STUDIES ON RENAL MUCOPOLYSACCHARIDES.

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DOCTOR OF PHILOSOPHY

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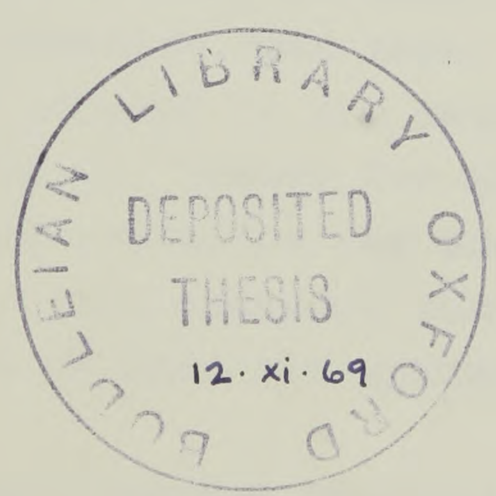

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ABSTRACT.

This thesis is an account of studies made on the distribution and nature of acid mucopoly - saccharides in the mammalian renal papilla. The papilla was found to be the only region of the kidney which has an appreciable extracellular space with acid mucopolysaccharide enmeshed in connective tissue fibres. Animals are unable to secrete urine which is either more concentrated or more dilute than plasma unless the tonicity of this region is greater than that of plasma. In the first part of this work (Chapter 2) the extent of extracellular acid mucopolysaccharide was correlated with the ability of the species to conserve water.

It was found that the papilla could be sharply delineated by staining the acid mucopolysaccharide within it. This had the advantage over the less distinct cut surface appearances in that it was possible accurately to measure the length of the papilla in relation to that of the cortex and medulla in stained sections. An empirical relationship between the development of the papilla and the ability to secrete hypertonic urine was obtained for a number of species:

$$U_{max} = 1.74 (M'/C') + 0.6 ,$$

where U_{max} is the maximum concentration in Osmols/litre

that can be achieved in the urine; M' is the length of the papilla, measured as the distance of the region containing extracellular acid mucopolysaccharide from the papillary tip towards the renal cortex; and C' is the continuation of this line through the medulla and cortex. The correlation coefficient for the six species for which figures for maximum urinary concentration were known was 0.9. The significance of this expression is discussed in Chapter 2 in relation to a similar equation put forward by Black in 1965.

The morphology of the connective tissue in the papilla of the guinea pig was examined by light and electron microscopy (Chapter 3) and by histochemistry (Chapter 4). The histological results were correlated with the electrophoretic and staining characteristics of acid mucopolysaccharides extracted from this region.

There was very little collagen of the adult type to be found in the papilla. The main fibrillar component was reticulin which was well demonstrated by silver impregnation and the periodic acid - Schiff technique. Within the reticulin mesh another class of PAS positive fibril was found which, in addition, stained positively for anionic groups. These fibrils could not be impregnated with silver. In the electron microscope the second class of fibril was seen not to be banded and to be smaller than the reticulin fibrils.

The histochemical results (Chapter 4) showed that the largest part of the amorphous extracellular acid mucopolysaccharide in the papilla was hyaluronic acid. In addition,two quantitatively small fractions were observed in the biochemical extracts which were associated with the extracellular acid mucopolysaccharide. Both these fractions stained metachromatically with thiazine dyes and were shown to incorporate radieactive sulphate. In sections metachromatic extracellular material was confined to the tip of the papilla: In this region of high in vivo osmotic pressures the interstiial cells also contained metachromatic granules. The exact nature of these two fractions was not elucidated, nor was an attempt made to identify the intracellular granules with its synthesis or destruction.

The results from the morphometry of the kidneys of fourteen species (Table 2/1) showed that desert species have long papillae which contain a limited amount of connective tissue. The metachromatic staining of extracellular acid mucopolysaccharides extends further towards the cortex in those species which can secrete highly concentrated urine. On the other hand, the papillae of animals which habitually produce dilute urine were squat in outline and contained abundant connective tissue. The tip of the papilla in these species was not found to stain metachromatically. The extent of metachromasia was thus found to be related to the development of high tonicity in vivo, and thus to be most prominent in that part of the papilla where the sodium concentration is greatest in life.

It was observed histologically that acid mucopolysaccharide was associated with the lateral spaces between collecting tubule cells in the guinea pig. It had been suggested by Ginetzinsky in 1958 that the material found between these cells in the rat constituted the barrier between fluid in the collecting tubule lumen and the interstitium. He postulated that antidiuretic hormone released hyaluronidase from the apex of the collecting tubule cell and that the enzyme then digested the inter - cellular cement permitting tubular fluid to equilibrate osmotically with the interstitium.

The material between collecting tubule cells in the guinea pig was extracted and information about its nature was obtained from its behaviour in electrophoresis and from its staining characteristics. It was found to be a very soluble acid mucopolysaccharide which contained sulphate and PAS positive groups, both of which features distinguish it from hyaluronic acid. The histological technique had thus to be modified to preserve as much as possible of the material in the thin (3 μ) wax sections required for the optimal demonstration of this region in the light microscope. The fixative developed to give the most satisfactory retention of acid mucopolysaccharide at the same time as preserving cytological structure reasonably well was as follows:

Formol (40% CHO)	100 ml
Cellosolve	225 ml
Distilled water	45 ml
Aminoacridine hydrochloride	1.6 gm.

After fixation the tissue blocks were passed directly into pure cellosolve: Rinsing in aqueous solutions was avoided. Aminoacridine hydrochloride is soluble in cellosolve, hence excess precipitant was removed during dehydration without running the risk that acid mucopolysaccharide might be leached out. The blocks were cleared in the usual way and then embedded in ester wax which was found to be superior to paraffin wax for a number of reasons discussed in Chapter 4.

Radioactive sulphate was used to trace the synthesis of acid mucopolysaccharide by the collecting tubule cells. After injection of the label, radioactivity was first seen in sections to be associated with PAS positive, amylase resistent granules in the Golgi regions of the collecting tubule cells: After longer intervals radioactivity became associated with granules near the apical and lateral borders of the cells. The Golgi region as demonstrated morphologically (Chapter 3) was always seen to be between the nucleus and apical or lateral borders of the cell and never between the nucleus and the base. Similarly, material which had incorporated radioactive sulphate was never seen to pass across the base of a collecting tubule cell. The reasons for associating the development of an extensive Golgi region with the synthesis of acid mucopolysaccharide by these cells is discussed in connection with the results published by others working with embryonically similar gut cells.

It proved possible by autoradiography to identify positively the material in the biochemical

extract that had come from the collecting tubule cells. Autoradiography of tissue sections one hour after injection of the label showed that radioactivity was limited to the collecting tubule cells: Similarly, only one band in the electrophoretic separation of acid mucopolysaccharides extracted from the papilla after this interval contained radioactivity. By noting how this band stained before and after the action of enzymes upon the material within it, it was concluded that the material associated with collecting tubule cells had the following features:

1) It contained protein which could not be digested by papain.
2) It contained a limited amount of 'neutral' polysaccharide which could be stained by the PAS routine. It could not be digested by amylase.
3) It stained as strongly at pH 1 with Alcian Blue as it did at pH 3.5: This indicated that its anionic groups were sulphate and that carboxyl groups played little part in the staining.
4) It incorporated radioactive sulphate.
5) The acid mucopolysaccharide component was partially degraded by commercial bovine testicular hyaluronidase.

In Chapter 5 the changes in the morphology of acid mucopolysaccharides in the interstitium and between collecting tubule cells that arise when guinea pigs are dehydrated, injected with ADH or hyaluronidase, or given a water load are recorded. In animals given a water load the structural fibrils of the papillary interstitium become widely separated and, at the same

time, the apparant space between tubules is increased. However, morphometry showed that the actual volume of the interstitium did not alter significantly whether the animal was water laden, deprived of water or injected with ADH. The significance of this observation is discussed in view of the contrary reports concerning changes in interstitial volume: In the present study it was concluded that hydration of the acid mucopolysaccharide gel trapped within the fibrils accounted for their expanded appearance in diuresis.

In antidiuresis papillary tubules were closely packed and a number of thin walled vessels, which were not normally observed by light microscopy, appeared to be pulled open. This observation could be explained by contraction of interstitial cells under the influence of ADH, or by an increased force exerted on certain fibrils in the interstitium as a result ~~in~~ of local changes in the physical properties of acid mucopolysaccharides. No evidence could be found in this study to support active contraction of the interstitial cells. A model (Fig. 6/3) is presented to explain how a system of fibrils and hyaluronic acid could enable water to be removed from the papilla in antidiuresis without a reduction in the tonicity of the papilla as a whole.

The difference in volume of collecting tubule cells in hydration and dehydration was measured. In both cases the basal width of the cells averaged 9.4 μ under the conditions of measurement. The average length of the cells in hydration was 13 μ falling to 10 μ on

ADH injection or water deprivation. The change from columnar to cuboidal shape decreased the cell volume by 23%.

Autoradiographic evidence was obtained which showed that the acid mucopolysaccharide associated with the apex of the collecting tubule cell was depolymerized in antidiuresis. The removal of a layer of acid mucopolysaccharide from this site could account for most of the volume change noted in sections not stained for acid mucopolysaccharides. It was possible that there was also some shrinkage of the cytoplasmic volume by osmosis in antidiuresis but the evidence for this was equivocal. There was nothing in the results from this study to support the hypothesis that hyaluronidase was released by apocrine secretion from the collecting tubule cells.

'Granules' of a size which could just be resolved optically (ie. of ¼ to ½ μ in dia.) could be seen between collecting tubule cells (fig. 4/6). These 'granules' contained anionic groups which stained in the same way as the material at the apex of the cells. The resolution that is possible with light microscopical autoradiography was insufficient to prove that these particles incorporated sulphate in parallel with the apical material. The arrangement of the Golgi region in these cells (fig. 3/12) suggested that secretion took place into the lateral intercellular spaces as well as across the apex. However, granules were never seen between the cells in the electron microscope, and it is suggested that the granular appearance of this

region in the light microscope arises from the plane of section of the cellular interdigitations.

The amount of acid mucopolysaccharide between collecting tubule cells was reduced when guinea pigs were deprived of water or given ADH. Removal of apical material with preservation of that between cells, or vice versa, was never observed. This result is discussed in Chapter 6, and a model is put forward to explain how such a system could regulate the flow of water across the collecting tubule wall.

The evidence arising from studies by other workers which suggests a role for a hyaluronic acid / hyaluronidase system in the physiology of the kidney is reviewed in Chapter 6: This, and the criticism of the hypothesis of Ginetzinsky (1958) is discussed in the light of findings in the present study. It is concluded that renal acid mucopolysaccharides are involved in maintaining the constancy of the 'milieu interieur' of the papilla, which determines that of the blood and ultimately of the whole body. It is thought that an understanding of the actual mechanisms by which they does this has probably been delayed by the modern emphasis on cellular activity with the result that the functional significance of connective tissue has tended to be overlooked.

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ABSTRACT.

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CHAPTER 1: INTRODUCTION.

The countercurrent theory of urinary concentration has become accepted virtually to the exclusion of earlier theories involving water secretion or absorption. While the main events taking place along the length of an 'average nephron' have been established and have withstood frequent review (Smith, 1956; Pitts, 1963; Black, 1967) much remains to be discovered about the actual mechanisms operating at each stage. In the present study one of the more fundamental mechanisms which has evolved in terrestial mammals, that of conserving water, is investigated.

Since the end of the last century some cases of excessive body water loss have been associated with damage to the renal medulla: Conversely, the ability to conserve water was known to depend upon the size of the papilla and the development of a high osmotic pressure within it (Cushney, 1926). In this account modern physiological views as to how papillary tonicity is built up are first summarized, and then the relationship between papillary structure and the maximum urinary concentration that can be achieved is examined for a number of species. Aspects of the fine structural morphology of the guinea pig papilla are discussed in relation to the handling of water by this region of the kidney. A subsequent chapter deals with some of the physical properties of the extracellular material which

would be expected profoundly to influence the flow of water in the papilla, but which is not visualized in routine microscopy. The changes in morphology and histochemistry that accompany the flow of water between tubules in the papilla in different physiological states are then recorded. The final chapter brings together some of the results and advances suggestions as to how they may interact to determine whether a molecule of water is more likely to be lost down the lumen of a collecting duct, or whether it will be returned to the 'milieu interieur'.

<u>Summary of Current Physiology:</u> Water together with solutes but not plasma protein is forced by hydrostatic pressure from the blood through ultrafilters in the glomeruli. Some of this water will be returned by osmosis as the fluid flows down the proximal tubule lumen since blood that has flowed through the glomeruli now surrounds the tubule and is at some 40 mm Hg lower in hydrostatic pressure than arterial blood. Salts returning to the blood along their concentration gradients, or actively pumped by the tubule cells, must be accompanied by more water since the tubular fluid is known to be isotonic with blood plasma (Marsh & Solomon, 1965). Together these mechanisms probably account for the resorption of 80% of the filtered water (ie. some 100 ml/min in man).

Fluid leaving the proximal convoluted tubule then flows down the thin limb of Henle's loop where it

is subjected to the increasing osmotic pressure of the papillary interstitium, as can be judged by the crenation of red blood cells in figs. 3/4 & 3/5. There is no hinderance to the passive flow of water out of the tubule. This permits the lumenal fluid to reach a tonicity approaching that of the interstitium. The cells of the ascending portion of the loop of Henle are more substantial: They contain mitochondria placed along basal infoldings which suggests that they actively extrude sodium from the lumenal fluid. However, in this part of the loop the accompanying outward movement of water is restricted. The relative loss of sodium from the ascending limb enables fluid which may be as much as 200 mOsm/l lower in osmotic pressure than plasma to be delivered to the start of the distal convoluted tubules.

If the distal convoluted tubules and the collecting ducts are impearmeable to the outward flow of water then urine of a lower tonicity than plasma is excreted. In this respect the development of an active ascending limb of Henle's loop in the medulla is essential for the production of hypotonic urine.

However, if the distal convoluted tubules and collecting ducts are rendered permeable to the outward flow of water as in antidiuresis then water will leave the distal tubules by equilibration with plasma in the cortex. It will similarly leave the collecting tubules in the medulla and papilla as the lumenal contents equilibrate with the increasing osmotic pressure outside the tubule. This process is further augmented by active sodium extrusion in the distal

convoluted tubules and probably also in the collecting tubules. Thus the development of a long hair pin arrangement of the loops of Henle in the papilla functions equally to conserve water by delivering hypotonic fluid to the cortex at the same time as permitting a build up of osmotic pressure in the papilla so that hypertonic urine may be secreted.

That there is an osmotic gradient from cortex to papillary tip was demonstrated for the rat by Wirz, Hargitay and Kuhn in 1951; and subsequent workers have found this to be substantially true for all the animals so far investigated. The hair pin arrangement of the loops of Henle implies that no cell layer has to withstand an osmotic difference of, say, 600 mOsm/l across its borders which would represent the maximum concentration that can be achieved in the urine of man on one side with plasma on the other: The fluid within most of the length of the ascending limb from which sodium is pumped has an osmotic pressure in excess of that in the cortex since water was withdrawn from it as it passed down the descending limb. It thus follows that the greater the length over which this pumping takes place the higher can the tonicity of the fluid at the apex be, although at each stage the pump works against a constant small osmotic gradient.

Because water is drawn out of the descending limb of Henle's loop, and enters neither the ascending limb nor the collecting duct, it is thought to leave the papilla in the vasa recta since the interstitial osmotic pressure remains at a constantly high level.

The papillary tonicity is not reduced even in antidiuresis when the volume of water passing through the interstitium must be at its greatest (Zain-ul-Abedin, 1967). Thus, when the kidney is producing concentrated urine, the blood leaving it should be diluted. However, since the total renal blood flow is in excess of 1 litre per minute and the volume of urine produced is 0.3ml/min under these conditions, it would be difficult to detect a drop in tonicity between the renal artery and vein.

It is possible on anatomical grounds to believe that some of the water lost from the descending limbs might find its way to efferent lymphatics. In support of such a view was the demonstration by Mayerson (1963) that obstruction of the renal efferent lymph flow in the dog resulted in a threefold increase in flow of dilute urine. Mayerson believed that water, with solute isosmotic for the particular level of the medulla, could move into the vasa recta only if there was an oncontic pressure gradient between the interstitium and the plasma since the hydrostatic pressures in the capillaries and the interstitium were probably equal. He held that the function of the medullary lymphatics was the maintenance of a relatively low oncontic pressure in the interstitium, which permitted the establishment of a gradient with the higher oncontic pressure within the vasa recta.

Mayerson obtained his results by cannulating the lymphatic channels in the capsule of dog kidneys, and thus he was unable to prove that the lymph did in fact originate in the medulla. However, Rawson (1949) had demonstrated the existence of lymphatics in the human

kidney by following the route of a cancerous permeation: Judged by the diameter of the vessels in the papilla the flow of lymph appeared to be from the apex to the its base. If this were so, lymph flow could act in the way suggested by Mayerson.

<u>The Role of the Collecting Tubules:</u> A crucial observation arising from micropuncture of the mammalian kidney was that the fluid in the distal convoluted tubule was either hypotonic or, in antidiuresis, isotonic to plasma. It was never hypertonic, although urine produced in antidiuresis could have osmotic pressures in excess of 1,000 mOsm/l (Walker, Bott, Oliver and MacDowell, 1941). Thus, either water must be abstracted from the urine as it flows down the collecting tubule or salt added.

Catheterization of the actual collecting ducts by Hilger (quoted by Pitts, 1963) enabled him to compare inulin concentrations near the cortex with those in samples collected near the duct openings. The higher values found in the latter indicated that water was abstracted rather than that salt was added as the urine flowed down the collecting tubule. More recently evidence has been obtained which suggests that sodium might even be pumped out of the collecting tubule. The measureable transtubular potentials are probably established in this way: It has also been found that collecting tubule sodium chloride concentration is always lower than that in the interstitium (Marsh and Solomon, 1965; Marsh, 1966).

Unfortunately it has not been established that the amount of water abstracted from the collecting tubules is greater in antidiuresis than it is in water diuresis. Hypotonic urine is normally produced in diuresis, but if the normal mechanisms are interferred with to restrict the amount of fluid reaching the start of the collecting tubules (eg. by reducing the glomerular filtration rate) then the urine produced becomes hypertonic. Similarly, in stop flow experiments on water diuretic animals, damming up the very hypotonic collecting tubule fluid for 25 mins is sufficient to make it become hypertonic.

In antidiuresis, where it might be expected that removal of water from the collecting tubule lumen would be at its maximum, it has been found that very little water, about 3 ml/min, is reabsorbed in man (Mills, 1963). In maximum diuresis in man some 20 ml/min of urine are produced, and this is usually taken to be only slightly less than the volume of fluid presented by the distal convoluted tubules to the collecting tubules. Reabsorption of two or three millilitres of water from twenty millilitres of hypotonic fluid would have a negligible effect in raising its osmotic pressure compared with the reabsorption of the same volume of water from, say, five millilitres of isotonic fluid which the distal convoluted tubules pass on each minute to the collecting tubules in antidiuresis. There are no figures available which would enable a decision to be made as to whether the osmotic gradient across the collecting tubule cell is greater or less in water diuresis although it is known that

the osmotic pressures in both lumen and interstitium are reduced. Thus the explanation of a high tonicity in the urine produced in antidiuresis need not involve an alteration in the collecting tubule permeability to water provided the osmotic gradient continues to exist between lumen and interstitium.

Nonetheless, Ganote, Grantham, Moses, Burg and Orloff (1968) have elegantly demonstrated that vasopressin applied to the exterior surface of cortical collecting tubules isolated from the rabbit does cause a net increase in water reabsorption along an osmotic gradient. In the absence of an osmotic gradient vasopressin still causes an increased diffusional water permeability which can be measured by bathing one of the tubule surfaces with isotopically labelled water. If these observations obtained on cortical tubules apply also to those in the medulla then in antidiuresis it could be expected that more water would leave the collecting tubule lumen along a constant osmotic gradient. It is possible that the increased water permeability in antidiuresis might outweigh any changes in osmotic gradient as regards the production of a final hypertonic urine.

An ingenious suggestion as to how the permeability to water could be varied in the collecting tubules was put forward by Ginetzinsky in 1958. He observed that, in histological sections, there was less 'hyaluronic acid' between collecting tubule cells in antidiuresis: He suggested that ADH acted through the release of hyaluronidase which then removed the water-

proof intercellular cement, enabling water more readily to flow down the osmotic gradient into the interstitium. In support of this hypothesis was his observation that the measured hyaluronidase activity was high in urine produced during antidiuresis, or following ADH injection and undetectable in the urine produced during a water diuresis.

Dicker and Eggleton (1960) also measured the viscosity reducing effect of urine on samples of hyaluronic acid. They found that the hyaluronidase activity of human urine fell in water diuresis and rose again on restoration of normal flow. Two subjects with (an inherited) diabetes insipidus of renal origin excreted no hyaluronidase activity following injection of ADH, while normal subjects or those with a diabetes of pituitary origin excreted the enzyme following the hormone injection.

On the other hand Berlyne (1960) was unable to find proof in human subjects that ADH acted through the release of hyaluronidase. Berlyne noted that the viscosity reducing activity of hyaluronidase was increased in solutions containing high concentrations of salt. To overcome this problem Berlyne measured the viscosity refucing activity in human urine to which salt had been added to give a final constant concentration (0.1 M NaCl). When this was done he could find no relationship between the minute volume production of urine and its hyaluronidase like activity. Returning to the results of Ginetzinsky (1958) Berlyne calculated the quantity of enzyme released in unit time and came to the conclusion that it was independent of the level of diuresis.

These criticisms were later countered by Ginetzinsky himself (Ginetzinsky, 1961), who claimed that the difficulty of varying electrolyte concentration could equally be overcome by dialysis of the urine, which would remove the low molecular weight solutes. He maintained that if this was done the measured activity of hyaluronidase in antidiuresis was still above control values. Neither worker made a direct comparison with the method of the other and it is open to speculation how Berlyne could attain a final concentration as low as 0.1 M NaCl in urine produced in antidiuresis, when it would be expected to have a tonicity approaching 1,000 Osm/l.; and similarly it is possible that the larger surface area of the dialysis membrane used by Ginetzinsky in diuresis could have adsorbed (or lost) more enzyme.

The measurement of hyaluronidase activity by observing the rate of fall in viscosity of pure hyaluronate solutions is a difficult proceedure with error introduced by

i) Small changes in temperature;
ii) The effect of the concentration of electrolytes present as well as the strength and type of buffer employed; &
iii) The possible presence of hyaluronidase inhibitors in the preparation being tested.

Corbascio and Dong (1966) avoided some of these difficulties by assaying undepolymerized hyaluronic acid by the turbidity produced when it is added to standard

~~to standard~~ solutions of horse serum. In the present context these workers incubated the samples of urine under investigation with a known concentration of bovine vitreous humour hyaluronate and then assayed the amount depolymerized in a given time. At the same time an aliquot of the same sample was boiled to destroy any enzyme that it might have contained and, when cool, a known amount of testicular enzyme added and the apparant activity measured: This provided a control to take into account the effect of electrolytes and inhibitors.

The results obtained by Corbascio and Dong (1966) support the hypothesis of Ginetzinsky (1958): Hydrated rats injected with vasopressin showed significantly increased hyaluronidase activity in their urine in spite of an antidiuresis. In rats not given a water load a rise in enzyme activity could be induced by vasopressin, but this was was not always directly related to the final volume of urine excreted.

