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A study of excretion in the slug Agriolimax reticulatus

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A STUDY OF EXCRETION IN THE SLUG, AGRIOLIMAX RETICULATUS (MULLER)

BY

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A thesis submitted to the University of Wales in candidature for the degree of Philosophiae Doctor.

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Introduction

Agriolimax reticulatus (Müller) is a Stylommatophoran Pulmonate Gastropod of the family Limacidae and is commonly known as the grey field slug. It is extremely widespread and numerous both in Britain and elsewhere in temperate regions, and is an important agricultural pest.

Work has been carried out in this laboratory (Zoology department, U.C.N.W.) on various aspects of feeding and digestion, (Runham, 1969; Walker, 1969), reproduction, (Bayne, 1966, 1967, 1968; Runham and Laryea, 1968; Bailey, 1970), and the nervous system, (Laryea, 1970 unpublished) of A. reticulatus. Elsewhere work involving this slug has been carried out in other fields e.g. Ecology, (e.g. Hunter, 1968), Parasitology, (e.g. Brooks, 1966), Behaviour, (e.g. Newell, 1966).

The term excretion, as applied to animals concerns the removal of "waste metabolic material" from the body. As such it is only related to defecation when products of metabolism are present along with unabsorbed food in the faeces. Substances which may be classed as waste products of metabolism are many and varied, ranging from carbon dioxide and water to quite complex organic molecules like uric acid.

The dividing line between a waste product and a secretion which has a postive biological function, is not always easy to draw. The moulting of homoiotherms at the onset of climatic changes is a case which readily illustrates

this point: undoubtedly moulting serves the purpose of giving an animal a coat more suitable to the prevailing environmental conditions, but the hair or feathers lost in the process could also be classed as excretory products. Under drought conditions water is a valuable commodity, however, when an animal is in contact with solutions hypotonic to its body fluids, water must be disposed of. Although water (=osmo-) regulation is often considered in a category of its own, it is closely linked with excretion, and water availability is usually directly related to the nature of the normal excretory products of a particular animal.

Excretion is often defined in a much narrower sense than that outlined above; namely, the elimination of waste nitrogenous metabolic material. The validity of this view is that the nitrogenous products of protein catabolism are not easily stored in large amounts, nor are they readily convertible to forms which can be easily stored. On the other hand, breakdown products of carbohydrates and fats which are the other major organic dietary constituents of animals are more readily stored and anyway, can be relatively rapidly used in respiratory metabolism, especially in an active animal.

Nitrogenous breakdown products, especially the more soluble ones, are often toxic and therefore must be constantly eliminated from the body. If one disregards water and carbon dioxide as excretory products, then nitrogenous compounds are often the only other chemicals, apart from secretions, which are being eliminated in any significant concentration. Hence the frequent use in

Biology of the narrower definition of excretion. In the present study, excretion is viewed for the most part in the narrower sense.

Although nitrogenous material may be a minor constituent of various secretions, one organ is usually specialised for the collection and excretion of the nitrogenous waste products of metabolism. In Molluscs, this organ is termed the nephridium or kidney. It is with the kidney and connected organs that this work is concerned.

Work connected with excretion has been carried out on a wide variety of Gastropods, (e.g. Helix lactea, Speeg and Campbell 1968; Achatina fulica, Martin et al. 1965; Pila globosa, Lal and Saxena 1952; Strophocheilus oblongus, Tramell and Campbell 1970 and De Jorge et al. 1969; Helix pomatia, Cepea vindobonensis, Helicogona arbustorum, Jezewska et al. 1963, 1964; Some marine prosobranchs, Duerr 1968, Baribault 1968; Lymnaea stagnalis, Friedl and Bayne 1966 and Bonga and Boer 1969; Lanistes baltemia and Eremia desertorum, Haggag and Fovad, 1968), and a number of papers review the work in this field, (e.g. Potts 1968; Gostan 1965; Needham 1935). However, not until recently (Jezewska 1969) has any work relating to details of excretion in slugs been carried out. Here, Jezewska reported the nephridial excretion of purines by slugs (Limacidae) and snails (Helicidae).

Snails of the genus Helix have been popular experimental animals for many years. In Helix pomatia, the physiology of

excretion, the ultrastructure of the nephridium, and the nature of the excreta have been studied by Vorwhol (1961), Bouillon (1960), and Jezewska (1963) respectively. In common with other-pulmonates, Helix pomatia excretes mainly purines (uric acid, xanthine, and guanine), but as Vorwhol has shown, it can also produce a hypotonic urine when water is plentiful. Jezewska has shown that Limax maximus also excretes these three purines (1969). Otala lactea and Helix aspersa have been shown to excrete gaseous ammonia which is volatilised through the shell, (Speeg and Campbell 1968). Since partial, or even full, uricotelism (or "purinotelism") seems to be the rule in terrestrial invertebrates, one would assume that Agriolimax reticulatus is no exception; certainly, A. reticulatus has more need to conserve water than Helix, since it possesses no external shell.

From his work on H. pomatia, Vorwhol makes the supposition that haemolymph filters through the nephrocyte epithelium from the surrounding blood space into the kidney lumen. In such a system, purines carried to the kidney in the haemolymph, could presumably be removed from the haemolymph filtrate by the nephrocytes, en route. Vorwhol has shown fairly conclusively that the properties of the liquid in the kidney lumen are consistent with the properties that one would expect a haemolymph ultrafiltrate to possess. However, the kidney lumen is confluent with the pericardial cavity via the renopericardial canal, and there

appears to be no conclusive proof that the liquid in the kidney is not derived from the pericardial fluid.

Concretions accumulate within the nephrocytes of Helix and these are released at intervals to pass out of the excretory system via the ureter with some fluid. The ureter appears to modify the kidney liquid, by the resorption of salts and water. Certainly the ureter cells have characteristics in common with water resorbing cells elsewhere in the animal kingdom, e.g. cells of the avian salt gland, (Ernst and Ellis, 1969), and an adequate blood supply for such a purpose.

One might expect that Helix and Agriolimax would share much in common as far as general metabolism is concerned except, possibly, where metabolism is affected directly or indirectly by hibernation. Active individuals of A. reticulatus can be found at all times of the year, and no type of dormancy has been observed. Thus it would obviously not be necessary for A. reticulatus to possess a kidney of accretion, while Helix must accumulate some excretory material over each period of hibernation or aestivation. Nevertheless, perhaps in other respects the excretory physiology of A. reticulatus will prove to be similar to that of H. pomatia.

Before the details of the functioning of any organ system can be studied, it is obviously necessary to determine the anatomy and general physiology of that system. The purpose of this study is to provide such a basic survey of the structure and function of the excretory system of A. reticulatus.

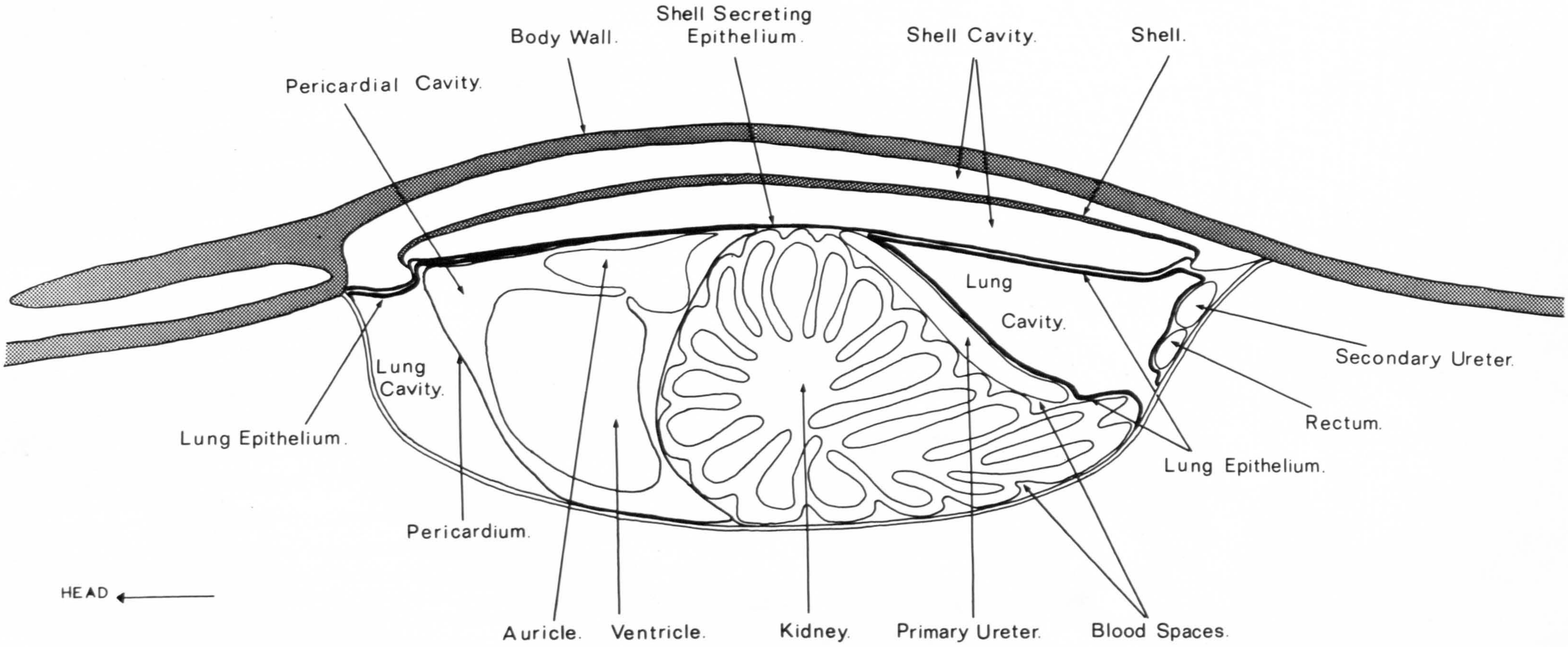
Section 1.

The Anatomy, Histology and Ultrastructure of the Excretory System.

Materials and Methods

Unless otherwise stated, slugs used in all dissections and experiments were collected from an area within a three mile radius around Bangor, and were kept in the laboratory in plastic containers at room temperature. A small wad of cotton wool soaked in water was kept in each container to prevent dessication, and the slugs were maintained on a diet of carrot, supplemented with lettuce. These conditions must be borne in mind when considering the results from experiments of a chemical nature, which could be subject to change according to diet, although slugs which had been kept in the laboratory for more than two or three days were not used for such experiments. The lid of each container was either perforated with air holes, or else consisted partly of fine mesh nylon netting.

The anatomy and morphology of the excretory system was studied by means of dissection, injection and wax sectioning. Slugs were anaesthetised using solid carbon-dioxide, (Bailey, 1969). To investigate the blood supply to the excretory organs Indian-ink and "Chromopaque" were injected with a hypodermic syringe. When injected animals needed to be sectioned to investigate the fine detail of the blood system, a small amount of gelatin ($1_g/50ml$), was added to the



DIAGRAMMATIC LONGITUDINAL SECTION THROUGH THE CENTRE OF THE MANTLE REGION TO SHOW THE RELATIVE POSITIONS OF THE MANTLE ORGANS.

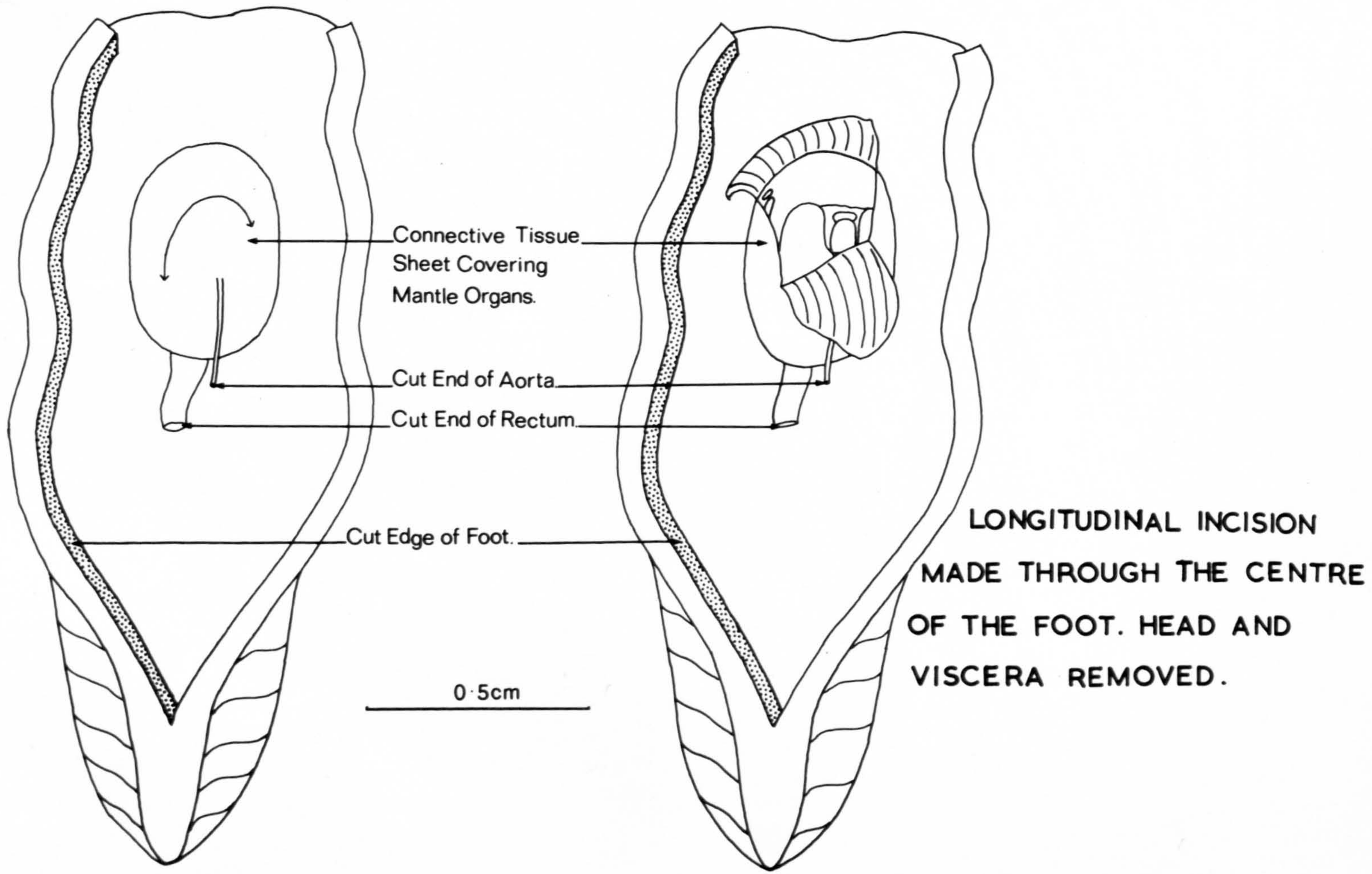
The lung cavity lies directly beneath the shell cavity, and is bordered by the kidney, heart, ureter, and rectum. A horizontal sheet of connective tissue and muscle fibres lies beneath these structures, isolating them from the rest of the viscera. The edges of this sheet join the lateral and dorsal body wall at the same points as the posterior two thirds of the mantle.

The cavity is completely lined with a simple squamous epithelium. It opens to the exterior by the pneumostome, which is situated just above the right hand edge of the mantle, about one third of the way from its posterior end. The actual lung surface is characterised by the presence of an extensive plexus of venous vessels beneath the lung epithelium. The lung surface lines the whole of the cavity, except the pericardial wall and those parts of the connective tissue sheet not covered by the kidney. (Fig.1)

The Kidney.

The kidney of A. reticulatus is a sac-like structure which protrudes into the postero-ventral portion of the lung cavity. (Figs. 2 & 3). It is bilobed anteriorly, and the heart lies between these two lobes. The bilateral symmetry of the kidney is broken by the presence of a right posterior lobe, which lies in the floor of the posterior right-hand corner of the lung cavity. The horizontal sheet of connective tissue and muscle fibres which isolates the lung cavity from the viscera, extends beneath the kidney. The kidney is overlaid dorsally and anteriorly by lung

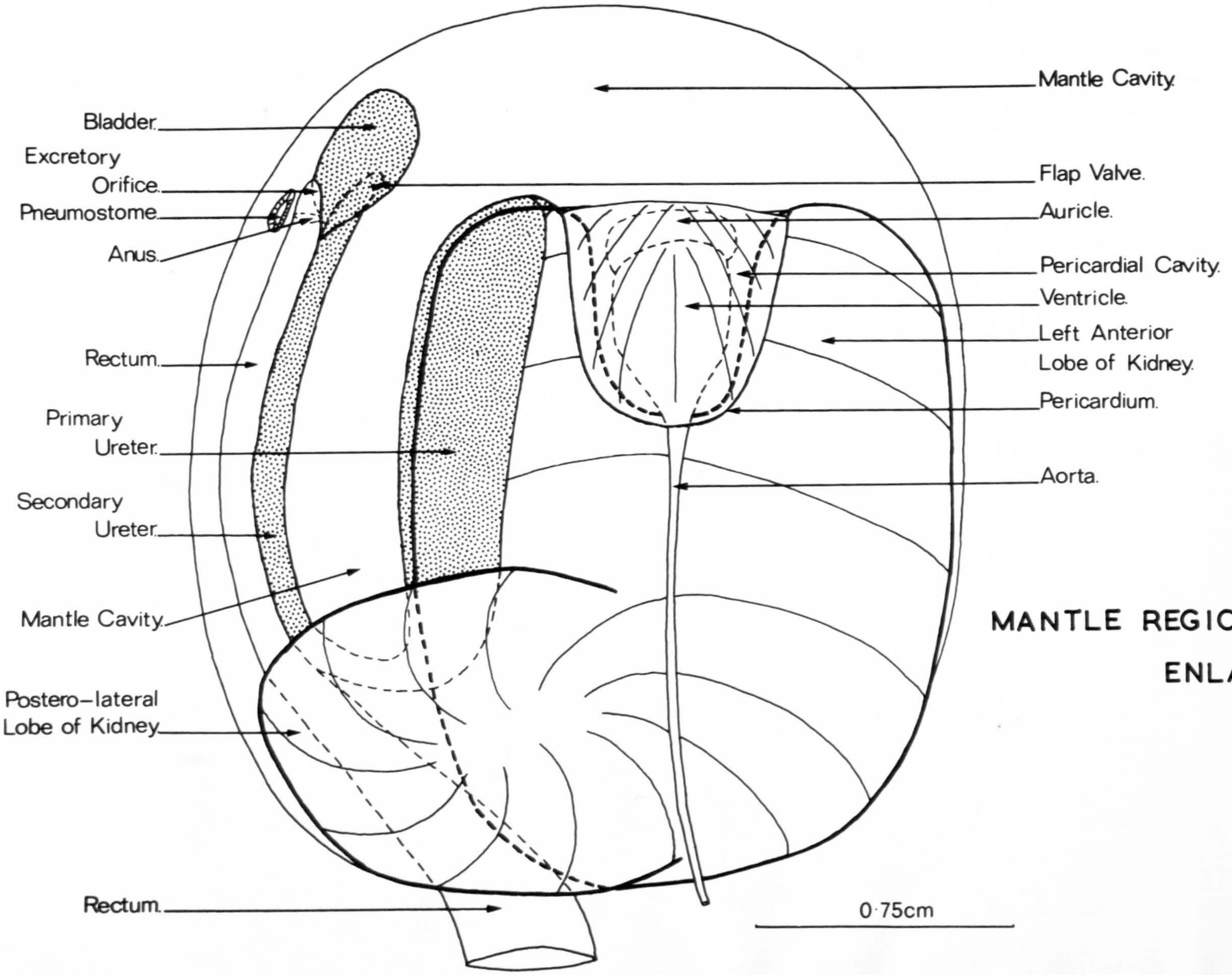
FIGURE 2.



LONGITUDINAL INCISION MADE THROUGH THE CENTRE OF THE FOOT. HEAD AND VISCERA REMOVED.

STAGES IN THE VENTRAL DISSECTION OF A. RETICULATUS TO EXPOSE THE MANTLE ORGANS.

FIGURE 3.



MANTLE REGION

ENLARGED.

epithelium and posteriorly a ridge of lung tissue connects the dorsal surface of the kidney to the roof of the lung cavity. The kidney epithelium is simple and columnar, and it is thrown into a series of deep folds which are aligned predominantly in a longitudinal direction.

The renopericardial canal is well defined in Agriolimax (Fig.4) It connects the pericardial cavity and the right anterior lobe of the kidney at the most postero-ventral part of the junction between them.

The Ureter.

The ureter is divided into two parts, the primary and secondary ureters. Whether these two are of different ontogenetic origin is not known, but they are histologically distinct. The secondary ureter opens into a sac or bladder, which in turn opens to the exterior at the top of the cloacal slit. This bladder is apparently characteristic of the Limacidae (Hyman, 1967).

Primary Ureter.

The primary ureter opens by a small aperture from the anterior end of the right anterior lobe of the kidney. There is no apparent valve or sphincter, but it is evident that some closing mechanism is present, since injection media will not enter the kidney by this aperture when they are injected along the ureter. The primary ureter lies beneath the lung epithelium and adjacent to the kidney. It stretches to the posterior end of the kidney, covering most of the dorsal nephridial surface posterior to the

FIGURE 4.

Light micrograph, T.S. Kidney and Heart

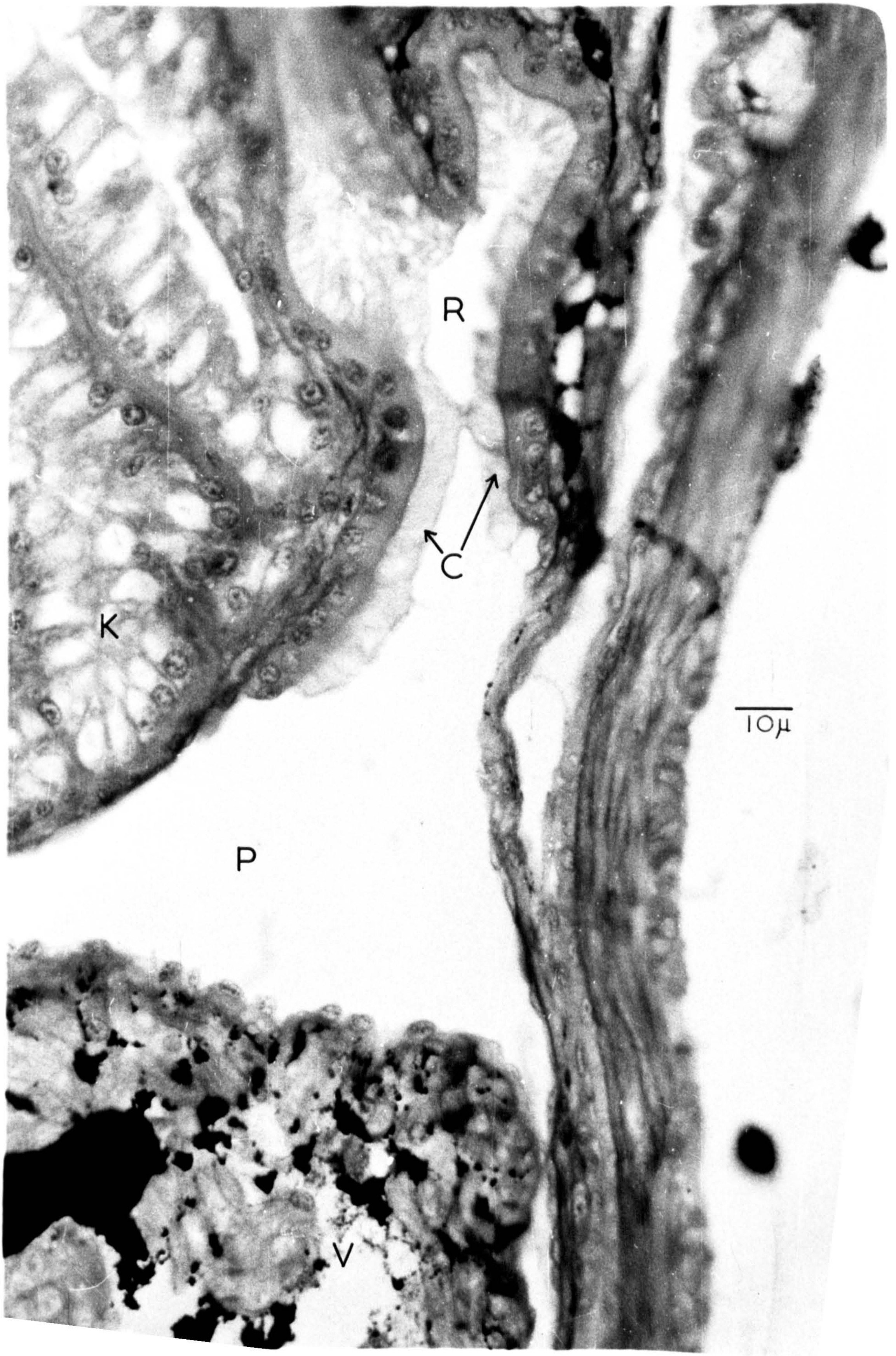
C : Cilia

K : Kidney

P : Pericardial Cavity

R : Renopericardial canal

V : Ventricle


R
C
K
P
V
10μ

heart. Anterior to the heart it covers the right anterior lobe of the kidney only. In its normal state the primary ureter is filled with fluid in which small white granules move around at a high speed. In cross section, however, the ureter wall is seen to be quite highly folded. This folding presumably allows the ureter to expand to accommodate larger volumes of fluid.

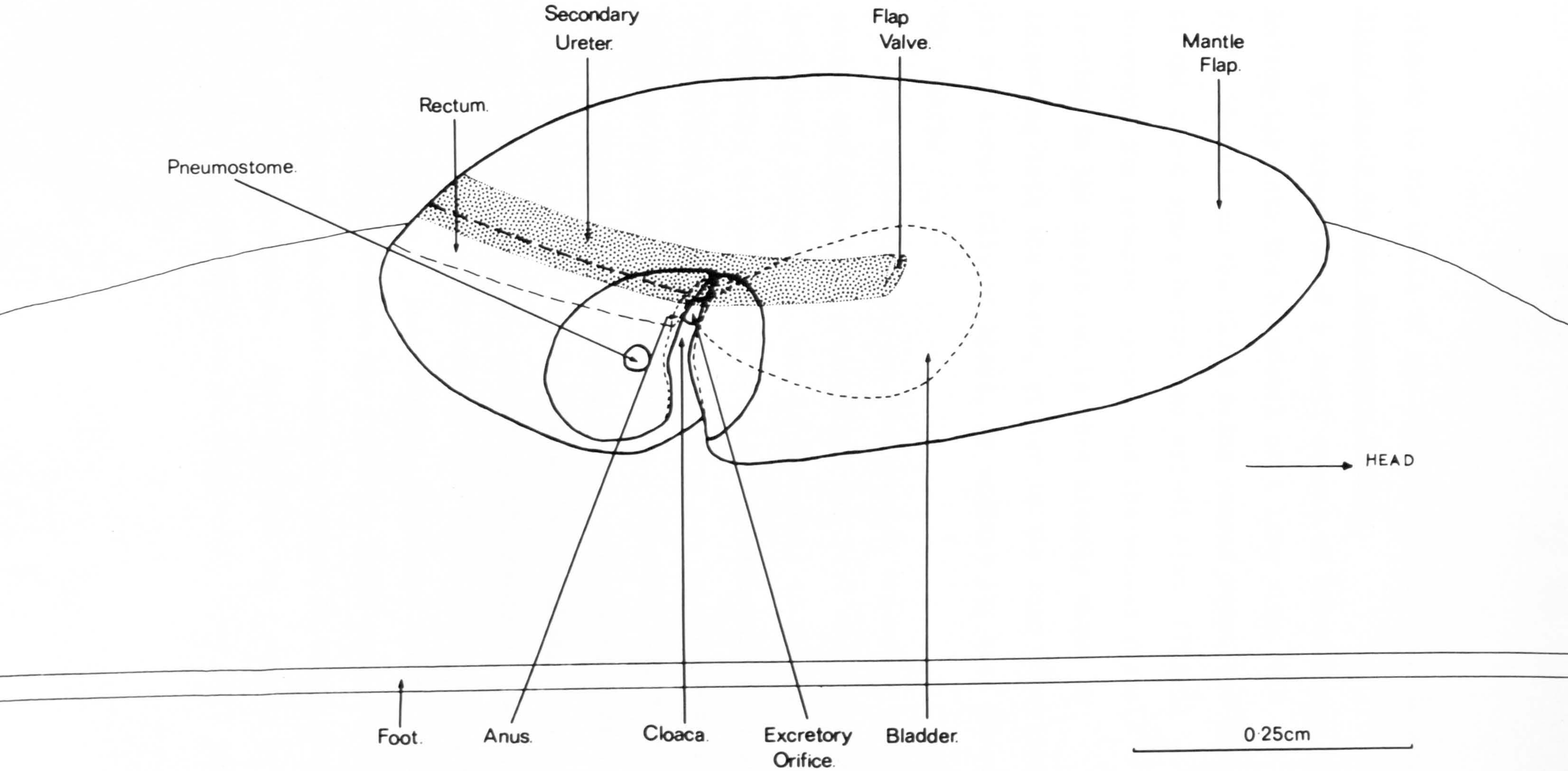
Secondary Ureter.

The primary ureter is continuous with the secondary ureter at the posterior end of the kidney. The secondary ureter is situated beneath the lung epithelium on the right-hand side of the lung cavity, and is closely applied to the dorsal surface of the rectum. It opens into the bladder just above, and anterior to, the pneumostome. (Fig.5) A flap of tissue (the "flap valve") closes off the end of the secondary ureter, and probably prevents the contents of the bladder from passing back into the ureter.

The Bladder.

The wall of the bladder is quite extensively folded to allow for expansion. The bladder extends anteriorly from the flap valve for a short distance beneath the lung epithelium, and also extends posteriorly to open into the cloacal slit by a circular aperture in the lateral body wall adjacent to the anus. In the live animal the thin bladder wall presses against the inside edges of this aperture, possibly because of the hydrostatic pressure of the body fluids. This has the effect of closing the bladder outlet. A bridge of lung tissue across the lung cavity connects the

FIGURE 5.



MANTLE REGION DRAWN TO SHOW THE APPROXIMATE POSITIONS OF THE URETER, BLADDER AND RECTUM BENEATH THE MANTLE TISSUE.

bladder to the walls of the primary ureter and kidney.

Blood Supply to the Excretory Organs.

The injection of a small amount of Chromopaque or Indian-ink into the haemocoel of a live slug, via the foot, results in the fluid being pumped round the whole of the blood system before the animal dies. (Fig.6) However, the arterial system and the venous vessels leading to the heart can be more clearly shown by injecting into the aorta, either in the same direction as the normal flow of blood, or against it, back through the heart.

The connective tissue sheet which separates the lung cavity and excretory system from the rest of the viscera, is loosely trabecular, and is permeable to blood. Little connective tissue exists between the external walls of the longitudinal kidney folds and gaps can be seen between the folds in sections of the kidney. However, the fold apices, distal to the kidney lumen, are rather more firmly bound together with connective tissue. Thus, longitudinal sinus-like channels are formed by the folds of the kidney. These channels readily expand when fluid is injected into the blood system. Undoubtably, injection causes some of the connections between the folds to break when the channels expand, because an extra volume of fluid is being introduced into the blood system. Nevertheless the channels between the folds are unlikely to be artifacts, since they can be

FIGURE 6.

Light micrograph. T.S. Kidney region of an animal injected with Indian Ink : blood spaces are black. (The kidney is infected with a Trematode parasite.)

A : Auricle

K : Kidney

P : Primary ureter

R : Renopericardial canal

T : Trematode parasite

V : Ventricle

A
P
P
T
T
K
K
R
100µ

observed in sections of uninjected animals under the light microscope and also in electron micrographs. Lymphocytes may also be observed in electron micrographs in these spaces. The kidney and ureter thus lie virtually within the haemocoel, and the kidney folds provide a means of channeling blood back into the heart. The blood returning from the haemocoel via the kidney folds enters the right hand side of the auricle.

In dissected specimens, the wall of the lung appears as a network of fine vessels extending over the kidney. This network also covers the rectum, ureter and bladder, and here the vessels are more prominent. The network is not apparent in transverse sections of injected animals, where in fact, no discrete lung vessels can be seen, except in the roof of the lung cavity, where they run in a longitudinal direction. If a large excess of fluid is injected into the haemocoel, cross sections show that even these vessels disappear, or rather merge with the general haemocoel.

Thus under normal conditions in the live animal, blood is returned to the heart via channels which are kept discrete by the blood itself, but blood can permeate all around the mantle organs.

The lung and kidney epithelia are separated only by connective tissue and muscle fibres. Thus the blood channels formed by the folding of the kidney epithelium are in fairly intimate contact with the network of lung vessels.

FIGURE 7.

Dissection to show the Sinuses (cut open) in the body wall. The viscera have been removed.

A : Auricle

K : Kidney

L : Lung

LPS : Left pallial sinus

R : Renopericardial canal

RPS : Right pallial sinus

V : Ventricle

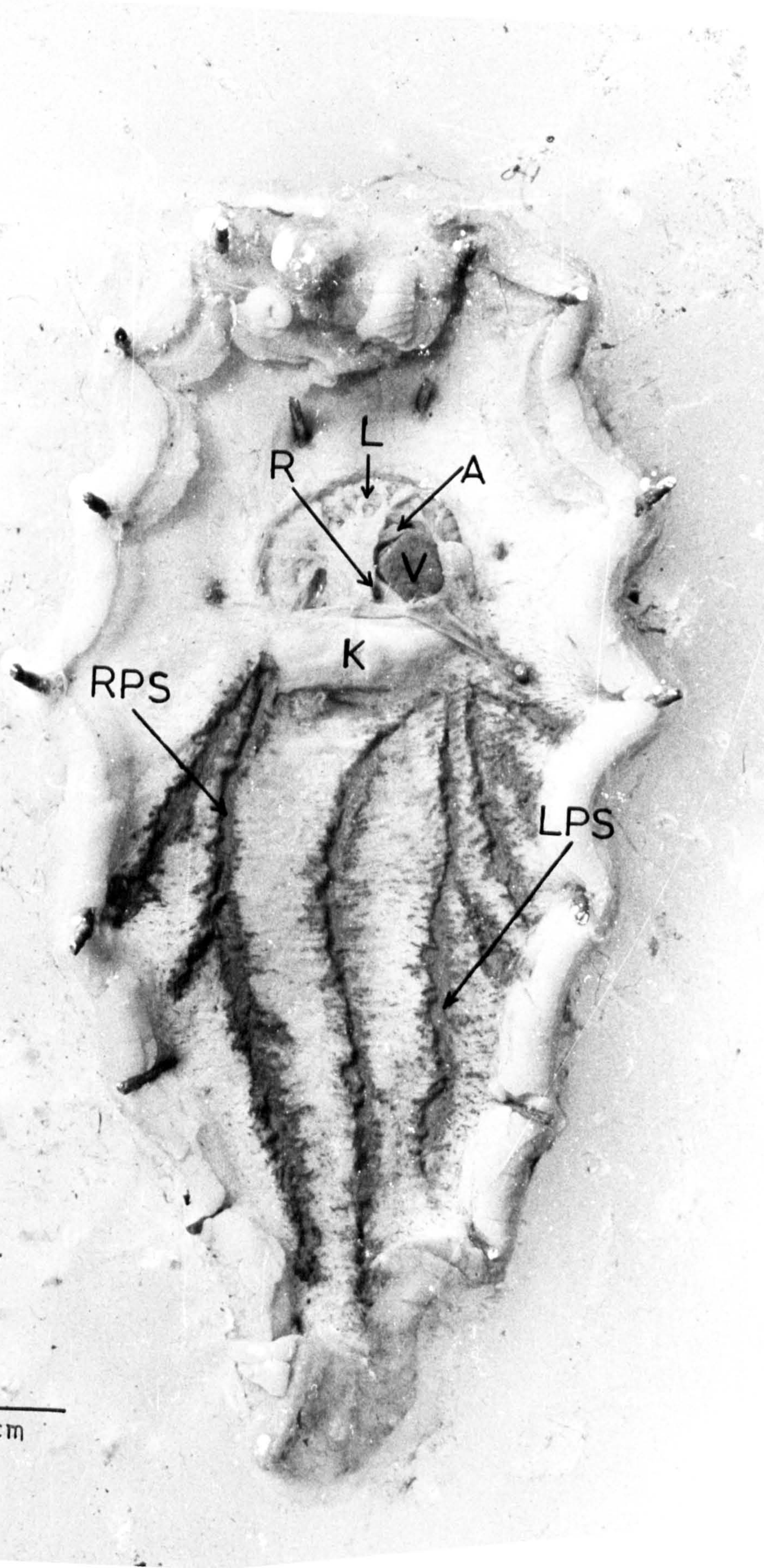
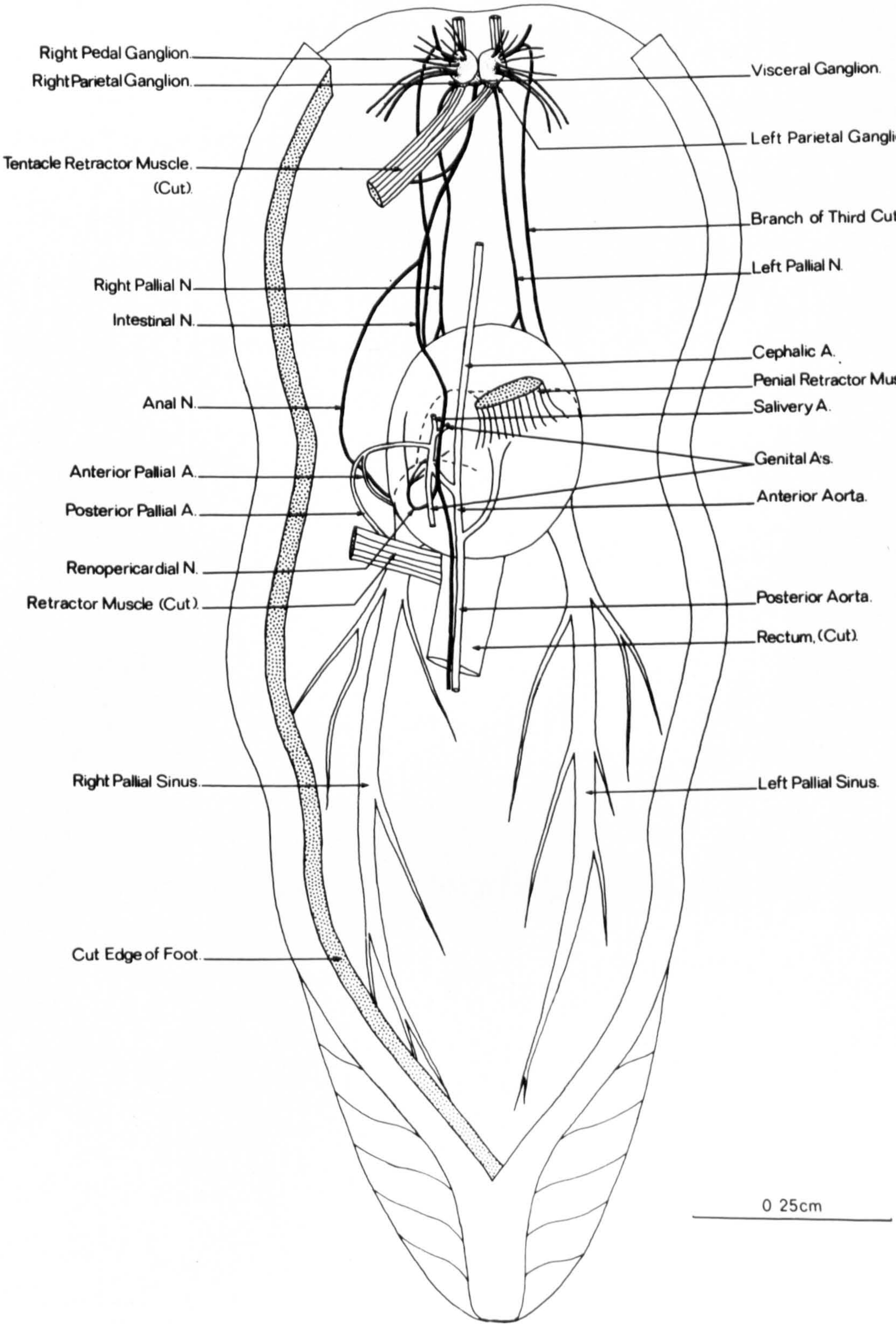

L
R
A
V
K
RPS
LPS
0·25cm

FIGURE 8.

VENTRAL DISSECTION TO SHOW THE INNERVATION AND BLOOD SUPPLY TO THE MANTLE REGION.



However, the vessel disappears when the liquid dissecting medium is squirted onto it with a Pasteur pipette. The occurence of this artefact is due to the following circumstances. Firstly, the injection medium tends to leak out from the blood sinuses and the hypodermic needle into any available space. Secondly, around the lateral circumference of the lung cavity, there is a natural groove formed where the connective tissue sheet composing part of the floor of the cavity, meets the dorsal and lateral body walls. Any injection medium which does not leak out, tends to collect in this groove, and give the impression that a ring vessel is present. Small sinuses leading into the lung are most prevalent in the connective tissue immediately beneath the lateral body wall on either side of the lung cavity. Very little blood enters the lung from its anterior edge. In demonstrating the venous system by injection back through the heart, the lung vessels present less resistance to pressure from the hypdermic than the venous channels formed by the folding of the kidney epithelium. However, on injection into the right pallial sinus, the kidney channels are the first to fill with the injecting medium. It may be that Agriolimax can regulate its flow of venous blood to give preference either to the lung or to the kidney.

