsular adhesions and hypercellularity. Note the proteinaceous casts in the renal tubules. H & E x 200.





varying degree of glomerular scarring ranging from 5% of glomeruli showing > 50% scarring (Dog No. W967) to 11% showing > 50% scarring (Dog No. W927); in the latter case, as many as 13% of glomeruli were completely obsolescent. Many protein casts were still to be found in the tubules. In addition, Dog No. W927 killed at 21 days showed a moderately severe diffuse tubulo-interstitial nephropathy characterised by necrosis of proximal tubular epithelium and infiltration by neutrophils, lymphocytes, plasma cells and macrophages.

30 - 40 days:

Segmental areas of capillary thrombosis with exudation of fibrin into the urinary space was still present in many glomeruli; likewise patchy mesangial hypercellularity and capsular adhesions were also observed. The percentage of glomeruli showing obsolescence was higher than in dogs killed previously (Fig. 60) (20% in Dog No. W974). Nevertheless, many glomeruli were normal in appearance.

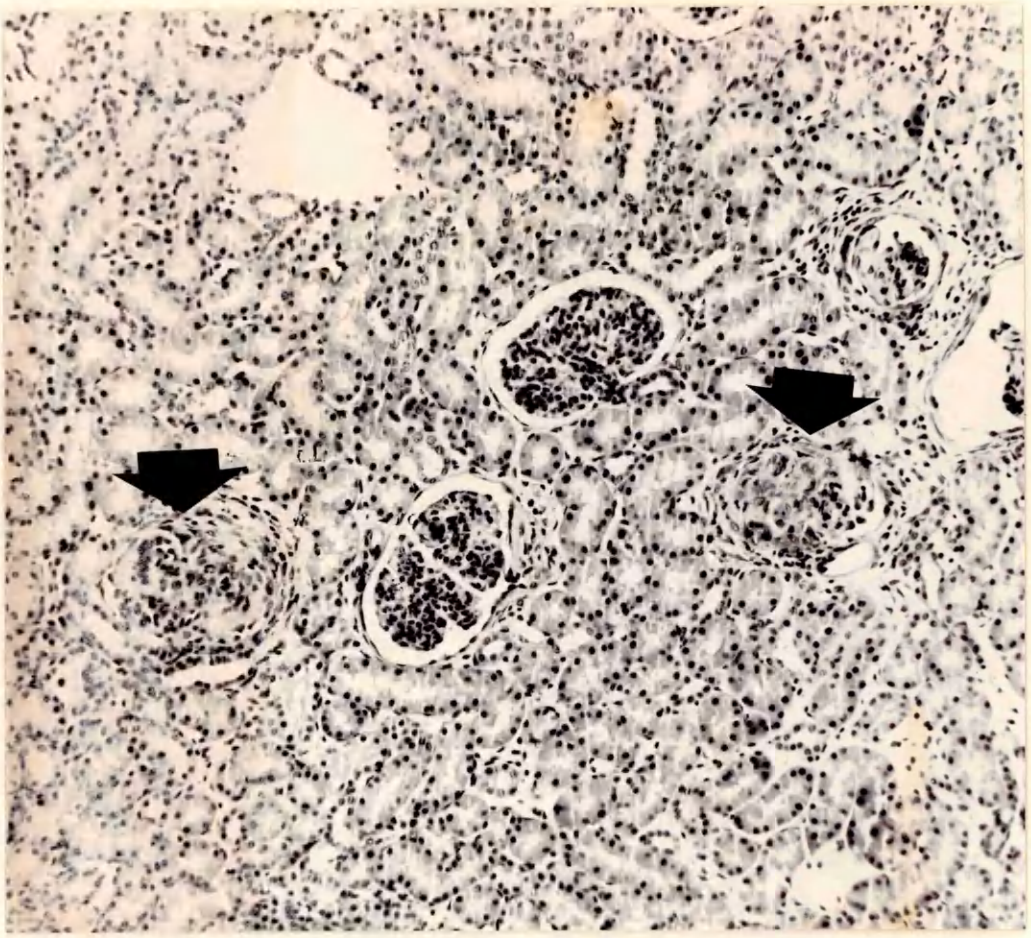
60 days:

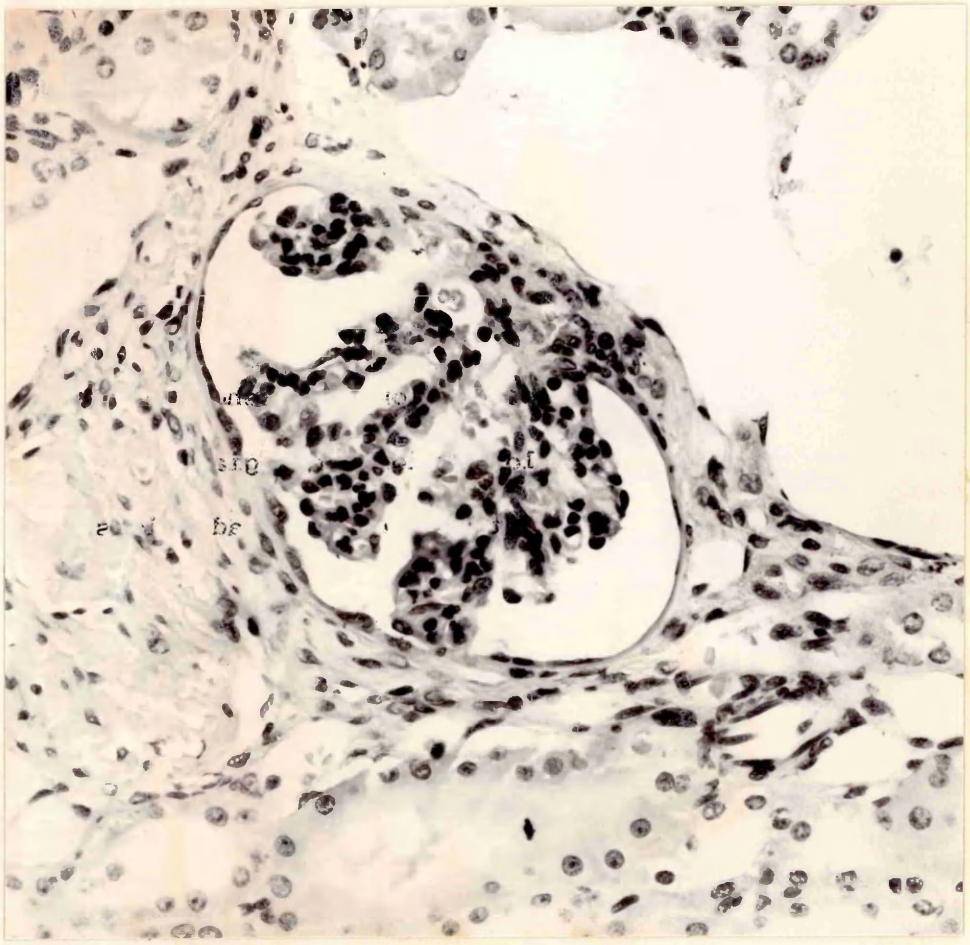
Although most glomeruli had returned to normal, some glomeruli still showed segmental areas of capillary thrombosis. Similarly, mesangial hypercellularity and capsular adhesions were still present in some instances (Fig. 61) and some glomeruli showed distinct lobulation.

The percentage of glomeruli showing < 50% scarring was 8% (Dog No. W975). In Dog No. W970, however, all glomeruli were normal.

Fig. 60. NTN: 30 days. Two glomeruli are hypercellular, while other two are obsolescent (arrows). H & E x 100.

Fig. 61. NTN: 60 days. A glomerulus showing accentuated lobulation, mesangial hypercellularity and capsular adhesions. H & E x 250.





80 days:

Most glomeruli were now within normal range and capillary thrombosis could not be found at this stage. Mesangial hypercellularity was segmental and confined to only a few glomeruli (Fig. 62). The percentage of glomeruli showing $>$ 50% scarring was 3% and only 1% of glomeruli were completely obsolescent.

Transmission electron microscopy:

In the two dogs examined 30 minutes after administration of NTabs, the glomeruli were ultrastructurally normal.

One - 24 hours:

As early as one hour neutrophils appeared in the lumen of the glomerular capillaries and in some instances were in direct contact with the GBM (Fig. 63), having displaced the overlying endothelium. Alterations on the epithelial aspect of the GBM were also recognised; here patchy fusion of the foot processes was observed although most visceral epithelial cells appeared normal.

Two - five days:

At this time, the most prominent features were swelling and detachment of the endothelial lining of the capillaries (Fig. 64), together with mesangial hypercellularity and increase in mesangial matrix (Fig. 65). Pseudopodia of mesangial cell cytoplasm were often observed bulging into the axial regions of the loops (Fig. 66) and in so doing displacing the endothelial cells.

In every case killed at this time, deposits of fibrin could always be found in some capillary loops (Fig. 67) and, where the lumen

Fig. 62. NTN: 80 days. Note the prominent segmental mesangial hypercellularity. One μm plastic section. Toluidine blue x 250.

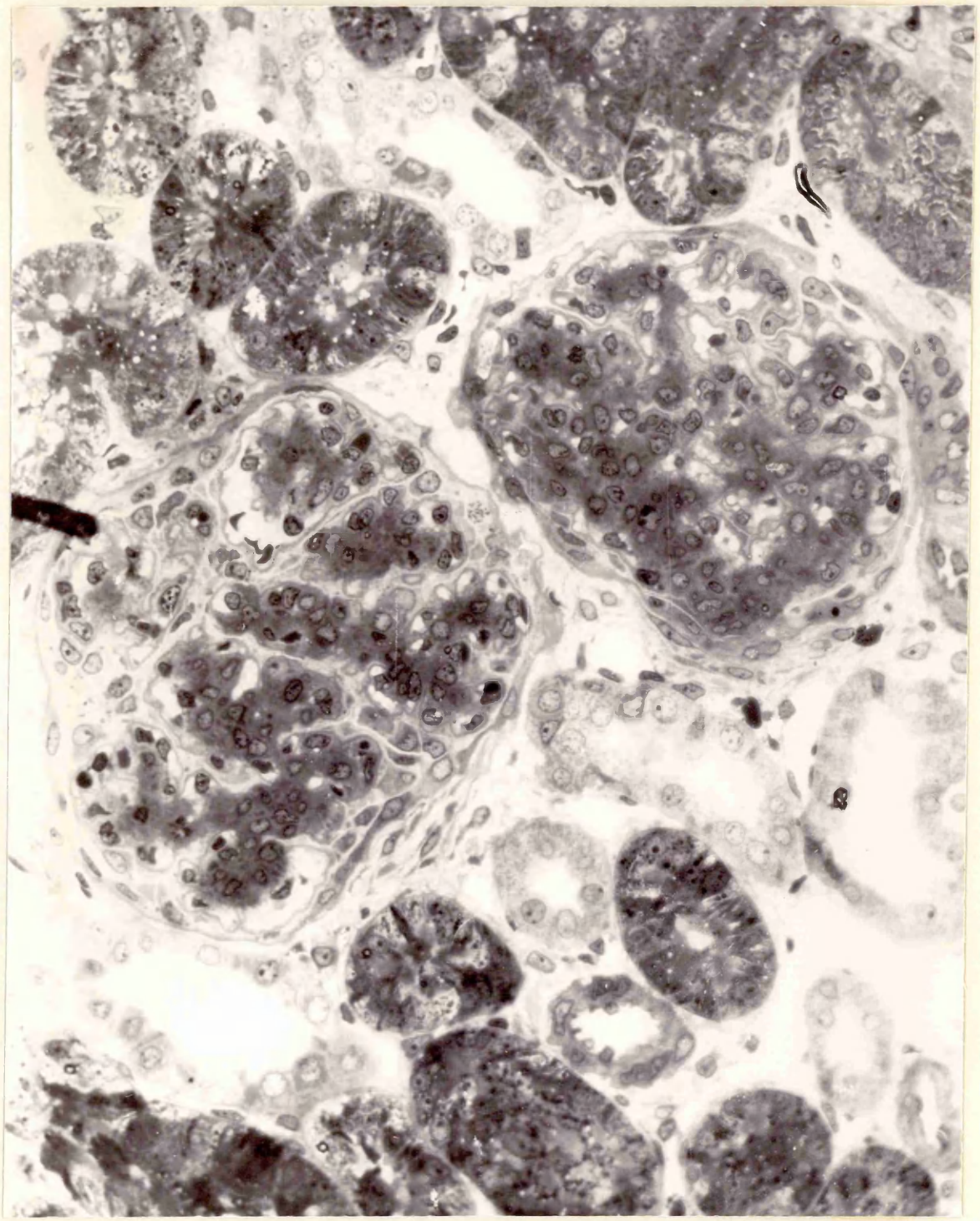
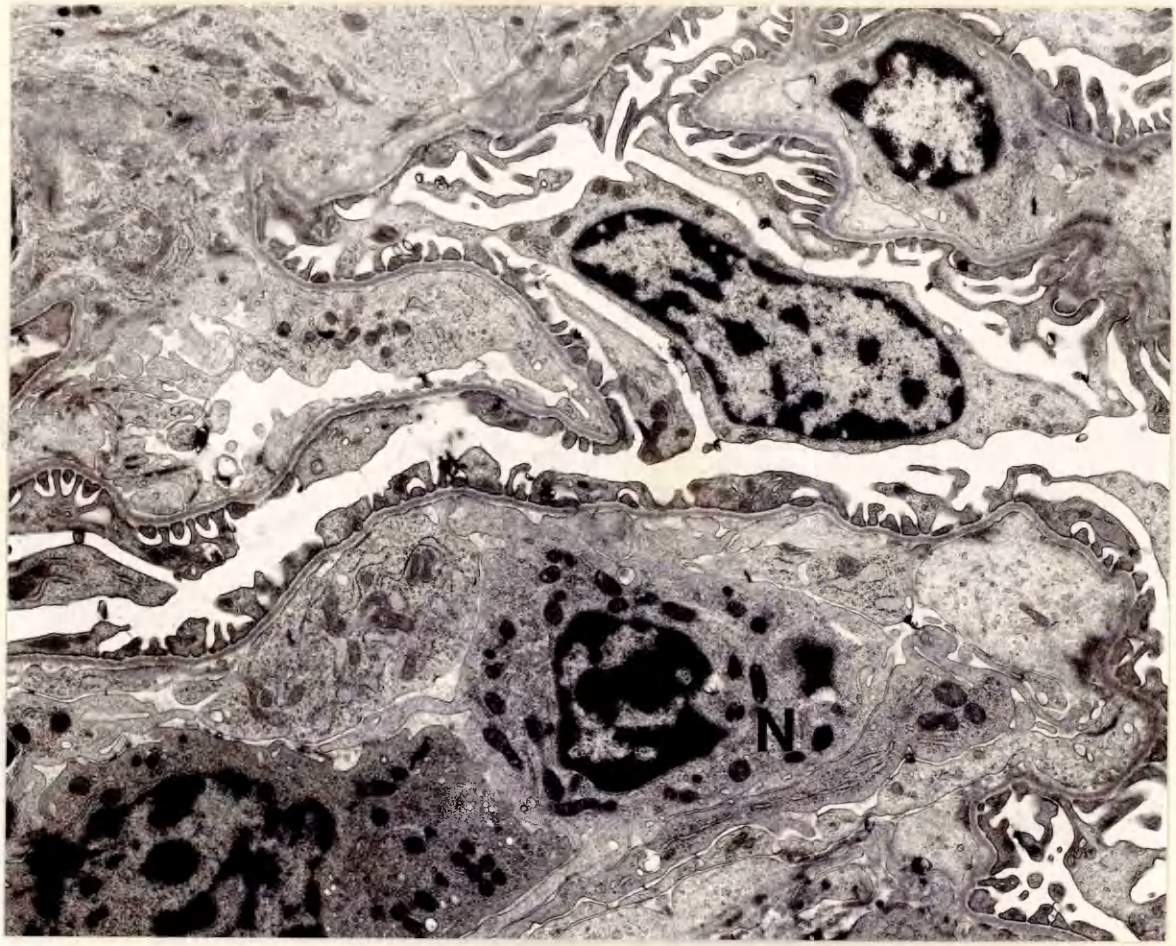


Fig. 63. NTN: one hour. Neutrophil (N) within the capillary lumen; note the direct contact of its pseudopodia with the GBM. TEM x 8,000.

Fig. 64. NTN: two days. A glomerulus showing part of a mononuclear cell in direct contact with denuded GBM. TEM x 9,000.


N

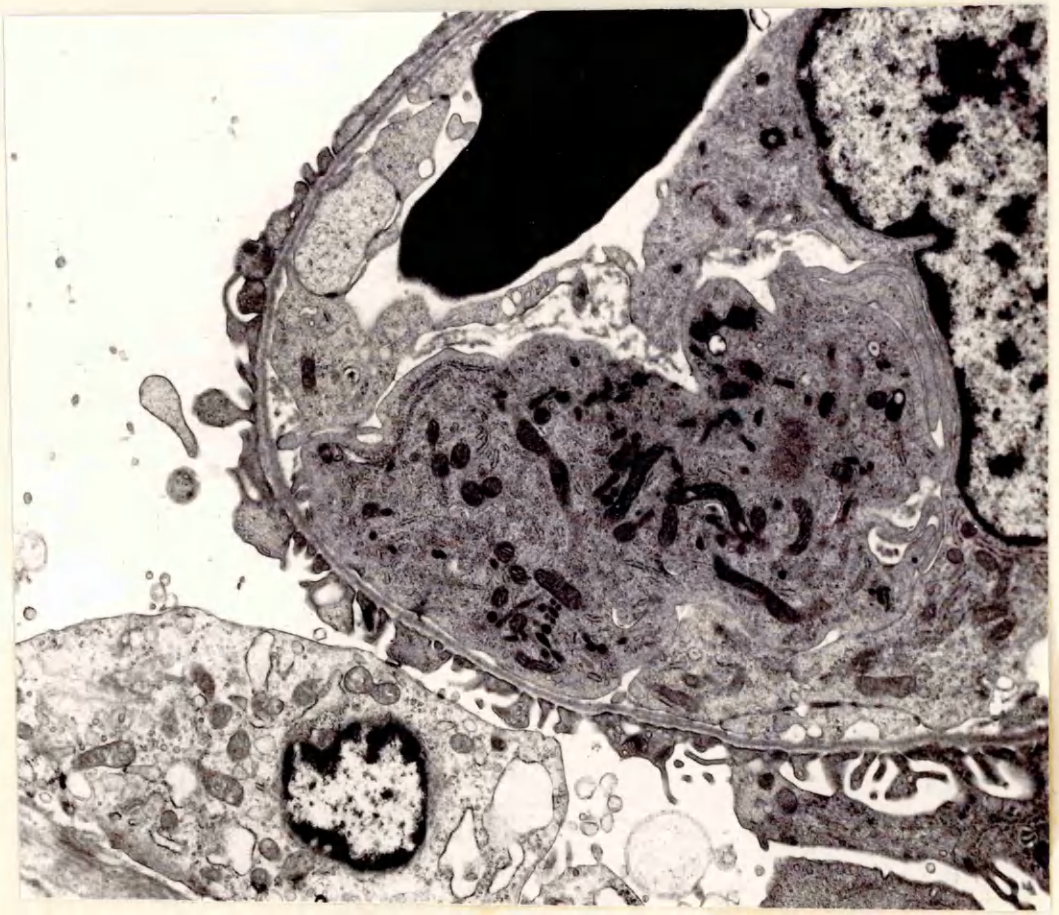
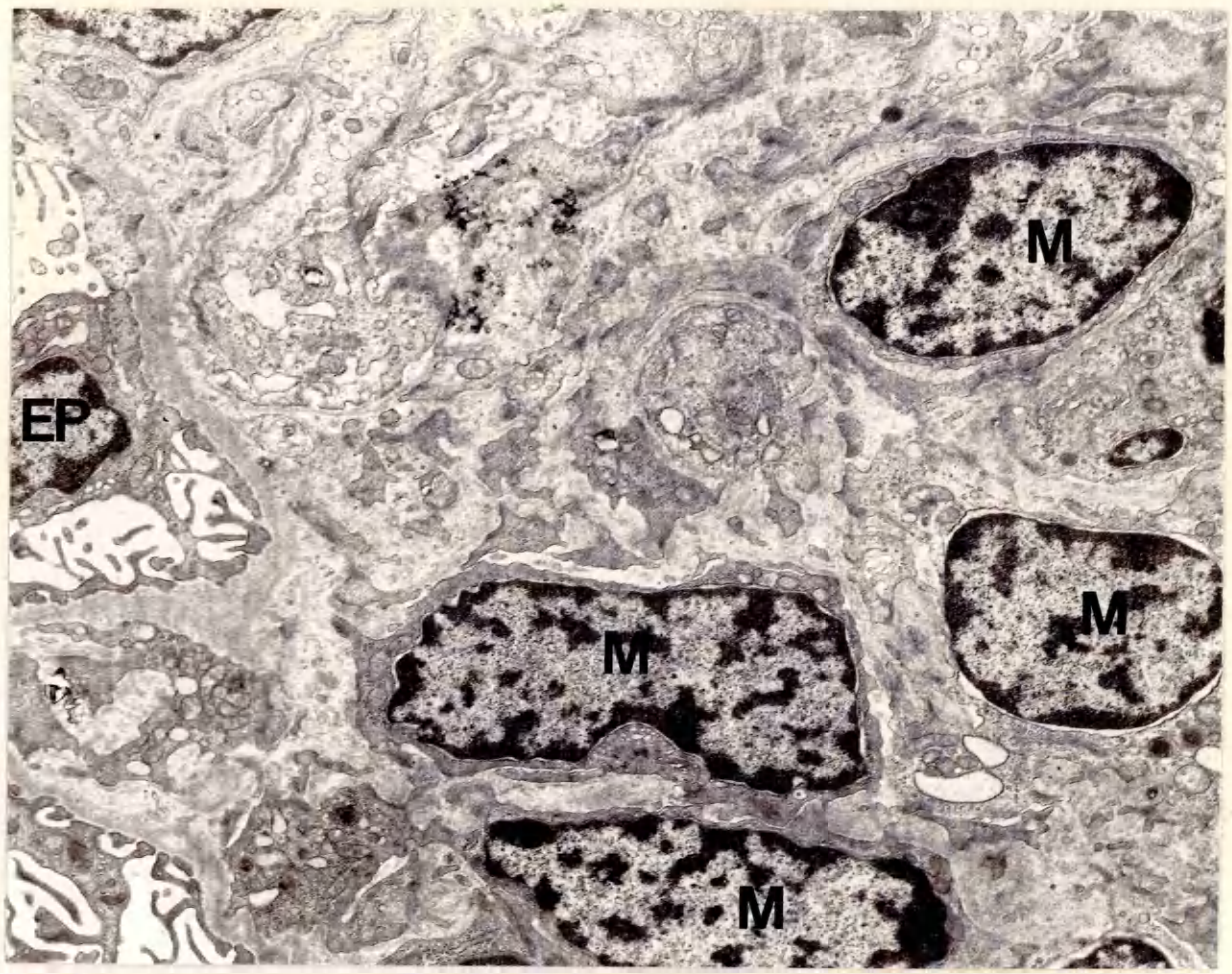
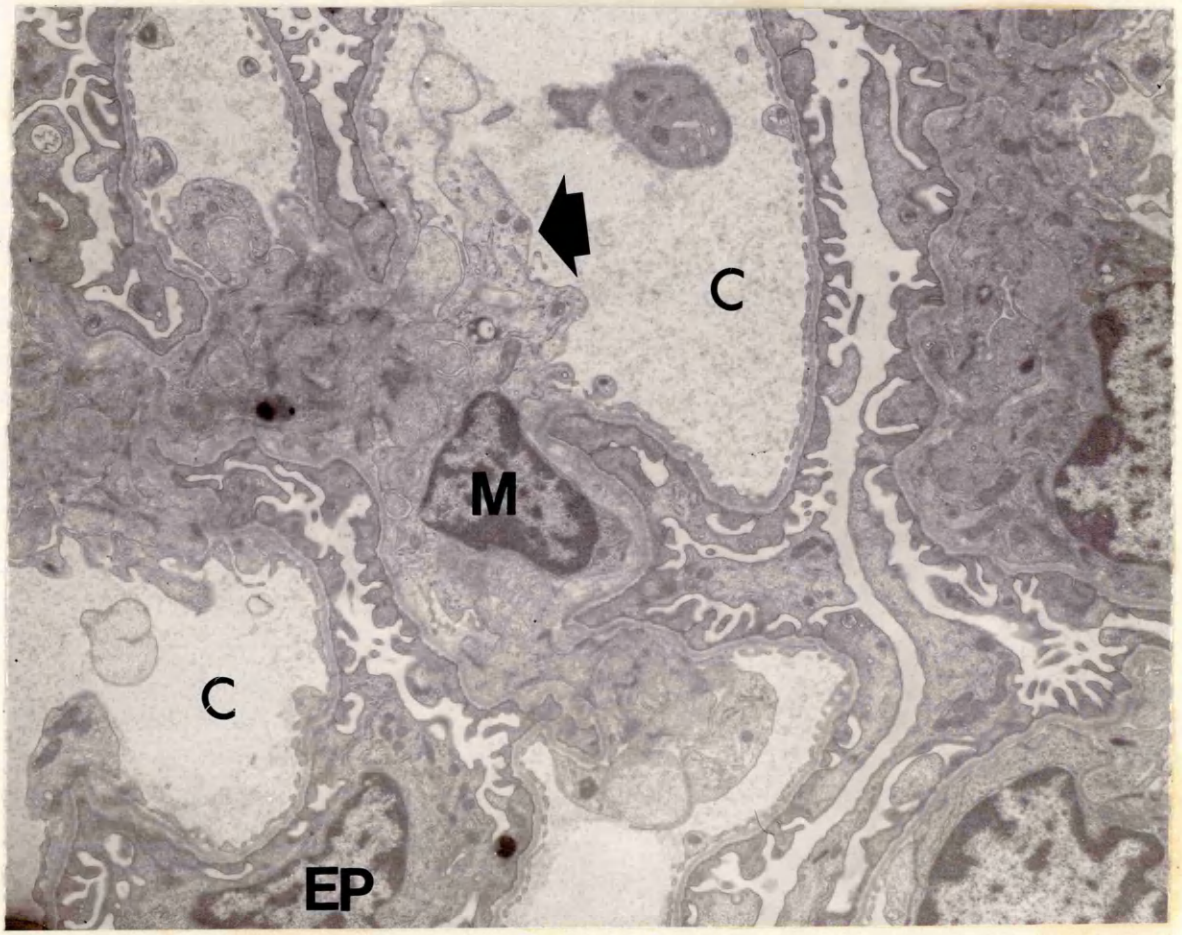


Fig. 65. NTN: five days. A glomerulus showing mesangial hypercellularity. Mesangial cells (M), visceral epithelial cell (EP). TEM x 9,500.

Fig. 66. NTN: two days. A glomerulus showing mesangial cell cytoplasmic pseudopodia bulging into the axial region of the loops (arrow). Capillary lumen (C), mesangial cell (M), visceral epithelial cell (EP). TEM x 8,000.


M
EP
M
M
M


C
M
C
EP

of the capillary was thrombosed with loss of the endothelium, fibrin could also be seen extending into the urinary space. Fusion of the epithelial cell foot processes was often extensive.

Seven - 15 days:

The above structural changes were now more severe and fibrinous deposits in the loops and the urinary space were common. Furthermore, circulating mononuclear cells could occasionally be seen pushing their way into the mesangium (Fig. 68). By day 15, distinct expansion of the subendothelial space with loose electron-lucent material was observed (Fig. 69). In addition to widespread fusion of the epithelial cell foot processes, many epithelial cells showed extensive surface microvilli.

19 - 21 days:

One dog (Dog No. W966) died 19 days after administration of NTabs and kidney material was not available for ultrastructural examination.

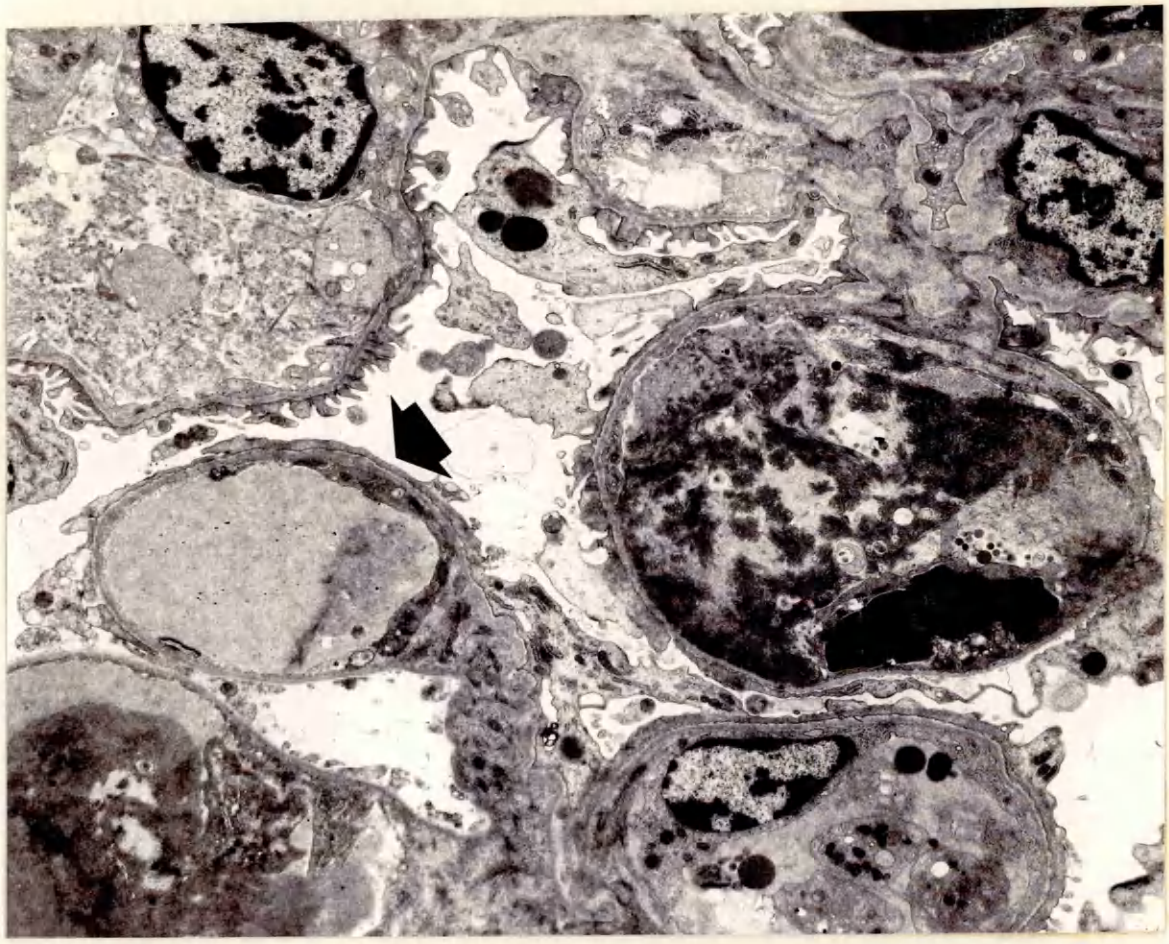
At 21 days, all the above mentioned changes were still evident, but there was more extensive expansion of the subendothelial space. In addition, localised electron dense deposits were occasionally observed in the expanded subendothelial spaces (Fig. 70).

30 - 40 days:

At this stage less fibrinous deposits were to be found in the capillaries and urinary spaces, but occasionally neutrophils could still be observed in direct contact with denuded GBM. Electron dense deposits were also found in expanded subendothelial as well as in

Fig. 67. NTN: five days. Glomerular capillary loop showing thrombosis. Dense fibrinous material (F) is occluding the capillary. The fenestrated endothelial lining has disappeared. There is also marked fusion of foot processes (arrow). TEM x 4,000.

Fig. 68. NTN: seven days. Circulating mononuclear cell (arrow) infiltrating the mesangium (M). The capillary loop contains cytoplasmic debris. TEM x 7,000.