Further support for the general scheme was put forward by Thorn, Knudsen and Koeford (1961) who gave rats intravenous injections of testicular hyaluronidase. It was noted that, after a delay, this resulted in a reduction in urine flow accompanied by a rise in the osmolarity in the urine produced. These workers, however, considered that the delay of at least twenty minutes between injection and observation of its effect was overlong in view of the more rapid action of injected vasopressin.

Three points can be raised in connection with the reservation expressed by Thorn et al (1961):

i) The material between collecting tubule cells was reported by Ginetzinsky (1958) to stain metachromatically with toluidine blue, which makes it unlikely to have been hyaluronic acid. Thus the testicular enzyme used by Thorn et al may not have been acting on its optimal substrate.

ii) The injected enzyme would have to travel the whole length of the nephron before it reached its site of action in the collecting tubule, whereas it is possible that any enzyme released in response to antidiuretic hormone has a local action.

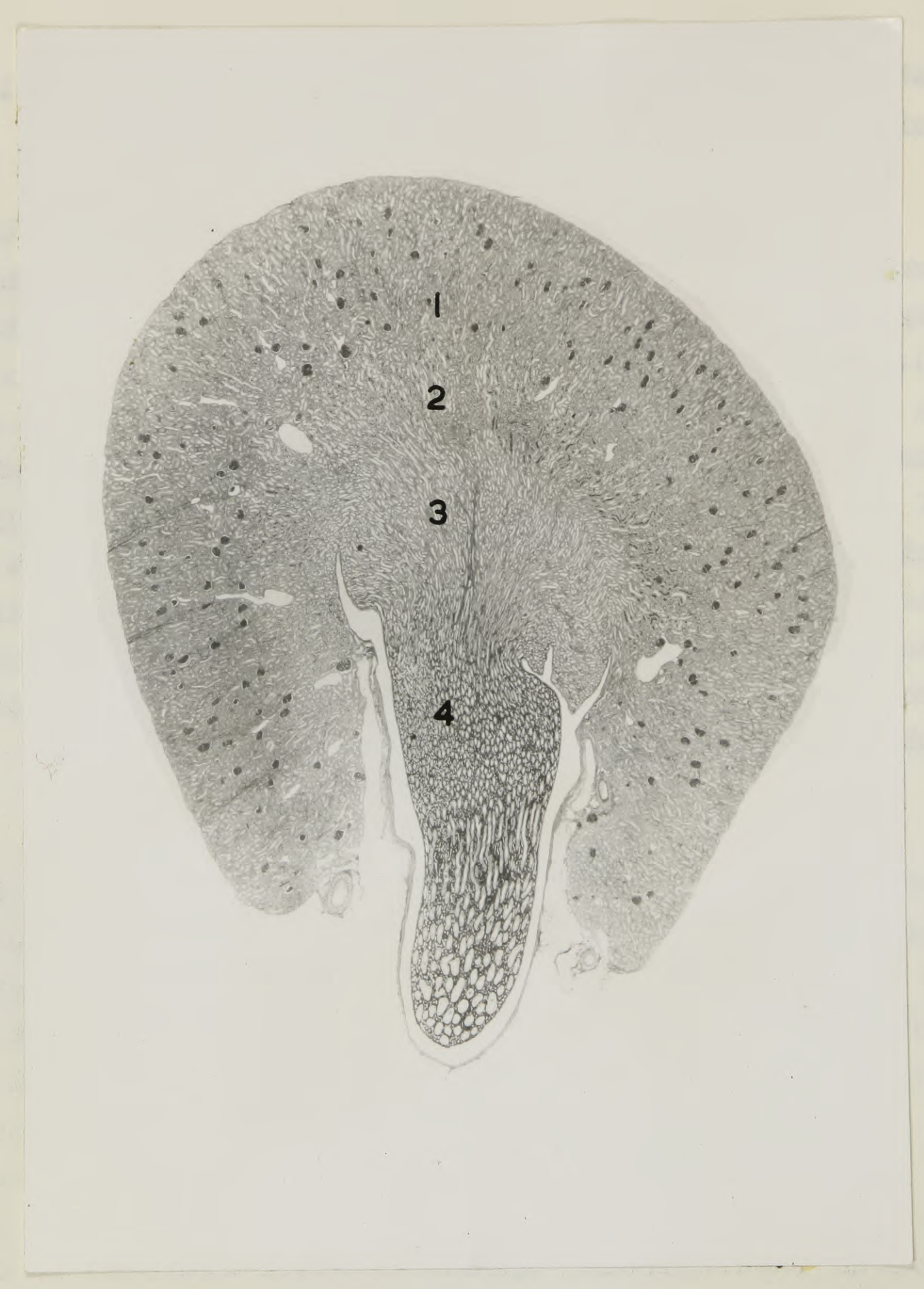
iii) Cobbin and Dicker (1962) and Dicker and Elliot (1963) have isolated two hyaluronidases from the kidney, neither of which has the same spectrum of activity as testicular hyaluronidase. The injection of a 'foreign' enzyme less active at this site could equally account for the delay in producing an effect.

Acid Mucopolysaccharide in the Interstitium: At the same time that he described the changes in the intercellular material which followed the administration of antidiuretic hormone, Ginetzinsky (1958) drew attention to the presence of a non-metachromatic network of acid mucopolysaccharide in the interstitium. When Dicker and Franklin (1966) isolated chondroitin sulphate and hyaluronic acid from the medulla of pigs, sheep and dogs it became possible that the metachromatic intercellular 'waterproofing'

material was chondroitin sulphate and that the interstitial acid mucopolysaccharide was hyaluronic acid.

In the present study this question has been examined with some care. The observation that the papilla is the only region of the kidney that contains an appreciable amount of connective tissue is taken up in Chapter 2. The development of this region in a number of species is examined in relation to the ability of that species to conserve water. The morphology of this region in the guinea pig is examined by light and electron microscopy as well as by histochemistry in Chapter 3. The acid mucopolysaccharides were extracted from the papilla and their staining and electrophoretic behaviour investigated and reported in Chapter 4.

In the final two chapters an attempt is made to relate what is known of the properties of these molecules to the physiology of the papilla by observation of the morphological effects of administering a water load, antidiuretic hormone or hyaluronidase to guinea pigs. The notion is entertained that the emphasis on apparent destruction of the intercellular cement in antidiuresis might be misplaced. It could equally be a priori that mucoid material is secreted into the lateral intercellular spaces in water diuresis in order to prevent the flow of sodium from the interstitium into the collecting tubule lumen: It is known that hyaluronic acid resists the flow of water through it (Day, 1952) while chondroitin sulphate impedes the movement of cations (Joseph, Engel and Catchpole, 1952).



Hamster Kidney x12 Stained for Acid Muco-polysaccharides (Mowry). Regions: 1, Outer Cortex; 2, Inner Cortex; 3, Medulla; 4, Papilla.

Fig. 2/1.

CHAPTER 2: THE DEVELOPMENT OF THE PAPILLA IN RELATION TO MAXIMUM URINARY CONCENTRATION.

Selection of Material: In order to study the development of the renal papilla in different species, fresh kidneys were processed in the manner outlined in Appendix 2/1. Sections were cut in a plane which as nearly as possible contained the full length of the loops of Henle. Sections obtained in this way, however, can be misleading unless the observer has knowledge of the gross anatomy of the kidney in a given species. For example, some animals have curved and often slightly twisted papillae which extend beyond the main renal mass into the ureters. Thus sections cut longitudinally or transversely might not contain the plane of curvature. An effect of this can be seen in the photograph of the hamster kidney (Fig. 2/1): At the base of the papilla tubules have been sectioned longitudinally as they have been again in a more central portion but, in the remaining regions, they have been cut obliquely.

A relationship between the actual volumes of the cortex and papilla can only be deduced from those cases where the papilla approximates to a conical shape (eg. as in the case of the guinea pig and rabbit). It is not possible from a two dimensional representation to tell whether a cross section passes through a cone rather than a ridge. The dissection of the dog kidney

Dissection of the Dog Kidney.

Fig. 2/2. Ureteric View of the Area Cribrosa showing fused Pyramids.

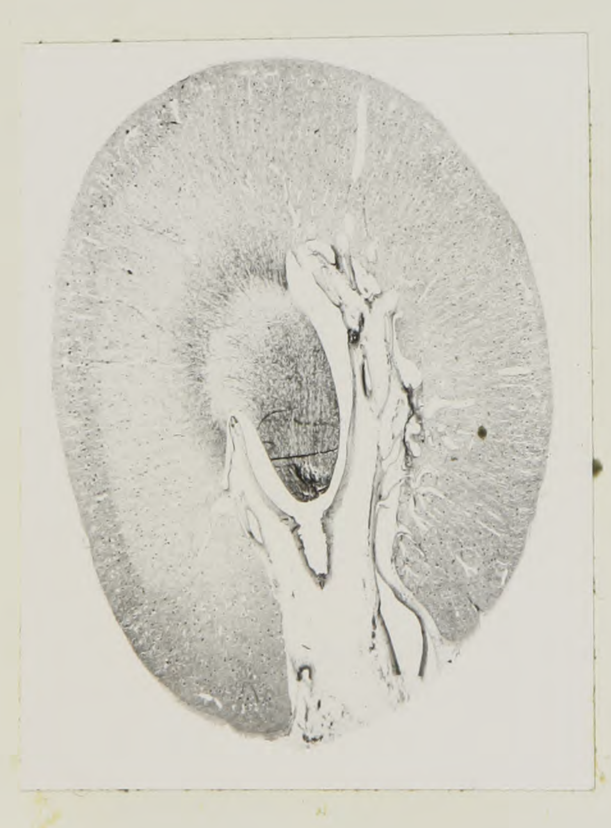


Fig. 2/3. Transverse Section. Mag. 2.5.

Fig. 2/4. Longitudinal Section. Mag. 3.

(Figs. 2/2 to 2/4) shows how this problem arises.

A further complexity is introduced where kidneys have more than one papilla (eg. cow or the primates). Sections taken through the median plane of one papilla tend to over-emphasize the extent of the cortex, part of which is related to papillae not shown.

However, it is the product of factors influencing the diffusion of water from the collecting tubule lumen to that of the vessel that removes it from the papilla and the total number of such paths which determines the ability of the kidney to produce highly concentrated urine. It is thought that the sections selected probably give a fair indication of both these factors.

<u>Definition of Regions in the Kidney:</u> Previous attempts have been made to relate quantitatively the ratio of renal medulla to cortex with the maximum concentration that can be achieved in the urine of a given species. For example, Black (1965) collected these parameters for sixteen species and plotted them against each other (redrawn as Fig. 2/18 a), and obtained the equation:

$$U_{max} = 1.06 (M/C) - 0.51 \qquad (1)$$

where U_{max} was the maximum concentration that could be

Species Variation.

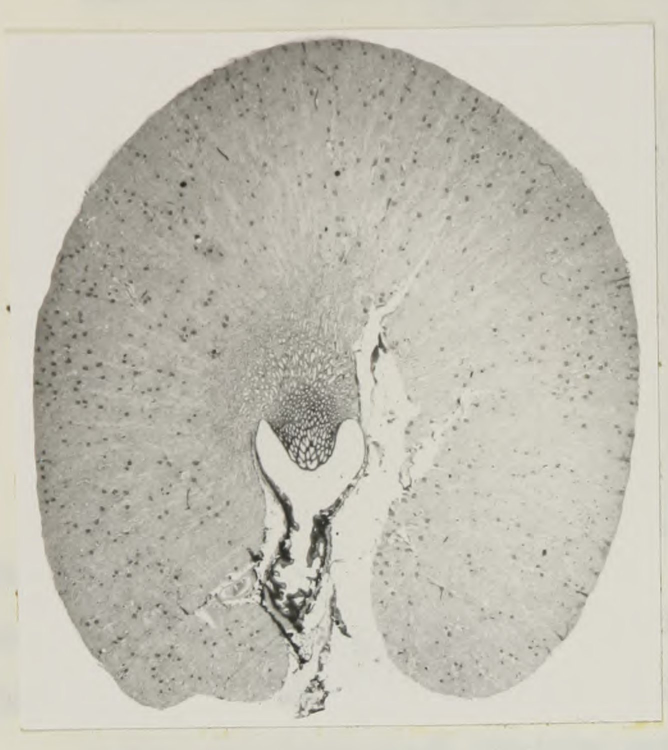
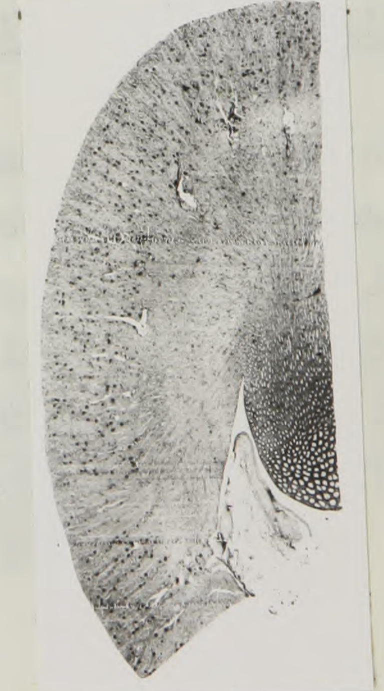


Fig. 2/5 Coypu ×3.9

Rhesus Monkey ×5 Fig 2/6

Fig. 2/7 Man × 3.3



Rabbit × 4 Fig. 2/8

Species Variation

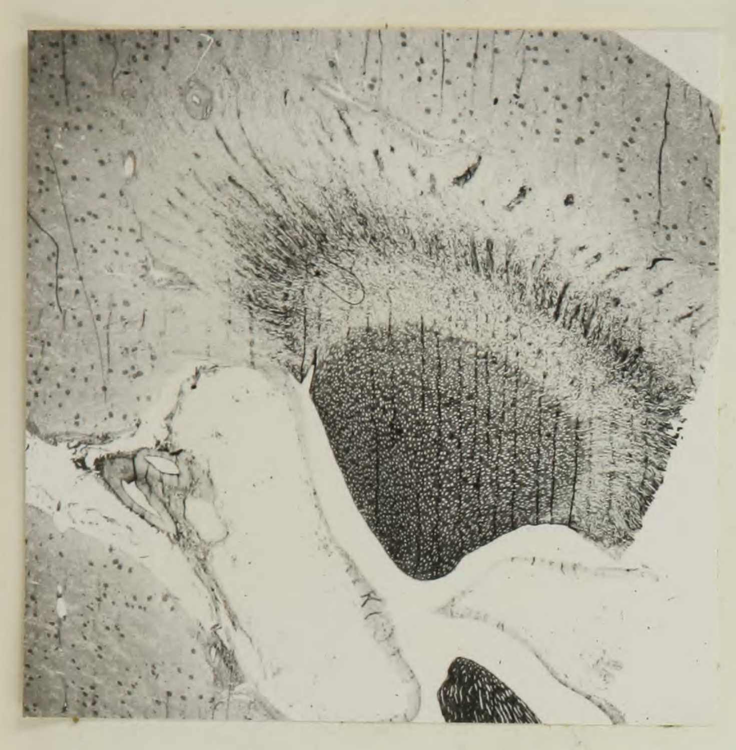


Fig. 2/9 Ankole Cow x 4

African Wild Pig . 2/10
x 2.3.

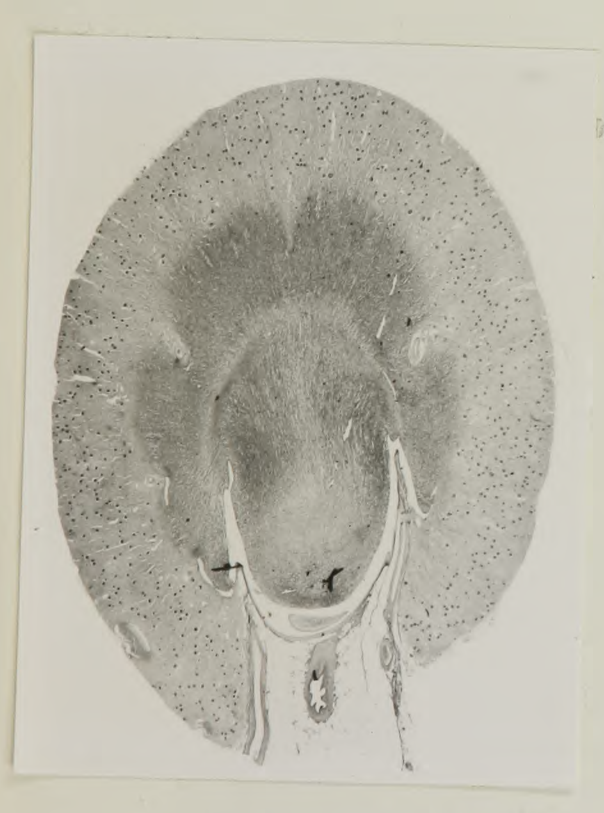
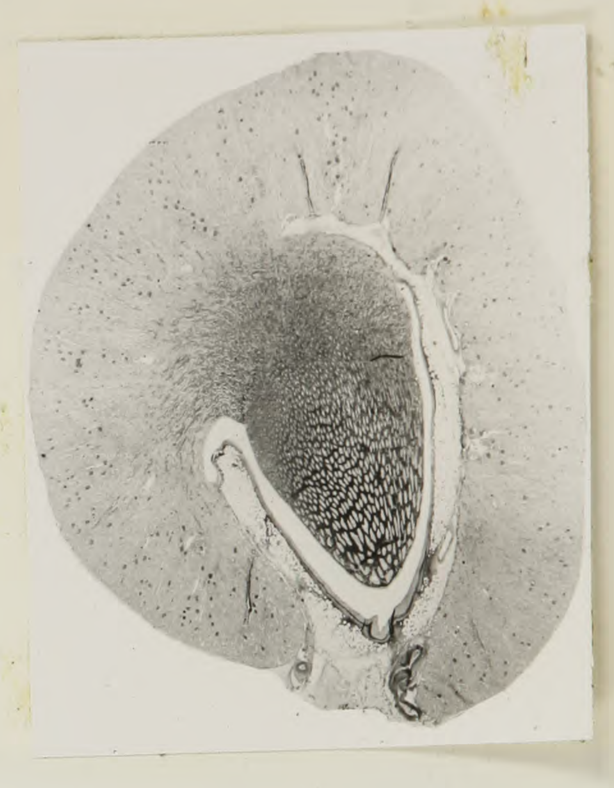


Fig. 2/11 Cat X 3.3

Rat X 8.5 Fig. 2/12.

Species Variation.

Fig. 2/13 Sheep ×2



Guinea Pig. ×7 2/14

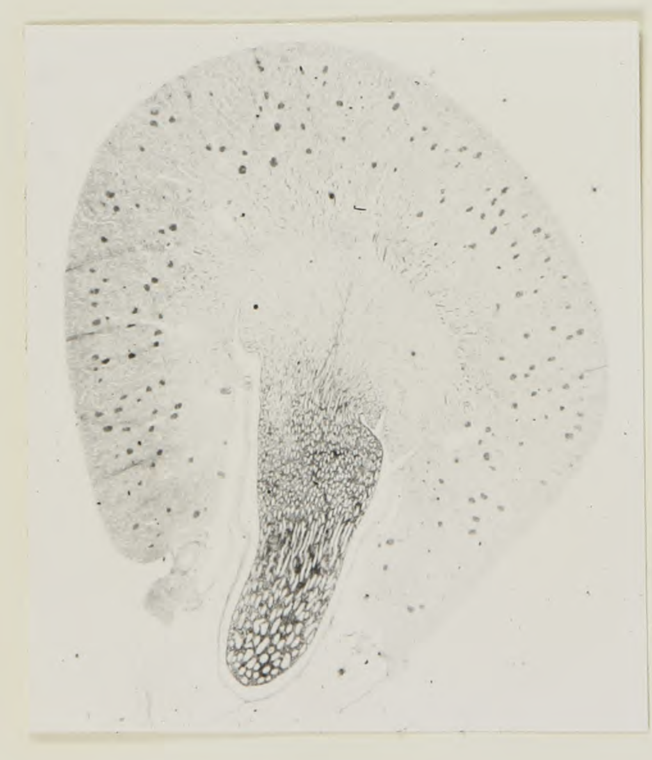
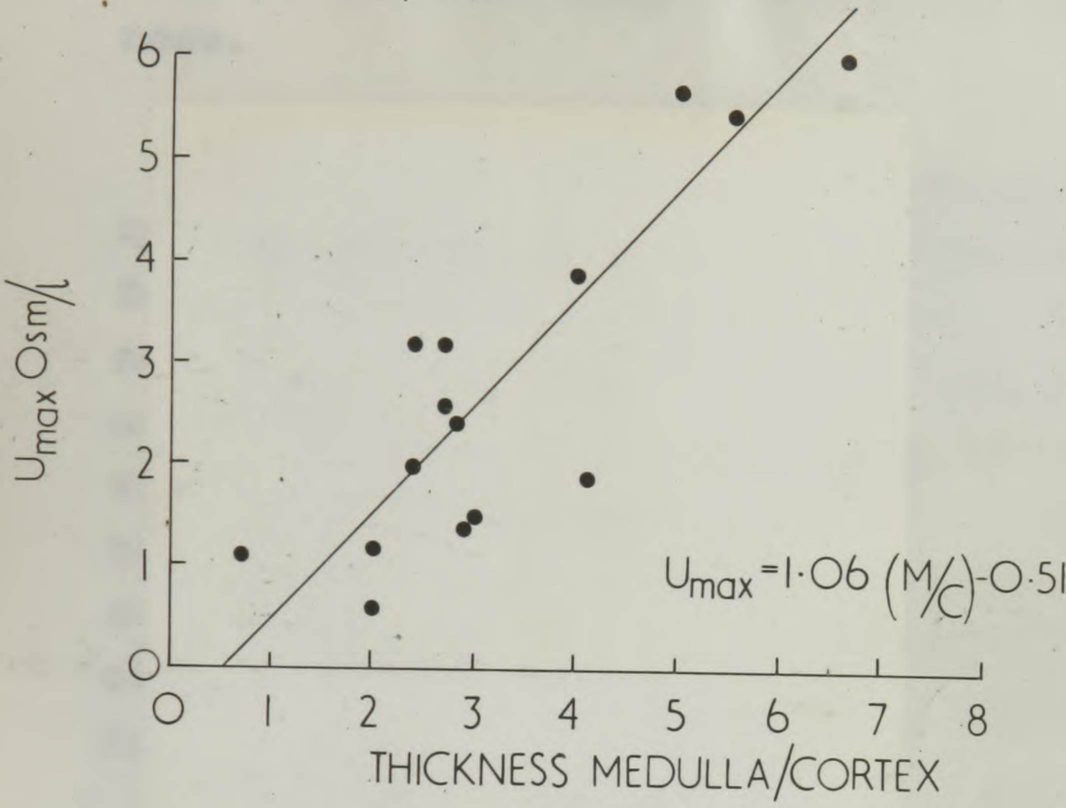


Fig. 2/15 Hamster ×8.5

Mouse ×10 Fig. 2/16



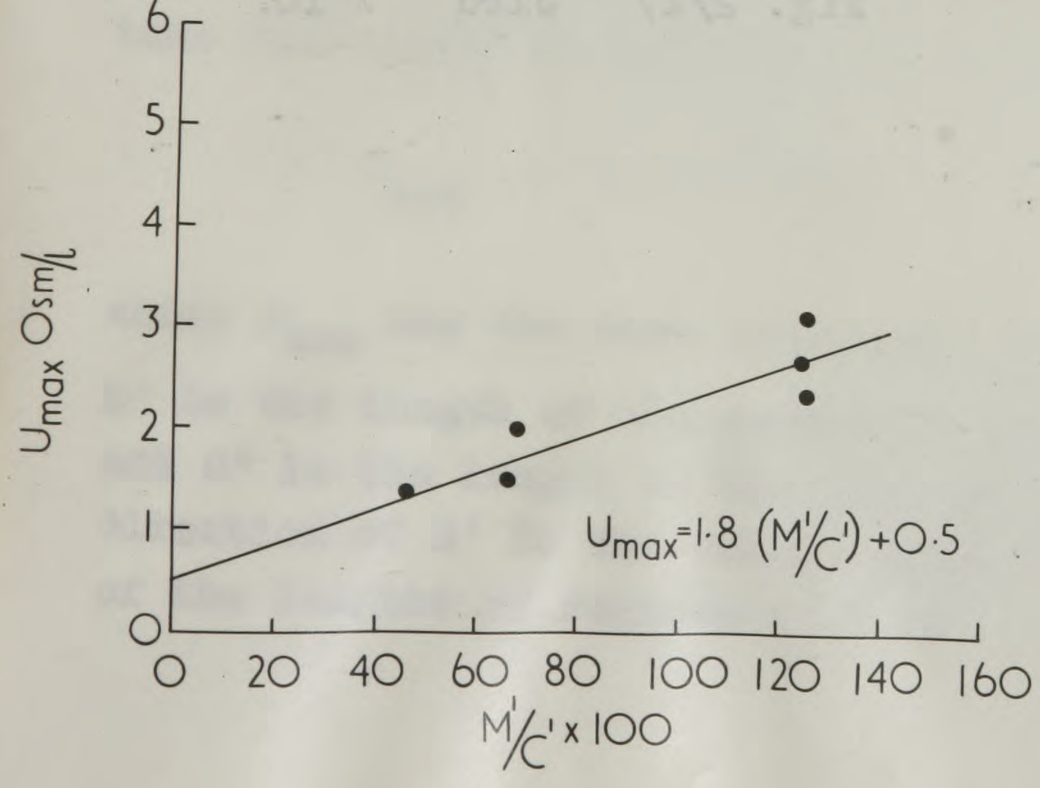


Fig. 2/18

Species Variation.