The heart lies assymetrically within the pericardial cavity. The sinuses from the lung and kidney enter the auricle where the pericardium meets the dorsal surface

of the right anterior lobe of the kidney. Valves are present between the auricle and ventricle, and between the ventricle and the aorta. At the posterior ventral edge of the pericardium, the ventricle gives rise to the aorta, which then passes through the connective tissue sheet beneath the kidney.

An arterial supply to the kidney is lacking, but the anterior pallial artery does supply fine branches which terminate in the regions of the secondary ureter and bladder.

The Nerve Supply to the Exretory Organs.

The nerve supply to the excretory system is sparse, although a few fibres can be seen in cross sections, running through the connective tissue around the kidney and ureter.

Nerve branches, too fine to follow under the dissecting microscope, terminate in the region of the bladder, from the right pallial nerve, and the secondary ureter, from the anal nerve. (Fig.8) A fine branch of the intestinal nerve, the renopericardial nerve, supplies the connective tissue sheet where the anterior edge of the posterior kidney lobe meets the right anterior lobe of the kidney. This innervation is very close to the renopericardial canal. However, it is not known if the cilia of the canal are under nervous control.

1B. The Histology and Ultrastructure of the Excretory System.

The renal cells of A. reticulatus are very similar in structure to those of Helix pomatia, described by Bouillon in 1960.

The Kidney.

The nephrocytes are columnar and can be upto 30μ in length. They lie on a basal lamina, about 600Å thick. The basal plasmalemma is convoluted to form many irregular, interdigitating processes. (Figs. 9 & 10) The basal lamina does not intrude between the convolutions, but lies immediately beneath them. Beneath the basal lamina there is a narrow zone of collagen fibres, which abuts directly onto the blood space.

Each nephrocyte has a basal nucleus, with a prominent nucleolus, and either a large apical vacuole, which may take up as much as two thirds of the total cell volume, or a large number of small apical vacuoles. (Fig. 9) The contents of some of the large vacuoles may be either partially or wholly crystallised . (Fig. 11) Logically, this condition suggests that the small vesicles fuse to form a large 'mature' vacuole within the nephrocyte, but this has yet to be proved.

Mitochondria are fairly numerous and are predominantly basal in position. They are mainly spherical to cylindrical in shape, with a diameter of about 0.5μ , but a few are dumbell-shaped, and may have a length of up to 1.5μ. Their cristae are well defined, and are aligned transversely . Most of the cristae completely span the mitochondrion.

FIGURE 9.

E. M. Nephrocytes.

B : Blood Space

ER : Endoplasmic reticulum

KL : Kidney lumen

M : Microvilli

MT : Mitochondria

N : Nucleus

NL : Nucleolus

V : Vacuole

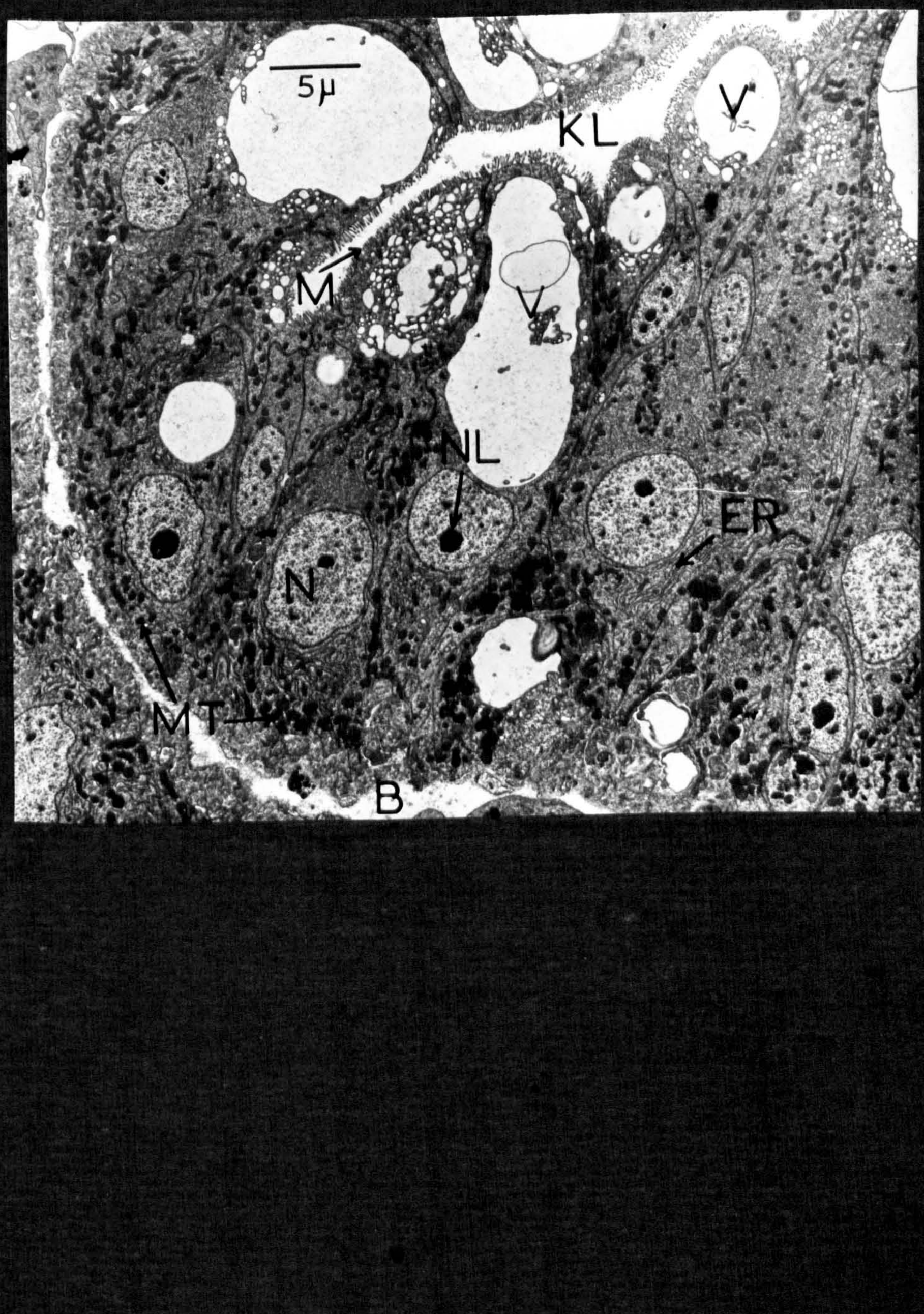

5μ
KL
V
M
V
NL
ER
N
MT
B

FIGURE 10.

E. M. Nephrocyte

a) Cell Base

BL : Basal lamina

CF : Collagen fibres

CBP : Convolutions of basal plasmalemma

b) Edge of Apical Vacuole

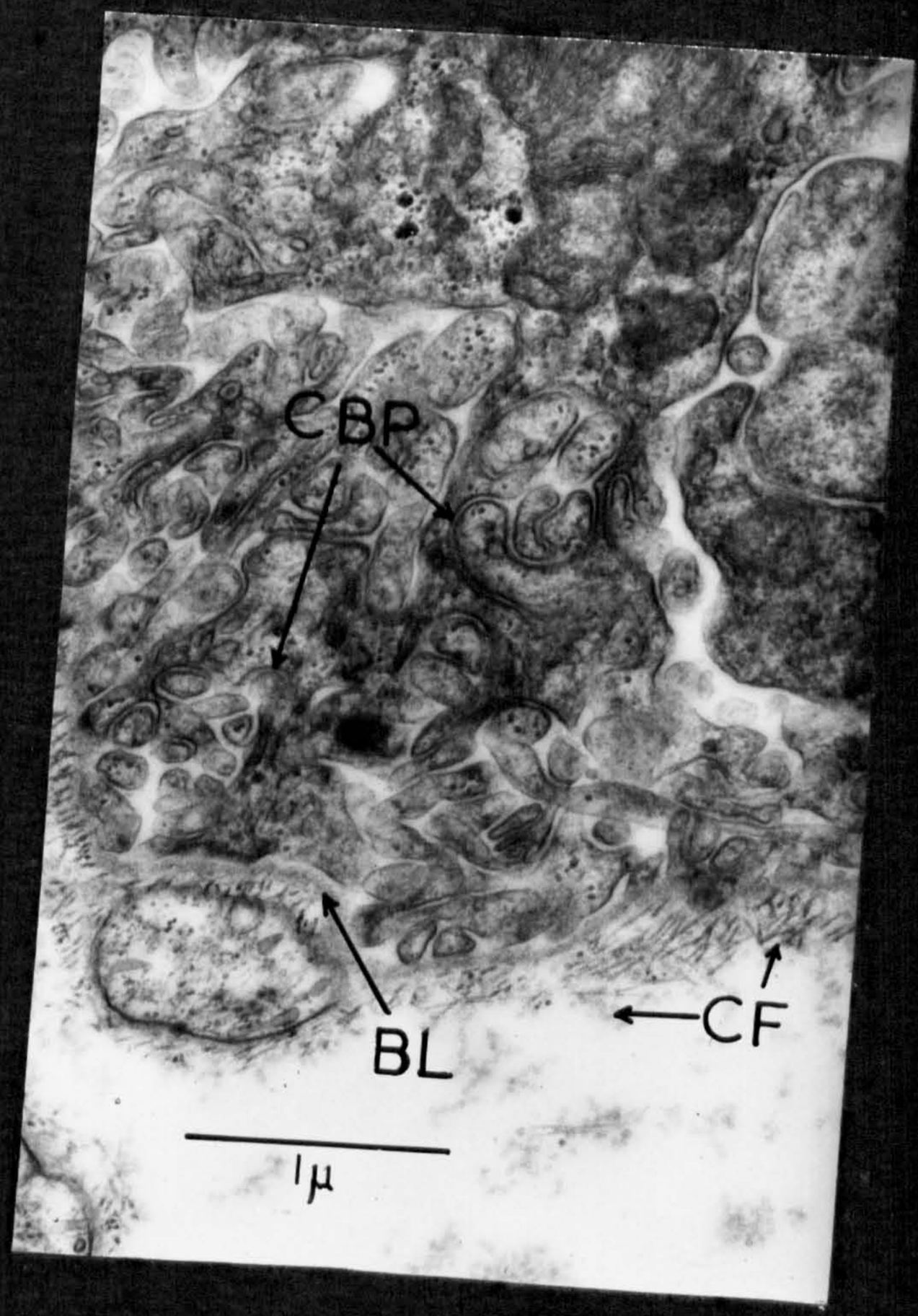
G : Glycogen

GA : Golgi apparatus

LP : Lateral plasmalemma

MT : Mitochondria

V : Vacuole


CBP
BL
CF
1μ

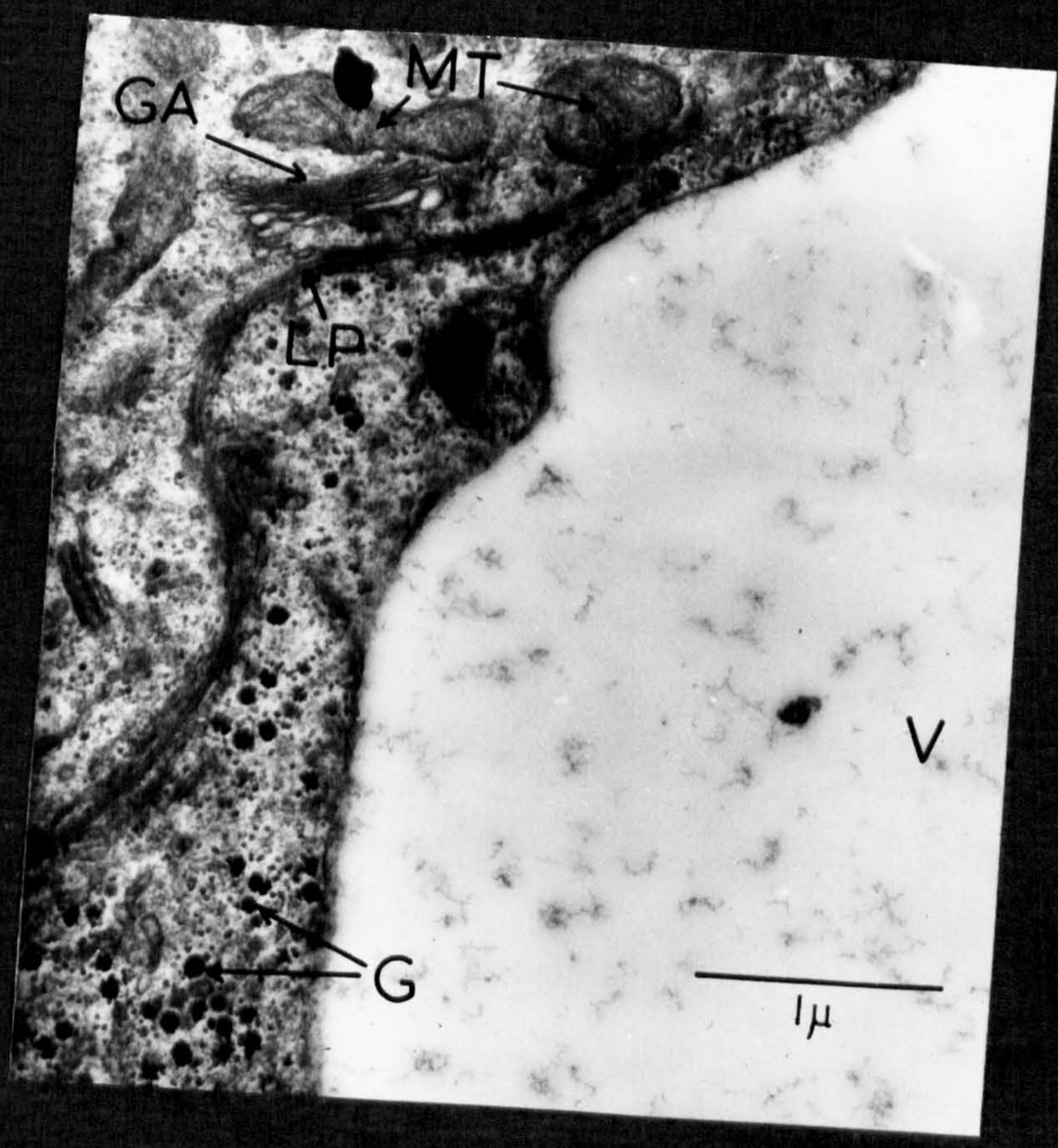

GA
MT
LP
V
G
1μ

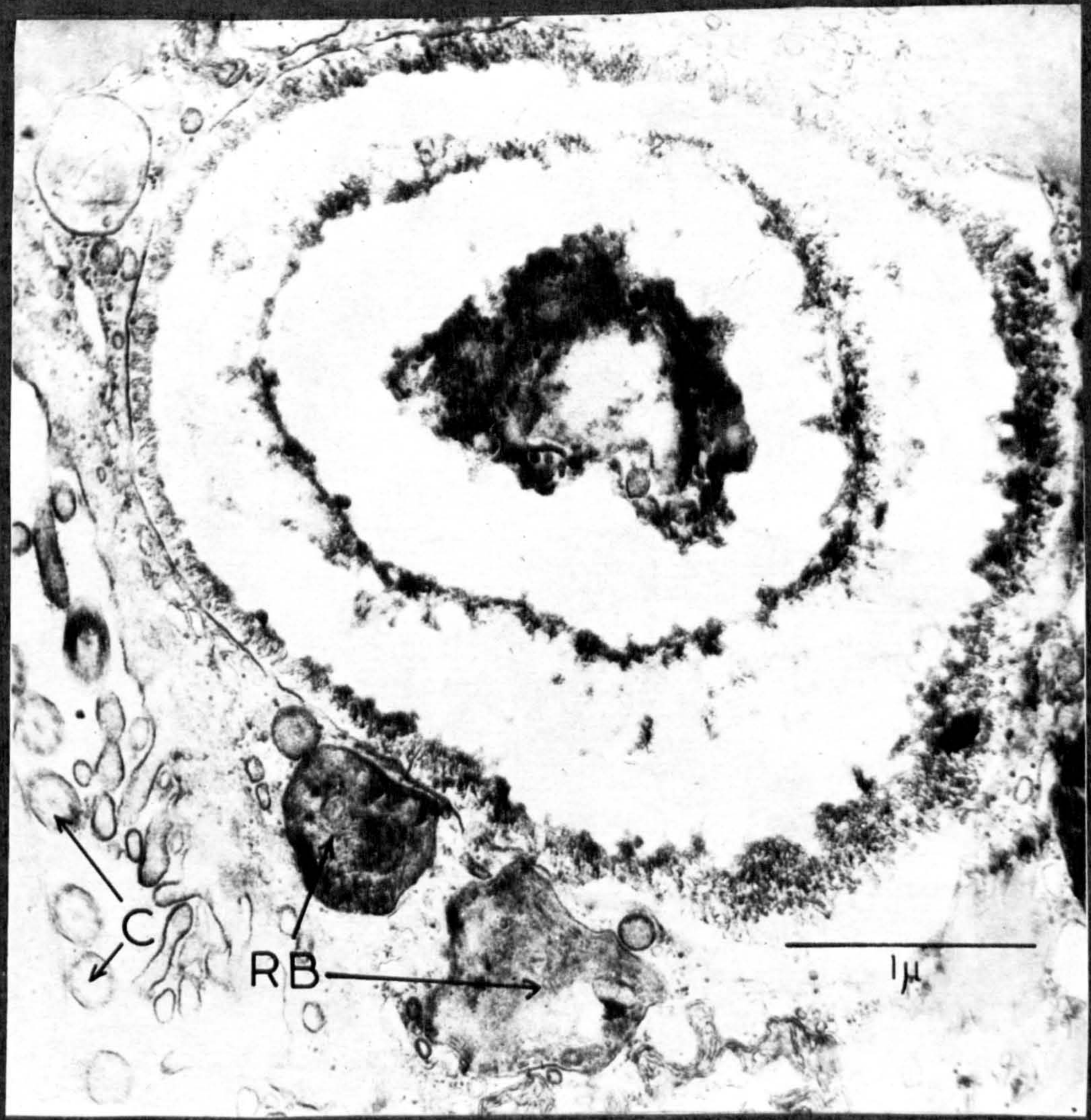
FIGURE 11.

E.M. Primary ureter lumen :

nephrocyte concretion in cell debris.

C : Cilia

RB : Residual Bodies


C
RB
1μ

Both α- rosette and β- granule forms of glycogen are present. These are fairly well dispersed within the cytoplasm, but are slightly more numerous between the mitochondria. Golgi bodies are also present but are not numerous. They have the classical structure of four to eight flattened cisternae arranged in a stack, and they generally lie close to the walls of the nephrocyte. (Fig.10b)

Each nephrocyte has an apical brush border, about 0.8μ thick. Both rough and smooth endoplasmic reticula are present. The rough reticulum is particularly noticeable in the region of the nucleus, where it occurs as aggregations of filamentous cisternae. Free ribosomes are also present. but it is often difficult to distinguish them from β-glycogen. Smooth reticulum occurs thrcughout the cytoplasm, and takes the form of small vesicles which vary in diameter from 500 to 2,000 Å.

There are no pronounced intercellular spaces; the nephrocytes being separated by a gap of about 150 Å.

The Primary Ureter

The primary ureter is lined with a simple epithelium of two cell types. (Fig.12) For convenience I have termed these "pyramidal" and "columnar". Both types of cell are highly irregular in form, due to the folding of their plasmalemmas, but they do conform very generally to these two basic shapes. The columnar cells form the greater portion of the epithelium.

The pyramidal cells can be found throughout the epithelium, but they occur mainly at the luminal apices of the epithelial

FIGURE 12

Light micrograph.

T.S. Kidney and primary ureter.

B : Blood spaces

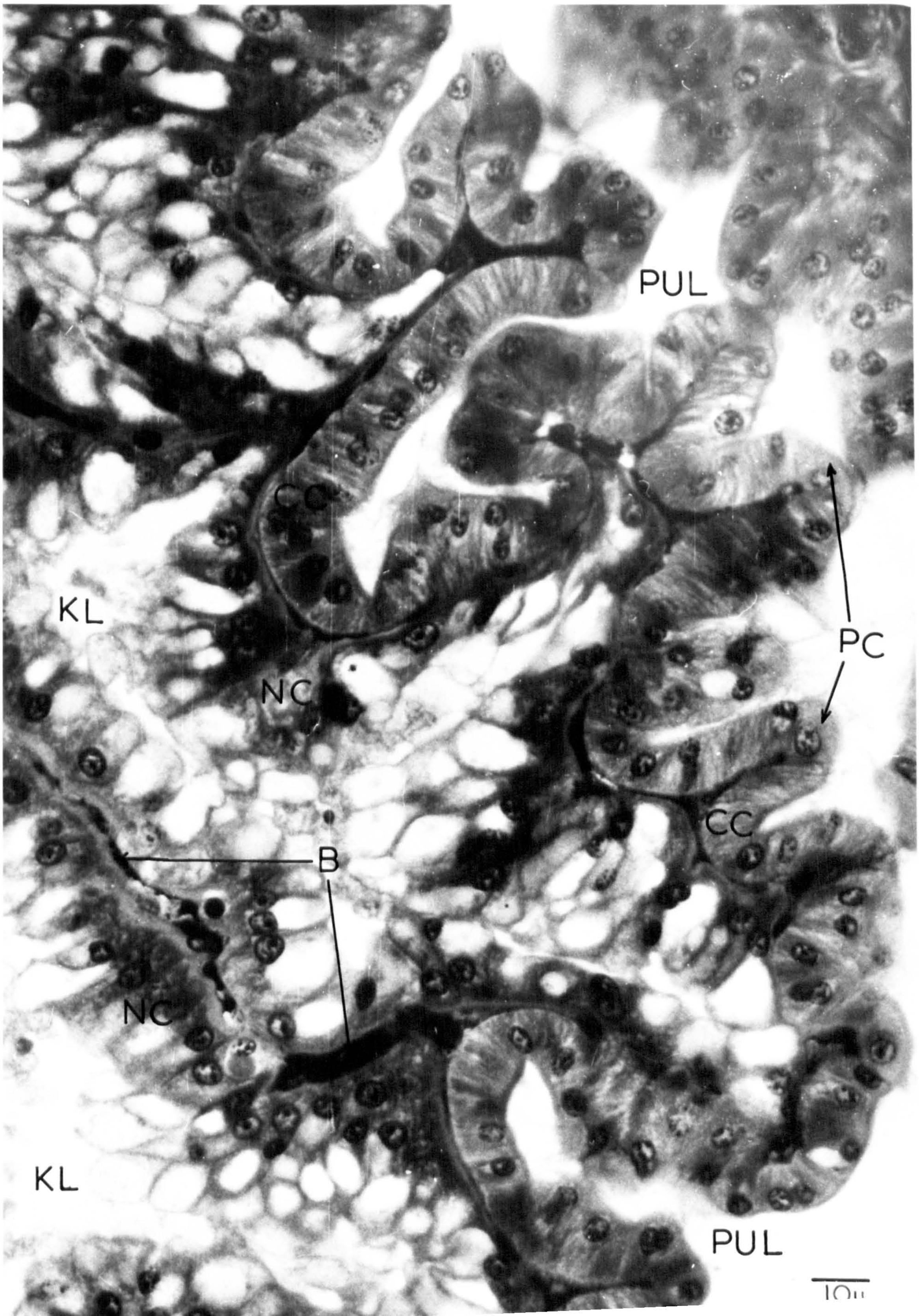
CC : Columnar cells

KL : Kidney lumen

NC : Nephrocytes

PC : Pyramidal Cells

PUL : Primary ureter lumen


PUL
KL
PC
NC
CC
B
NC
KL
PUL

folds. In fact, a single pyramidal cell usually forms the apex of a fold.

Columnar cell type.

The most prominent feature of these cells is their dense population of mitochondria. The mitochondria are intimately associated with the cell membrane, which is deeply and intricately folded both laterally and basally. (Figs. 13a & 14a) This situation, which involves a massive increase in the surface area of the cell, occurs in numerous cell types which are involved with the active transport of ions, e.g. cells of the avian salt gland (Doyle, 1960), and of the vertebrate proximal tubule (Rhodin, 1958). The term " β-cytomembranes" has been fairly widely used to describe such a system of membranes formed by the folding of the plasmalemma. The cell processes formed by this folding interdigitate in both an intra-, and inter-cellular fashion, and the interdigitations are often so complex that it is extremely difficult to distinguish the boundaries of adjacent cells. These processes also interdigitate, but to a much lesser extent, with processes from the pyramidal cells.

The β-cytomembranes are aligned predominantly in the vertical direction and often extend as far as the apical plasmalemma. A few of the mitochondria are irregular in shape, but most of them are cylindrical, being up to 1.5μ in length, and the β-cytomembranes tend to follow their contours. The mitochondrial cristae are numerous and well defined, and mitochondrial granules are often evident. Vesicles formed by rough endoplasmic reticulum are common apically and also

FIGURE 13.

E.M. Primary ureter

a) Columnar epithelium with pyramidal cell.

CC : Columnar cells

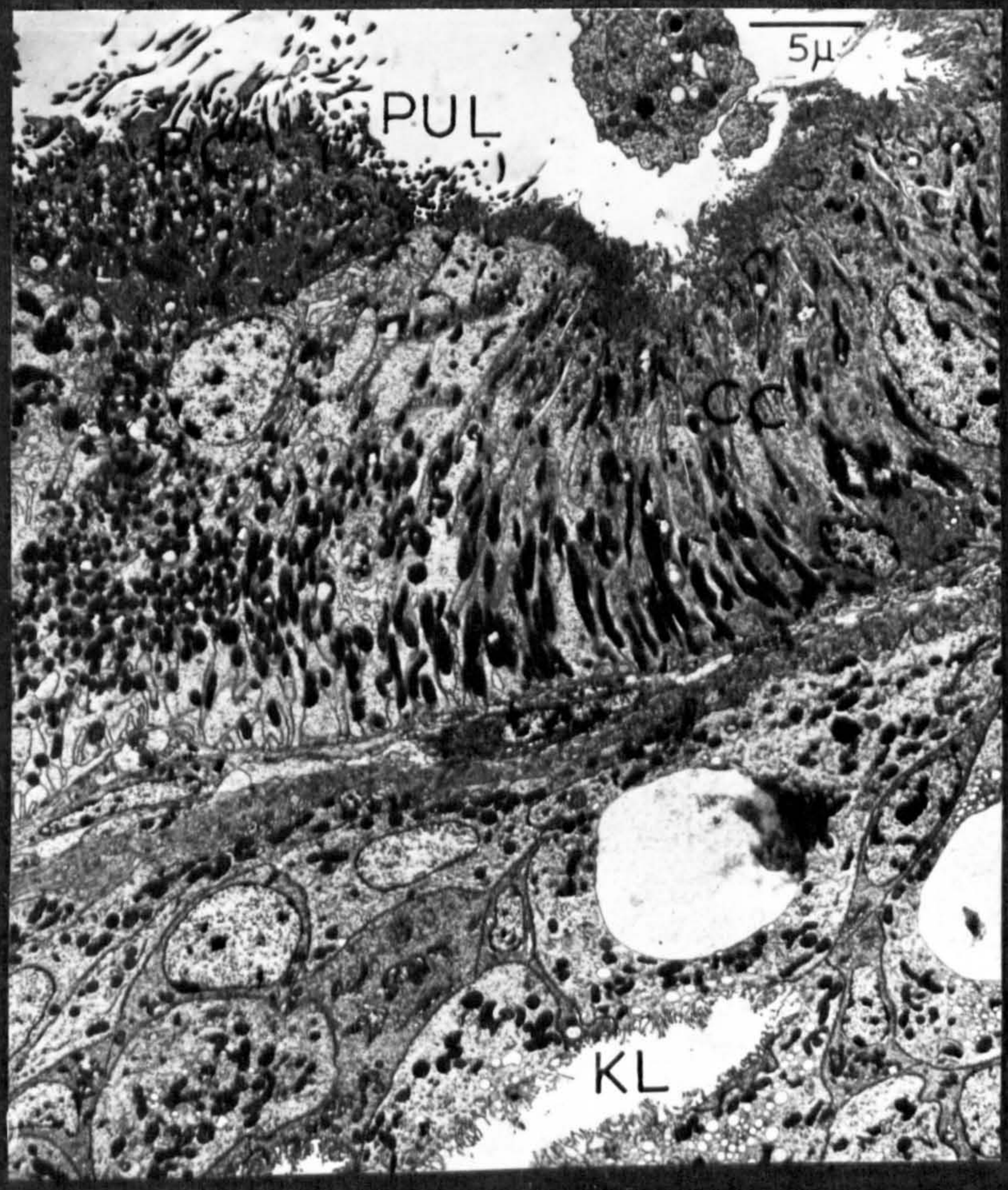
KL : Kidney lumen

PC : Pyramidal cell

PUL ; Primary ureter lumen.

b) Pyramidal cell.

CR : Cilia rootlets


5μ
PUL
CC
KL

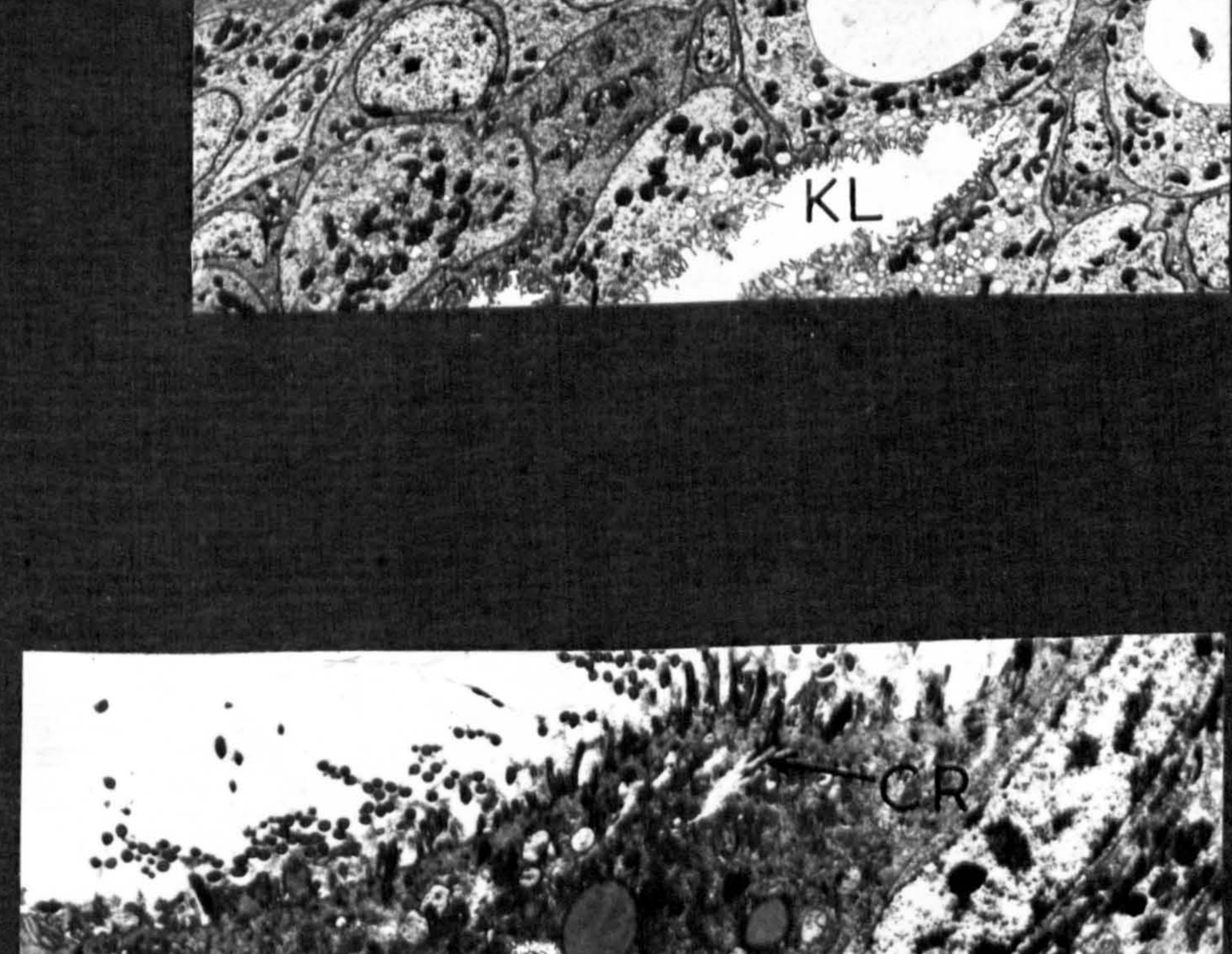

CR
2μ

FIGURE 14.

E.M. Primary ureter

a) Columnar cell base

B : Blood space

BC : β - cytomembranes

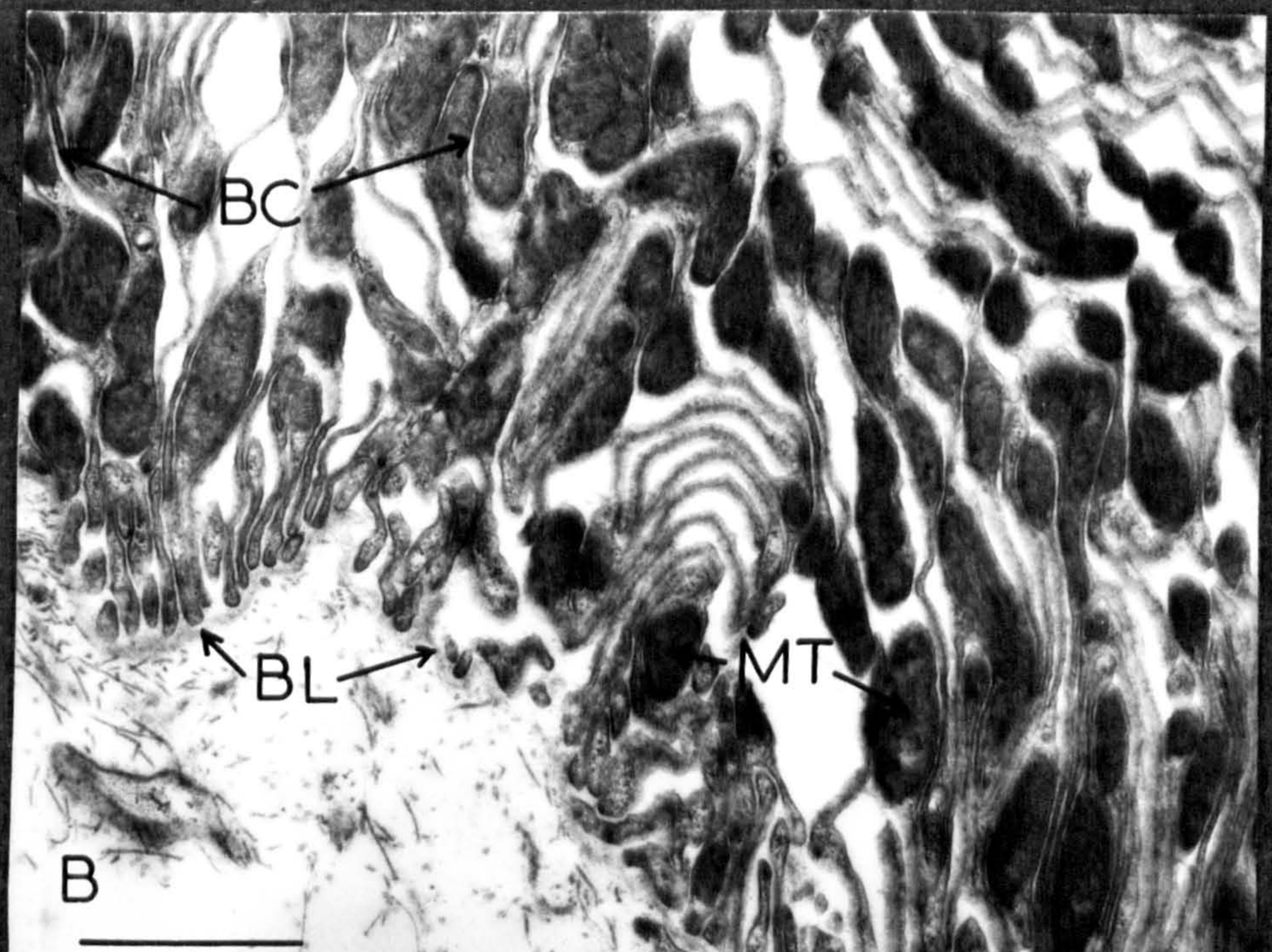
BL : Basal lamina

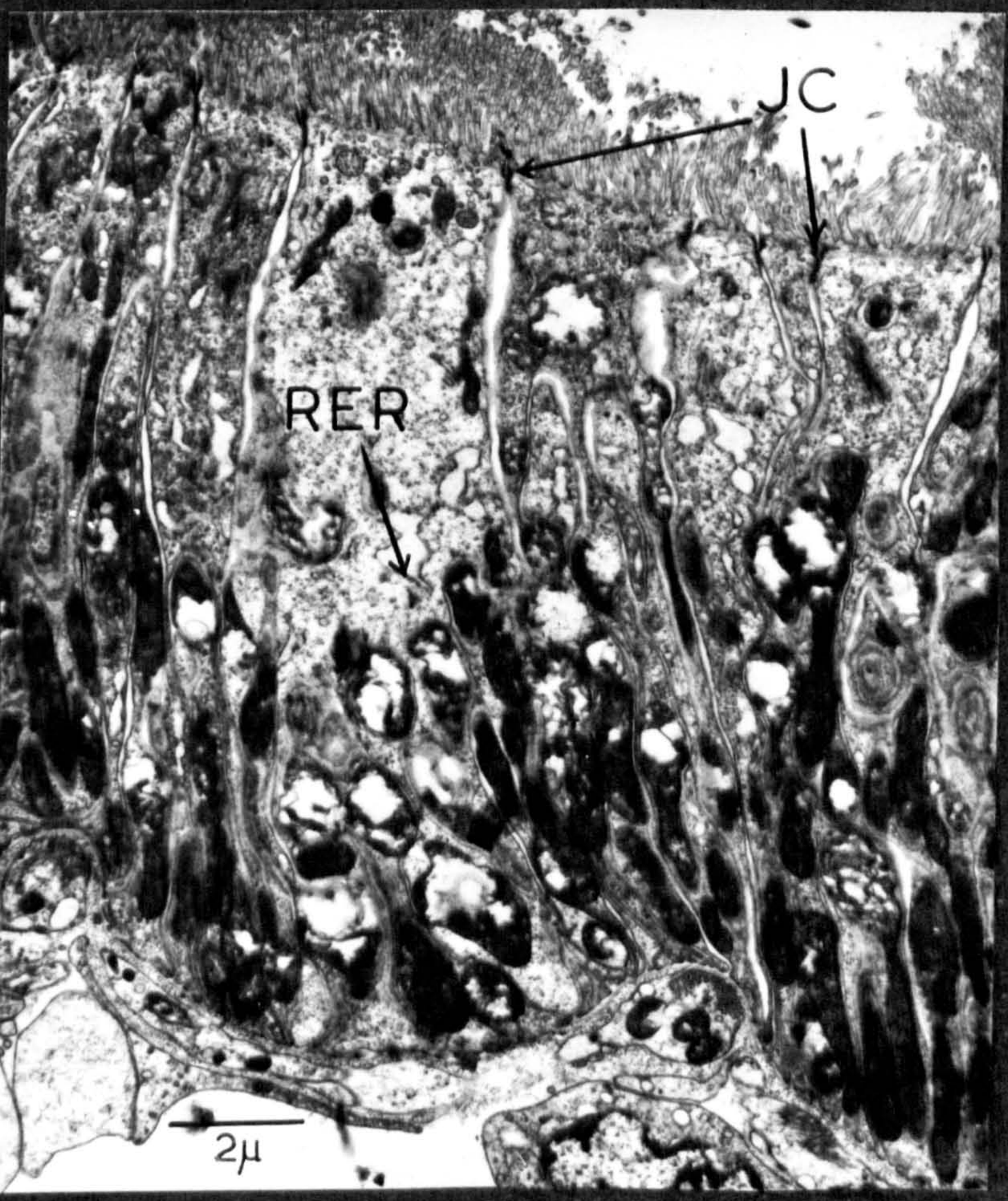
MT : Mitochondria

b) Columnar cell apices

JC : Junctional complexes

RER : Rough endoplasmic reticulum


BC
BL
MT
B
1μ


JC
RER
2μ

between the β-cytomembranes. Free ribosomes are also common in the cytoplasm. Another constituent of these cells is glycogen which occurs as β-particles scattered in the cytoplasm around the mitochondria.

Nuclei tend to be apical in position, and are generally closely surrounded by β-cytomembranes, but they do sometimes lie within an area of clear cytoplasm. Their nucleoli are less prominent than those of the nephrocyte nuclei, and their condensed chromatin content tends to be more peripherally placed, rather than uniformly distributed in the nuclear matrix.

The apical plasmalemma is modified to form a dense layer of microvilli which is, on average, 1 μ thick. (Fig.14b)

The cells are joined at their apical surfaces by conspicuous junctional complexes. As with the nephrocytes, a basal lamina and a narrow zone of collagen fibres separate the ureter from its surrounding blood space.

Pyramidal cells.