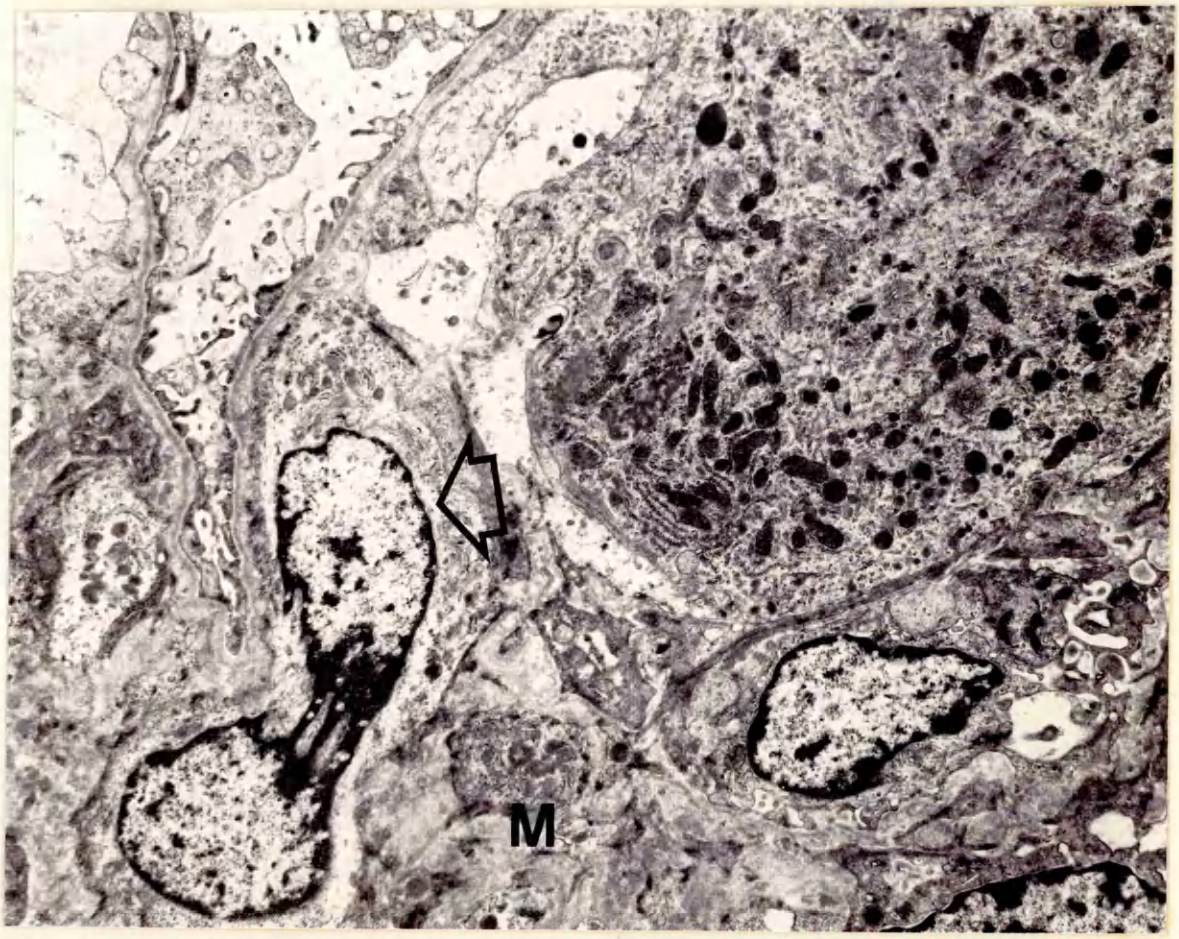
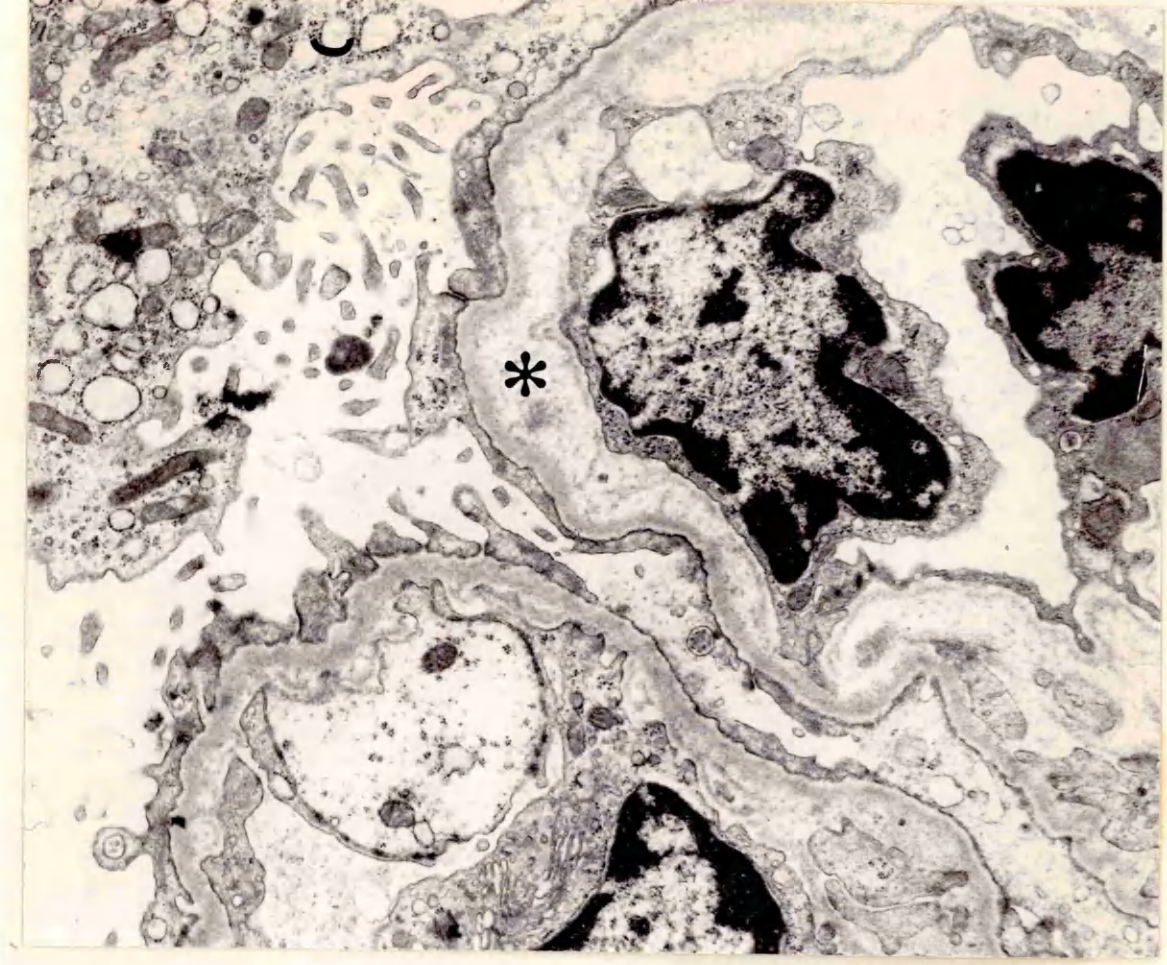
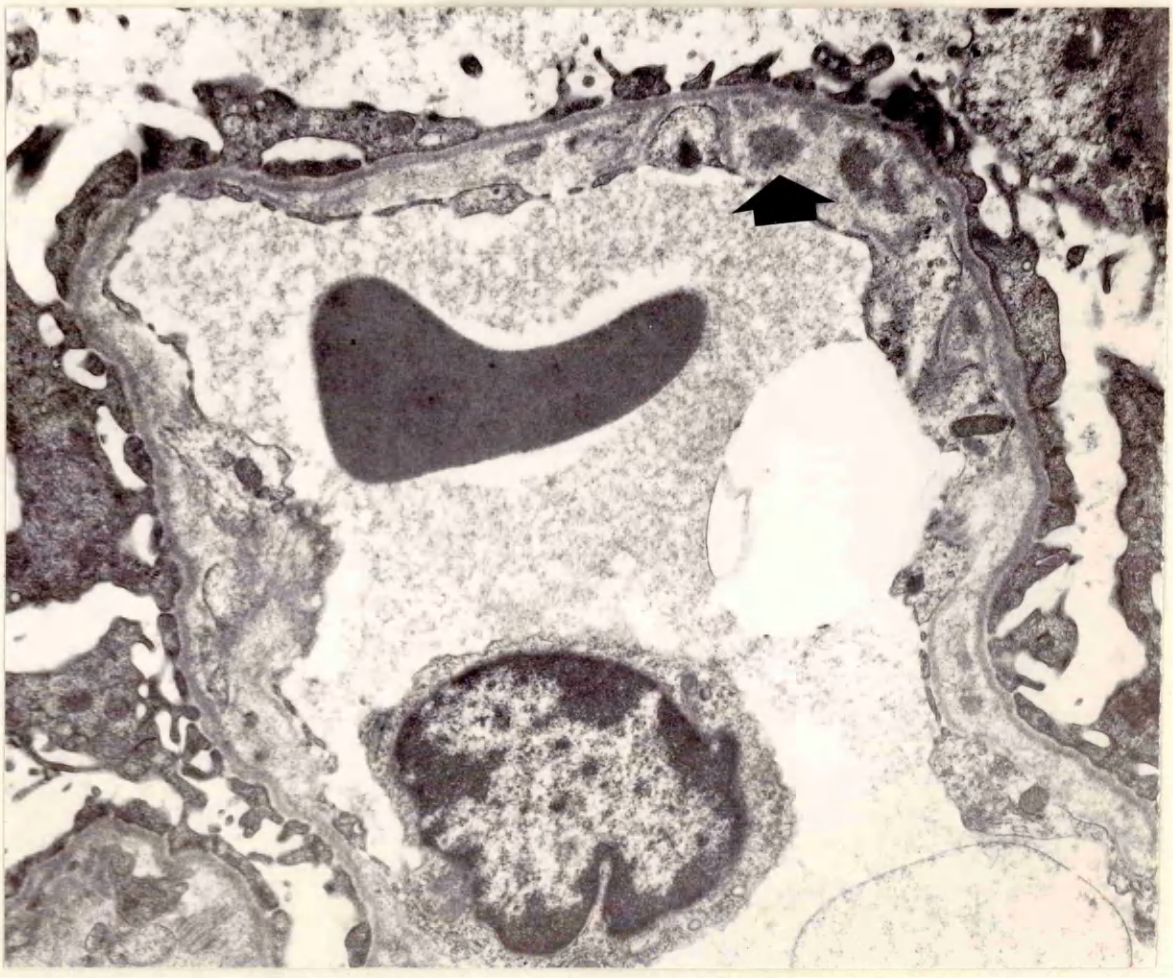

M

Fig. 69. NTN: 15 days. Note the distinct expansion of the subendothelial space with loose electron-lucent material (asterisk). TEM x 6,800.

Fig. 70. NTN: 21 days. A glomerular capillary loop, showing expansion of subendothelial space with localized electron dense deposits (arrow). TEM x 6,800.





mesangial areas. Epithelial cells still showed considerable activity with many surface microvillous projections although, by 40 days, the orderly arrangement of epithelial foot process was largely restored. The most striking persisting alterations at this time, however, were the patchy expansions of the subendothelial space still containing loose electron lucent material, mesangial hypercellularity and increase in mesangial matrix.

60 - 80 days:

Although most of the glomerular capillaries were now considered to be within normal range, some loops still showed detectable patchy expansion of the subendothelial space (Fig. 71). Hypercellularity of the mesangium and increase in mesangial matrix still persisted in some glomeruli. No electron dense deposits were found at this time.

Scanning electron microscopy:

30 - 60 minutes:

Very few alterations in the surface topography of the glomerular capillaries were evident at this early stage of nephrotoxic nephritis. In a few glomeruli, however, the primary cytoplasmic processes of the podocytes appeared thicker and flatter and had a roughened surface; there were also areas where the normal orderly arrangement of the foot processes was lost (Fig. 72). Some visceral epithelial cells appeared to have more surface microvilli than normal.

Examination of glomerular casts, moreover, showed the leakage of cast material into Bowman's space had occurred in approximately 10% of glomeruli (Fig. 73). Otherwise, the outline of the glomerular

Fig. 71. NTN: 80 days. Focal expansion of the subendothelial space can still be detected (arrow). TEM x 5,500.

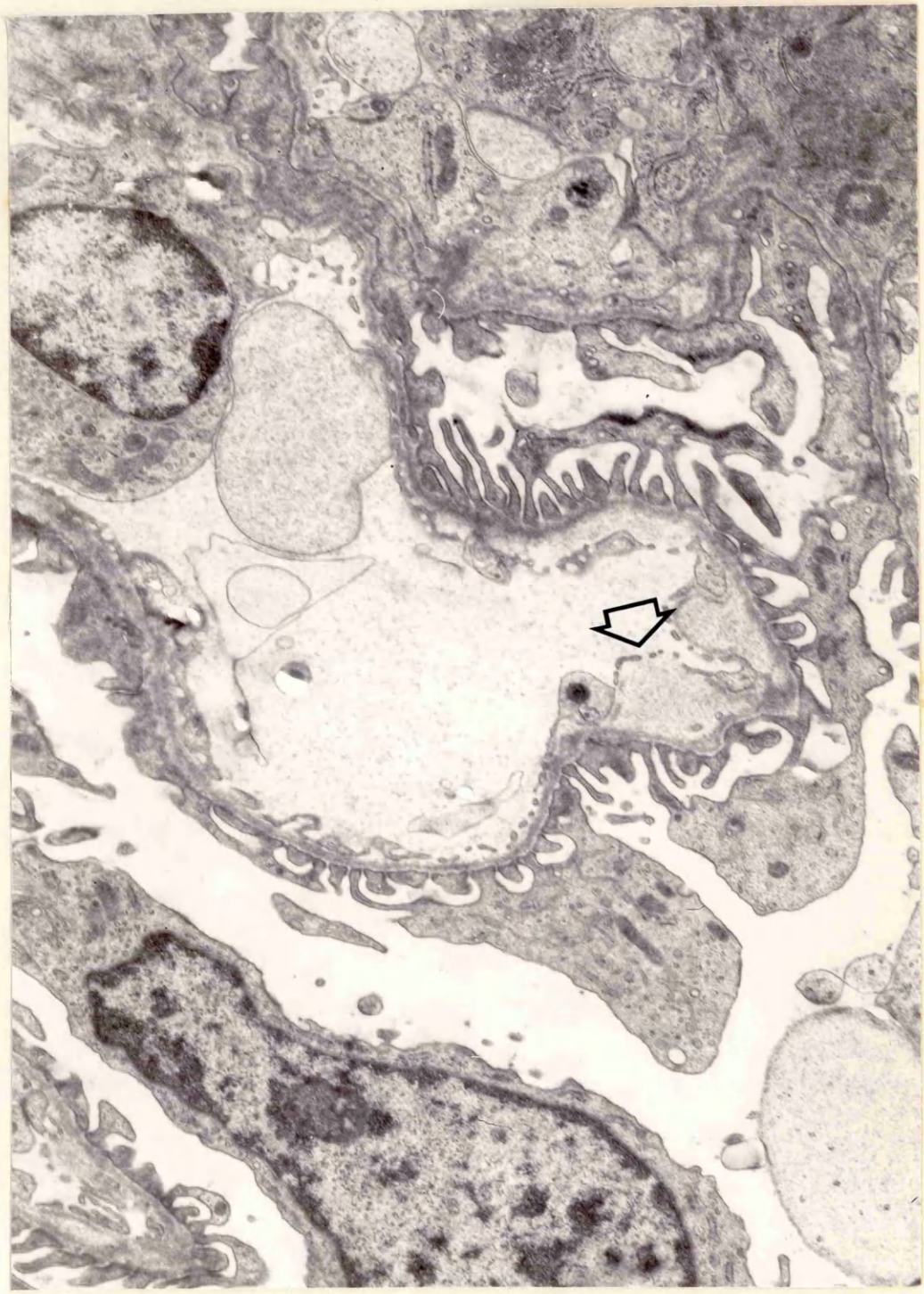
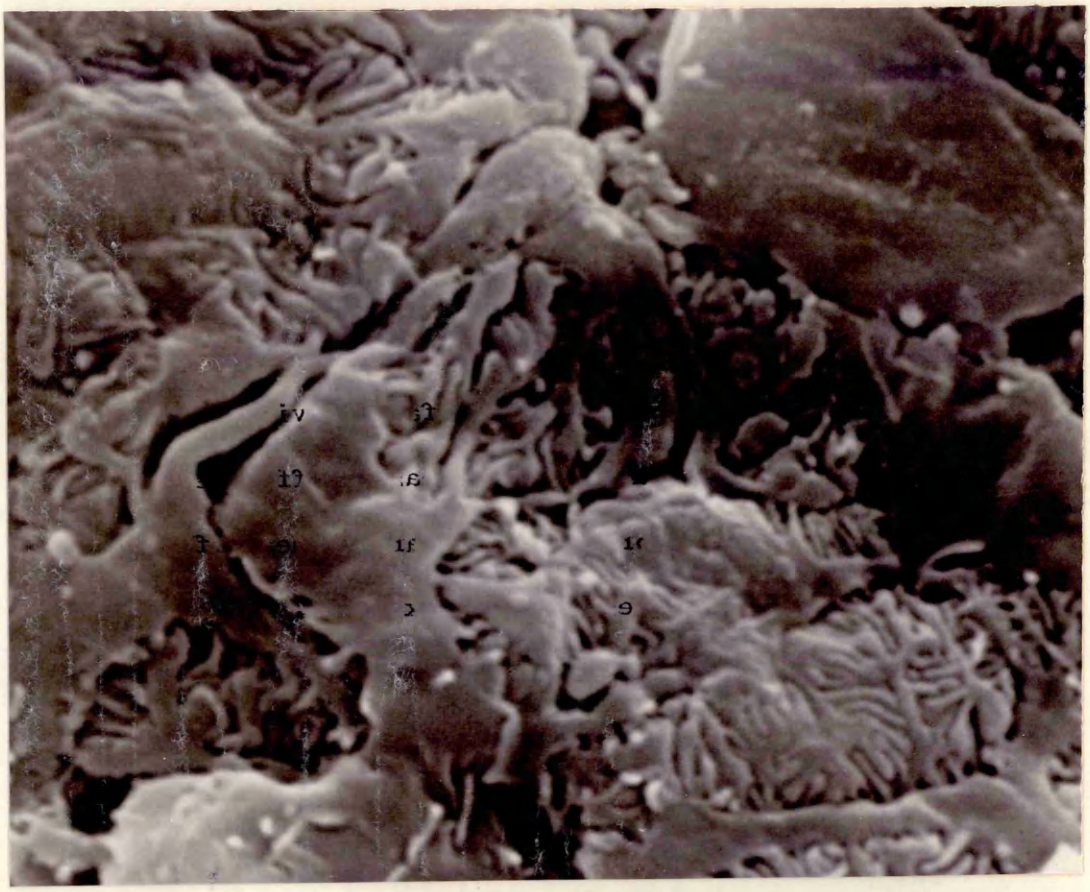
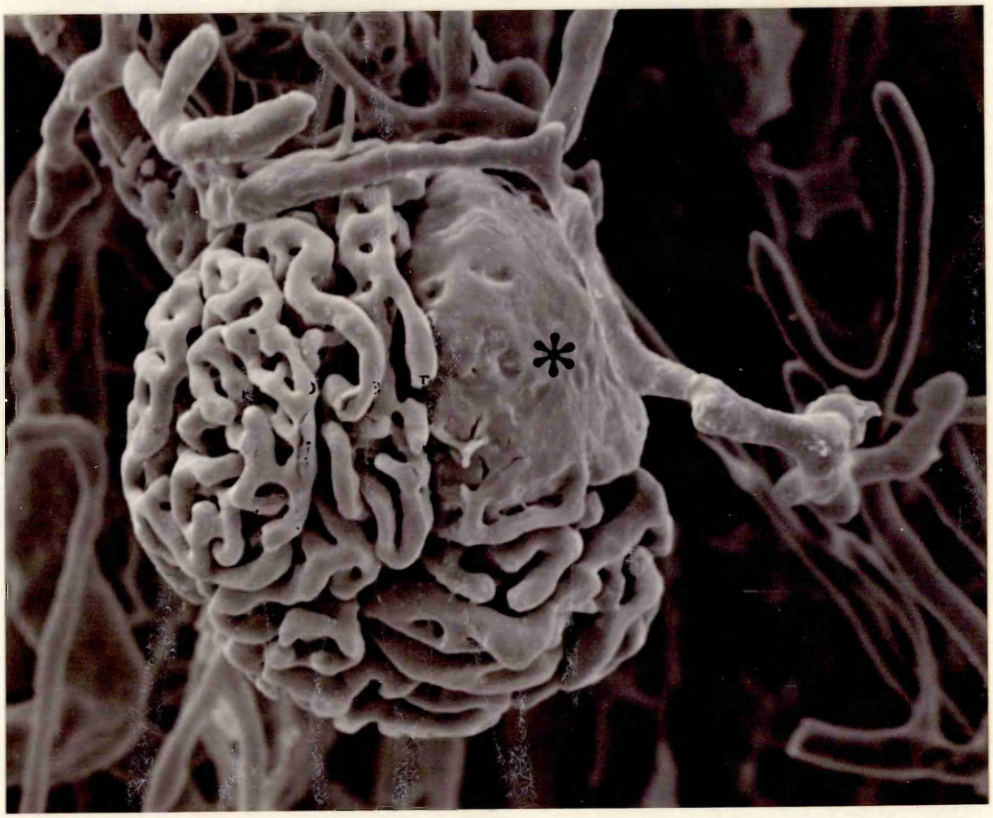


Fig. 72. NTN: one hour. Surface of visceral epithelium showing areas of effacement of the normal orderly arrangement of foot processes. SEM x 2,500.

Fig. 73. NTN: one hour. Tensol cast, showing leakage of cast material into urinary space (asterisk). SEM x 320.





capillaries appeared normal.

Four - six hours:

At this time, the cell bodies of the visceral epithelial cells were swollen and the primary and secondary cytoplasmic processes were thickened and flattened, with a roughened, pitted surface. Obliteration of the foot processes was now more extensive (Fig. 74).

Examination of renal casts again showed leakage of cast material in approximately 10% of glomeruli. Furthermore, the glomerular capillaries now had a roughened appearance and there appeared to be constrictions and dilations of the vessels in comparison to the uniform diameter of the glomerular capillaries which was characteristic of control animals (Fig. 75).

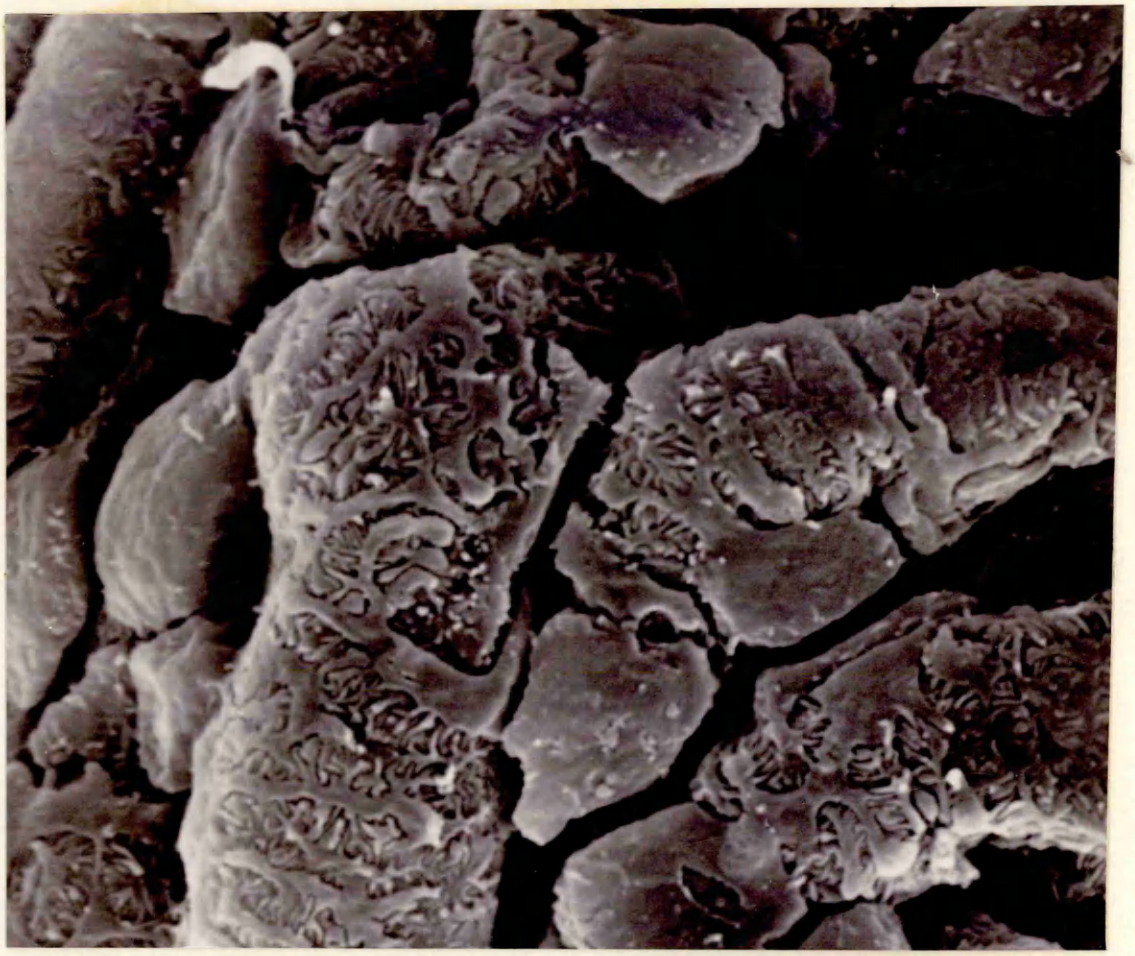
24 - 48 hours:

There was now widespread effacement of the epithelial cell foot processes giving the surface of the capillaries a roughened and wrinkled appearance (Fig. 76). Erythrocytes and strands of fibrin-like material were found in the urinary spaces. However, some glomerular capillaries were less severely affected with only patchy fusion of the foot processes.

In renal casts, the orderly arrangement of many glomerular capillaries was altered and many seemed to have disintegrated altogether. Up to 50% of glomeruli showed leakage of cast material (Fig. 77) and, in addition, many capillaries showed alteration in their diameter with localised constrictions and dilatations.

Fig. 74. NTN: four hours. Visceral epithelium showing thickened flattened primary and secondary cytoplasmic processes and obliteration of foot processes.
SEM x 2,500.

Fig. 75. NTN: four hours. Casts of glomeruli showing roughened capillary surfaces.
SEM x 320.



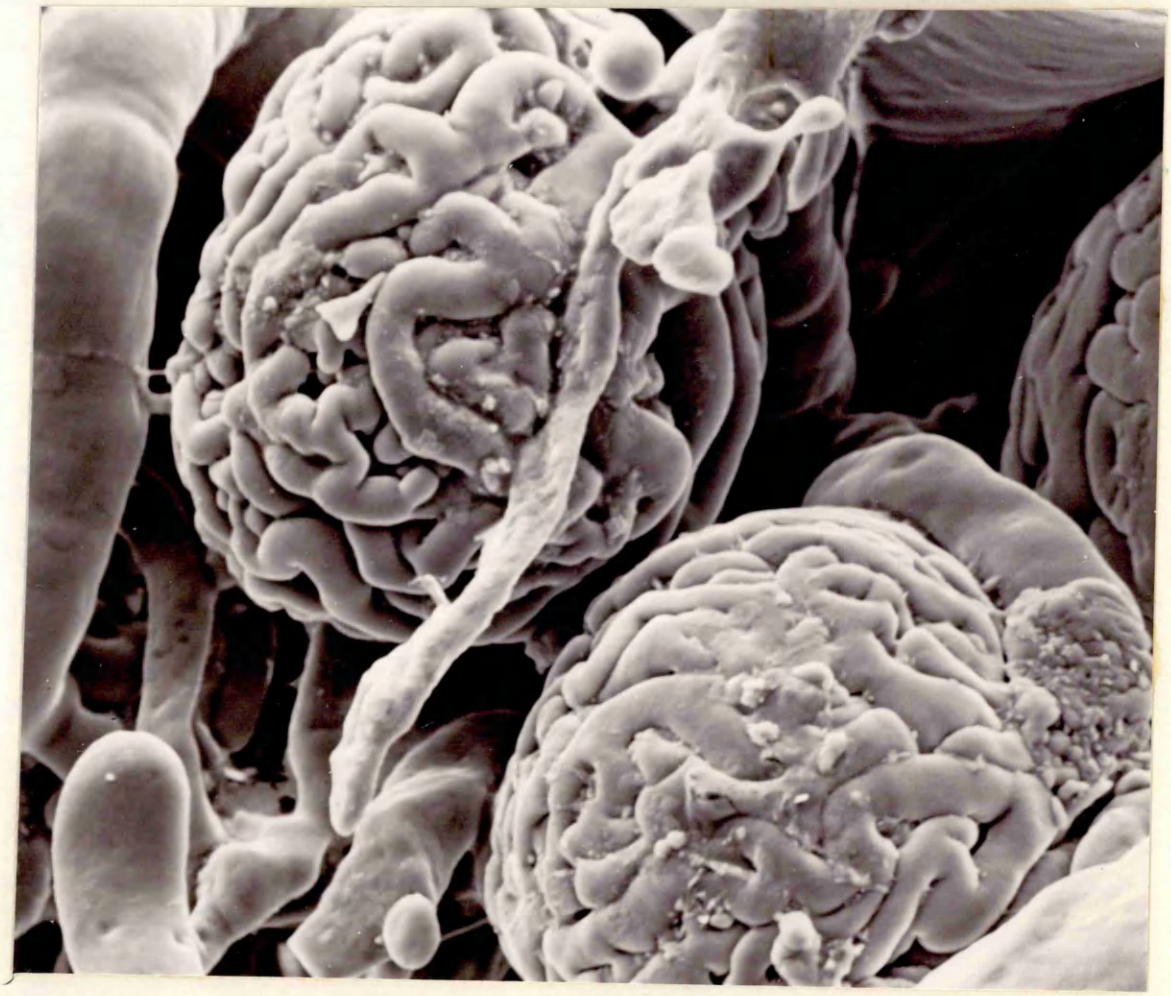
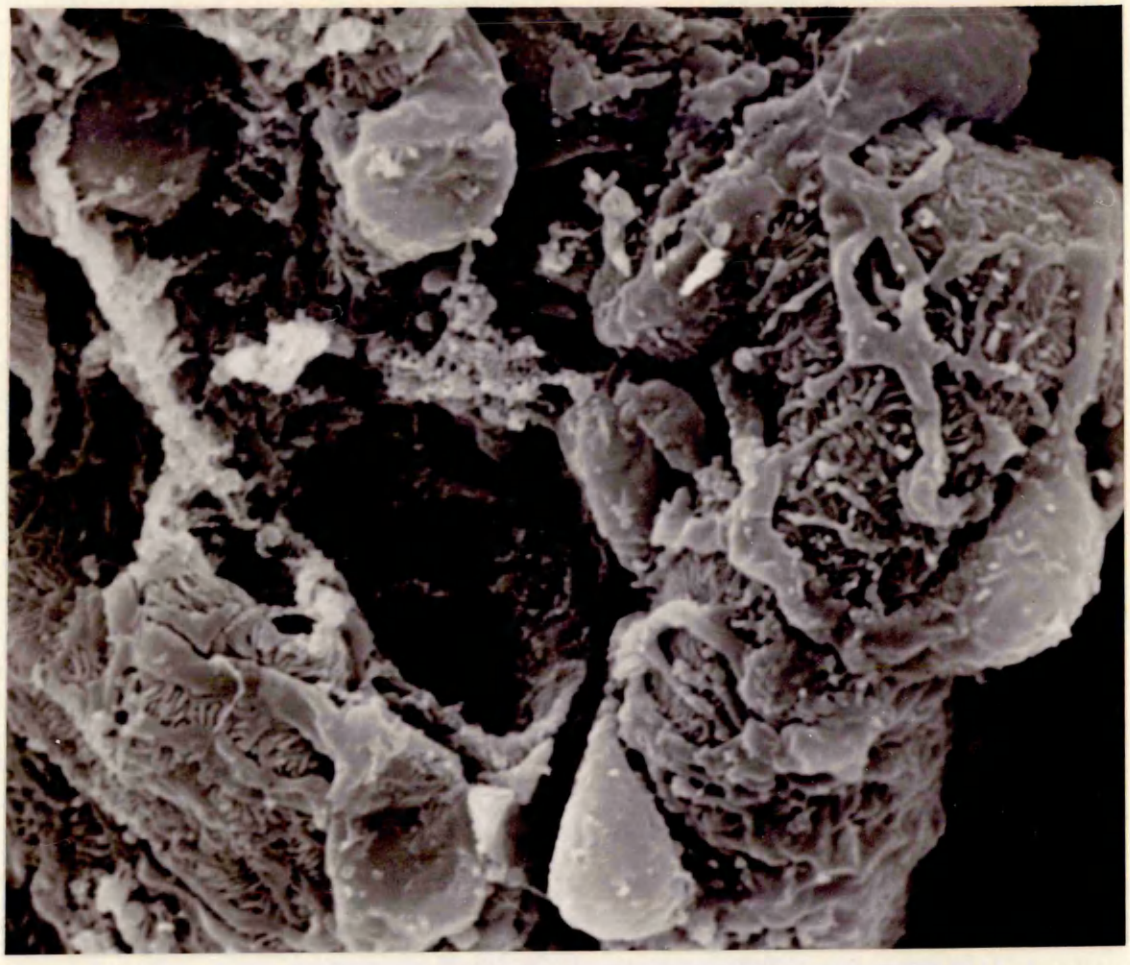
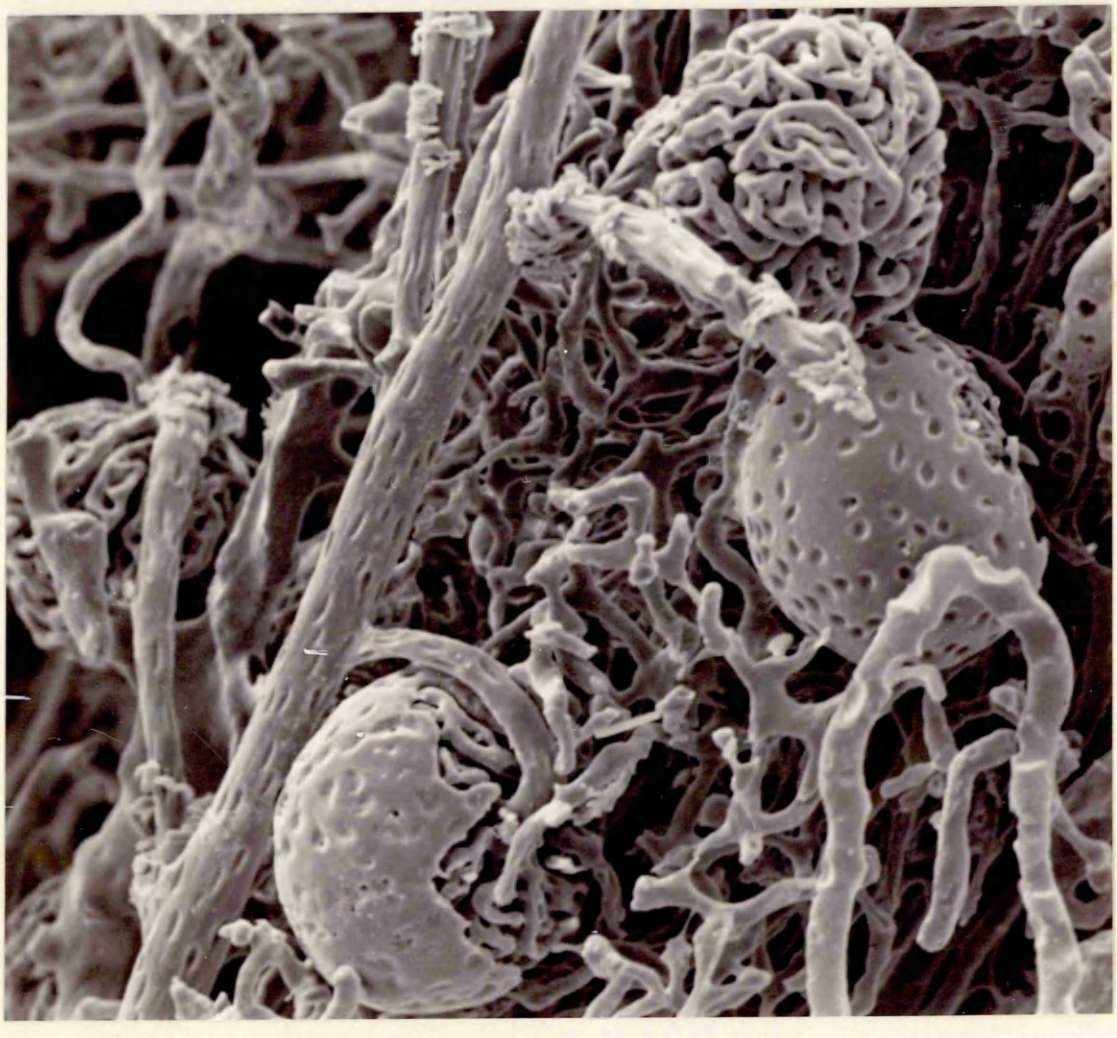


Fig. 76. NTN: 24 hours. Visceral epithelium showing widespread effacement of the foot processes. SEM x 2,500.