Fig. 2/17 Jird x 10.

achieved in the urine, measured in Osmols/litre; and (M/C) was the ratio of medullary to cortical thickness.

Black chose total medullary thickness, as opposed to the inner zone thickness considered by Schmidt-Nielsen and O'Dell (1961) to indicate concentrating ability, because 'the distinction between outer and inner zones of medulla is more arbitary than that between medulla and cortex'. This is certainly the case for cut surface appearances, or for routinely stained sections of kidney, but it was found in this study not to be true for sections stained specifically for acid mucopolysaccharides.

Figure 2/18 (b) has been constructed using the data provided by Black (1965) for maximum urinary concentration, but with the substitution of the ratio of the thickness of the papilla to that of the rest of the kidney in the ratio of 'medulla to cortex'. The best line drawn by eye gives the equation:

$$U_{max} = 1.8 (M'/C') + 0.5 \qquad \text{(ii)}$$

where U_{max} has the same connotation as in equation (i), M' is the length of the papilla (region 4 in fig. 2/1) and C' is the length of the line continued along the direction of M' to the renal capsule (ie is the sum of the lengths of regions 1, 2 and 3 in fig. 2/1).

A statistical treatment of these values (Appendix 2/2) gives the equation:

$$U_{max} = 1.74 \, (M'/C') + 0.6 \qquad \text{(iii)}$$

with a correlation coefficient of 0.9.

Equations (ii) and (iii) differ from that proposed by Black (i) particularly in the sign of the constant. Equation (i) states that as the ratio of M/C tends to zero, eg. as is the case of animals such as the coypu, then the maximum urinary concentration tends to a negative figure, which is clearly impossible. Equations (ii) and (iii) imply that kidneys with papillae of negligable length are unable to concentrate urine to an osmolarity much greater than that of blood. The small degree of concentration that these kidneys can achieve may represent the maximum osmotic gradient against which a single layer of cells can pump.

Development of Connective Tissue Between Tubules:

Transverse sections of papillary tips were used to estimate the relative area occupied by the interstitial connective tissue by the point counting method illustrated in Appendix 2/3. In general a large value for the relative interstitial area indicated that the tubules were widely separated: It does not follow that there need/be more acid mucopolysaccharide present in this space.

Table 2/1.

Species	M'/C' 100	U_{max} in Osm/1	Relative Areas in Papilla: Coll. T	L. of H. & V. R.	Inter-stitium
Coypu	25	-	52	7	40
Rhesus	43	-	-	-	-
Man	46	1.4	25	15	60
Rabbit	66	1.5	45	17	38
Dog	68	2.0	19	21	60
Ankole Cow	72	-	20	23	56
Afr. Pig	100	-	25	18	56
Cat	124	2.7	24	17	58
Rat	125	2.4	34	18	48
Sheep	125	3.2	38	27	36
Guinea Pig	131	-	50	11	39
Hamster	134	-	44	16	39
Mouse	139	-	55	25	20
Jird	193	-	37	17	47

Figures for maximum concentration of urine taken from Black (1965).

Several broad patterns could be seen in the results (Table 2/1). Carnivores had relatively large papillae, much of which was given over to the interstitial components (about 60%). Herbivores tended to have papillae in the middle range as regards volume of the papilla, about half of which was composed of interstitium. Desert species fed on grain had long thin papillae in which the interstitium accounted for only 20 to 50 per cent of the volume.

It was noticed that the higher the concentration of urine that could be produced the more prominent became the interstitial cells. In the desert rodents, for example, these cells gave the papillary interstitium a characteristic ladder - like appearance (fig. 2/24). The arrangement of interstitial cells in such animals prompted Vimtrup and Schmidt-Neilsen (1952) to suggest that they could be contractile and thus possibly play some part in regulating papillary extracellular volume. However, it seems equally possible on morphological grounds to associate these cells with the synthesis of the interstitial acid mucopolysaccharide. Reasons are advanced later in this chapter for taking the view that the acid mucopolysaccharides probably provide a barrier to the free flow of water within the papilla: In this case the sandwiching of layers of cells with the acid mucopolysaccharide in the long papillae of desert species, which develop very high osmotic pressures at the tip, might act to prevent the flow of water from the base of the papilla to dilute the apex.

Interstitial Reticulin

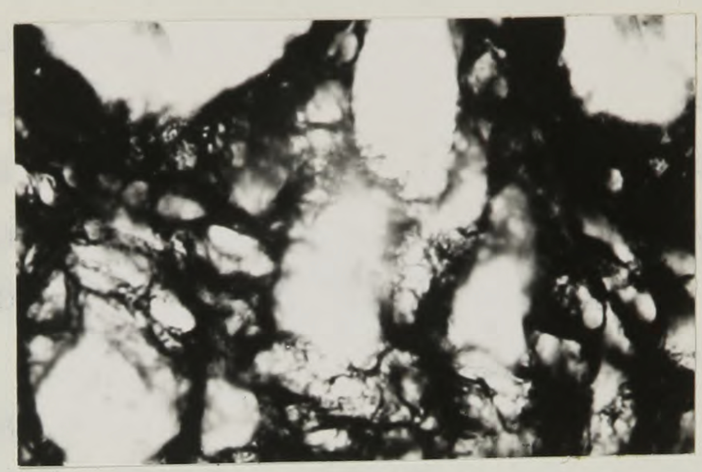


Fig. 2/19 Coypu

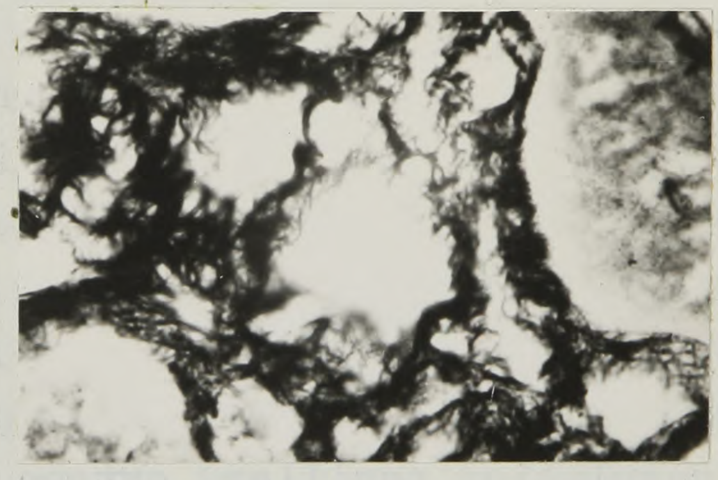


Fig. 2/20 Rabbit

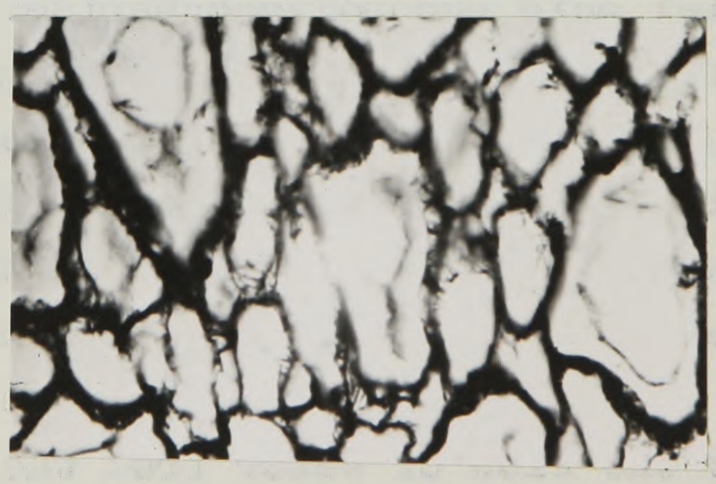


Fig. 2/21 Rat

Transverse sections, all magnified 800.

It was particularly noticeable in the present study that the animals with a low concentrating ability had papillae with a diffuse interstitium with fine interlacing reticulin fibrils between well separated tubules (eg. fig. 2/19). As the concentrating ability increased among species so did the tightness of this net (eg. fig. 2/20), with a compactly woven 'stocking' around the collecting tubules becoming especially prominent. The space between tubules also became less (fig. 2/21).

The density of staining for acid mucopolysaccharides followed a similar trend. Staining was relatively pale in the more aquatic animals and very dense in the desert species, (figs. 2/22 to 2/24). That the same component in the interstitium was not being demonstrated by the reticulin and acid mucopolysaccharide staining was indicated by the different morphology, which is more fully discussed in the fourth chapter. Further, the acid mucopolysaccharide staining could very considerably be reduced by washing the sections in water before staining, while reticulin is not extracted by such a proceedure.

Physiological Implications: A similar arrangement of acid muco - polysaccharide enmeshed in a fibrillar frame work exists in almost all of the less specialized connective tissues. Depending upon the site, four main functions have been attributed to such a system, and these have been re - interpreted in terms of renal function as follows:

Interstitial Acid Mucopolysaccharides

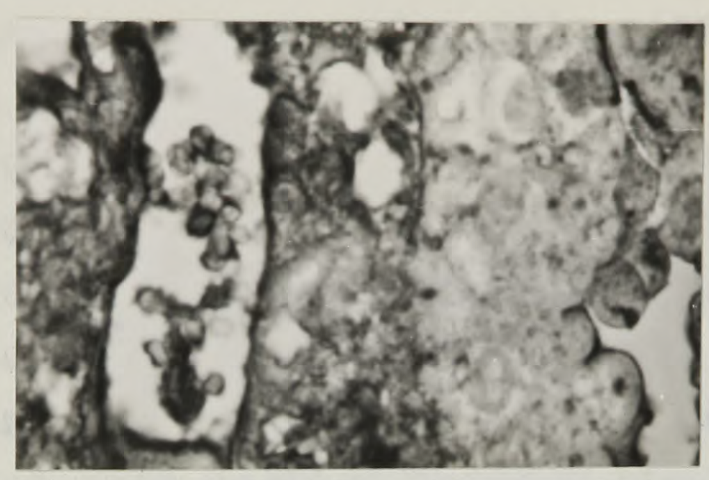


Fig. 2/22 Coypu.

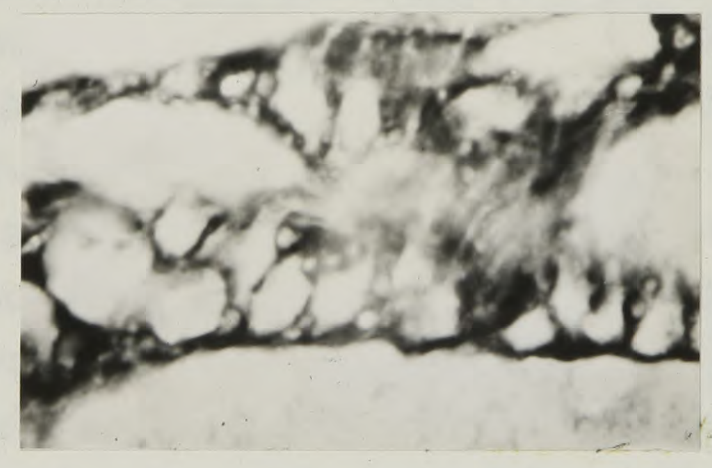


Fig. 2/23 Rabbit.

Fig. 2/24 Rat.

Transverse Sections, Colloidal Iron Stain, 800.

i) The mucopolysaccharide might block possible channels for the flow of water within the reticulum. For example, Day (1952) found that the hyaluronic acid / collagen system of fascia impeded the flow of water through it. Hyaluronidase acted greatly to increase the flow of water. Day suggested that this was the result of depolymerization of the acid muco - polysaccharide component since the flow of water could similarly be reduced if the 'pores' were subsequently blocked by starch, and increased once more if the starch was digested away by amylase. Seifter, Baeder and Dervinis (1949) studied a similar arrangement in the bladder wall of the rabbit. They found that hyaluronidase increased the permeability to water, while aqueous extracts of the adrenal cortex caused the urinary bladder to become waterproof even in the presence of the enzyme.

The work of Seifter et al. (1949) is of interest on three counts. First, the embrology of the bladder wall and the collecting tubule wall is the same, so they might be expected to share similar properties. Second, Ginetzinsky (1958) observed that there was a high hyaluronidase activity in the urine in antidiuresis, and that this was associated with the removal of acid mucopolysaccharide (between collecting tubule cells), which led him to suggest that removal of hyaluronic acid enabled water in the collecting tubule lumen to equilibrate with the papillary interstitium. Third, cortisone is held by some workers to have a direct action in the kidney in opposition to antidiuretic hormone and thus to increase the waterproofing of the distal convoluted and collecting

tubules. Presumably the adrenal cortical extract of Seifter et al. (1949) contained this hormone. The mechanism whereby this extract abolished the effect of hyaluronidase was unknown. It is thus interesting to note that in a review of hyaluronidase inhibitors Mathews and Dorfman (1954) mention that cortisone inhibits hyaluronidase in vitro. (It is even possible that cortisone has a further action in vivo in connection with the stabilization of lysosomal membranes, which might inhibit the release of acid hyaluronidases in addition.)

ii) The effect of acid mucopolysaccharides in the papillary interstitium might be to impede the movement of certain solutes within it: This was shown by Ogston and Sherman (1961) to arise when hyaluronic acid was confined between two membranes through which solutes could readily pass. If the interstitial mucopolysaccharide acted in a similar way to that found in the symphysis pubis then it would also tend to bind potassium while letting sodium pass through it relatively freely.

Anohher way of looking at this property is to think of the excluded volume that the acid mucopolysaccharide gives rise to in the papilla. Thus water molecules entering the papillary interstitium would tend to become hydrogen bonded to the acid mucopolysaccharide, which would cause the polymer to occupy a larger volume and at the same time to leave a smaller total volume of solvent. In this way, the acid mucopolysaccharide might be able to maintain the osmotic pressure of the interstitium

in the face of an apparant dilution.

iii) The arrangement of reticulin and acid muco - polysaccharide might be able to resist mechanical pressure and so keep open certain tubules in the papilla. A similar arrangement of connective tissue has been said to have this property in the umbilical cord (Fessler, 1960) The situation is also analagous to the mechanism invoked by Florey and Pullinger (1935) to explain how lymphatic channels could remain open in the ear of a mouse when the tissue was distended by inflammatory oedema.

In the last chapter the special arrangement of fibrils around certain tubules is discussed and the possibility is raised that changes in the physical properties of the mucopolysaccharide xxx in the immediate vicinity of two tubules might make the flow of water between them either more or less, and this xxx aspect might be influenced by mechanical forces developed in the fibrils anchored around the tubules.

iv) Finally, it has been suggested that a number of possible biochemical situations exist where it appears that the action of an enzyme can be regulated by the degree of polymerization of a component in a polyanion - polycation system. The inhibition of the depolymerisation of hyaluronic acid by hyaluronidase in the presence of heparin is an example (Spensley and Rogers, 1954). However, until the functions of the extracellular colloids in the renal papilla are established, it would be premature to speculate upon the possible effect of enzymes upon these colloids in terms of renal physiology.

There are three ways in which the morphological investigation of these aspects of acid mucopolysaccharide function in the renal papilla has been delayed. Cytochemical methods which will allow of demonstration of acid mucopolysaccharides as a class in the electron microscope are only now being developed and it will be at this level of resolution at least that answers will be obtained. Some of the molecules involved are large enough to enter the province of light microscopy: A molecule of hyaluronic acid with a molecular weight of about 4×10^6 could have a x length of as much as 5 u in theory (Gardner, 1965). However, even in light histochemistry relatively little information about the nature of the molecules that would be intelligible to the biochemist has been forthcoming.

Electron microscopy employing present day techniques is also logistically unattractive as a tool for the investigation of the problem: The questions posed require that changes should be observed in the papilla of experimental animals in which the level of hydration has been altered. Thus a statistically reliable number of experimental blocks of tissue would have to be processed with the minimum of variation in technique, quality of reagents, embedding medium etc., which is clearly unreasonable.

It will be appreciated from Fig. 4/14 and the comparison between figures 4/6 and 4/7 that the cellular elements do not exhibit uniform activity throughout the papilla. Thus any comparisons between the tissues taken under different physiological conditions would involve the almost serial processing of whole papillae.

In the present study electron microscopy has been used to demonstrate the fine structural anatomy of the papilla of the guinea pig. However, acid mucopolysaccharides are not revealed by the conventional electron microscopical techniques and recourse was had to light microscopic histochemistry to reveal details about some of the groupings of the molecules occupying large clear areas in the electron micrographs. The histochemical techniques employed were derived from those which have been used by others to study connective tissue in other regions. Optimism has become tempered with time and most of the limitations of the methods have been realized. In the present work an attempt at precision has been made by extracting the acid mucopolysaccharides so that their properties could be examined more rigorously than is possible in tissue sections, where their distributions overlap. The results from these approaches are given in the next two chapters, while the fifth chapter deals with morphological changes which accompany altered levels of hydration in the experimental animal.

NAMING OF PARTS

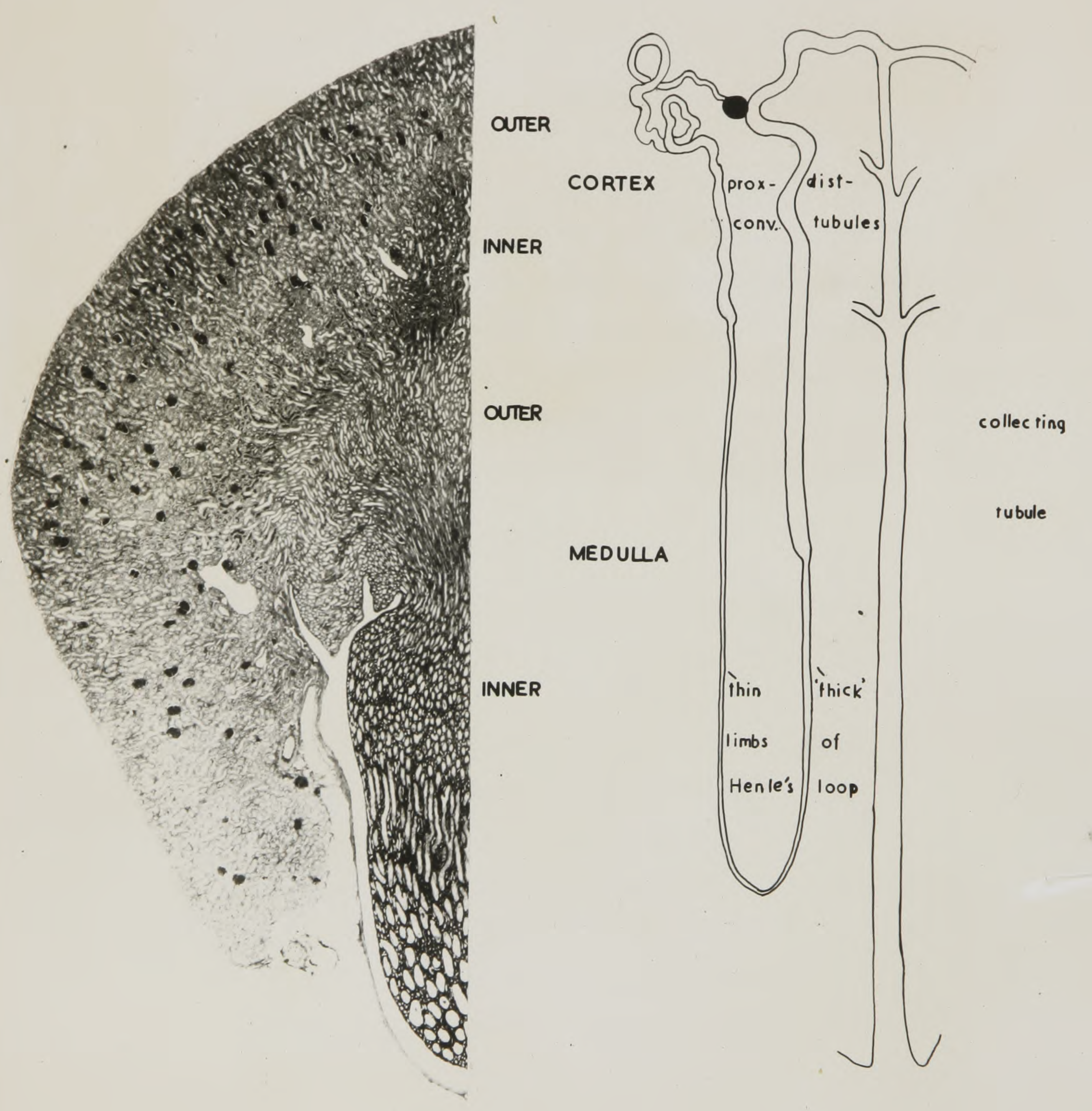


fig 3/1

fig 3/2

CHAPTER 3: THE MORPHOLOGY OF THE GUINEA PIG PAPILLA WITH REFERENCE TO THE MOVEMENT OF WATER WITHIN IT.

A. The Collecting Tubule Cell: The adult mammalian kidney has a dual origin. There is an 'inert system of drainage ducts (ureter, pelvis, calyces, papillary ducts and straight collecting tubules) derived from a bud growing off the mesonephric duct' with 'each secretory unit, or nephron, differentiated from the substance of the caudal end of the nephrogenic cord' (Arey, 1954).

While in histology it is permissable to be uncertain about possible functions of structures that can be seen, it is unjustifiable to deny the existence of those that may not. The impression of 'inertness' has arisen from the feeble manner in which collecting tubule cells take up dyes used in routine histology. The micrographs of rabbit kidney stained by the Masson trichrome technique (Figs. 3/3 to 3/5) provide a good example. From their origin in the cortex, the collecting tubule cells stand out on account of their pale staining and their well defined lateral borders. They possess few mitochondria and have no lumenal brush border. Similarly, in the medulla, they contrast strongly with the granularity of the cells in the thick limb of the loop of Henle (Fig. 3/4).