These cells approximate very closely to a pyramidal shape. The widths of their apices, (pyramid bases), may be up to 18 or 20 μ, and the widths of their bases, (pyramid apices), are often less than 1 μ. The luminal face of each cell is ciliated, and cilia rootlets may be seen in the apical cytoplasm. (Fig.13b) Cilia are presumably responsible for the rapid movement of granules in the fluid contained in the primary ureter, which may be observed in dissected live specimens. Microvilli are found between the cilia, but are few in number compared with those at the surfaces

of the columnar cells. The lateral surfaces of the pyramidal cells are often irregular, but lack the profuse interdigitations of the columnar cell membranes. Some infolding of the basal membrane does occur, however. Junctional complexes at the luminal apices are again conspicuous, and the usual lamina and collagen fibres are present basally.

Cell nuclei are central, and may contain either homogeneous nucleoplasm, or fairly discrete aggregations of condensed chromatin.

This cell type contains far fewer mitochondria. They are more ovoid than cylindrical in shape, concentrated apically rather than basally, and their cristae are not so well defined as those of the columnar cell mitochondria. A small number of vacuoles is usually present, in the apical cytoplasm. Their size varies between approximately 0.1 and 1μ. β-glycogen particles are fairly abundant throughout the cytoplasm, and are often concentrated in aggregations basally. Endoplasmic reticulum is not abundant, but it is present mainly in the region of the nucleus, as rounded and elongated vesicles, which may be rough or smooth. Free ribosomes are also present. Several golgi bodies may be found in the region apical to the nucleus. (Fig.15a)

Discrete bundles of what appear to be extremely fine filaments (one filament is approximately 40 Å in diameter), may be seen running longitudinally and transversely through the cytoplasm. These bundles are probably tonofibrils and can be up to 0.4μ in diameter, and over 1μ long. They occur basally, and can extend up around the nucleus. Sections through cilia may

FIGURE 15.

E.M. Primary ureter : pyramidal cells

a) Nuclear region

FF : Region of fine filaments

IC : Intracellular cilia

N : Nucleus

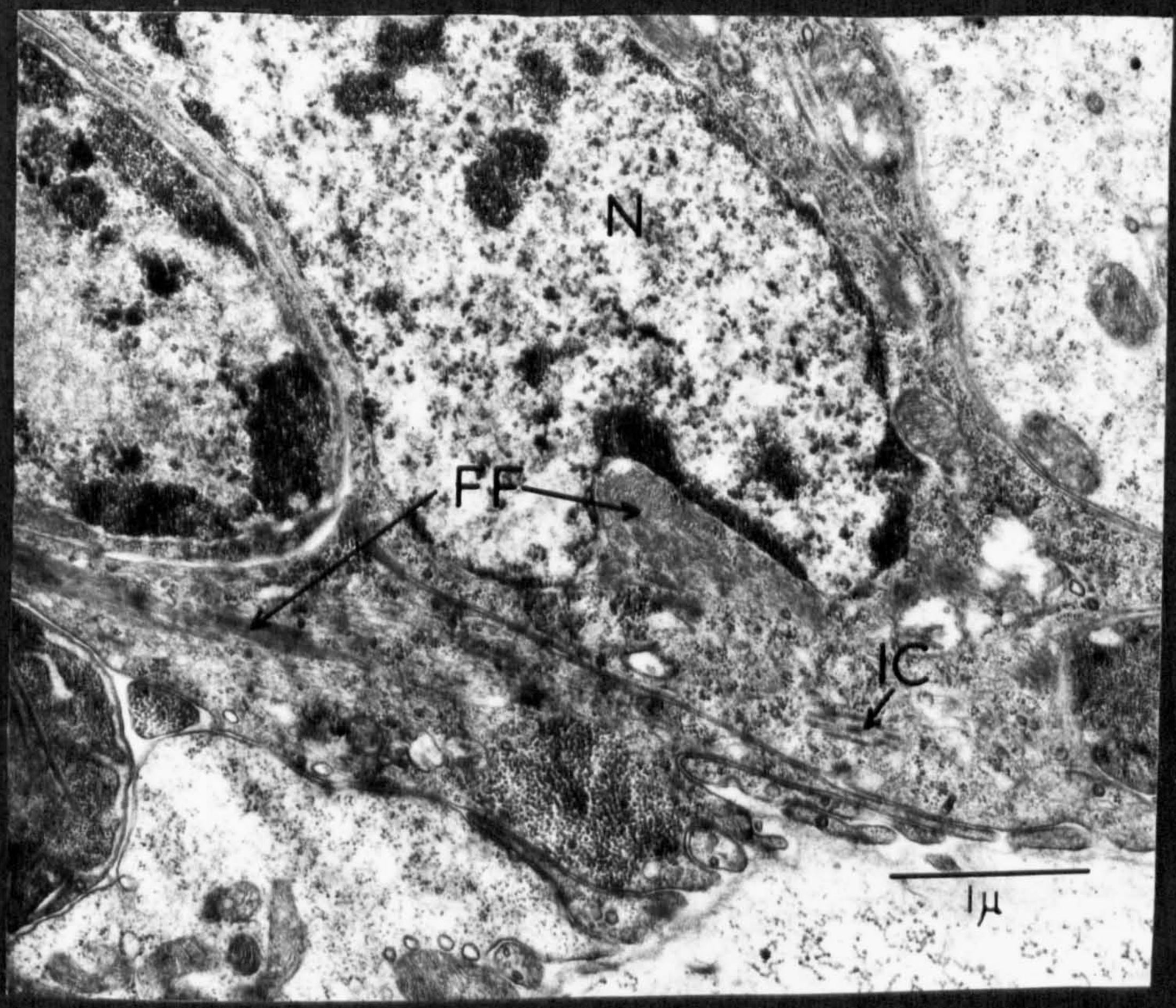
b) Apical region

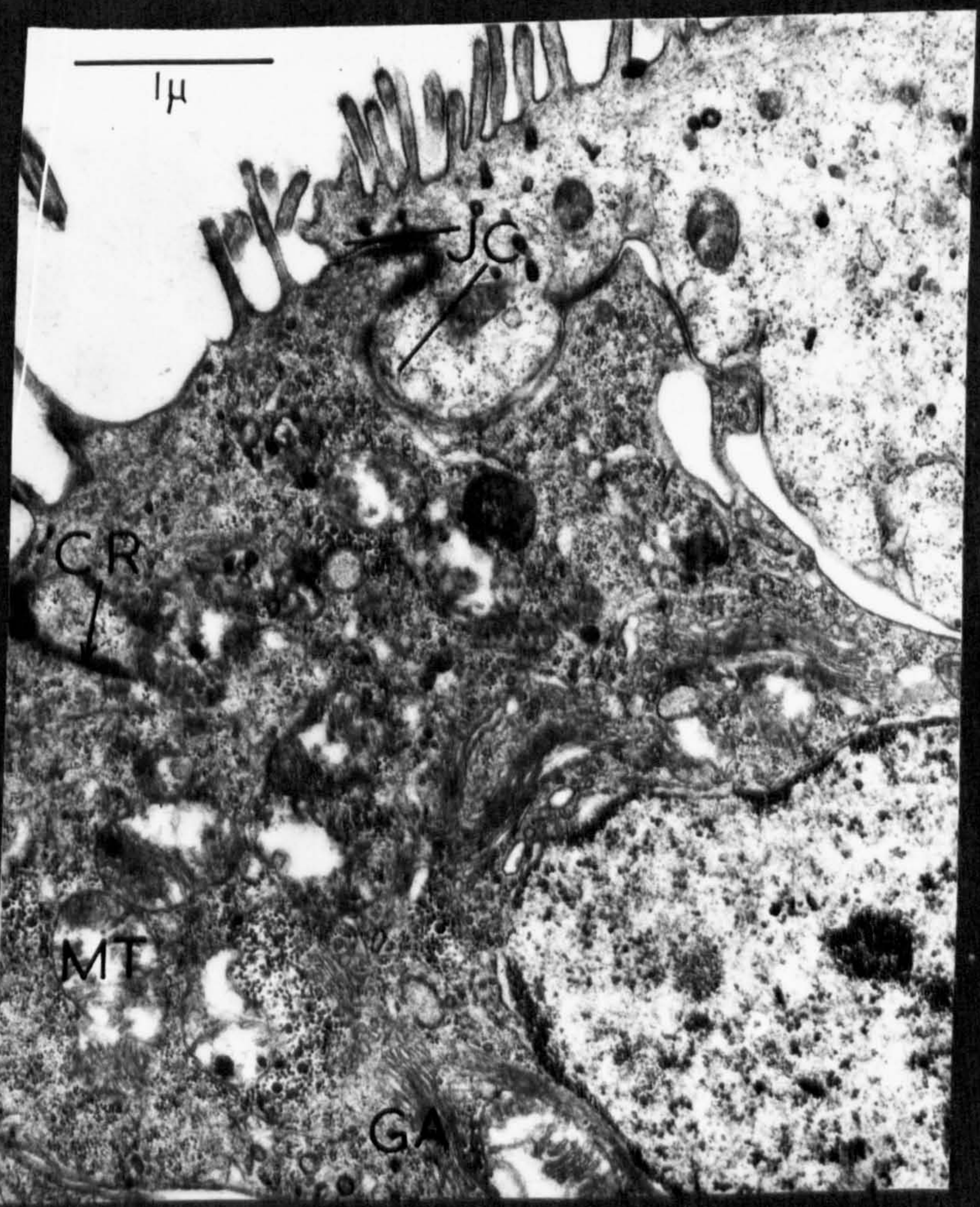
CR : Cilia rootlet

GA : Golgi apparatus

JC : Junctional complex

MT : Mitochondrion


N
FF
IC
1μ


1μ
JC
CR
MT
GA

sometimes be observed intracellularly (Fig.15b).

Pyramidal cells have a very variable appearance. Healthy looking cells have the appearance described above; other pyramidal cells, however, may be observed disintegrating into the lumen of the primary ureter. Such cells have different characteristics. They appear to have lost most of their cilia. They also contain large numbers of vacuoles and lysosome-like residual bodies. Their nuclei have a much more amorphous nucleoplasm, and a single large nucleolus. There are many intracellular cilia, and the apical plasmalemma may be broken or distorted in several places. Where mitochendria are at all recognisable, the cristae appear to be in a state of disintegration.

<u>The secondary Ureter</u>

As with the primary ureter, the secondary ureter is also lined with a simple epithelium consisting of two cell types, ciliated and non ciliated. Both types are rather irregular in shape and size.

The great majority of cells are non ciliated.

The secondary ureter is surrounded by more muscle fibres and connective tissue than the primary ureter. (Fig. 16a) Although the wall is folded, thus allowing for expansion, its surface area is not nearly as large as that of the primary ureter.

<u>Non-ciliated cells</u>

These cells are easily recognisable due to the inter-cellular spaces which measure up to 1μ in width. (Figs.17 & 18) The irregularities of the cell surface result in some

FIGURE 16.

E.M. Secondary ureter : non-ciliated cells.

a) Cell base

B : Blood space

CF : Collagen fibres

N : Nucleus

SM : Smooth muscle

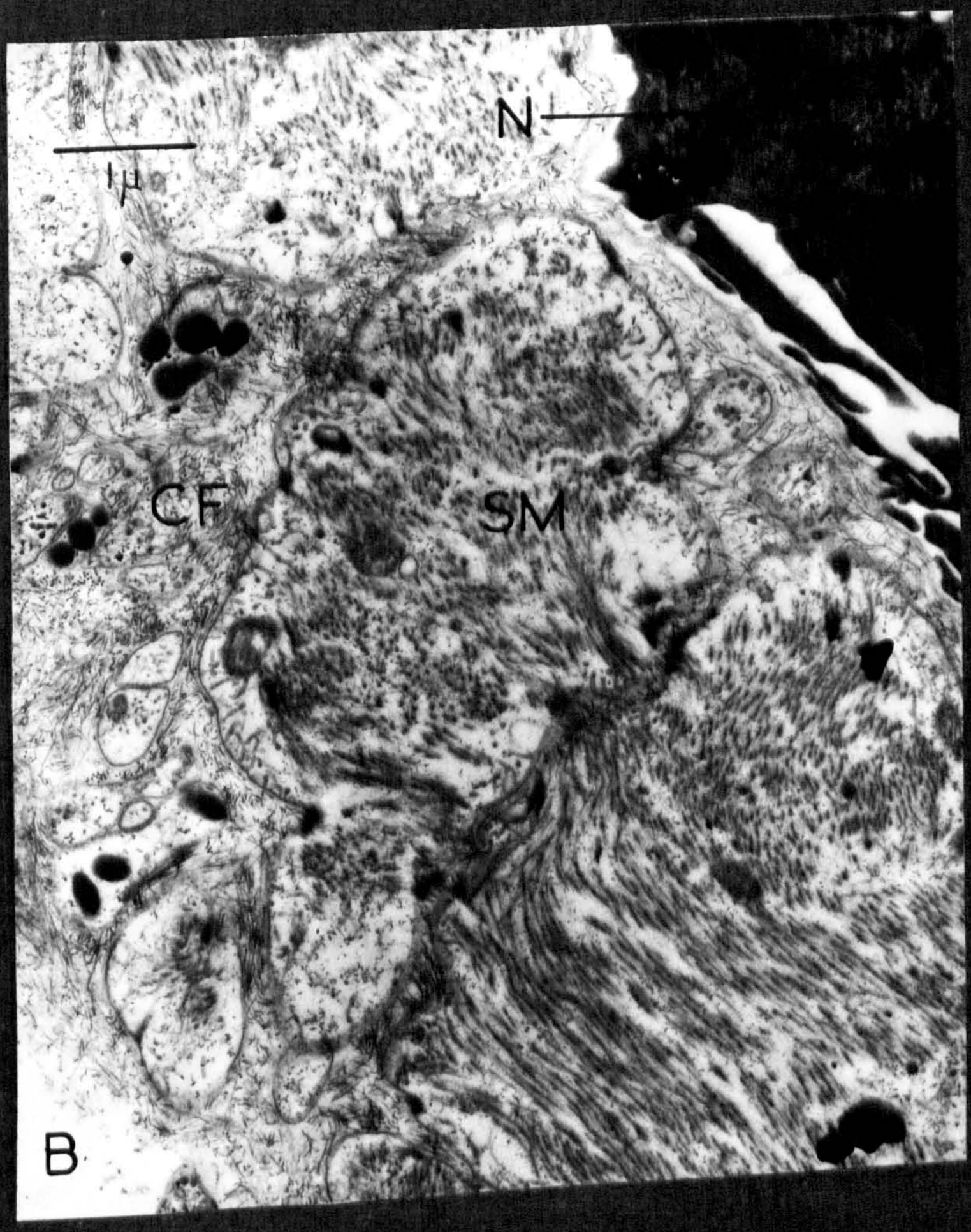
b) Cell apex

G : Glycogen

MT : Mitochondria

P : Pinocytosis?

UL : Ureter lumen


1μ
N
CF
SM
B

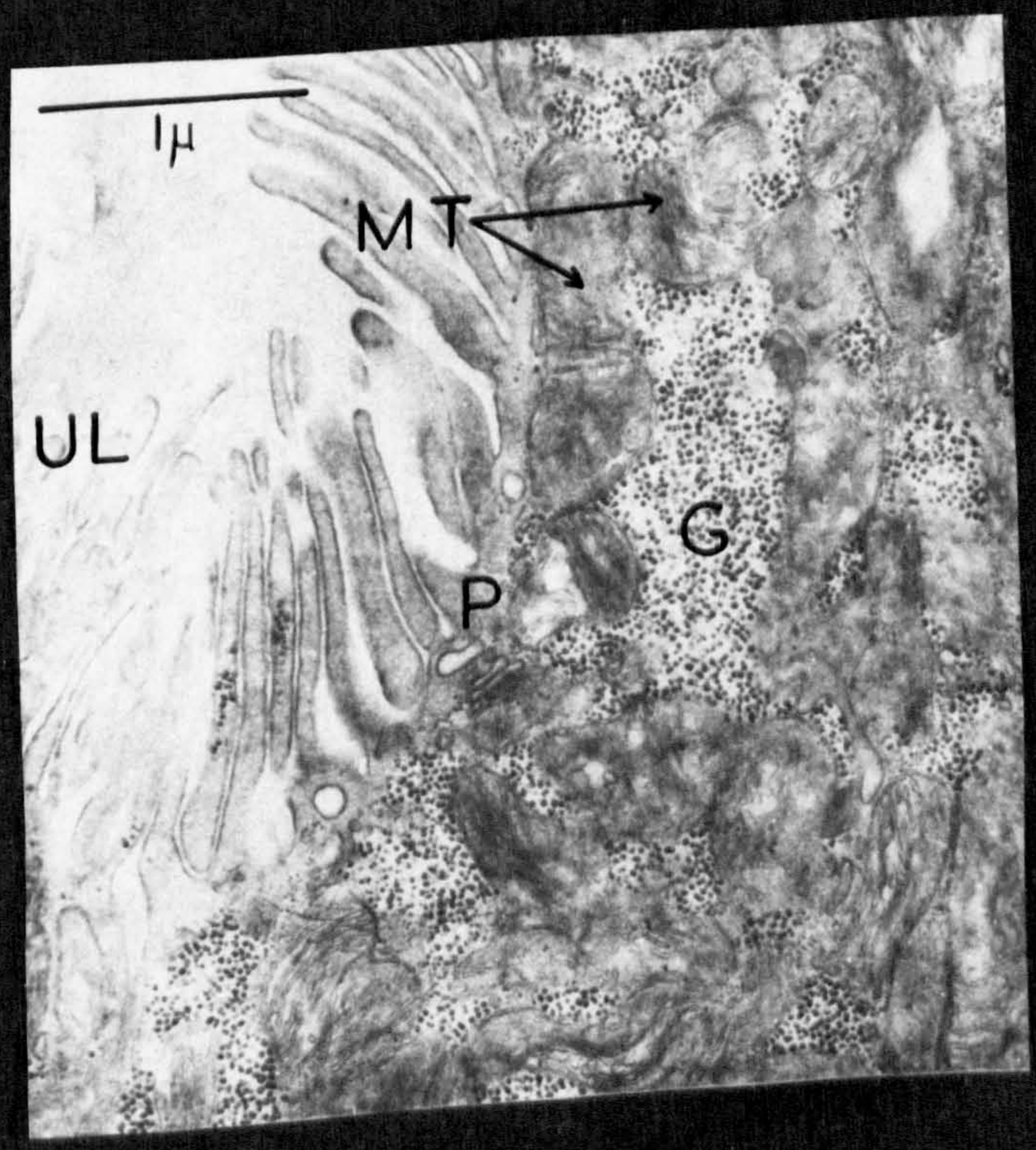

1μ
MT
UL
G
P

FIGURE 17.

E.M. Secondary ureter.

Non-ciliated cell : apex.

G : Glycogen

GV : Golgi vesicles

MT : Mitochondria

UL : Ureter lumen

FIGURE 18.

E.M. Secondary ureter.

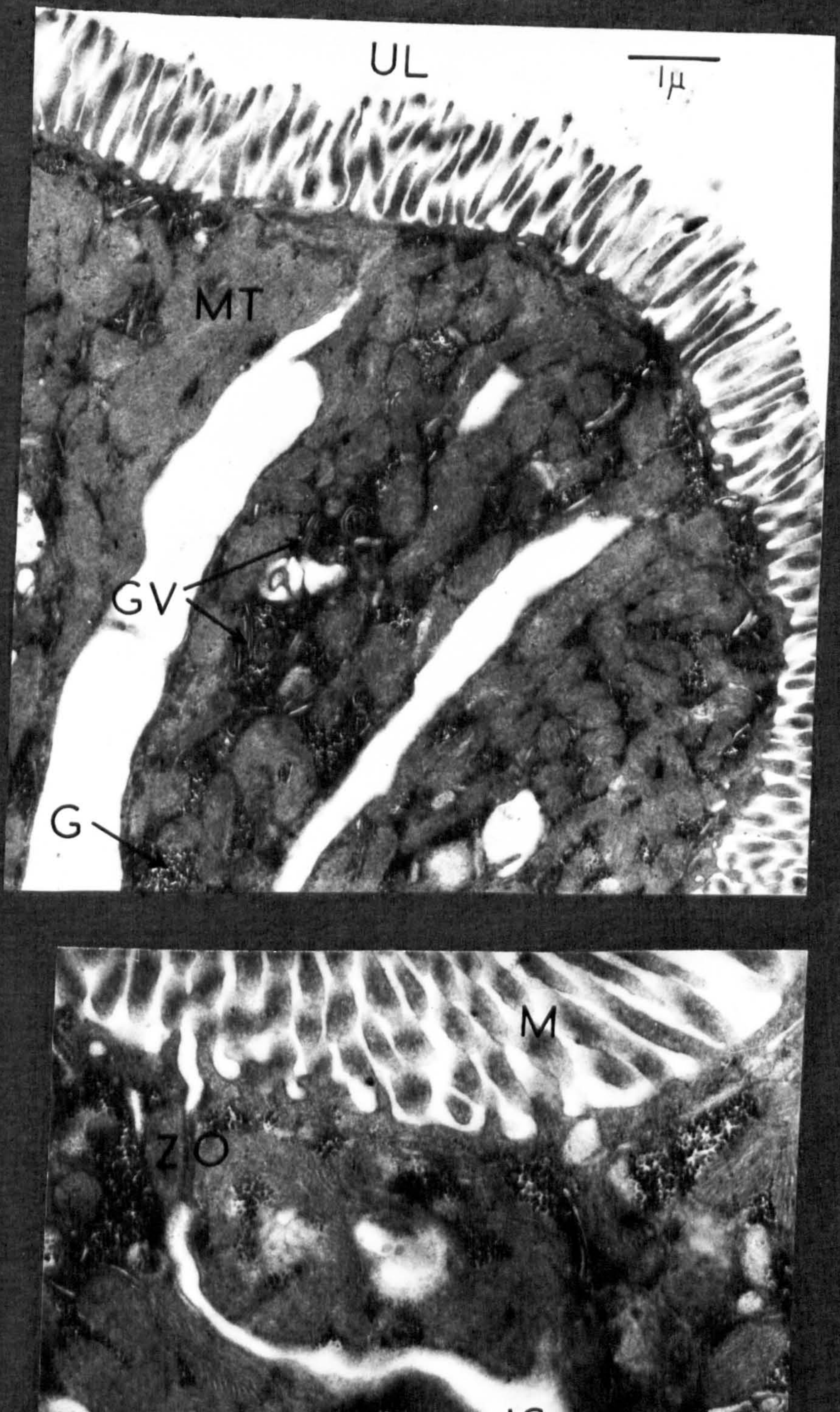
Non-ciliated cells.

B : Blood space

IS : Intercellular space

M : Microvilli

ZO : Zona occludens


UL
1μ
MT
GV
G
M
ZO
IS
1μ
B

interdigitation between the plasmalemmas of adjacent cells, but the folding is simpler than that in the primary ureter. Prominent junctional complexes close off the inter-cellular spaces at the luminal surface of the ureter, but the only barrier between these spaces and the blood space surrounding the secondary ureter consists of a few collagen fibres and the thin basal lamina on which the cells lie.

Nuclei are central to basal in position and often have extremely irregular outlines, thus increasing their surface area to volume ratio. Nucleoli are fairly prominent. The condensed chromatin content lies peripherally in close contact with the nuclear membrane. The cells have a brush border between 1 and 1.5μ thick. (Fig.16b) Each cell is packed with mitochondria, between which are very high concentrations of glycogen, in β-granule form. (Fig.16b) The mitochondria are mostly ellipsoidal or cylindrical in shape (diameter up to 0.75μ and length up to 2μ), but a variety of shapes and sizes occur. Cristae are well developed.

The only other obvious inclusions are narrow vesicles, usually less than 400Å in diameter and up to 4μ long. They are often straight sided, but may also be smoothly curved. Sometimes they occur in small groups (up to 7), where they are arranged in a similar way to the vesicles of a golgi body.

Ciliated Cells

The protoplasm of these cells is much less electron dense than that of the non-ciliated cells. (Fig.19) Their shapes are often pyramidal (with the pyramid base next to the lumen), but they do vary quite considerably. A few microvilli are present, and a number of apical vacuoles can usually be seen in the cytoplasm. Nuclei are cenral to basal in position and contain a fairly homogeneous nucleoplasm. Mitochondria are quite numerous throughout the cytoplasm, but are slightly more concentrated in numbers apically. Small concentrations of β-granules of glycogen are usually present throughout the cytoplasm in close proximity to the mitochondria. The usual basal lamina and collagen fibres are present beneath the cells.

Bladder

This again is lined by a simple epithelium consisting of the same two cell types as those found in the secondary ureter, along with a third type of cell. (Fig.20) Cells of this third type are fairly well distributed throughout the epithelium but are not quite as common as the non-ciliated cells. They have the following characteristics :- Each cell is pyramidal (Figs. 20, 21a & b), and situated with the pyramid apex adjacent to the bladder lumen. The cells are usually surrounded by the non-ciliated cells and have a small area of free surface at the bladder lumen, with a brush border of a thickness similar to that of the non-ciliated cells. The nucleus is apical. It has a prominent nucleolus and a number of discrete patches

FIGURE 19.

E.M. Secondary ureter.

Ciliated cell.

AV : Apical vacuoles

C : Cilia

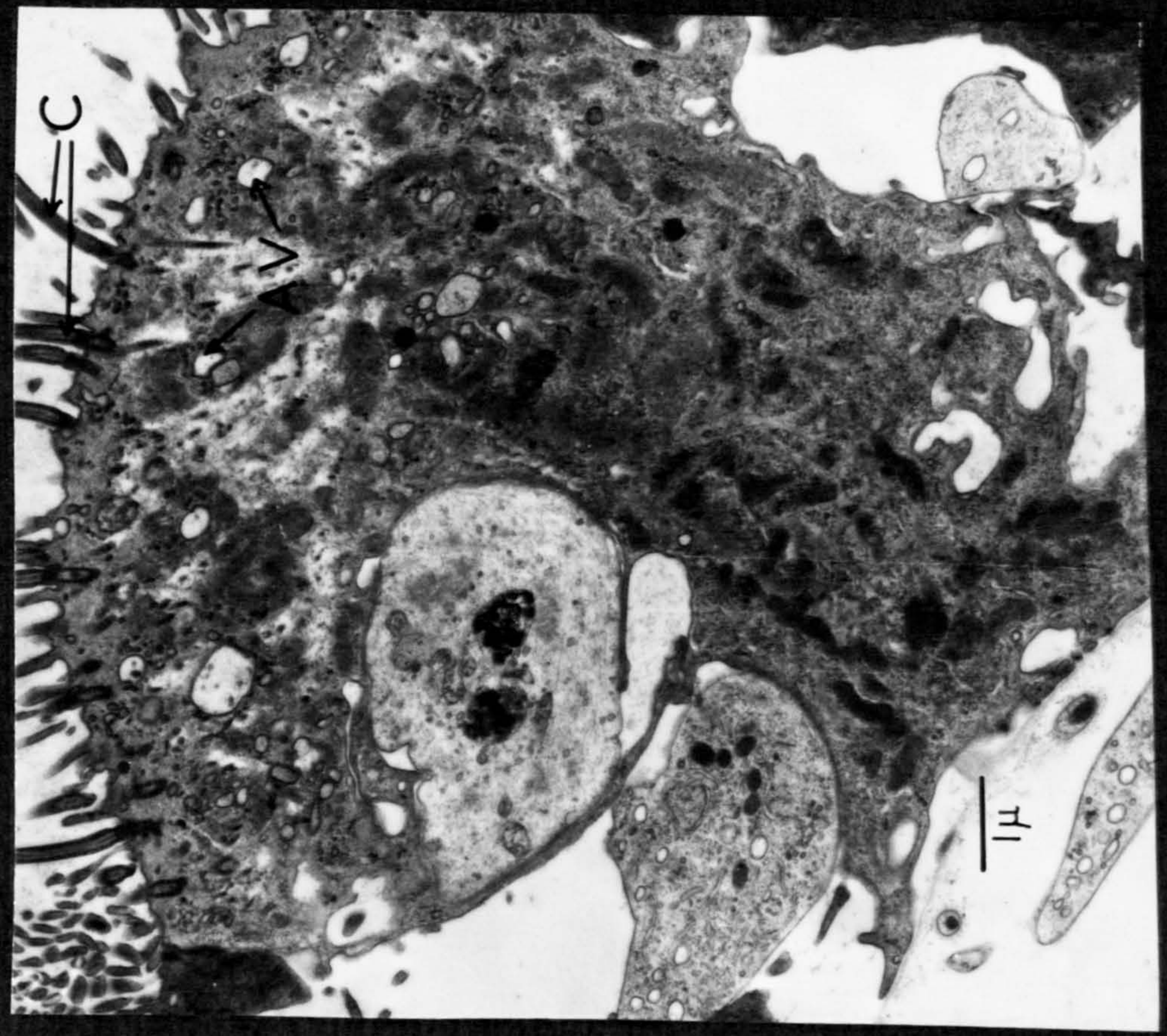

C
1μ

FIGURE 20.

E.M. Bladder. Low Power Scan.

B : Blood space

C : Cilia

CC : Ciliated cell.

NCC : Non-ciliated cell.

R : Rectum

TC : Third cell type.

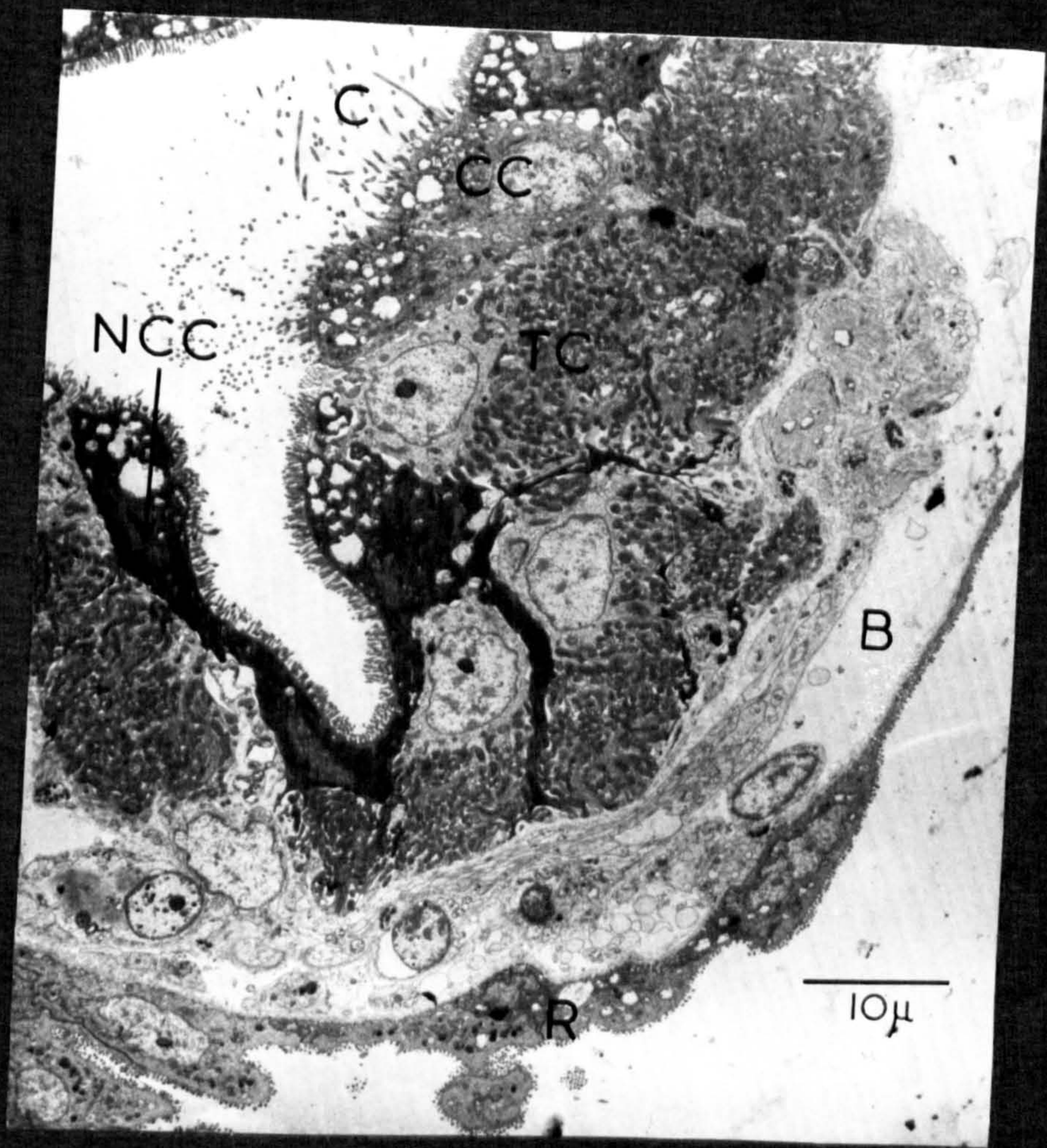

C
CC
NCC
TC
B
R
10μ

FIGURE 21.

E.M. Bladder. Third cell type.

a) N : Nucleus

b)BL : Bladder lumen

BC : β-cytomembranes

MT : Mitochondria.

N : Nucleus

NL : Nucleolus

1μ

N

BL

NL

N

MT

BC

2μ

of condensed chromatin in the nucleoplasm. Small vesicles and free ribosomes may be found in the apical cytoplasm. A few mitochondria may be found apically around the nucleus. The most prominent feature of the cell is the presence of β-cytomembranes, in the lower two thirds of the cell. These form many intricate folds enclosing mitochondria. There is, however, only very slight interdigitation of the lower parts of these cells with the non-ciliated cells. For the most part, the intercellular space between the two cell types is preserved. The mitochondria are mainly ellipsoidal to cylindrical in shape, and may be up to 2μ in length. Cristae are quite well formed and tend to run obliquely across each mitochondrion. The basal lamina and collagen fibres do not follow any of the contours of the plasmalemma, but lie directly beneath the whole cell.

Although the ciliated and non-ciliated cells of the bladder are basically similar to those of the secondary ureter, they differ in one or two respects. The non-ciliated cells do not contain as much glycogen as their counterparts in the secondary ureter. Also the cristae of their mitochondria are often not so well formed. These cells generally seem to be in a less active state than those of the secondary ureter. Mitochondria in the ciliated cells of the bladder often have poorly defined cristae, like those of the non-ciliated cells. Both cell types are more often seen in states of apparent disintegration than their counterparts in the secondary ureter.

Glycogen in the α-particle form can sometimes be seen in the ciliated bladder cells, but does not seem to be present in this form in the ciliated cells of the secondary ureter. Ciliated cells only become abundant near the exit of the bladder. Here they occur on the inner ventro-lateral surface of the bladder in a broad band which is continuous with the ciliated epithelium of the cloacal slit.

Section 2.

The Relationship between Defecation and Voiding of Excreta

Introduction

The close proximity to each other of the anus and the excretory aperture in Agriolimax reticulatus, make it impossible to be sure from which opening voided materials emerge when studying the live animal. Material from both apertures leaves the body by passing down the cloacal slit to the foot. From here it passes along the foot together with mucus secreted by the body wall, and is eventually left behind on the substratum. A typical evacuation consists of from one to several pellets, of different textures and colours, (depending on the nature of the food), along with a small collection of whiteish yellow concretions bound loosely together with mucus. The concretions are generally the last portion of the faecal string to emerge and were presumed to be the excreta. This has also been assumed by other authors, (Pallant, 1970, Jezewska, 1969). This short experiment was performed partly as a check on the validity of this assumption, and partly to find out the relationship between defecation and excretion. Usually only one collection of concretions is voided in a single twenty four hour period, although several faecal strings may be voided during the same period of time. Concretions are sometimes voided without the accompaniment of faecal pellets.

Materials and Methods

The experiment was carried out in two parts.

1) Groups of slugs were observed from time to time over a period

of days. Any slug which was in the process of voiding concretions was collected. Its kidney was immediately dissected out, and a portion of the kidney epithelium mounted in liquid paraffin on a glass slide. The slugs' voided concretions were also collected and mounted in the same way. In all, twenty slugs were studied. Concretions were visible within the nephrocytes and were approximately spherical in shape, as were also the voided concretions. The average diameters (to the nearest 0.25μ) of concretions from each collection, also from the kidney of each animal involved, were calculated. This was done using a light microscope and graticule, previously calibrated with a micrometer slide. About 100 concretions were measured from each collection.

2) Slugs were narcotised using solid carbon dioxide. A small amount (less than 0.01 ml) of a 50/50 blue chromopaque/water mixture was injected with a hypodermic syringe into the posterior part of the kidney or primary ureter. It was possible to insert the needle into the kidney or ureter via the pneumostome and mantle cavity. During injection, pressure was applied to the hypodermic until chromopaque just appeared at the pneumostome. The needle was then withdrawn and excess chromopaque was washed out of the mantle cavity with water. The slugs were then left to recover in separate pots. The reappearance of any chromopaque was noted. As a check on the accuracy of penetration of the needle, a group of slugs were first injected and then sectioned to determine exactly where the

chromopaque was accumulating. It was not important whether the chromopaque originally entered the lumen of the kidney or the primary ureter, since the contents of the kidney pass into the ureter anyway. In all, thirty-four slugs were injected.

Results.

1) As can be seen from table I, the concretions from both sources are directly comparable in size. In sixteen out of twenty cases (and also comparing the averages), the average "voided concretion" diameter is greater than that of the concretions in the nephrocytes.

Concretions can be seen in the kidny of Agriolimax quite clearly under the dissecting microscope as tiny white dots. From previous dissections of large numbers of slugs, an impression had been formed that the kidney always contained very large numbers of concretions. However, during this experiment, it was noted, that the kidney, in every case, contained relatively few concretions. These experimental animals were dissected immediately after excretion while previously, animals had been dissected during the normal working day.

2) Out of thirty-four slugs injected, eight injections were unsuccessful, i.e. the chromopaque never reappeared, presumably because all the injected chromopaque had leaked out into the mantle cavity immediately after injection, and so was washed out along with the excess chromopaque; one slug died; two slugs produced chromopaque from the cloacal slit at a different time from defecation; in twenty-three cases the voiding of chromopaque and of faeces was synchronous.

TABLE I

AVERAGE DIAMETER OF CONCRETION (μ)

SLUG NO.	EXCRETORY MASS	NEPHROCYTES
1	13.5	14.25
2	8.25	7.25
3	11.5	11.0
4	15.0	8.0
5	13.5	7.5
6	18.0	9.25
7	15.0	12.0
8	13.5	16.5
9	13.25	7.0
10	13.25	10.0
11	13.5	10.25
12	14.0	16.0
13	13.25	7.5
14	9.25	8.5
15	17.0	15.25
16	9.0	7.25
17	12.25	16.0
18	15.25	8.25
19	14.25	13.5
20	9.25	8.25
Average	13.03	10.67

The chromopaque took anything from a few hours to two days to reappear.

Slugs which had been injected and then sectioned showed chromopaque to be present in the primary and the secondary ureter, and in the kidney.

Conclusions.

The comparable sizes of concretions from the "excretory mass" and from the kidney cells of the same slug are one indication that the voided concretions do in fact come directly from the kidney. The fact that the voided concretions are slightly larger on average than their counterparts within the kidney cells, could indicate that a single concretion must reach a particular maximum size before it is released from the kidney.

Although a subjective observation, the fact that the kidney of a slug appeared relatively "empty" soon after voiding concretions, suggests that it might be usual for most of the kidney contents to be expelled each time voiding occurs.

The fact that chromopaque injected into the kidney or ureter of Agriolimax generally reappeared with the faeces, is a strong indication that excretion and defecation are usually synchronous.

It would appear from the above conclusions that the whitish-yellow concretions found with faecal strings from Agriolimax reticulatus are originally voided from the nephrocytes and, in fact, constitute the excreta.

Section 3.

An Investigation of the Excretory Products

Introduction

The products of excretion have been identified in a number of Molluscs, (e.g. Jezewska 1969; Duerr 1968; Speeg and Campbell 1968), but not as yet in *A.reticulatus*. A number of techniques were used to investigate the excretory products of this animal.

Materials and Methods

1) The murexide test for purines was carried out on macerated kidney material and freshly collected excretory masses.

2) Thin layer chromatography was used for the preliminary identification of kidney contents and of excreta. 20 by 20 cm glass plates were thoroughly washed and dried; then hand or machine spread with a 0.25 mm layer of MN cellulose 300, (Machery Nagel & Co.). Three solvent systems recommended for purines were tried: n-butyl formate/formic acid/water, 10/4/1, + 0.05% w/v sodium formate (Blundstone 1963); butanol/formic acid/ water, 77/13/10; butanol/acetic acid/water, 20/3/7 (Jezewska, Gorzkowski and Heller 1963). The latter was found to be the most effective. The best results were obtained when blank, spread plates had been pre-run in the appropriate solvent, and "activated", (by heating at 105°C for half an hour), just before an experimental run. Extracts were made of the excretory material by shaking single kidneys or excretory masses with 0.1 ml (or less) of solvent. The solvent used was a 10% solution of 10% v/v 0.880 ammonia in isopropyl alcohol in water, (Smith 1958). Excreta dissolves readily

in this solution. Samples of kidney extracts were run on plates alongside solutions of xanthine, guanine, uric acid, hypoxanthine, and a mixture of all four compounds dissolved in the same solvent. This was also done with samples of excretory mass extracts. The samples were spotted onto the plates with capillary tubing and the spots were dried with a hair drier. If necessary further drops of solution were applied to the plates, on the same spots, until the dry spots were clearly visible in ultra-violet light. Each run was continued until the solvent line had almost reached the top of the plate, (up to eight hours). Plates were air dried in a fume cupboard, and viewed in ultra violet light. Any spots present were ringed. It was possible to see the spots in ordinary light by spraying the plate with saturated silver nitrate solution and then leaving the plate in an atmosphere of ammonia vapour until the spots developed. This method, however, was found to be very messy and the spots, once formed, tended to deteriorate quite rapidly.