Fig. 77. NTN: 48 hours. Two glomeruli show leakage of cast material into the urinary space. Imprints of parietal epithelial cells can be observed. SEM x 160.





Three - five days:

At this stage of glomerular injury the surface changes in the visceral epithelial cells were maximal. To varying degree, most glomeruli were affected. There was widespread obliteration or loss of the foot processes resulting in a roughened sheet-like appearance of the epithelial cell membrane (Fig. 78). Strands of fibrin-like material as well as erythrocytes were found in large quantities in the urinary space.

In renal casts there was generalised disorganisation of capillary arrangement with leakage of cast material in approximately 50% of glomeruli (Fig. 79). The surface of remaining capillaries was roughened.

Seven - 14 days:

The pattern of changes was at this stage essentially the same as those described above. The degree of destruction of the foot processes, however, varied from capillary to capillary and from glomerulus to glomerulus and microvilli on the surface of visceral epithelial cells were especially numerous (Fig. 80). Erythrocytes and fibrin-like material could still be observed in the urinary space. In renal casts, many glomeruli showed disorganisation of capillaries and the surface of the capillary casts was often roughened; leakage of cast material was still commonly observed (Fig. 81). Nevertheless some glomerular casts were relatively normal or showed only minor constrictions or dilatations.

21 - 30 days:

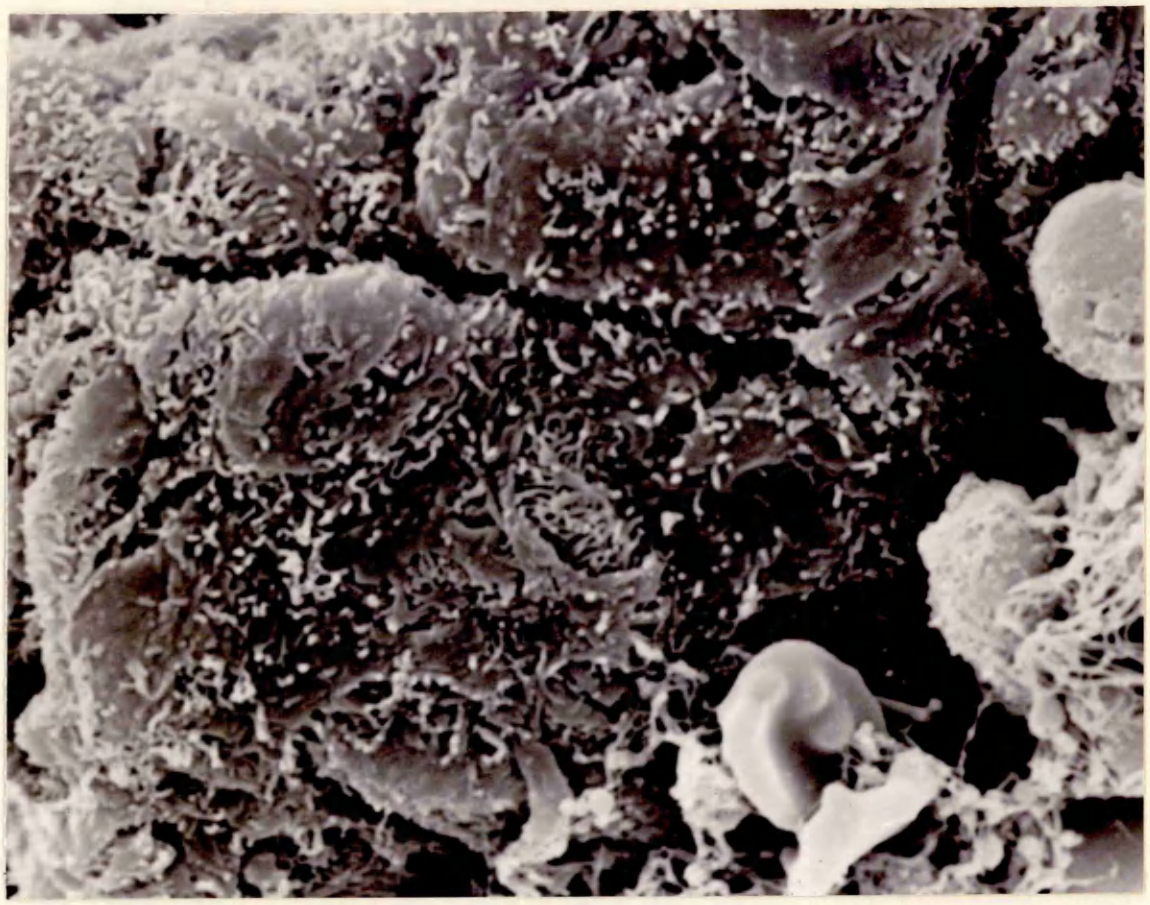
The general architectural organisation of the visceral

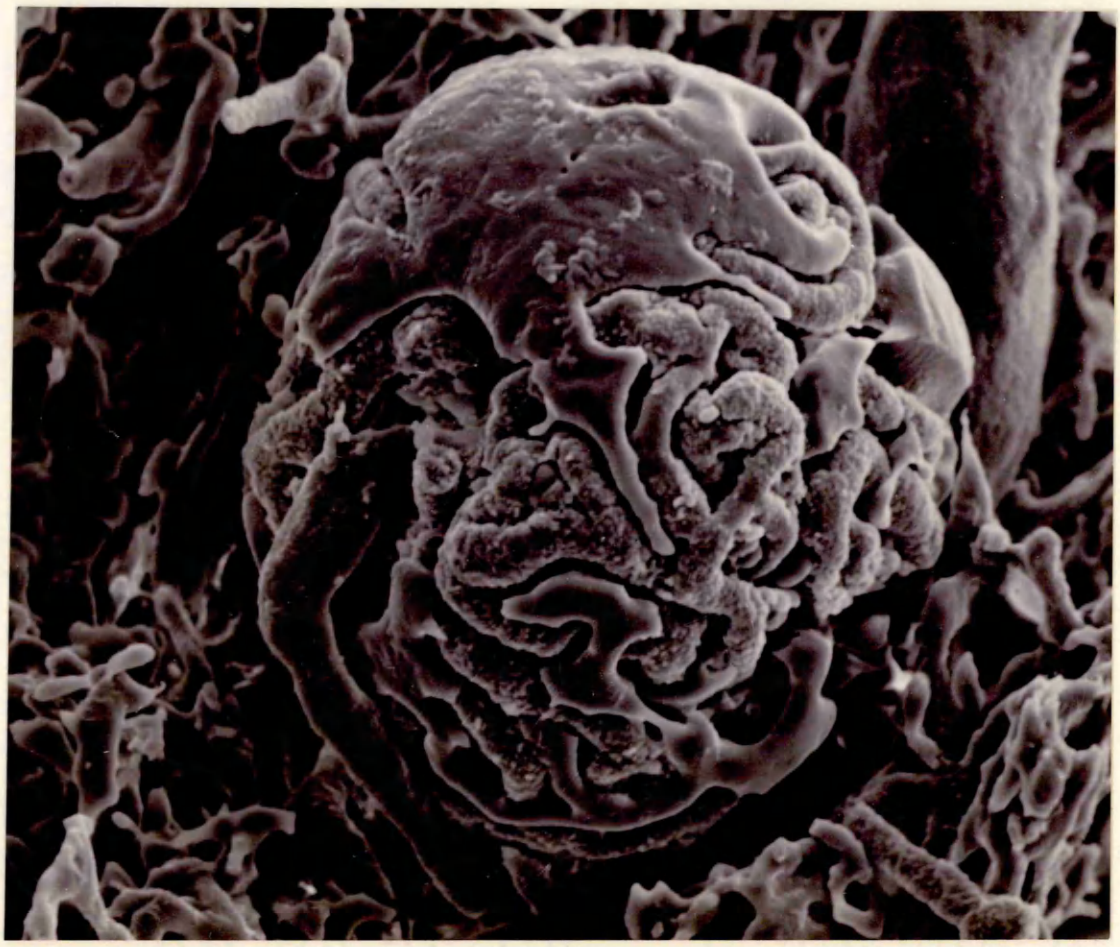
Fig. 78. NTN: three days. The epithelial covering of the capillaries has lost its characteristic configuration resulting in a roughened sheet-like appearance. SEM x 2,500.

Fig. 79. NTN: five days. All the glomeruli in this preparation show leakage of cast material into the urinary space. SEM x 160.

Fig. 80. NTN: 14 days. The glomerular surface epithelium shows dense microvilli; a few erythrocytes can be seen in the urinary space. SEM x 2,500.

Fig. 81. NTN: seven days. A glomerulus is showing disorganisation of capillaries and their surfaces are roughened; there is also leakage of cast material into the urinary space. SEM x 320.





epithelial lining of the glomerular capillaries was still altered to varying extent. There was distinct variation in the degree of effacement of foot processes from capillary to capillary, even in the same glomerulus (Fig. 82). Indeed at 30 days some glomeruli appeared within normal range (Fig. 83).

Glomerular casts varied in form. Some appeared normal while, in others, leakage of cast material still persisted. Distinct lobulation of some glomerular tufts was observed; others were shrunken with narrowed or dilated blindly ending capillaries, presumably reflecting poor filling of partially scarred glomeruli (Fig. 84).

40 - 80 days:

Overall, most of the glomeruli were within normal range. Although patchy areas of obliteration of the foot processes could always be found in a few glomeruli, only rarely was fibrin-like material observed in the urinary space. Occasional shrunken and distorted glomeruli were also observed. Leakage of cast material was still observed in a relatively few glomeruli ($<$ 10%) as late as 80 days after administration of NTS (Fig. 85). Most of the glomeruli, however, now appeared normal (Fig. 86) although a few shrunken tufts with blindly ending capillaries could still be seen (Fig. 87).

Immunofluorescence findings:

These are summarised in Table 5.2. Striking linear deposition of rabbit globulin on the GBM was found as early as 30 minutes after administration of NTabs (Fig. 88). At this time all glomeruli without exception showed 4+ linear deposition which was maintained until 14 days, after which time the intensity of deposition

progressively decreased. By 60 days, some glomeruli still showed areas of linear deposition which persisted until 80 days (Fig. 89). Granular deposition of complement (C_3) was not detected until three days (Fig. 90). This pattern persisted up to 60 days fluctuating between 1+ and 2+. At 80 days, deposition of C_3 was not found.

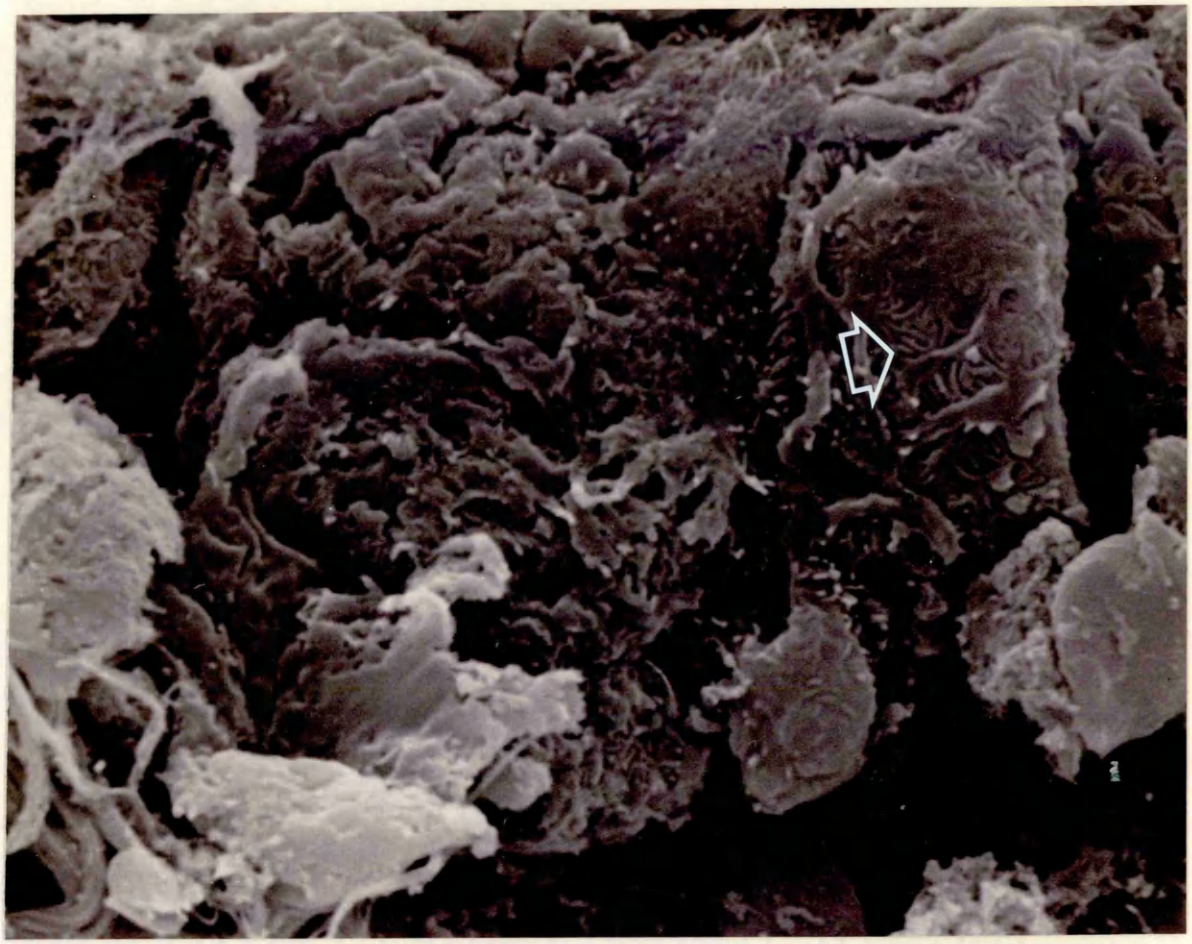
IgG deposition was not seen until five days when irregular linear deposits were found (Fig. 91). At 30 days the deposition was maximal and thereafter IgG fluorescence gradually diminished but was still present at 60 days. IgG was not detected at 80 days. Sections stained with rabbit antidog fibrinogen showed lumpy deposits of fibrin from two days onwards. The peak of deposition was between three and seven days (Fig. 92) and thereafter irregular deposits were found up to 60 days.

Control animals:

The morphological features of the control dogs were similar to those described in Chapter 3. In glomerular casts, only very rarely ($<$ 1%) was leakage of cast material into Bowman's capsule observed. With immunofluorescence, however, three control dogs showed minimal granular deposition of IgG and C_3 at 10 and 14 days after inoculation of normal rabbit serum; in these dogs not all glomeruli were involved. None of the controls showed linear deposition of rabbit globulin.

Fig. 82. NTN: 21 days. Visceral epithelial lining of the glomerular capillaries showing widespread effacement of foot processes. Note, however, the appearance of a small area of relatively normal foot processes (arrow). SEM x 2,500.

Fig. 83. NTN: 30 days. A glomerulus is showing surface morphology which is within normal range. SEM x 2,500.



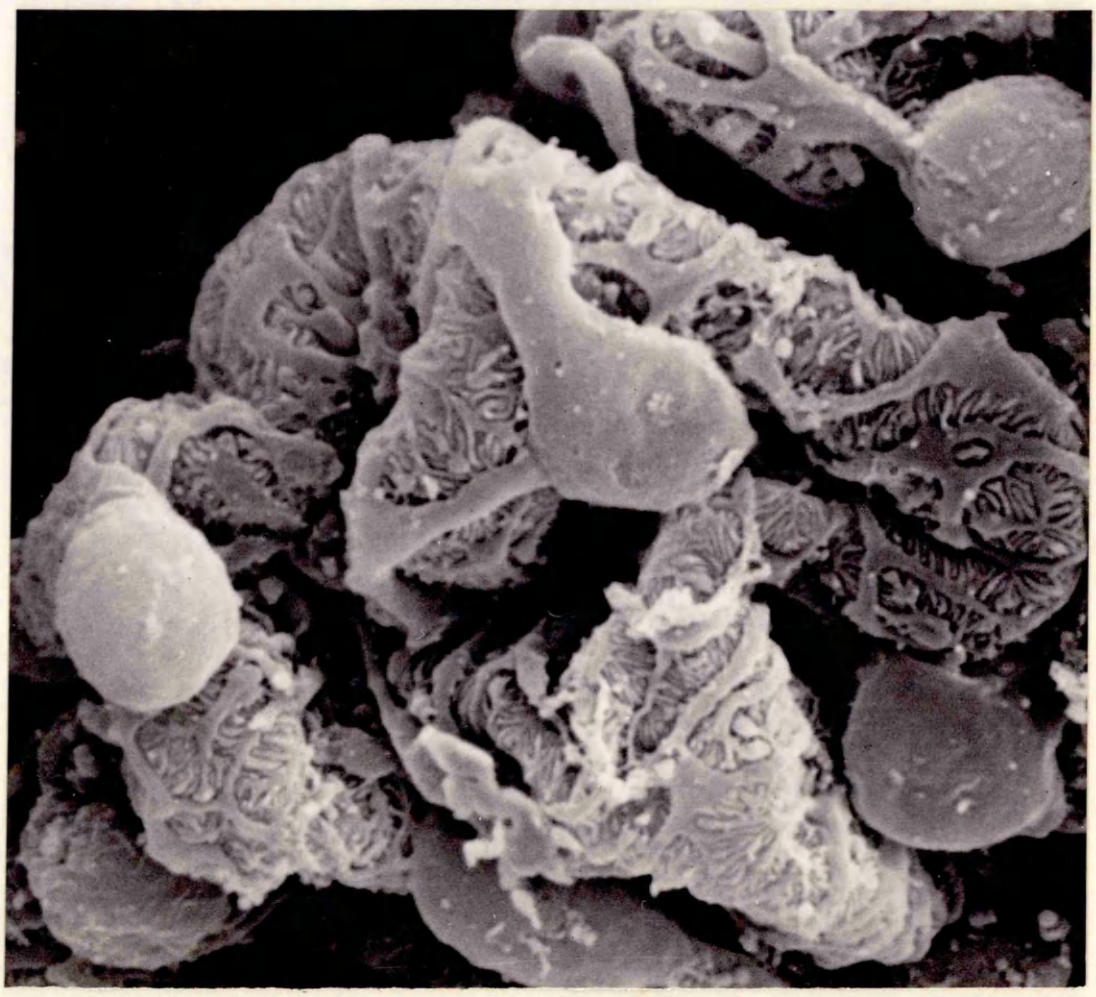
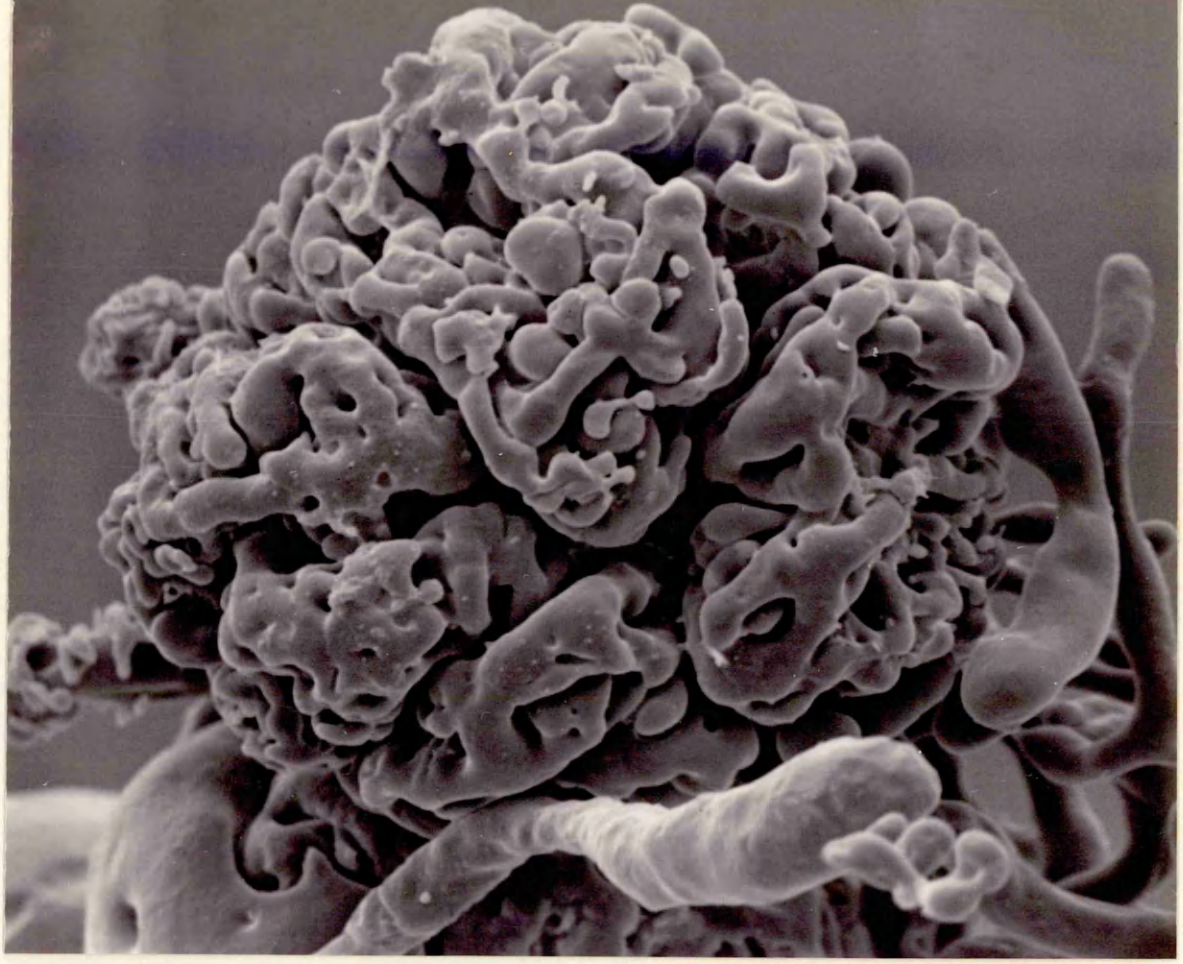


Fig. 84. NTN: 30 days. A glomerular tensol cast, showing disorganization of the glomerular capillaries. SEM x 320.

Fig. 85. 60 days. There are still a few glomeruli showing leakage of cast material into the urinary space. SEM x 180.



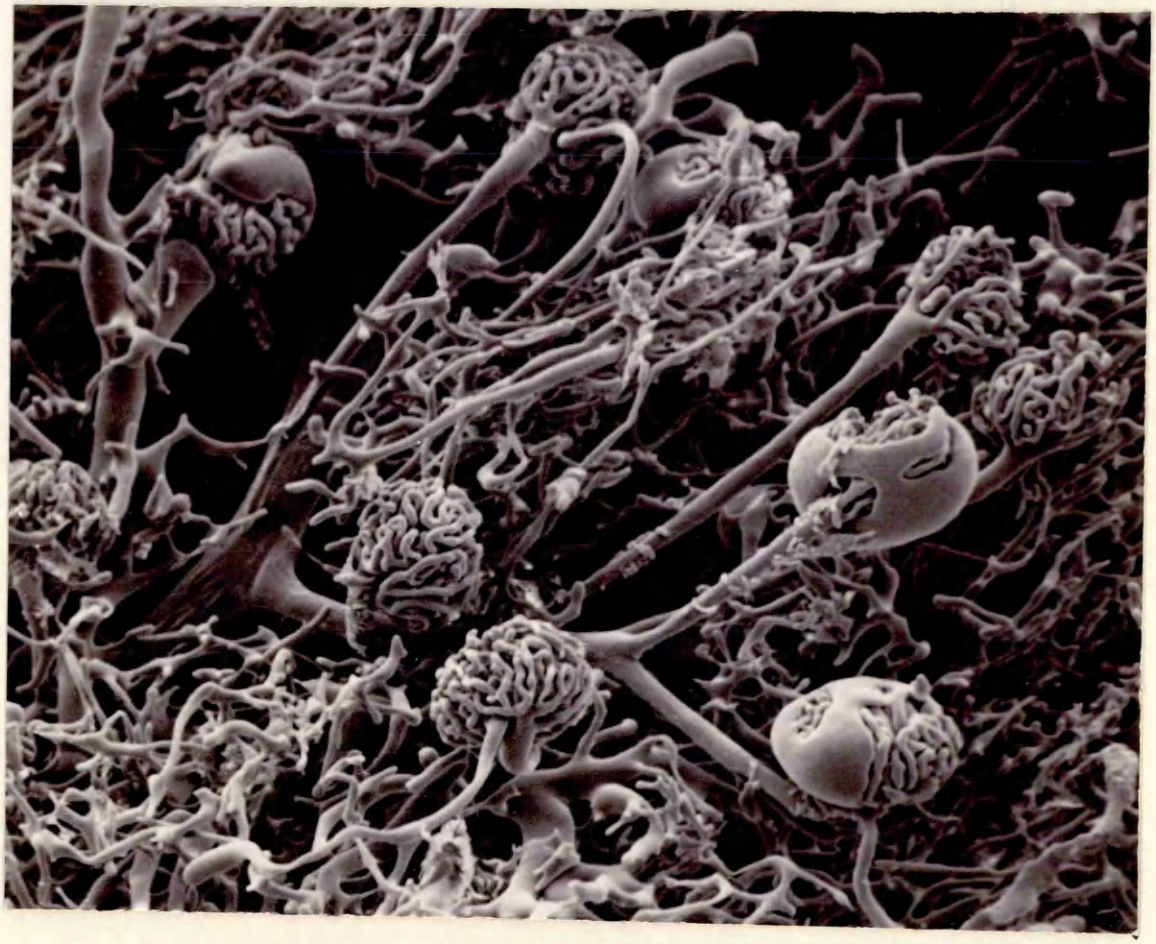
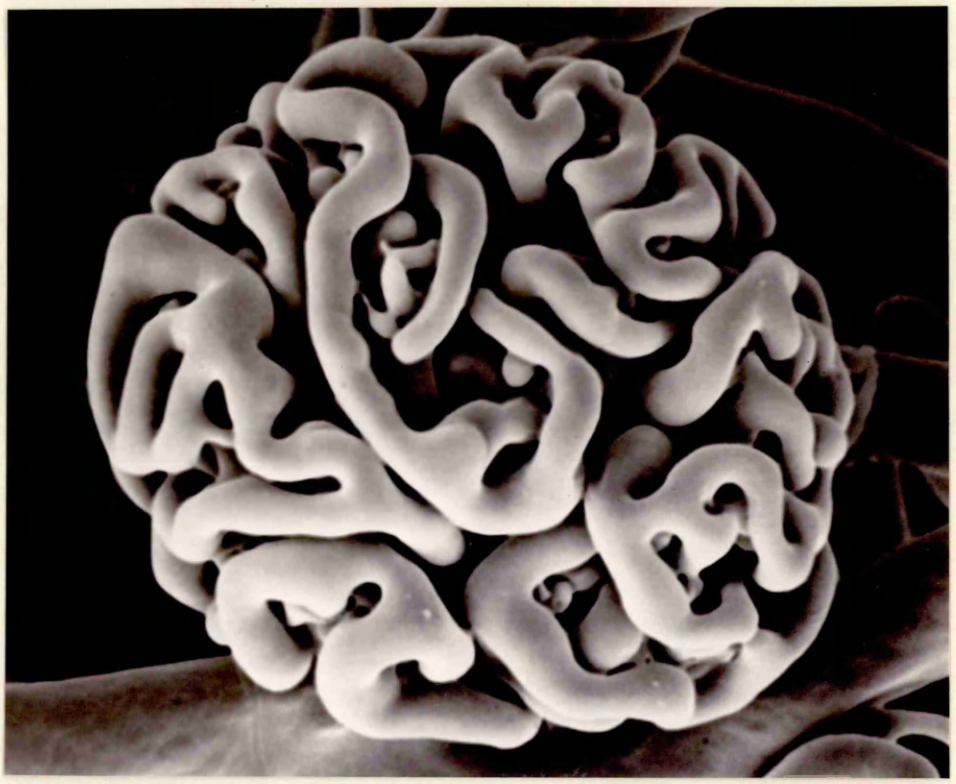


Fig. 86. NTN: 80 days. A glomerulus showing normal capillary arrangement. SEM x 320.

Fig. 87. NTN: 80 days. A shrunken glomerular tuft with blindly ending capillaries. SEM x 320.