The Collecting Tubule at Three Levels.

Cortex
(1)

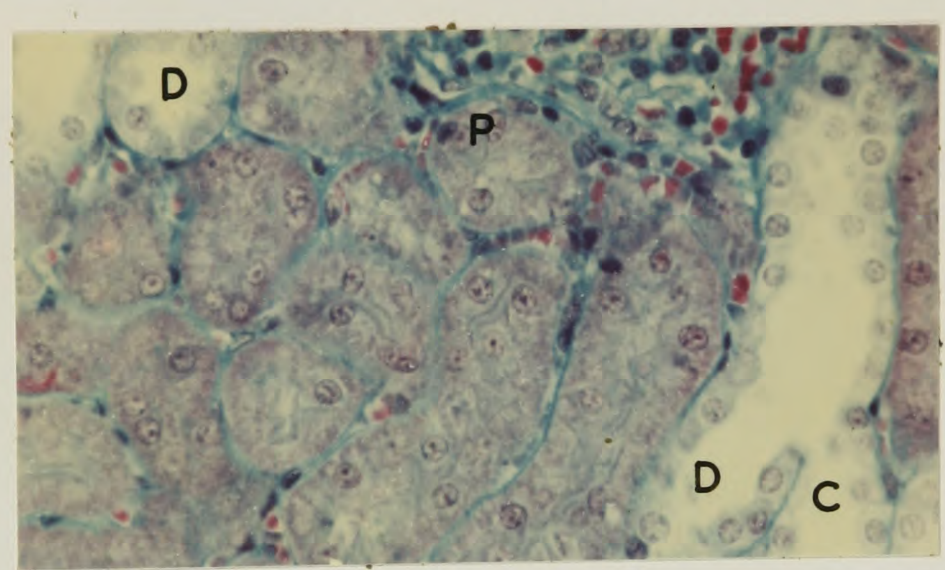


Fig. 3/3.

Medulla
(3)

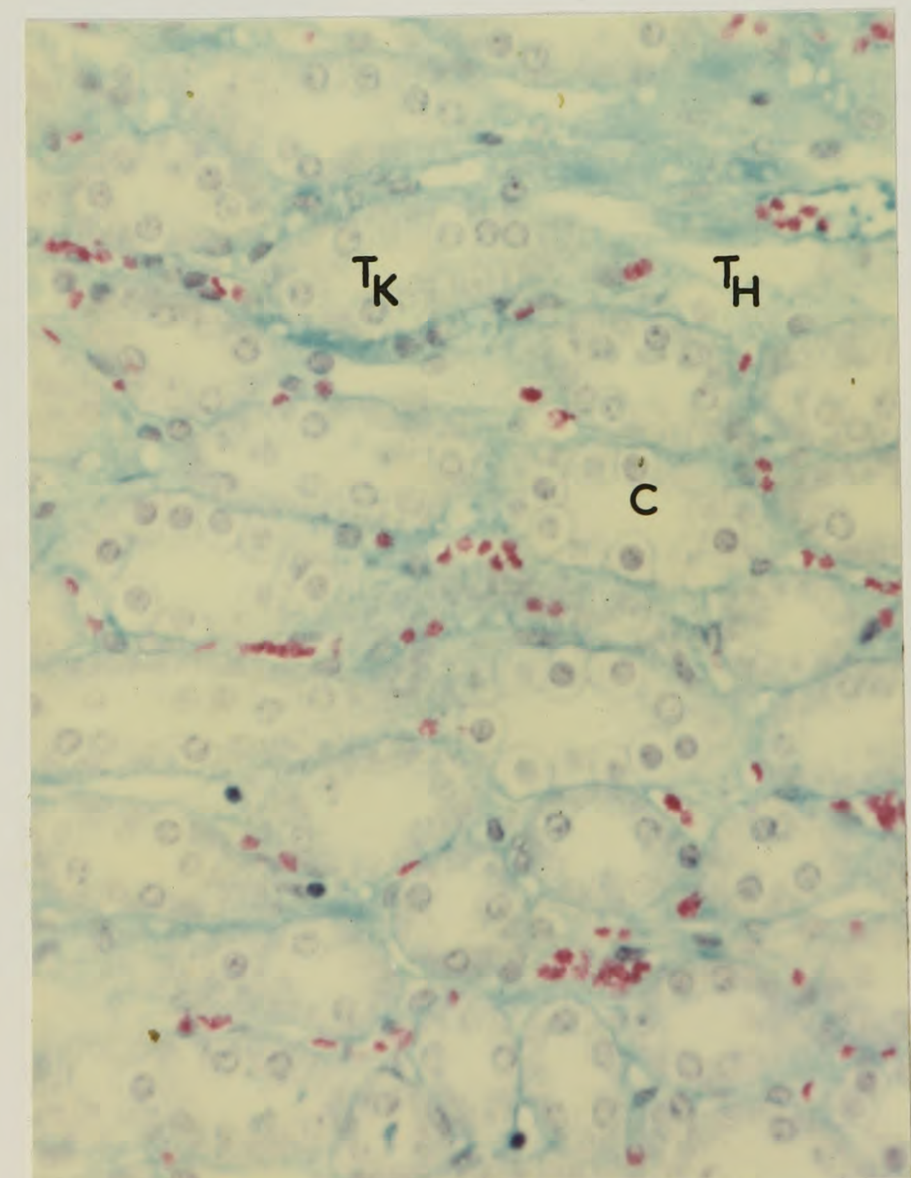
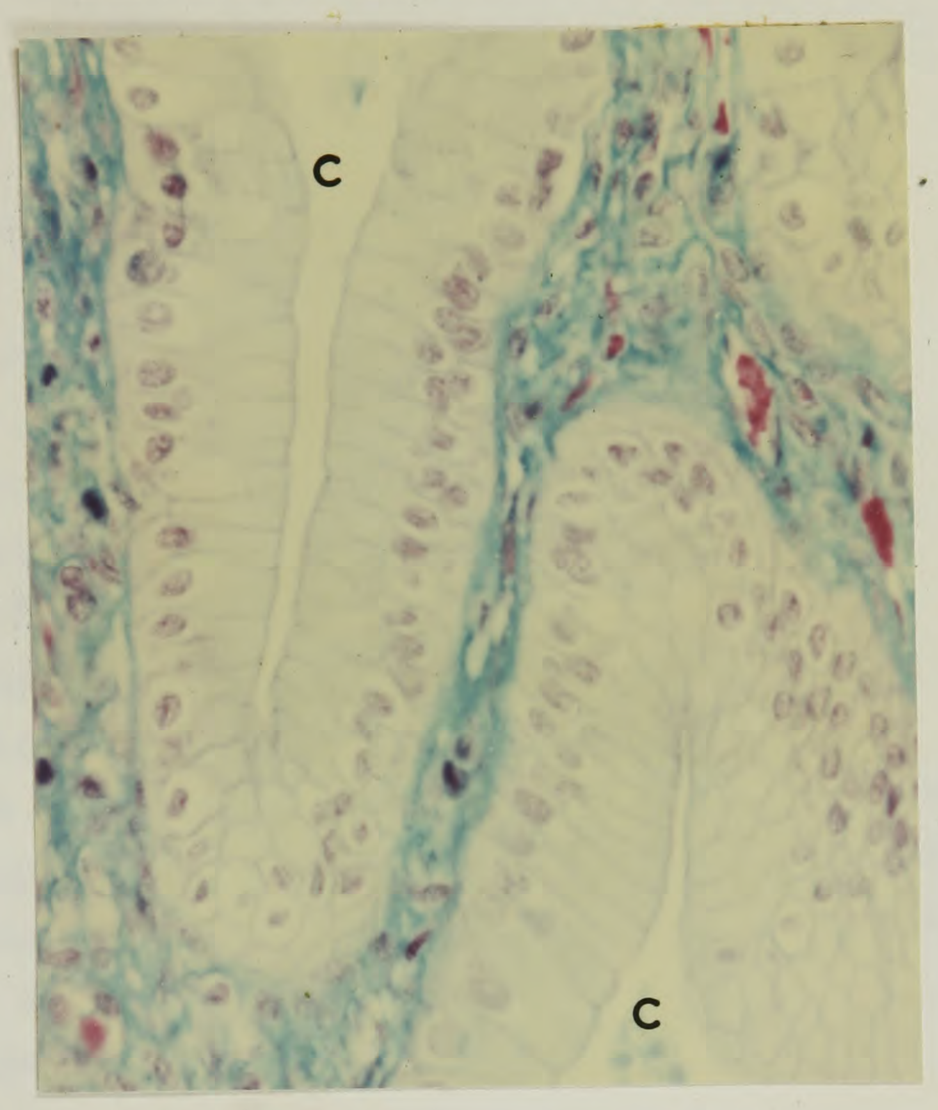


Fig. 3/4.

The Collecting Tubule at Three Levels.



Papilla
(4)

Fig. 3/5

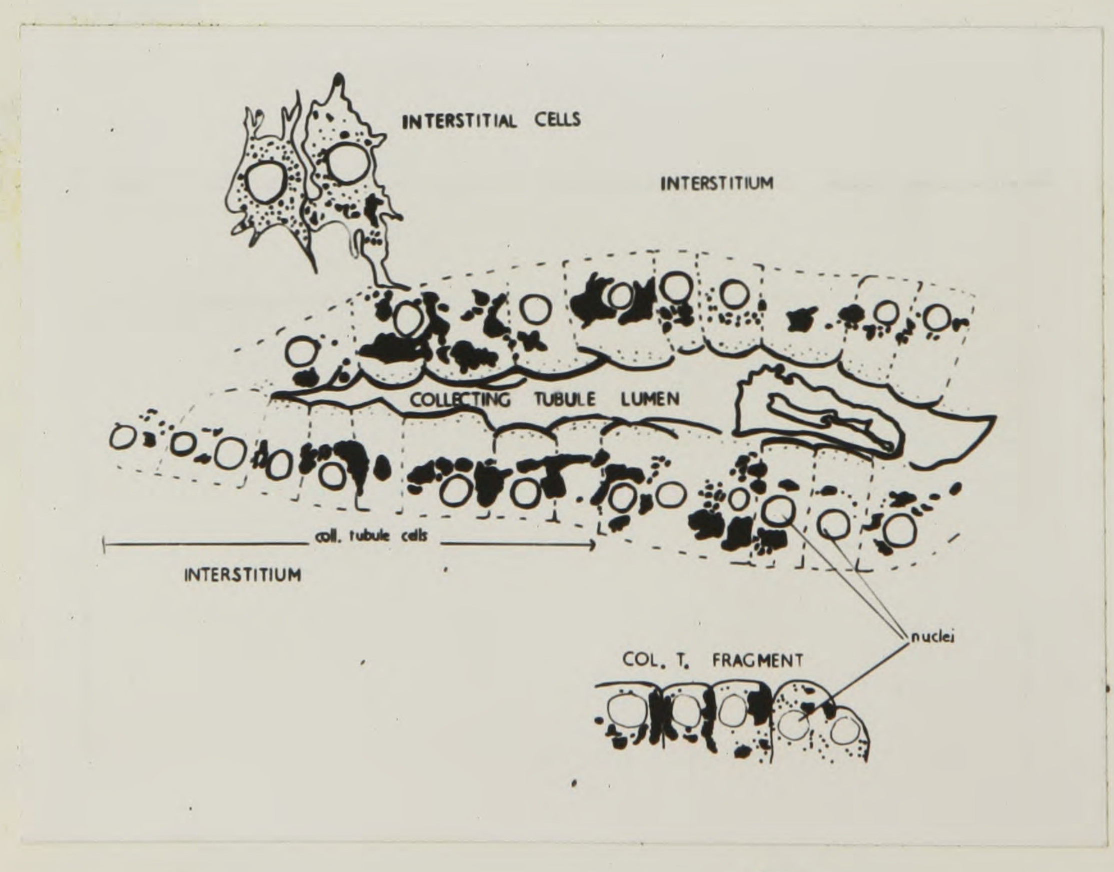
Modified Masson Trichrome Stain.   Mag. Appr. 800

P = Proximal Convoluted Tubules.
D = Distal Convoluted Tubules.
C = Collecting Tubule, near origin in Fig. 3/3.
T_k= Thick (Ascending) Limb of Henle's Loop.
T_h= Thin Limb of Henle's Loop.

Note red blood cells become crenated as the tip of the papilla is approached: At the same time the collecting tubule cells become columnar.

Darkground Appearances.

(a)



(b)

Fig. 3/6. Mag. 500.

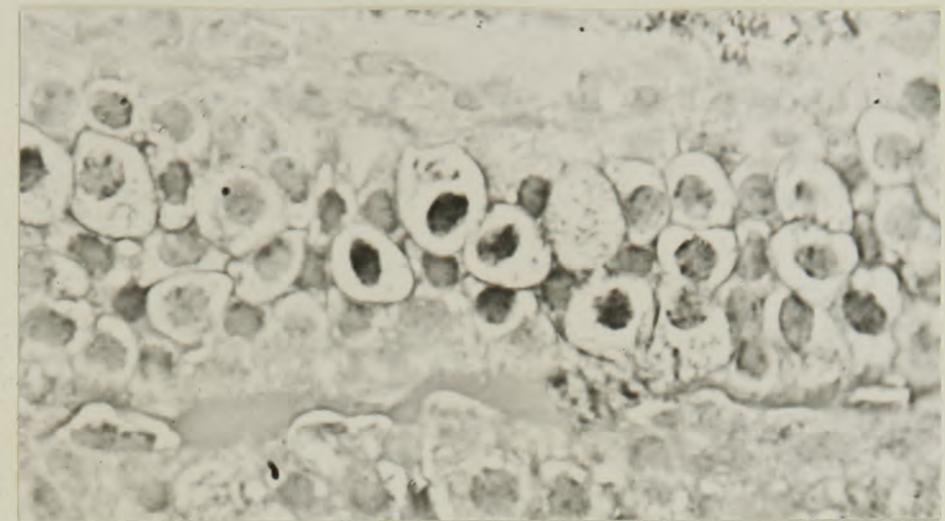
Even so, there are three discordant features: First, their embryological origin does not preclude an active function; it is the same as the cells which are known to pump salts and water actively from the gut lumen. Second, their nuclei are always large and have the prominent nucleoli characteristic of 'active cells'; they are therefore unlike the nuclei of cells impermeable to water in the upper layers of transitional epithelium which have pyknotic nuclei. Finally, the cells increase in height from cortex to papilla, where they are surrounded by the highest osmotic pressures and might be considered to be the least active. Thus in the present study an attempt has been made to reappraise the supposedly inactive part played by these cells in the physiology of the kidney. It was possible to observe these cells in vivo and to correlate some of their appearances with those seen in the static snapshot poses achieved by electron microscopy and histochemistry.

i) The Appearance of Collecting Tubule Cells 'In Vivo':

Tips of guinea pig renal papillae were gently homogenized in warm 2% saline to minimise osmotic swelling of the cells. A drop of the brei was then placed on a slide held in a simple warming device like that suggested by Schafer (1877); a coverslip was added, and the tissue fragments observed under darkground illumination. Groups of cells could readily be identified as having come from collecting tubules by virtue of their size and shape. It was thus possible to observe these cells with their full complement of intracellular ~~organelles, compounds etc.~~ components in a condition more akin to their natural state than that represented by frozen sectioning, freeze-drying or the paraffin

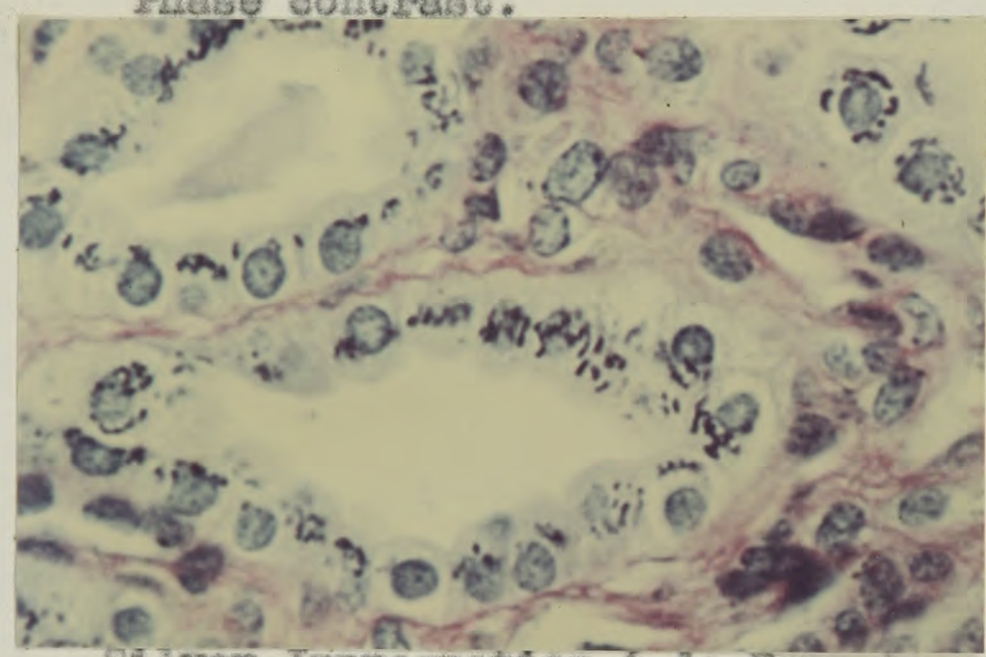
Golgi Region Appearances

Fig. 3/7



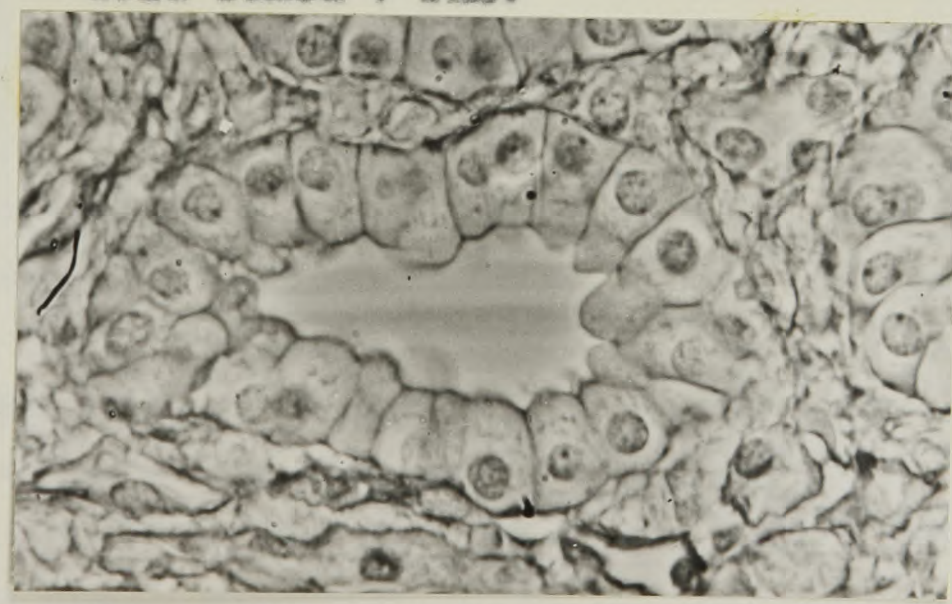
Homogenate + formol - saline.
Phase contrast.

Fig. 3/8



Silver Impregnation (da Fano),
Gold Toned + PAS.

Fig. 3/9



Phase Contrast Unstained
Dewaxed Section.

All Mag. 500

Golgi Region Appearances.

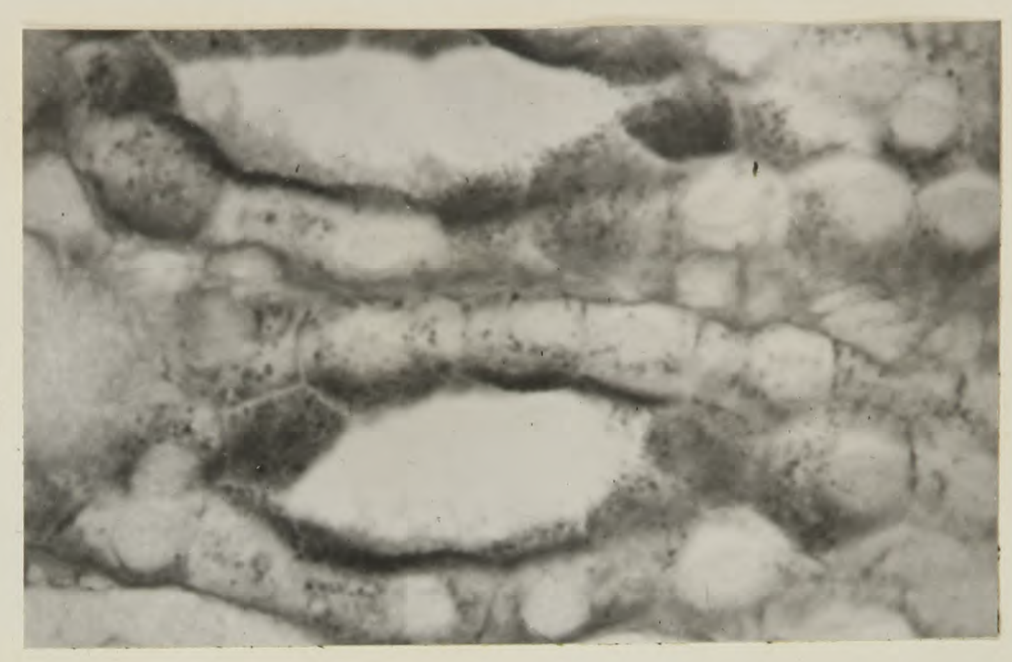


Fig. 3/10. Adjacent Section to Fig. 3/9.
Alcian Blue, pH 1 : Sulphate groups only.

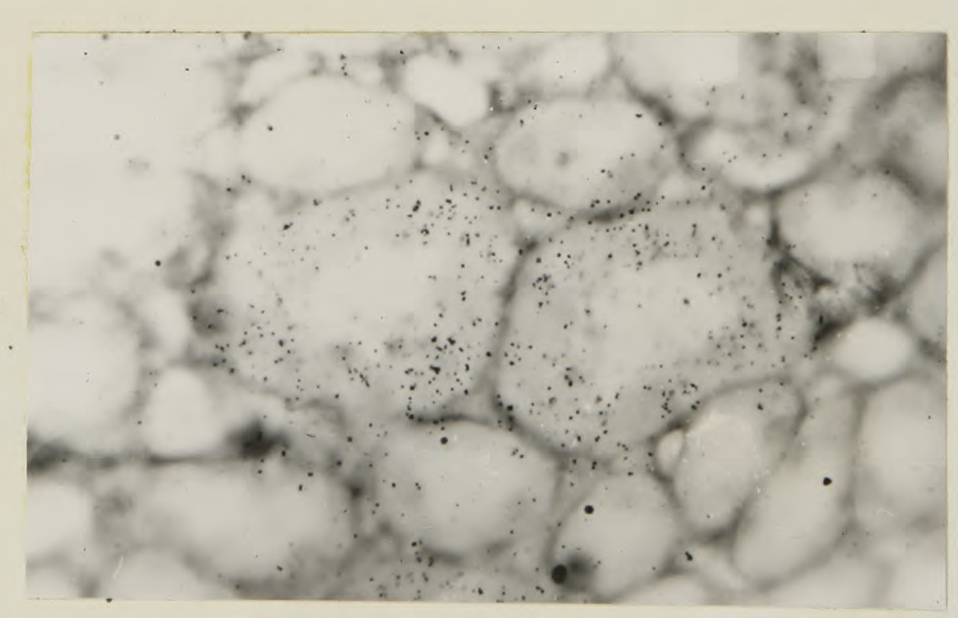


Fig. 3/11. Autoradiograph 1 hour after
Injection of $S^{35}O_4^{=}$. PAS.