3) As a check on results obtained with thin layer chromatography, the constituents of the excreta were examined spectrophotometrically. Extracts of kidney and of excretory masses were separated into their constituents by T.L.C. The same method as that already described was used, but instead of spotting, narrow lines of an extract were spread onto each plate by means of a syringe, and each line was dried with a hair drier before a second application. Half the extract from two dissected kidneys or half of that from three excretory masses was found to be sufficient to give a reading on the spectrophotometer. The bands obtained on the cellulose

were scraped into a small piece of filter paper in a filter funnel and were eluted using 6 ml of saturated lithium carbonate solution for each band. The eluates were poured into 5 ml quartz spectrophotometer cells and were then scanned between the wavelengths of 200 and 300 mμ in an ultraviolet spectrophotometer, (Unicam S.P.800). Eluates from blank areas of cellulose on the plates, equivalent in size to the areas covered by the bands, were used as blanks inthe 'photometer. Solutions of known constitution were also scanned as comparators for the unknowns. Dilute hydrochloric acid and dilute sodium hydroxide were used to adjust the pH of the solutions so that the absorbances of known and unknown solutions could be measured at similar pH's.

4) The faecal material (without the excreta), from ten slugs was collected over a 24 hour period. This material was macerated with the same solvent as that used for the excreta. After centrifuging the sample, the supernatant was examined chromatographically for the presence of purines.

5) It has been suggested that calcium is a constituent of the concretions found in the kidney. A histochemical test for calcium carbonate and phosphate was therefore carried out. Animals were fixed in 50/50 formalin/ethanol, (to prevent any calcium from dissolving out of the tissues), sectioned at 5μ, and the sections stained with Neutral Fast Red (Pearse 1960). It was necessary to remove the shells of the slugs before sectioning to prevent the blocks from cracking on the knife edge.

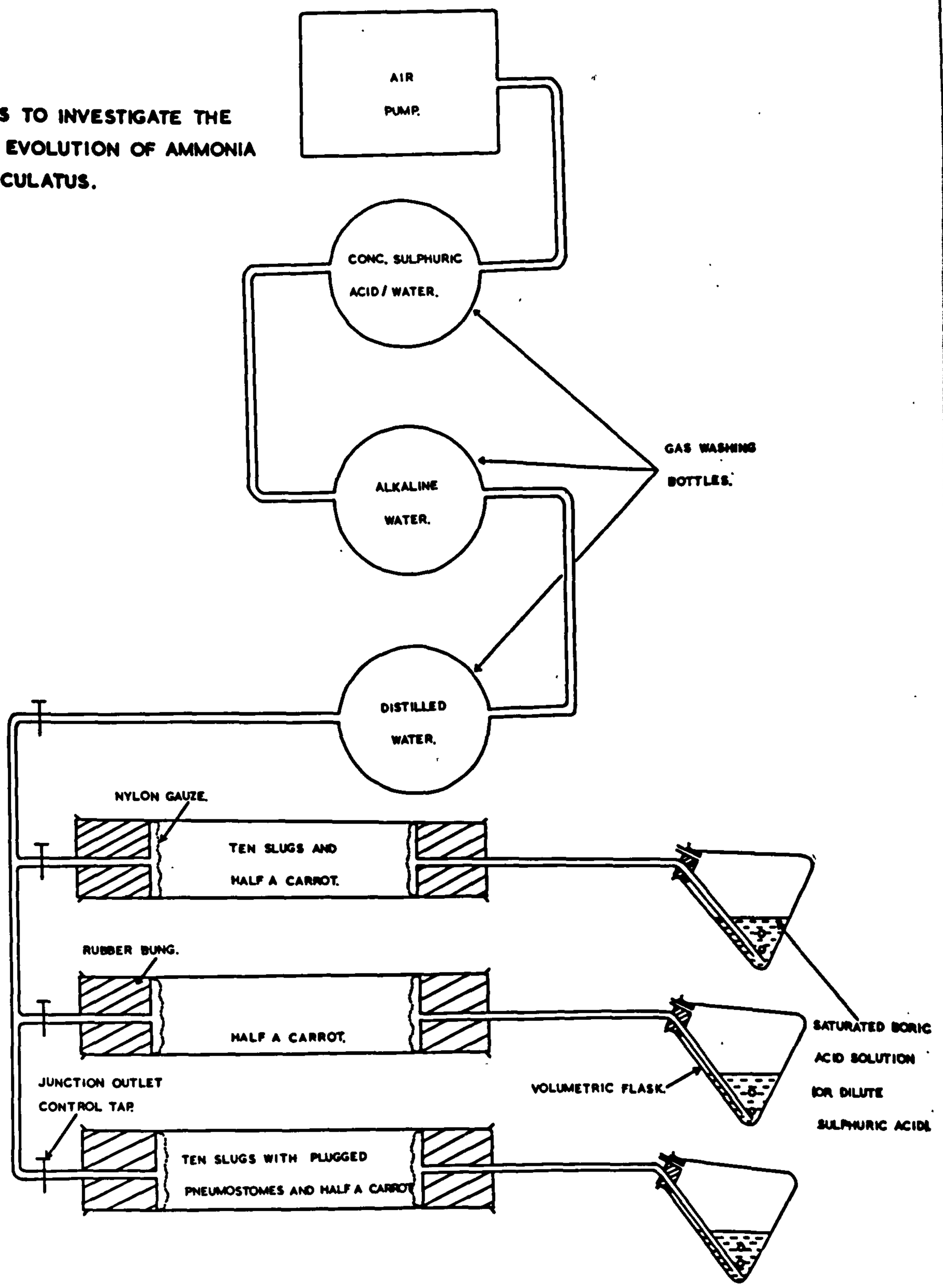
The voided excreta was also tested for the presence of calcium using flame photometry, in case calcium was present in some form other than its carbonate or phosphate. Kidneys were dissected out of six animals, and the kidneys and animals were dried and weighed. Excreta was also collected, dried and weighed. The kidneys were well macerated with 10 ml of solvent, (10% solution of 10% v/v 0.880 ammonia in isopropanol, in water, the whole solution containing 600 p.p.m. lanthanum chloride). The excreta were also dissolved in 10 ml of this solution. Solutions of known concentrations of calcium chloride, (between 1 and 100 p.p.m. calcium), were made up in the same solvent and, using the flame photometer, a calibration curve was constructed for calcium. To ascertain whether uric acid had any effect on readings, again using the same solvent, a 0.0275% solution of uric acid was made up and tested on the photometer. The extracts of kidneys and of excreta were also tested on the photometer and, using the calibration curve, their calcium contents were calculated. Because of the deliquescent nature of solid calcium chloride, the stock solution of known calcium content was made up, using a weighed amount of calcium carbonate dissolved in its equivalent of dilute hydrochloric acid. A problem was encountered in getting all the solutes to dissolve thoroughly. This was overcome by adding 10% v/v acetic acid to all solutions.

6) Helix lactea and H. aspersa have been shown to evolve ammonia gas (Speeg and Campbell, 1968). To find out if this were also true for A. reticulatus, the following

experiment, (based on the Kjeldahl method of nitrogen estimation), was performed. Air was pumped, by a "Rena 100" fish tank pump, through three gas wash bottles connected in series (Fig.22). Three glass tubes were connected to the last wash bottle by a four way plastic junction. The first wash bottle contained a 50/50 solution of concentrated sulphuric acid/water, the second, alkaline water, (distilled water with a few drops of sodium hydroxide solution added), and the third, distilled water. The three tubes were each about 1" diameter and 6" long and were stoppered with a rubber bung at each end. Each bung was pierced with a narrow bore glass tube to give an inlet and outlet and a circular piece of nylon gauze was placed over the inside of each bung. Each of the three outlet tubes let into a volumetric flask containing 20 ml of saturated boric acid solution with 10 drops of Tashiro's indicator added. Each flask was bunged and the bung pierced to leave a narrow outlet open to the air. Polypropylene tubing was used throughout for connecting purposes. The four way junction outlets could be controlled by means of a tap on each outlet. Into the first tube were placed ten slugs and half a small carrot (cut lengthways): into the second a half carrot by itself, and into the third, ten slugs each with their pneumostome blocked and half a carrot. Each tube contained the same net weight of carrot. The technique for blocking the slugs' pneumostomes was as follows: A mixture of 50/50 paraffin wax/liquid paraffin was prepared. Slugs which had been narcotised with solid carbon dioxide were allowed to recover sufficiently to open

FIGURE 22.

APPARATUS TO INVESTIGATE THE POSSIBLE EVOLUTION OF AMMONIA BY A. RETICULATUS.



their pneumostomes. Using a warmed Pasteur pipette, the melted wax mixture was squeezed into the mantle cavity until it exuded from the pneumostome. The wax set almost immediately on leaving the pipette and so formed an effective plug. The slugs were then given an hour or two to recover before being put into the tube. The apparatus was connected up and the pump started so that a regular stream of bubbles passed through the boric acid solution in each flask. The apparatus was left running for approximately 48 hours. After this time, $\frac{N}{70}$ hydrochloric acid was titrated against the boric acid in each flask, (0.5ml $\frac{N}{70}$ HCl ≡ 100μg nitrogen). Any ammonia evolved by the slugs was thus absorbed by the boric acid and estimated by titration. The flask from the tube containing no slugs was used as a blank. The whole experiment was repeated with dilute sulphuric acid replacing the boric acid, and ammonia nitrogen was then estimated by Nesslerisation.

<u>Results and Conclusions.</u>

1) Both excreta and kidneys gave a strongly positive reaction to the murexide test.

The murexide test is usually specified as a test for uric acid. However, when tried on other purines, it gave positive results, although the colours produced were not as clear cut nor as consistent as with uric acid. A positive result with excreta and kidneys thus showed the presence of purines, but was not specific as to which.

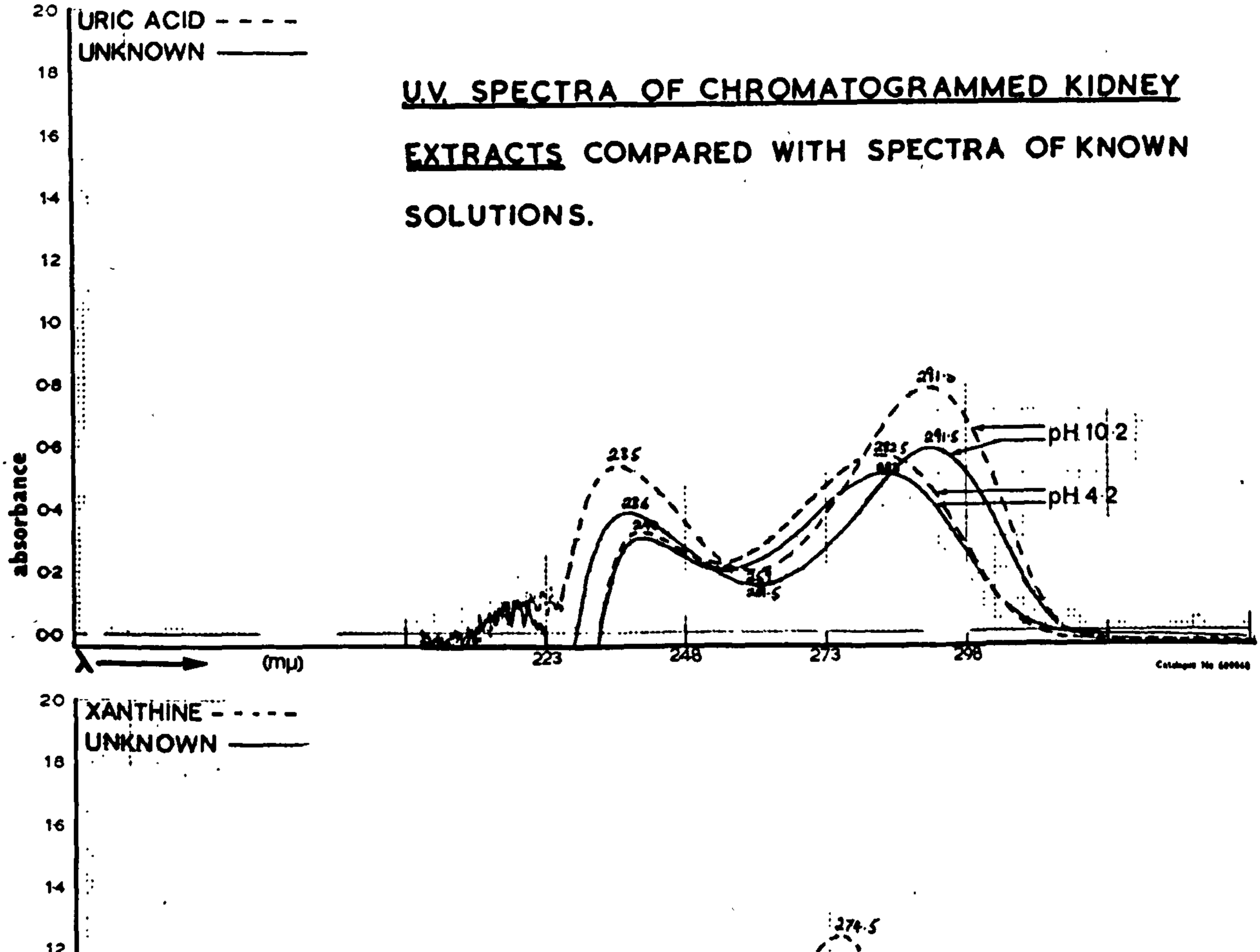
2) On chromatograms of both kidney and excreta extracts, three spots were visible in ultraviolet light. The R_F values of the spots were similar to those of uric acid, xanthine and guanine. A spot with an R_F similar to that of guanine was often not obtained, but spots with R_F's similar to those of uric acid and xanthine were always present.

From thin layer chrometography alone, it would have appeared that uric acid and xanthine were the main purines present both in the kidney and in the excretory masses, and that guanine was present occasionally.

3) Separating relatively large amounts of kidney extract by thin layer chromatography resulted in only two bands appearing under ultraviolet light. (Fig.23) These bands gave U.V. absorption spectra which were very similar to those obtained with known solutions of uric acid and xanthine. (Table II)

With excreta extracts intially, three bands were obtained, but subsequently, only two. (Fig.24) The upper and lower of the initial three bands gave U.V. absorption spectra similar to those of xanthine and uric acid respectively. (Table III) The middle band, however, did not give an absorption spectrum similar to that of guanine. If anything, its spectrum was similar to that of uric acid. Since the peaks on the spectrum obtained did not correspond to those of any known U.V. absorbing compound, the eluate was re-chromatographed. Two bands appeared under U.V. light and their eluates gave U.V. absorption spectra which now correspond fairly well with those of uric acid and xanthine. (Fig.25)

FIGURE 23.



U.V. SPECTRA OF CHROMATOGRAMMED KIDNEY EXTRACTS COMPARED WITH SPECTRA OF KNOWN SOLUTIONS.

TABLE II

Absorption maxima	pH	Absorption minimum	Sample
236 & 291.5mμ	10.2	259.5mμ	Lower band eluate
235 & 291.5mμ	10.2	259 mμ	Uric acid solution
240 & 283 mμ	4.2	255 mμ	Lower band eluate
240 & 282.5mμ	4.2	254 mμ	Uric acid solution
240 & 275 mμ	10.0	256.5mμ	Upper band eluate
240 & 274.5mμ	10.0	256 mμ	Xanthine solution
N.C.+ & 268 mμ	5.5	N.C.	Upper band eluate
250 & 268 mμ	5.5	256.5mμ	Xanthine solution

+ N.C. = Not concentrated enough to give a clear reading at this pH.

TABLE III

Absorption maxima	pH	Absorption minimum	Sample
234 & 288mμ	10.4	257.5mμ	Lower band eluate
234 & 292mμ	10.4	259 mμ	Uric acid solution
229 & 280mμ	1.1	251 mμ	Lower band eluate
229.5 & 283mμ	1.1	253 mμ	Uric acid solution
240 & 275mμ	10.1	255.5mμ	Upper band eluate
240 & 276mμ	10.1	255.5mμ	Xanthine solution
N.P.+ & 269mμ	7;2	N.T.+	Upper band eluate
N.P.& 269mμ	7.2	N.T.	Xanthine solution

+ N.P. & N.T. = No clear peak and no clear trough at this pH.

FIGURE 24.

U.V. SPECTRA OF CHROMATOGRAMMED EXCRETA EXTRACTS COMPARED WITH SPECTRA OF KNOWN SOLUTIONS.

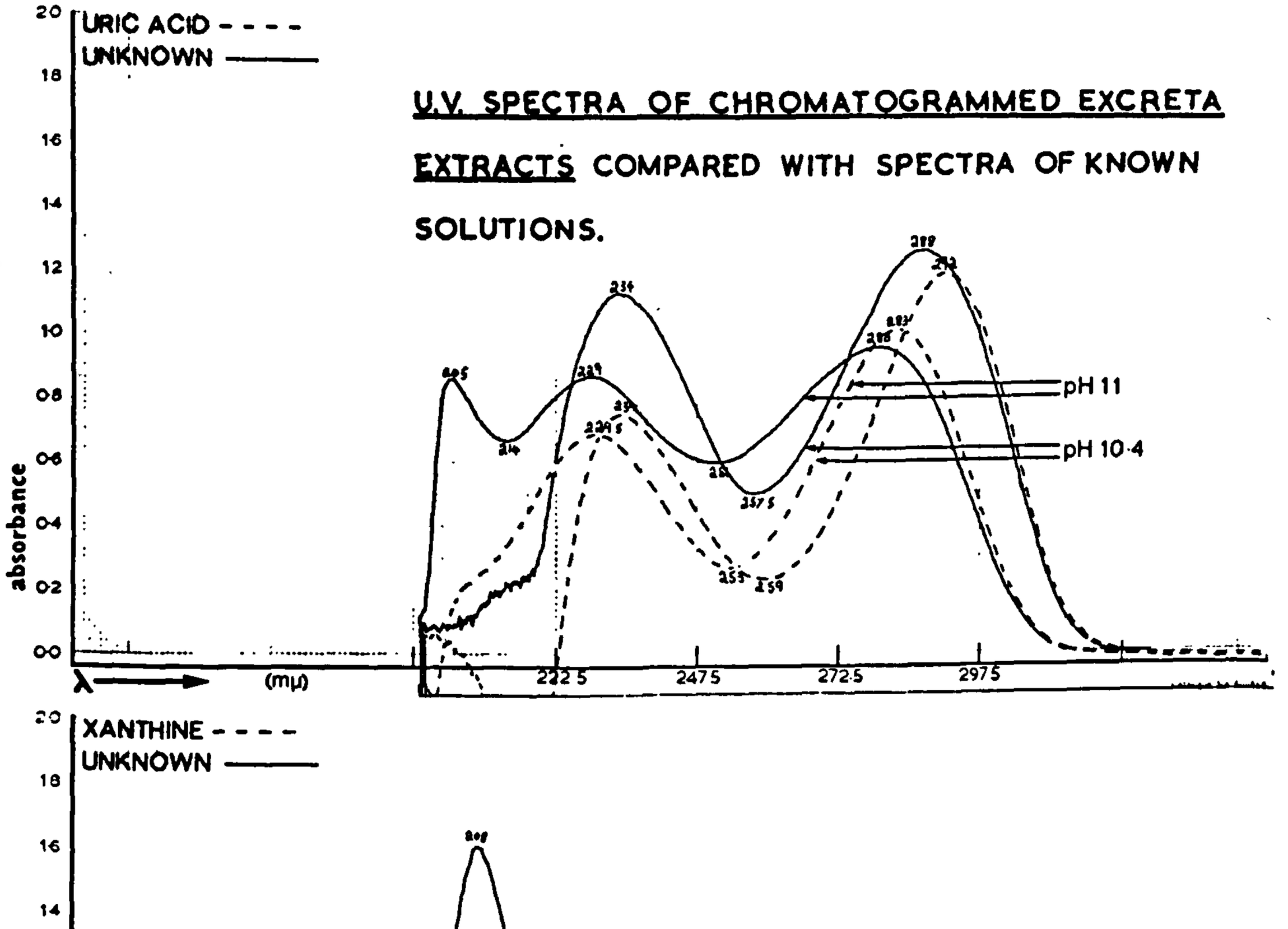
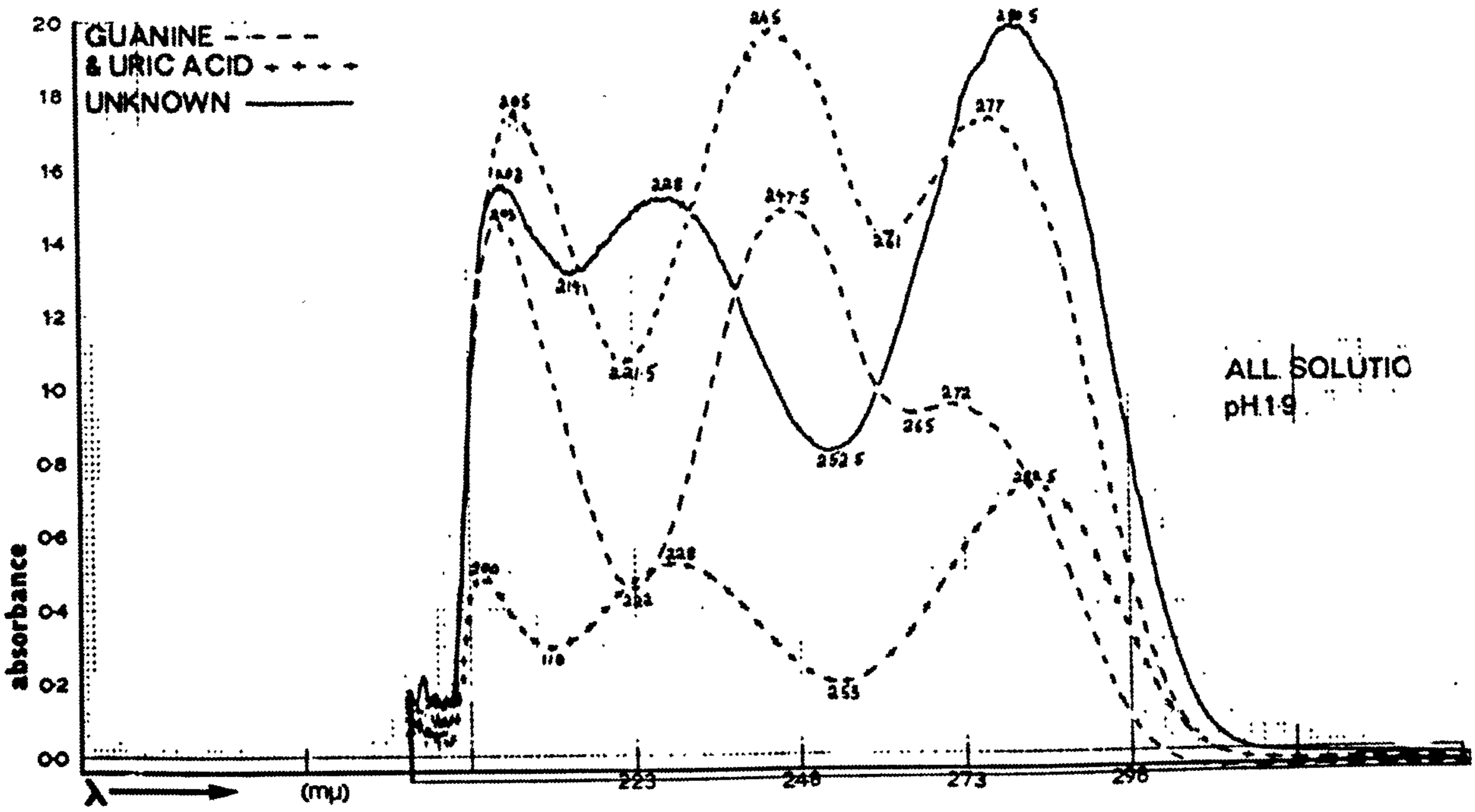
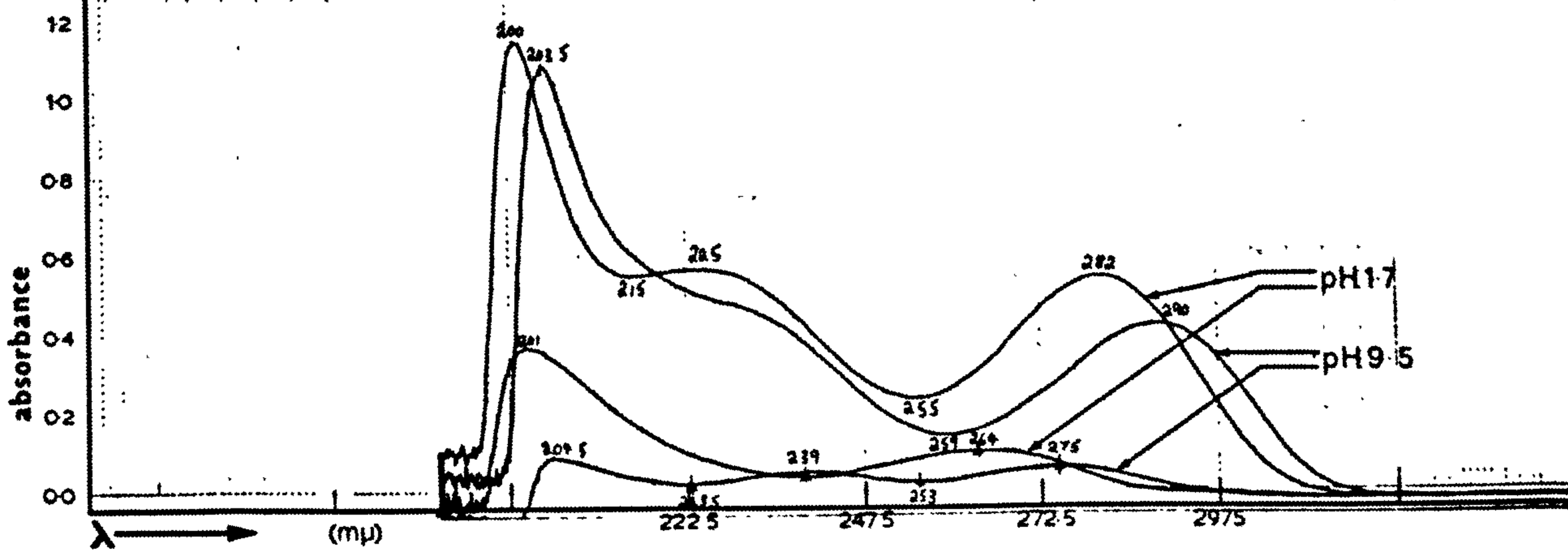


FIGURE 25.



MIDDLE BAND ELUATE FROM CHROMATOGRAMMED EXCRETA EXTRACT:
ABOVE, SPECTRUM COMPARED WITH SPECTRA OF KNOWN SOLUTIONS;
BELOW, SPECTRA OF TWO RESULTANT ELUATES AFTER
RECHROMATOGRAMMING THE ORIGINAL ELUATE.



According to U.V. spectrophotometry, the only purines present in the kidney and in the excreta are uric acid and xanthine. If guanine is present at all, its concentration is too low for it to be detected by T.L.C. The presence of a third band after T.L.C. of the initial excreta extract may have been due to incomplete separation of xanthine and uric acid, i.e. where the extreme edges of these two bands overlapped centrally, although the concentration of each one was too low for them to be seen separately in U.V. light, together they appeared as a narrow third band. Subsequent separations were presumably more efficient.

4) Purines were not detectable by thin layer chromatography in the extract from 5.83mg (dry weight), of faecal material. It would appear that purines are not excreted via the alimentary canal in A. reticulatus.

5) In the histochemical test the kidney and ureter both gave a negative reaction for calcium carbonate and phosphate. As a control, glandular cells in the skin on the same slides gave positive reactions. (Calcium is present in the mucus produced by the skin.)

The presence of uric acid did not affect the results obtained with flame photometry. 5.2 mg (D.W.) of excreta were collected and this was found to contain 0.072mg of calcium. The animals from which the kidneys were taken weighed o.3495g (D.W.) Calcium thus formed less than 2% by weight of the excreta, and less than 0.5% by weight of the kidneys.

Calcium is not present in any significant concentration in the excretory system of A. reticulatus. The presence of a slightly greater proportion of calcium in the excreta than in the kidney is probably due not only to the fact that the kidney consists of material other than excretory products, but also to the secretion of some mucus by the skin as the excretory masses pass over it.

6) By the modified Kjeldahl technique, in 49½ hours, the ten non-plugged slugs (D.W.0.5128g) were found to produce 158 g nitrogen, while the ten plugged slugs, (D.W. 0.5767g) produced 140 g nitrogen. Using Nesslerisation, in 46¾ hours, ten non-plugged slugs (D.W.0.5776g) produced 31.0 g Nitrogen, and ten plugged slugs (D.W. 0.6942g) produced 17.5 g Nitrogen. The amount of ammonia nitrogen released via the mantle cavities of the slugs was therefore 0.036 g/slug/hour (By the boric acid method), and 0.029 μg/slug/hour (by the Nessler method). i.e. 1.32 and 0.61 g of ammonia nitrogen/g (D.W.) of slug/hour, respectively.

The reaction of Nessler's reagent with ammonia ions is fairly specific. Boric acid solution, however, may possibly absorb gases other than ammonia, which could affect its titration with dilute hydrochloric acid. Thus, although the results from both methods of estimation are well within the same order of magnitude, the one obtained by Nesslerisation is probably the most accurate. The amount of ammonia nitrogen eliminated via the mantle cavity of A. reticulatus is low compared with the amount of nitrogen excreted in purine form (Section 8, page 60).

Section 4.

Determination of the Freezing Points of Blood and Pericardial Fluid in Specimens of A. reticulatus, Over a 24 hour Period.

Introduction

In A. reticulatus the pericardial cavity is in direct communication with the kidney lumen via the renopericardial canal.

It was thought that measurement of the solute concentrations of the blood and of the pericardial fluid of animals, over a twenty four hour period, might shed some light on the process of purine accumulation in the kidney.

The freezing point of a dilute solution directly reflects the concentration of its solutes. Thus the freezing points of blood and pericardial fluid samples were measured.

Method.

Twenty six slugs were collected and were starved for one day. The slugs were then allowed to feed on fresh carrot for a single two hour period on each of the following two days. On the fourth day the slugs were again starved, and on the fifth day the slugs were allowed to feed for three hours immediately before the experiment was started. This procedure was carried out so that the slugs' feeding rhythms were as closely in phase with each other as possible. The slugs were then sacrificed at hourly intervals in order that samples of blood and pericardial fluid could be taken for freezing point determinations. Samples were obtained in the following way: Each slug was anaesthetised using

solid carbon dioxide. The mantle was then dissected away and the shell lifted away from the shell gland. A fine Pasteur pipette was then pushed through the roof of the mantle cavity, through the pericardium and into the pericardial cavity. Pericardial fluid was automatically drawn into the pipette by capillarity. A drop of this fluid was expelled under liquid paraffin in a watch glass. Very small portions of this drop were then drawn up into a capillary tube along with small portions of liquid paraffin, so that the pericardial fluid and liquid paraffin appeared alternately in small segments along the capillary tube. Each end of the tube was then sealed with plasticine. To collect a blood sample, a slit was made in the foot of the anaesthetised animals and, again using a Pasteur pipette, blood was taken direct from the haemocoel surrounding the viscera. Blood samples were treated in the same way as samples of pericardial fluid. The freezing point of each sample was determined (using the apparatus described by Ramsey and Brown, 1955), as quickly as possible after obtaining the sample. (Table IV)

The last feeding period was terminated at 1300 hours, i.e. just before the first animal was sacrificed.

The results are expressed graphically (Figs. 26 & 27). In order to obtain the best fitting curve and to eliminate some of the inevitable variation, each point was plotted after it had been recalculated, i.e. each new point was the average of the original point and the one on either side of it. Point 1 was obtained from the average of (point 1 x 2) + point 2, and point 22 from the average of point 21 + (point 22 x 2). The whole averaging procedure was carried out four times.

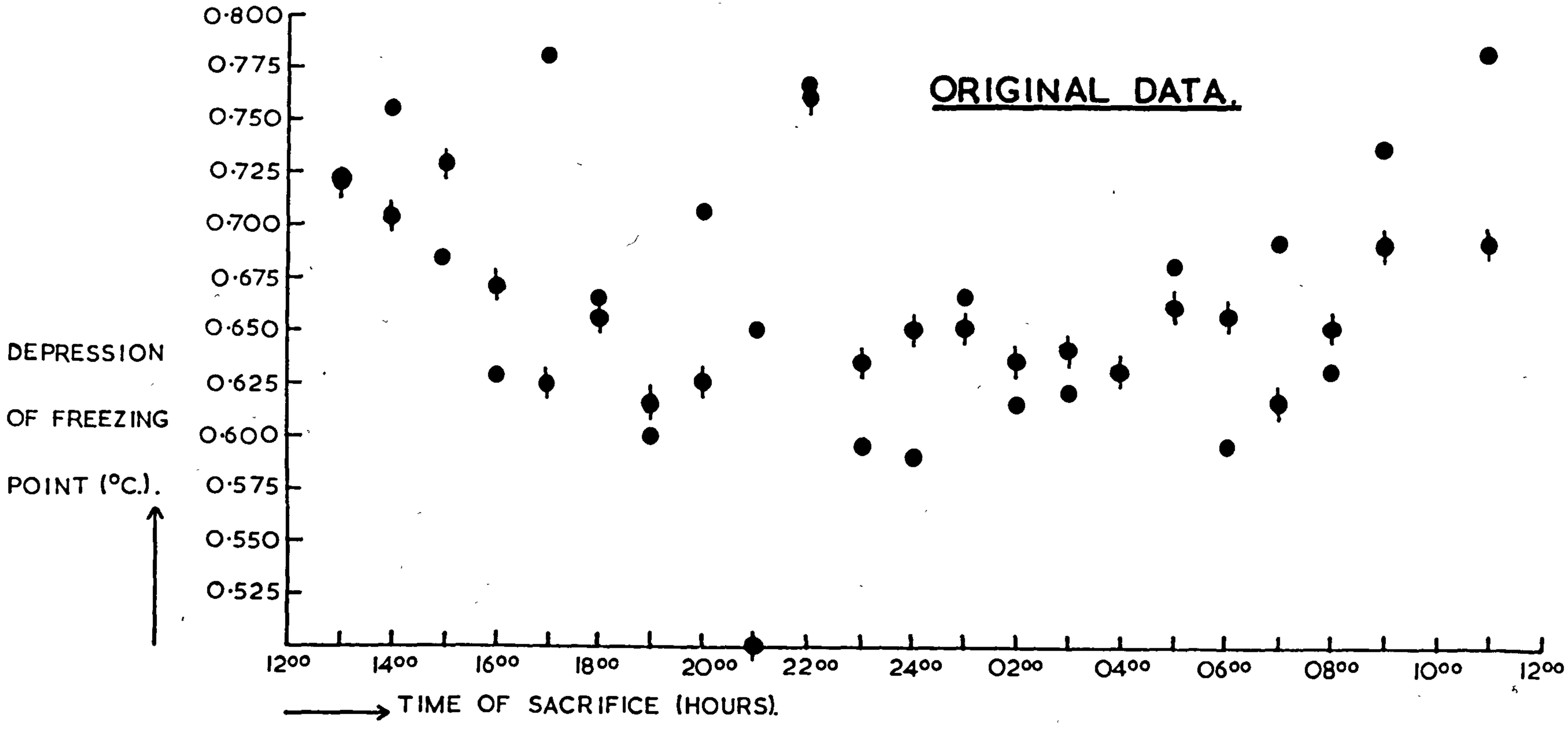
TABLE IV

Slug No.	Time of Sacrifice	Depression of Freezing Point in degrees Centigrade	
		Pericardial Fluid	Blood
1	1300	0.720	0.720
2	1400	0.705	0.755
3	1500	0.730	0.685
4	1600	0.670	0.630
5	1700	0.625	0.780
6	1800	0.655	0.665
7	1900	0.615	0.600
8	2000	0.625	0.705
9	2100	0.500	0.650
10	2200	0.760	0.765
11	2300	0.635	0.595
12	2400	0.650	0.590
13	0100	0.650	0.665
14	0200	0.635	0.615
15	0300	0.640	0.620
16	0400	0.630	
17	0500	0.660	0.680
18	0600	0.655	0.595
19	0700	0.615	0.690
20	0800	0.650	0.630
21	0900	0.690	0.735
22	1000	0.690	0.780
	Average	0.655	0.674

N.B. There was a natural mortality of four slugs, hence only twenty-two results were obtained.

FIGURE 26.

SOLUTE CONCENTRATIONS (REPRESENTED BY DEPRESSIONS OF FREEZING POINTS) OF THE BLOOD AND PERICARDIAL FLUID MEASURED OVER A TWENTY FOUR HOUR PERIOD.



● BLOOD

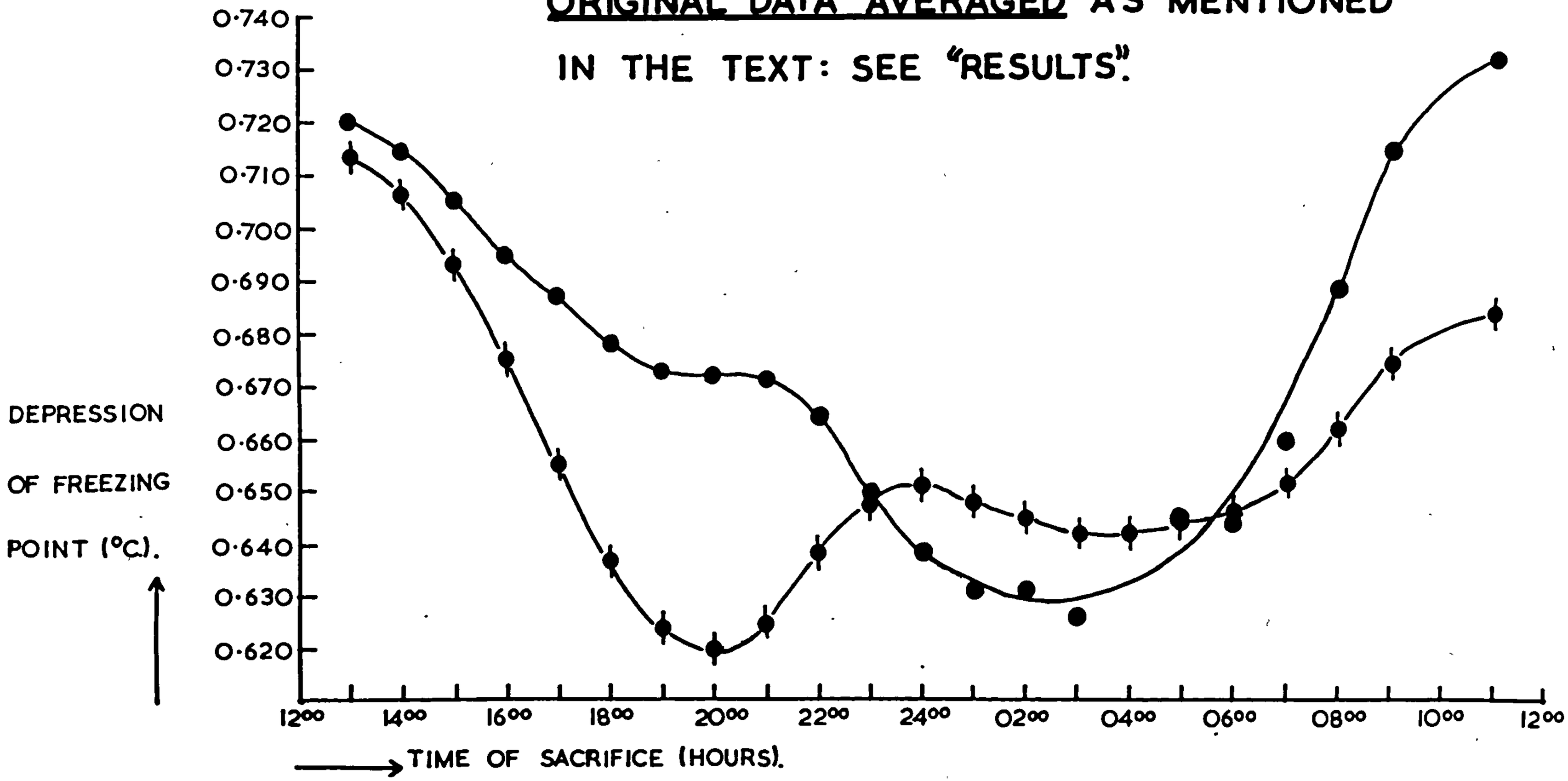
PERICARDIAL FLUID

FIGURE 27.

● BLOOD

⊕ PERICARDIAL FLUID

<u>ORIGINAL DATA AVERAGED</u> AS MENTIONED IN THE TEXT: SEE "RESULTS".



Results and Conclusions.

Because of their high molecular weights, blood colloids (e.g. haemocyanin and other blood proteins) will have little effect on the freezing point of the blood. Thus, any changes which may have occured in their concentration during this experiment will presumably have had negligible effects on the results.