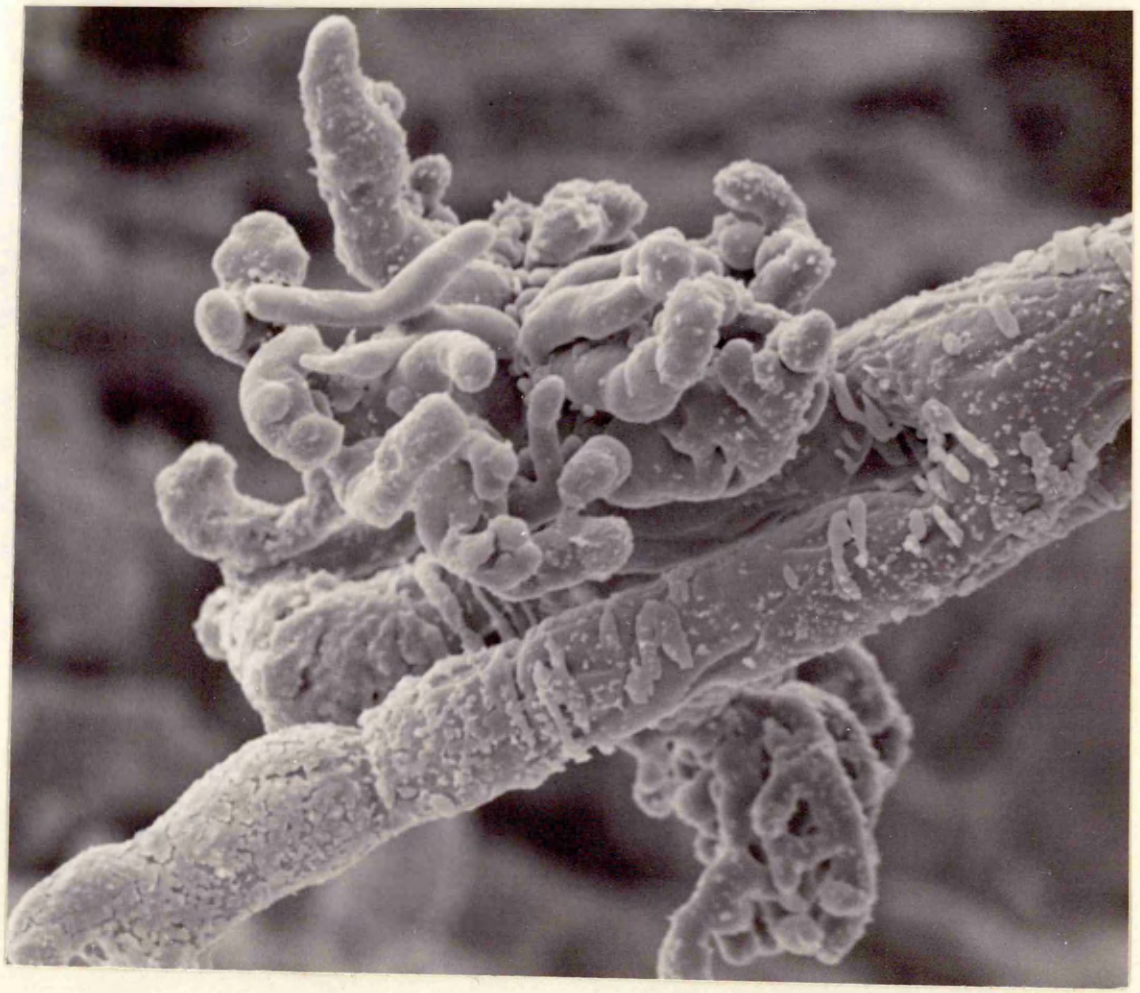
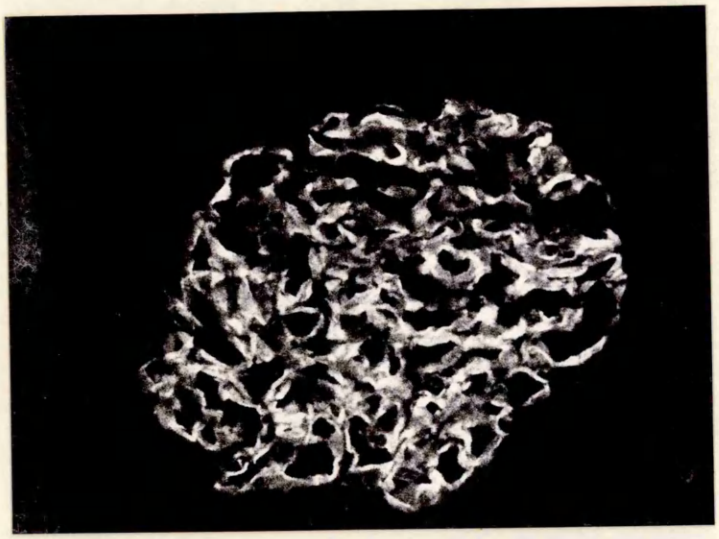
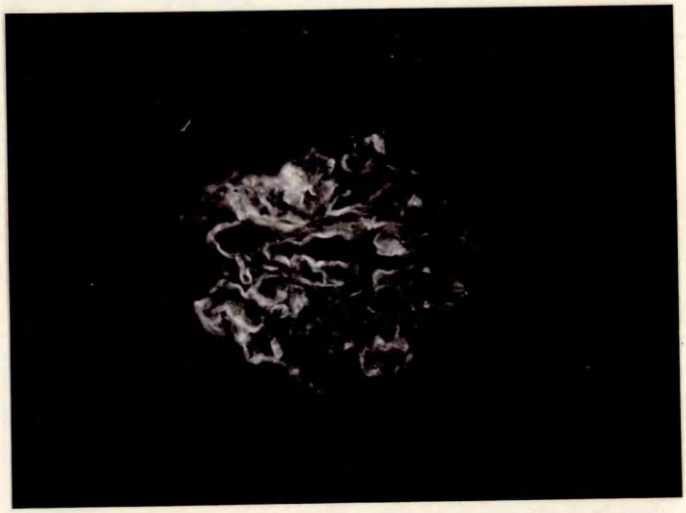


Fig. 88. NTN: 30 minutes. Glomerulus stained with FITC labelled - sheep antirabbit globulin (SAR). Note the linear fluorescence of the GBM. Immunofluorescence x 170.

Fig. 89. NTN: 80 days. Note the persistence of linear fluorescence of SAR along the GBM. Immunofluorescence x 120.

Fig. 90. NTN: three days. Glomerulus stained with FITC conjugated rabbit antidog complement (C_3). There is irregular granular fluorescence of the basement membrane. Immunofluorescence x 170.





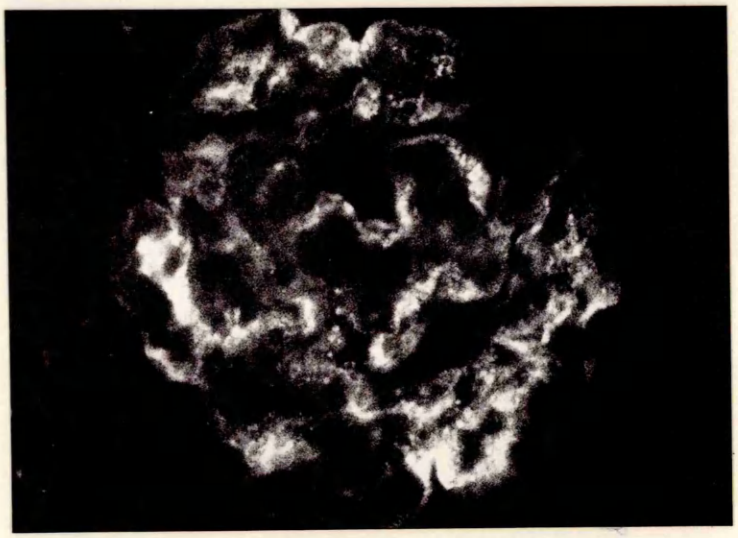


Fig. 91. NTN: five days. Glomerulus stained with FITC conjugated rabbit-antidog IgG. Note the irregular distribution of fluorescence.
Immunofluorescence x 170.

Fig. 92. NTN: seven days. Glomerulus stained with rabbit antidog fibrinogen. Note its lumpy deposits of fibrin.
Immunofluorescence x 170.

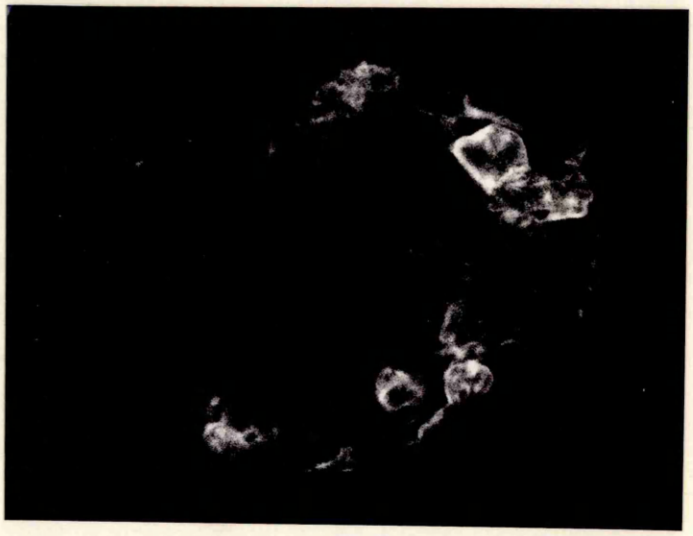


TABLE 5.1

EXPERIMENTAL NTN IN DOGS: HISTOLOGICAL FINDINGS

Dog No.	Day Killed	Fibrin Loops	Fibrin U.S. *	Capillary Thrombosis & Necrosis	Mes. Hyper. **	Neutrophils	Adhesions	Scarring < 50%	Scarring > 50%	Scarring Obsol. ***	Normal
W940	30 mins.	-	-	-	-	-	-	-	-	-	100%
W959	30 mins.	-	-	-	-	-	-	-	-	-	100%
W939	1 hour	-	-	-	-	1+	-	-	-	-	100%
W958	1 hour	-	-	-	-	-	-	-	-	-	100%
W926	4 hours	-	-	-	-	1+	-	-	-	-	100%
W957	4 hours	-	-	-	-	1+	-	-	-	-	100%
W937	6 hours	-	-	-	-	1+	-	-	-	-	100%
W938	6 hours	-	-	-	-	1+	-	-	-	-	100%
W935	1 day	-	-	-	-	1+	-	-	-	-	100%
W936	1 day	-	-	-	-	1+	-	-	-	-	100%
W934	2 days	-	-	-	-	1+	-	-	-	-	100%
W943	2 days	1+	-	1+	1+	3+	1+	-	-	-	100%
W956	2 days	-	-	-	-	-	-	-	-	-	100%
W925	3 days	1+	2+	1+	2+	2+	-	-	-	-	100%
W955	3 days	1+	1+	1+	1+	1+	-	-	-	-	100%
W961	3 days	1+	1+	1+	2+	2+	1+	-	-	-	100%
W941	5 days	1+	1+	1+	3+	1+	1+	-	-	-	100%
W942	5 days	1+	2+	2+	3+	1+	2+	-	-	-	100%
W928	7 days	1+	3+	1+	3+	1+	3+	15%	6%	3%	76%
W292	14 days	-	2+	1+	3+	1+	3+	17%	10%	6%	67%
W967	15 days	-	1+	1+	3+	-	3+	11%	5%	4%	80%
W966	19 days	-	1+	-	3+	-	3+	9%	10%	7%	74%
W927	21 days	2+	3+	1+	3+	-	3+	9%	11%	13%	67%
W974	30 days	-	1+	1+	3+	-		2%	8%	20%	70%
W976	30 days	-	1+	1+	3+	-	3+	2%			98%
W969	40 days	1+	1+	2+	-	1+	-	-	-	8%	92%
W973	40 days	-	-	-	2+	-	1+	-	-	-	100%
W970	60 days	-	-	1+	-	1+					100%
W975	60 days	-	-	1+	2+	-	1+	3%	8%	-	89%
W971	80 days	-	-	-	1+	-	-	2%	3%	1%	94%
W972	80 days	-	-	-	1+	-	-	2%	3%	1%	94%

* Urinary Space ** Mesangial Hypercellularity *** Obsolescence

Lesions graded 1+ to 3+ according to severity.

TABLE 5.2

IMMUNOFLUORESCENCE FINDINGS IN EXPERIMENTAL NTN IN DOGS

Dog No.	Time Killed	Rabbit Globulin	IgG	C3	Fibrinogen
W940	30 mins	++++	-	-	-
W959	30 mins	++++	-	-	-
W939	60 mins	++++	-	-	-
W958	60 mins	++++	-	-	-
W926	4 hrs	++++	-	-	-
W957	4 hrs	++++	-	-	-
W937	6 hrs	++++	-	-	-
W938	6 hrs	++++	-	-	-
W935	24 hrs	++++	-	-	-
W936	24 hrs	++++	-	-	-
W934	48 hrs	++++	-	-	+
W943	48 hrs	++++	-	-	-
W956	48 hrs	++++	-	-	-
W925	3 days	++++	-	+	++
W955	3 days	++++	-	-	-
W961	3 days	++++	-	++	++
W942	5 days	++++	++	-	-
W941	5 days	+++	-	-	++
W928	7 days	++++	++	+	++
W929	14 days	++++	+	+	++
W967	15 days	+++	-	+	+
W966	19 days	+++	-	++	+
W927	21 days	++++	++	++	++
W974	30 days	+++	+	++	+
W976	30 days	++++	++	+	+
W969	40 days	++++	+++	++	+
W973	40 days	++++	++++	++	+
W975	60 days	+++	+++	+	-
W970	60 days	+++	++	-	+
W971	80 days	++	-	-	ND
W972	80 days	++	-	-	ND

ND = Not Done

Lesions graded + to ++++ according to severity.

TABLE 5.3

BIOCHEMICAL FINDINGS IN EXPERIMENTAL NTN IN DOGS

Dog No.	Time Killed	Urine Protein* mg/100ml	Blood Urea mmol/l	
			Pre-inoculation	Necropsy
W940	30 min	12.5	3.6	10.5
W959	30 min	10.0	2.8	2.8
W939	60 min	4.5	2.8	3.1
W958	60 min	0.0	2.3	2.4
W926	4 hrs	550.0	3.8	N.D.
W957	4 hrs	0.0	N.D.	3.3
W937	6 hrs	0.0	3.6	3.3
W938	6 hrs	0.0	3.0	2.4
W935	24 hrs	0.0	4.7	6.2
W936	24 hrs	315.0	3.8	5.5
W934	48 hrs	325.0	1.1	5.1
W943	48 hrs	N.D.	2.6	2.8
W956	48 hrs	0.0	4.1	4.3
W925	3 days	475.0	2.5	3.1
W955	3 days	90.0	1.8	3.9
W961	3 days	125.0	2.6	2.6
W942	5 days	125.0	4.0	4.1
W941	5 days	60.0	2.8	9.9
W928	7 days	106.0	2.9	5.8
W929	14 days	80.0	2.9	5.9
W967	15 days	662.5	2.8	28.3
W966	19 days	5.0	1.9	2.1
W927	21 days	0.0	3.4	10.3
W924	30 days	10.0	1.2	9.1
W976	30 days	10.0	1.8	6.1
W969	40 days	1.0	1.7	5.8
W973	40 days	0.0	3.4	5.8
W975	60 days	0.0	7.5	14.8
W970	60 days	0.0	5.5	1.7
W971	80 days	2.0	1.2	14.7
W972	80 days	6.0	1.5	7.8

* Urine protein levels at necropsy only.

ND = Not done.

Discussion:

The few reports which deal with the sequential pathology of NTN in dogs (Bevans et al, 1955; Movat et al, 1961) have described the lesions induced by injection of serum prepared against whole kidney or placental suspensions. The only comprehensive histological, ultrastructural and immunofluorescence study of experimental NTN in dogs using serum raised against dog GBM was reported by Wright et al (1973[a]).

The present study was designed to build on the earlier work of Wright and co-workers by carrying out a more extensive investigation of the sequential morphologic events in glomeruli damaged by nephrotoxic serum and to lay special emphasis on the ultrastructural changes which were relatively poorly documented in previous reports.

The earliest observable histological event was the appearance of neutrophils in lumina of the glomerular capillaries by one hour after administration of NTabs. This was followed by swelling of the tuft with occlusion of capillaries and, by two days, segmental capillary thrombosis and necrosis and mesangial hypercellularity. The severity of the histological lesions was most pronounced on days five and seven. Thereafter, the exudative lesions diminished while glomerular scarring became progressively noticeable.

In the later stages of the disease mesangial hypercellularity, lobulation of the glomerular tuft and persistence of a few obsolescent glomeruli were the only major observations. The overall histological changes were similar to those recorded by Movat et al (1961) but these workers did not observe obvious light microscopic

changes till 11 days, when they reported that all glomeruli were affected to some degree, with swelling, hypercellularity and crescent formation the main features. However, Wright et al (1973[a]) in their more comprehensive study reported early neutrophil infiltration by four hours (compared to one hour) in the present study.

In the present study, severe capillary thrombosis and necrosis was not observed until five or seven days whereas in the experiment of Wright et al (1973[a]), severe glomerular capillary thrombosis and necrosis was noted as early as two days. This disparity in results may reflect differences in the inherent nephrotoxicity of the rabbit serum as, in both studies, the volume of NTabs used was similar.

With the TEM, the first ultrastructural changes were observed by one hour when neutrophils were found in the glomerular capillaries and in some instances some of these cells were in direct contact with the GBM. This change was accompanied by fusion of epithelial foot processes. The ultrastructural changes were most marked, however, on days three - seven and this period was characterised by intraluminal fibrin deposits, by extensive fusion of foot processes, necrosis of the endothelial lining and mesangial hypercellularity. There was some evidence that circulating mononuclear cells emigrating into the mesangium were contributing to the overall hypercellularity of the glomeruli. This is in accord with current opinion on the role of these cells in glomerular hypercellularity in human and experimental animal glomerulonephritis (Atkins et al, 1981).

The remaining notable feature of the ultrastructural alteration was the expansion of the subendothelial space with electron

lucent material. This was not found until 15 days and persisted until the end of the experiment at 80 days. Furthermore, between 19 and 40 days, localised electron dense deposits were also to be found in the expanded subendothelial spaces and in the mesangial matrix. Similar subepithelial deposits have been reported by Albini et al. (1979) who suggested that they represent GBM - anti GBM immune complex deposition superimposed on the existing NTN.

Although the general structural features reported in the present work were in some ways similar to those described by Wright et al (1973^a) there were a number of differences. Firstly, in the present study, the appearance of neutrophils in the glomerular capillaries by one hour was not described by Wright et al (1973^a) until four hours. Secondly, the subendothelial deposits described by Wright et al (1973^a) as sometimes completely encircling the capillary loops were not observed in the present study until 14 - 21 days and only localised deposits were observed. Thirdly, the electron dense deposits found by 19 days in the present work were not recorded by Wright and co-workers.

In the present study, the detailed ultrastructural changes in the glomeruli as observed by the SEM were recorded for the first time in experimental NTN in the dog. Alteration to the normal orderly arrangement of the foot processes was noted at 30 minutes after administration of NTabs and, at this early stage, arterial casts showed leakage of cast material in approximately 10% of glomeruli. By 24 hours in addition to obliteration of foot processes, strands of fibrin like material were observed in the urinary spaces. By three days, more severe changes characterised by extensive fusion of foot processes and complete loss of normal podocytic architecture

were evident. In renal casts up to 50% of glomeruli showed leakage of cast material and by 14 days glomerular casts revealed roughened capillary outlines which probably corresponded to the endothelial destruction found with TEM.

Although some glomeruli still showed obliteration of foot processes and leakage of cast material at 30 days, other glomeruli had returned to normal. The percentage of normal glomeruli increased gradually towards the end of the experiment and at 80 days most glomeruli showed normal surface morphological arrangement of epithelial foot processes.

In these later stages of the experiment, arterial casts showed shrunken glomeruli with stunted blindly ending capillaries, which presumably represented partial glomerular scarring. The only other brief report on the use of SEM in NTN was that of Buss and Lamberts (1975) who, in their rat model, did not find changes in the visceral epithelial cells until 12 days after administration of NTabs when they recorded irregularity of second and third order processes.

The immunofluorescence patterns were similar to those described by Wright et al (1973[a]). Rabbit globulin was detected in the glomeruli as early as 30 minutes after administration of NTabs and had a striking linear distribution. This persisted until 15 days; thereafter, the intensity of fluorescence diminished and although all glomeruli were involved some only showed patchy segmental fluorescence. Faint linear deposition, however, was still present in some glomeruli at 80 days. Complement (C_3) could not be detected until three days when weak linear fluorescence was observed. The intensity of fluorescence was never as striking as that for rabbit globulin and persisted only up to 60 days. The autologous phase of the disease

was characterised by irregularly linear deposition of host IgG; this was detected by five days in contrast to the findings of Wright et al (1973[a]) who did not detect IgG until seven days. As with C_3 the intensity of fluorescence was not as clear cut as that for rabbit globulin but nevertheless persisted until 60 days. Lumpy deposits of fibrin were not detected until two days but were still found in the later stages of the experiment at 60 days. Unlike the fluorescence patterns of rabbit globulin, C_3 and host IgG, deposits of fibrinogen were always focal as well as segmental.

CHAPTER 6

EXPERIMENTAL SERUM SICKNESS GLOMERULONEPHRITIS IN DOGS

During the last three decades the increasing utilization of immunohistochemical and electron microscopic techniques in the study of renal disease has clearly established that glomerulonephritis (GN), the most important renal disease of man, is immunological in origin. Deposition of circulating immune complexes or, alternatively, *in situ* formation of complexes are thought to play a vital pathogenetic role in a variety of primary glomerular disorders in both man and animals (Osborne *et al*, 1977; Couser and Salant, 1980; McCluskey, 1983).

The role that these immune complexes play in the induction and progression of GN has been the subject of many experimental studies particularly serum sickness where artificially induced circulating immune complexes lodge in and subsequently cause damage to the renal glomerulus. For both the morphologist and immunologist, experimental serum sickness has been the first and most promising laboratory model for the study of the effect of deposition of immune complexes in the glomerulus (Dixon *et al*, 1958).

The renal glomerulus is particularly susceptible to the effect of circulating immune complexes and this vulnerability is related to its unique anatomical and functional properties, aspects of which have already been discussed in Chapter 3.

In 1903, Von Pirquet and Schick first introduced the term "Serum Sickness" when describing an illness in human patients eight - 12 days after receiving heterologous serum; they claimed that antibody raised by the recipient patient reacted with the injected heterologous protein. Most of the subsequent information on serum sickness has been obtained from an experimental model system in which experimental animals are injected with heterologous protein such as

bovine serum albumin (BSA). Although serum sickness can be produced by injecting a sufficient amount of any heterologous protein into rabbits or indeed into other experimental animals, BSA has been the most frequently used antigen. In general, the dosage and duration of administration of antigen determines the type and extent of glomerular injury (Germuth et al, 1967), although Isaacs and Miller (1982) have provided convincing evidence of an interaction between antigenic size and electrical charge in governing the degree of immune complex deposition and subsequent degree of glomerular damage.

A variety of injection schedules have been employed to induce immune complex-mediated GN in experimental animals. A single intravenous injection of a large dose of BSA produces an acute, usually transient, form of GN - so called "one-shot serum sickness" (Germuth, 1953; Dixon et al, 1958), whereas multiple injections of BSA given over a prolonged period of many weeks often results in a more chronic form of serum sickness (Dixon et al, 1961; Germuth et al, 1967). Although the "acute" one-shot and "chronic" multiple dose forms of serum sickness remain the most commonly used model system, there are a number of other more recently introduced variant models. Leaving aside experimental Heymann nephritis associated with intrinsic renal (tubular) antigens (Heymann et al, 1959; Glassock et al, 1968) the main features of the four main model systems involving non-renal antigens and which are employed to induce serum sickness will now be briefly summarised:

1. Acute "one-shot" serum sickness GN:

As already indicated, this form of experimentally induced GN is usually induced by a single large intravenous injection of heterologous

protein, usually purified BSA, into rabbits (Germuth, 1953; Dixon, 1967). Following a rapid period of equilibration between intra and extravascular compartments for 24 - 48 hours after inoculation when two-thirds to three quarters of the injected BSA disappears from the circulation, there ensues a slower rate of antigen disappearance representing catabolism of the residual circulating BSA. By the fourth or fifth day the animal begins to produce antibodies against BSA, an event which marks the beginning of the immune phase of antigen elimination. Dixon et al (1958) and Dixon (1967) have shown that it is circulating soluble complexes formed in a state of antigen excess that are capable of localising in and causing damage to the renal glomeruli. In situations of antibody excess, the complexes are larger and poorly soluble and most of these complexes are phagocytized by the mononuclear phagocytic system, and, there is rapid elimination of circulating BSA which is generally accomplished between 10 - 14 days after injection (Albini et al, 1979).

The typical glomerular proliferative changes observed during "one-shot" serum sickness have been described by Dixon et al (1958) and Cochrane and Koffler (1973) who reported swelling and proliferation of endothelial and mesangial cells, hypertrophy of epithelial cells and detachment of endothelial cells from the underlying GBM.

In 1977, Easley and Halliwell listed the ultrastructural changes in "one-shot" serum sickness as irregularity in thickness of the GBM, increase in mesangial matrix and mesangial hypercellularity, endothelial hypertrophy, fusion of epithelial foot processes and electron dense deposition in mesangial and, to a lesser extent,

subepithelial areas. They suggested that ultrastructural changes tend to occur prior to any hypercellularity detectable by light microscopy. They also observed that, during the early stages, there was no correlation between the morphologic changes and loss of functional integrity of the GBM as manifested by proteinuria.

The characteristic immunofluorescence patterns of experimental serum sickness have been described by Germuth and Rodriguez (1973) and Cochrane and Koffler (1973) who reported deposits of antigen, IgG and C_3 scattered along the GBM. The immune deposits detected in the kidney by immunofluorescence have been shown to correspond with electron dense deposits observed with the electron microscope (Fish et al, 1966).

2. Chronic Serum Sickness GN:

It is well recognised that renal lesions resembling some kinds of human GN can be produced experimentally in animals by long-term administration of foreign proteins (Dixon et al, 1961; Germuth et al, 1967).

In this model system, repeated (usually daily) intravenous injections of heterologous protein into experimental animals (again usually rabbits and BSA have been used) produces after two - three months a chronic, immune complex mediated GN often in the form of membranous nephropathy, although crescentic and mesangiopathic forms of GN have also been noted.

Albini et al (1979) have summarised the two main experimental protocols in this system:

a) Daily doses of a constant amount of BSA tends to produce small

soluble complexes which, in a percentage of rabbits with low levels of antibody, are able to cross the GBM and localise in subepithelial sites. In rabbits with intermediate levels of circulating antibody the larger, less soluble, complexes localise in the glomerular mesangium and produce a less severe "mesangiopathic" form of GN. Germuth et al (1977) have also suggested that the incidence of membranous GN in rabbits is dependent on the dosage of antigen administered; constant low doses of antigen produces membranous GN in approximately 50% of rabbits.

b) Adjustment of the antigen dose to maintain constancy of antigen excess theoretically is more likely to lead to circulating immune complexes small enough to become deposited in subepithelial sites along the GBM and hence induce more serious renal lesions in the form of membranous nephropathy.

Regardless of which of the two methods are employed membranous nephropathy, associated with prominent deposition of immune complexes along the GBM is the usual but not invariable feature of chronic serum sickness nephritis (Dixon et al, 1961; Germuth et al, 1972; Kuriyama, 1973).

Advances in renal immunology and physiology have altered traditional concepts of the pathogenesis of immune complex GN. Whereas glomerular immune deposits were once thought to arise primarily by deposition of circulating preformed soluble immune complexes (Cochrane and Koffler, 1973), it is now known to be equally possible that they form in situ. Several investigators have now convincingly demonstrated in situ immune complex formation (Batsford et al, 1980; Oite et al, 1982; Ward et al, 1984).

The recent discovery that the glomerular wall acts as a charge selective barrier to circulating molecules (Brenner et al, 1978; Venkatachalam and Rennke, 1978) repelling anionic and attracting cationic molecules has important theoretical implications for the pathogenesis of immune complex GN. Border et al (1981) in describing the effect of antigenic charge on the rat glomerulus, noted that cationic BSA developed deposits confined to the mesangium, whereas anionic BSA resulted predominantly in capillary wall deposits. On the other hand, Gallo et al (1983) reported that in mice differently charged immunogens induced distinctly different patterns of immune complex formation in that highly cationic antigen formed predominantly subepithelial deposits whereas less cationic antigen predominantly formed mesangial deposits. However, requirement of a cationic antigen in the formation of subepithelial immune complexes in the rabbit glomerulus was observed by Ward et al, (1984).

In chronic serum sickness, the characteristic glomerular deposition of IgG and C_3 along the capillary walls, observed by immunofluorescence (Cochrane and Koffler 1973), can be identified ultrastructurally as electron dense subepithelial and intramembranous deposits. It is these deposits which cause the characteristic membranous thickening of the glomerular capillary walls which gives the name to membranous nephropathy. There are associated alterations in the visceral epithelial cells most of whose foot processes are effaced and epithelial cytoplasm forms a continuum along the GBM.

3. Accelerated serum sickness GN:

In this recently introduced model of serum sickness, experimental rabbits are sensitized subcutaneously with BSA emulsified with Freunds complete adjuvant and subsequently given one or more

intravenous injections of large doses of BSA. The aim of this model was to establish a more rapidly developing and progressive form of immune complex GN. The first extensive study of glomerular lesions in accelerated serum sickness in rabbits was carried out by Shigematsu and Kobayashi (1976) who observed that the glomerular changes were proliferative rather than membranous in nature and that the proliferative changes at the onset of proteinuria as early as 12 days after sensitization with BSA, were mainly due to accumulation of monocytes and neutrophils in the capillary lumen. The subendothelial space was expanded with proteinaceous amorphous material and local exfoliation of endothelial cells was accompanied by direct contact of monocytes with the inner surface of the GBM. Electron-dense subendothelial deposits were observed only on rare occasions. The same workers also reported localised fusion of foot processes and swelling and proliferation of mesangial cells.

4. Passive serum sickness GN:

Many investigators have attempted to induce serum sickness by administration, usually daily for three - five days, of preformed antigen-antibody complexes prepared in antigen excess (McCluskey and Benacerraf, 1959; Benacerraf et al, 1960).

Although Cochrane and Koffler (1973) had reported difficulty in obtaining consistent results using a mouse BSA model, Okumura et al (1971) employing a BSA model in mice and Wright et al (1974) using an adenovirus model in dogs for the preparation of soluble immune complexes, have both demonstrated consistent deposition of passively administered preformed complexes, as detected by immunofluorescence and electron microscopy, and histological changes in the glomeruli.

McCluskey et al (1960) described the main histological changes in mice with passive serum sickness. These began two - three days after the first injection of complexes and gradually regressed during the following two weeks. This course of events indicated that the recipient host's own immune response played no part in the initial pathogenesis of the glomerular lesions. The latter were characterized by infiltration of neutrophils with swelling and hypercellularity of glomerular (mesangial) cells.

With the electron microscope, passively administered complexes appear to localize only in small quantities in mesangial regions and beneath the endothelium although these may be associated with severe proliferative changes (Okumura et al, 1971). Immunofluorescence patterns following single or repeated injections of preformed complexes were described by Okumura et al (1971) who noted granular or lumpy deposition of BSA and host IgG localized predominantly in the mesangium.

Spontaneous immune-complex GN in dogs:

Early workers such as Monlux (1953) and Kirk et al (1959) have both suggested that spontaneous canine GN is uncommon. However, in the light of recent advances in the diagnosis and understanding of GN in animals particularly through the use of renal biopsy methods, immunofluorescence and electron microscopic techniques, a large number of reports of spontaneous canine GN have now accumulated in the literature and the disease is now considered to be a common occurrence (Kurtz et al, 1972; Osborne and Vernier, 1973; Murray and Wright, 1974; Lewis, 1976; Mueller-Peddinghaus and Trautwein, 1977; Wright et al, 1981). Most if not all, of the reports of spontaneous GN

in dogs are associated with the deposition of immune complexes in subepithelial and intramembranous sites and to a lesser extent in the mesangium. True anti-GBM GN has not so far been recorded in the dog.

Despite the similarity of spontaneous immune complex GN in the dogs with human forms of the disease, most experimental work on immune complex GN has been carried out in laboratory animals particularly the rabbit, mouse and rat. Apart from nephrotoxic (anti-GBM) nephritis (Wright et al, 1973[a]) the dog has not previously been used as a model for study of immunologically-mediated glomerular injury.

Although experimentally induced immune complex GN has been described in dogs with canine adenovirus (Wright et al, 1974; Wright and Cornwell, 1983) and Dirofilaria immitis (Casey and Splitter, 1975), infections there are no reported studies on experimentally-induced serum sickness type GN using a non-replicating antigen. The purpose of this section of the work was to study for the first time, the reaction of the dog glomerulus to various forms of experimentally-induced serum sickness induced by BSA.

Materials and Methods:

46 young collie-cross puppies aged 14 - 20 weeks and weighing five - seven kg were divided into three experimental groups to induce different forms of serum sickness glomerulonephritis.

Group I: "One-shot" serum sickness GN:

11 dogs received an intravenous injection of BSA (crystalline fraction V, (Sigma Chemical Company Limited, London) in PBS, according to the protocols described as follows:-

Subgroup I: Seven dogs received a single dose of six gm BSA intravenously and were killed at four, seven, 10, 15 (three dogs) and 20 days.

Subgroup II: Two dogs received a single intravenous injection of 12 gm BSA; one dog was killed at 10 days and the other at 15 days.

Subgroup III: Two dogs received four successive daily intravenous doses of six gm BSA and killed on days 10 and 15 respectively.

Subgroup IV: A further two dogs were given a single dose of 5 ml of PBS intravenously and killed on days 10 and 15.

Group II: Accelerated serum sickness GN:

Subgroup I: 11 dogs were included in this experiment. All were immunized with BSA in the following manner:-

100 mg BSA dissolved in 2 ml PBS (pH 7.2) and mixed with 2 ml of Freunds complete adjuvant (Miles Research Products Division, Elkhart, Indiana) was injected subcutaneously on multiple sites on each dog. Subsequent doses of BSA and intervals between immunization

and death are summarized in Table 6.2.

Subgroup II: Control animals:

Four dogs received 2 ml of Freunds complete adjuvant subcutaneously and killed at 10 (two dogs), 15 and 25 days after injection.