Both Mag. 500.

routines of conventional histology. A further advantage of the method was that particles too small to be resolved by transmitted light microscopy were shown up.

The collecting tubules were seen to shine brightly as did material that had come from the tubule lumen (Fig. 3/6). As was pointed out by Frey-Wyssling (1948) the cytoplasm itself was optically clear in the ultramicroscope. While it is impossible to deduce the shape of a structure from its antipoint, the larger juxtanuclear particles were strikingly similar in their distribution to those seen in sections stained for acid mucopolysaccharides (Figs. 3/10 and 4/5 & 4/6), or those in tissue impregnated with silver by the da Fano technique (Gatenby and Painter, 1937) to demonstrate Golgi organelles (Fig. 3/8).

ii) The Extent of the Golgi Apparatus: Despite the early feelings of Baker (1949) that investigations into the nature of the Golgi elements were not of 'the calibre that would be recognized in any other branch of science', Gatenby (1955 (a) and (b))was able to observe the existence of a specialized juxtanuclear region in ultracentrifuged living cells. This was found to correspond with a fraction which could be isolated and examined biochemically by homogenization and centrifugation of cells. Further, the regions which could be impregnated by silver in sections corresponded roughly with the distribution of vacuoles in living cells and were found by Lacy and Challice (1956) to have a morphological counterpart in the electron microscope: To prove the point these workers

The Golgi Region.

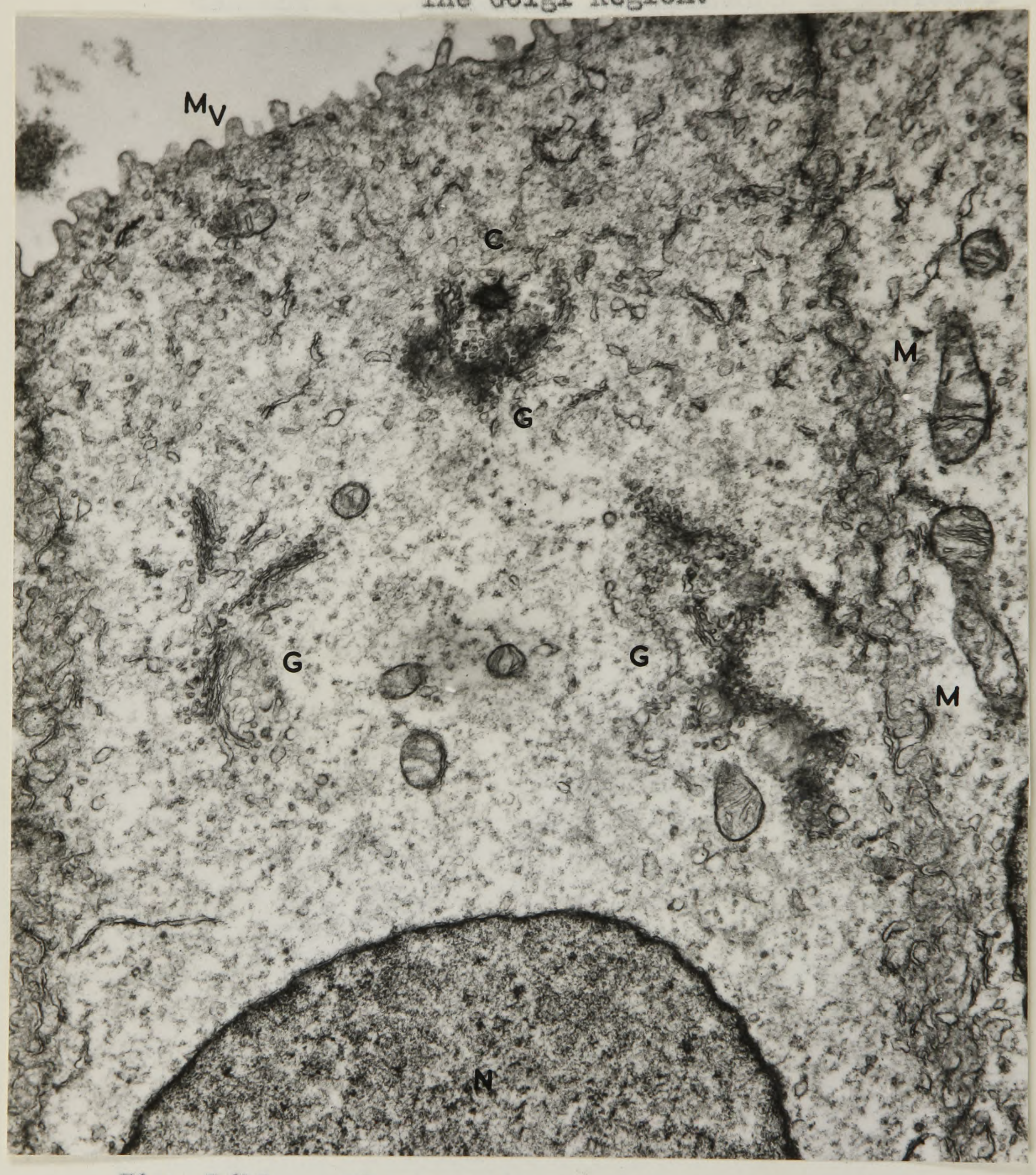


Fig. 3/12. Electronmicrograph Mag. 15,000.
M_V = Microvilli; C = Centriole; G = Golgi Region;
M = Mitochondria, near lateral border; N = Nucleus.

prepared electron microscopic sections of silver impregnated tissue and showed a perfect coincidence of the two.

The extent of the Golgi region in the collecting tubule cells has an importance in the present study because of the association between this region and the synthesis of acid mucopolysaccharides. For example, electron microscopical methods using colloidal iron (Wetzel, Wetzel and Spicer, 1966), or colloidal thorium (Berlin, 1967), to show up acid mucopolysaccharides have demonstrated the presence of polysaccharide precursors in the Golgi region of similar (gut) epithelial cells. Neutra and Leblond (1966 (a) and (b)) have tracked the synthesis of acidic mucins by this organelle by electron autoradiographic studies.

Figures 3/6 to 3/12 and 3/14 illustrate the various appearances of the Golgi organelle in guinea pig collecting tubules. It can be seen in the unstained preparation viewed by phase contrast (Figs. 3/7 and 3/9). It appears to be extensive in preparations successfully impregnated with silver (Fig. 3/8). It is associated with positive staining for sulphate groups (Figs. 3/10, ~~3/11~~ and 4/5) and with the incorporation of injected radioactive sulphate (Fig. 3/11). In the electron microscope the Golgi region is seen to occupy a large portion of the cytoplasmic volume between the collecting tubule nucleus and the lumen, and between the nucleus and the lateral intercellular space (Fig 3/12 and 3/17): Under no circumstances could it be found, or induced to take up a position, between the nucleus and the base of the cell.

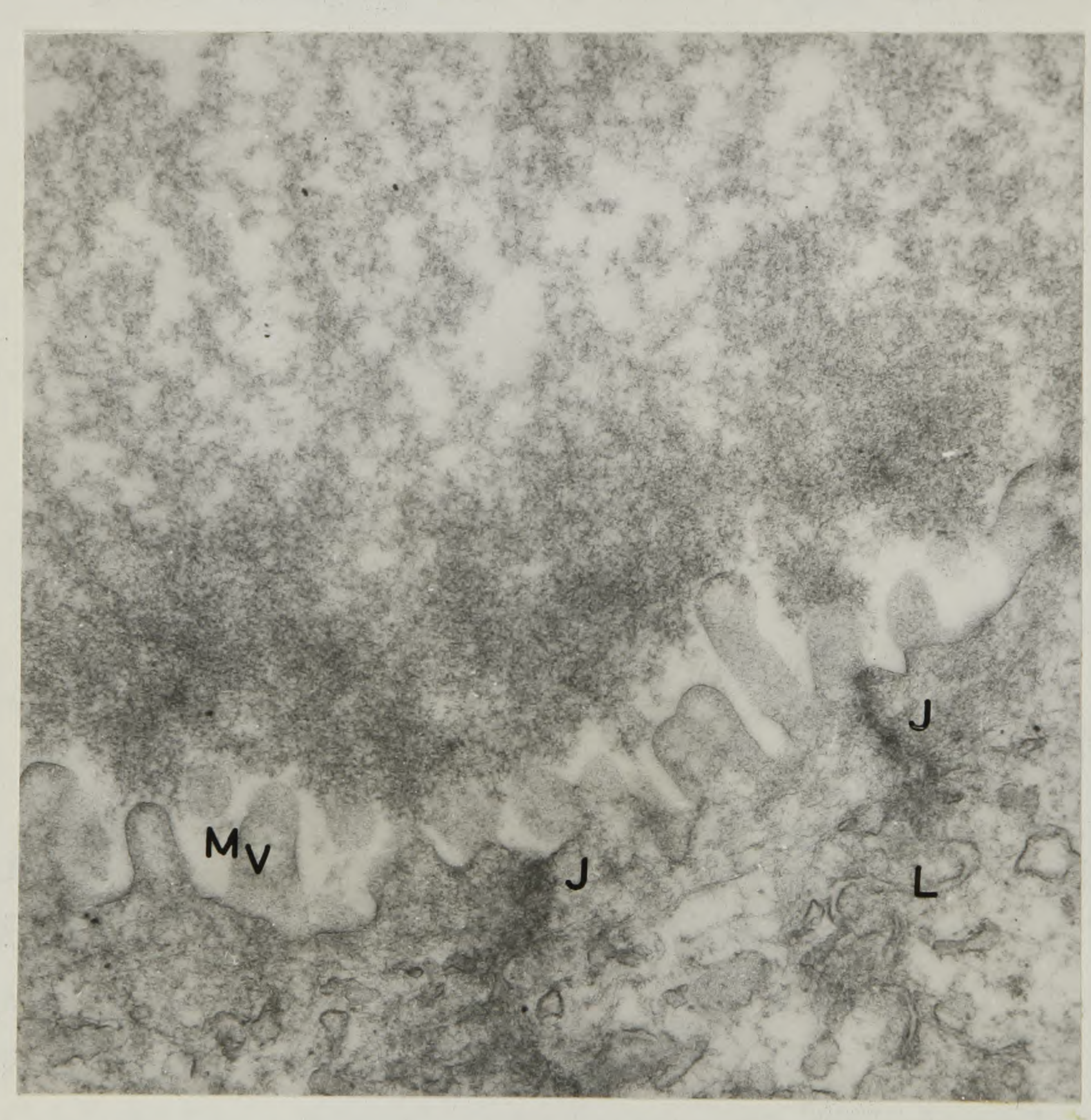


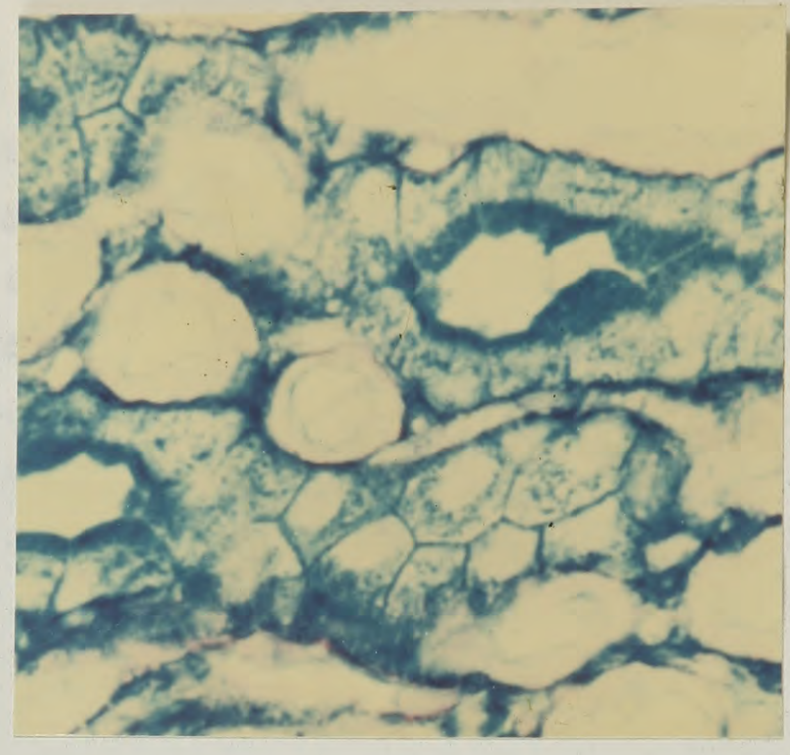
Fig. 3/13. Electronmicrograph Mag. 120,000. 300 mu Thick Layer of Lumenal fuzz. M_V = Microvilli; J = Junctional Complexes; L = Lateral Intercellular Channels.

The proven association between the Golgi region and acid mucopolysaccharide synthesis suggests that it may be important in the synthesis of the intercellular cement, which is central to the scheme of Ginetzinsky (1958). In this respect, the electron microscopic appearances are compatible with the secretion of products into the tubule lumen as well as into the lateral intercellular spaces. The presence of junctional complexes at the apical ends of the lateral intercellular spaces (Figs. 3/13 and 3/16) makes it unlikely that the material found between the cells (Fig. 3/17) could come from the lumen of the tubule and is much more likely to have been produced by the more lateral aspects of the Golgi complex (Fig. 3/16).

iii) The Nature of the Outer Surface: The electron microscope demonstrates the existence of microvilli along the lumenal border of the collecting tubule cells. These are about ½ μ long and about half this in diameter : In size they correspond roughly to the lateral cytoplasmic interdig - itations between the cells (Fig. 3/12). While these microvilli undoubtedly increase the surface area of the cells they cannot do so to the extent that they do in, say, the small intestine where they are much more tightly packed.

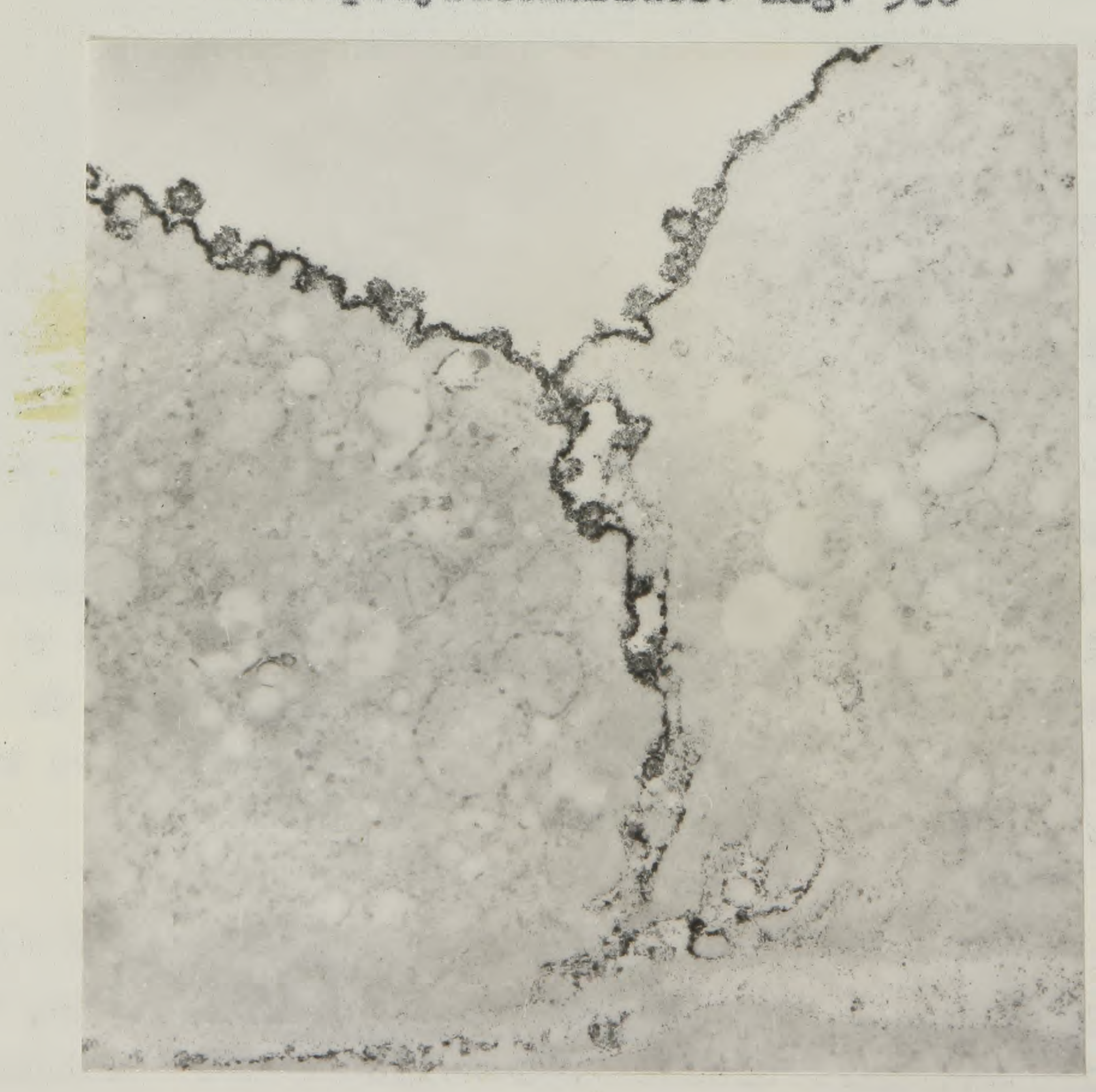
These microvilli were found to possess a surface coat (Fig 3/13) similar to, but more dense than, that on absorptive gall bladder epithelium (the 'antennulae microvillares' of Yamada (1955)) or on the intestinal

Fig. 3/14



Colloidal Iron Stain for Acid Mucopolysaccharides. Mag. 500

Fig. 3/15



Electronmicrograph of Tissue Treated with Colloidal Iron. Mag. 10,000.

epithelial cell brush border, (Ito, 1965). The strong binding of colloidal iron at this site in material processed both for light microscopy (Fig. 3/14) and electron microscopy (Fig. 3/15) is compatible with an outer coating of an anionic polysaccharide at this site. Thus this material might represent the so-called 'glycocalyx' described by Bennett (1963) which is believed to be more or less well developed around all cells.

iv) The Lateral Borders: A further simularity between collecting tubule cells and those of other epithelia known to transport water (eg. those of the gut and gall bladder) lay in the presence of junctional complexes at the apical end of the lateral borders of adjacent cells (Figs. 3/13 and 3/16). At the point of contact nearest to the tubule lumen, the outer leaflets of adjacent plasma membranes fuse to form a quintiple layered structure similar to the 'zonula occludens' described by Farquhar and Palade (1963). Below this there are usually two or more desmosomes followed by a tortuous intercellular gap: Near the apex this has a width of about 30 mμ but it widens to as much as ten times this towards the base of the cell. Because of its tortuosity the length of the intercellular gap is usually more than double the cell height (ie. a cell 18 μ long might have an intercellular space at its lateral border which approaches 40 μ in length).

If the tight junctions behave as those which look the same between endothelial cells do, then molecules

Fig. 3/16.
Mag. 15,000

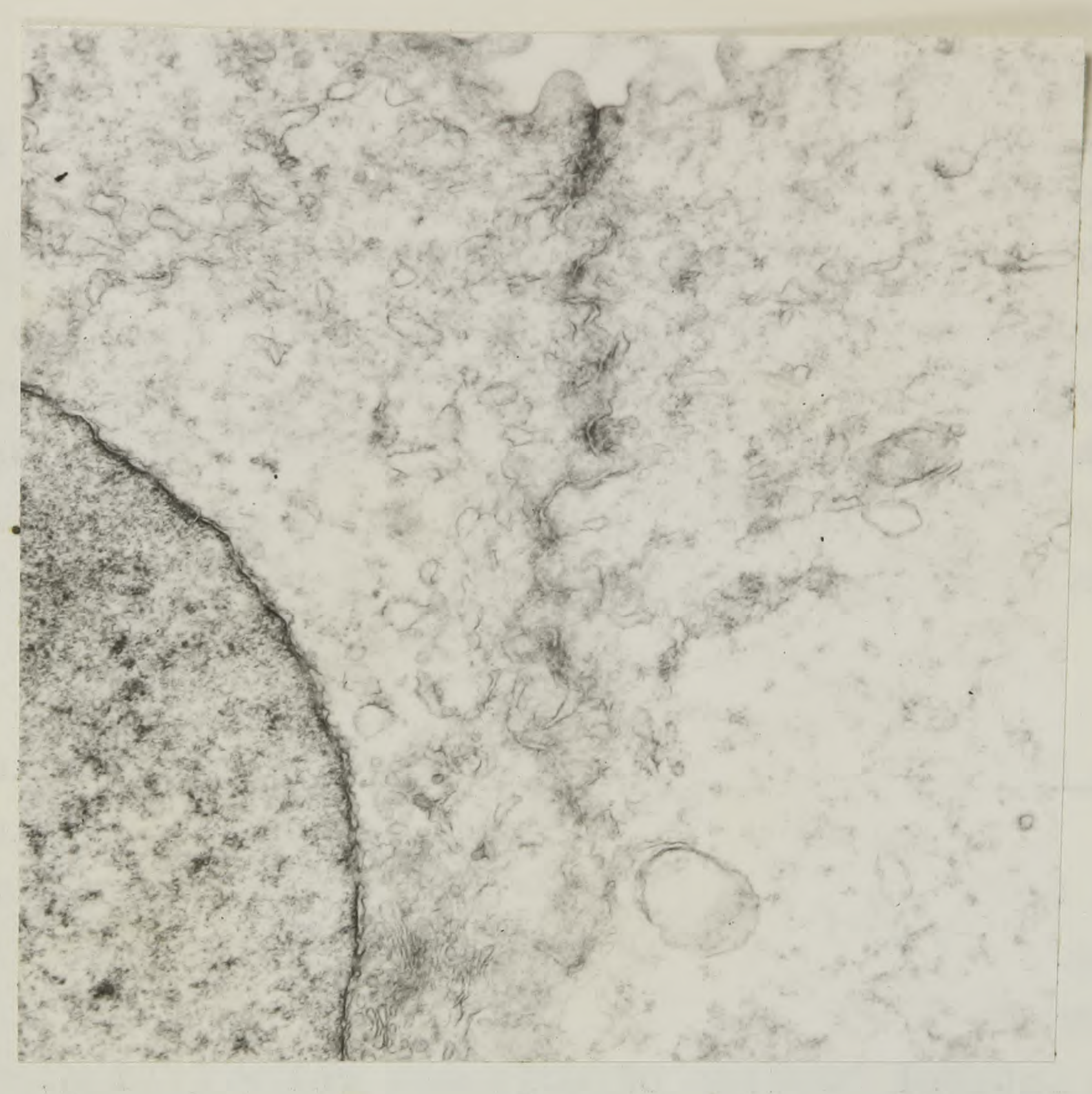
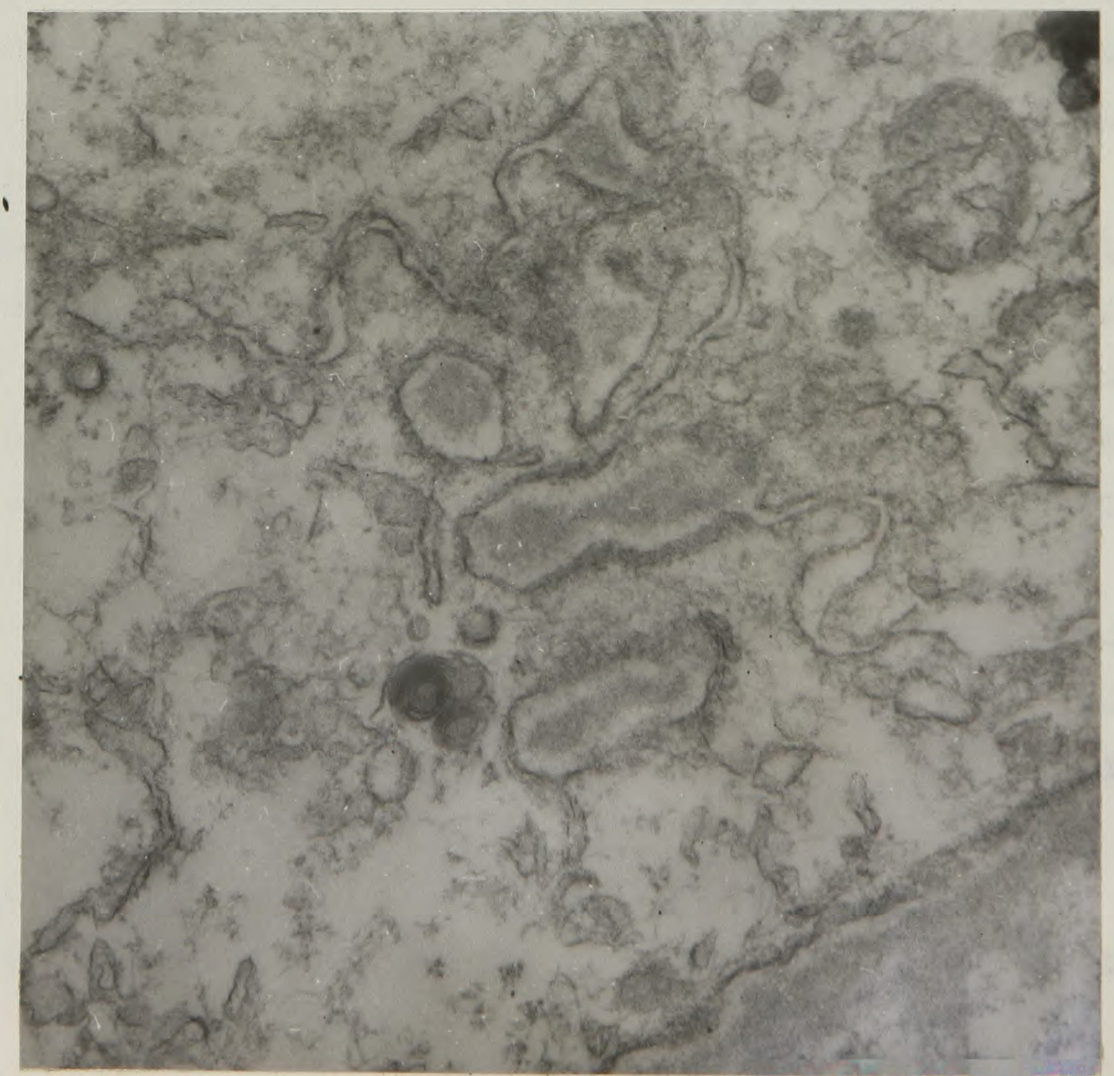