There appears to have been quite a marked fluctuation in the concentration of blood solutes over the twenty-four hour period, the lowest concentrations being found between 9 p.m. and 5 a.m. An approximately similar, but narrower fluctuation may also be seen in the concentration of the solutes in the pericardial fluid. Here, however, the trough is much broader, extending from 6 p.m. to 7 a.m. It is different to assess the precise effects of the feeding programme (described in the method) on the results of this experiment. However, it would appear that diurnal rhythms of blood and pericardial fluid solute concentrations do exist.

A. reticulatus, rests during the day and is active at night. It therefore has a diurnal feeding rhythm. Under normal circumstances fluctuations in solute concentrations of the blood and pericardial fluid might be connected with this feeding rhythm. Alternatively they may be related more to activity than feeding, e.g. the removal of glucose from the blood by respiring muscle tissue during activity, would result in a lessening of the depression of freezing point of the blood; mucus production during locomotion could also affect blood solute concentration.

Since the fluctuation in concentrations of solutes in the blood is similar to that in the pericardial fluid, it is likely that these phenomena are in some way related.

Although there is an apparent difference of approximately 0.02^{o} C between the average freezing points of blood and pericardial fluid, the number of samples measured is probably too small to be sure that this represents any real difference between the two. If the difference were real, it would indicate that the blood contained a slightly higher concentration of solutes in true solution (as opposed to colloidal sol state), than the pericardial fluid, and that a solute gradient thus existed between the two solutions.

Section 5.

Investigation of the Removal of Uric Acid from the Blood Using Radioactive (^{14}C-) Uric Acid

Introduction

The removal of foreign particulate material from the haemolymph of Bullia and Helix has been investigated by Brown (1965 and 1967). This apparently takes place via the amoebocytes, which take up such material and migrate with it along various routes to the exterior. Apart from this, little work appears to have been done on the actual removal of substances from the blood of Gastropods and their subsequent excretion.

Uric acid has been identified and its quantity estimated in the haemolymph of both Helix (Jezewska, 1968) and Strophocheilus (De Jorge, 1965). I have found a concentration of approximately 1mg/100ml of uric acid in the blood of A. reticulatus. (This estimation was carried out on the dialysate from 0.6ml of haemolymph, by paper chromatography and ultraviolet spectrophotometry. The haemolymph was obtained from a number of slugs, and the estimation was carried out during a previous experiment.)

It is logical to assume from the facts available, that most of the uric acid which makes up the excreta is not actually produced in the kidney, but is transported to the kidney by the blood from elsewhere in the body. This experiment was therefore carried out in order to examine some of the characteristics of the removal of uric acid from the blood.

Method.

Twenty-five slugs were collected and each one was weighed. The slugs were anaesthetised together in a large plastic washing-up bowl, using solid carbon dioxide. Each slug was then injected, via the foot, with 1μc of ^{14}C-labelled uric acid. The uric acid was administered in a solution containing 0.7g sodium chloride and 0.15g sodium bicarbonate per 100 ml of distilled water. The injection was performed using an "Agla" syringe clamped onto a bench stand, and 1μc of uric acid represented 0.01ml of solution, or 3.266μg of uric acid. The slugs were all injected between 10.10 and 10.30 a.m. After the slugs had been allowed to recover they were sacrificed following re-anaesthetisation with solid carbon dioxide, at known time intervals. Samples of blood and pericardial fluid were then taken. The pericardial fluid sample was collected by dissecting away the top of the mantle, lifting the shell away from the shell gland, and inserting a fine Pasteur pipette through the shell gland and the pericardium into the pericardial cavity. The drop of fluid so obtained was ejected under liquid paraffin, and then drawn up into a 1μlitre microcap with a drop of liquid paraffin on either side of it. The bore of the microcap was assumed to be constant. The total length of each microcap tube was measured using vernier callipers. The length of tube occupied by the sample was also measured (from meniscus to meniscus of the sample) and from the two measurements the volume of the sample was calculated. A sample of blood was obtained direct from the haemocoel by making a slit in the

foot and filling a ten μlitre microcap with the blood thus exposed. Each sample was washed into a Dreyer's agglutination tube with 0.1ml of NCS solubilizer. The tubes were bunged, marked with a diamond pencil and agitated by vibration in order to mix the samples thoroughly. Each sample was later added to a mixture of carrier toluene, POPPOP and dimethyl POPPOP, and counted (β-emission) on a Unilux lll radiation counter, at a window setting, lower to upper of 0.5 to 9.9 and range F/300. Each sample was counted for forty minutes and a figure representing counts per minute (CPM) was obtained in each case.

In order to obtain some measure of the accumulation of ^{14}C- uric acid in the kidney, a sample of fluid was extracted from the lumen of the kidney of each sacrificed animal and treated in a similar way to the samples of pericardial fluid. (Counts from such samples could only give an approximate estimation of the concentration of ^{14}C- uric acid in each kidney because the samples contained a variable number of uric acid concretions which are always present in the kidney lumen. The samples would also be certain to contain a small volume of blood which would have been released from the blood spaces around the kidney during collection of the sample.)

The background radiation (averaging 35 C.P.M.) was subtracted from each result obtained. Since the slugs used were of different sizes, their blood volumes must also have differed, and this will have affected the dilution of the injected ^{14}C- uric acid. Thus, after subtraction of the

background count, each result was multiplied by the wet weight of the slug concerned. (It is assumed that the volumes of the blood, pericardial cavity and kidney increase in direct proportion to the weight of the animal.)(Table V)

Results and Conclusions.

From the graphs of radioactivity (i.e. uric acid concentration) in the blood, against time of sacrifice, (Figs. 28 & 29), it would appear that uric acid is at first removed very rapidly from the blood, since the counts per minute drop from 1062 to less than 100 insome animals, in about four hours. Regrettably, no results are available for the period between 22.00 hours on the day of injection and 09.00 hours the following morning. However, the uric acid concentrations in the samples which were taken around 10.00 hours do indicate that after the initial rapid fall off in concentrations, a steady level of uric acid is maintained (represented by a count of around 30 emissions per minute per μlitre per unit weight in the blood samples). The most likely shape of the curve of blood radioactivity plotted against time of sacrifice is readily seen from the graph where the values of the blood counts have been averaged in threes to eliminate some of the variation. A similar treatment of the values of the pericardial fluid counts (repeated twice because of the greater variation in these values than those of the blood), results in a curve on the graph of pericardial fluid radioactivity against time of sacrifice, which approximates fairly closely in shape to the former curve. However, there are some differences between the two curves. Firstly, it would seem that uric acid disappears

TABLE V

Slug No.	Time of Sacrifice	Wet Weight of Slug (g)	(C.P.M./μl Pericardial Fluid) × Wet Wt. Slug	(C.P.M./μl Blood) × Wet Weight of Slug	(C.P.M./μl Kidney fluid) × Wet Weight of Slug
1	10.55	0.6981	692	1062	47025
2	11.15	0.6707	41	262	
3	11.40	0.7139	9977	321	38612
4	12.00	0.6040	252	253	36574
5	12.20	0.5379	523	131	45052
6	12.40	0.6747	224	185	28355
7	13.00	0.6139	154	108	52010
8	13.20	0.5991	3174	243	8178
9	13.40	0.7572	188	127	41475
10	14.00	0.5305	28	59	24515
11	14.20	0.7522	27	108	16246
12	14.40	0.5518	194	90	14993
13	15.00	0.4439	1013	54	73307
14	15.20	0.7638	113	112	82799
15	15.40	0.8156	29	92	73885
16	16.00	0.6460	11	146	45872
17	16.20	0.6485	17	84	56292
18	16.40	0.3751	1937	67	125429
19	17.00	0.6657	81	123	25299
20	17.20	0.6044	97	124	30734
21	21.00	0.4203	0	39	21762
22	21.20	0.4245	43	47	39360
23	09.55	0.8074	0	28	14271
24	10.15	0.7976	0	25	58031
25	10.35	0.5847	0	34	

FIGURE 28.

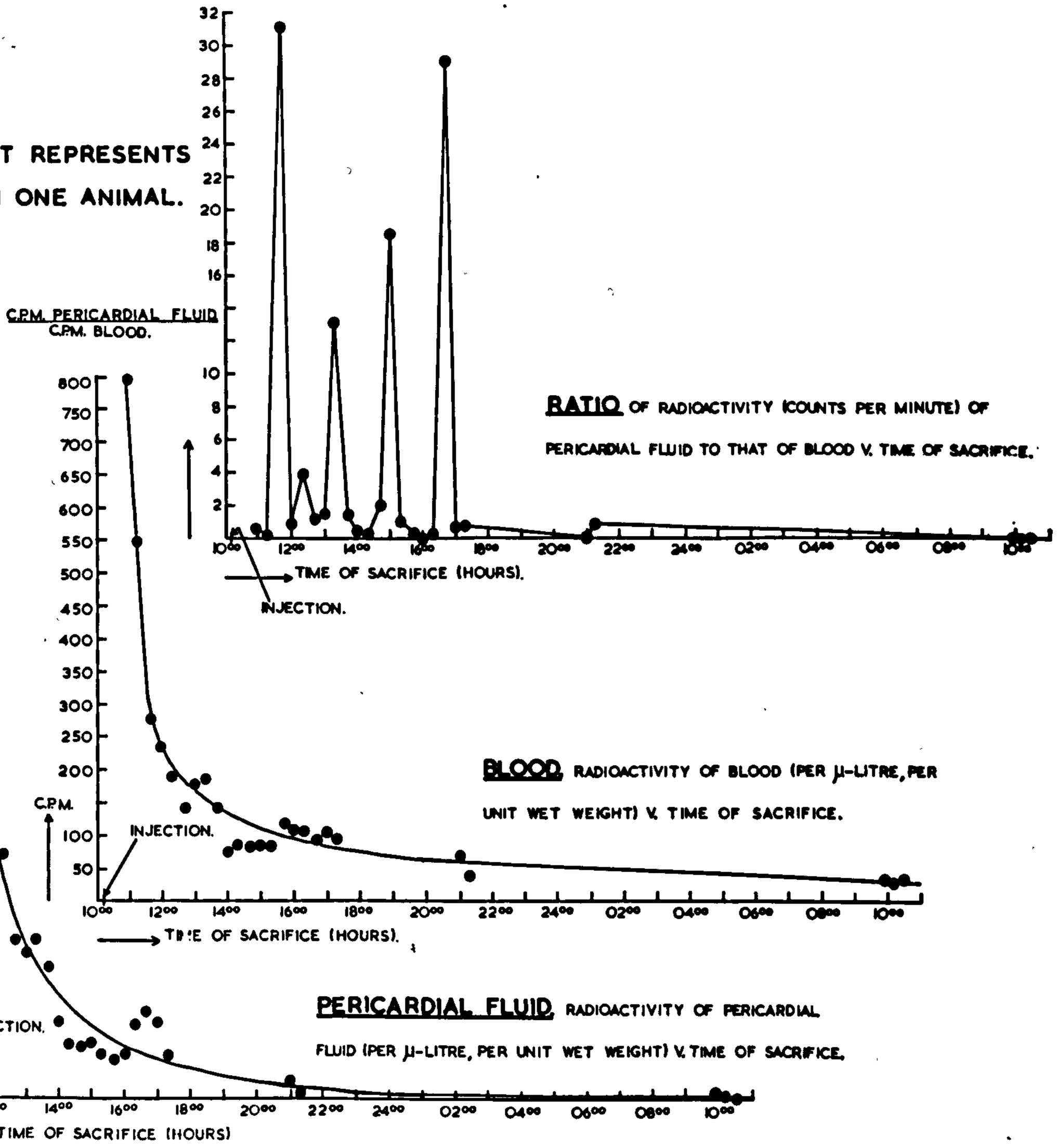
RADIOACTIVITY PER μ-LITRE OF FLUID EXTRACTED FROM SACRIFICED SLUGS WHICH HAD BEEN INJECTED PREVIOUSLY WITH ^{14}C-URIC ACID V. TIME OF SACRIFICE OF EACH SLUG.

EACH POINT REPRESENTS DATA FROM ONE ANIMAL.

FIGURE 29.

THE RELATIONSHIP BETWEEN CHANGE IN RADIOACTIVITY OF PERICARDIAL FLUID AND OF BLOOD. DATA USED IN THE LOWER TWO GRAPHS HAVE BEEN AVERAGED AS MENTIONED IN THE TEXT: SEE "CONCLUSIONS".

EACH POINT REPRESENTS DATA FROM ONE ANIMAL.



completely from the pericardial fluid at about the same time that a steady level of uric acid in the blood is beginning to be maintained. Secondly, it is apparently possible for the uric acid to reach very much higher concentrations in the pericardial fluid than in the blood, under the conditions of the experiment, (9977 C.P.M. in the pericardial fluid compared with 1062 C.P.M. in the blood.) While the concentration of uric acid in the blood shows a fairly steady decline, four "peaks" of uric acid concentration (i.e. four samples which show a reading of over a thousand C.P.M.), can be seen in the pericardial fluid of each of four individual slugs at various time intervals after injection. Obviously, the possibility that radioactive uric acid in solution in the kidney lumen could pass into the pericardial cavity via the renopericardial canal cannot be ruled out. However, the fact that C.P.M. /μ-litre of pericardial fluid was zero in four of the last five samples, whilst radioactivity was still present in the blood, and the kidney fluid samples, indicates that contamination of the pericardial fluid from either of these sources was not a regular occurence during sampling. It would seem that the blood, rather than the kidney fluid is perhaps more likely to be the immediate source of the ^{14}C- uric acid in the pericardial fluid, since the graph of C.P.M. in the pericardial fluid v. time of sacrifice is similar to that for the blood C.P.M.v. time of sacrifice, whereas the graph of kidney fluid C.P.M.v. time of sacrifice shows very little pattern. Also, the amounts of ^{14}C- uric acid found in the kidney are much greater than those found in the blood or the

pericardial fluid. However, too few results are available to be sure of this conclusion. The comparison of blood and pericardial fluid uric acid concentrations can best be seen in the graph of the ratio of C.P.M. in the pericardial fluid to C.P.M. in the blood against time of sacrifice. In this graph it is apparent that for the most part this ratio approximates to one, except for the four "peaks" already mentioned. The ratio, of course, falls to zero as the uric acid level in the pericardial fluid drops to zero.

Inaccurate though the size of the kidney extract samples may have been, some valuable conclusions may still be drawn from these results. Firstly it is evident that a high concentration of uric acid builds up very rapidly in the kidney lumen as the compound is removed from the blood, (47025 C.P.M. in the kidney fluidcompared with 1062 C.P.M. in the blood within twenty five minutes after injection). Secondly it seems that most of the ^{14}C- uric acid found in the kidney enters within the first twenty five minutes after injection since, except for one sample, (No.18), the concentration of ^{14}C- uric acid in the kidney never reaches even twice the amount found in the first kidney sample. Thirdly, there does seem to be a very slight positive correlation between C.P.M. in the kidney fluid and time of sacrifice up to about six hours after injection, after which the points are too widely spaced to draw any conclusion. One would expect that the concentration of radioactive uric acid would diminish as concretions of uric acid were released into the ureter, prior to voiding.

Section 6.

The Fate of ^{14}C- labelled Uric Acid Injected into The Haemocoel of A. reticulatus.

Introduction.

It has been shown (section 5, page 43) that uric acid injected into the blood stream of A. reticulatus is rapidly removed by the kidney. Using autoradiography, this investigation was carried out to try to locate the site of removal of uric acid from the blood and to shed more light on the process of the accumulation of uric acid in the kidney.

Materials and Methods

Six slugs were used in the investigation. Five of the slugs were injected with ^{14}C-uric acid dissolved in saline and one was injected with saline alone, as a control. The saline consisted of 0.7g sodium chloride (A.R.grade) plus 0.15g sodium bicarbonate (A.R.grade) per 100 ml of distilled water. The slugs were not anaesthetised. Using an Agla syringe they were injected with 0.01 ml of either the uric acid or the saline solution, via the foot. The uric acid saline contained 100μc of ^{14}C-uric acid (326.6μg) per ml.

The slugs were sacrificed at intervals of $\frac{1}{2}$, 1, 5, 30 and 90 minutes. The control animal was sacrificed at about 100 minutes. The animals were subjected to the following procedure: Each was first put into a deep freeze compartment (at -20^{o}C) for about one minute in order to halt its metabolism as quickly as possible. The kidney and heart were then quickly removed from the animal and immersed in Arcton cooled

with liquid nitrogen. This technique froze the tissues rapidly, while preventing any disruption of the cells by ice crystals, (This can occur if tissues are immersed directly into liquid nitrogen). The tissues were freeze dried for four days in a Speedivac Pearse Tissue Dryer. The tissues were then vacuum embedded in degassed paraffin wax and sectioned at 5μ. The sections were laid out on black paper in a perspex box and fixed for four minutes in the vapour from heated paraformaldehyde. The vapour was made by gently heating solid paraformaldehyde in a small glass receptacle. Sections were laid out in the dark room on slides which had been previously cleaned with alcohol and coated with A.R.10 (Kodak) stripping film. The sections were pressed down with the thumbs and the slides were placed in museum specimen jars. Black paper was taped around the jars and they were then sealed in black polythene bags, each bag containing a small pot of anhydrous calcium chloride. The bags were then stored in a deep freeze compartment at about -25°C. For dark room procedures, using A.R.10 stripping film, a 15 watt safe light with a Wratton No.2 filter was used.

The slides were left for three days exposure and then taken back to the dark room for further treatment. They were removed from the jars and dipped in a solution of 10% cellulose acetate in butanone. They were then covered to prevent the butanone from evaporating too quickly, and left to dry for a few hours in the dark. This served to stick the sections to the film more securely. When dry, the slides were dewaxed in xylene (5 minutes), hydrated (absolute alcohol - 5 mins., 90% alc. - 3 mins., 70% alc. - 3 mins., distilled water - 3 mins.) and

developed (4 mins) in Kodak D19 developer. They were then washed (30 seconds) in distilled water, fixed in Kodak Acid Fixer (10 minutes) and washed again in slow running tap water (10 minutes). They were then stained with Mayer's Haemalum (2 minutes), blued in tap water, dehydrated and mounted in DePeX. The slides were examined with a light microscope.

The procedure outlined above incorporated generally accepted techniques for autoradiography of soluble materials.

Results

The labelling could be clearly seen but was only found in any concentration, under the nephrocytes. The sections of the slug sacrificed after half a minute, and of the control slug, showed no labelling, but the "one minute sections" and all subsequent ones did show labelled nephrocytes. In some of the one minute sections about a third of the nephrocytes were labelled. The silver grains were present at very high density under nephrocyte vacuoles and rapidly thinned out further away from these areas. The effect under low power (X40) was that a large black spot appeared to cover the apical part of the vacuole in each labelled nephrocyte. The labelling was clear enough to enable one to see the outline of the whole kidney on parts of the slides even where the sections had been washed off during process of the film.
In sections of the slugs sacrificed after one minute the proportion of nephrocytes which were labelled increased until, in some of the 30 and 90 minute sections, all that could be seen was a black patch of irregular density surrounded by completely unlabelled parts of the sections. This patch

represented the area covered by the nephrocyte epithelium; all other parts of the sections were unlabelled.

Although under low power each dense black spot could be attributed to a single nephrocyte vacuole, under high power the grains which were densely packed in the centre of a vacuole label gradually thinned out to a perimeter beyond the confines of the vacuole. Thus, in a heavily labelled section, the whole area covered by the nephrocyte epthelium had a homogeneous low density covering of grains with a number of much denser centres equivalent to the nephrocyte vacuoles present.

Conclusions.

It was shown (section 5 page 43) that uric acid injected into the blood system of A. reticulatus could be found in the kidney fluid within 25 minutes after injection. The results of this investigation show that injected uric acid can be localised actually within the nephrocyte vacuoles within one minute after injection.

The injected uric acid became concentrated so rapidly within the nephrocyte vacuoles, that the labelled vacuole contents completely dominated the sections, and radiations from the ^{14}C- uric acid within the vacuoles also affected the film in the area surrounding each vacuole. The uric acid was concentrated so much by the nephrocytes that the areas of film affected by the vacuole contents overlapped to a large extent and it was impossible to localise any specific sites where ^{14}C- uric acid passed into the nephrocytes.

From the pattern of labelling observed (i.e. under low power a dense black spot under the apical region of a vacuole), it seems probable that the ^{14}C- uric acid accumulating in the vacuoles was being deposited round a concretion, and that on the finished slides, the concretions were no longer visible since they had been dissolved and leached out of the vacuoles by the processing after exposure of th film.

Section 7.

The Measurement of Fluid Pressure in the Aorta and Pericardial Cavity of A. reticulatus.

Introduction.

An attempt was made to estimate the variations in fluid pressure within the aorta and pericardial cavity of Agriolimax reticulatus during "normal" heartbeat. For a number of reasons it was not possible to make any measurements of absolute pressures with the equipment available at the time. Results were difficult to obtain due to the small size of A. reticulatus.

Measurements of blood pressures have been made before in both Helix (Schwartzkopff, 1954) and Patella (Jones, 1970), but as far as is known no measurements have been made in any slug as yet. The greatest difficulties were those concerning the small size of the animal. For instance, the smaller the diameter of the canula inserted into the appropriate part of the slug for the measurement of pressure, then the weaker was the pressure signal, (the narrower the canula diameter, the greater is the canula internal surface area in proportion to its volume, and hence the greater is its resistance to the passage of any contained fluid). In fact, a small hypodermic needle was used as a canula and its performance as part of the link between the slug and the pressure transducer appeared to be just within the limits of the experimental set-up. Other difficulties were fairly obvious ones, concerned mainly with the mechanical manipulation of the canula within the body of the slug. There are also obvious criticisms as to the validity of results obtained

with a canula in the body of a live animal; dissection to reach the desired portion of the body is equivalent to very serious injury and is bound to have affected the results in some way; a tight seal was never obtained around the edges of the canula and, once in position, any disturbance of the canula resulted in complete loss of its effectiveness; the use of an anaesthetic to keep the slugs still during measurement of pressure must also have affected the results. Nevertheless, the "In Vivo" condition of measurement is presumably more desirable than measurements taken on an excised heart, since, in the latter case, conditions would be even more unnatural.

Method.

For each attempt at measurement, a slug was anaesthetised using solid carbon dioxide. Measurements were made during the short space of time (about five minutes) just before the recovery of full locomotory powers of the slug in question. Generally speaking, between five and ten minutes exposure to the carbon dioxide vapour was sufficient to provide a reasonable period of time for measurement, although this varied according to the slug. The slugs were operated on immediately after anaesthetisation, in order to insert the canula (hypodermic needle) into the appropriate place. Successful insertion of the canula was very difficult to achieve, and after insertion, any slight movement of the recovering slug resulted in the canula being jolted out of position.

To record pressure in the arterial system, the free portion of the mantle was dissected away after anaesthetisation,

and a slit was made in the dorsal body wall just anterior to the lung cavity. The hypodermic needle was inserted into the anterior aorta and from there, pushed along into the aortic stem so that the tip of the needle was just in front of the ventricle outlet. For the measurement of pericardial fluid pressure variation, the top of the mantle was dissected away and the shell was removed. The hypodermic needle was then pushed through the shell gland, the lung, and the pericardial membrane into the pericardial cavity. No attempt was made to tie the needle in position with either type of measurement because, during trials, tying had not proved possible without dislodging the needle. It was assumed that, at least to some extent, the elasticity of the tissues would serve to seal off the slit made by the insertion of the needle. The needle was manouevred into position by means of a two way racking device held in an ordinary bench clamp. The needle was joined via a ground glass connector (consisting of the removed tip of a Tuberculin Syringe barrel) to a piece of polythene tubing about 30 cm. long. The bore of the tubing was about 5mm. and its walls were fairly rigid to reduce any possible damping effects which the tubing might have had on the pressure signal. The tubing was connected to a pressure transducer (type S.E.4-81 Mk.2 serial no. 138 ... S.E. Laboratories). The signal received at the transducer was amplified using the E.M.M.A. 4000 system. Units of the system used were a carrier amplifier, type S.E.4192, and a D.C. amplifier type S.E. 4190. Results were recorded on ultra-violet sensitive paper with an S.E. U.V. recorder 3006.

After a trace was obtained, the paper was stabilised with Kodak Linagraph Stabilising Laquer. The hypodermic, polythene tubing and transducer were filled with Hedon-Fleig saline, and this was also used as a perfusion liquid to keep moist the dissected(and therefore exposed) parts of the slug. A recording attempt was usually terminated by the slug crawling away from the end of the hypodermic needle.

All pressure measurements were carried out with the settings on the E.M.M.A. system as follows:-

Carrier Amplifier Gain .....M

Amplifier Gain .....Maximum

Base Shift 0

Filter ..... Off position

Attenuation 1

At these settings, a vertical movement on the recording paper of ± (i.e. above or below the base line) 2.6mm represented a variation of ± 1 cm. of water pressure.

The base line usually showed a certain amount of drift. This was rarely marked enough to have a critical effect on a recording of pressure variation, though it was sufficient to prevent the measurement of absolute pressures. It was difficult to cut down background noise, since the apparatus was extremely sensitive to vibration. Interference at 50 c.p.s. was always present and was presumable due to mains A.C.

Results.

Frequency of heartbeat was very variable, as would be expected in the circumstances. Frequencies as low as 1.3/sec. and as high as 6.6/sec. were recorded from the canulated aorta. (Fig.30)

Each peak in the recording from the canulated aorta directly reflects a single contraction of the ventricle. The maximum pressure variation recorded in the aorta was approximately 5.4 cm. of water.

Each double peak in the intra-pericardial recording is explained as follows:- the first peak represents the expansion of the auricle (by drawing in blood from the venous sinuses) caused by the presence of negative pressure in the pericardial cavity; the slight dip after the first peak represents auricular systole; at the second peak blood has been transferred to the ventricle; the last dip represents ventricular systole, and may create a negative pressure in the pericardial cavity. The maximum pressure drop recorded in the pericardial cavity was approximately 2.5 cm. of water (at ventricular systole). The pressure drop at auricular systole was usually less than 1 cm of water.

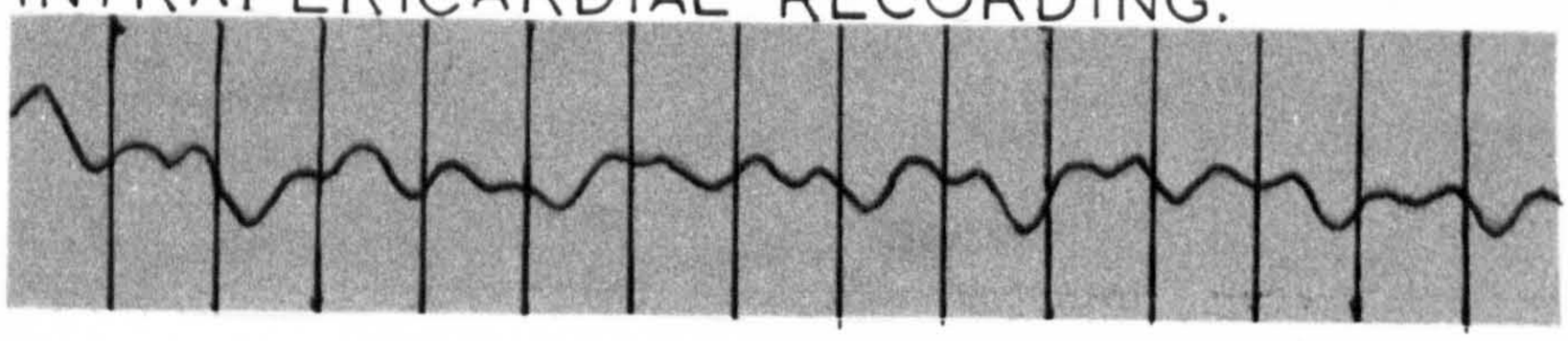
Conclusions.

It would not be legitimate to draw any conclusions from this attempt at measurement of fluid pressure variation because of the necessarily unnatural conditions pertaining to the experimental animals. (Ideally, a device which could be placed "in situ" without too much disruption of the slugs' tissues, and which would allow continuous monitoring of pressure without

FIGURE 30.

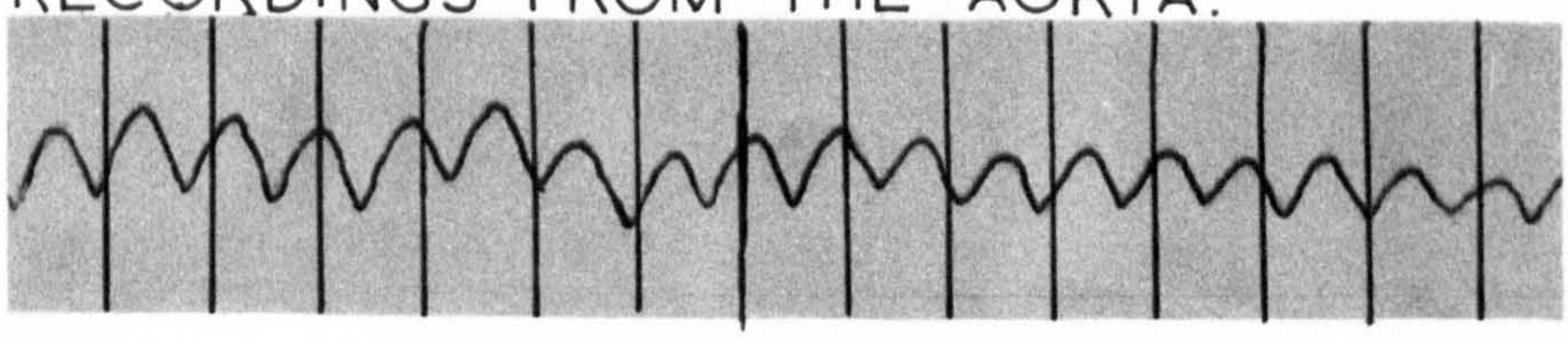
PRESSURE RECORDINGS FROM THE BLOOD SYSTEM OF A. RETICULATUS.

INTRAPERICARDIAL RECORDING.

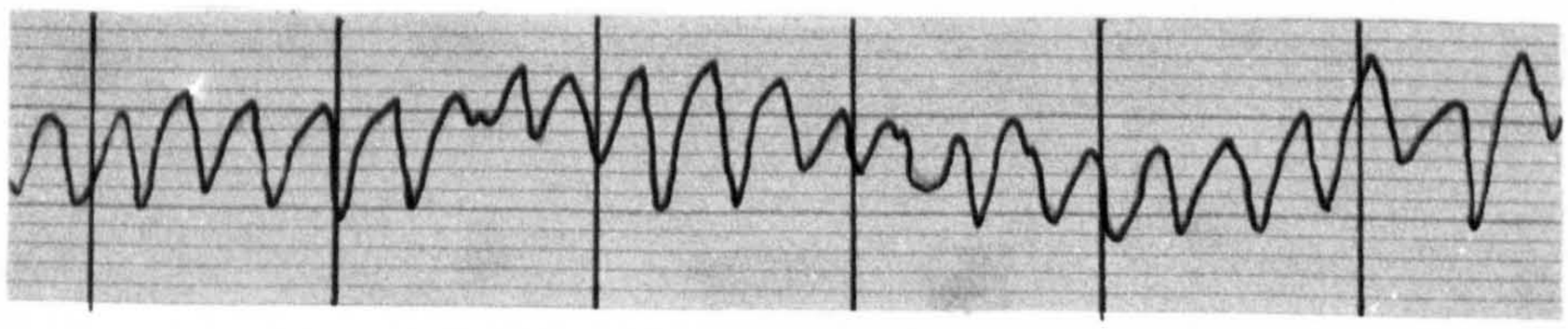


RECORDINGS FROM THE AORTA.

1.



2.



VERTICAL LINES REPRESENT ONE SECOND INTERVALS.

further interference with the slugs, would be desirable.) Nevertheless, some inferences can be made.

In nature, there would be no leaks in the vascular system. Hence the actual arterial blood pressure variation is probably rather higher than the maximum recorded pressure variation. The same will probably also apply to the measured pressure variation in the pericardial cavity. That the experimental animals were still under the influence of the anaesthetic will doubtless have had an effect - probably to decrease the values obtained.

On average there is no difference in height between the two components of each double peak in the intra-pericardial recording. So, despite the apparent lack of a valve between the auricle and the venous sinuses, more or less all the blood in the auricle is presumably passed on into the ventricle at auricular systole, and little, if any is passed back into the venous side of the circulation; positive pressure in the sinuses probably prevents any significant backflow of blood.

The timelag between ventricular systole and the filling of the auricle during auricular diastole (which may allow a negative pressure to build up in the pericardial cavity), is probably due to the contraction of the ventricle which stretches the auricle and prevents it from filling up with blood until the ventricle relaxes. It is regrettable that no findings which would elucidate the function of the reno-pericardial canal were made. However, the above inferences do not preclude the possibility that the reno-pericardial canal is a source of kidney fluid.

Section 8.

Experiment to Ascertain Whether a Definite Rhythm of Excretion Occurs in A. reticulatus

Introduction

Voiding of excreta has been shown to occur approximately once daily. From observations during previous experiments it was apparent that a single animal took only a matter of days to produce an amount of excreta roughly equivalent in magnitude to the dry weight of the kidney, and also that the kidney of an animal immediately after that animal has voided excreta usually contained very few concretions. The kidney was therefore thought to have quite a high turnover rate of excretory material whose fluctuations in concentration might well correspond to a measurable diurnal rhythm. The following experiment was carried out to test this hypothesis.

Materials and Methods.

A number of slugs were collected, kept in plastic pots, and fed on cauliflower. The slugs were examined from time to time. Whenever a slug was observed to be voiding excreta, the slug was put into a clean glass tube and the time was noted. Each tube had a perforated lid and contained food and wet cotton wool. After ameasured period of time in a clean tube, each slug was anaesthetised with dry ice, and its kidney (along with the wall of the primary ureter - see Fig.3), cleanly dissected out and put into a small (0.5 x 5cm.) glass tube which had been labelled with a diamond marker

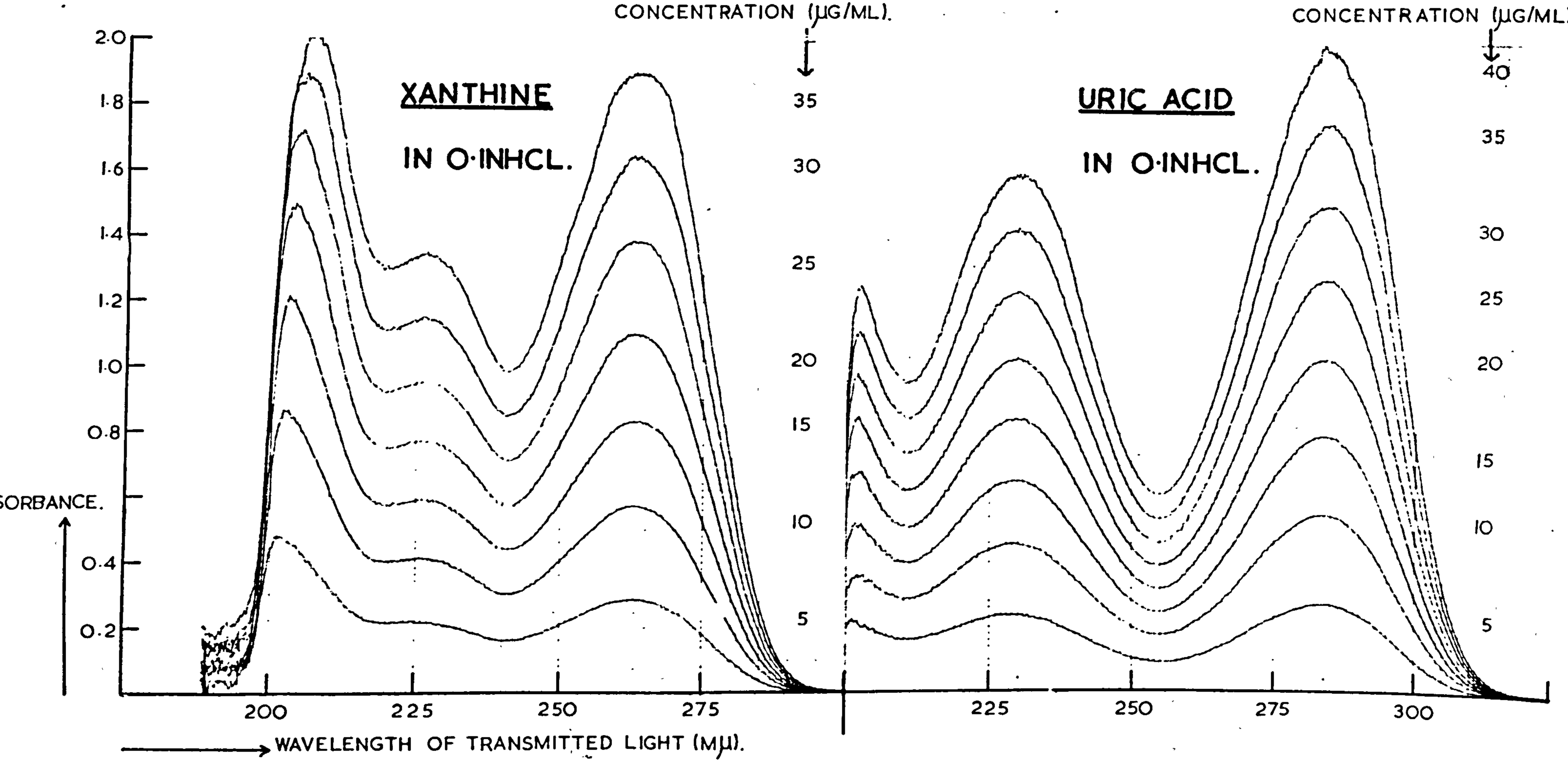
and pre-weighed. The remains of the slug were placed on a weighed cover slip and dried to constant weight at about 100°C. The kidney in the tube was twice frozen (by placing the tube on a block of dry ice) and immediately thawed after each freezing. This was done in order to disrupt the nephrocyte membranes. The kidney was finally frozen for a third time, freeze dried overnight in a Speedivac Pearse Tissue Drier, and its dry weight determined. 0.3ml of solvent (ammoniacal, 10% isopropanol) was then added to the tube and the kidney was macerated in this solution (using a glass rod whose tip had been roughened on a Carborundum stone) for three minutes. The suspension was centrifuged at 5000 r.p.m. for three minutes. 0.2ml of the supernatant was spread in a narrow band on Whatman No.3 chromatography paper and chromatographed using butanol/acetic acid/water, 20/3/7 as solvent. The supernatant was spread on the paper by hand, using a 0.2ml constriction pipette, and each application was dried with a hair drier before subsequent applications were made. The tip of the constriction pipette was sufficiently narrow to prevent the application of too wide a band on the paper. Paper chromatography was found to be quite adequate and was quicker than the thin layer chromatography which had been used previously. After chromatography the paper was air dried and examined under U.V. light. The two bands of uric acid and xanthine (see section 3 page 36) were cut out of the paper and each shaken with 5ml of 0.1N hydrochloric acid in a clean specimentube. The strips were left to soak overnight and the tubes were

shaken again before using the solutions the following day. This gave quantitative elution of the purines (Chargaff and Davidson, 1955). Blank strips, approximately the same size as each purine band, were cut from the chromatogram and these were treated in a similar way to provide blank solutions. The purine eluents (each with its appropriate blank) were scanned on a U.V. spectrophotometer (Unicam S.P.800) and the U.V. absorbance was read off from the resultant peak on the graph nearest the wavelength of 300 mμ. The molecular extinction coefficients for xanthine and for uric acid are both constants. Therefore both chemicals obey the Beer-Lambert law and their concentrations in solution can be estimated by the degree to which they absorb ultra-violet light. Calibration curves (of absorbance against concentration) were constructed for xanthine and uric acid using solutions of known concentrations, (Figs. 31 and 32). The concentrations of the unknown solutions were read directly from these curves according to their absorbance. The percentage recovery which could be expected after chromatography was determined by chromatogramming solutions of known concentrations, eluting them and finding their absorbances, and comparing the expected with the actual results. The actual concentrations of uric acid and xanthine in the kidney could thus be found.