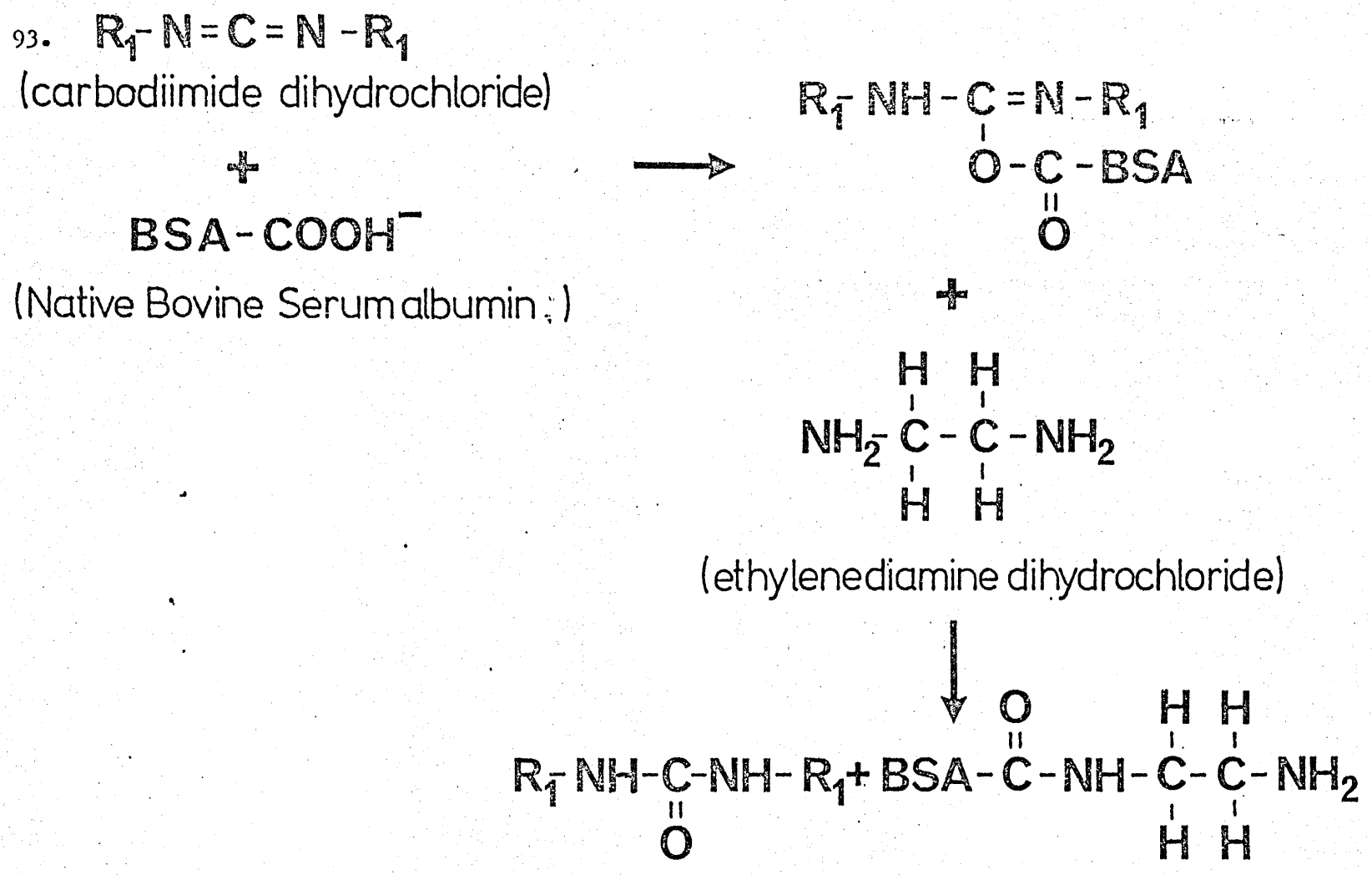
A further two dogs were immunized with 100 mg BSA together with 2 ml Freunds complete adjuvant and were killed 10 and 15 days respectively.

Group III: Chronic serum sickness GN:

Antigen preparation and characterization:

Crystalline fraction V BSA was used unmodified as native (anionic) as in Group I and II and also as substrate for the preparation of charge-modified cationic BSA. Cationization was carried out according to a modification of the method of Danon et al (1972) and Border et al (1982) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and crystalline ethylenediamine dihydrochloride (Sigma Chemical Company Limited, London). A solution of 330 gm ethylenediamine dihydrochloride in two litres distilled water was prepared. To this solution was added two litres distilled water containing 25 gm native BSA followed by 10 gm water soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide dihydrochloride and the final volume made up to five litres. The pH was adjusted to 4.75 by 1N HCl and the solution allowed to react overnight at 25°C (Fig. 93). The reaction was then terminated by the addition of 300ml 4M acetate buffer, pH 4.75, to react with any remaining carbodiimide. The BSA solution was dialized extensively against deionized water for 72 hours, its

Fig. 93.


R1-N=C=N-R1
(carbodiimide dihydrochloride)
+
BSA-COOH⁻
(Native Bovine Serum albumin)
R1-NH-C(=N-R1)-O-C(=O)-BSA
+
NH2-CH2-CH2-NH2
(ethylenediamine dihydrochloride)
R1-NH-C(=O)-NH-R1 + BSA-C(=O)-NH-CH2-CH2-NH2

volume reduced tenfold by dialysis against polyethylene glycol (B.D.H. Chemicals, Dagenham, Essex) and subsequently lyophilized and stored at -20°C.

The isoelectric point (pI) of each native and cationic BSA preparation was measured by isoelectrofocusing in thin layers of polyacrylamide gel according to the method of Eckersall and Conner (1984) using an LKB flatbed isoelectrofocusing unit (LKB Instruments Inc., Rockville, Maryland). Protein bands formed by native BSA at pH 4 - 5.1 and cationised BSA at pH 9.3- 9.5 (Fig. 94).

The molecular size of cationic BSA was compared to that of native BSA by Sephacryl S-300 gel filtration chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). The buffer employed was 0.1 mmol/l phosphate pH 7.0 plus 0.15 mmol/l NaCl. 100 mg of native BSA was applied to a column (90 x 2.6 cm) of sephacryl S-300 and after 20 fractions of 3 ml each had eluted 100 ml of cationised BSA was also applied. The samples eluted with an interval of 20 fractions and therefore showed no alteration in molecular weight due to the cationization process (Fig. 95).

The chloride content, as indicative of any chemical contaminations remaining after dialysis was determined by a chloride meter EEl 920, (EEl Halstead, Essex, England). Dialysis was continued until the chloride content of the sample after lyophilisation was within the physiological range ($<$ 100 mmol/l). This took up to three days of dialysis.

The BSA content was measured by spectrophotometry assuming a molar extinction coefficient of $E_{280}^{1\%} = 6.6$ for, BSA at 280 nm and showed 85% by weight of BSA in the final end product.

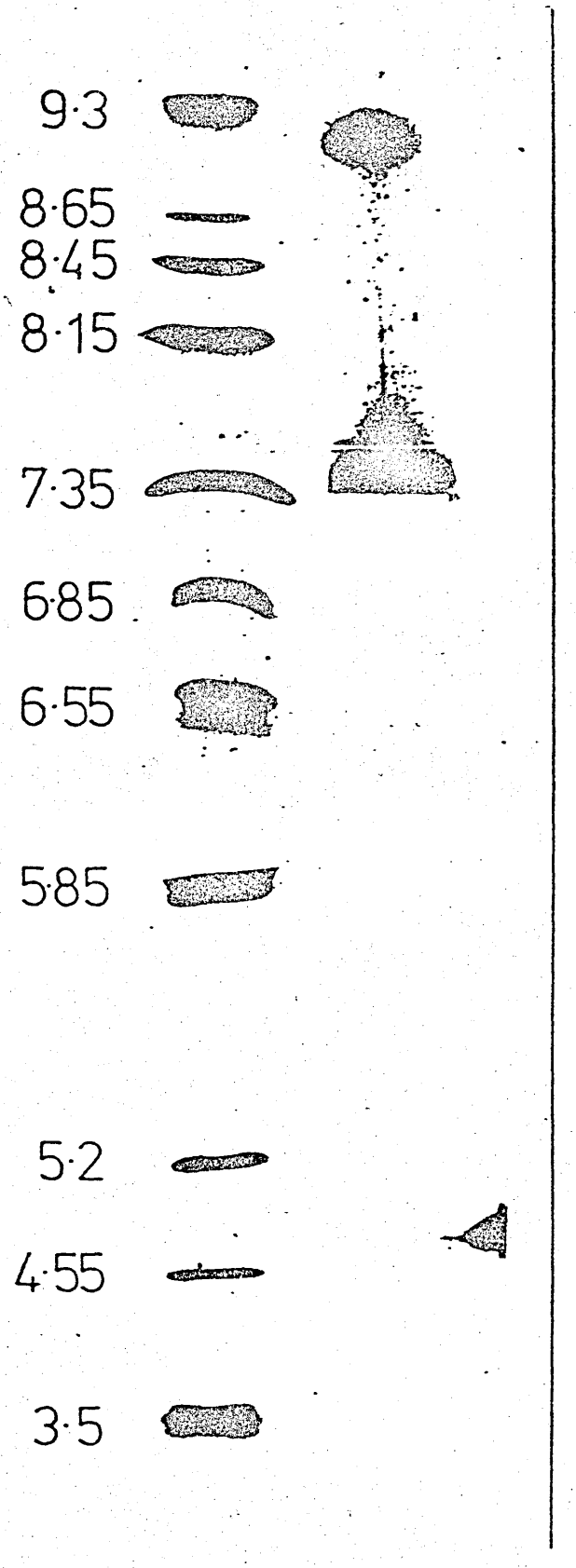
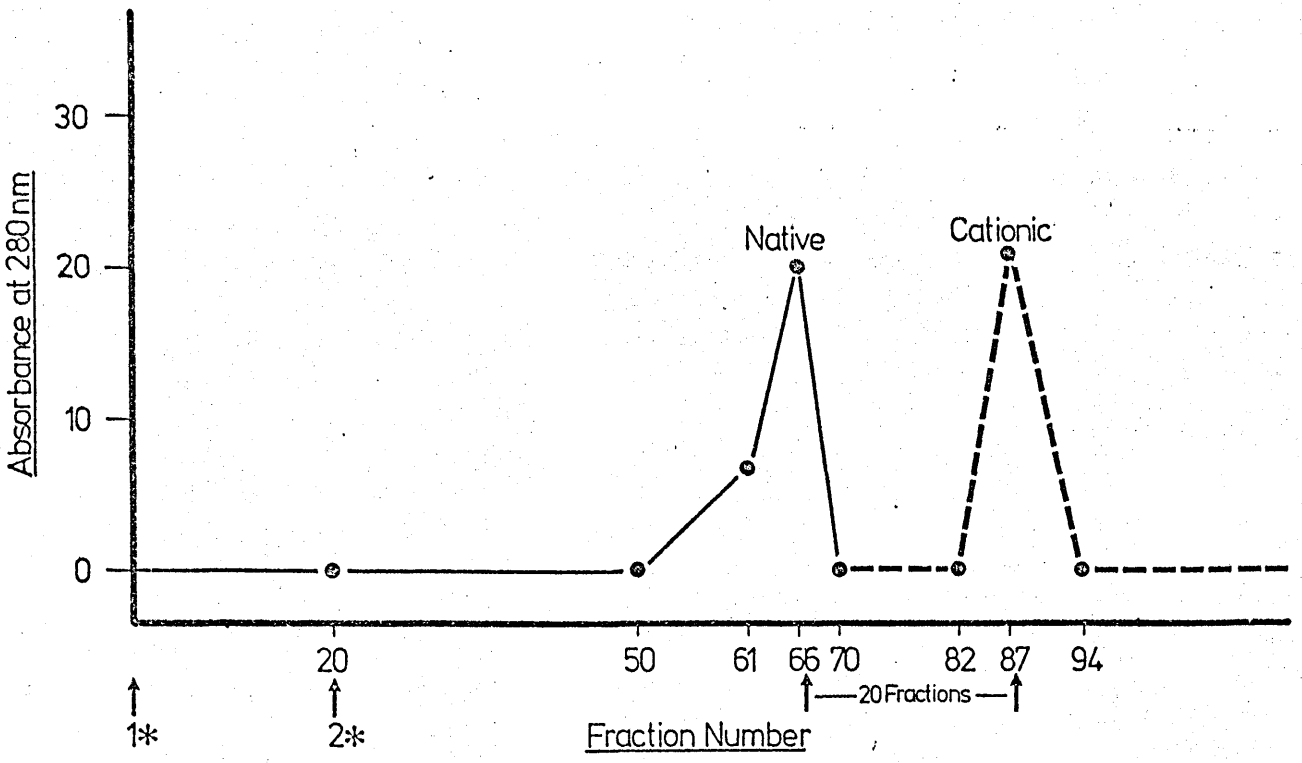


Fig. 94. Protein bands formed by cationic BSA and native BSA after isoelectrofocusing in polyacrylamide gel. cationic BSA Pl. 9.3 native BSA Pl. 4.55

Fig. 95. Comparison of change of modified BSA to Native BSA by gel filtration chromatography



Native (100mg) in 3ml Buffer + 2ml 50% Glycerol.

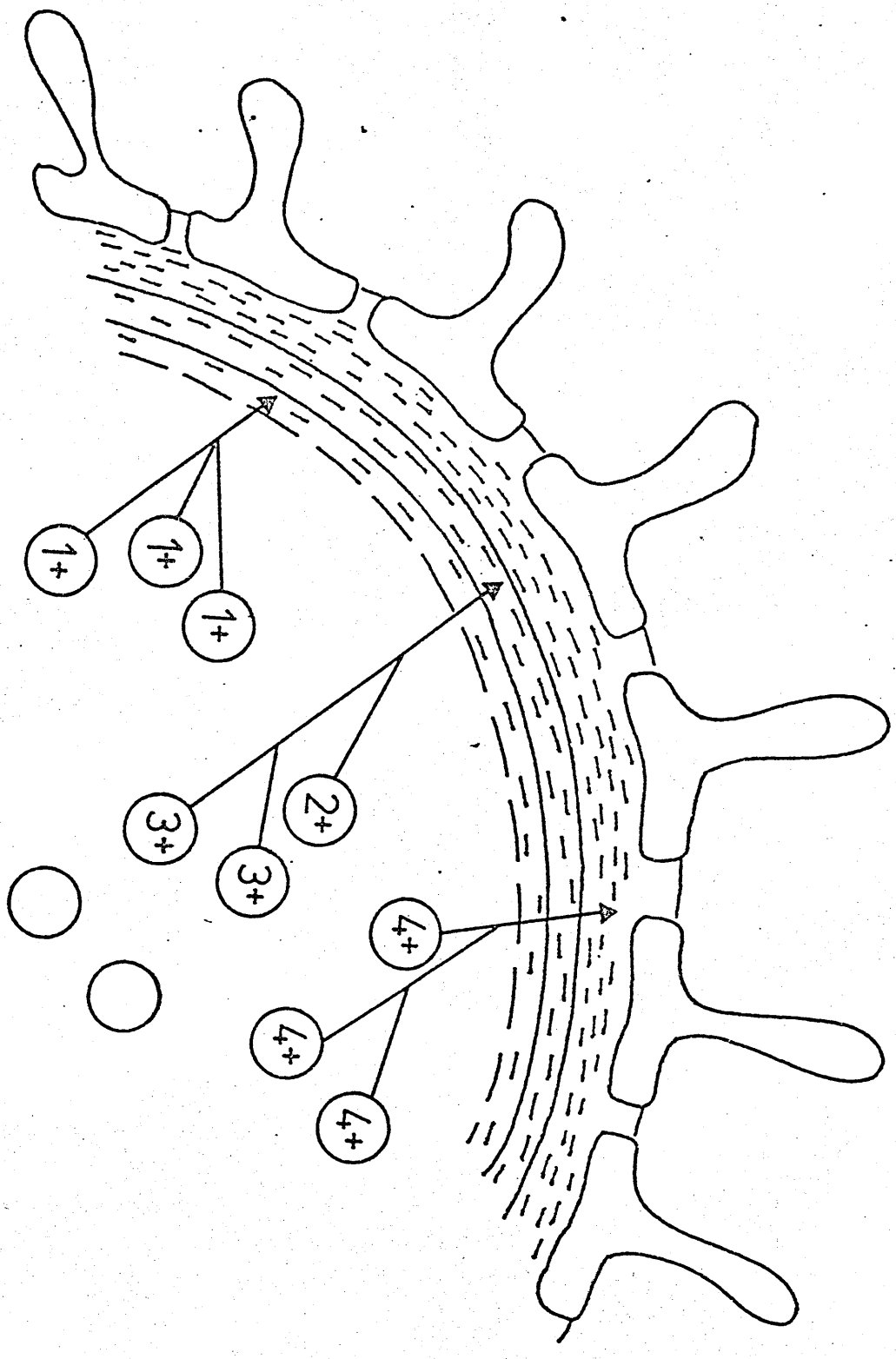
Cationic (100mg) in 3ml Buffer + 2ml 50% Glycerol.

1* = Native BSA applied

2* = Cationised BSA applied.

Flow Rate = 24ml/h Fraction Volume = 3ml

Fig. 96. A diagram showing the anionic sites of GBM.



Subgroup I: 12 dogs were immunized with an intravenous injection of five mg of cationized BSA containing one µg of endotoxin (Sigma Chemical Company Limited, London) as adjuvant in PBS (pH 7.2). One week later, intravenous injections of 120 mg of cationized BSA were begun and continued on a daily basis (five days/week) for four weeks, at which time (five weeks after initial immunization) the dogs were subjected to percutaneous renal biopsy, urine and blood examinations. The BSA injections were continued for the following three weeks at the end of which time no more BSA was administered and the dogs were sacrificed at intervals (see Table 6.1).

In addition, in order to establish fixation of cationized BSA to the glomeruli, three dogs were immobilized and then anaesthetized as described in Chapter 2. The abdominal cavity was opened, a 12 gauge cannula was inserted into the renal artery and five ml of PBS containing 10 mg cationized BSA was injected. All three animals were killed 20 minutes later.

Subgroup II: Two dogs were immunized intravenously with five mg native BSA and one µg of endotoxin in PBS (pH 7.2). One week later, daily injections of 120 mg of native BSA were begun and continued as described for the animals in Subgroup I.

Subgroup III: Two dogs were immunized with five µg cationic BSA as above and daily intravenous injections of 2 ml PBS (pH 7.2) were given in place of BSA as described for the animals in Subgroup I.

Production of antisera:

Antisera to BSA was raised in rabbits. These were immunized with 50 mg BSA emulsified in Freunds complete adjuvant injected at multiple subcutaneous sites. Two booster doses were given at three

week intervals. The rabbits were bled from the marginal ear vein 10 - 15 days later.

Ammonium sulphate precipitation of IgG:

25 ml of a saturated solution of chilled ammonium sulphate was slowly added to 50 ml serum. This was centifugated at 3,000g for 30 minutes at 4°C. The supernatant was discarded and the precipitate redissolved and dialysed extensively in phosphate buffered saline (PBS, pH 7.4).

Preparation of fluorochrome conjugated anti-BSA:

Anti-BSA antibody was conjugated to fluorescein isothiocyanate (FITC) (Calbiochem, San Diego, U.S.A.) by adding dry FITC to a solution containing 10 mg protein/ml in 0.15 mol/1 NaCl and 10% by volume of carbonate buffer adjusted to pH 9.5. This mixture was mixed for one hour at 4°C.

Purification:

The unreacted fluorescent material was removed by gel filtration in a Sephadex G-25 column. Filtration was carried out at 4°C. The column volume was approximately six times the sample volume. The eluate was then dialysed against PBS for 48 hours at 4°C.

Peroxidase-antiperoxidase (PAP) method:

Specimens from biopsies and necropsies of chronic serum sickness GN were examined with the PAP method, according to minor modifications of the technique outlined by Sinclair et al (1981).

Method:

Three µm paraffin sections were dried overnight at 37°C, dewaxed in xylene and hydrated. The prewarmed sections were digested with 0.1% protease type VII (Sigma Chemical Company, London) in Tris buffer brought to pH 7.8 with 0.1N NaOH. The solution was warmed to 37°C and the pH checked again. The digestion process lasted for 30 - 45 minutes, then the digestion was terminated in cold running tap water for 10 minutes and the sections transferred to two changes of phosphate buffered saline PBS (pH 7.4) over 10 minutes. Non-specific reaction was blocked by exposing the sections to normal swine serum (NSS) diluted 1/5 (all dilutions were performed in 1% ovalbumin in PBS) for 10 minutes. The excess swine serum ~~serum~~ was drained off and the primary antisera rabbit anti-dog IgG (1/800) was applied for 30 minutes. Then the sections were washed in four changes of 1% NSS in PBS over 20 minutes.

The endogenous peroxidase activity was blocked with a fresh 0.5% solution of hydrogen peroxide in methanol for 10 minutes; then the sections were washed in 1% NSS in PBS for 10 minutes. The sections were incubated with unlabelled swine anti-rabbit IgG (1/20) for 30 minutes and washed in four changes of 1% NSS in PBS for 20 minutes. They were then incubated with rabbit PAP soluble complexes (1/50) for 30 minutes and washed in four changes of 1% NSS in PBS for 20 minutes. The sections were then treated with a fresh solution of 0.05% 3-3-diaminobenzidine (DAB) (Sigma Chemical Company Limited, London) in 0.01% hydrogen peroxide in PBS. The reaction was monitored microscopically up to 5 minutes. Finally, the sections were washed in running tap water, counterstained with Harris's haematoxylin, dehydrated, cleared and mounted.

Acute "one-shot" Serum Sickness GN:

All dogs which received a single large dose of BSA survived the experiment and were clinically normal when killed. At necropsy, no gross abnormalities were found in any kidney. Only two dogs (W997, W1002) showed mild proteinuria (56 mg/100ml and 75 mg/100ml respectively). The other dogs had normal protein concentrations in the urine (i.e. $<$ 50 mg/100ml). Blood urea levels were all within normal range (i.e. $<$ 6 mmol/l).

Light microscopic findings:

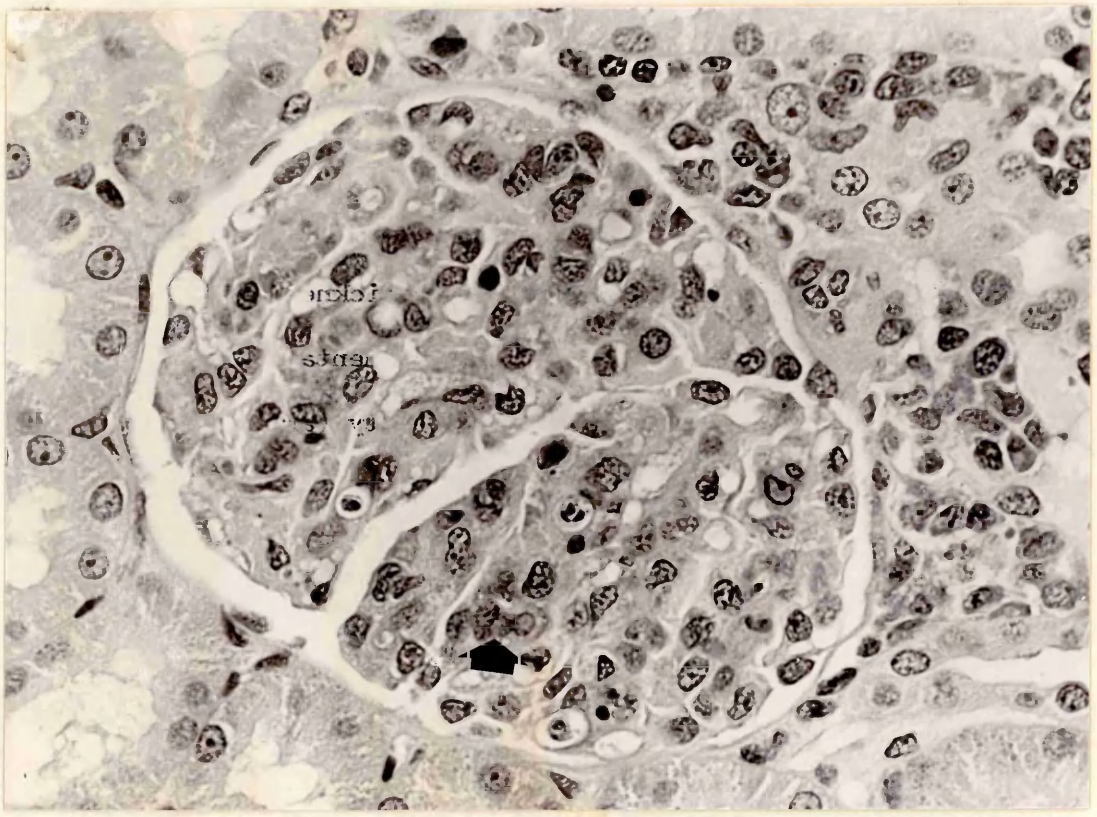
Focal glomerular changes were observed in over 80% (9/11) of animals and, as detected by light microscopy, these varied from animal to animal and from glomerulus to glomerulus. Some glomeruli were entirely normal while, in others, there was segmental mesangial hypercellularity (Fig. 97); these lesions were observed as early as seven days after injection of BSA. In two animals (W997, W1002) more severe, although still focal, glomerular lesions were observed; these consisted of swelling of the tuft, occlusion of glomerular capillaries due to striking mesangial hypercellularity and expansion (Fig. 98) and periglomerular infiltration by lymphocytes and plasma cells.

Ultrastructural findings:

With the TEM, scattered electron dense deposits were observed in the mesangium in four of the 11 dogs (Fig. 99). Other mesangial changes, characterized by increased mesangial matrix and cellularity with active pseudopodia invading the axial region of the capillary loops, were also noted (Fig. 100). Swollen endothelial cells and

Fig. 97. Acute "one-shot" serum sickness GN. A glomerulus showing segmental mesangial hypercellularity (arrow) H & E x 400.

Fig. 98. Acute "one-shot" serum sickness GN. Two glomeruli are showing swelling of the tuft, occlusion of glomerular capillaries and mesangial expansion and hypercellularity. H & E x 250.



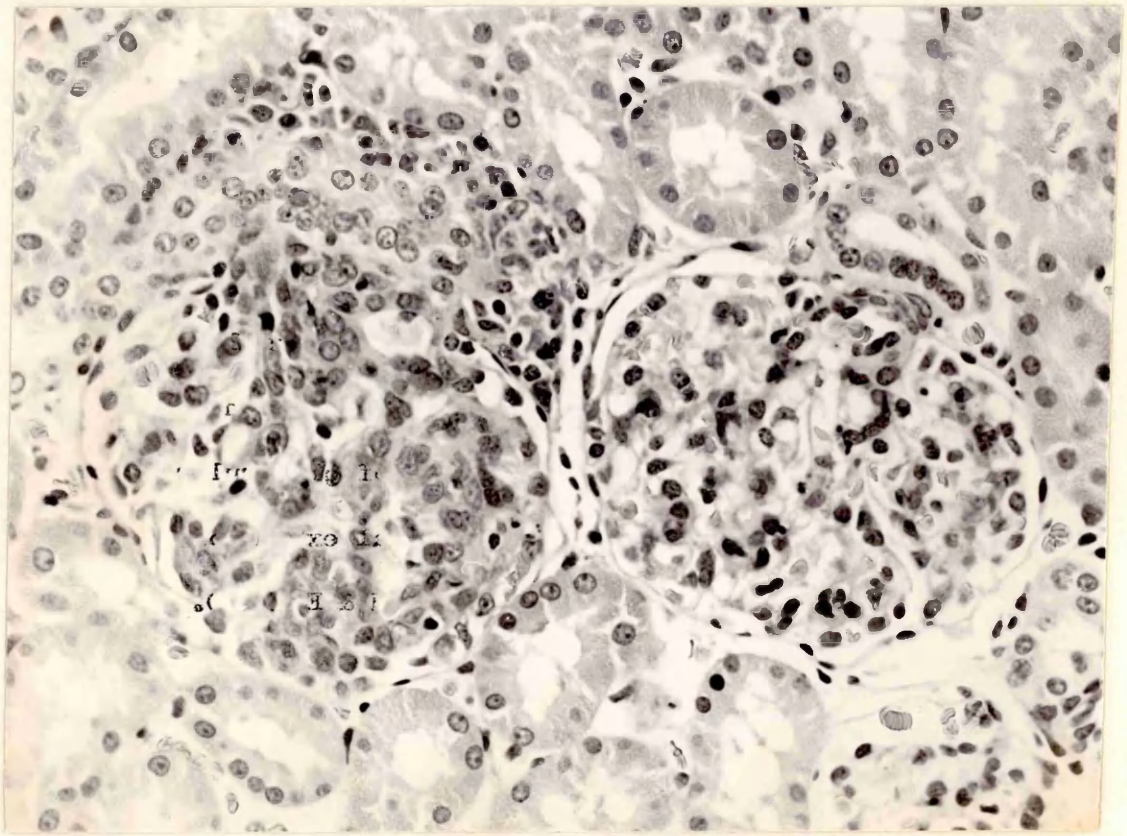
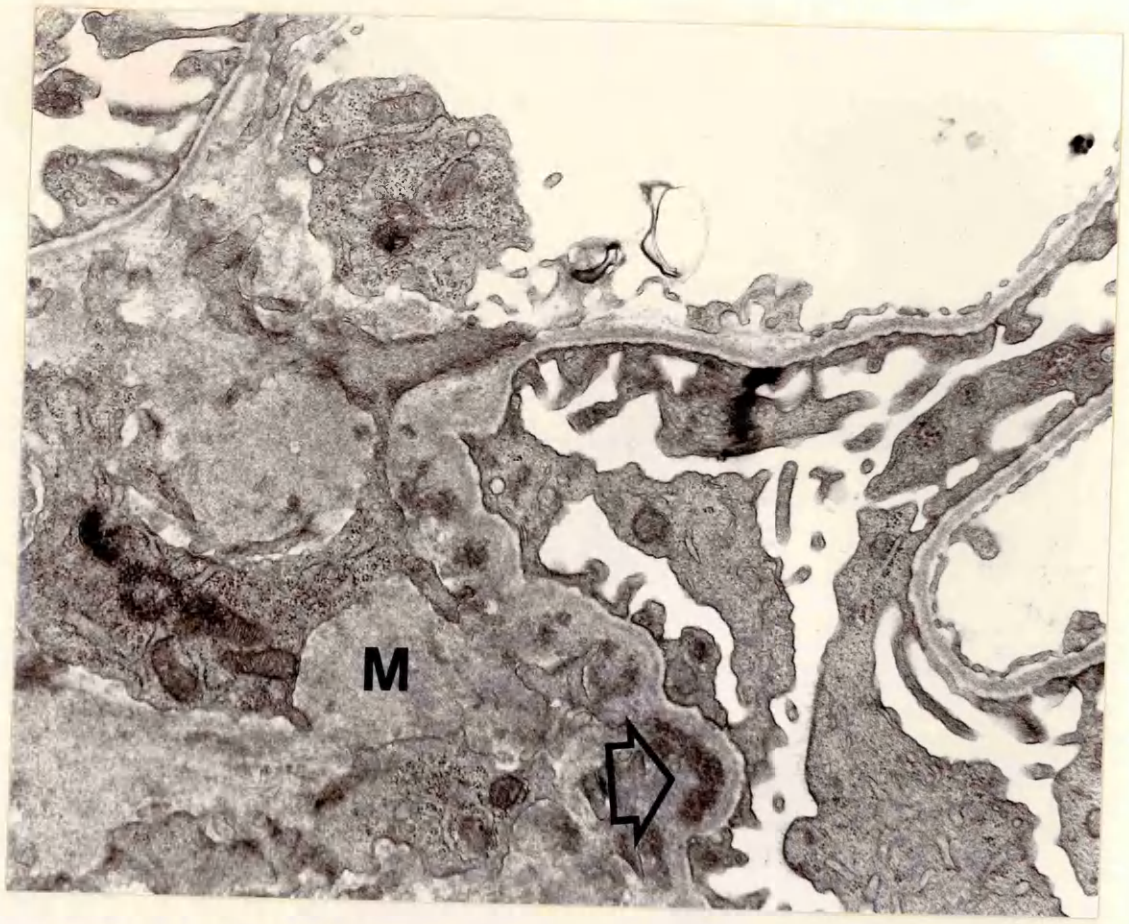
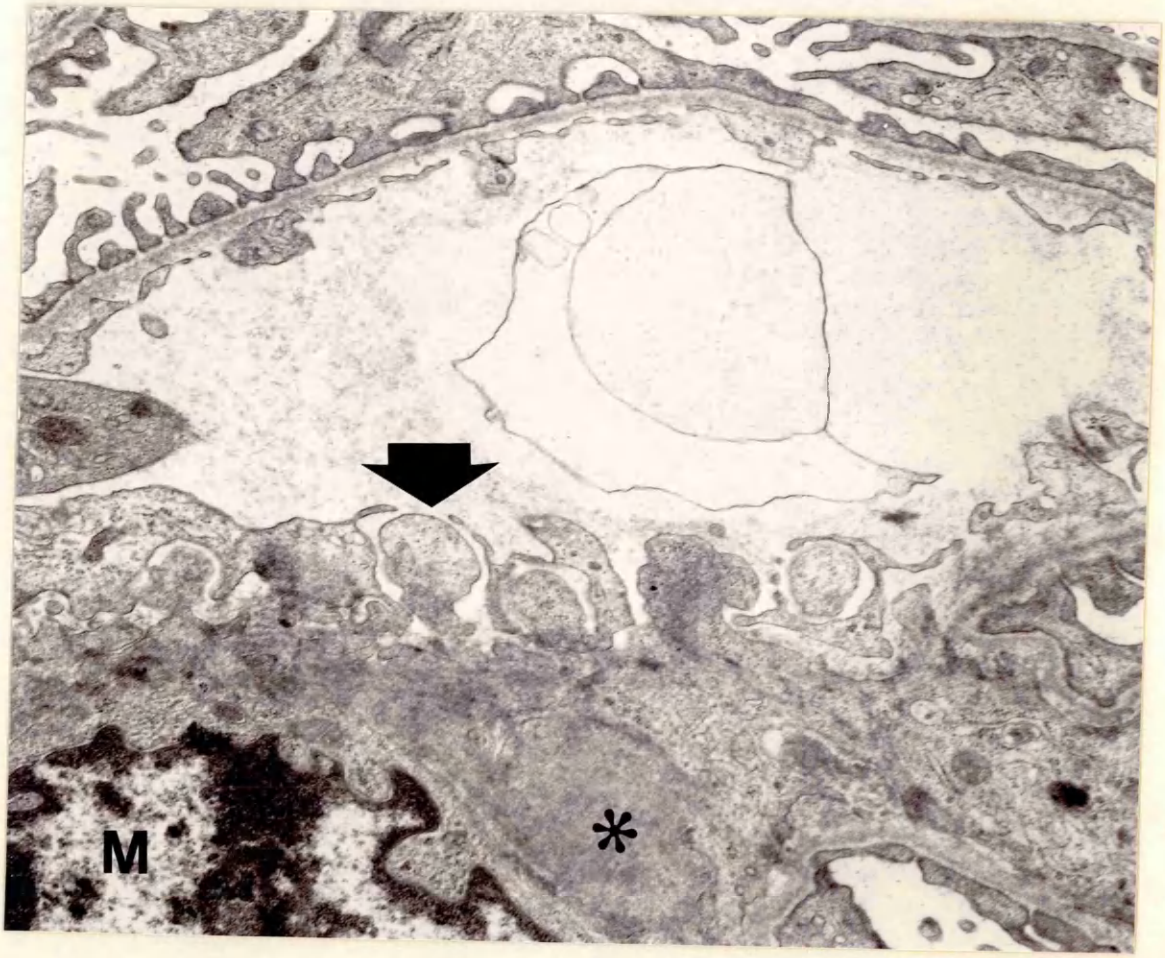


Fig. 99. Acute "one-shot" serum sickness GN. Electron dense deposits (arrow) in the mesangium. Mesangium (M). TEM x 6,800.