Fig. 3/17.
Mag. 30,000



below 20 to 70 Å in diameter might be expected to pass between the cells at this point, but not molecules larger than about 105 Å (Jennings and Florey, 1967). The permeability of the tight junction is of some interest since it was suggested by Ginetzinsky (1958) that hyaluronidase was released into the urine from the collecting tubule cells by apocrine secretion: It was then believed to depolymerize the intercellular cement. Thus it is possible that hyaluronidase might be able to pass the tight junction and gain access to the lateral border. However, there is no reason to think that it could do so with great rapidity in vivo since there cannot be any sort of pressure within the lumen of the collecting tubule to force it in an outward direction.

The material occupying the tortuous intercellular spave external to the junctional complexes has an amorphous appearance with an electron density similar to that of the 'fuzz' found on the surface of the microvilli. At both these sites the material is separated from the outer, electron dense leaflet of the plasma membrane by an electron clear gap of approximately 20 mu, across which run fine threads seemingly to attach the material to the cell surface.

Hydrolysis of the anchoring threads could be envisaged as a mechanism for increasing the effective lateral separation of collecting tubule cells. Alternatively, hydrolysis of the amorphous material might bring them together. Whichever is the case, attention is drawn to the fact that this intercellular distance exceeds the usual gap of 15 - 20 mμ between adjacent epithelial cells (Robertson, 1959).

Arrangement of Mitochondria.

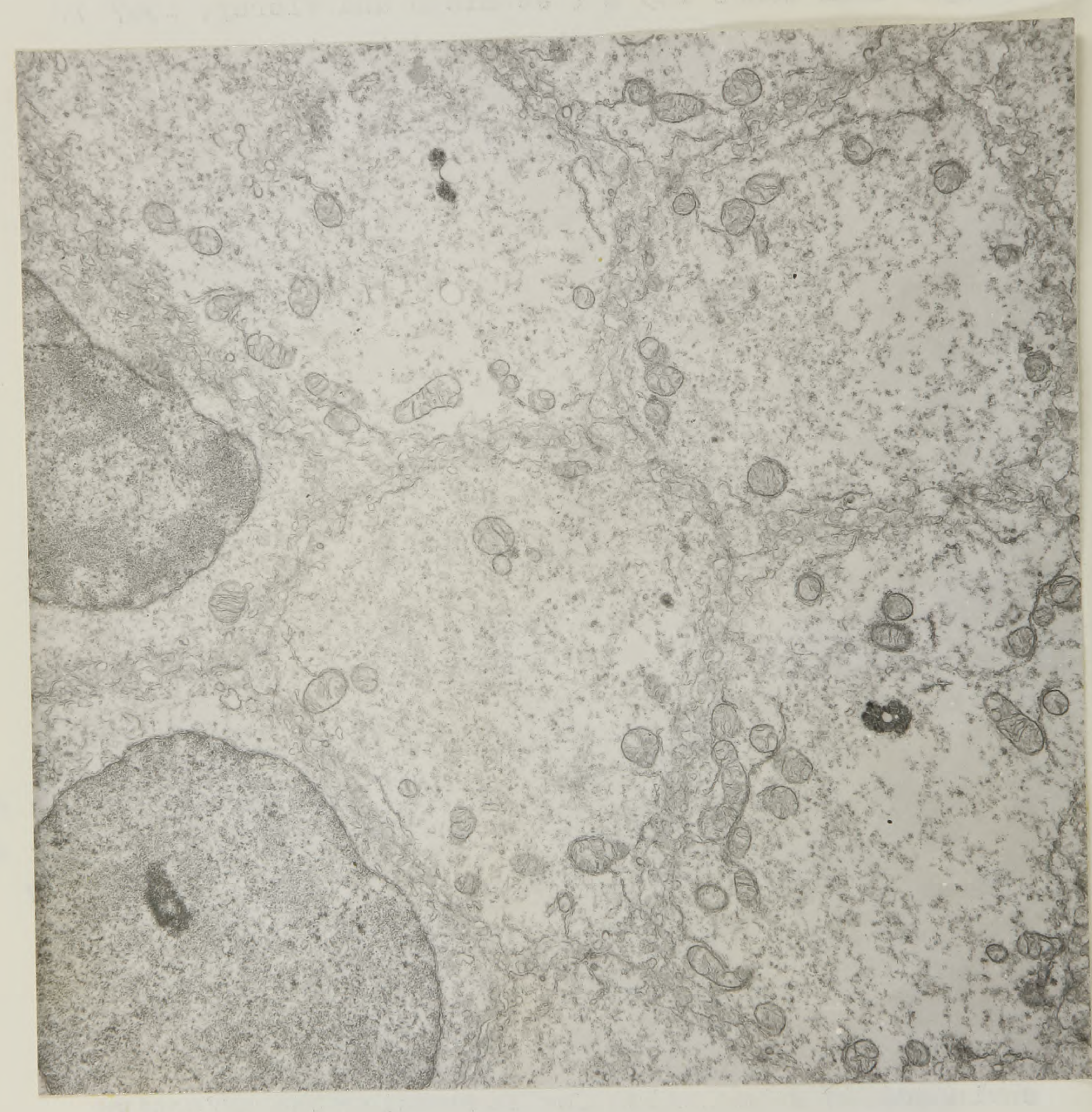


Fig. 3/18. Collecting Tubule Cells Transversely Cut. The Mitochondria are to be found near the cell membrane at the lateral intercellular spaces. Mag. 9,000.

Vesicles.

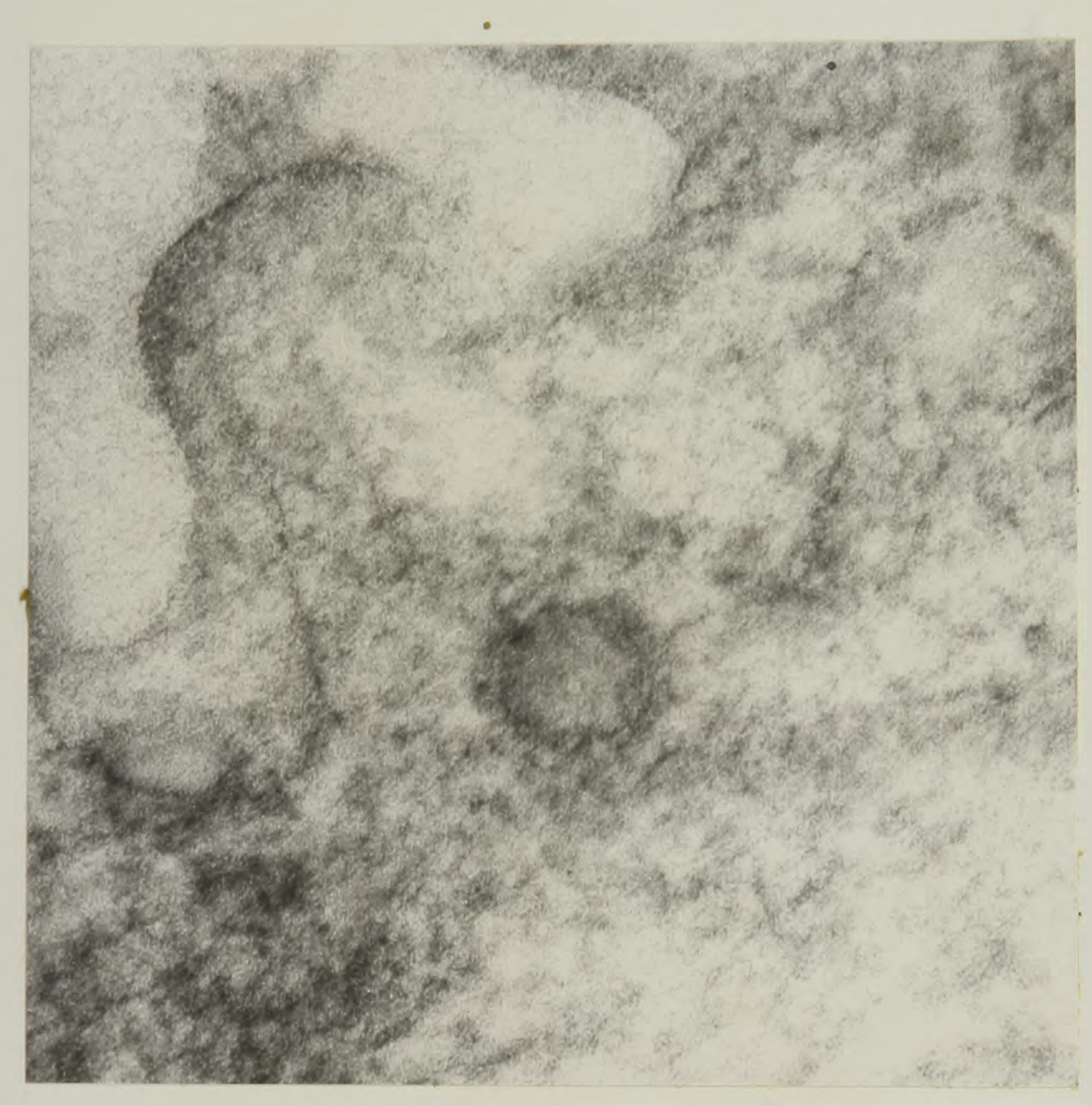


Fig. 3/19. Mag. 150,000

Vesicles.

Fig. 3/20.
Mag. 150,000.

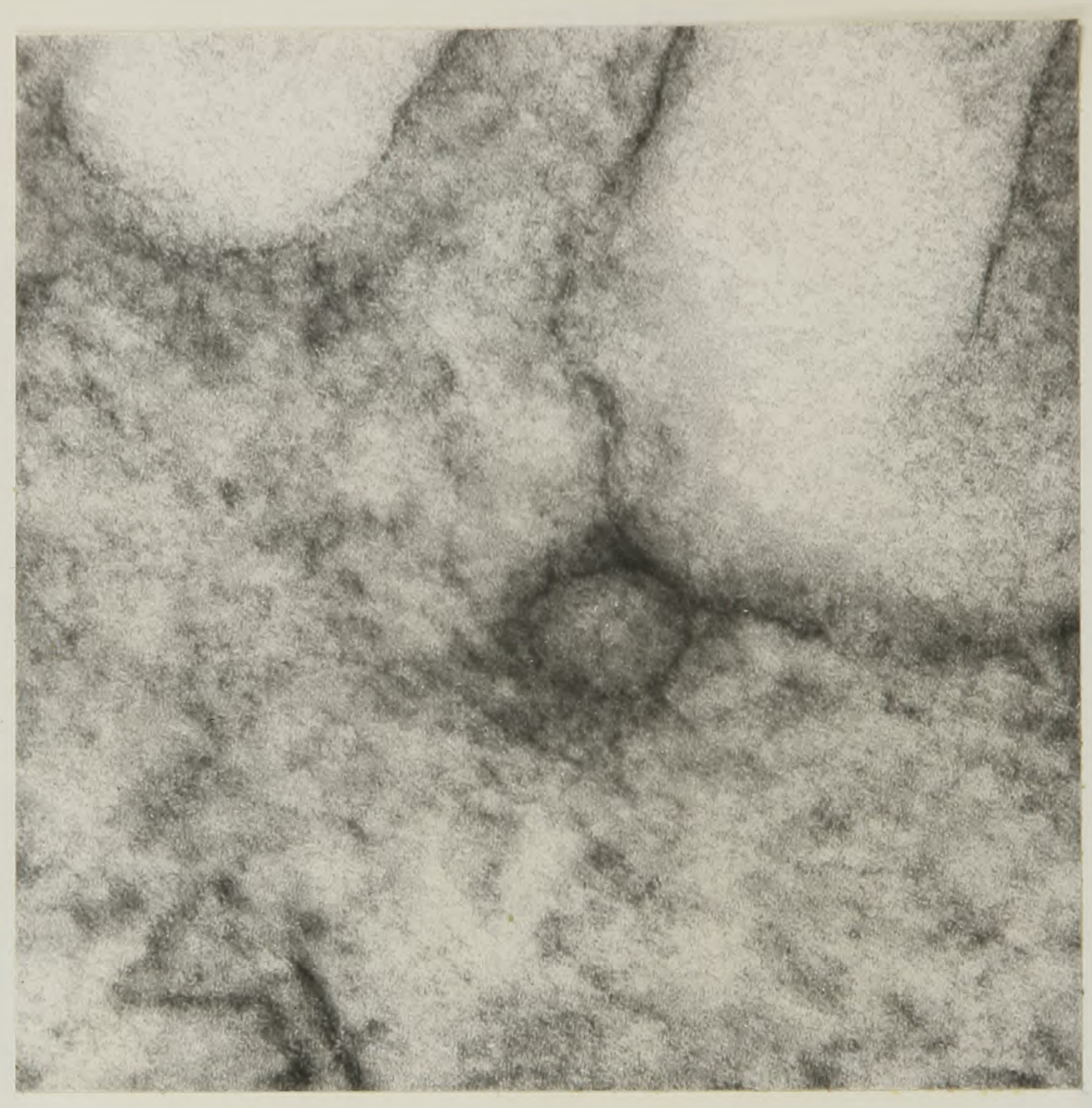
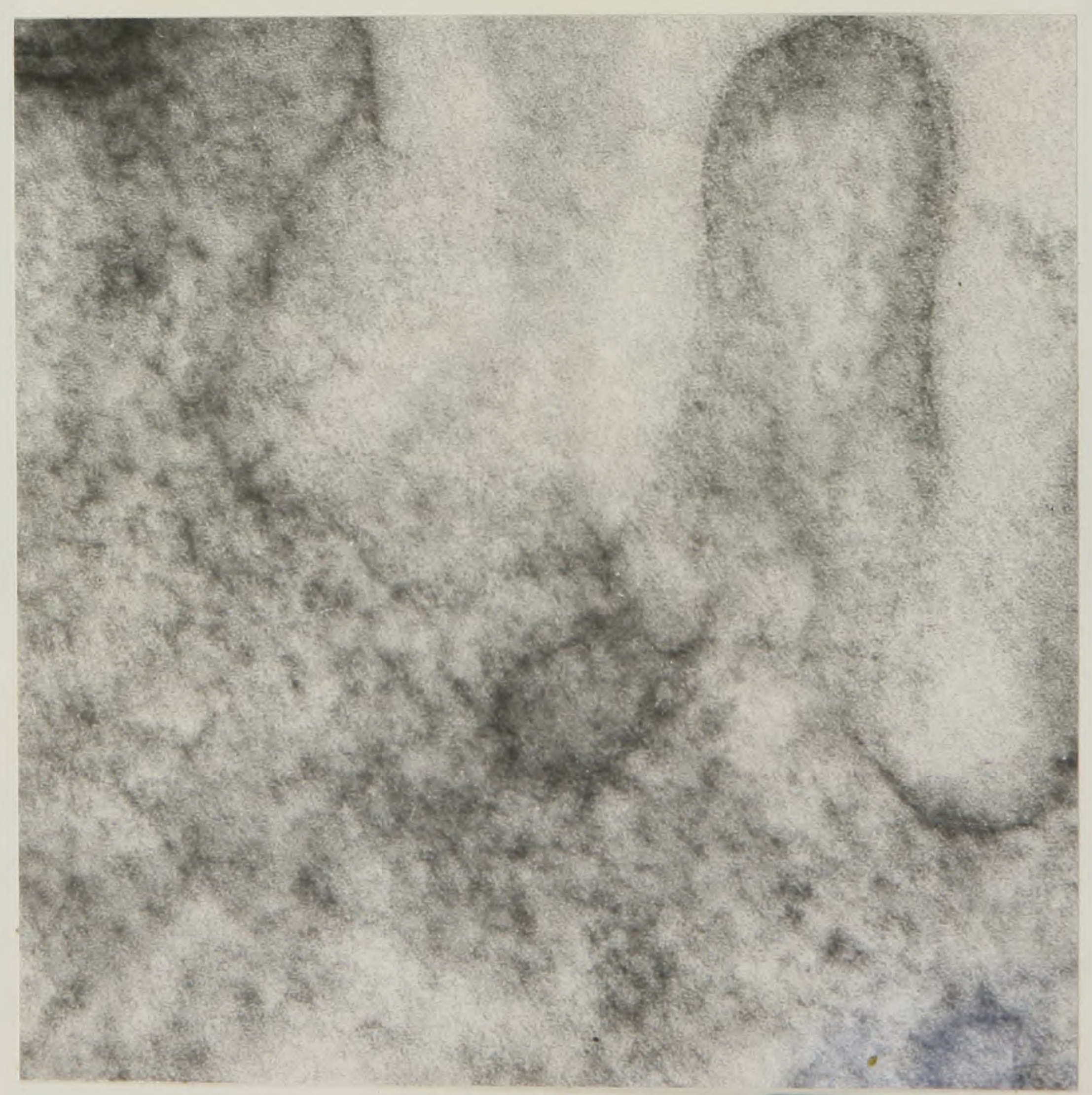


Fig. 3/21.
Mag. 150,000.



Enclosed Vesicles.

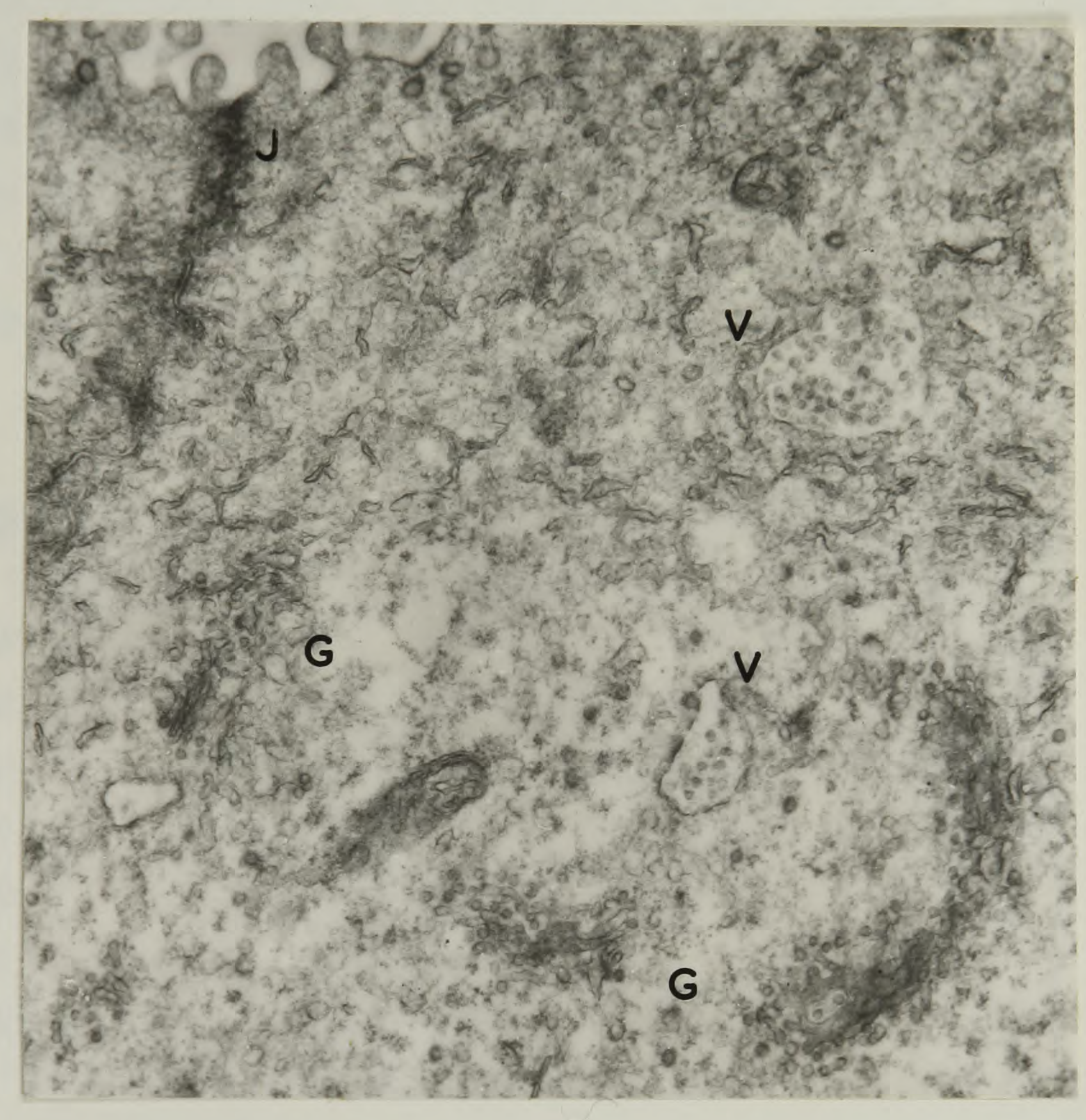


Fig. 3/22. Mag. 30,000.