In order to discover the relative amounts of uric acid and xanthine in the excreta, a 24 hour sample of the excreta from each of four batches of slugs (three slugs in each

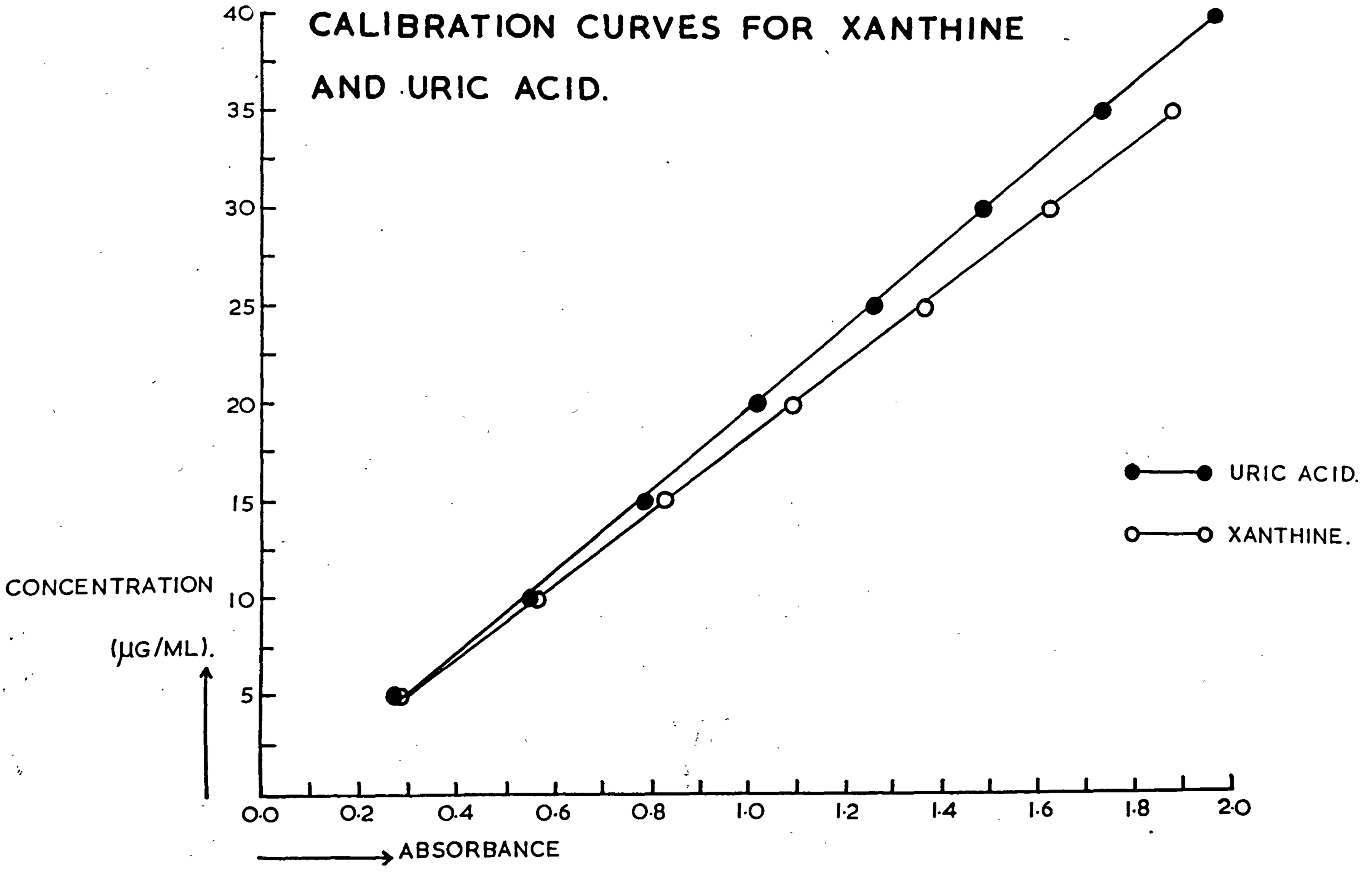
FIGURE 31.

U.V. Spectra of solutions of xanthine and uric acid for the calculation of calibration curves.



(LENGTH OF LIGHT PATH: 10MM).

FIGURE 32.


CALIBRATION CURVES FOR XANTHINE AND URIC ACID.
CONCENTRATION (µG/ML).
40
35
30
25
20
15
10
5
0·0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
ABSORBANCE
URIC ACID.
XANTHINE.

batch) was collected, freeze dried, weighed, dissolved in ammoniacal isopropanol solution, and then treated the same way as kidney extracts. The kidneys were also dissected out of the animals after a known period of time for dry weight and purine determinations. This was carried out as before except that batches of three, rather than single kidneys, were involved, and hence only a quarter of each extract was chromatogrammed to obtain approximately the correct concentration to give a readable result on the spectrophotometer.

Results and Conclusions

All the results are recorded in tables VI and VII, and some of the data from the tables are compared graphically in Figs. 33-36 inclusive. Table VI shows data concerning the amount of purine found in the kidney of each of a number of slugs, sacrificed at different time intervals after voiding excreta, during a 24 hour period. Table VII shows data concerning the relative amounts of purines found in 24 hour samples of excreta.

Basing calculations on the averages from the sets of results, the following data may be derived from the tables:

Table VI

The total amount of purine in the kidney per gram (dry weight) of body tissue was 1528 μg (i.e. 1127 μg of uric acid plus 401 μg of xanthine); on average purine forms approximately 10% of the dry weight of kidney tissue (i.e. 7.5% uric acid plus 2.5% xanthine). Since it is shown below that diurnal rhythm of excretion probably

TABLE VI

Slug No.	Time after voiding excreta, when sacrificed (hours).	Dry wt. of kidney.	Dry wt. of slug plus kidney.	Wt. of uric acid in kidney.	Wt. of xanthine in kidney	%(wt.) of xanthine plus uric acid in kidney.	%(wt.)of uric acid in kidney.
1	17.5	570	23250	103	51	27	18
2	23	480	27580	39	17	12	8
3	13	680	40960	250	59	45	37
4	0.5	780	59580	49	8	7	6
5	19.5	770	38080	98	25	16	13
6	16.5	690	42580	82	27	16	12
7	1.5	230	21540	15	8	10	6
8	12	1090	59120	174	32	19	16
9	14	540	41680	115	25	26	21
10	20	560	39790	18	8	5	3
11	22	890	59540	40	13	6	4
12	24	600	42870	12	8	3	2
13	9	700	27990	3	4	1	0.5
14	8	730	48280	26	11	5	3
15	6	450	33950	15	4	4	3
16	7	690	44870	36	17	8	5
17	5	380	36160	18	11	7	5
18	2	370	38690	3	4	2	1
19	1	620	21950	16	8	4	2
20	3	820	92560	3	4	1	0
21	4	440	38850	1	2	1	0
22	10.5	490	28520	19	8	6	4
23	16.5	500	29960	169	30	40	34
24	17	200	18600	1	1	1	0
25	17	590	40320	30	21	9	5
26	9	580	37660	4	5	1	1
27	10.5	710	36800	0	5	1	0
28+	8.5	580	40666	17	11	5	3
29+	17.5	607	36617	91	38	21	15
30+	17	483	33800	9	6	3	2
31+	10	817	54487	19	9	3	2
Av.	11	605	39913	45	16	10	8

All weights are in μg, and are corrected to the nearest whole number where appropriate.

+ The results for the starred numbers represent averaged results for four groups of three slugs each, (used to estimate the amounts of xanthine and uric acid in the excreta).

Times are recorded to the nearest half hour.

TABLE VII

Batch no.	Dry Wt. of 24 hour sample of excreta.	Dry Wt. of slugs plus kidneys.	Dry Wt. of kidneys.	Wt. of uric acid in excreta.	Wt. of xanthine in excreta.	Wt. of total purine in excreta.
1	520	109850	1820	460	98	558
2	540	122000	1740	408	83	491
3	430	101400	1450	381	102	483
4	550	163460	2450	453	55	508
Average per slug	170	41392	622	142	28	170

All weights are in μg and are corrected to the nearest whole number where appropriate.

Each line of figures (except for the last line) represents the pooled results from three slugs.

occurs in A. reticulatus, these totals are probably not very meaningful (amounts of purine in the kidney vary quite considerably at different times of the day: as little as 1%, e.g. slug no.13, or as much as 45%, e.g. slug no.3, can be found). However, the totals are included for comparison with results obtained by other workers, (e.g. Jezewska et.al., 1963).

Table VII

The average amount of purine in the excreta is 99% (i.e. 83% uric acid plus 16% xanthine); the average rate of purine production in A. reticulatus is 169 µg/g body tissue/hour; total purine nitrogen excreted by the kidney is 58 µg/g body tissue/hour (i.e. 48 µg as uric acid plus 10 µg as xanthine).

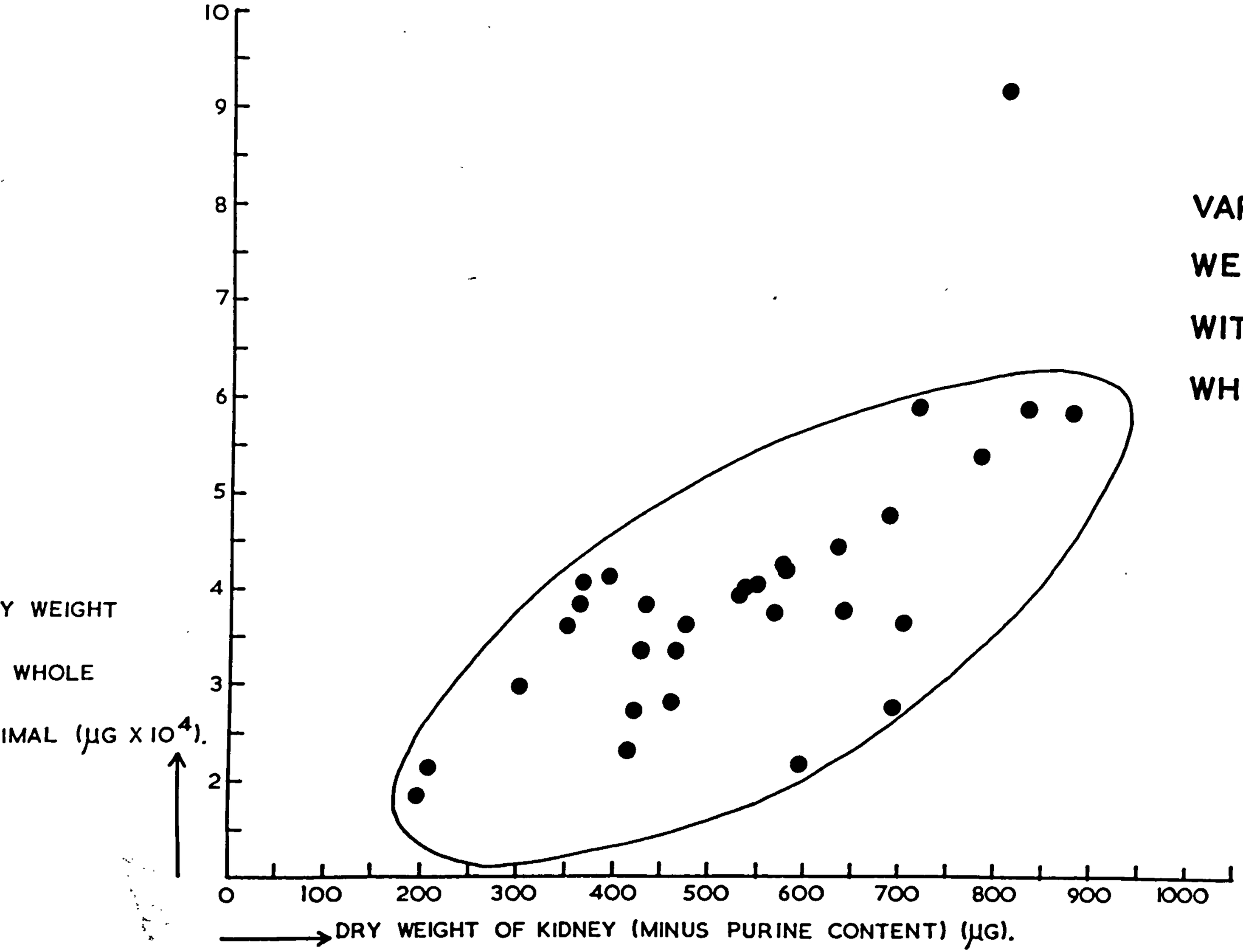
The average proportion of xanthine to uric acid in the kidney was found to be 1:3, (2.5:7.5). In the excreta, this was found to be approximately 1:5.2, (16:83). It is possible that this reflects some change in proportion that takes place after the purine has left the kidney, e.g. some of the xanthine might be converted to uric acid in the ureter, (in the excretory cycle of birds, xanthine is a precursor of uric acid).

Thus it appears from the tables that the excreta consists almost entirely of purine, the greater part of which is uric acid. (Less than 2% of the excreta consists of calcium, and at least 45 times as much nitrogen is excreted in the form of purine, as that released in the form of ammonia, see section 3, pages 37 and 38.)

As would be expected, the graph of kidney dry weight against dry weight of the whole animal shows a strong positive correlation, (Fig.33). However, there is a fairly wide variation between individual kidney contents. This variation evidently has a physiological basis, since excretory matter, (purine), can constitute as much as 45% or as little as 1% of the dry weight of the kidney, (see Table VI).

The nature of this physiological variation is readily explainable in terms of a diurnal excretory rhythm. This is illustrated in Fig.34 which shows the amounts of purine found in each kidney plotted against the number of hours after voiding excreta. There is a very high turnover rate of purine by the kidney of A. reticulatus. As can be seen from the results shown in table VII, a single kidney could release more than its own dry weight of purine in only four days. A slug usually voids excreta only once in twenty four hours, and though it was obviously not possible to show a rhythm in any one individual slug by continuously measuring the purine content of its kidney, a twenty four hour rhythm did emerge from the aggregation of data from a number of slugs. A wide variation in kidney purine content did of course occur between slugs sacrificed fairly close together in time. However this variation was eliminated by plotting the number of hours after voiding excreta, (rather than the actual time of day or night), against kidney purine content. Further variation due to slight difference in individual cycles of accretion were eliminated by an averaging procedure:-

FIGURE 33.



VARIATION OF THE DRY WEIGHT OF THE KIDNEY WITH THAT OF THE WHOLE ANIMAL.

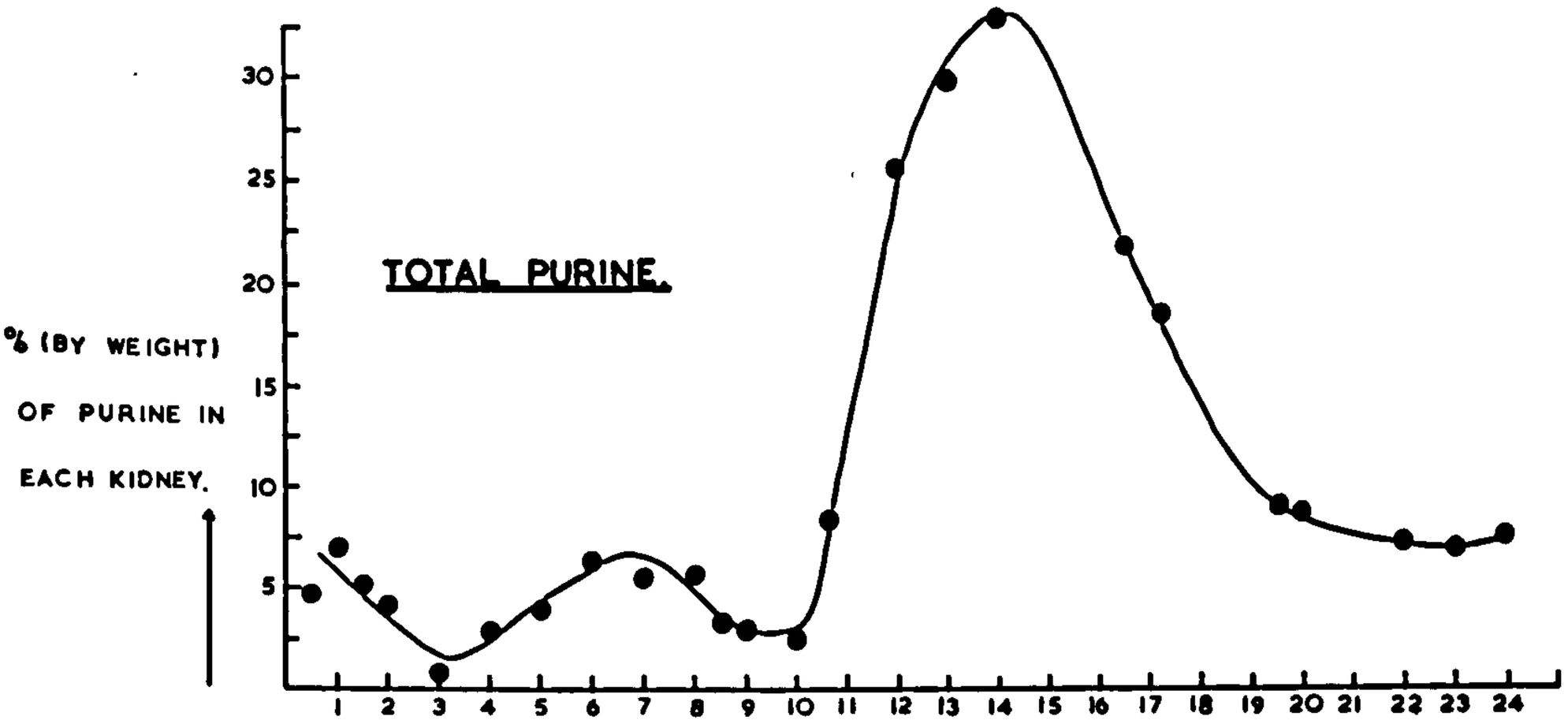
Any measurements of kidney purine content which fell within a quarter of an hour of each other were averaged, and the measurements themselves were then averaged in threes, (the first point being produced by averaging the thirty-first, the first and the second points, and the last point by averaging the thirtieth, the thirty-first and the first points). It is regrettable that more readings at the "critical" times were not obtained. However, the readings which were obtained appear to be sufficiant to show the basic trends of excreta accumulation by the kidney of A. reticulatus.

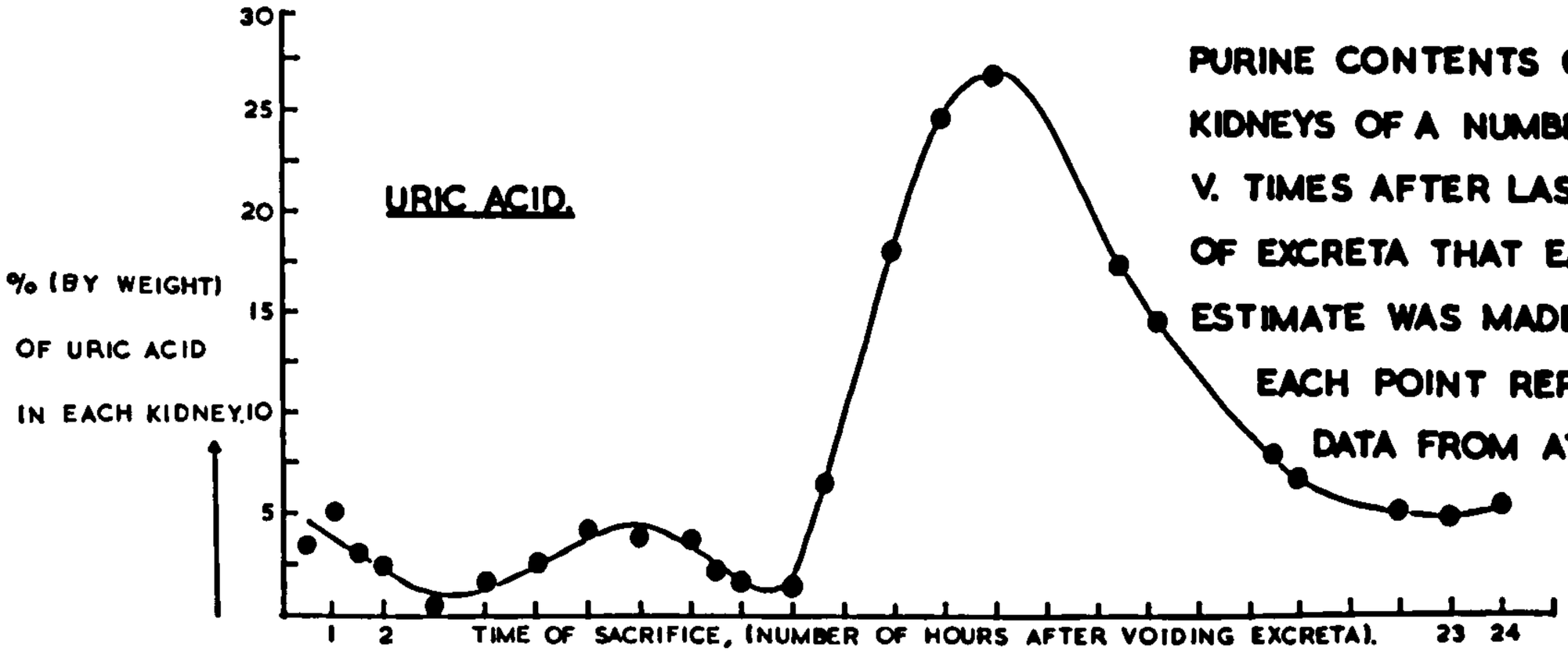
It is quite evident that a peak of purine accumulation does occur between twelve and seventeen hours after voiding an excretory mass, (Fig.34). The peak is also evident in the separate graphs for uric acid and xanthine showing that both purines accumulate at similar rates. The latter point is also borne out by the positive correlation between the amounts of xanthine in each kidney plotted against the percentage uric acid, (Fig.35). This graph also shows that the greater the purine content of the kidney, the greater is the variation in the ratio of xanthine to uric acid in the kidney.

The significance of the occurence of a minor peak of purine accumulation between six and eight hours after voiding, is obscure at the present time.

It is apparent from the graphs (Fig.34), that purine accumulates rapidly over a short period of time (between 10 and 14 hours after voiding in this experiment), and is released rapidly also over a short period (14 to 19 hours

FIGURE 34.





PURINE CONTENTS OF THE KIDNEYS OF A NUMBER OF SLUGS V. TIMES AFTER LAST VOIDING OF EXCRETA THAT EACH PURINE ESTIMATE WAS MADE.

EACH POINT REPRESENTS DATA FROM AT LEAST ONE ANIMAL.

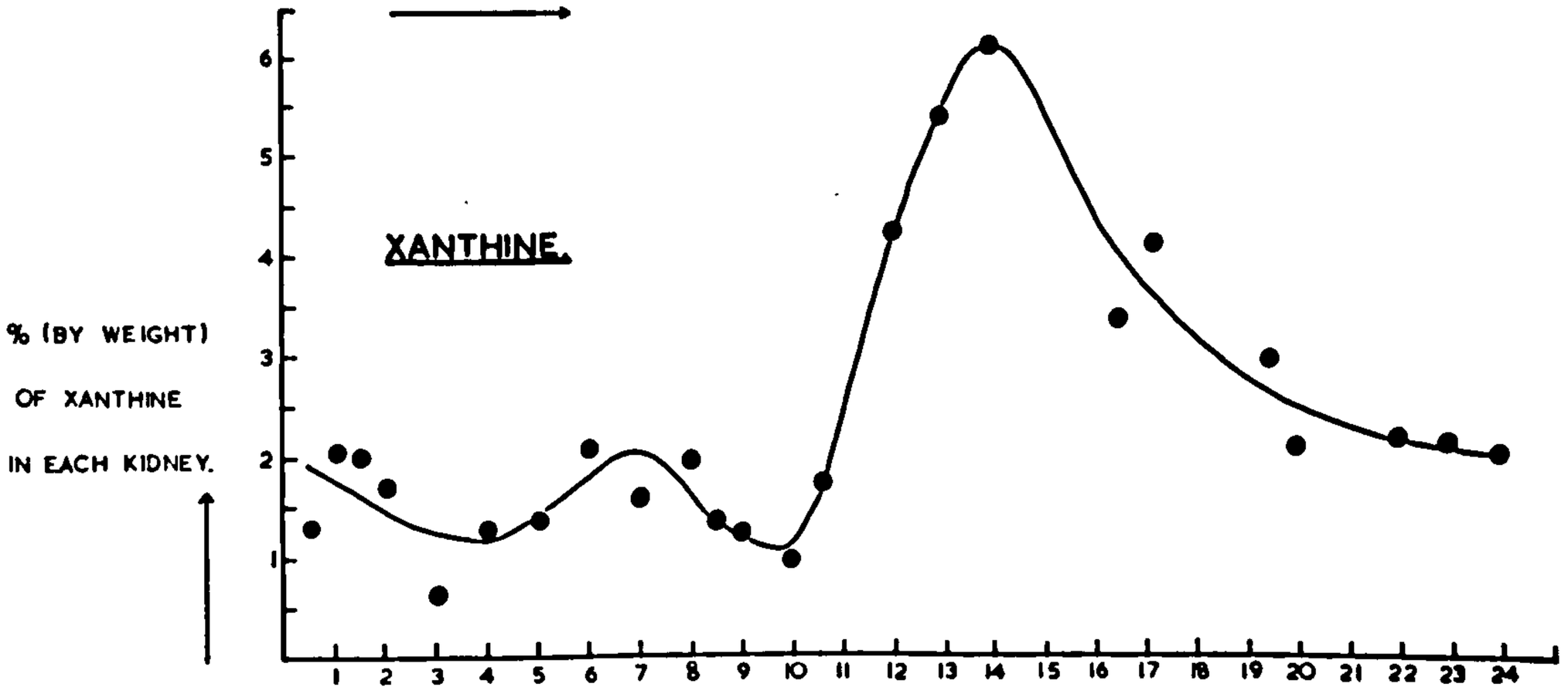
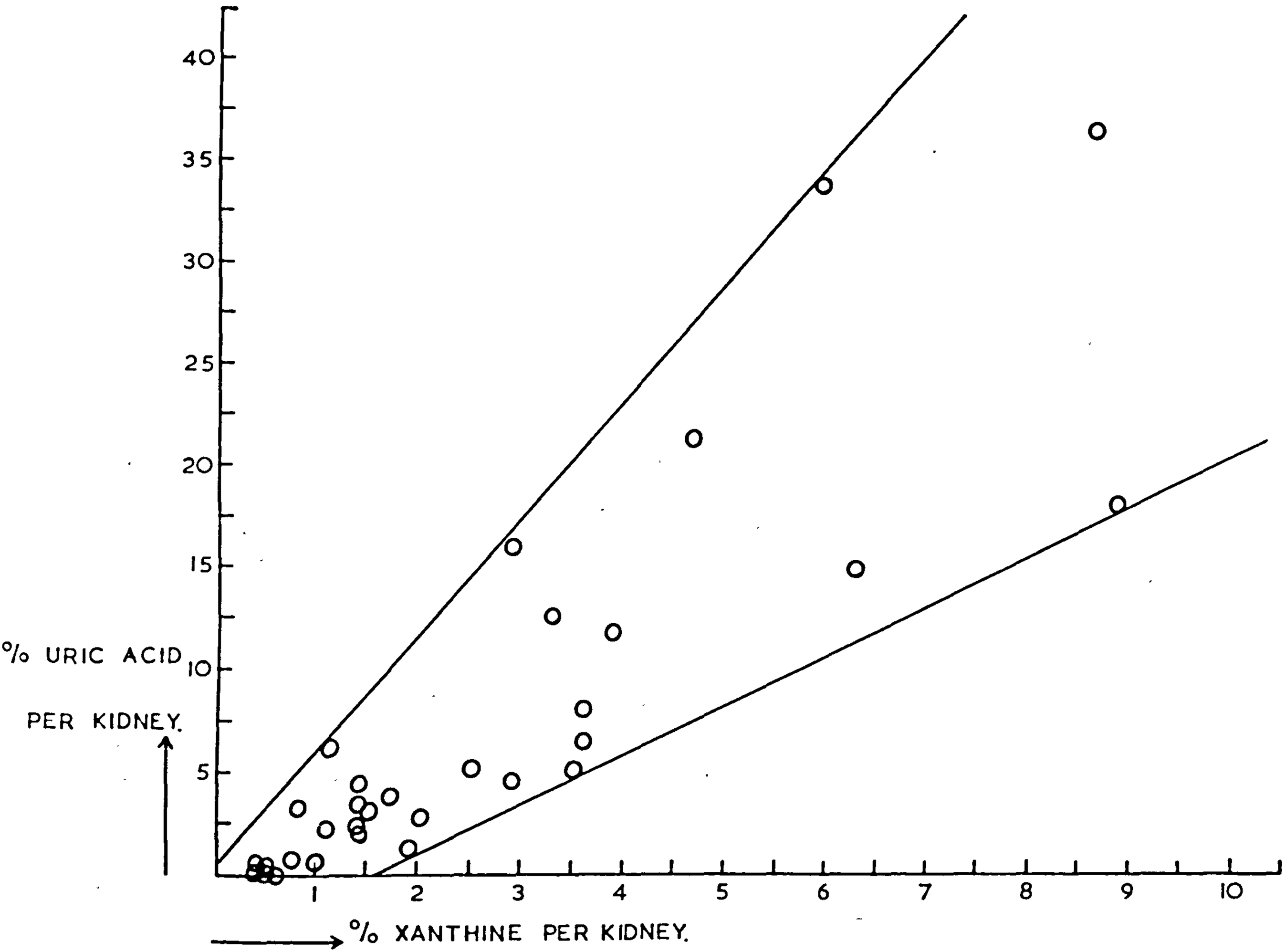


FIGURE 35.



COMPARISON OF THE AMOUNTS OF XANTHINE AND URIC ACID IN THE KIDNEY.

after voiding), almost immediately after maximum kidney content is reached. However, the downward gradient of the curve from the peak is shallower than its former upward gradient: it is evident that uric acid can be removed from the blood very rapidly (see section 5, page 46), but whether all the nephrocytes receive purine more or less simultaneously has not been elucidated; nevertheless, individual nephrocytes must reach maximum capacity for purines at different times according to their individual characteristics; the results suggest that there is a gradual release of purine from the kidney, rather than a sudden release in response to a stimulus affecting the whole kidney at one time. A possible inference is that each nephrocyte reaches its full capacity for purine and this then constitutes the stimulus for the release of its purine, or perhaps the release of the whole nephrocyte, into the kidney lumen. Since actual voiding of excreta takes place at a single point in time during a 24 hour period, the purine must be stored (probably in the bladder) until this time. Presumably, the next voiding would occur approximately 24 hours after the previous one. The original voiding generally took place during the late afternoon or early evening, but the time of voiding did vary between 11.00 hrs. and 00.15 hrs. Maximum kidney content thus occurs during the early part of the day.

Section 9.

Structural Changes in the Kidney of A. reticulatus during the Diurnal Rhythm of Excretion.

Introduction

It is apparent (see section 8, page 60) that a diurnal excretory rhythm is present in individuals of Agriolimax reticulatus. The aims of the present investigation were:

1. To find out if there was a relationship in time between the rhythms of a number of slugs collected from one particular habitat.
2. To observe with the light and the electron microscope the appearance of kidneys containing different amounts of excretory material at different stages of the diurnal cycle.

Light Microscope Work.

Materials and Methods.

All the animals used were collected within about half an hour one evening from the vertical surface of a wall. The animals were maintained on carrot. The wall was about five feet high and had been built to support a grass bank which came right to the top of it on its east side. A cement path ran along the base of the wall on its west side and the west surface was shaded by a terrace of houses running parallel to the wall about five feet away from it. Large numbers of A. reticulatus could usually be found on this surface of the wall, especially after rain.

Slugs were sacrificed at approximately twenty minute intervals over a twenty four hour period starting at 17.35 hrs. on the day after collection.

The mantle and mantle organs (including the kidney) were removed with scissors and mounted immediately in a viscous sol· (10g of sodium carboxymethylcellulose (B.D.H.) per 100ml of distilled water) on a freezing-microtome block. The tissue was then quenched by lowering the block into liquid air. 8μ frozen sections of the tissue were cut on a cryostat and picked up on a square cover slip. Using watch glasses as reagent containers, the sections were then fixed in absolute methanol (1½ mins.), stained with methylene-blue (30 secs.), rinsed in distilled water, dehydrated in two changes of absolute alcohol (30 secs. and 2 mins.), cleared in xylene (2 mins.), and mounted in DePeX, (Pearse, 1960). This method of producing stained sections was used in order to preserve as much of the original kidney structure as possible. The stained sections were examined with a light microscope.

Granules in the kidney occured either as single, isolated concretions, or as distinct groups of small concretions. Although cell boundaries could not be seen, it was evident from the distribution of the groups of small concretions that each group represented the excretory contents of a single cell. Measurements of concretions were made using an oil-immersion objective and a calibrated graticule. Where the concretions occurred in groups, each group was measured as if it were a single concretion. The diameters of between 150 and 200 concretions were measured from a section of each slug. To standardise measurement as far as possible, all concretions within the circular field of the graticule were measured in any one field of view, Each quadrant of the field was covered thoroughly by moving the eyepiece in a clockwise direction

and measuring each concretion in turn as the scale on the graticule passed over it. Most concretions were approximately spherical in shape, but different shapes did occur frequently. Where a concretion was not spherical, the first diameter which the graticule scale passed over was measured. Using this procedure, it was assumed that any irregularities in the shapes of the concretions would cancel themselves out over the whole range of measurements in each sample.

There were quite large variations in the numbers of nuclei and concretions visible in sections from different slugs. Generally, the greater the number and the larger the size of the concretion then the more nuclei were observed in any one section. In every field of view in which concretions were measured, the numbers of nuclei and concretions were counted. The ratio of concretions to nuclei in any one kidney thus gave a very rough measure of what fraction of nephrocytes contained concretions.

The average size of concretion, and the approximate fraction of nephrocytes which contained concretions were calculated for each slug, (Table VIII). A graph of concretion size versus time of sacrifice was plotted (Fig.36). The results were also displayed to show the average size of concretion per slug, arranged in ascending order, (Fig.37). The latter was then used to refer back to the slides and to ascertain what differences there were between kidneys containing small concretions and those containing large ones.

TABLE VIII

Sample Number	Time	Average diameter of concretion (μ)	Concretion: nephrocyte ratio
1	17.35	8.5	0.63
2	18.00	---	---
3	18.15	9.9	0.96
4	18.35	5.7	0.61
5	18.55	7.5	0.76
6	19.10	5.9	0.47
7	19.30	5.4	0.19
8	19.55	7.1	0.62
9	20.20	7.2	0.50
10	21.15	6.6	0.46
11	21.15	7.6	0.86
12	21.40	7.6	0.83
13	22.00	7.2	0.79
14	22.20	8.0	0.70
15	22.55	8.5	0.99
16	23.15	7.2	1.17
17	23.45	6.2	0.65
18	24.00	7.8	0.73
19	00.20	7.0	1.00
20	00.45	6,4	0.74
21	01.05	6.2	0.69
22	01.25	4.8	0.60
23	01.45	6.0	0.59
24	02.05	6.8	0.87
25	02.30	6,3	0.45
26	02.50	7.5	0.86
27	03.15	8.0	1.03
28	03.45	---	---
29	04.20	6.9	0.48
30	04.40	5.8	0.96
31	05.05	5.8	0.51
32	05.30	5.6	1.04
33	05.50	7.7	0.74
34	06.10	7.1	0.55
35	06.30	6.5	1.00
36	06.50	6.6	0.72
37	07.05	6.3	0.47
38	07.30	6.3	0.74
39	07.50	7.6	0.99
40	08.10	7.6	0.91
41	08.30	7.5	0.79
42	08.50	7.2	0.84
43	09.10	6.1	0.61
44	09.30	5.3	0.42
45	09.50	8.3	0.48
46	10.10	10.4	2.93
47	10.30	7.0	0.62
48	10.50	7.4	0.67
49	11.05	6.8	0.67

TABLE VIII continued....

Sample Number	Time	Average diameter of concretion (μ)	Concretion: nephrocyte ratio
50	11.35	6.9	0.67
51	11.55	6.7	0.61
52	12.15	6.1	0.65
53	12.35	8.3	1.65
54	12.55	7.0	0.78
55	13.15	9.3	1.12
56	13.35	8.1	0.82
57	13.55	8.4	1.12
58	14.20	7.3	0.67
59	14.40	9.2	1.98
60	15.00	7.3	0.69
61	15.20	7.3	1.25
62	15.40	7.8	0.40
63	16.00	4.6	0.46
64	16.20	6.2	0.71
65	16.40	6.0	0.59
66	17.00	7.0	1.12
67	17.20	6.2	0.75

Sample numbers 2, and 28, were lost during processing.

FIGURE 36.

AVERAGE SIZES OF GRANULES FOUND IN THE KIDNEYS OF SLUGS SACRIFICED AT INTERVALS OVER A TWENTY FOUR HOUR PERIOD.

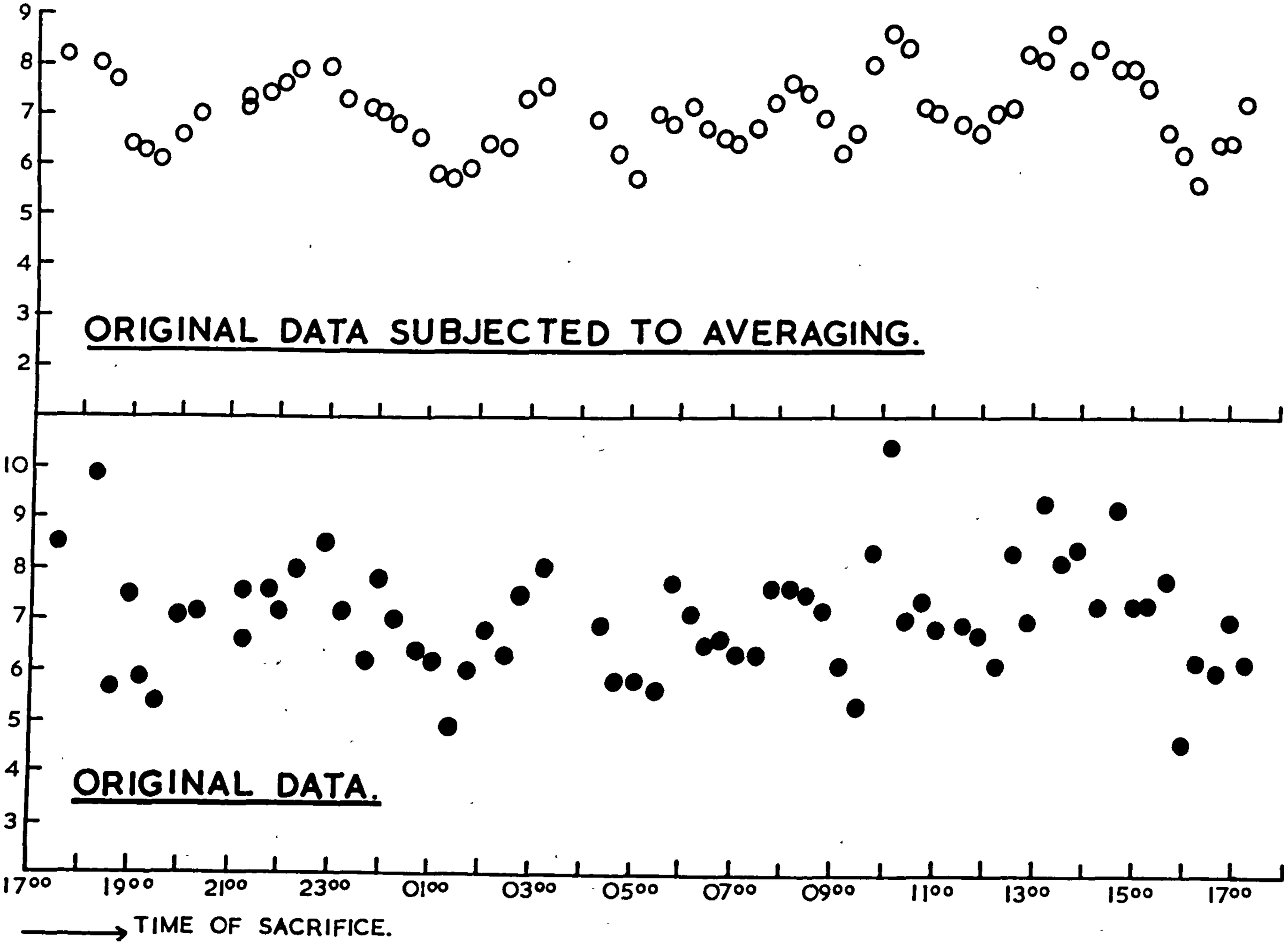
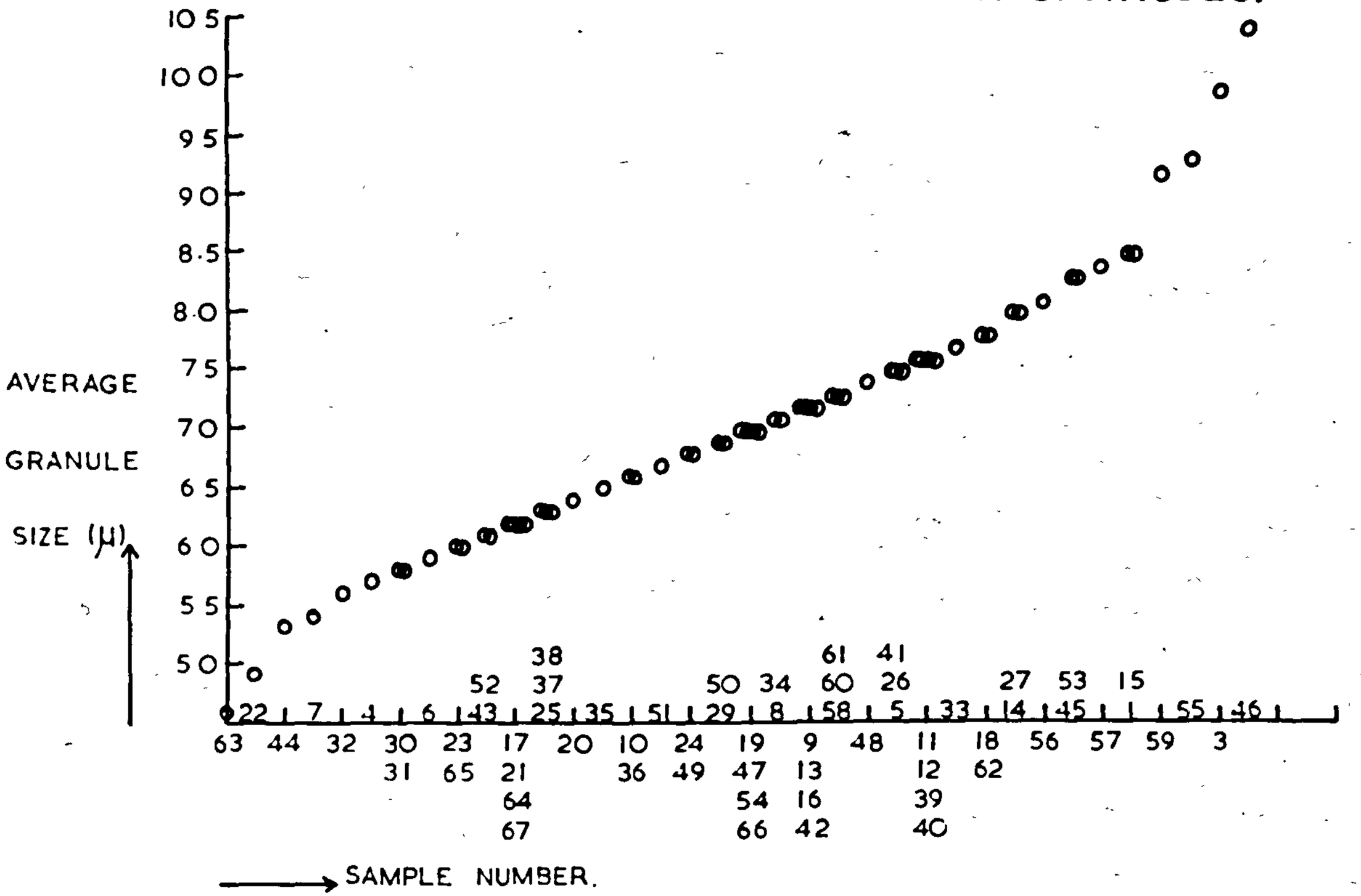


FIGURE 37.