Fig. 100. Acute "one-shot" serum sickness GN. Note the active pseudopodia invading the axial region of the capillary loop (arrow) and electron dense deposits in the mesangium (asterisk). TEM x 6,800.


M


M
*

infiltration of polymorphonuclear cells often combined to occlude the lumen of capillaries. To a lesser extent localized subendothelial expansion and partial fusion of the epithelial foot processes were also recorded.

With the SEM, the most obvious feature in the most severely affected glomeruli was the presence of numerous surface microvilli and blebs on the visceral epithelium and swelling and effacement of the normally orderly interdigitating arrangement of the foot processes of the visceral epithelium (Figs. 101, 102). In every animal, however, many normal glomeruli were still to be found. with corrosion casts, most glomeruli showed normal capillary arrangement. In all animals, however, a few glomeruli (approximately 10 per cent) showed localized dilation of capillaries and leakage of cast material into the urinary space (Fig. 103).

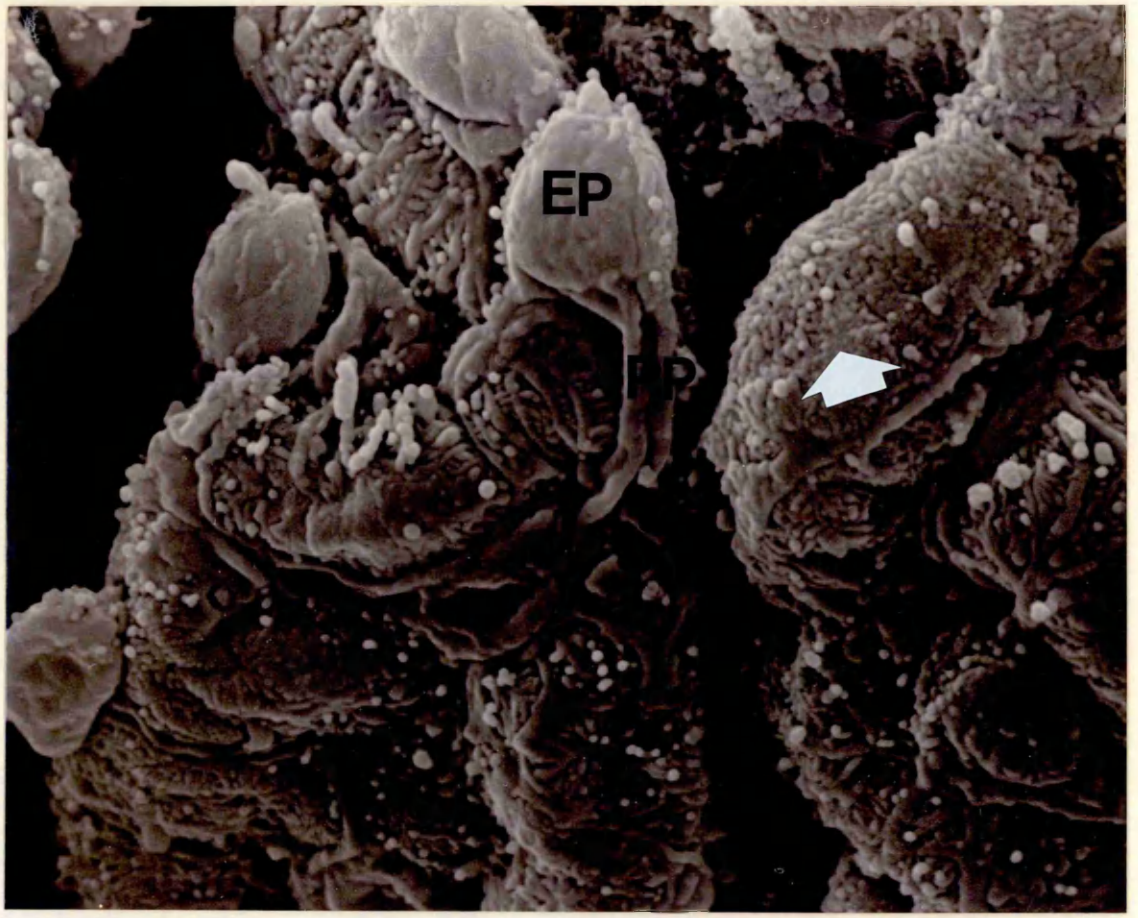
The immunofluorescence studies are summarized in Table 6.1. Ten of the 11 dogs showed granular deposition of C_3 mainly confined to the mesangium but also along the capillary walls (Fig. 104). Patchy linear deposition of C_3 along the tubular basement membrane were also noted in these animals (Fig. 105).

Seven of the 11 dogs had segmental granular deposition of IgG mostly localized in the mesangium (Fig. 106), although some deposits appeared to be related to the capillary wall. BSA was not detected.

Control Animals: (Subgroup IV):

No histological or ultrastructural changes were observed; likewise IgG and C_3 were not detected in the glomeruli.

Figs. 101, 102. Acute "one-shot" serum sickness GN. Visceral epithelial cells showing roughened irregular surface and alteration in the normally well organized arrangement of foot processes (arrows). EP = epithelial cell body; PP = primary processes; SP = secondary processes. SEM x 2,500, 5,000.


EP

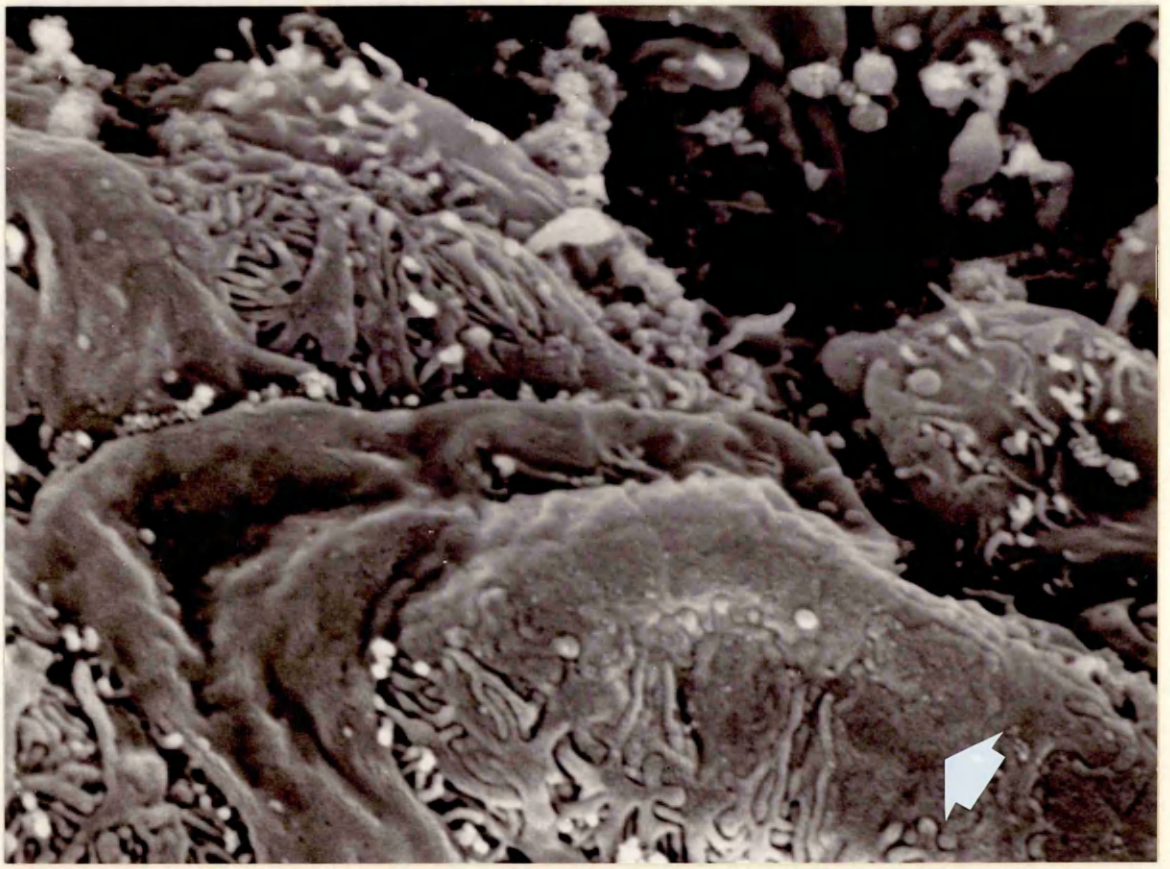


Fig. 103. Tensol casts of three glomeruli can be seen. One is normal, a second shows marked leakage of cast material into the urinary space while, in the third, the capillaries are poorly filled and blindly ending.
SEM x 160.

Fig. 104. Acute "one-shot" serum sickness GN. Note the granular, mostly mesangial, deposition of C_3. Immunofluorescence x 170.

Fig. 105. Acute "one-shot" serum sickness GN. Note the patchy linear deposition of C_3 along the tubular basement membrane. Immunofluorescence x 170.

Fig. 106. Acute "one-shot" serum sickness GN. Note the granular segmental deposition of IgG confined to mesangial areas. Immunofluorescence x 170.

TABLE 6.1

EXPERIMENTAL "ONE-SHOT" SERUM SICKNESS

Dog No.	Dose of BSA	Day Killed	Glomerular Lesions*	Immunofluorescence**		Electron Dense Deposits
				IgG	C_3	
W1006	6G	4	-	-	-	No
W1005	6G	7	1+	-	1+	No
W996	6G	10	1+	-	1+	No
W1000	12G	10	1+	-	1+	No
W998	24G*	10	1+	1+	2+	Yes (Mes.)
W997	6G	15	3+	1+	2+	Yes (Mes.)
W1002	6G	15	2+	1+	1+	No
W1004	6G	15	1+	2+	2+	Yes (Mes.)
W1001	12G	15	1+	1+	1+	No
W999	24G*	15	-	1+	1+	No
W1003	6G	20	1+	1+	2+	Yes (Mes.)

* Divided into 4 daily doses of 6G.

** Lesions judged to 1+ to 4+ according to severity.

Accelerated Serum Sickness GN:

All dogs in this group survived the experiment and were clinically normal when killed. At necropsy no gross abnormalities were found in any kidney. Only one dog (W1013) showed significant proteinuria (105 mg/100ml); the other dogs had normal protein concentration in the urine (i.e. < 50 mg/100ml) blood urea levels were also within normal range (i.e. < 6 mmol/1).

Light microscopic findings:

Mild glomerular changes were observed in eight of the 11 experimental dogs (Table 6.2). These were focal in nature and characterized by swelling of the tuft, occlusion of capillaries and segmental mesangial expansion and hypercellularity (Fig. 107). In addition, one dog (W994) showed segmental and even global areas of necrosis in a few glomeruli (Fig. 108).

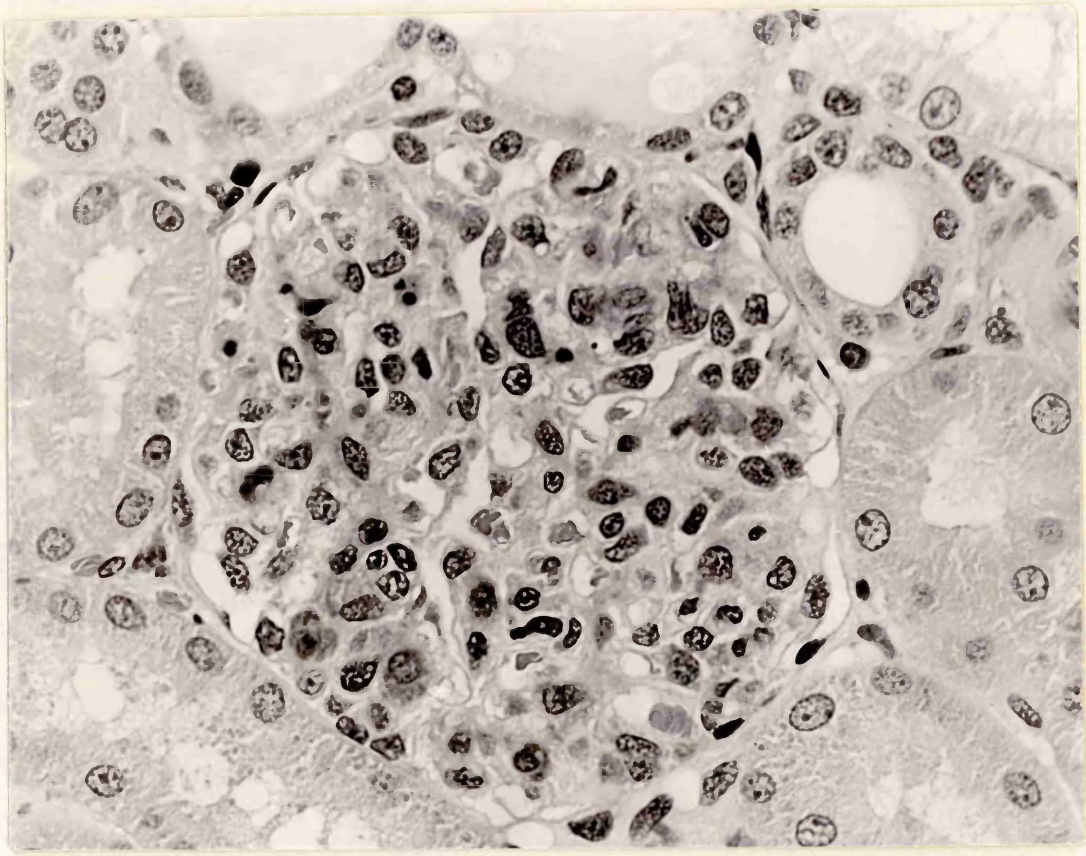
Extraglomerular lesions in the form of necrosis of the wall of some interlobular and afferent arterioles, the presence of hyaline casts in tubules (Figs. 109, 110) and focal periglomerular and perivascular infiltration of plasma cells, macrophages and lymphocytes were also noted.

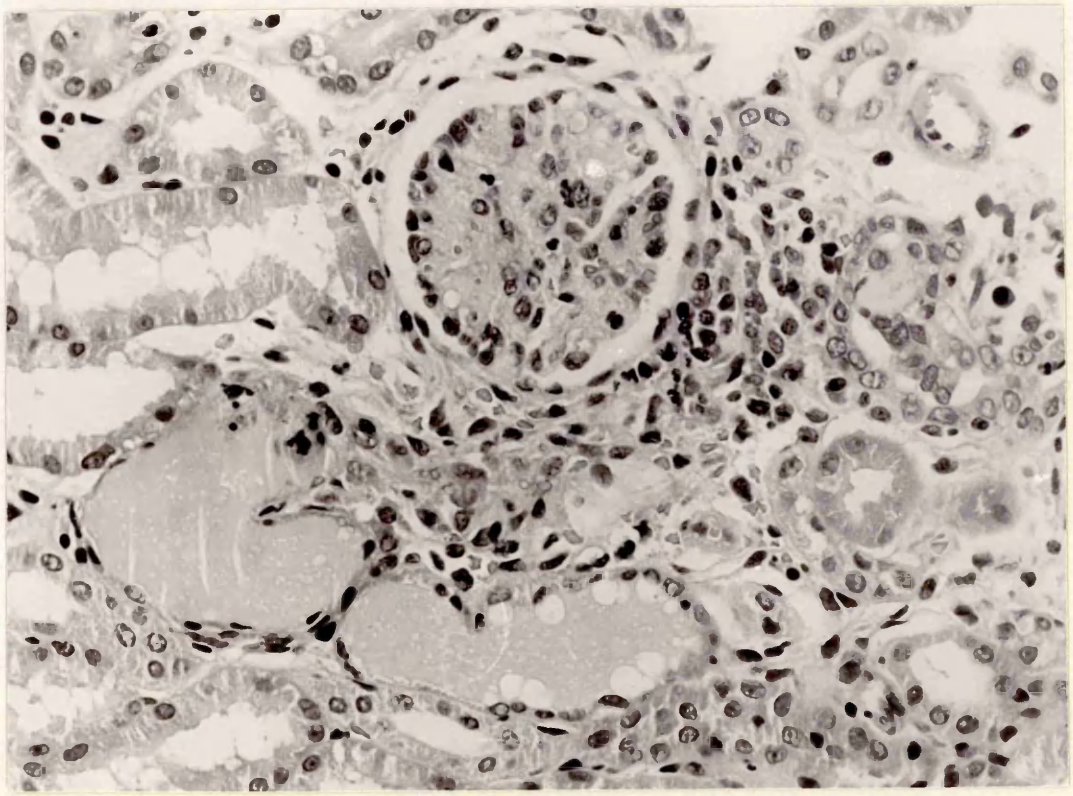
Ultrastructural findings:

With the TEM, six of the 11 dogs showed scattered mesangial electron dense deposits (Figs. 111, 112). The deposits corresponded to the immunofluorescence findings as set out below (Table 6.2). In addition, localised expansion of the subendothelial space with occasional detachment of swollen endothelial cells were also observed.

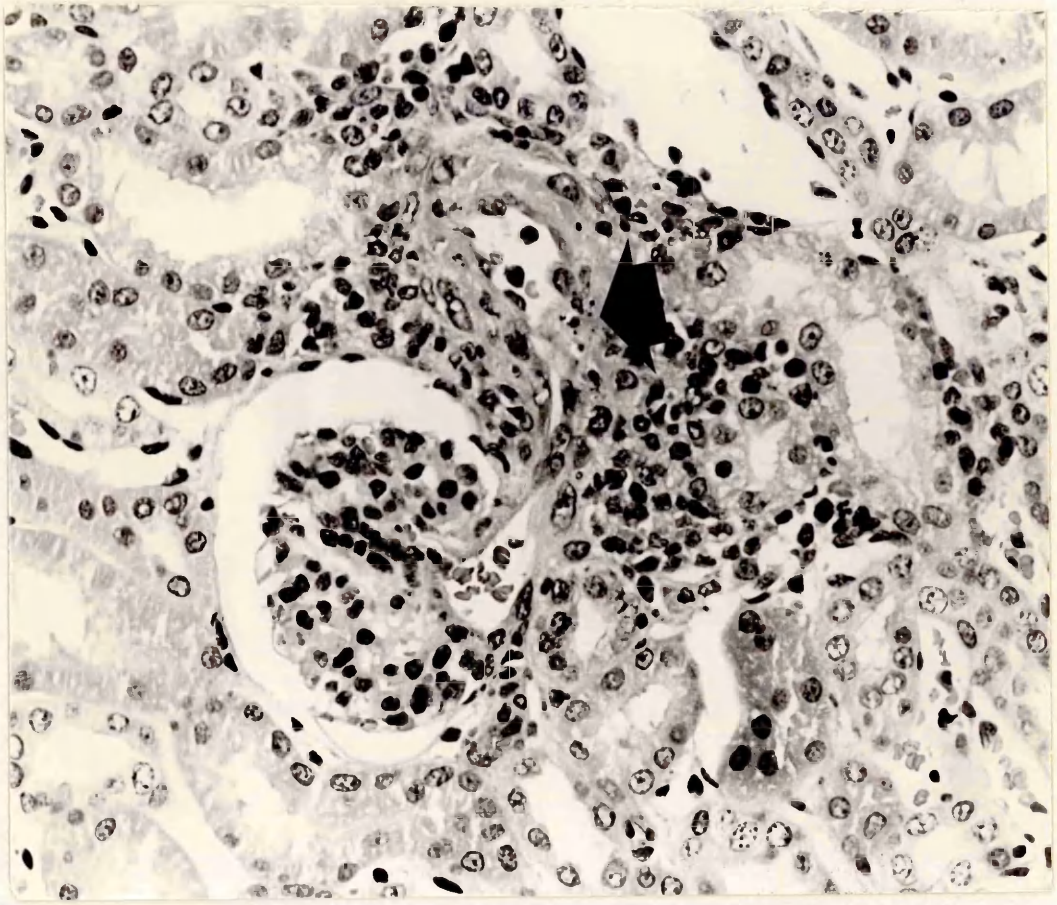
Fig. 107. Accelerated serum sickness GN.
A glomerulus shows swelling of the tuft and segmental mesangial expansion and hypercellularity.
H & E x 400.

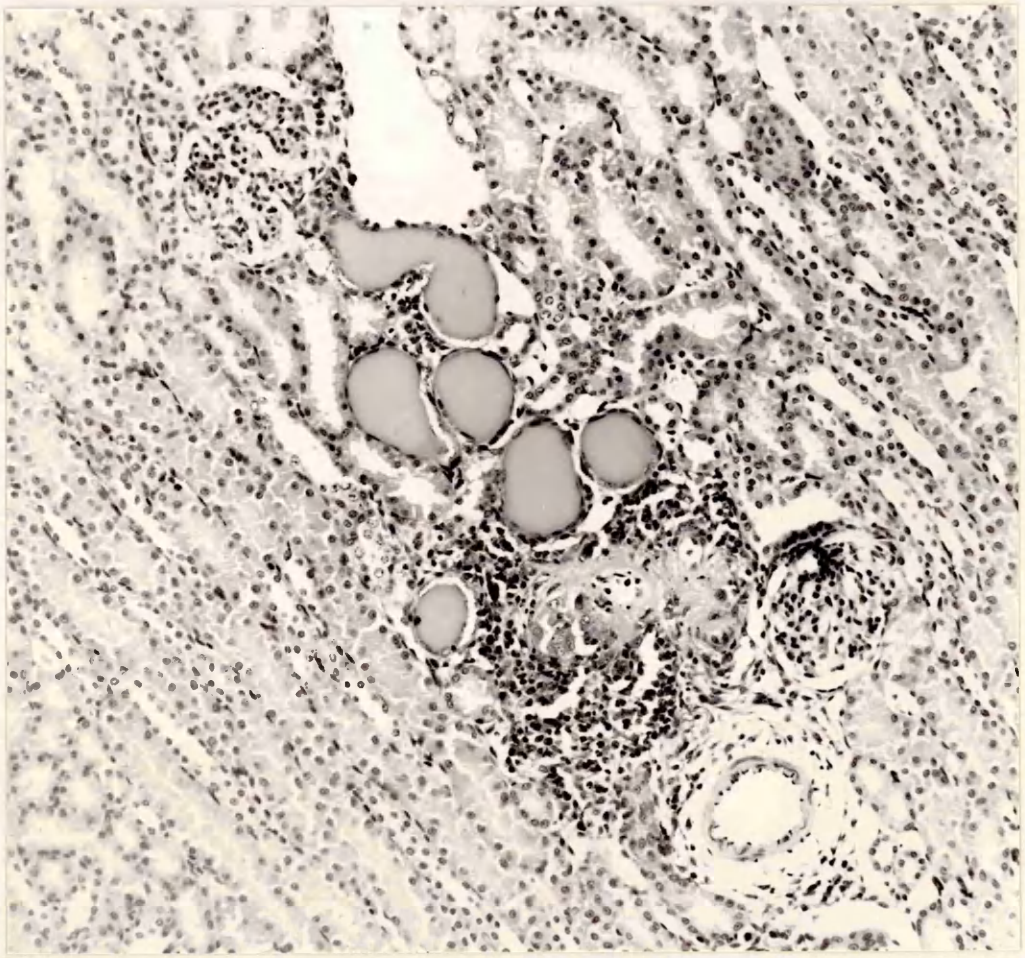
Fig. 108. Accelerated serum sickness GN.
A glomerulus shows global necrosis. Note the periglomerular infiltration by lymphocytes and plasma cells and the protein casts in the adjacent tubules. H & E x 180.



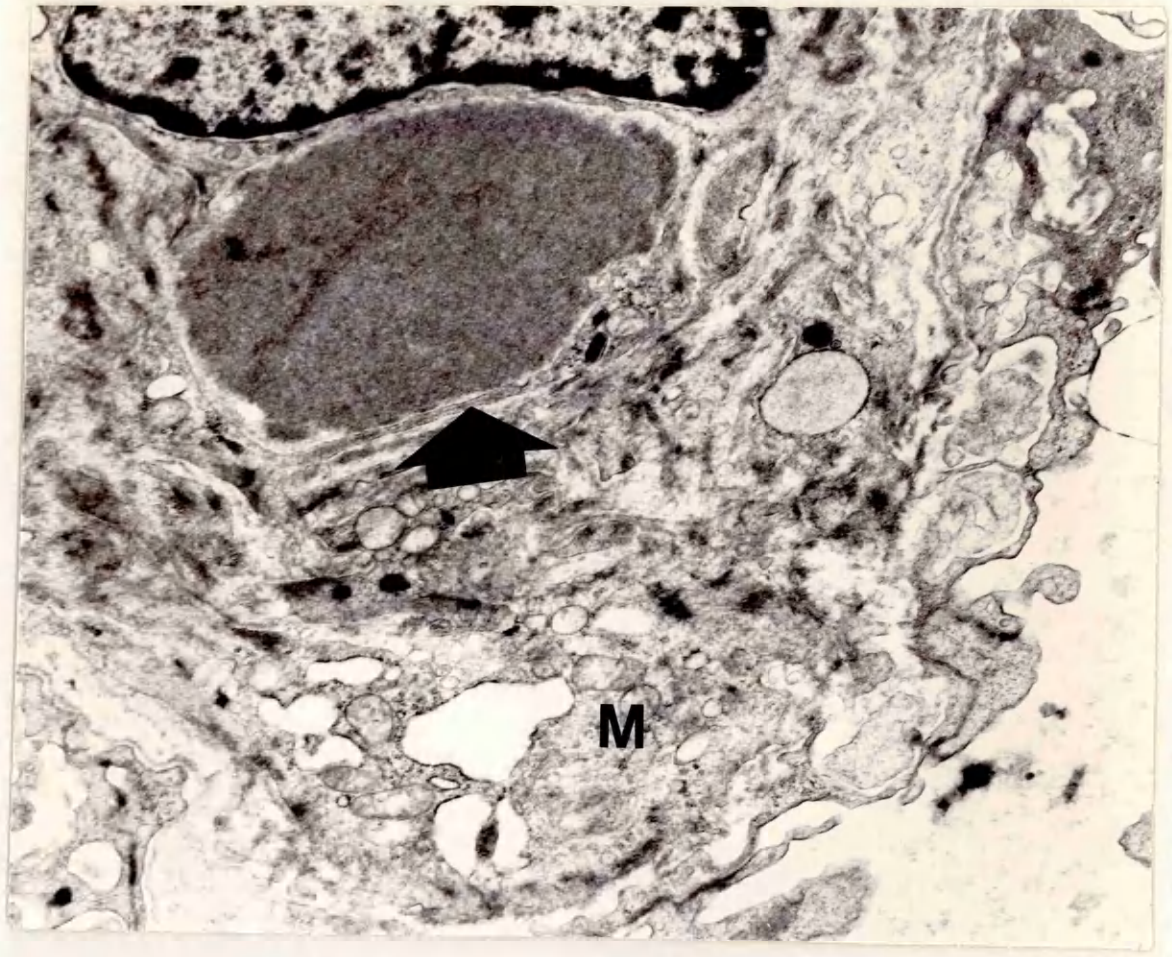


Figs. 109, 110. Accelerated serum sickness GN. Note the necrosis of an afferent arteriole (arrow) and presence of hyaline casts in the tubules. H & E x 180, 120.





Figs. 111, 112. Accelerated serum sickness GN. Note the electron dense deposit (arrow) in the mesangium. E = endothelial cell; M = mesangial cell. TEM x 8,000, 8,000.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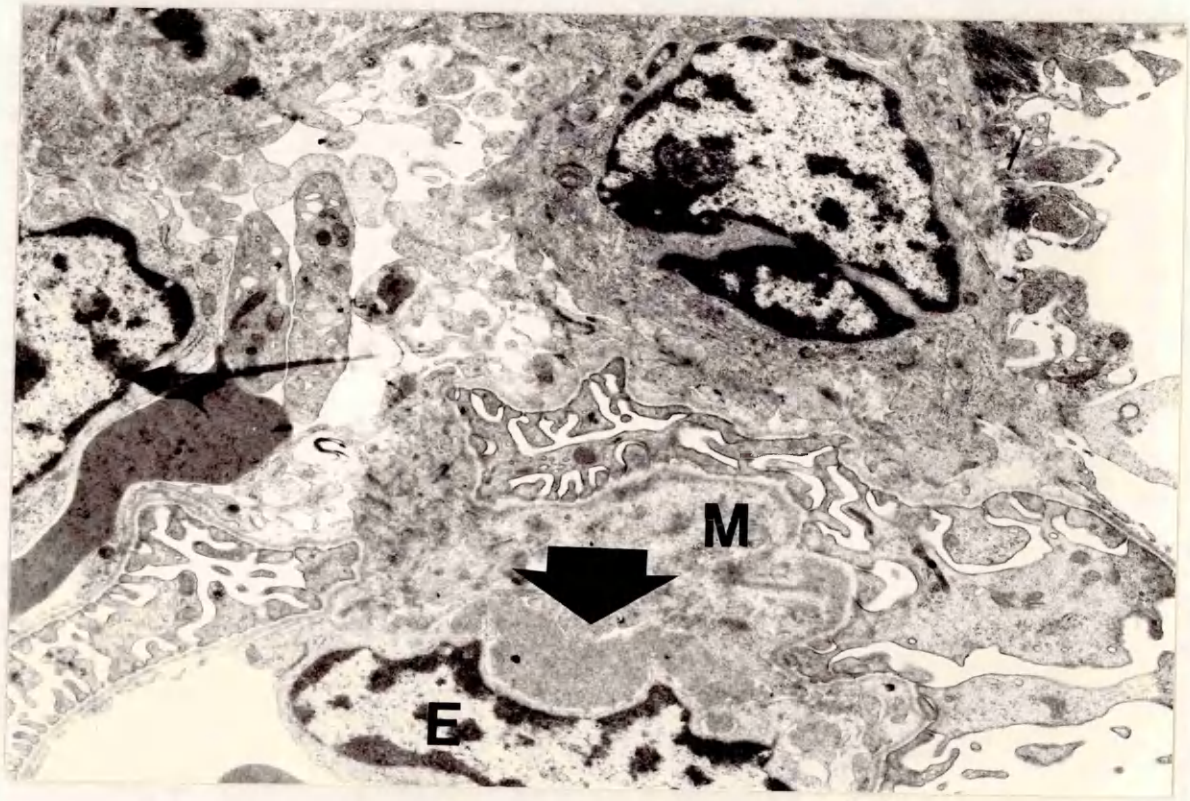

M
Б

All 11 dogs, but particularly those with electron dense deposits, showed varying degrees of mesangial expansion and hypercellularity (Fig. 113) and mesangial pseudopodia were often to be seen pushing their way into the capillary lumina, in so doing displacing the endothelial cells (Fig. 114). Circulating monocytes, mononuclear cells, polymorphonuclear leukocytes were sometimes found lodged in the capillaries. Fusion of epithelial foot processes was minimal or absent. With the SEM, swelling and partial effacement of foot processes with the appearance of surface blebs on the epithelial cell body were common findings in all 11 dogs (Fig. 115).