V = Vesicles.
J = Junctional Complex.
G = Golgi Region.

Enclosed Vesicles.

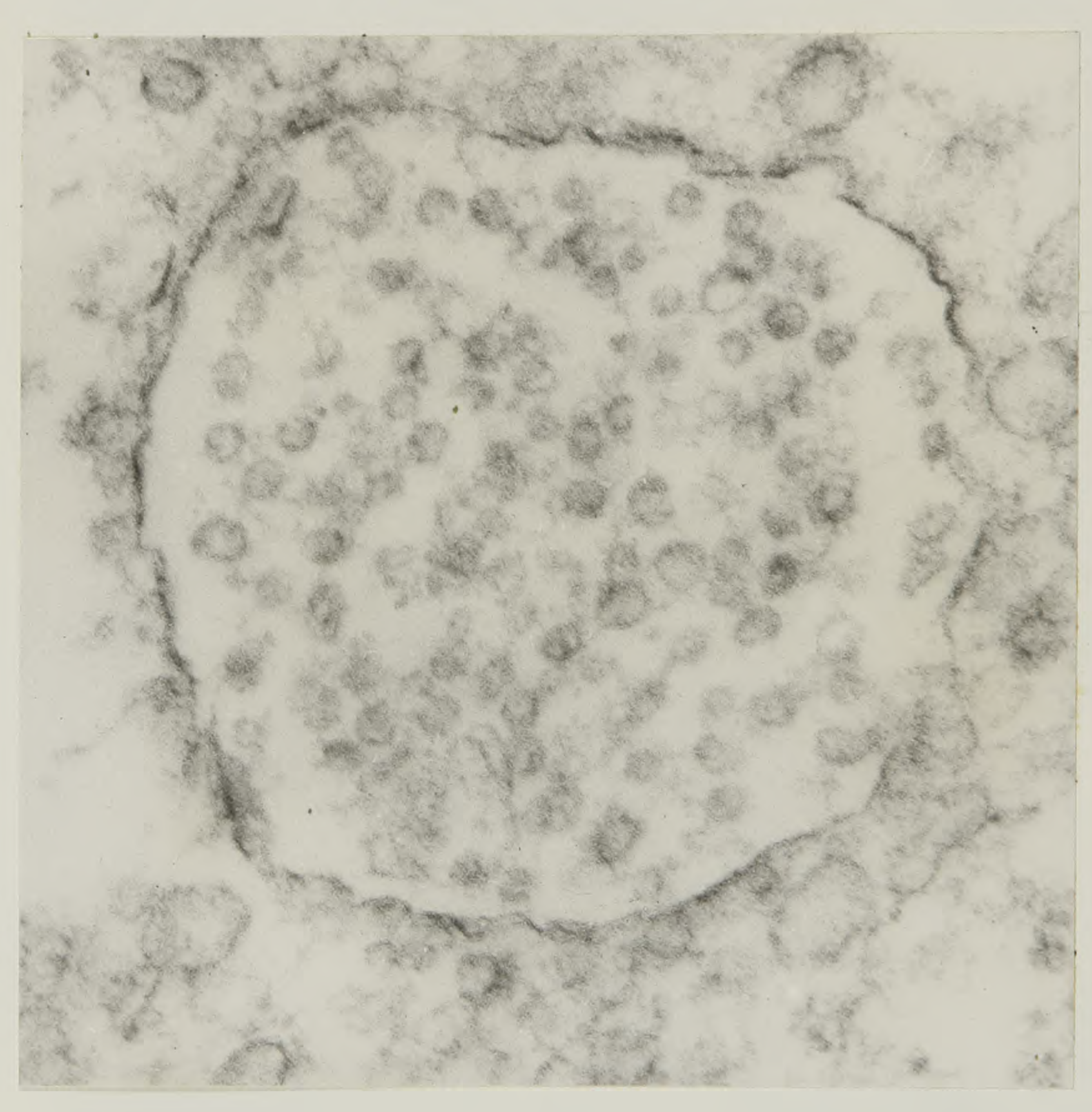


Fig. 3/23. Mag. 60,000.

Total dia. of large vesicle is about 1 μ. That of the smaller vesicles is about 60 mμ.

Transverse sections taken near the base of the collecting tubule cells showed a peripheral arrangement of the mitochondria adjacent to the plasma membrane (Fig. 3/18). This arrangement, which is also seen in striated duct cells, oxyntic cells and renal convoluted tubule cells, suggests active sodium transport. However, it might be significant in this case that each mitochondrion appears also to share a relationship with a fragment of endoplasmic reticulum.

v) Vesicles: Vesicles (70 mu in dia.) were frequently noted in communication with the tubular lumen (Figs. 3/19, 3/20 and 3/21). At other sites such structures have been associated with the absorption of large molecules (eg. in the proximal convoluted tubules; Fawcett, 1965) although in the electron microscope there can be no direct demonstration as to whether they have an absorptive or secretory function.

Collections of enclosed vesicles were also noted (Figs. 3/22 and 3/23). Two or three such structures could usually be found, usually between the Golgi complex and the tubule lumen, in a section only 50 mμ thick. The individual vesicles have a diameter of about 60 mμ: The collection of vesicles in Fig. 3/23 has a total diameter of 1μ and it can thus be calculated to contain as many as 100 of the smaller vesicles within its volume.

Aryl Sulphatase.

Fig. 3/24. COLLECTING TUBULE; Mag. Appr. 1,000 (Water Immersion). Aryl sulphatase activity demonstrated by the method of Rutenburg, Cohen and Seligman (1952).

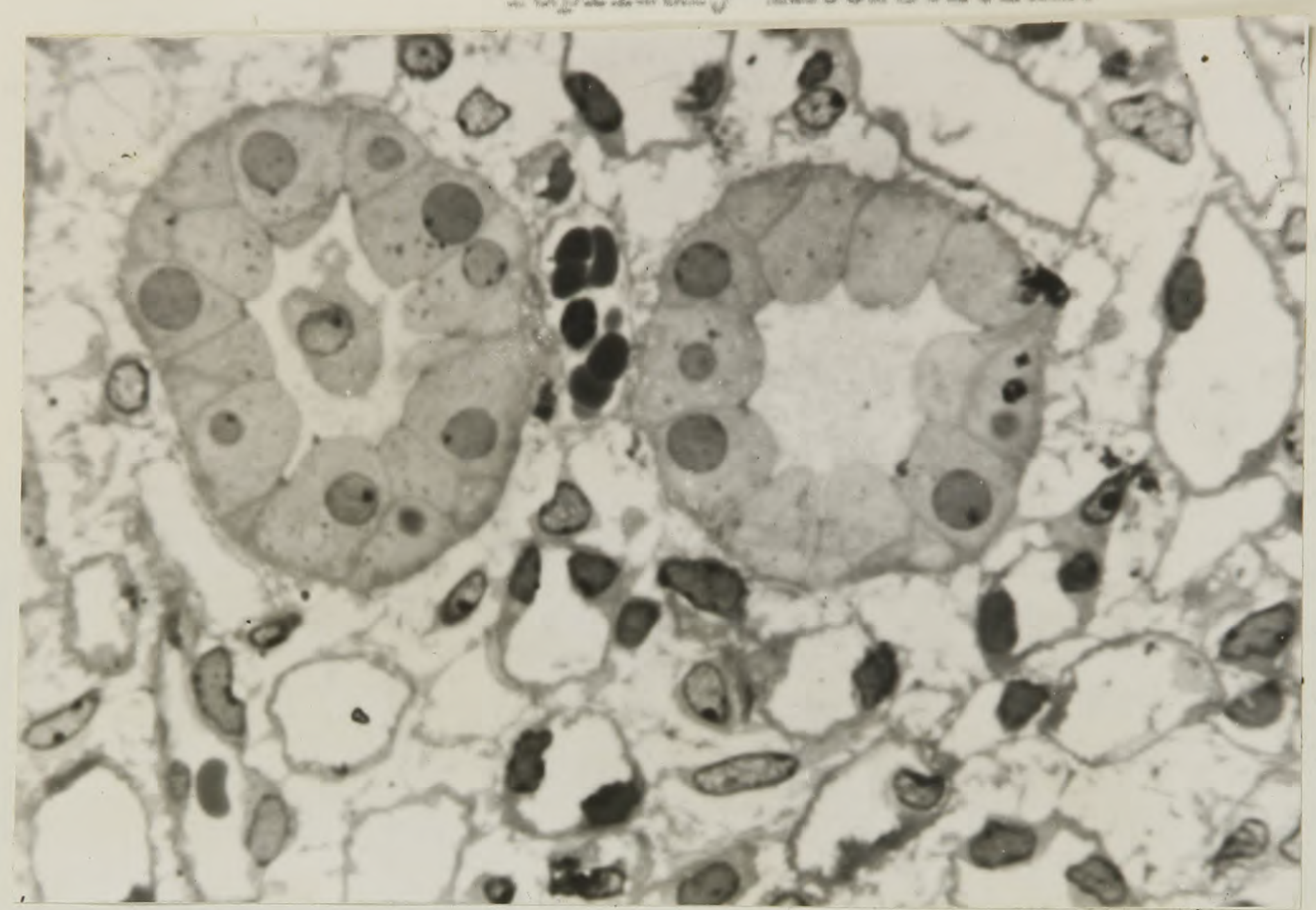
Dicker and Franklin (1966) biochemically demonstrated the existence of an acid hyaluronidase in kidney tissue and, in the present study, it was found that the collecting tubule cells showed weak acid phosphatase and aryl sulphatase activity (Fig. 3/24). It is thus tempting to suggest that the multivesicular bodies could be lysosomes. However, the activity of the usual lysosomal enzyme, acid phosphatase, was not impressively demonstrated by the conventional histochemical methods applied to collecting tubule cells: Unless these 'lysosomes' have a high ratio of hyaluronidase - for which there is as yet no histochemical method - to other hydrolases it is difficult to conceive of a very active role for them in the physiology of the kidney.

In conclusion, then, it will be appreciated that the relatively new techniques of histochemistry and electron microscopy have done much to dispel the impression of 'inertness' associated with these cells. The limited methods available to light microscopists at a time when physiologists were elucidating the functions of each portion of the nephron probably gave rise to a widespread impression that the collecting tubules have relatively little function.

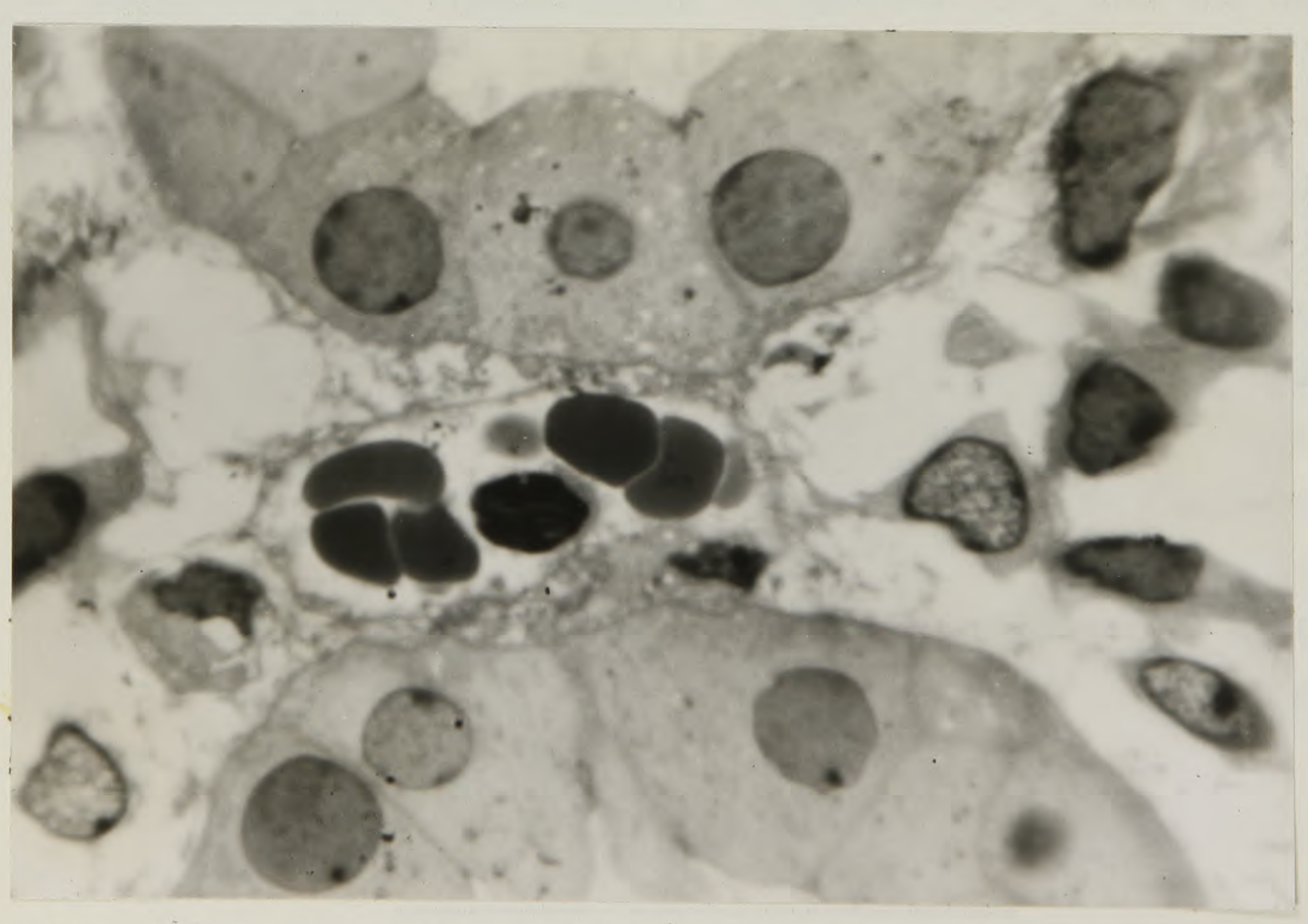
B. The Interstitium.

i) The Cellular Component. The fine cytoplasmic processes of the cells in the interstitium of the renal papilla prevents an adequate

Papillary Interstitium.



(a)
Mag.
1,000



(b)
Mag.
3,000

Fig. 3/25. Light micrographs of Araldite sections illustrating some of the fine processes of the interstitial cells, and proximity of collecting tubules to a vasa recta. The clear intertubular spaces are filled with hyaluronic acid. Richardson's stain.

study by light microscopy of the whole morphology of the cells even in thin sections (Fig. 3/25). It is even more difficult to appreciate the three dimensional ramifications of these cells from the electron micrographs (Figs. 3/26, 3/30 to 3/33). It is thus not easy to understand how the structural elements seen within an interstitial cell might function in relation to that cell, the extracellular colloid around it, or to the whole complex of tubules within which it lies. Without doubt these cells regulate the separation between certain tubules in the papilla, but histology is unlikely to reveal whether this is by chemical means (eg. by the release of enzymes to destroy the extracellular colloid with subsequent resynthesis etc), which would determine the resistence offered to the flow of water and ions, or by physical means (eg. by contraction in response to vasopressin, pulling certain tubules more closely together). Thus, the following description is a deliberate simplification dealing with the interstitial cells in so far as they show any evidence of a regulatory function over the extracellular acid mucopolysaccharides.

It was found by light microscopical methods that there was only one ~~true population of~~ essential cell type within the guinea pig papillary interstitium. There was, however, a spectrum in the histochemical staining of acid mucopolysaccharide within the cells such that those in the medulla might be compared to fibroblasts while those at the papillary tip had more in common with mast cells (Figs. 4/12 & 4/13): This gradation was continuous and at neither end were the appearances of the interstitial cell incompatible with its having either of the implied functions.

Nearly always these cells were seen in the electron microscope to be surrounded incompletely by 'basement membrane' material which was characteristically multilayered at several points (Fig. 3/31). This appearance became more striking when the animal was given a water load before it was killed: It was rarely seen in animals deprived of water (eg. Fig 3/32). The Golgi regions always seemed to be extensive in water loading experiments, but by no means absent if the animal was deprived of water: A difficulty arises in the interpretation of this aspect by virtue of the small volume of a whole cell visualized in an electron micrograph and from the considerable variation between cells. However, such appearances suggest that the interstitial cells are active in mucopolysaccharide synthesis, and that they thus control some of the physical properties of the extracellular space.

It was not possible by the use of light microscopic autoradiography to observe the synthesis of acid mucopolysaccharides by these cells since their cytoplasm is too attenuated to allow a distinction to be made as to whether radioactivity is intra - or extracellular. While the granules within these cells at the papillary tip do stain metachromatically, radioactivity following sulphate injection never appeared in the interstitium before it did in the collecting tubules: Thus, if these cells do incorporate sulphate they must do so more slowly. Alternatively, the possibility is not excluded that the intracellular granules were derived from the extracellular material.

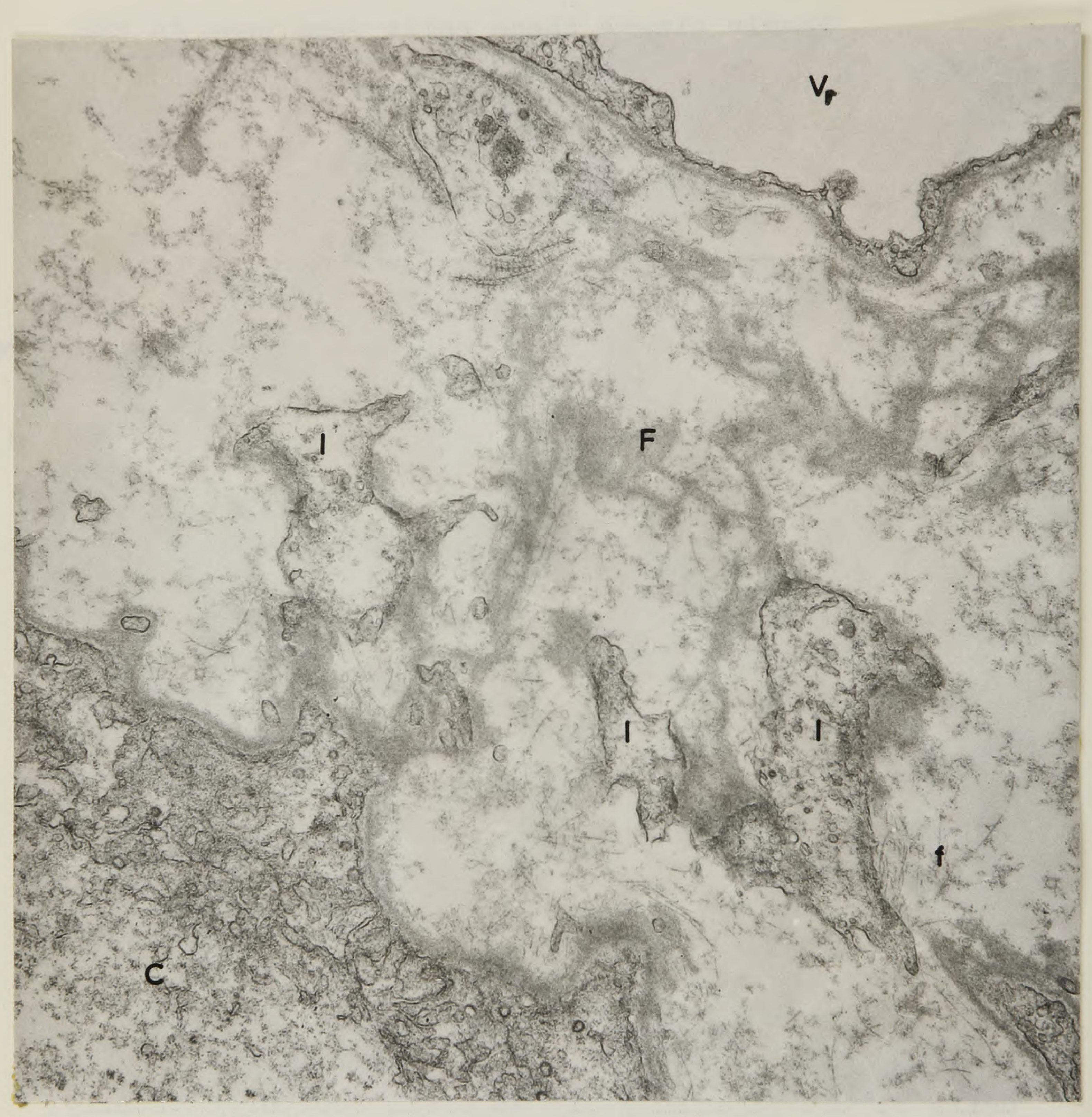


Fig. 3/26. Mag. 15,000.

V_r = Vasa Recta. C = Collecting Tubule Cell.
F = Coarse Fibrils.
f = Fine fibrils. I = Part of Interstitial cell.

It was impossible in this study to detect any gross alteration in the configuration of the interstitial cells themselves when the level of hydration of the animal was altered. This was largely due to the difficulty experienced in trying to define what should be the morphology and size of an interstitial cell under normal conditions.

ii) <u>The Fibrillar Component:</u> When a water molecule has reached the basal end of a collecting tubule it has a relatively long way to travel before it gains access to the lumen of a vessel which will carry it away from the papilla (Fig 3/25). This is quite a different situation to that which obtains in the cortex where the post - glomerular vessels lie in close relation to the convoluted tubules.

The interstitium across which the molecule has to travel may be as much as 6 to 10 μ thick. It is composed of hyaluronic acid (cf. Chapter 4) confined in a felt-like mesh of two types of fibril. One class of fibril can be impregnated with silver (Figs. 2/19 to 2/21), is PAS positive (Fig 3/8, 3/28 and 3/29), can be made (orthochromatically) basiphilic by immersion of sections in chlorosulphonic ~~acid~~ acid (Pearse, 1960) (Fig. 3/27), and has a characteristic banded structure seen in the electron microscope (Fig. 3/26). All these properties identify it as reticulin. It was seen to have a definite pattern around certain thin walled tubules within the guinea pig papilla (Figs. 3/27 to 3/29): In a transverse

Reinforced Tubules.

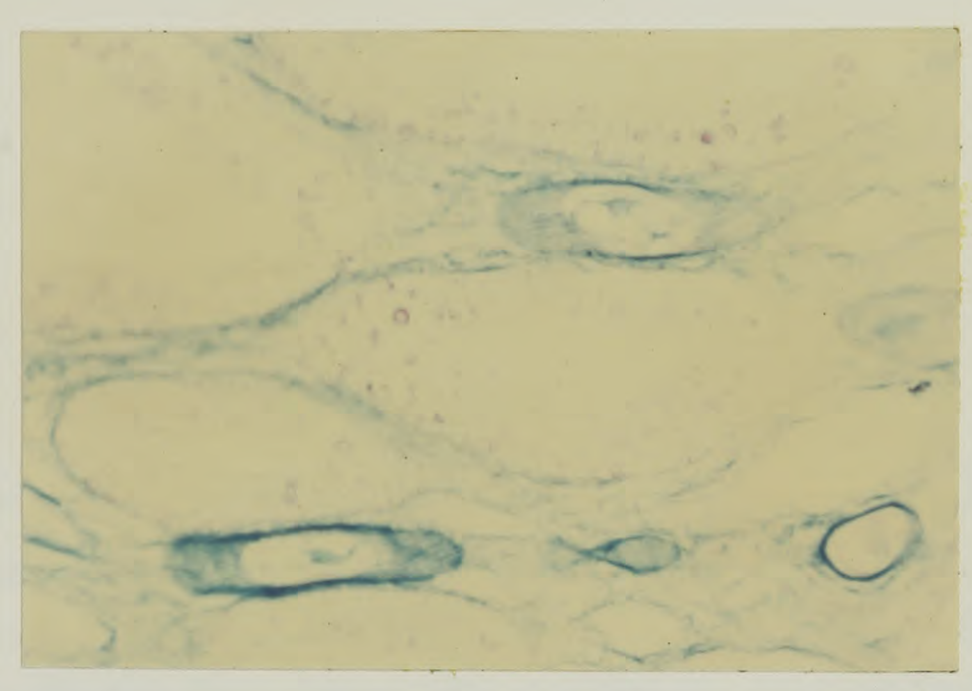


Fig. 3/27. Tissue Sulphated by Immersion in Chlorosulphonic acid and then stained in Azur A. One 'reinforced tubule' has been sectioned in three places, and another appears at top centre. The winding stains orthochromatically. Collecting tubule glycogen is rendered metachromatic.