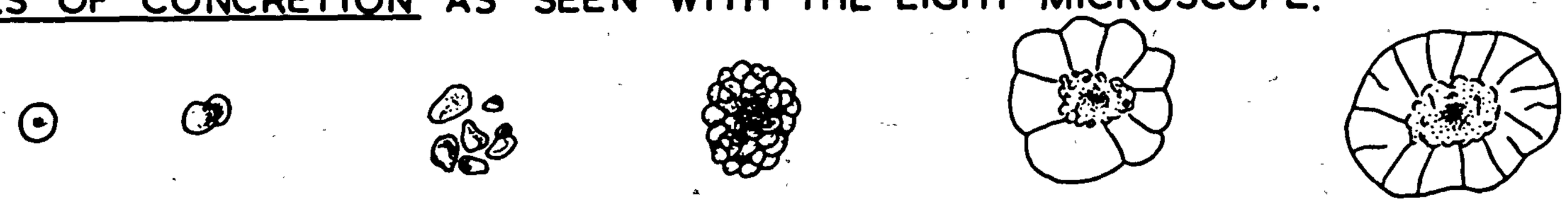
SAMPLE NUMBERS ARRANGED IN ASCENDING ORDER ACCORDING TO THE AVERAGE SIZE OF THEIR GRANULES.



N.B.

SAMPLE NUMBERS 2 AND 28 MISSING

SERIES OF TYPES OF CONCRETION AS SEEN WITH THE LIGHT MICROSCOPE.



DRAWN FROM

SAMPLE NUMBERS:	63	63	44	6	55	46

Results

The graph of concretion size versus time of sacrifice shows that there was only a slight tendency for the rhythm of accumulation of excretory material in the kidneys of the slugs to be synchronised with each other: only ten out of the sixty-five points fall above 8μ but nine of these do occur over the nine hours between 9.30a.m. and 6.30 p.m.

The maximum average concretion diameter was 10.4μ. This compares favourably with the average size of concretion (10.7μ) found in the kidneys of slugs which had recently voided excreta, (see section 2 page 28). In the present investigation however, concretions greater than 13μ in diameter were not found, whereas previously, (section 2), concretions up to 16.5μ diameter were observed in the kidney.

The minimum average diameter of concretions in a kidney was 4.6μ and individual concretions less than 1μ in diameter were observed.

There may, to some extent, be a continual release of larger concretions from the kidney into the primary ureter. However, the sections showed fairly clearly that most concretions tend to reach a maximum size at roughly the same time in any one kidney, and that at any specific time most concretions are of similar sizes.

Different kidneys varied in appearance. Some of the kidneys contained few concretions, e.g. sample no.7, whilst others contained very large numbers e.g. sample no.46. Different stages in development of the concretions can also be seen in the sections. Kidneys with very high average concretion diameters e.g. sample no.'s 46, 3, contain almost

entirely large single concretions. About ¾ of the nephrocytes in kidneys "lower down" the series (Fig.37), e.g. sample no.'s 55, 59, contain single, large concretions and ¼ contain groups of many, small, closely packed concretions. Concretions can also be seen in these samples whose outer contours are irregularly scalloped, unlike the smooth outline of the larger concretions. Such concretions become more common 'further down' the series; e.g. sample no.'s 15, 53, contain roughly equal numbers of the three types of concretion so far mentioned. In the middle of the series, groups of small concretions, rather than single, isolated ones, become more frequent e.g. sample no.'s 11, 16, 36 contain mostly groups of concretions. Right at the bottom of the series, the groupings of concretions are smaller in diameter e.g. sample no.'s 7, 44, and the concretions in each group are fewer and more widely spaced out. Single, small, isolated concretions become commoner lower down the series and in e.g. sample no.'s 22, 63, they make up a large portion of the sample.

Electron Microscope Work.

Materials and Methods

Slugs were collected, kept in plastic pots and maintained on carrot. The slugs were observed from time to time and any which were voiding excreta were isolated from the others and kept in separate pots. Thesd slugs were sacrificed at known time intervals after they had excreted and part of the kidney of each slug was dissected out for electron microscope study. Thin sections were prepared for the electron microscope in the same way as described in section one. The times of sacrifice (after voiding excreta) were 0, 3½,

7, 10, 13, 17, 24 and 28 hours. The "28 hour" slug was starved from the time that it excreted to the time that it was sacrificed. Photographs of the sections were examined and any differences were noted.

Results.

Sections from the first slug (sacrificed immediately after excretion) showed mainly nephrocytes with small apical vacuoles, (less than 2 μ diameter), (Figs. 38a, 38b and 39a). Sections from the next four slugs ($3\frac{1}{2}$-13 hours) all showed kidneys with the majority of nephrocytes having at least one large apical vacuole, (up to 13 μ diameter), and often quite large numbers of smaller vacuoles, (about 1 μ or less in diameter), associated with it (Figs. 39a, 40b, 41a and 41b). Nephrocytes from the later sacrificed slugs, (17 and 24 hours) contain far fewer small vacuoles, and the single large apical vacuole in each nephrocyte reached a maximum length of about 25 μ: large vacuoles tended to be ovoid rather than spherical in shape (Figs. 42a, 42b, 43a, 43b and 44a). Sections of the "28 hour" kidney showed some nephrocytes with contents similar to those of the 0-hour kidney, and some with contents similar to those of the $3\frac{1}{2}$ hour kidney (Fig.44b). Quite large amounts of cell debris could also be seen in the lumen of the 28 hour kidney (Fig.45). Such debris included nuclei, degenerating mitochondria, residual bodies, membranes and microsomes.

Differences other than those of number and size of vacuole were also observed between sections of different kidneys:... Concretions were only seen in single large vacuoles and thus were most frequently found in sections of the 17 and 24 hour

FIGURE 38.

E.M. Nephrocytes at different stages of the twenty-four hour cycle.

a) 0 hours. KL : Kidney Lumen

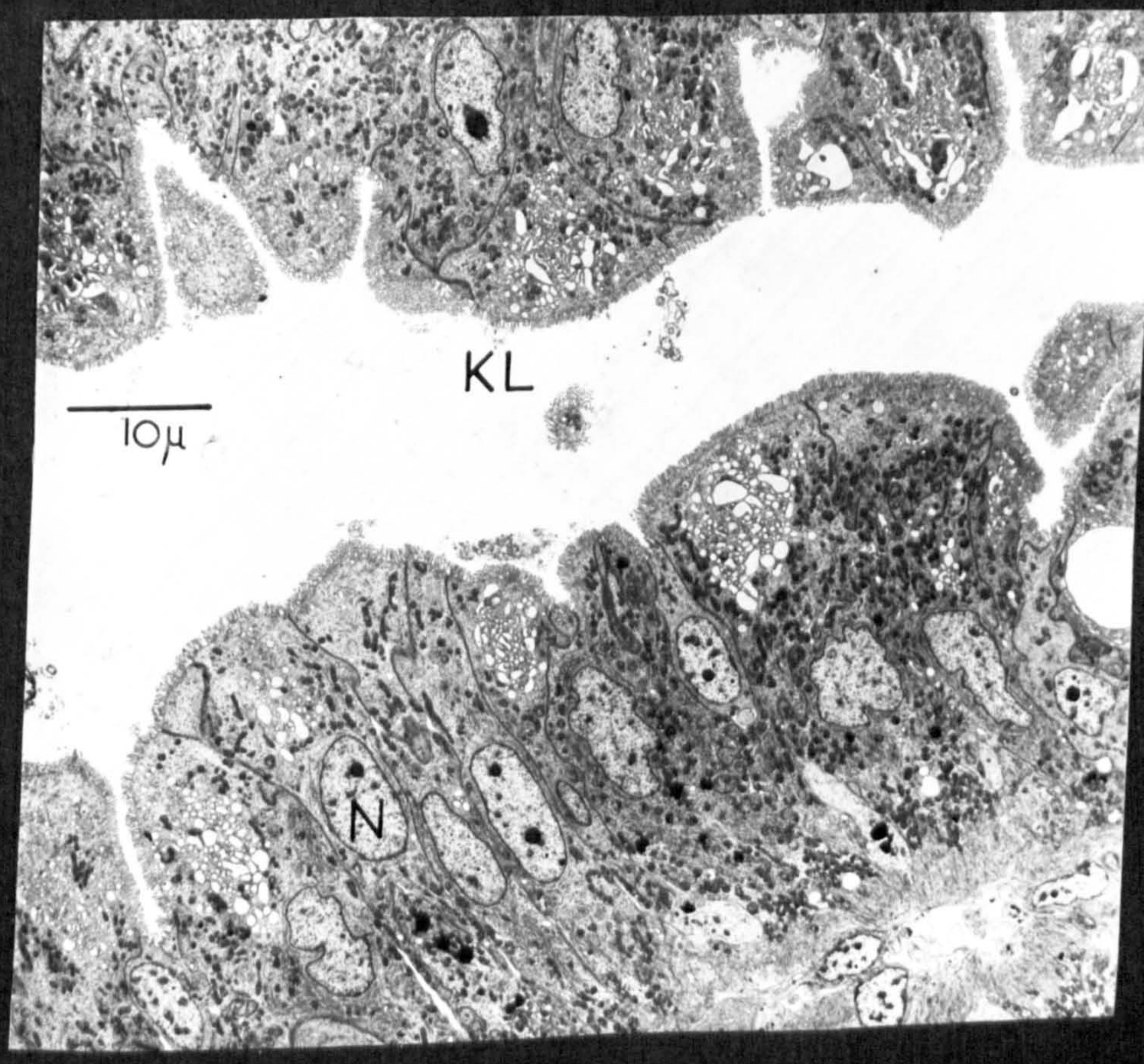
N : Nucleus

b) 0 hours. AV : Apical vacuoles

KL : Kidney lumen

N : Nucleus

RER : Rough endoplasmic reticulum


KL
10μ
N

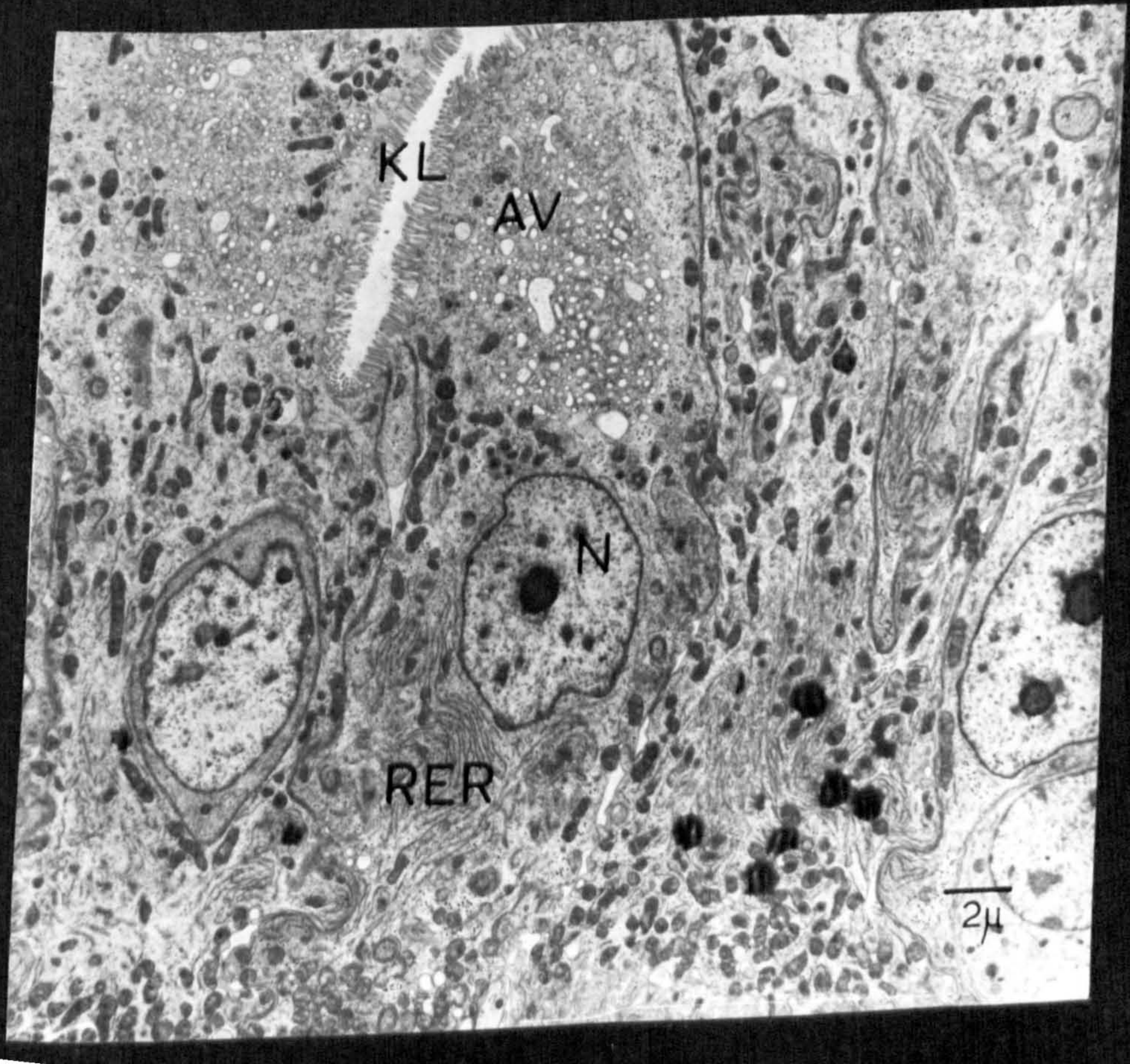

KL
AV
N
RER
2μ

FIGURE 39

a) 0 hours

AV : Apical vacuoles

KL : Kidney lumen

b) 3½ hours

KL : Kidney lumen

VF : Vacuoles fusing

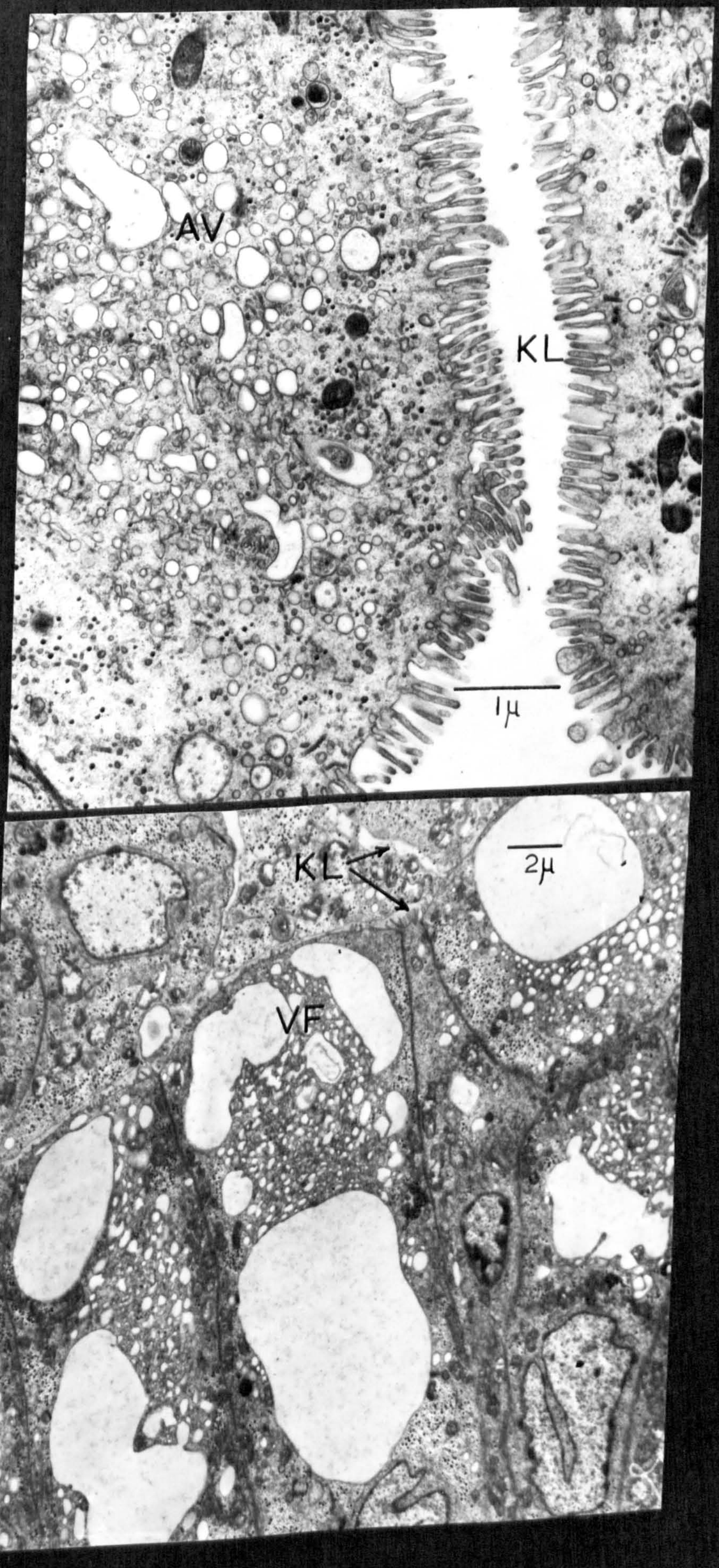

AV
KL
1μ
KL
2μ
VF

FIGURE 40

a) 7 hours

C : Concretion

KL : Kidney lumen

N : Nucleus

b) 10 hours

KL : Kidney lumen

KL

C

N

2μ

KL

KL

KL

2μ

FIGURE 41.

a) 13½ hours

KL : Kidney lumen

V : Vacuole

b) 13½ hours

RB : Residual body?

V : Vacuole

KL
V
2μ
1μ
V
RB

FIGURE 42.

a) 17 hours.

C : Concretions

KL : Kidney lumen

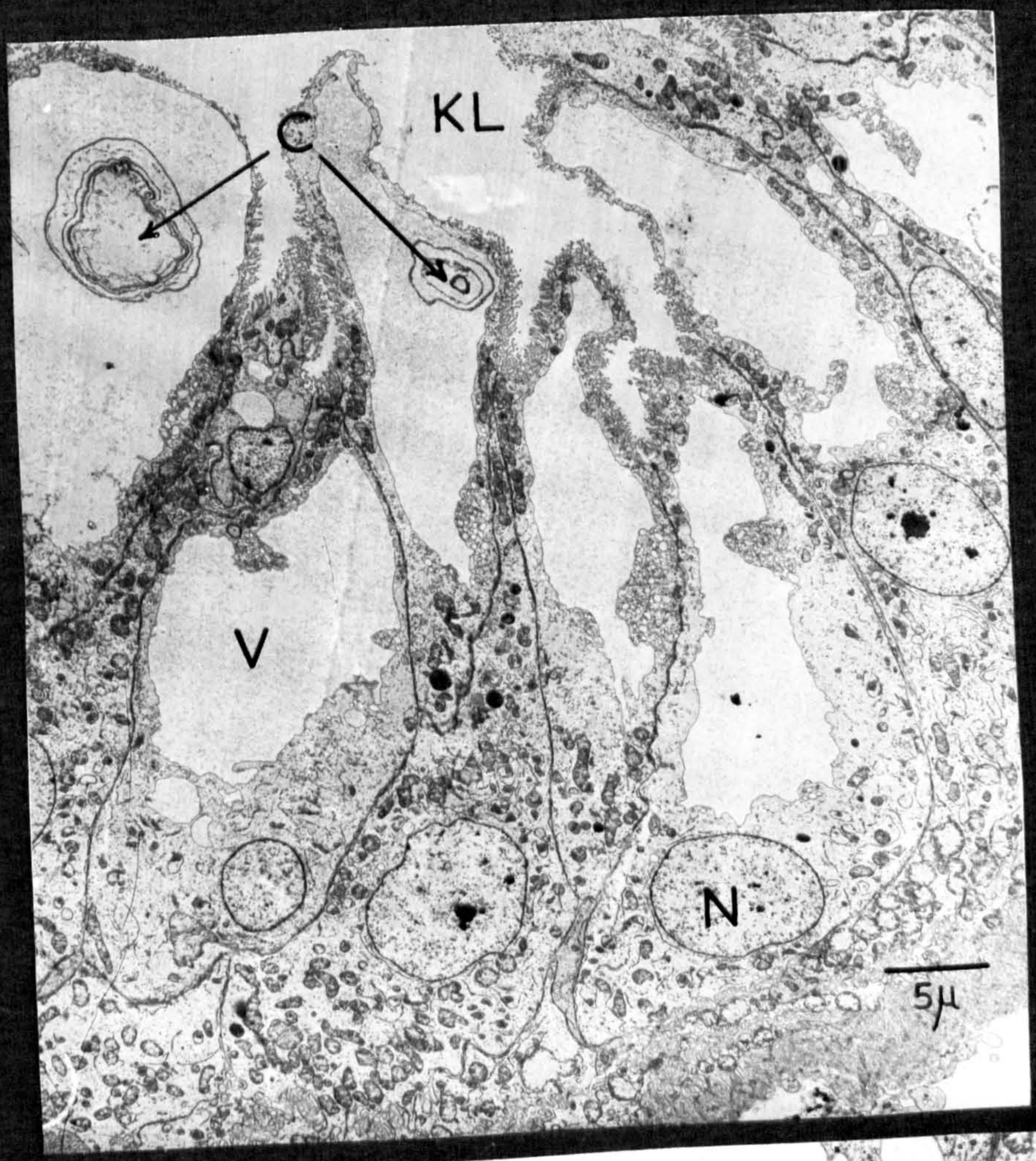
N : Nucleus

V : Vacuole

b) 17 hours.

N : Nucleus

V : Vacuole


KL
C
D
V
N
5μ

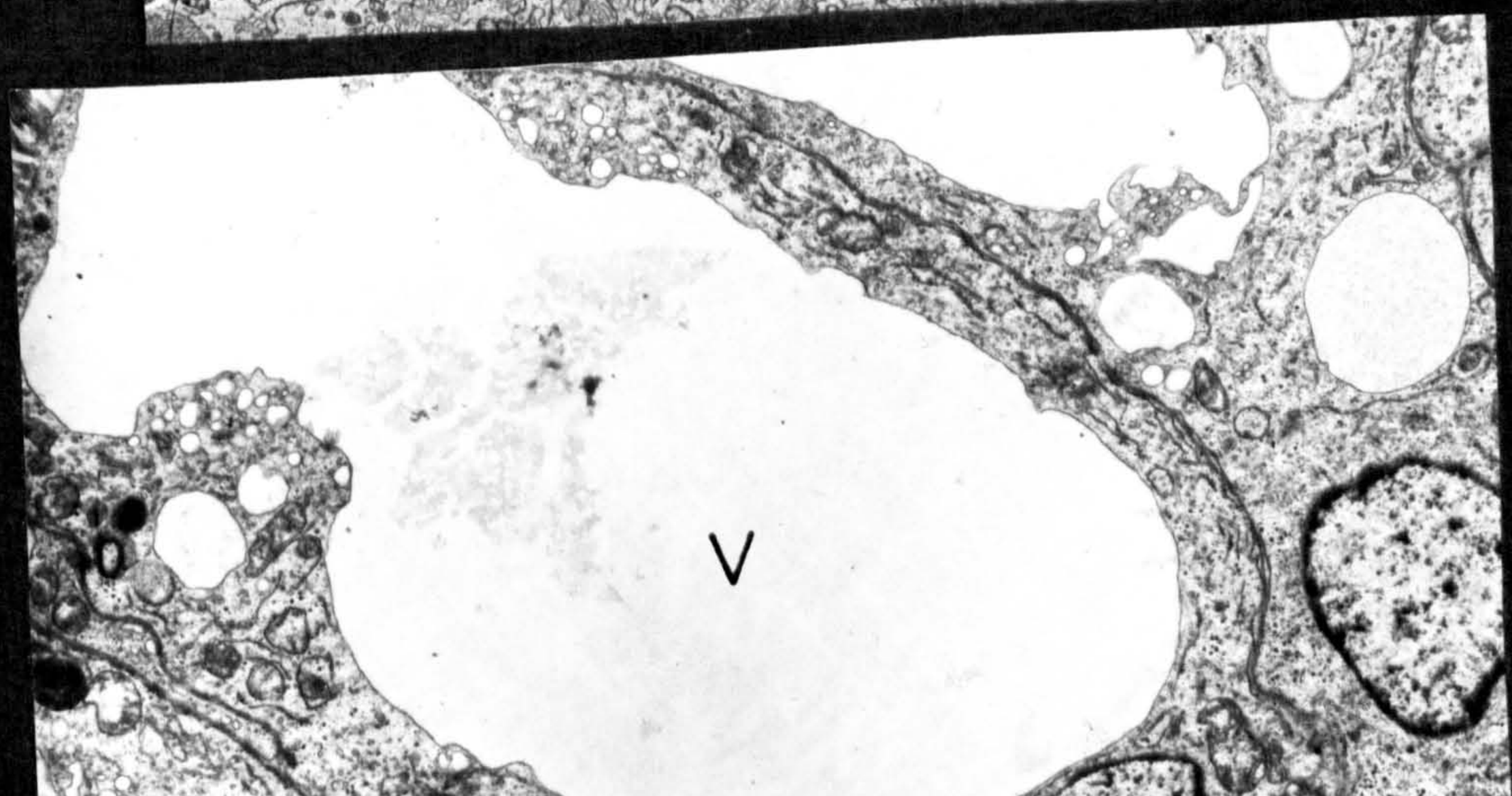

V
N
2μ

FIGURE 43

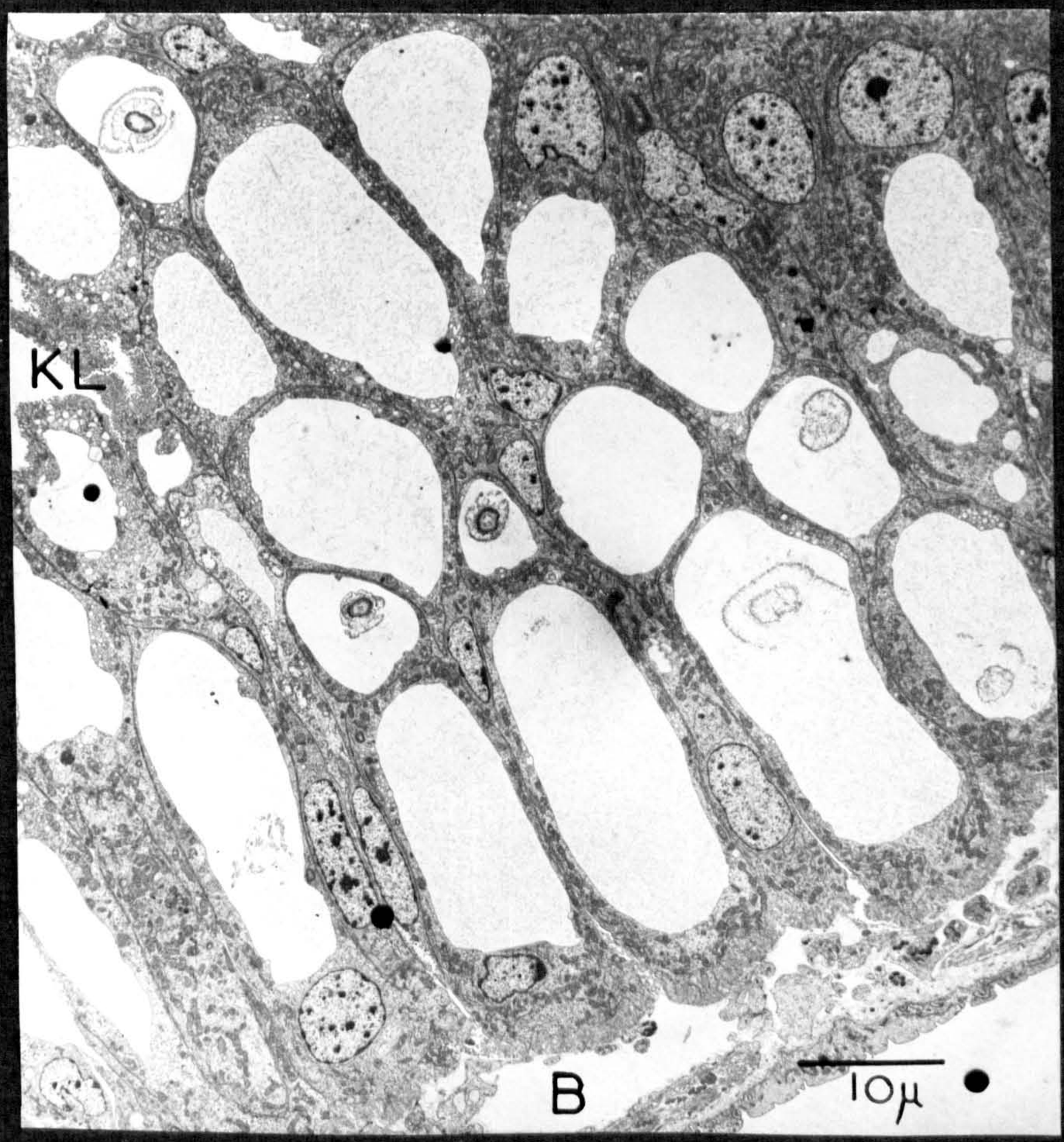
a) 24 hours.

B : Blood space

KL : Kidney lumen

b) 24 hours.

C : Concretion


KL
B
10μ

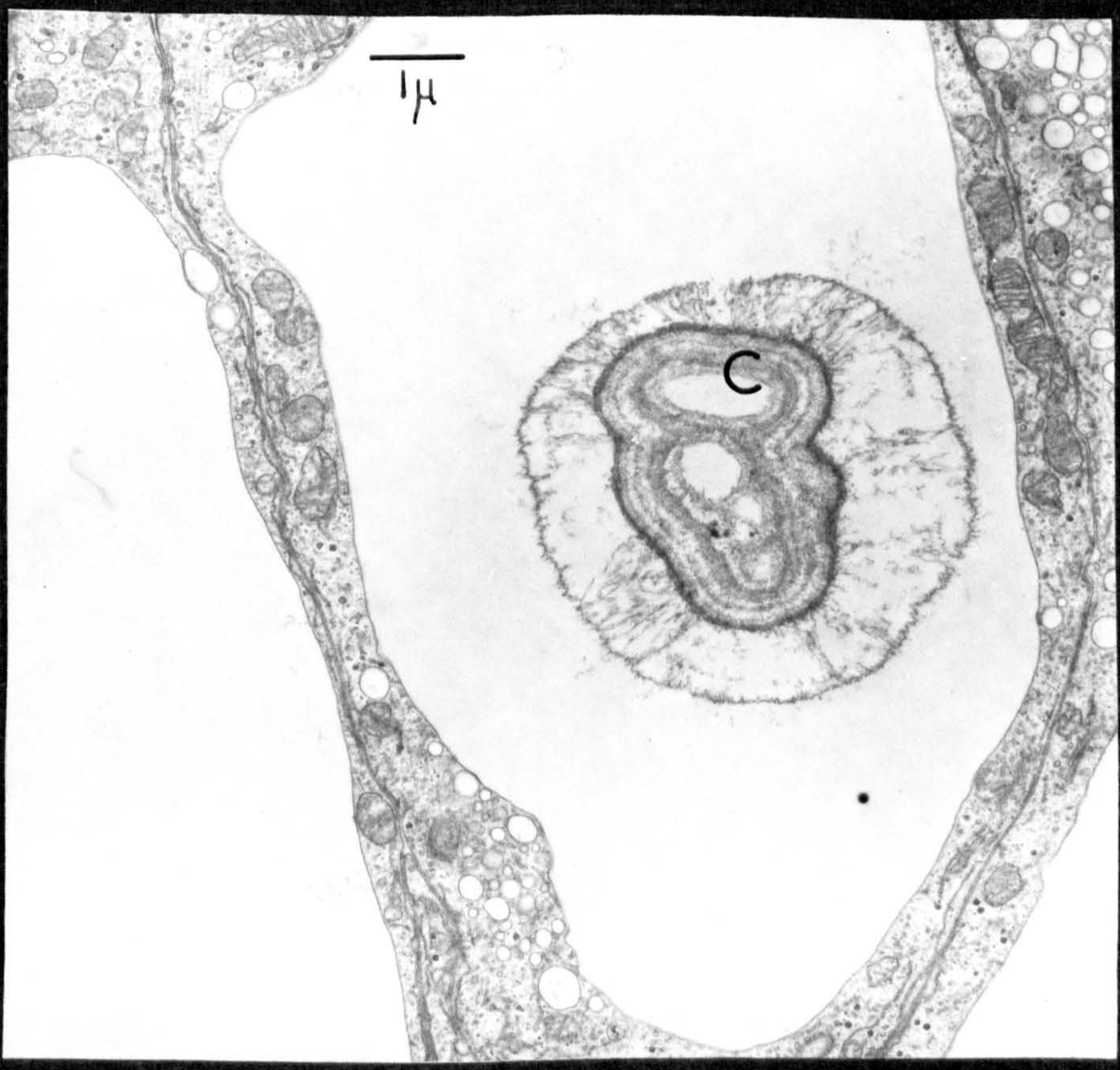

1μ
C

FIGURE 44

a) 24 hours.

AV : Apical vacuoles

G : Glycogen

LP : Lateral plasmalemma

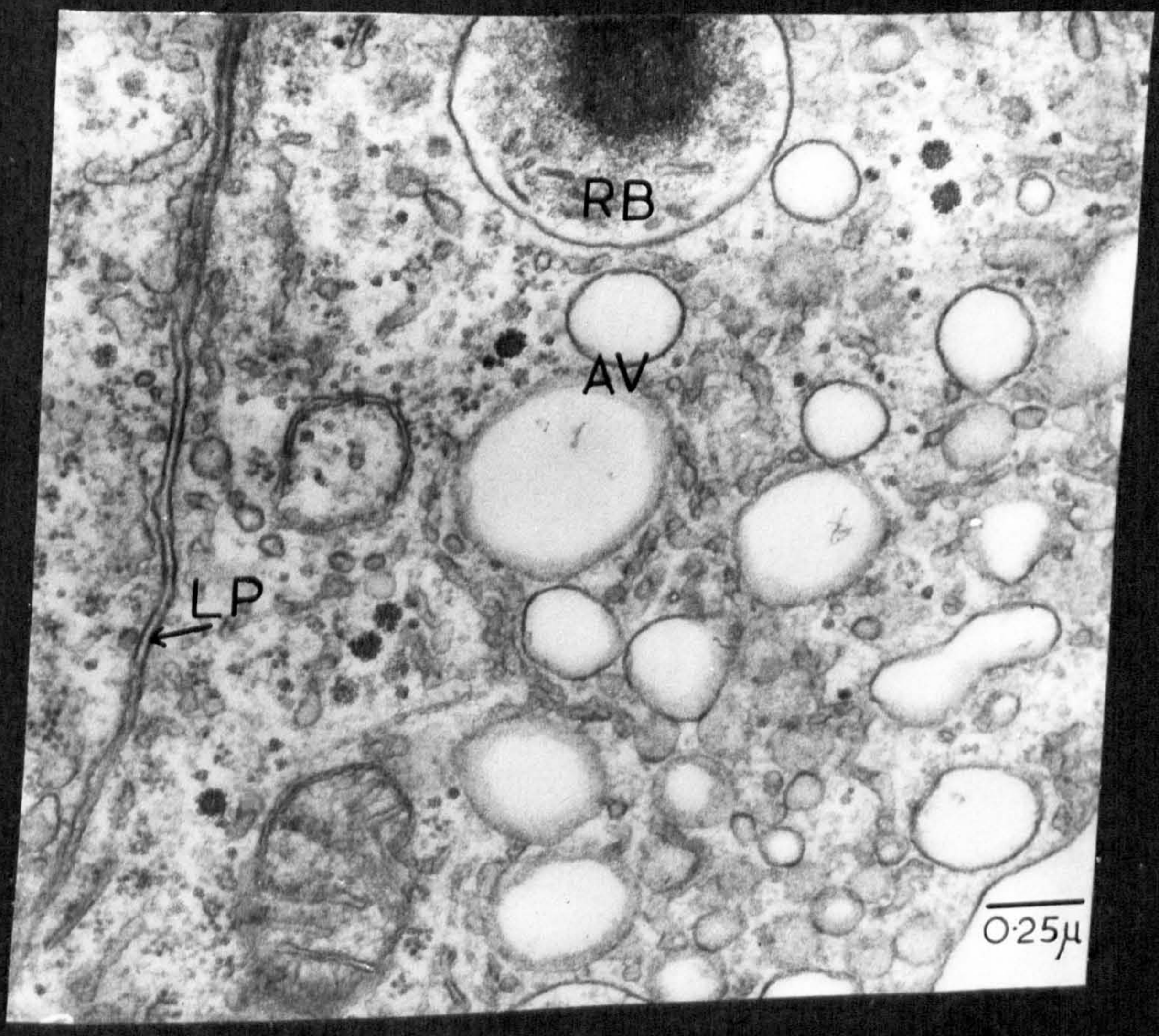
RB : Residual body

b) 28 hours.