Immunofluorescence findings:

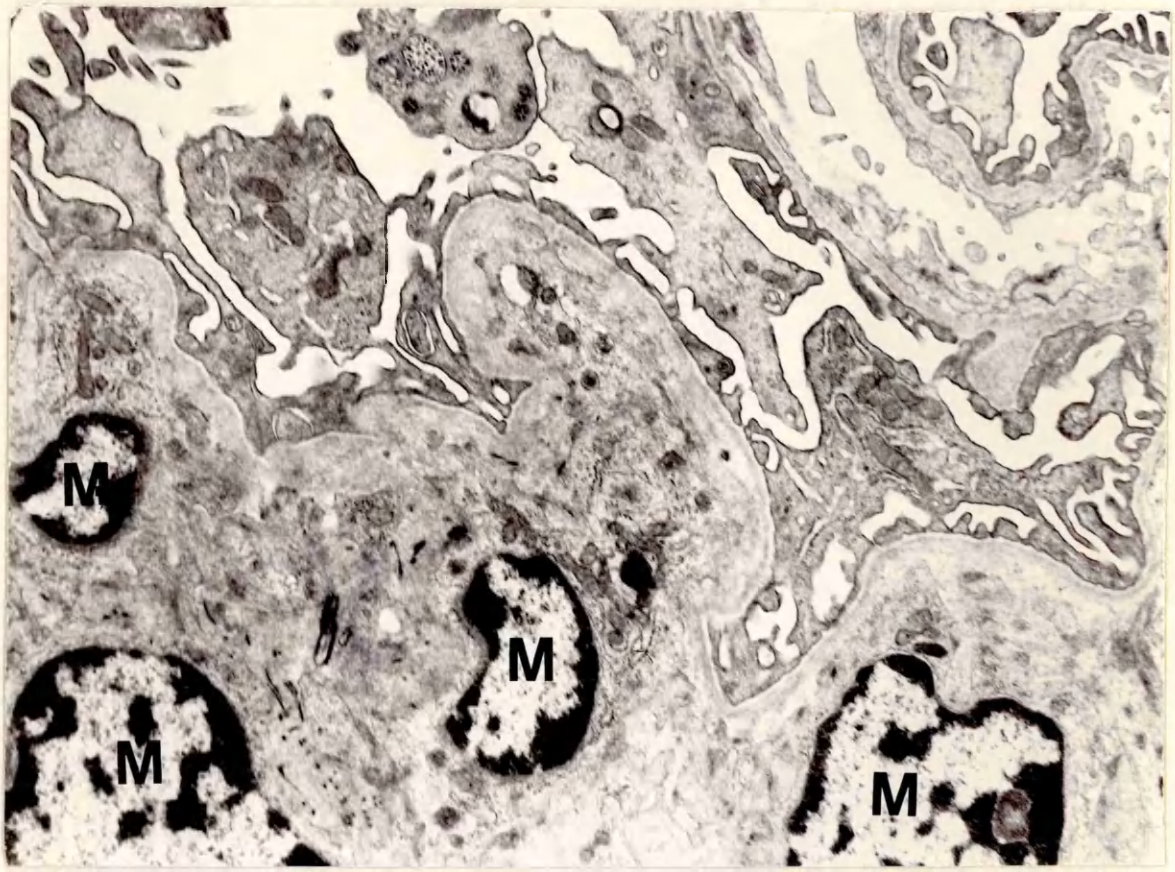
The results are summarized in Table 6.2. Five of the 11 experimental dogs showed granular deposition of IgG which was confined to the mesangial region (Fig. 116). Deposits of IgG were also observed at the hilar region of some glomeruli, and in vessel walls. C_3 was detected in 10 of the 11 dogs and again was mainly mesangial (Fig. 117). Granular deposition of C_3 was also found in vessel walls (Fig. 118) and patchy linear fluorescence of the TBM was a frequent occurrence. BSA was not detected.

Control Animals: (Subgroup II):

No histologic or ultrastructural changes were observed; likewise no IgG, BSA or C_3 were detected in the glomeruli.

Fig. 113. Accelerated serum sickness GN. Note the mesangial expansion and hypercellularity. M = mesangial cell. TEM x 6,800.

Fig. 114. Accelerated serum sickness GN. Note the active mesangial pseudopodia (arrow) displacing the endothelial cell (E). TEM x 6,800.


M
M
M
M

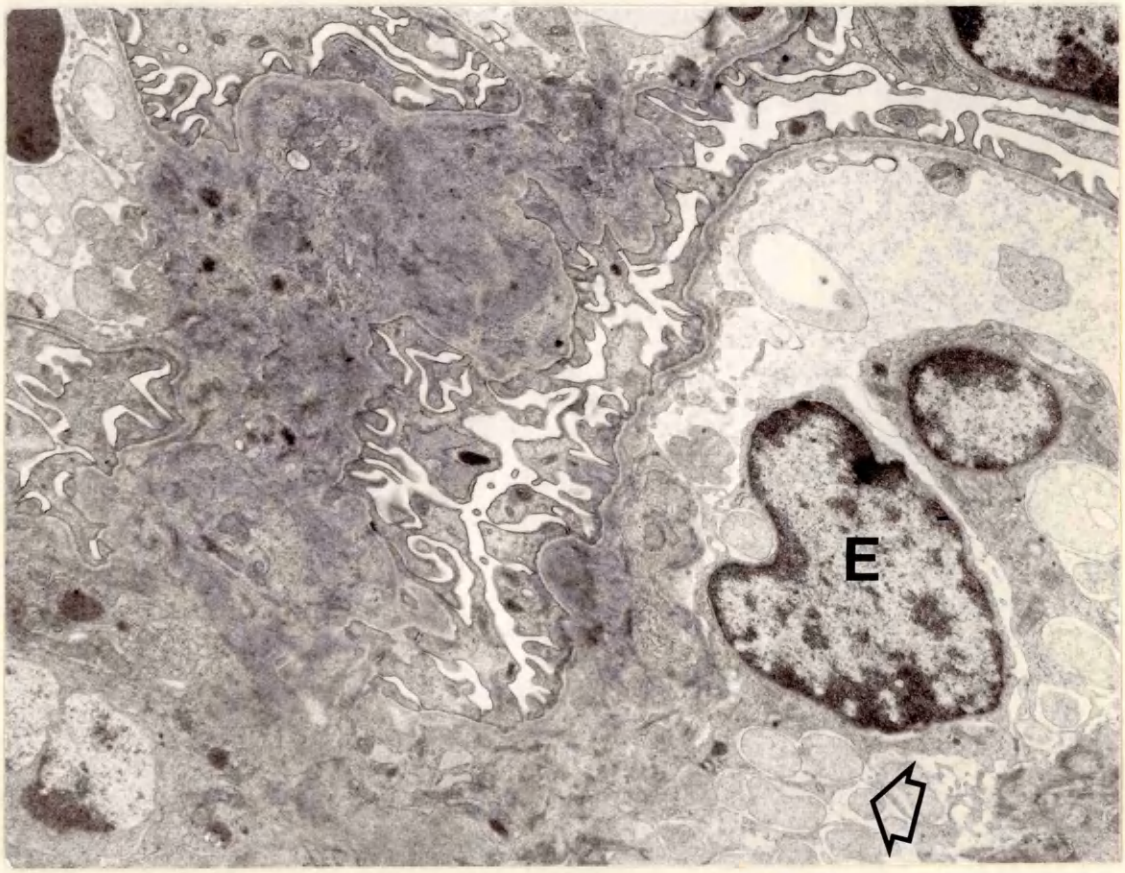

E

Fig. 115 Accelerated serum sickness GN. Note the partial effacement of foot processes (arrow). SEM x 2,800.

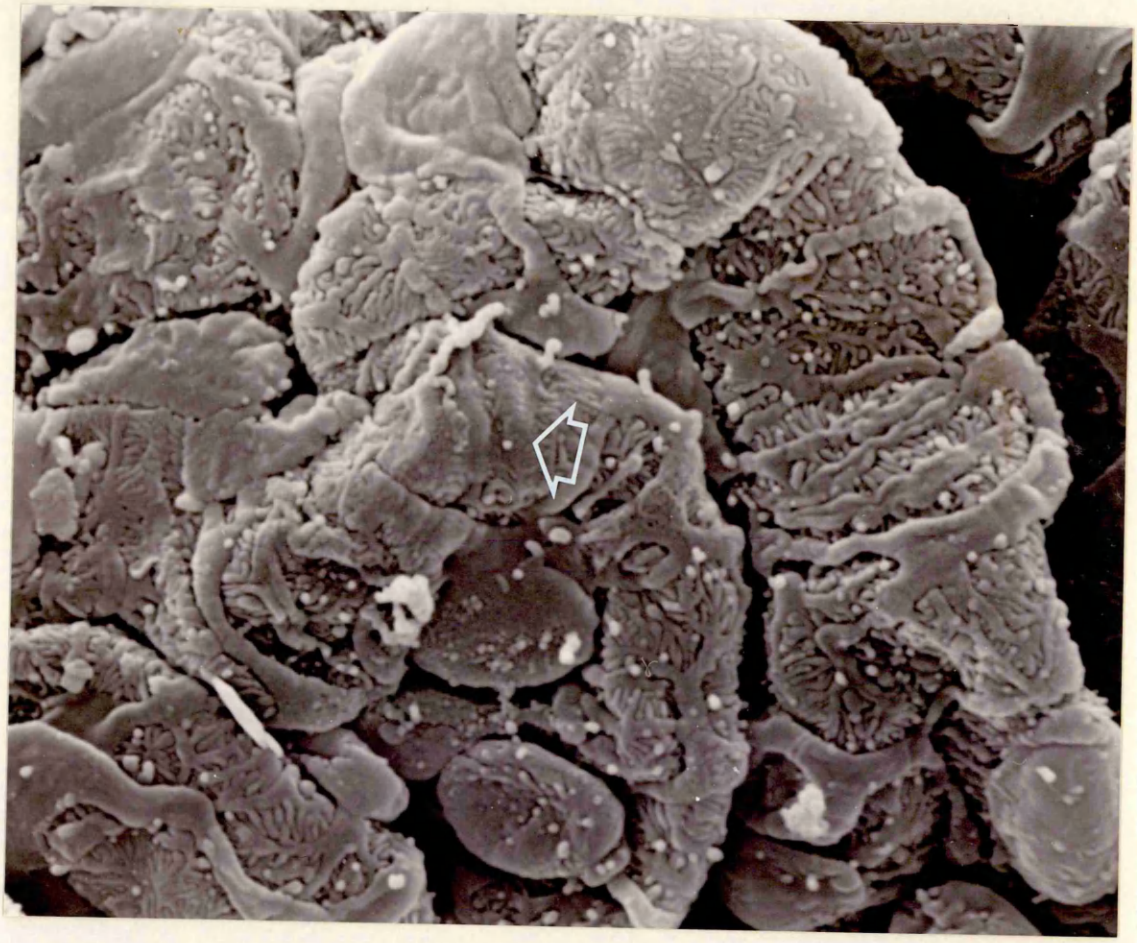


Fig. 116. Accelerated serum sickness GN. Note the granular deposition of IgG in the mesangial region. Immunofluorescence x 170.

Fig. 117. Accelerated serum sickness GN. Note the mesangial deposition of C_3. Immunofluorescence x 170.

Fig. 118. Accelerated serum sickness GN. Note the granular deposition of C_3 in the wall of an interlobular artery. Immunofluorescence x 170.

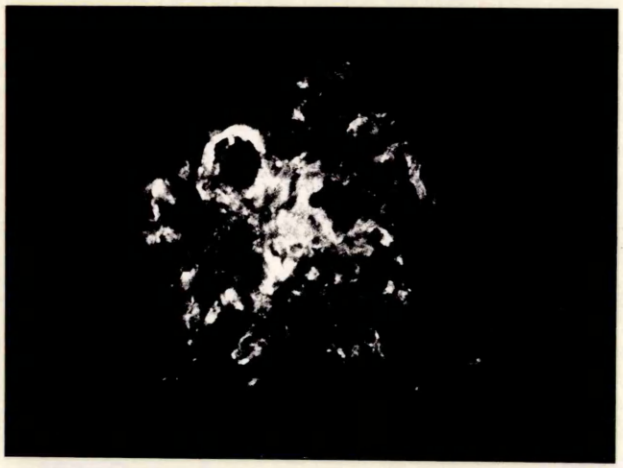


TABLE 6.2

EXPERIMENTAL ACCELERATED SERUM SICKNESS

No.	Sensitising Dose S.C.	Intravenous Dose 1 (4 days)	2 (7 days)	Day * Killed	Glomerular Lesions **	Immunofluorescence** IgG	C_3	Electron Dense Deposits
W984	100 mg	4G	-	10	-	-	1+	No
W983	100 mg	4G	2G	10	-	-	-	No
W990	100 mg	6G	-	10	-	2+	3+	Yes (Mes.)
W991	100 mg	6G	6G	10	1+	3+	3+	Yes (Mes.)
W994	100 mg	6G	-	10	2+(V)	-	1+	Yes (Mes.)
W995	100 mg	6G	-	15	1+(V)	-	1+	No
W1018	100 mg	12G	12G	15	1+	1+	1+	Yes (Mes.)
W1017	100 mg	12G	12G	20	1+	-	1+	No
W1013	100 mg	12G	12G	25	1+	1+	1+	Yes (Mes.)
W1016	100 mg	12G	12G	25	1+	1+	1+	Yes (Mes.)
W1012	100 mg	12G	12G	30	1+	-	1+	No

* Days post sensitising dose

** Lesions judged 1+ to 4+ according to severity

V Also showed vascular lesions

Mes. = Mesangial

Chronic Serum Sickness GN:

Subgroup I: Cationic BSA experiment:

From the Table 6.3 it can be seen that three dogs (W1031, W1032 and W1033) died after 12 injections of BSA at the third week of the experiment and a further dog (W1034) died at four weeks due to post biopsy bleeding. Only four dogs showed significant proteinuria (W1032, W1033, W1034, W1035; 489, 200, 210, 55 mg/100ml respectively). Three of these included the dogs that died early in the experiment. Only one dog (W1037) showed a raised level of blood urea (13.7 mmol/l).

Light microscopic findings:

The three dogs which died at three weeks and the three dogs biopsied at four weeks showed no histological changes. One dog (W1044) killed at three weeks, however, showed mild mesangial expansion and a further dog (W1043) killed at four weeks had early thickening of the capillary loops (Fig. 119).

With one exception, all dogs killed seven weeks and onwards showed diffuse membranous thickening of the loops together with mild mesangial expansion and hypercellularity (Fig. 120). One dog (W1036) killed at eight weeks showed no alterations in the glomeruli.

Ultrastructural findings:

TEM studies revealed subepithelial electron dense deposits in all the dogs injected with cationized BSA regardless of whether or not histological changes were found with the light microscope. The dogs which died were all sampled within two hours of death and

although autolytic degenerative changes were underway these did not prevent easy recognition of subepithelial deposits. At three weeks deposits were rather sparse and only one of the dogs biopsied at four weeks showed many deposits (Fig. 121). Thereafter, all dogs had numerous deposits resulting in thickening of the capillary walls (Fig. 122). Associated with the deposits there was widespread fusion of foot processes and frequent duplication of the lamina densa. Although mild mesangial expansion was found in some dogs, few electron dense deposits were also present.

With the SEM, numerous surface microvilli on the epithelial cell body and occasionally on the primary and secondary processes were observed. The most striking feature, however, was loss of the orderly interdigitation of the foot processes (Figs. 123, 124). Nevertheless, even in the most severely affected glomeruli, areas of normal foot process architecture could always be found.

Corrosion casts of the glomeruli were normal with no leakage of cast material into the urinary space.

Immunofluorescence and PAP findings:

All the dogs examined at three weeks and the dogs biopsied at four weeks had developed uniform granular deposits of IgG and C_3 along the capillary walls (Figs. 125, 126, 131, 132) and this persisted until the termination of the experiment at 10 weeks (Figs. 127 and 128). BSA was not detected in any animal in this study. One of the biopsied dogs (W1036) which showed heavy deposits of IgG and C_3 at the time of biopsy (four weeks) had somewhat lesser deposits when killed at eight weeks. BSA was detected with PAP (but not with immunofluorescence) only in the three dogs which received intravenal injection of

cationized BSA. (Figs. 133, 134).

Subgroup II: Native BSA experiment:

The two dogs in this group both remained clinically normal and showed normal urine protein and blood urea levels.

Light microscopic findings:

No histological changes were observed in the glomeruli in either animal.

Ultrastructural findings:

No electron dense deposits were detected in these dogs and apart from slight mesangial expansion and hypercellularity the glomeruli appeared to be normal.

Immunofluorescence findings:

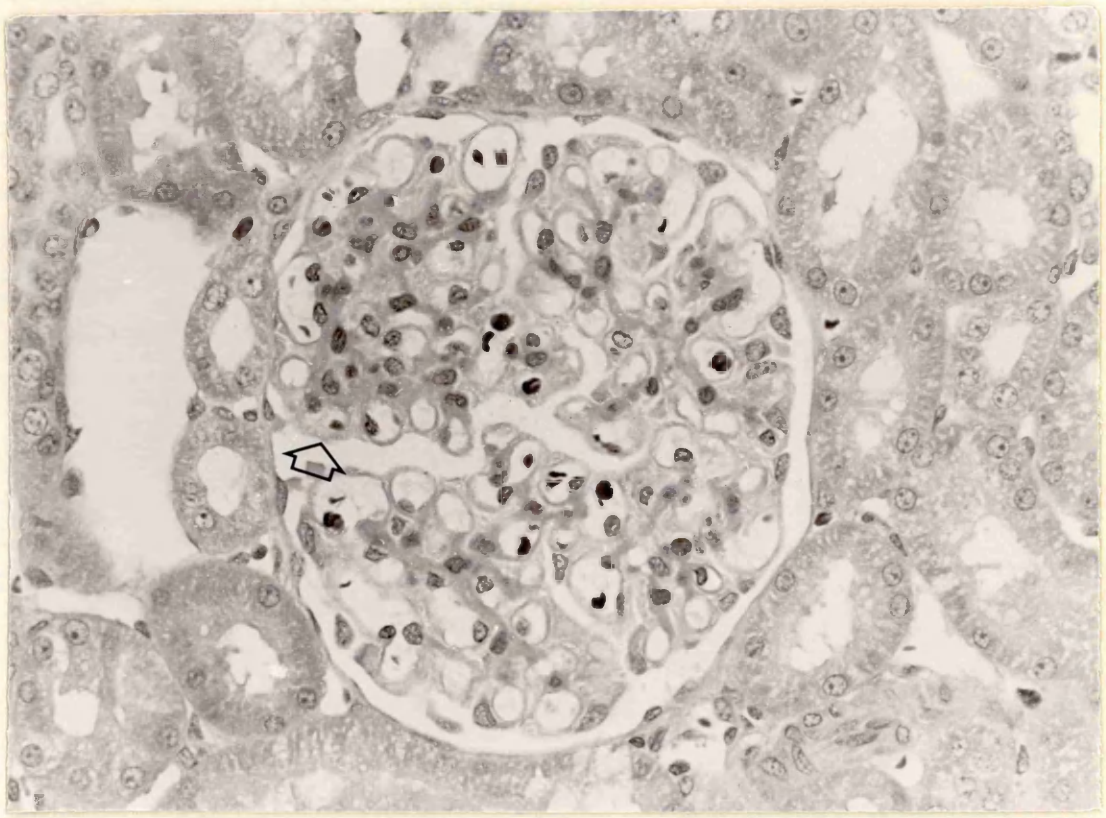
Both dogs showed sparse glomerular deposits of IgG and C_3 (Figs. 129, 130), these were confined to the mesangial region. BSA was not detected.

Controls: (Subgroup III):

No histological and ultrastructural changes were observed; likewise no IgG, BSA or C_3 were detected in the glomeruli.

Fig. 119. Chronic serum sickness GN.
Note the early segmental thickening
of the capillary loops (arrow).
H & E x 250.

Fig. 120. Chronic serum sickness GN.
A glomerulus showing diffuse
membranous thickening of the
loops and mild mesangial
expansion and hypercellularity
at 10 weeks. H & E x 250.



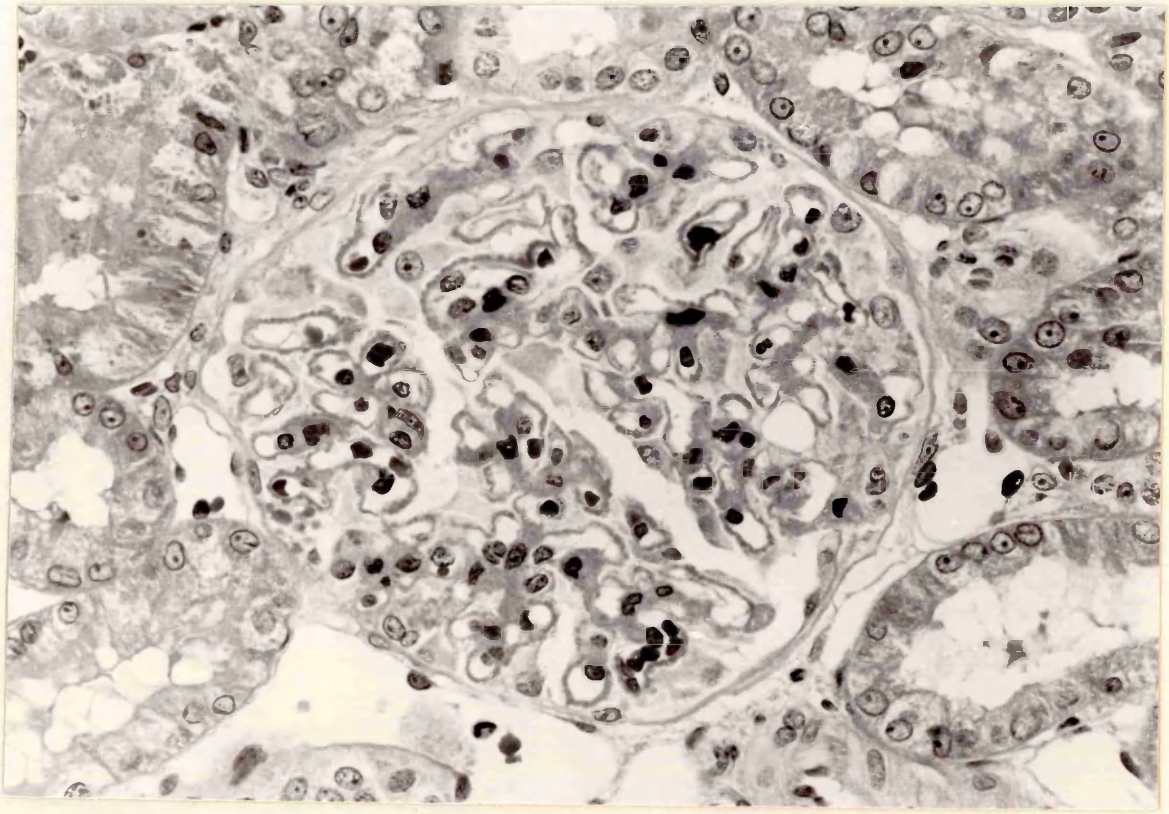
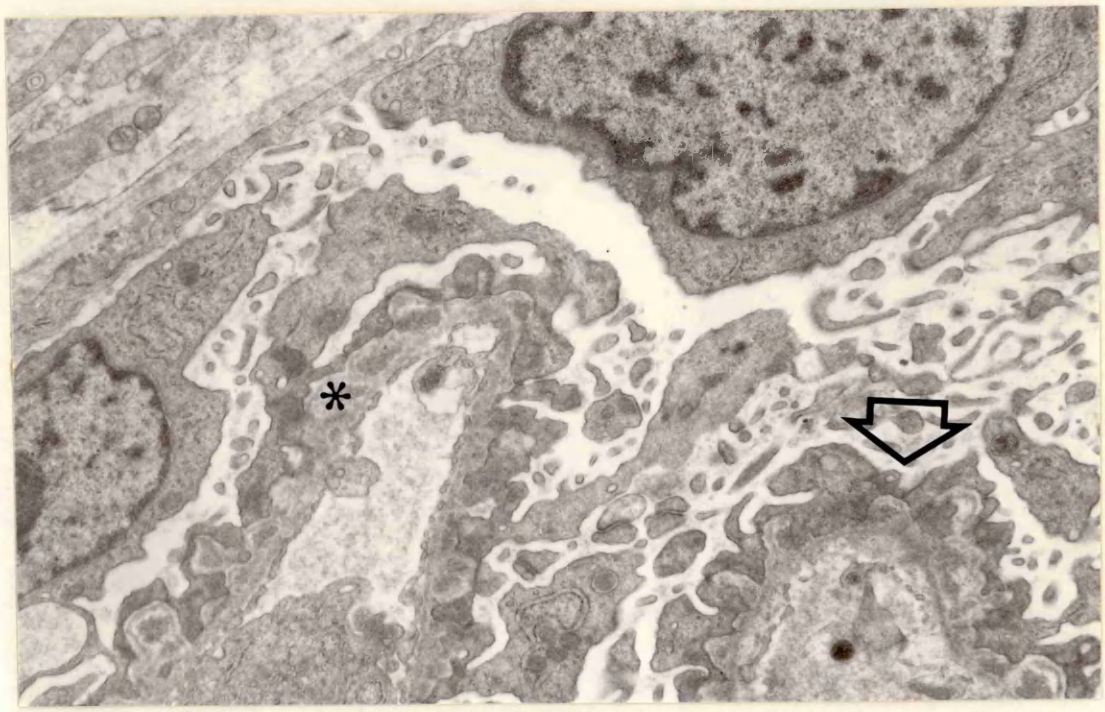
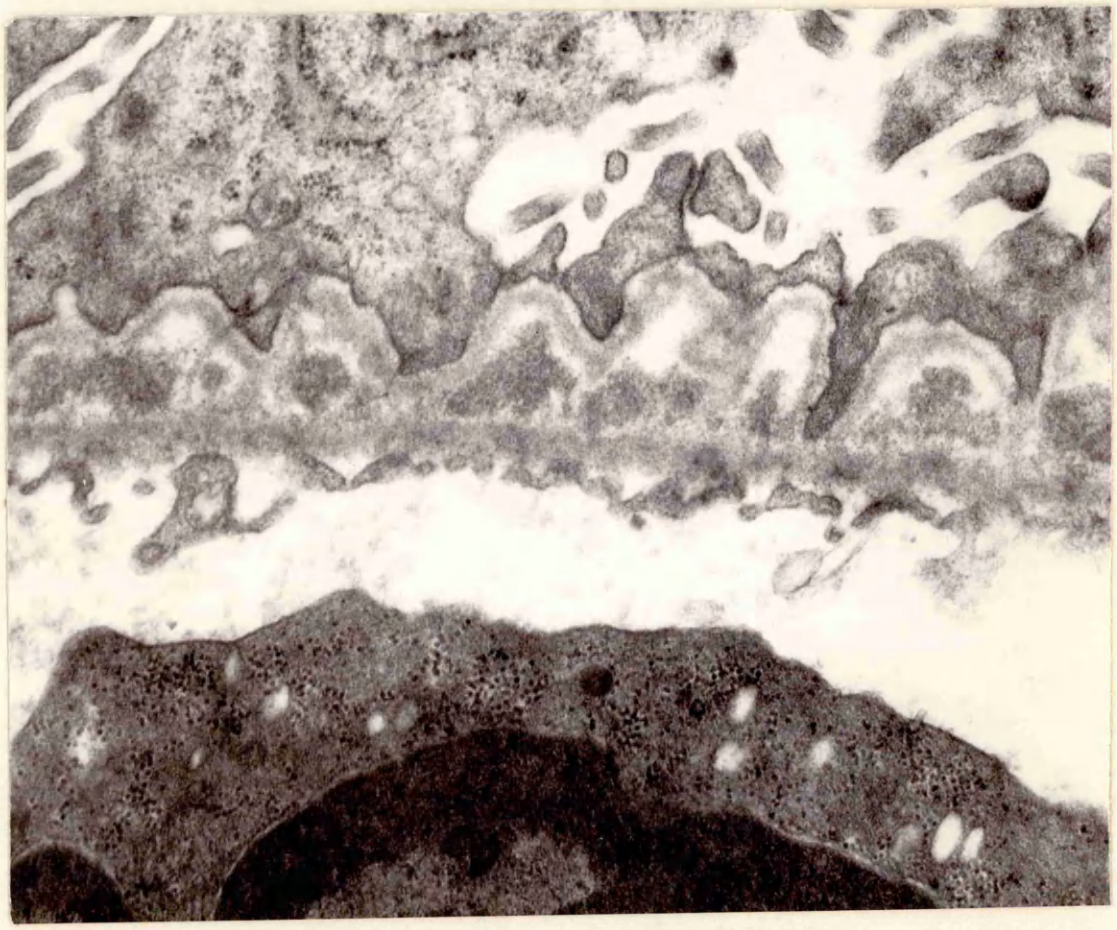


Fig. 121. Chronic serum sickness GN.
Note the subepithelial deposits (asterisk) and fusion of epithelial foot processes (arrow).
Biopsy, 4 weeks. TEM x 5,500.

Fig. 122. Chronic serum sickness GN.
Necropsy specimen from the same dog killed at 10 weeks. Note the heavy deposits in the subepithelial space, duplication of the lamina densa and fusion of epithelial foot processes.
TEM x 10,000.





Figs. 123, 124. Chronic serum sickness GN.

The surface epithelium has lost its orderly interdigitating foot processes and there are many surface microvilli (arrow). EP = epithelial cell body; PP = primary process; SP = secondary process.

SEM x 2,500, 5,000.

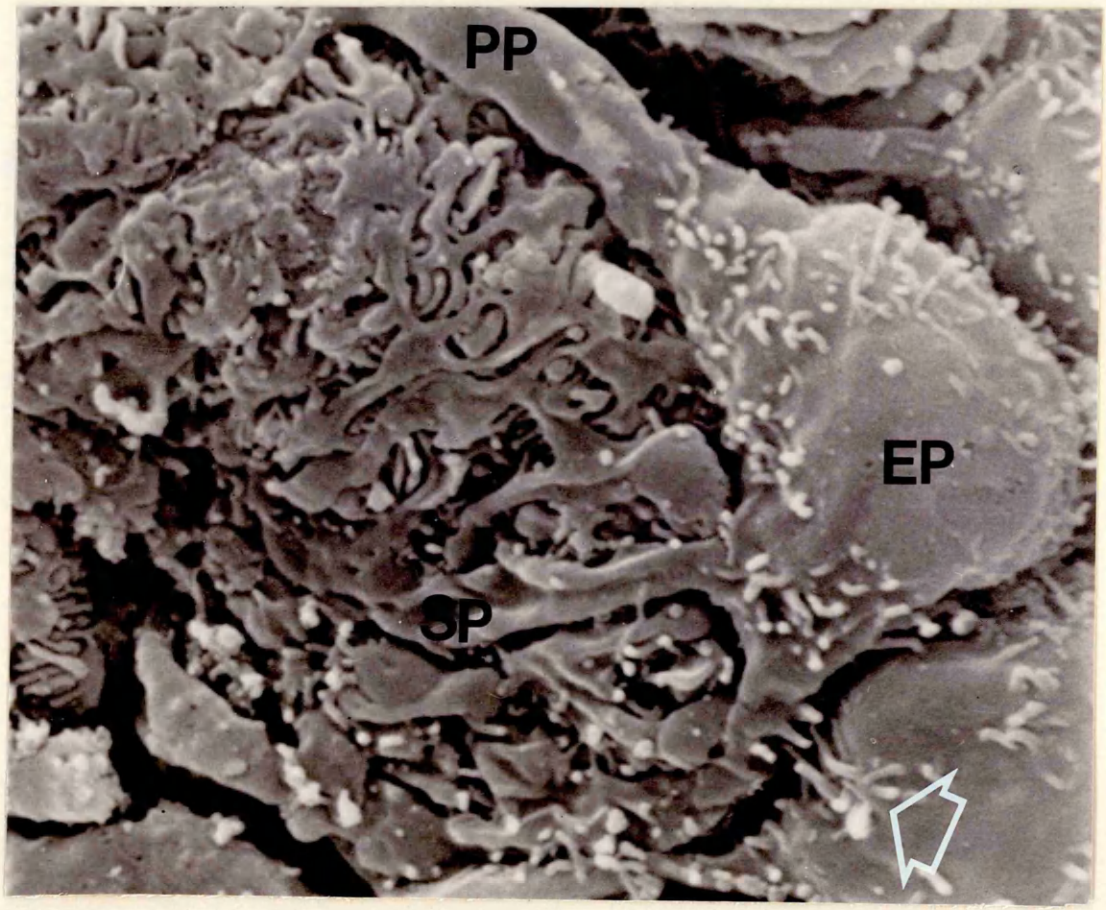
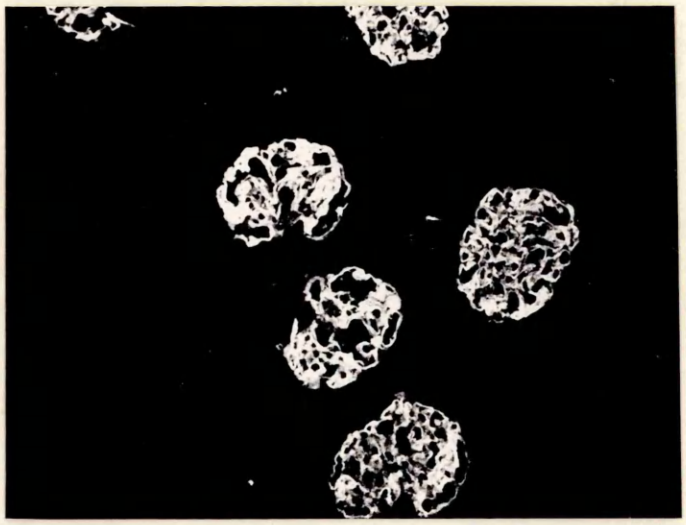

PP
EP
SP

Fig. 125. Chronic serum sickness GN, four weeks. All the glomeruli show uniform heavy deposition of IgG along the glomerular capillary wall. Immunofluorescence x 80.

Fig. 126. Chronic serum sickness GN, four weeks. A glomerulus shows heavy granular deposition of C_3 along the capillary wall. Immunofluorescence x 180.

Fig. 127. Chronic serum sickness GN, 10 weeks. Note the persistent uniform heavy deposition of IgG. Immunofluorescence x 180.