KL : Kidney lumen

N : Nucleus

V : Vacuole


RB
AV
LP
0·25μ

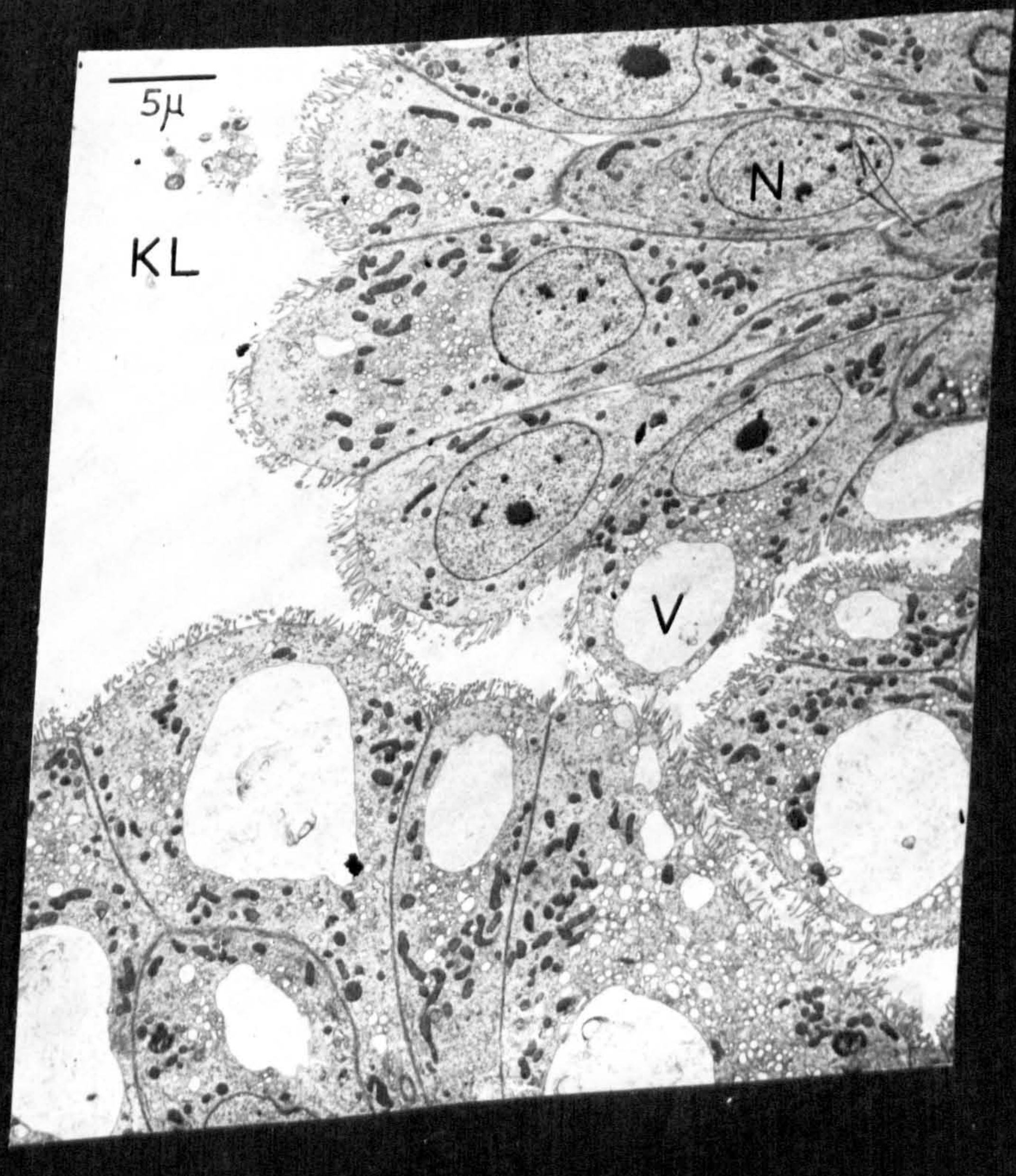

5μ
KL
N
V

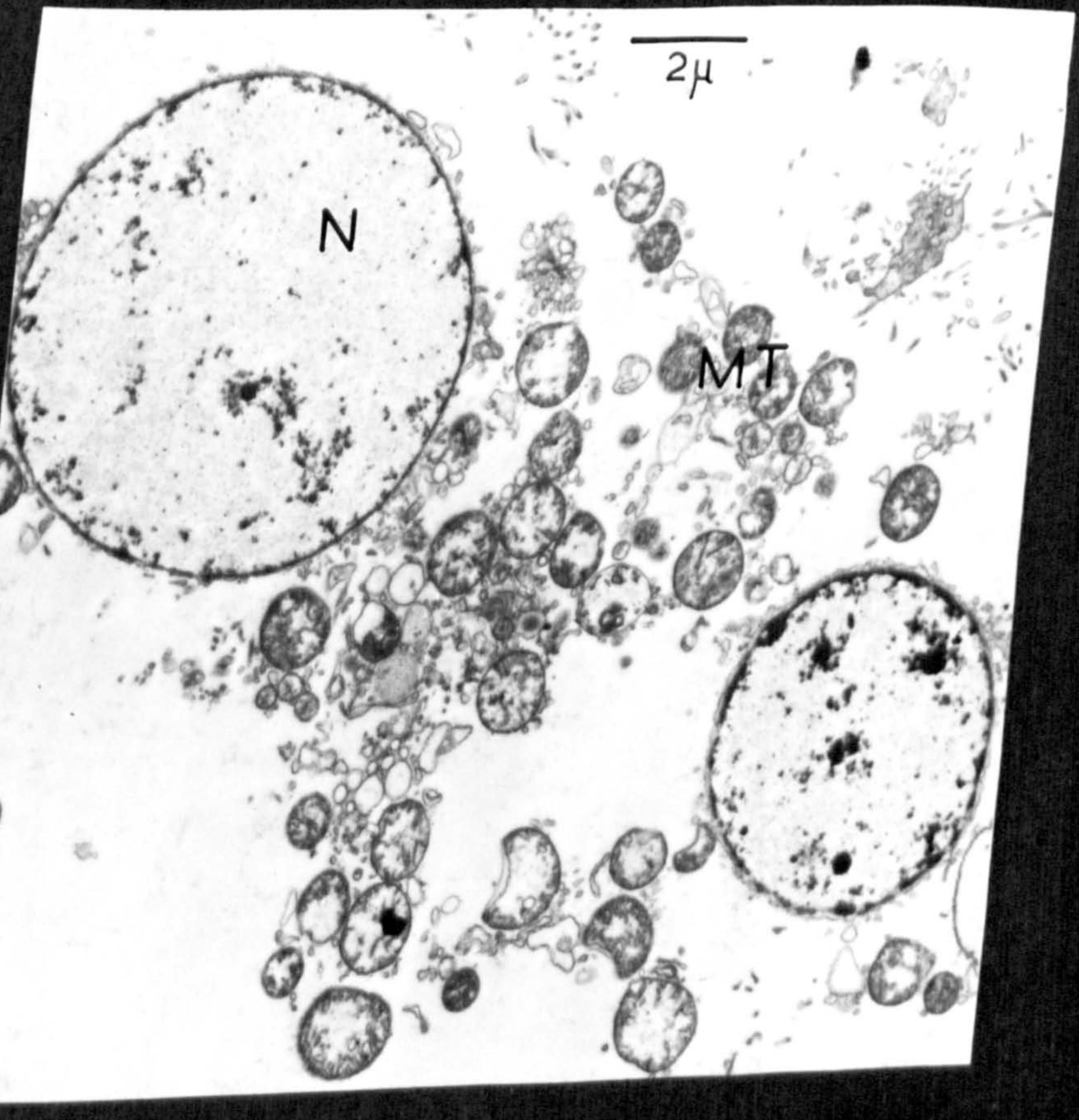
FIGURE 45

28 hours.

Cell debris (kidney lumen)

N : Nucleus

MT : Mitochondria


2μ
N
MT

kidneys. The concretions were most fully formed in the vacuoles of the 24 hour nephrocytes (Fig.43b), but cells with vacuoles in which the space was completely taken up by a concretion were not observed in any of the sections. However, such cells had been previously observed (see section 1, page 16). Transitional stages in the formation of concretions were found throughout the series of sections. It is fairly clear that the small apical vacuoles gradually fuse together to form a single large vacuole which may take up two thirds of the cell volume. Since this fusion may proceed from more than one centre within a cell, then more than one large vacuole can be formed. These large vacuoles eventually fuse into a single one within each cell. The homogeneous contents of the large vacuole have a very low density, but small, amorphous aggregations of material of a slightly higher electron density can be seen within the vacuole. The vacuole contents then become surrounded by a diffuse, irregularly shaped border of dense material about 4,000Å, or less, in width. After this, it appears that concentric layers of electron dense material are built up in succession from the outside inwards, each alternating with a layer of low electron density, until eventually a single large concretion is formed. The final concretions appear very similar in physical structure to those in the kidney of Helix pomatia (Bouillon, 1960). Under the light microscope, the concretions also appear to be similar in structure to those described by Lambertenghi in 1908 (after Gostan 1965).

Glycogen was present in all the cells but appeared to be most abundant in those cells where fusion of the small apical vacuoles was taking place, and least abundant in cells where this process was just beginning or ending.

Rough endoplasmic reticulum was quite discrete in cells showing the early stages of vacuole fusion, whilst in cells containing more "mature" vacuoles, the membranes of the reticulum were much more dispersed in the cytoplasm; the individual lengths of membrane were shorter and were not so frequently arranged parallel to each other in groups, as they were in cells with immature vacuoles. What appeared to be lysosomes were present in cells with completely immature vacuoles, and lysosome-like residual bodies were quite frequently found in cells with fully mature vacuoles. Irregularly shaped mitochondria with poorly defined cristae were also more common in cells with fully mature vacuoles.

Conclusions

Evidence from both light and electron microscopy suggests that over the 24 hour period between each evacuation of excreta in A. reticulatus, a gradual maturation of vacuoles occurs within the nephrocytes. Small vacuoles accumulate in each cell and these gradually coalesce to form a single large apical vacuole. The contents of the vacuole crystallise to form a concretion which is then released into the kidney lumen.

It appears, from the electron microscopy results, that crystallisation of a concretion does not begin until a single large apical vacuole has been formed within the cell. The

fact that all the immature vacuoles appeared as actual concretions in the light microscope sections is very likely to have been caused by the method of preparation of the sections: Immersion of the dissected kidney tissue into liquid air undoubtedly caused the immediate crystallisation of the vacuole contents; these crystals, which were presumably of high purine content, withstood any tendency to redissolve in the later stages of section preparation (the solubility of uric acid in alcohol and water is negligible). As inferred in the results, it was evident from the distribution of the concretions seen under the light microscope that there had been little, if any, disturbance of their positions, and it is therefore unlikely that there was any dissolution and recrystallisation of the concretions after their initial crystallisation in liquid air. It is evident that some condition exists within the nephrocytes which prevents the contents of immature vacuoles from crystallising until vacuole fusion is more or less complete. It is possible that a change in pH would be sufficient to effect crystallisation of vacuole contents, since uric acid is soluble in alkalis and insoluble in acids, However, it is not known whether this is the actual cause of crystallisation in the nephrocytes. Whatever the natural cause, it must take place in individual nephrocytes, rather than the whole kidney simultaneously, since a nephrocyte with a vacuole containing a crystal can often be seen adjacent to nephrocytes with "empty" vacuoles. Nevertheless it is true that within any one kidney most of the nephrocytes are at a similar stage of vacuole maturity. It is probable that the stimulus for crystallisation to occur

comes from the nephrocyte cytoplasm rather than the vacuole centre, since crystallisation seems to occur from the outside inwards.

It appears that vacuole changes during their maturation are concurrent with intracellular changes. The activity of the nephrocytes seems to reach a peak during the middle stages of vacuole maturation. Activity then decreases as crystallisation of the concretion commences.

Although cell debris can be found in the kidney lumen at the end of the maturation cycle, it would seem unlikely that whole nephrocytes always break down completely to release concretions into the kidney lumen. Cells in a state of mitosis were not seen in any of the sections. It seems probable that a concretion and part of the cell in which it was formed are cut off into the kidney lumen and that the whole cycle starts again in the remaining part of the cell.

The work with the light microscope showed that there was only a slight tendency for the diurnal excretory rhythms of slugs from the same habitat to be synchronous, and that the maximum average sizes of concretions occurred during the day, (9.30 a.m. to 6.30 p.m.).

Section 10.

Assessment of Cell Turnover in the Kidney of A.reticulatus.

Introduction

Tritiated thymidine has been used extensively to study movement and replacement of cells and interchanges between types in a cell population (e.g. Quastler and Sherman, 1959; Merzel and Leblond, 1969).

It has been shown that there is a very high turnover of excretory concretions by the kidney of A.reticulatus (see section 8, page 60). It has also been postulated that "mature" concretions are released from the nephrocytes that contain them, thus allowing the nephrocytes to restart their cycle of purine collection. However, it was not known to what extent nephrocytes released the concretions they contained or to what extent whole nephrocytes were cut off from the kidney epithelium along with their concretions to be replaced by new nephrocytes which would carry on the cycle.

Within the confines of the kidney, the only cells present are the nephrocytes. Hence the system only contains one component i.e. functional nephrocytes. If cell death occurs, and the system is to remain stable, then new nephrocytes must be derived from pre-existing ones.

In this investigation, tritiated thymidine and autoradiography were used to gain some idea of the rate of nephrocyte renewal.

Materials and Methods

Animals injected with tritiated thymidine were sacrificed at time intervals of 2,4,6,8,13 and 20 days after

injection, and wax sections of their kidney regions were prepared for autoradiography. The technique was similar to that used by Walker (1970).

Results

For the most part only nuclei were labelled. There appeared to be a fairly even distribution of labelled nuclei over the whole nephrocyte epithelium.

On average about 480 nephrocytes were counted from each animal and the percentages of labelled nuclei were noted. The numbers of labelled nuclei which were immediately adjacent to other labelled nuclei were also noted.

No. of days sacrificed after injection ...	2	4	6	8	13	20
% of total nuclei which were labelled ...	7.5	25.2	10.0	12.1	5.3	5.2
% of total nuclei which were labelled and in adjacent cells ...	2.6	8.4	4.1	2.2	0.8	1.2

During the greater part of the excretory cycle most of the nephrocytes are in a "functional" state, i.e. they are in some state of purine accumulation. Both in this investigation and during previous ones, no mitotic figures were observed. There appeared to be little appreciable falling off with the passage of time, of the amount of label per nucleus.

Conclusions

Since the orginal labels appeared to be more or less random throughout the kidney, it would seem that there is no fixed progenitor group of the nephrocytes e.g. at the bases or at the tips of the folds of the kidney epithelium, and thus any

nephrocyte could theoretically act as a progenitor.

Probably some of the labelled cells are adjacent merely by chance. However, even though mitoses were not observed, it is likely that some of these cells are labelled, and adjacent, because they are both daughter cells of the same labelled parent cell. Because - 1) such pairs of cells were relatively few, 2) mitoses were not observed and 3) the amount of labelling per cell did not appear to decrease much with time; cell turnover must be relatively low, and is certainly not of the same order of magnitude as the turnover of excretory concretions.

That the label was taken up at all, shows that at least the D.N.A. in the nephrocyte nuclei undergoes a duplication phase, albeit a rather slow one (none of the nuclei were ever <u>heavily</u> labelled), presumably in preparation for a mitosis.

Perhaps most, if not all, mitoses take place at a particular time in the excretory cycle. Certainly the most logical time for each nephrocyte to divide would be immediately after discharging its vacuole contents.

Section 11.

The Fate of "Thorotrast" Injected into the Pericardial Cavity and Kidney of A. reticulatus.

Introduction

It has been shown (sections 8 and 9) that a diurnal cycle of vacuole maturation takes place in the nephrocytes of A.reticulatus. Excretory products are presumably taken to the kidney via the blood, but the actual source of the liquid content of the vacuoles is somewhat obscure. The two obvious possibilities are firstly, the blood sinuses around the kidney and secondly the kidney lumen. The kidney lumen is confluct with the pericardial cavity via the reno-pericardial canal and it is thus possible for there to be some movement of liquid between the two cavities. Certainly, the renopericardial canal is very heavily ciliated; it would be of great interest to know in which direction these cilia normally beat.

Vorwohl (1961) states that in Helix pomatia the liquid in the kidney is most probably derived from the haemolymph by a filtration process across the kidney epithelium and he shows that the depression of freezing point for the haemolymph and kidney liquid are similar. He rules out the pericardial cavity as a source of kidney fluid, mainly on theoretical grounds. He maintains that the dimensions of the renopericardial canal in H.pomatia would probably not allow the rate of flow of liquid into the kidney lumen that he found to be possible. He determined this rate of flow by inserting a canula into the kidney and draining off the kidney fluid. Although relatively large volumes of kidney

fluid can be drained off using a canula, it does not however follow that equally large volumes of fluid pass continually through the kidney under natural conditions. Also, at times when plenty of water is available, H. pomatia produces a watery urine in addition to solid concretions, whilst A. reticulatus has never been observed to produce such a urine. Thus it is not necessarily true that the pericardial cavity cannot be the source of kidney fluid in A. reticulatus. However, neither can a filtration process across the kidney epithelium from the blood sinuses be ruled out.

Whatever the source of the liquid in the kidney, it is not certain whether this liquid is taken into the nephrocytes to form the vacuoles already described, or whether the vacuole contents come directly from the blood sinuses across the basement membrane and basal plasmalemmas of the nephrocyte

The purpose of this short experiment was to see if it was possible for pericardial fluid to pass into the kidney, and for liquid in the kidney lumen to be taken into the nephrocytes.

Materials and Methods

"Thorotrast" (a colloidal suspension of thorium dioxide) is x-ray opaque, emits β-radiation and has a small particle size suitable for tracing the movements of liquids at a cellular level. The particles can be readily identified with the electron microscope. The electron microscope was used to locate the Thorotrast in the tissues in this experiment

Two experimental animals were used. They were anaesthetised with solid carbon dioxide, the mantle tissue

was dissected away, and the shell was removed from each animal. Using a very fine Pasteur pipette, the shell gland was penetrated and Thorotrast was injected into the pericardial cavity of the first slug. In the second slug, Thorotrast was injected directly into the kidney lumen. In each case the amount of Thorotrast used was in the region of one microlitre. The shells of the slugs were replaced to prevent drying and the slugs were allowed to recover. They were then sacrificed $2\frac{3}{4}$ and $1\frac{3}{4}$ hours after injection respectively. A part of the kidney and primary ureter of each slug was dissected out and sections were prepared for the electron microscope (for methods, see section 1.).

Results.

As the electron micrographs show, Thorotrast was found in the cells of the primary ureter and kidney of both the injected animals.

Thorotrast was not seen in the lumen of either of the kidneys, but only in the nephrocyte vacuoles (Figs. 46a,46b and 47), and even then, very little of the injected Thorotrast could be found. In contrast to this, Thorotrast was present in relatively much larger amounts in the lumen of the primary ureter (Fig.48a) but had only been taken up by a few of the ciliated cells. In these cells the Thorotrast was usually located within the residual bodies (Figs. 48b, 49a and 49b).

Conclusions

Thorotrast did pass from the pericardial cavity into the kidney and primary ureter of one of the injected slugs. This is obviously insufficient evidence to state that movement of liquid in this direction is a normal occurrence.

FIGURE 46.

E.M. Thorotrast associated with a nephrocyte of an animal injected via the kidney.

a) M : Microvilli

MT : Mitochondrion

TH : Thorotrast.

b) G : Glycogen

JC : Junctional complex

KL : Kidney lumen

MT : Mitochondria

TH : Thorotrast

TH

M

MT

TH

0·25μ

G

KL

TH

MT

JC

0·5μ

FIGURE 47

E.M. Thorotrast in a nephrocyte vacuole of an animal injected via the pericardial cavity.

TH : Thorotrast

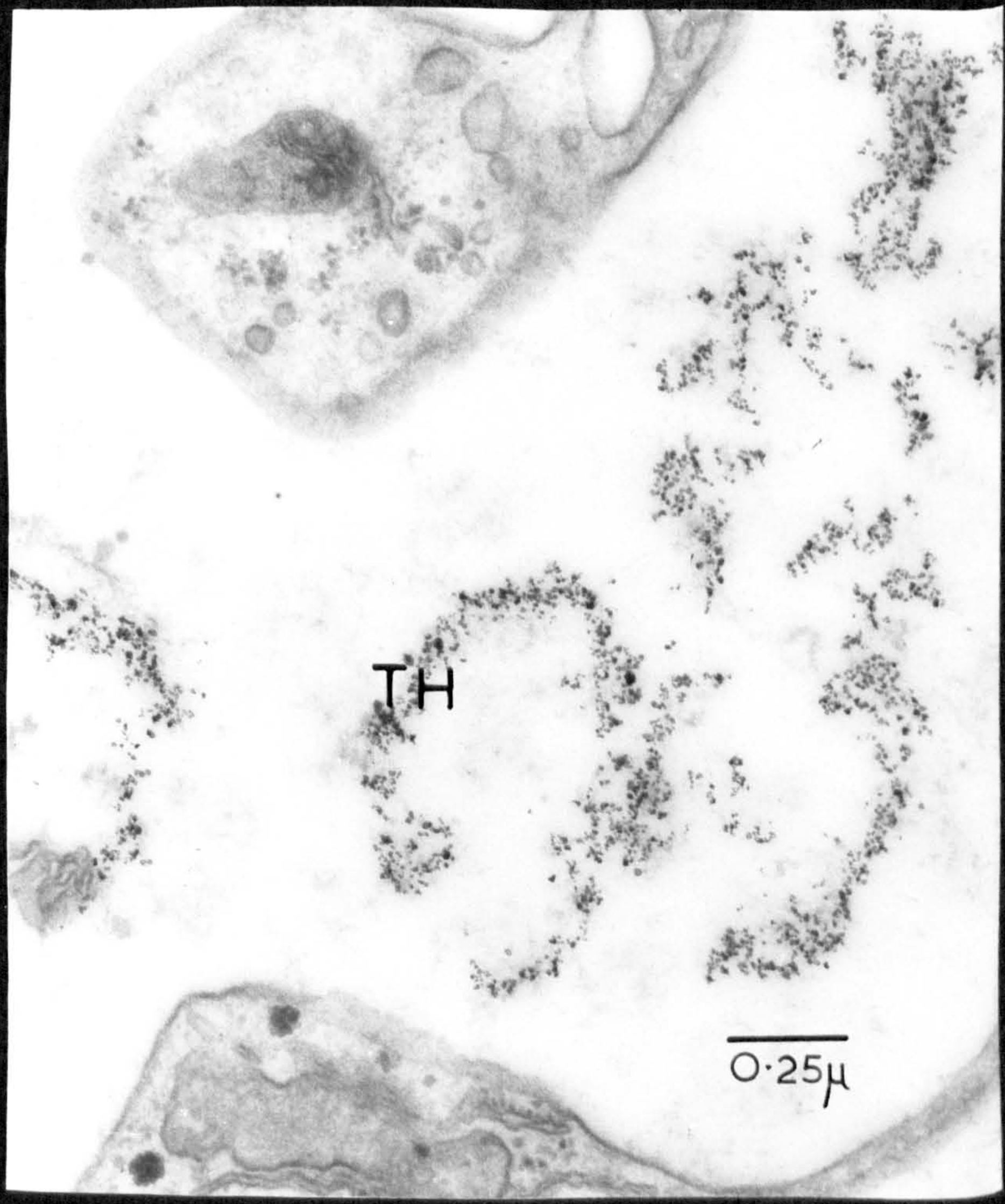

TH
0·25μ

FIGURE 48

E.M. Thorotrast in the primary ureter of an animal injected via the kidney.

a) Debris in the lumen

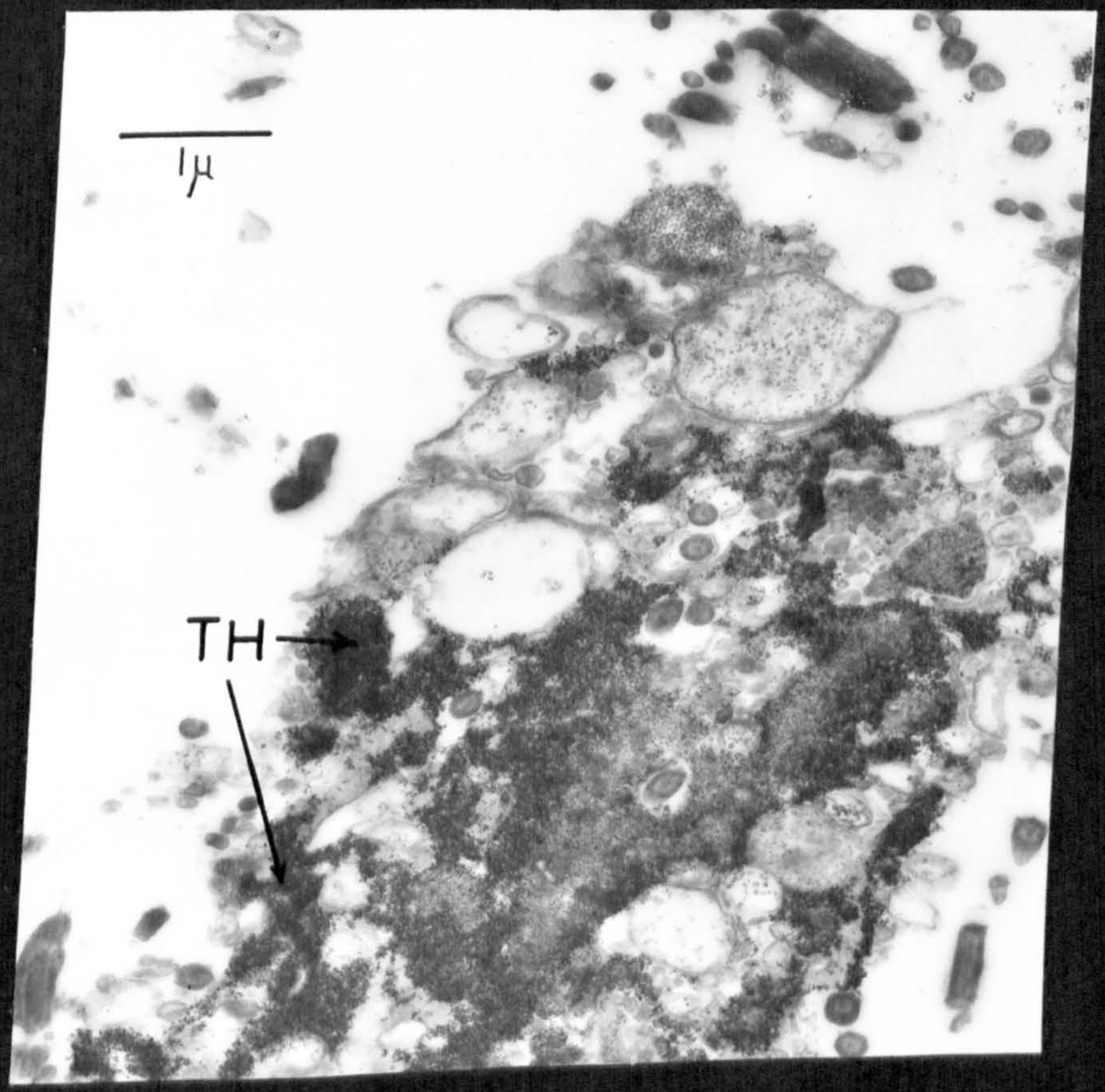
TH : Thorotrast

b) Ciliated cell.

M : Microvilli

PUL : Primary ureter lumen

TH : Thorotrast


1μ
TH

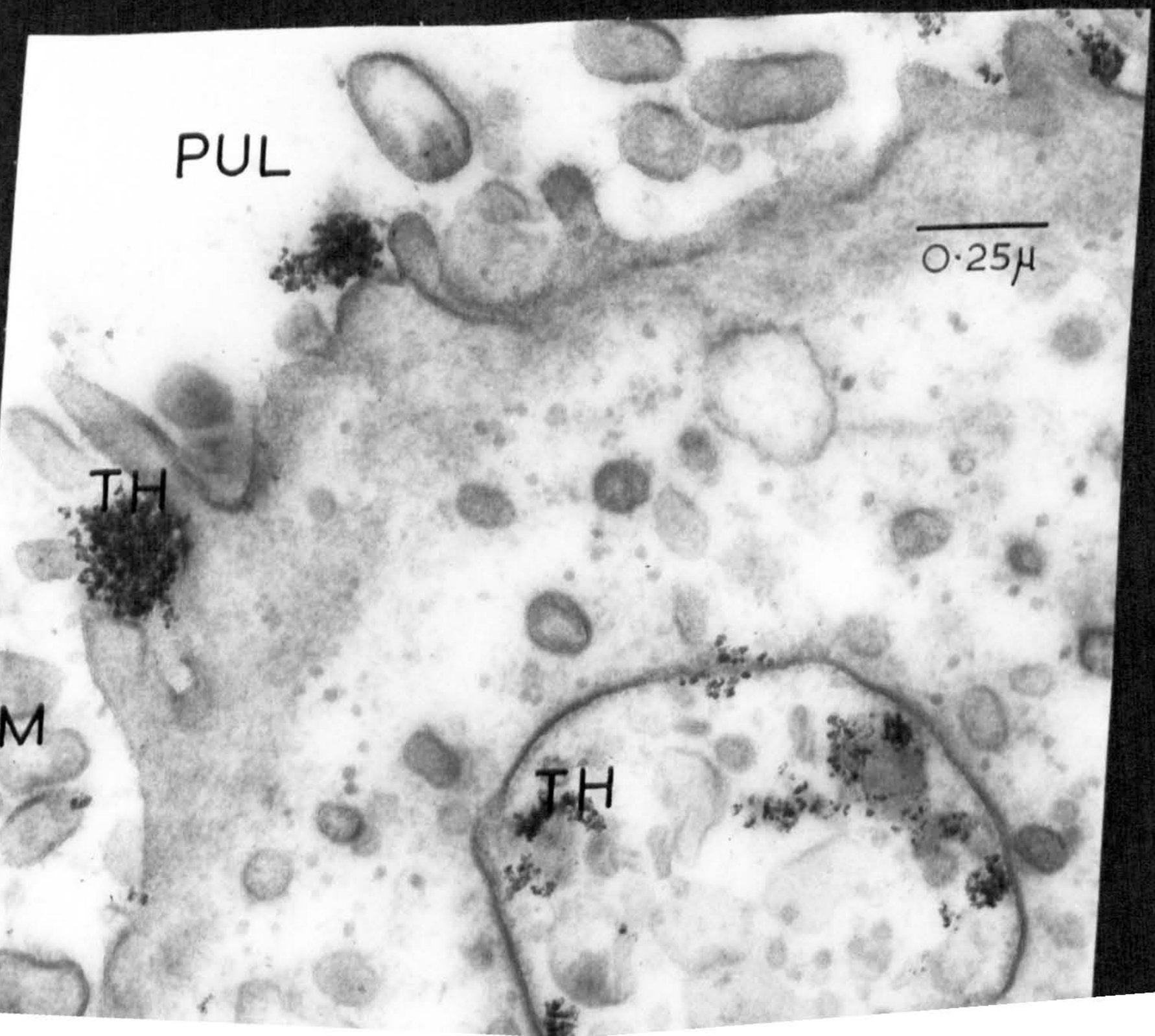

PUL
0·25μ
TH
M
TH

FIGURE 49

E.M. Thorotrast associated with ciliated cells in the wall of the primary ureter of an animal injected via the pericardial cavity

a) C : Cilium

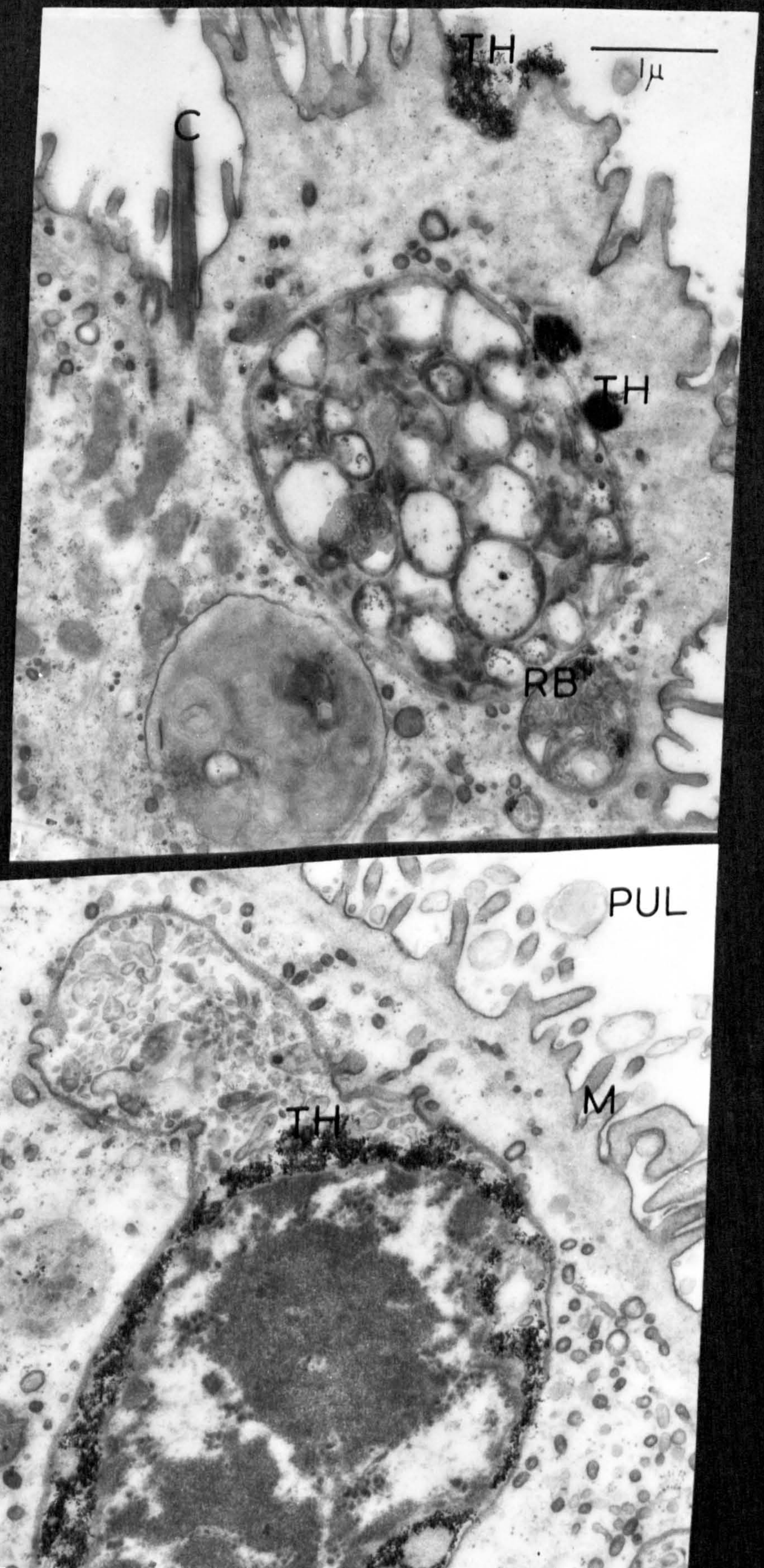
RB : Residual bodies

TH : Thorotrast

b) M : Microvilli

PUL: Primary ureter lumen

TH : Thorotrast


TH
1μ
C
TH
RB
PUL
0·5μ
TH
M

However, it does demonstrate that such a movement is possible.

Thorotrast was found in the vacuoles of intact nephrocytes. This indicates that at least part of the liquid content of the nephrocyte vacuoles comes from the kidney lumen.

Since relttively large amounts of Thorotrast were found in the primary ureter, it is apparent that movement of material from the kidney to the primary ureter is fairly rapid; less than three hours passed between the injection of Thorotrast into the pericardial cavity and its appearance in the primary ureter.

More work with Thorotrast along these lines would undoubtedly produce fruitful results.

General Conclusions

Although there are many points of similarity between excretion in Helix pomatia and in Agriolimax reticulatus, some differences have emerged. Excretion in both pulmonates is purinotelic but H. pomatia produces uric acid, xanthine and guanine as excretory products (Jezewska 1963), whereas A. reticulatus appears only to produce uric acid and xanthine. On occasion, H.pomatia will produce a watery urine (Vorwhol, 1961), while this has not been observed in A. reticulatus. As in H.aspersa and H.lactea (Speeg and Campbell, 1968), A. reticulatus was found to evolve small amounts of ammonia. Although the amounts evolved in A. reticulatus (approx. 24mg NH_3-N/kg tissue/ 24 hours), were greater than those evolved by H.lactea and aspersa (9.6 and 5.5mg/NH_3-N/kg tissue/24 houts respectively), during aestivation they did not contribute significantly to nitrogen output when compared with amounts of purine-nitrogen eliminated by A.reticulatus.

Vorwhol (1961) could not obtain sufficient pericardial fluid from H.pomatia to determine its depression of freezing point by the method which he employed, but he did measure and compare the depression of freezing points of the haemolymph and of a liquid which he was able to drain from the kidney lumen. He found that the strength of this kidney fluid varied. It was either isotonic or else hypotonic to the haemolymph: The maximum difference he detected betweenthe kidney fluid and the haemolymph was 18% of the value of the depression of freezing point of the haemolymph. In A.reticulatus the pericardial fluid was, on average,

hypotonic to the haemolymph, but measured over a twenty four hour period, it was seen that when the concentration of the haemolymph had dropped to its lowest measured value, the pericardial fluid was slightly hypertonic to it. Certainly it is likely that there are fluctuations in the concentrations of the blood and the pericardial fluids in A. reticulatus (as well as variations between individuals), and it is probable that these fluctuations are diurnal in nature.

Using a rhodium compound to represent osmotically active substances, Vorwhol showed that this tracer could be detected in the kidney fluid of H.pomatia within 5 minutes of its injection into the haemocoel. Using radioactive uric acid as a tracer, it has been shown in A.reticulatus that the passage of this chemical from the blood system to the kidney is equally rapid. In fact, by autoradiography, injected uric acid was localised in the nephrocytes of A.reticulatus within one minute after injection.

With tracer techniques, it has been shown that it is possible for substances to pass from the pericardial cavity to the kidney lumen, and from the kidney lumen to the nephrocyte vacuoles. It was also shown that the pattern of removal of injected uric acid from the blood was reflected in the changes in concentration of radioactive uric acid in the pericardial fluid of the same animals.

The work concerning a diurnal rhythm of accumulation and voiding of excreta in A. reticulatus has perhaps the most far reaching results, since the stage of the excretory cycle which a particular animal is in could profoundly affect the

results obtained in physiological investigations of certain aspects of excretion, (e.g. quantitative measurements of excretory products). However, whether such a rhythm is present in H.pomatia is at the moment a matter for speculation. Considering the size of the kidney in A. reticulatus there is a very rapid turnover of excretory material (almost all purine), though nephrocyte renewal is not at a correspondingly high level. Hence the nephrocytes must have quite a long metabolically active life. From an examination of the structural changes of the nephrocytes during the excretory cycle, it seems that each cell probably undergoes a continuous cycle of accumulation and expulsion of excretory products in a fairly well defined pattern. It is interesting to note that although the individual nephrocyte rhythms are not exactly synchronised, and though the rhythms of slugs from a similar habitat only show a slight tendency to be synchronised, nevertheless, the time of day when the maximum average concretion sizes can be seen in the kidney (9.30 a.m. to 6.30 p.m.) coincide approximately with the period when maximal blood and pericardial fluid osmotic concentrations can be measured.

In positioning and anatomy, the excretory system of A.reticulatus shows many points of similarity to that of Helix. In both animals, the kidney is a simple sac-like organ situated close to the heart and lung, with a duct leading to the exterior. In each animal, the kidney lumen is confluent with the pericardial cavity via the reno-pericardial canal, and the nephrocytes and the cells of the primary ureter of A.reticulatus are very similar to their counterparts in H.pomatia. (Bouillon 1960). There are some

obvious advantages in the structure of the excretory system of A.reticulatus: the "sharing" of blood as it flows back to the heart in the channels between the kidney and lung surface obviously facilitates good respiratory exchange, which is important in an organ as active as the kidney; the high degree of folding of the kidney epithelium produces a large surface area of active cells in a small volume, thus allowing a high turnover rate of excretory material; the wall of the ureter also has a large surface area and its cells show β- cytomembrane systems, both attributes of an absorptive epithelium; the cilia throughout the ureter will obviously aid movement of excretory material; the bladder allows the accumulation of solid excreta near to the cloacal slit (presumably over a period of time), so that it may be released at intervals, thus conserving the maximum amount of water; doubtless there are also many other points where the excretory system is structurally adapted to produce optimal functional efficiency.

There are a number of areas which have been touched on here where further work would certainly yield profitable results, e.g. the nephrocytes are a homogeneous group of very active, simple but specialised cells which perform their function with great efficiency and go through a similar cycle of events every 24 hours: the nephrocyte epithelium would thus be excellent material on which to base a study of cell physiology.

It is always true that in gathering information and finding the answers to various questions about a particular topic, many more questions are generated than are answered: What

is the mechanism of crystallisation of nephrocyte concretions? How is the blood purine content controlled? What are the reasons for the difference in proportion of xanthine to uric acid in the kidney and in the excreta? and what parts are played by the ureter and bladder in modifying the excreta?

However, one of the most evasive solutions at the moment seems to be that to the question concering the site of ultrafiltration of the blood. Evidence that the pericardial fluid can be ruled out as the major source of excretory material in H.pomatia is unconvincing, and is perhaps not applicable to A.reticulatus anyway. Results from the work on A.reticulatus are too few to draw any definite conclusionsone way or the other. However, a few interesting observations can be made: 1. Injected radioactive uric acid reached a very high concentration and dropped to zero in pericardial fluid samples, while the level in the blood also rapidly declined but only until it reached a basal level where it appeared to remain steady, and the level in the kidney remained fairly steady but at a comparatively high concentration: 2. The concentration of solutes in the pericardial fluid appears to follow very closely the blood concentration, (in this context it would be useful to know which solutes caused the pericardial fluid to be hypertonic to the blood during the experiment concerned with measurement of the depression of freezing points of the blood and pericardial fluid), and on average there is a (non colloidal) solute gradient from the blood down to the pericardial fluid: 3. As already mentioned, it appears to be possible for substances to pass from the pericardial

cavity to the kidney lumen via the renopericardial canal and from the lumen to the nephrocyte vacuoles. One could postulate from these observations that purine passes down a solute gradient from the blood to the pericardial cavity and is concentrated here to be eventually passed on via the renopericardial canal to the kidney lumen. Further work with tracers would undoubtedly be very valuable in elucidating the site of ultrafiltration of the blood, if such an ultrafiltration occurs.

Some aspects of the anatomy and physiology of the excretory system of A.reticulatus have been elucidated in this thesis. However very many questions remain to be answered both in anatomical and physiological spheres.

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SUMMARY.

The techniques of dissection, injection, light and electron microscopy were used to investigate the anatomy, histology and ultrastructure of the excretory system of A.reticulatus. Prominent features of the system are: the large concretions found in the nephrocytes; the β-cytomembrane systems and numerous mitochondria of the ureter and bladder cells; a well developed, ciliated renopericardial canal; the juxtaposition of the kidney and lung epithelia, and their common venous blood supply.

Uric acid and xanthine were identified by chromatography and spectrophotometry as the major nitrogenous excretory materials. Voiding of concretions discharged from the nephrocytes is approximately 24-hourly and is usually synchronous with defecation. Excretion of gaseous ammonia occurs at a very low level and the calcium content of the excreta is negligeable.

Depression of freezing point data indicate that the concentrations of non-colloidal solutes in the blood and pericardial fluid are closely related and show slight diurnal variation. Studies with radioactive (^{14}C) uric acid show that it moves rapidly from the blood into the nephrocytes, but the precise site of this occurence is still undetermined. The pericardial fluid could be the source of the uric acid in the nephrocytes. The use of Thorotrast indicates that material can pass from the pericardial cavity into the kidney lumen, and from here into the nephrocytes.

During heartbeat, the fluid pressure variation is at least 2.5 cm of water in the pericardial cavity and 5.4 cm of water in the aorta.

Quantitative measurements and the cytological changes undergone by the nephrocytes both illustrate the occurence of a diurnal excretory rhythm. There is a sequence of vacuole maturation culminating in the formation of a large concretion in

in each nephrocyte.

Turnover of excretory material in the kidney is rapid, (total purine nitrogen excreted is 58 µg/g body tissue/hour), but cell turnover is not.

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