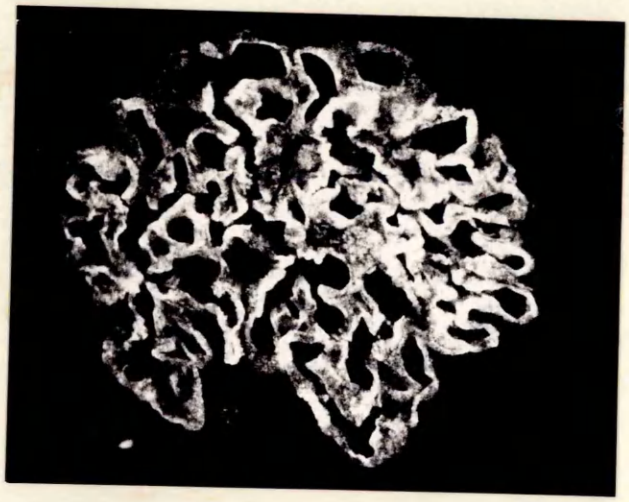


Fig. 128. Chronic serum sickness GN, 10 weeks. Note the C_3 deposition along the capillary walls. Immunofluorescence x 180.

Figs. 129, 130. Chronic serum sickness GN, (native BSA group). Note sparse mesangial deposition of IgG and C_3. Immunofluorescence x 180.

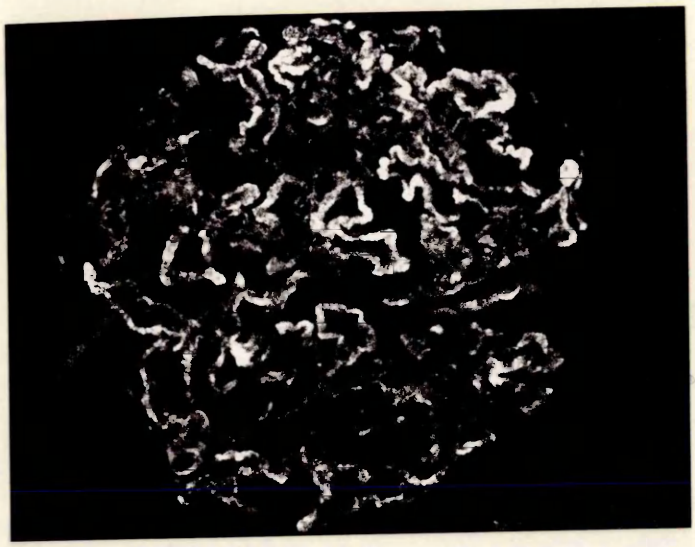
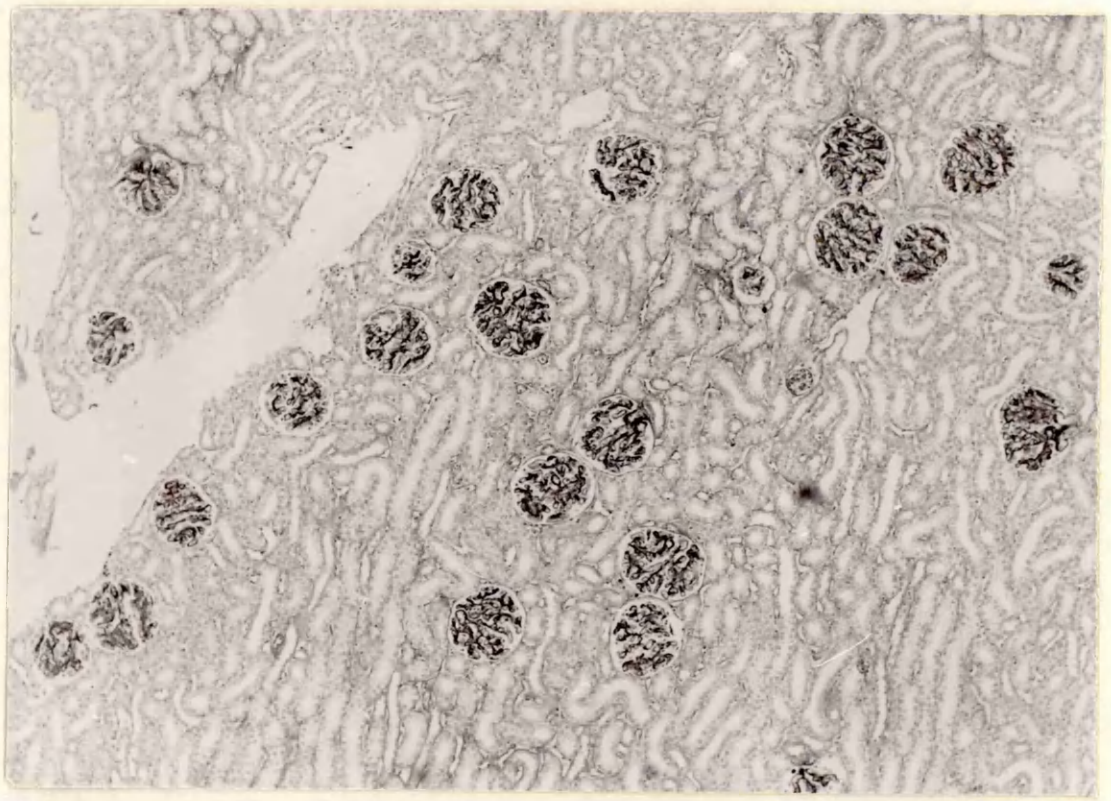
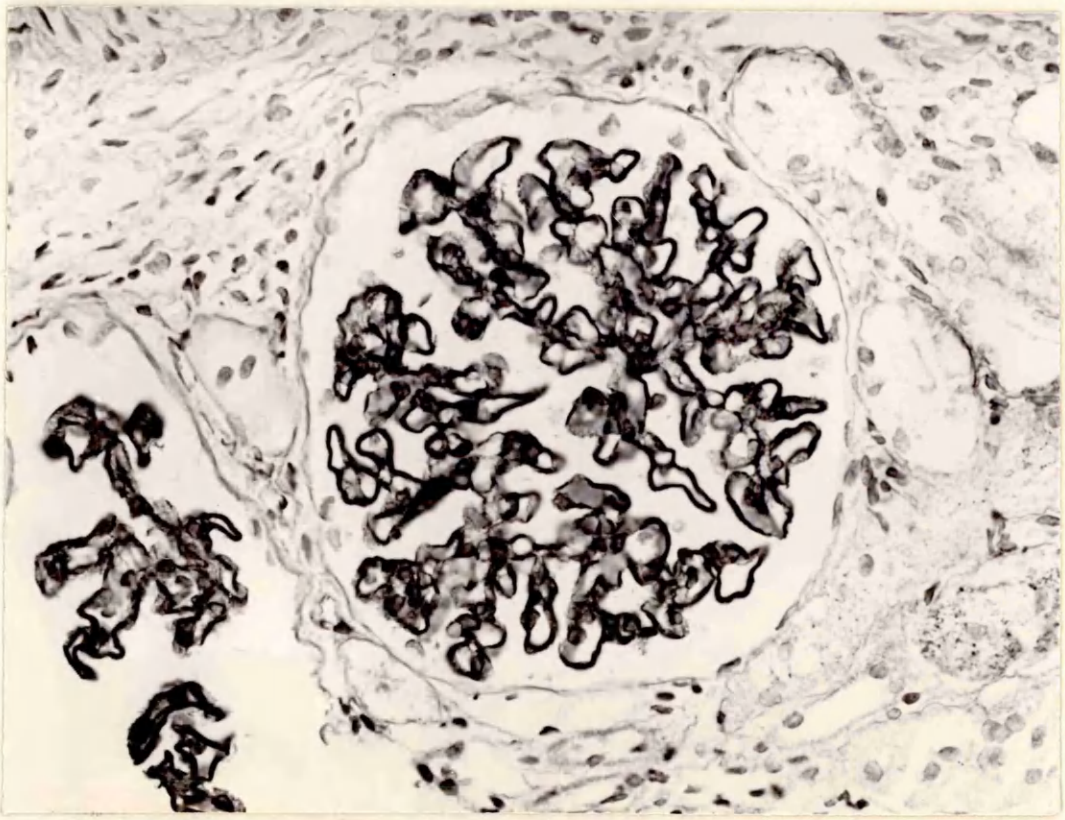


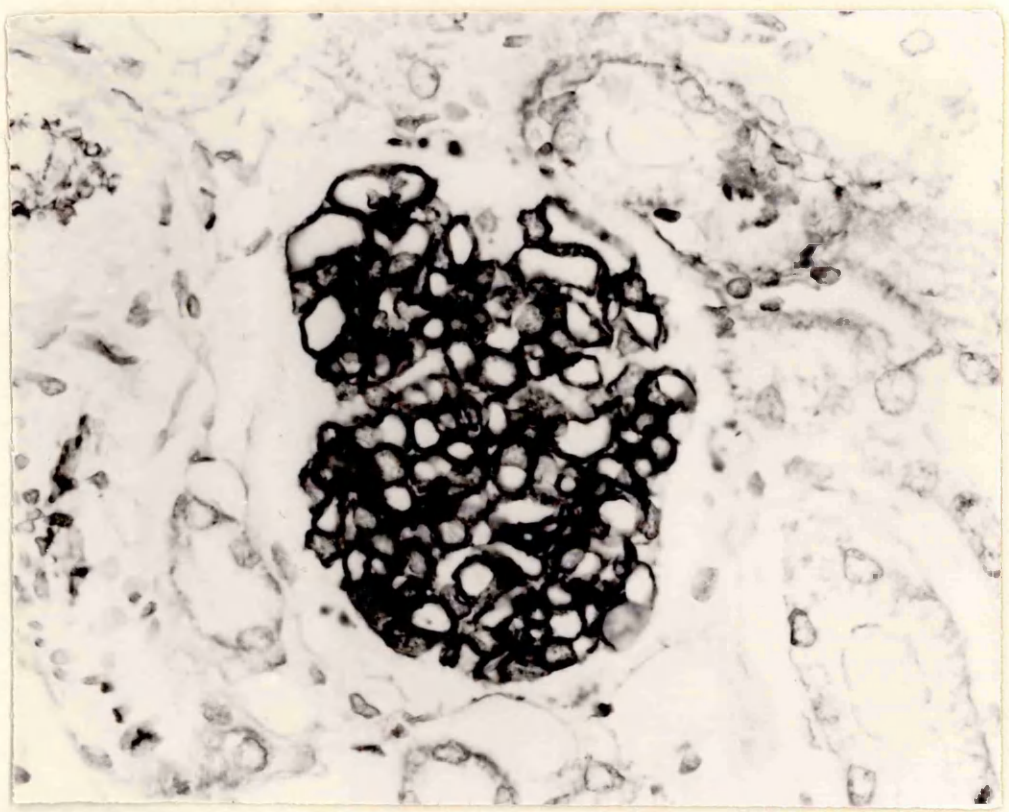
Fig. 131. Chronic serum sickness GN, four weeks. Note the heavy diffuse deposition of IgG. PAP x 40.

Fig. 132. Chronic serum sickness GN, four weeks. Note the heavy deposition of IgG along the capillary walls. PAP x 250.





Figs. 133, 134. Normal dog glomerulus showing binding of cationic BSA along the capillary wall. PAP x 200.



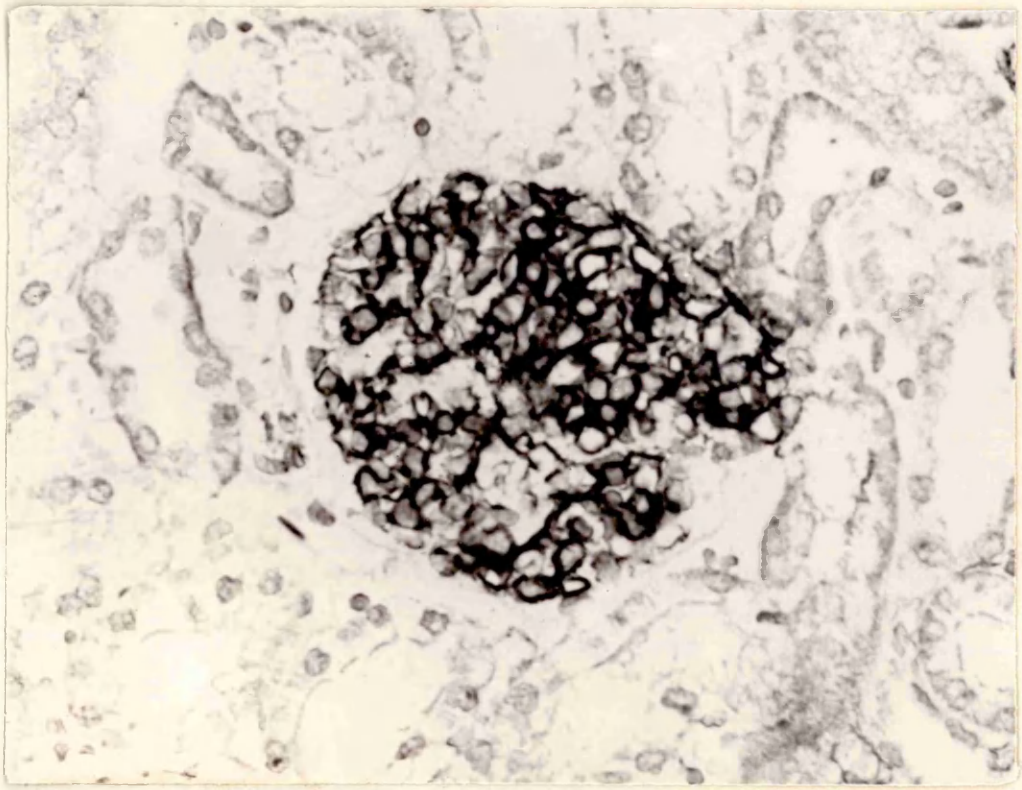


TABLE 6.3

EXPERIMENTAL CHRONIC SERUM SICKNESS AND CATIONIZED BSA

Dog No.	No. of Injections	Week Killed	Histological Lesions *	Immunofluorescence * IgG	Immunofluorescence * C_3	Electron Dense Deposits
W1031	12	3 (died)	-	4+	2+	Yes : few
W1032	12	3 (died)	-	4+	4+	Yes : few
W1033	12	3 (died)	-	4+	4+	Yes : few
W1044	12	3	1+ Mes. Exp.	4+	2+	Yes : few
W1043	22	4	1+ Loop thickening	4+	2+	Yes : many
W1034	22	4 (died)+	-	4+	2+	
W1035	37	7	2+ Loop thickening mes. exp.	4+	2+	Yes : many
W1048	37	7	3+ Loop thickening mes. exp.	4+	2+	Yes : many
W1036	37	8+	-	3+	1+	Yes : many
W1047	37	8	2+	3+	2+	Yes : many
W1037	37	10+	3+ Loop thickening mes. exp.	4+	1+	Yes : many
W1046	37	10	3+ Loop thickening mes. exp.	4+	1+	Yes : many

\+ Biopsied at 4 weeks

* Lesions judged 1+ to 4+ according to severity.

Discussion:

Although immune complex GN is the best documented form of spontaneous GN in man and his closest animal associates the dog and cat, all the serum sickness GN experiments reported to date have been carried out in the rabbit, rat and mouse.

In the present study, a series of serum sickness experiments were undertaken for the first time using the dog, an experimental animal unlike the rabbit, mouse or rat, subject to many of the spontaneous immune complex mediated forms of GN as seen in man.

The present investigation has shown that acute "one-shot" serum sickness GN following administration of BSA in dogs and characterized by focal proliferative glomerular changes is similar in some respects to that described in rabbits and reported by various workers (Dixon et al, 1961; Fish et al, 1966; Easley and Halliwell, 1977).

Dressman and Germuth (1972) in their study of experimental serum sickness in rabbits described a diffuse proliferative form of GN with focal glomerular necrosis. The latter lesion was not found in dogs and even in animals where glomerular lesions were present many glomeruli were histologically normal. Although the overall ultrastructural changes were in agreement with those described by other investigators, the anatomical distribution of the electron dense deposits in the glomeruli was at variance with previous reports.

Dixon et al (1961) were of the opinion that the deposits were rare and present along the luminal aspect of the GBM. On the other hand, Fish et al (1966) and Easley and Halliwell (1977) observed

numerous electron dense masses within the GBM on the epithelial side and fusion of processes of the overlying epithelium. In dogs, deposits were found only in the mesangium.

The study also afforded an opportunity to record for the first time the SEM observations in experimental serum sickness GN. These were characterized by the presence of numerous surface microvilli and blebs on the visceral epithelium and partial effacement of the foot processes, none of which changes were detectable by TEM.

Using immunofluorescence method, McCluskey and Vassalli (1969) mentioned a paucity of demonstrable immune aggregates in acute serum GN. Kniker and Cochrane (1965) however, reported extensive granular deposition of BSA, IgG and C_3 localized both in glomeruli and in arterial lesions in rabbits. Fish et al (1966) and Dixon et al (1961) have also detected large deposits of IgG but the former workers detected BSA only in a few rabbits. The present study showed deposition of IgG in 65% (7/11) and C_3 in more than 90% (10/11) of dogs; BSA however, was not detectable in any animal. The difficulty in demonstration of the antigen, BSA, in the deposits may be related to the fact that circulating free anti BSA antibodies may react with the BSA anti BSA aggregates lodged in the glomeruli thus adding to their size while at the same time covering the most of the available antigenic determinants of BSA (Albini et al, 1979).

The introduction of the accelerated serum sickness model by Shigematsu and Kobayaski (1976) was intended to establish a more serious form of experimental immune complex GN. Indeed, in their rabbit model, they did describe more progressive glomerular changes

than those normally associated with acute "one-shot" serum sickness. In their experimental rabbits they found severe glomerular hypercellularity due to accumulation of monocytes and polymorphonuclear cells, expansion of the subendothelial space and local exfoliation of endothelial cells leading to occlusion of capillary lumina was also a feature. Subepithelial electron dense deposits, local fusion of foot processes, and in more progressive cases, severe glomerular scarring and extracapillary fibrinous exudation were also noted. In the present dog model, however, a number of differences were found and little evidence was presented to show that, in the canine species, accelerated serum sickness offers a more useful model for the study of immune complex GN than "one-shot" serum sickness.

Electron dense deposits in the dog model were mesangial and only 55 per cent of dogs (6/11) showed these although mesangial hypercellularity was found in most animals; there was no glomerular scarring. With immunofluorescence less than half of the dogs showed deposits of IgG although 10 of the 11 dogs had C_3 deposition. Unlike the rabbit model, however, vascular lesions in form of necrosis of the tunica media of interlobular and afferent vessels were found.

Electrical charge as a factor influencing the glomerular localization of immune complexes, is emerging as an important new concept in renal immunopathology (Border et al 1982).

The classic studies of chronic serum sickness by Dixon et al (1961); Germuth et al (1967) and Germuth et al (1977) were performed before the elucidation of the glomerular wall as a charge selective barrier. Although membranous nephropathy occurs in some cases of experimental chronic serum sickness its occurrence is unpredictable; thus, the potential of using charged, i.e. cationized, antigens in the

induction of membranous nephropathy is only now being realized.

As membranous nephropathy is the most common form of spontaneous immune complex GN in dogs (Wright et al, 1981), the utilization of charged antigens in experimental chronic serum sickness offered an exciting opportunity to attempt to induce, for the first time, experimental membranous nephropathy in dogs. The present initial findings in dogs confirms those reported by Border et al (1982) in rabbits and indicated that the charge of administered antigen has a profound effect on the nature and severity of the renal lesion and that it plays a decisive role in the formation of subepithelial immune deposits and the production of membranous nephropathy.

It is important to note that, in the present work, all of the dogs receiving cationic BSA developed a diffuse glomerular lesion characterized by glomerular circumferential deposits of IgG and C_3 scattered along the capillary wall. These were found as early as three weeks, although only a few electron dense deposits, confined to the subepithelial space were detected at this time. By four weeks all dogs showed heavy IgG and C_3 deposition; nevertheless only two of these animals including one of the biopsy dogs had pronounced subepithelial deposits. All dogs, thereafter, killed between seven weeks and the termination of the experiment at 10 weeks had maintained the maximum level of granular IgG and C_3 deposition and all showed many subepithelial deposits. As in the acute "one-shot" serum sickness experiment BSA antigen was not detectable by immunofluorescence in any animal, despite the heavy deposition of IgG and C_3.

Overall, the immunofluorescence, histological and

ultrastructural features in the present experiment resembled those described in spontaneous canine membranous nephropathy (Wright et al 1981).

Although proteinuria was detected in four dogs in the third and fourth weeks of injection of cationic BSA, unlike the spontaneous cases of membranous nephropathy which characteristically show a heavy sustained proteinuria, there was no evidence of any significant protein leak into the urine in any of the experimental dogs receiving cationic BSA after cessation of daily injections, notwithstanding the degree of immune complex deposition and the widespread effacement of epithelial cell foot processes. Perhaps the dogs were killed before the morphologic expression of immune complex deposition had induced sufficient injury to the GBM. Further studies will be needed to examine the more long term effects of administration of cationized antigen.

The renal lesions induced by the administration of cationic BSA were significantly different, both quantitatively and qualitatively from those in dogs receiving native anionic BSA. In the latter animals, electron dense deposits were not found in subepithelial sites or in the mesangium.

Moreover, deposits of IgG and C_3 were much less intense than those of the dogs receiving cationized antigen and despite the absence of electron dense deposits appeared to be predominantly mesangial.

It must be accepted, however, that as only two dogs received native (anionic) BSA compared to 12 dogs which were given cationic BSA care must be taken in drawing direct comparisons between the two groups.

Further studies using more animals in the anionic group will be necessary to confirm the present initial and preliminary findings.

Whether or not the immune complex deposits in the dogs receiving cationized BSA were the result of deposition of circulating complexes or the formation of *in situ* complexes also remains unclear. Border *et al* (1982), however, using a rabbit model have provided evidence that binding of cationic BSA to the anionic GBM is a necessary prerequisite to formation of BSA-anti BSA antibody complexes at subepithelial sites. Similarly, Oite *et al* (1982) using a rat model with cationized human IgG as antigen, have clearly established that by artificially planting the antigen on the anionic GBM by perfusion of the renal artery, the subsequent administration of anti-human IgG antiserum stimulates an immune complex GN characterized by numerous subepithelial deposits.

CHAPTER 7

GENERAL SUMMARY AND CONCLUSIONS

In terms of investigation of renal function, the dog has been, and still is used, probably more than any other mammalian species. Yet, apart from man, the majority of morphological and immunopathological studies of the renal glomerulus has been carried out in the rat, mouse and rabbit. Furthermore, the recent surge of interest into spontaneous glomerular disease of the dog (Osborne and Vernier, 1973; Wright et al, 1981), has highlighted the importance of a real understanding of normal glomerular structure and the need to establish how the dog glomerulus reacts to various forms of immunological insult.

Chapter 3 of the work confirmed what little has been reported concerning the normal morphology of the dog glomerulus and added some new information with regard to methods of fixation (perfusion versus immersion) and embedding, thickness of section and variations on glomerular morphology between inner and outer cortical levels.

The comparison of fixation methods made in the present study show that, although both methods of fixation for both conventional light microscopy and electron microscopy, perfusion fixation appeared to be superior to immersion fixation with respect to patency of the glomerular capillaries, and allowed better differentiation of glomerular cells on light microscopy. The disadvantages of perfusion fixation was that the capillary lumina and urinary space were sometimes artificially widened and, while most glomeruli were evenly perfused some, especially in the outer cortex, did not seem to be perfused at all. In the present study fixative was infused with simple manual pressure although with a controlled flow rate. The use of a controlled pressure system might have given more uniform glomerular perfusion but would not normally be acceptable as a routine method of fixation.

The crucial role of section thickness in the study of the renal glomerulus was also underlined in the present study. In thick paraffin sections (six μm) many glomeruli appeared to be hypercellular and it was not always possible to identify the various glomerular cell types. The use of thinner sections (two-three μm) helped to overcome the problem of poorly patent glomerular capillaries often present with immersion fixation.

The present study also provided the first detailed SEM views of the normal dog glomerulus. As such, it provided essential baseline parameters for future SEM studies concerned with evaluating the morphological changes which accompany various kidney disorders. Most of the previous SEM studies had been carried out in the rat and rabbit kidney and there had been some controversy as to the organisation of the visceral epithelium particularly the arrangement of the tertiary processes. The present study confirmed the observations of Buss and Kronert (1969) in rats who noted that adjacent interdigitating foot processes arose either from different podocytes or from the same cell.

The presence of a few microvilli on the cell body as well as on the primary and secondary processes reported in this study in the dog has also been described in the rat and rabbit by Fujita et al (1970).

Although there is a growing interest in spontaneous GN in dogs only a few studies have been carried out on the autolytic changes occurring in the dog's glomerulus; to date, no detailed sequential histological and ultrastructural study has been undertaken. It was considered useful in the present study to record the early as well as late autolytic changes in the normal dog glomerulus in order to

establish the alterations in structure which might be expected to be due to autolysis when making a study of pathologic material. The earliest autolytic change observed by light microscopy occurred as early as two minutes after death when a small amount of tubular epithelial debris was found in the urinary space, albeit in a very few glomeruli. The degree of tubular reflux subsequently increased as the time of sampling after death lengthened although this has previously been shown to be influenced by some other factors such as ante-mortem ischaemia, the method of fixation and even palpation of the kidney at autopsy (Mullink and Feron, 1967).

From five minutes onwards, occlusion of capillaries as a result of endothelial and mesangial swelling gradually become more and more pronounced and this was complete by 24 hours.

The main feature of interest with the TEM was the remarkable preservation of the GBM and attached foot processes despite the early disintegration of endothelial cells and the cell bodies of visceral epithelial cells. Some foot processes still remained morphologically recognisable even as late as three - five days after death.

With SEM it was evident that glomeruli appeared to react at different rates to autolytic changes. The number of surface microvilli had markedly increased as early as two minutes after death on some visceral epithelial cells. These changes were more severe at 10 and 30 minutes when there was also widespread effacement of foot processes. Nevertheless, even at 24 hours after death some capillaries were still showing areas of normal morphology with good preservation of the orderly interdigitation of foot processes. On days three and five, however, no trace of the foot processes was found and the visceral

epithelium appeared as a roughened sheet of cytoplasm.

In the light of the results of TEM and SEM a number of important considerations have emerged. First, the rate at which autolytic changes occur in different glomeruli varies; thus although early changes were observed with the SEM at two minutes, areas of normal glomerular architecture were still maintained as late as 24 hours. Second, although early changes were identified by TEM at two minutes and cellular disintegration, particularly of endothelial cells, followed rapidly the GBM although swollen, remained intact and foot processes could still be observed as late as three - five days after death.

In Chapters 5 and 6 of the work the reaction of the dog glomerulus to two forms of immunologic injury was investigated.

In Chapter 5, the morphologic changes associated with nephrotoxic (anti-GBM) serum was studied while, in Chapter 6, three different models of experimentally induced immune complex GN were compared.

Although experimental NTN was induced in dogs as early as 1920 by Wilson and Oliver, the available literature indicates that all the subsequent studies have been fragmentary and brief; no detailed sequential investigation using combined light microscopy - immunofluorescence and electron microscopy (TEM and SEM) have been carried out.

The present study was designed to follow the sequential morphologic changes in the dog glomerulus from 30 minutes to 80 days after injection of NTS. The earliest event as established by light

microscopy was the appearance of neutrophils in the lumina of glomerular capillaries by one hour after administration of NTS. Although tuft swelling with occlusion of capillaries with sequential capillary thrombosis and necrosis and hypercellularity were observed at two days, the severity of the histological lesions was most pronounced on days five and seven. In the later stages of the disease, mesangial hypercellularity, lobulation of the glomerular tuft and persistence of few obsolescent glomeruli were the only major observations.

With TEM, the first ultrastructural changes were observed by one hour when neutrophils were found in the glomerular capillaries and in some instances some of these cells were in direct contact with the GBM. This change was accompanied by fusion of epithelial foot processes. The ultrastructural changes were most marked, however, on days three - seven and this period was characterized by intraluminal fibrin deposits, extensive effacement of foot processes, necrosis of the endothelial lining and mesangial hypercellularity. There was some evidence too of circulating mononuclear cells emigrating into the mesangium and contributing to the overall hypercellularity of the glomerulus. The remaining notable feature of the ultrastructural alterations was the expansion of the subendothelial space with electron lucent material which was not found until 15 days and persisted until the end of the experiment at 80 days. Furthermore, between 19 and 40 days, localized electron dense deposits were also to be found in expanded subendothelial spaces and in the mesangial matrix.

In the present study, the ultrastructural changes as observed by the SEM were recorded for the first time in experimental NTN in the

dog. Alterations in the normal orderly arrangement of the foot processes were noted at 30 minutes after administration of NTS and, at this early stage, arterial casts showed leakage of cast material in approximately 10% of glomeruli. By 24 hours, in addition to obliteration of foot processes, strands of fibrin-like material were observed in the urinary space. By three days, more severe changes characterized by extensive fusion of foot processes and complete loss of normal podocyte architecture were evident. In renal casts, up to 50% of glomeruli showed leakage of cast material. Although some glomeruli still showed obliteration of foot processes and leakage of cast material at 30 days, other glomeruli had returned to normal. The percentage of normal glomeruli increased gradually towards the end of the experiment and at 80 days most glomeruli showed normal surface morphological arrangement of epithelial foot processes. In the later stages of the experiment, however, arterial casts showed occasional shrunken glomeruli with stunted blindly ending capillaries, which presumably represented partial glomerular scarring observed on light microscopy.

The immunofluorescence patterns in the present study revealed nephrotoxic anti-GBM antibody (i.e. rabbit globulin) in the glomeruli as early as 30 minutes after administration of NTS and had a striking linear distribution along GBM. This persisted until 15 days; thereafter the intensity of fluorescence decreased. Faint linear deposition was, however, still present in some glomeruli at 80 days. Weak linear fluorescence of complement (C_3) was detected at three days and persisted only up to 60 days. Irregularly linear deposition of host IgG was detected at five days. Unlike the fluorescence patterns of rabbit globulin, C_3 and host IgG deposits of

fibrinogen were always focal as well as segmental.

The present study served to illustrate the remarkable reparative power of the canine glomerulus already noted by Wright et al (1973[a]). The nephrotoxic nephritis model, although so far without a comparative spontaneously occurring counterpart as occurs in man (Lerner et al, 1967) nevertheless has been shown to be a useful method of inducing diffuse glomerular disease and consequently is of special use in furthering the understanding of how a glomerulus reacts to non-lethal and non-progressive injury.

There is no doubt, however, that Chapter 6 of the work, in which a series of experiments were carried out to induce immune complex-mediated GN, offered an opportunity to study the sequential consequences of the arrival of immune complexes in the canine glomerulus, an event more akin to spontaneous GN in dogs than nephrotoxic nephritis. Thus a series of serum sickness experiments were undertaken, for the first time in the dog, an experimental animal unlike the rabbit, mouse or rat, subject to many of the spontaneous immune complex mediated forms of GN as seen in man (Murray and Wright, 1974; Mueller-Peddinghaus and Trautwein, 1977).

The present investigation has shown that acute "one-shot" serum sickness GN following administration of BSA in dogs was characterized by a mild focal proliferative form of GN with mesangial located electron dense deposits similar in many respects to that described in rabbits and reported by Easley and Halliwell (1977). The accelerated serum sickness GN model was marginally a more useful model for the study of immune complex GN than "one-shot" serum sickness as, although more severe individual glomerular lesions were

found the overall disease was focal in nature with many glomeruli histologically normal.

As membranous nephropathy is the most common form of spontaneous immune complex GN in dogs (Wright et al, 1981) the utilization of charged (cationised) antigens in an experimental chronic serum sickness model offered an exciting opportunity to attempt to induce, for the first time, experimental membranous nephropathy in this animal. It is important to note, in the present work, all of the dogs in the cationic group developed a diffuse glomerular lesion characterized by circumferential granular deposits of IgG and C_3 scattered along the capillary wall and electron dense deposits confined to the subepithelial space, findings not observed in the "one-shot" serum sickness or accelerated serum sickness experiments. These changes were detected as early as three weeks after administration of cationised BSA and thereafter, all dogs killed between seven weeks and the termination of the experiment at 10 weeks had maintained the maximum level of IgG and C_3 deposits and all had substantial subepithelial deposits.

Overall the immunofluorescence, histological and ultrastructural features in the present experiment resembled those described in spontaneous canine membranous nephropathy (Murray and Wright, 1974; Osborne and Vernier, 1973; Wright et al, 1981).

However, despite the degree of deposition of IgG and C_3, the presence of subepithelial deposits and widespread fusion of foot processes, there was no evidence of any significant protein leak into the urine in any of the experimental dogs receiving cationic BSA once daily injections had ceased. Perhaps the dogs were killed before

the morphologic expression of immune complex deposition had induced sufficient injury to the GBM to induce persistent proteinuria. Further studies are necessary to examine the more long-term effect of administration of cationised antigen.

It is also of interest to note that the renal lesions induced by the administration of cationic BSA were significantly different, both quantatively and qualitatively from those in dogs receiving native anionic BSA, in which animals immunofluorescence deposits were relatively sparse and electron dense deposits were not found. As only two animals were used in this group, in effect as controls for the cationised group, along with dogs receiving saline injections alone, it is perhaps important to note the small number of animals in this anionic group compared with the cationized dogs. Nevertheless, the overall severity of the glomerular lesions in dogs receiving cationized BSA compared to anionic BSA is similar to the findings of Border et al (1982) in rabbits.

Of importance is the fact that, for the first time in the dog, an experimental model is available for the study of immune complex-mediated GN a disease increasingly being recognised as a clinical problem in that animal.

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