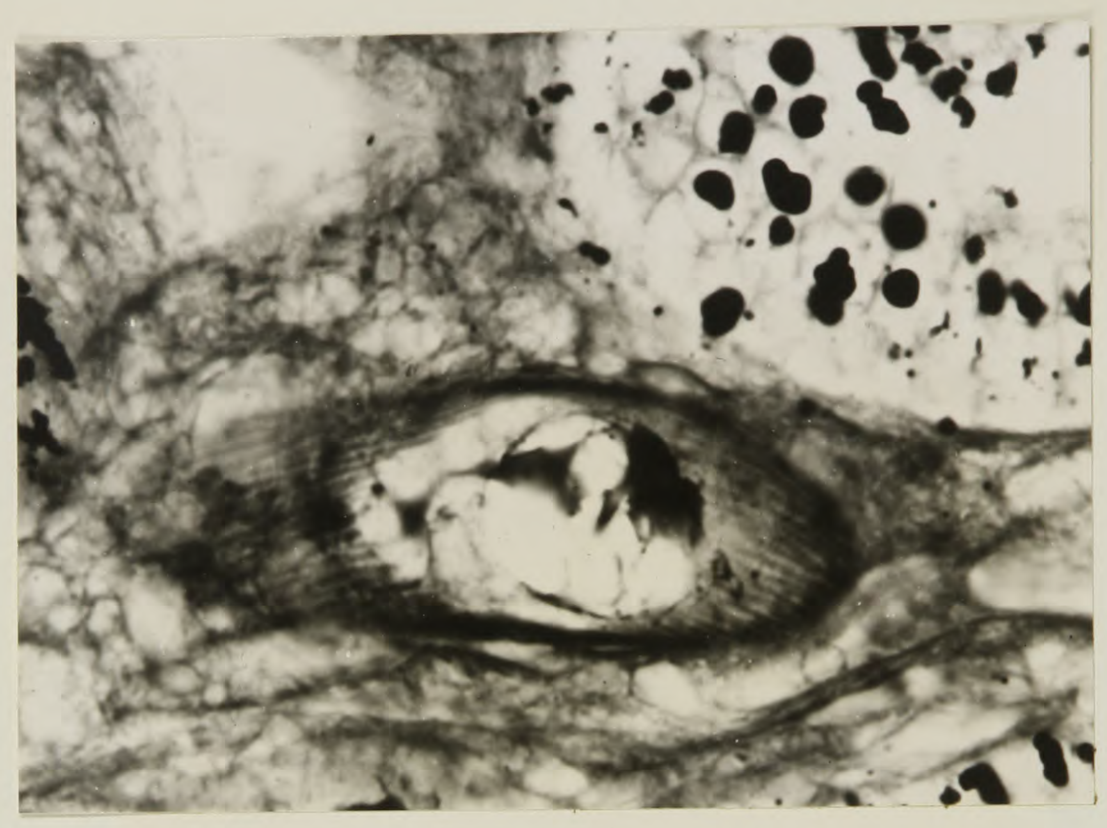


Fig. 3/28. Periodic acid - Schiff Stained.
(The use of acridine orange in the histological fixative has resulted in gross swelling of the collecting tubule glycogen.)

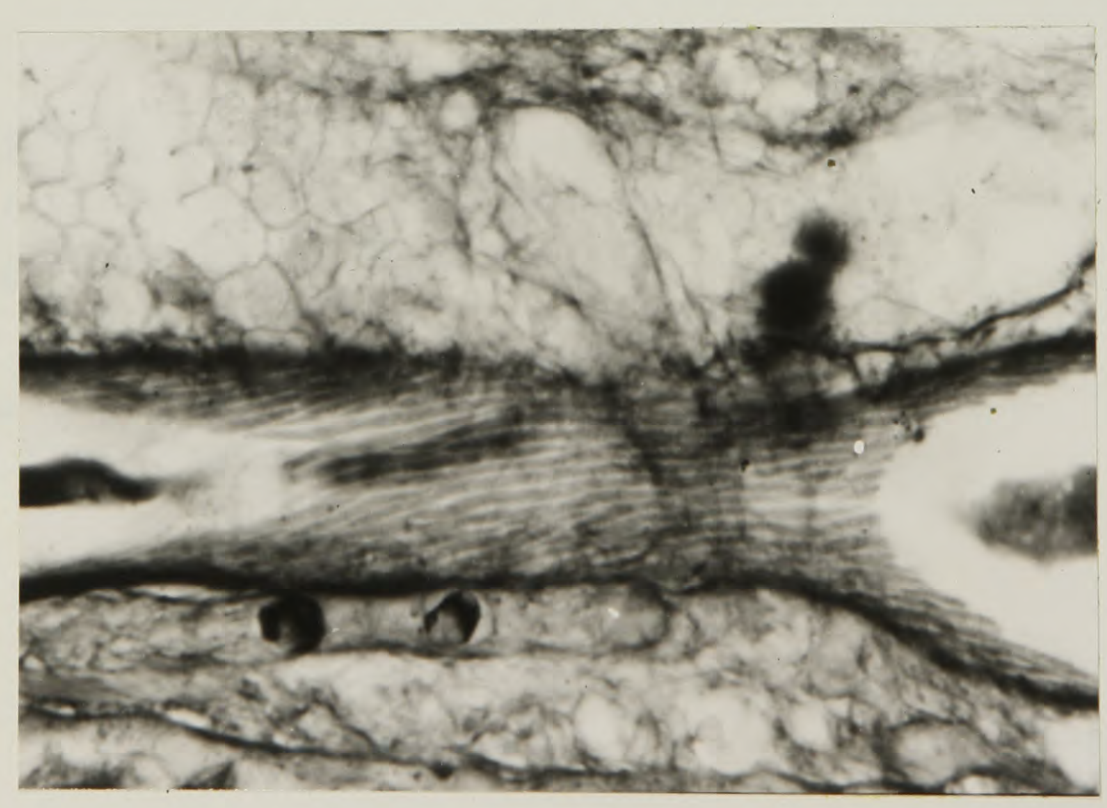


Fig. 3/29. Amylase Digestion followed by PAS.
Collecting tubule glycogen removed, but staining of winding unaltered.

section near the tip of the papilla, there would be roughly two tubules with this outer spiral structure to five collecting ducts and thirty six thin walled vessels (Ie. vasa rectae, loops of Henle and, possibly, lymphatics). It was a frequent finding that the lumen of these vessels would contain material that stained positively by PAS and for acidic mucins. The comparative rarity of this specialization of the reticulin made it difficult to identify with certainty the tubule which it surrounded. The best methods for reticulin demonstration in the light microscope are not the best methods for the preservation of morphology.

Within the reticulin was another class of fibril which did not have a banded structure as seen in the electron microscope, (Fig. 3/26), and was smaller in diameter. These very fine fibrils were seen in the light microscope to stain positively for sulphate groups (Fig. 4/7). They may also be the fibrils that stained for anionic groupings after the interstitium had been exposed to the action of hyaluronidase. In this case, however, it is not certain that all the hyaluronic acid associated with the reticulin had been digested, since there are more fibrils present and most are coarse, (Fig. 4/11).

Although interstitial cells often contained organelles which might be interpreted as enclosing immature fibrils (Fig. 3/32) the role of these cells in fibrillogenesis was not considered.

The Interstitium.

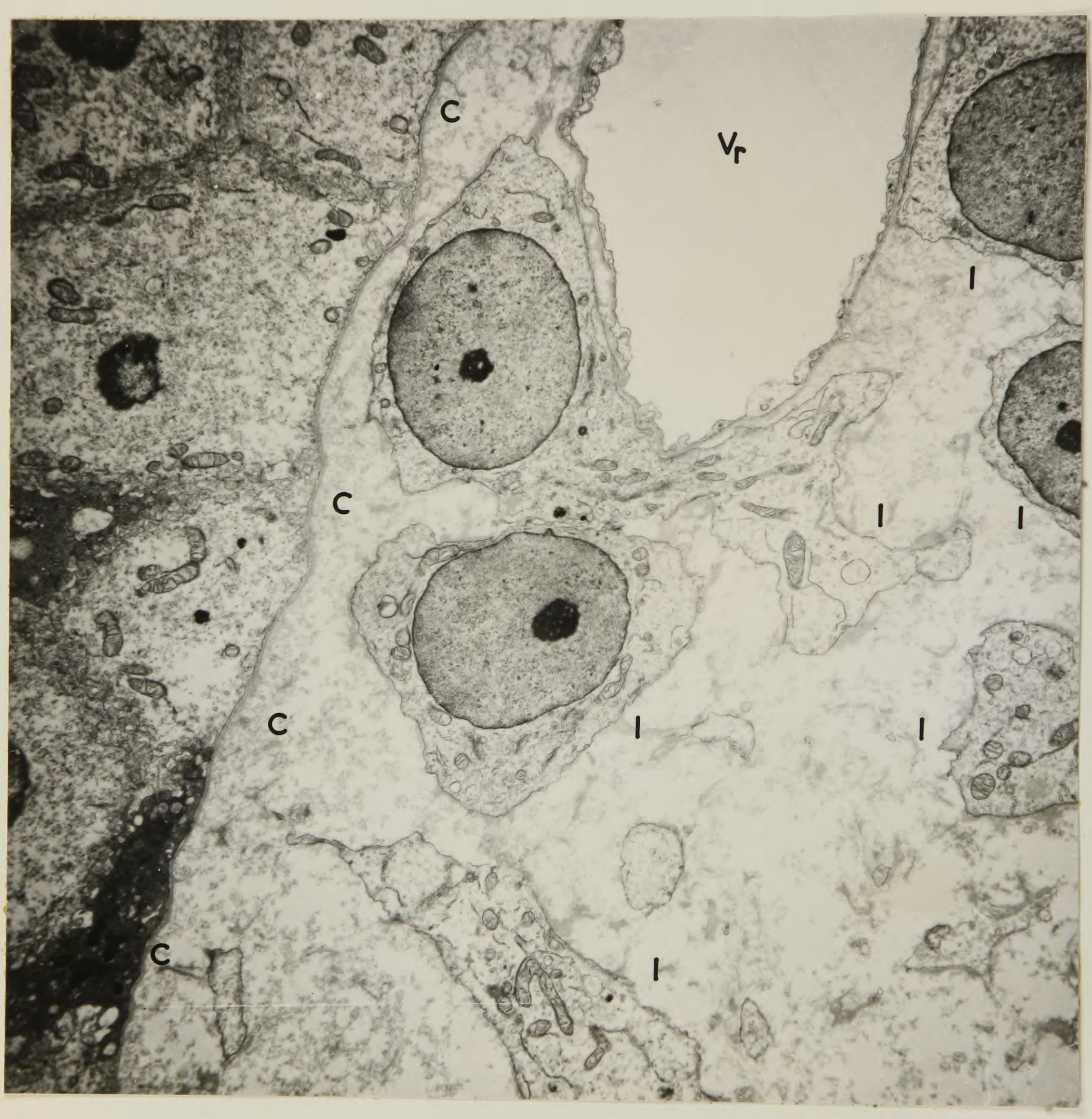


Fig. 3/30. Mag. 4,500.

V_r = Vasa Recta. I = Interstitial Cell.
C = Collecting Tubule.

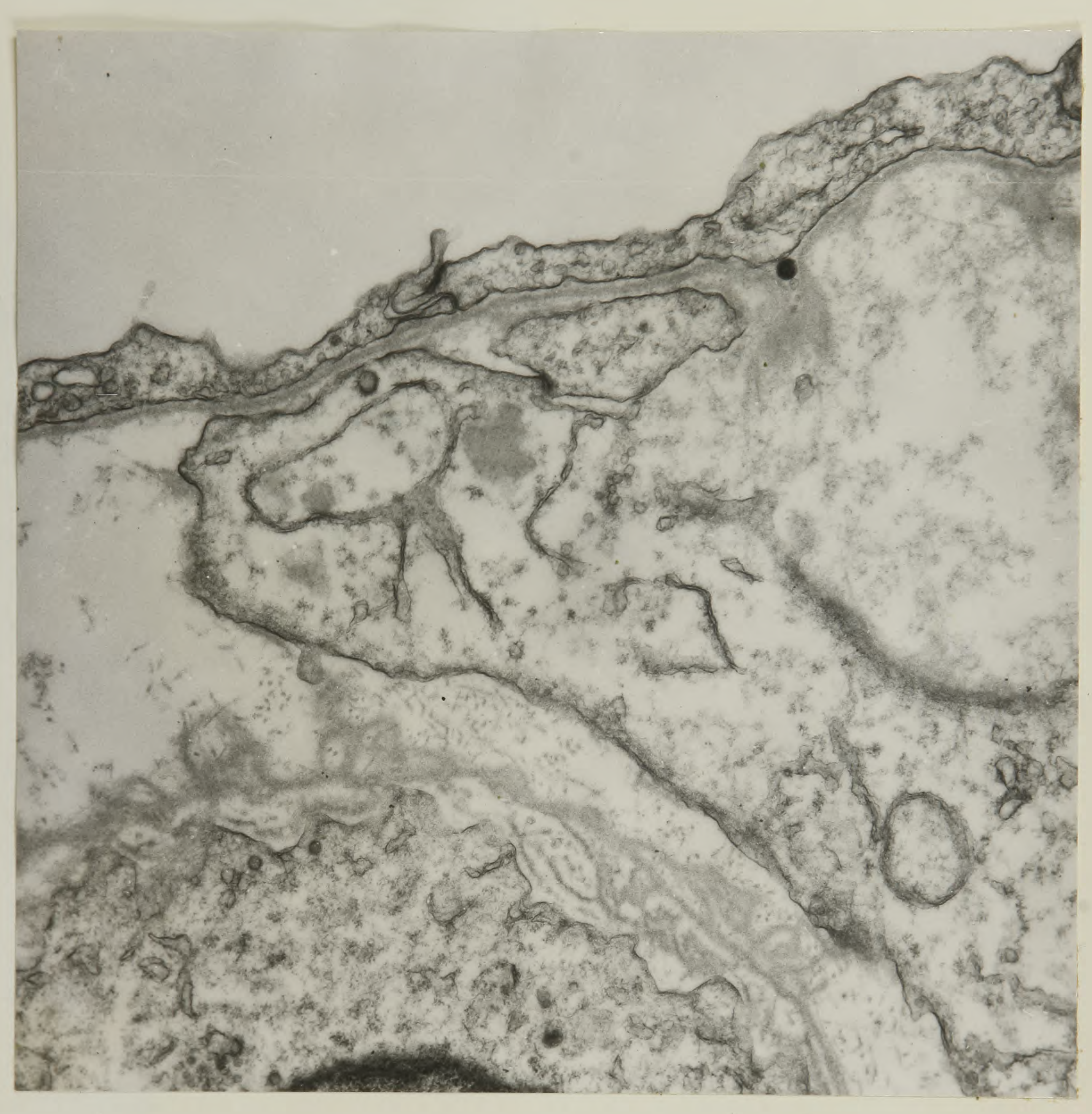
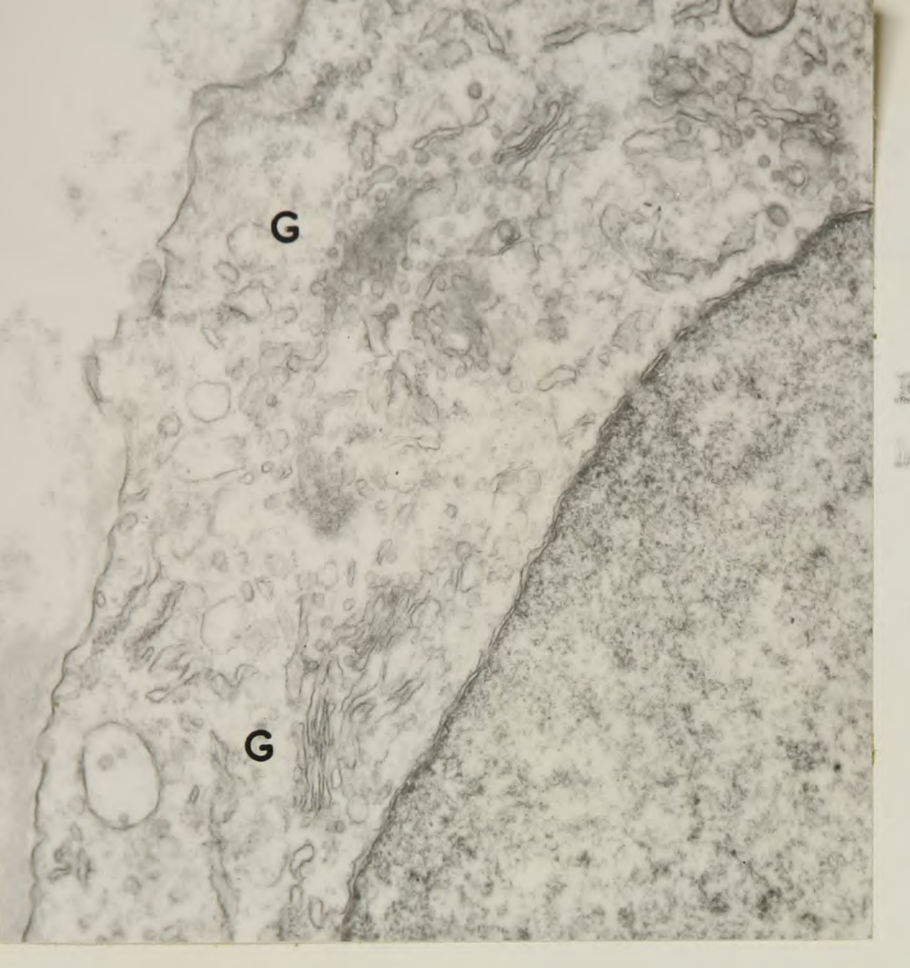


Fig. 3/31. Mag. 22,500.

INTERSTITIUM from a water laden guinea pig.
Note fibrillar basement membrane around lower interstitial cell, and also electron dense cylindrical body under basement membrane of Vasa Recta (? reinforcing fibril).



Interstitial Cells.

Fig. 3/32.
M. 15,000.

G = Golgi Region.
V_r = Vasa Recta.
C = Collecting Tubule.

Note fenestrae in the Vasa Recta.

Fig. 3/33.
M. 9,000.

C. The Loops of Henle.

It was not found possible to determine in electron microscopic sections of guinea pig papilla which tubules represented descending, and which ascending, limbs of the loop of Henle. Tubules that were not vasa rectae, in that they were never seen to contain red blood cells, were characterized by a low epithelium which projected into the lumen at the nuclear regions. The cells were elongated along the axis of the tubule and interdigitated with their neighbours: This gave a scalloped appearance to the edge of tubules transversely cut.

The loops of Henle possessed a continuous basement membrane which took on a fibrillar, multilayered aspect when the animal was given a water load before being killed. This was the only change that could be induced on hydration and dehydration: The cells were not seen to alter their volume nor to increase their activity as judged by the consistently small size of the Golgi region.

D. The Vasa Rectae.

The endothelial cells lining the vasa rectae were found to have numerous fenestrae of a maximum diameter of 0.1 μ, similar to those observed in vascular endothelium in, for example, endocrine glands. It is of

interest that filters with a pore diameter of this order are used biochemically to retain hyaluronic acid (Sandson and Hamerman, 1962). Chondroitin sulphate chains occupy much smaller domains and are easily filtered through millipore filters of 0.1 u size (Schubert, 1964). Hyaluronic acid is shown in the section of this thesis dealing with histochemistry to exist in the interstitium. An acid mucopoly saccharide bound to protein is produced by the collecting tubule cells. When the protein is removed from this complex, the polysaccharide moiety has properties in common with chondroitin sulphate. While there is also some sulphated acid mucopolysaccharide present in the interstitium, it does not appear to accumulate there and may well continually be leaving through these 'pores' into the vessel. This might account for the mucinous material seen within certain thin walled tubules as described (Figs. 3/27 to 3/29).

F. Observations by Other Workers.

Collecting Tubule Cells: During the course of this study there have appeared reports by other workers dealing with isolated aspects of the route that water has to follow in leaving the collecting tubule for the vasa recta. It is thought wisest to keep these observations separate from those described above since they deal with different species under different conditions, and the work in nearly all the cases has been undertaken with a different objective in mind.

Ganote, Grantham, Moses, Burg and Orloff (1968) used phase contrast microscopy to examine living cortical collecting tubules isolated from the rabbit kidney. The connective tissue which surrounds the collecting tubules in the medulla and papilla, the possible functions of which are considered in this thesis, have so far prevented them from being isolated in a satisfactory state. This is unfortunate since the osmotic gradient from tubule lumen to intercellular space is low in the cortex while it may be very high indeed in the papilla hence the cells in the two sites have to cope with rather different osmotic problems. In addition the supply of oxygenated blood to the cortical tubules is abundant, but the cells in the papilla working against the highest osmotic pressure are deprived of oxygen through short circuiting in the hair pin arrangement of the vasa recta.

However, for cortical tubules, Ganote et al demonstrated that vasopressin produced no changes in cell structure unless there was an osmotic gradient across the cell which led to a transepithelial bulk fol flow of water during the experiment; in which case there was an increase in the thickness of the cell layer, the lateral cell membranes became prominent and, if tissue was taken for electron microscopy at this time, many vacuoles could be seen in the apical cytoplasm. The results from this work suggest that, however vasopressin mediates an increased permeability to water, the flow of water out of the tubule is along part of the lateral intercellular spaces.

In the fifth chapter of this thesis it is recorded that the light microscopical evidence obtained from the study of guinea pig kidneys in anti-diuresis, when vasopressin was acting, indicates that there is some lateral separation of the collecting tubule cells at this time when the flow of water across the tubule wall is at its height. An increased lateral separation of the cells lining a cavity when fluid is moving across its wall has also been described in connection with the rabbit gall bladder (Tormey and Diamond, 1967). Diamond and Bossert have used the morphology of this situation to construct a methematical model for the transport of water across epithelia by the formation of a 'standing gradient' of osmotic pressure within the lateral intercellular space. These workers suggest how such a system could yield hypertonic fluids whose osmolarities were virtually independent of transport rate over a wide range. It is perhaps relevent to point out that, at the ultrastructural level, the anatomy of the gall bladder wall has much in common with that to the collecting tubule.

Johnson and Darnton (1967) made an ultrastructural study of the rabbit papilla in connection with the metabolism of glycogen. Since they studied animals in hydration and deprived of water some of their morphological results have a relevence in the present context. It was their conclusion that ' in spite of the fact that there was evidence of marked diuresis and antidiuresis, no ultrastructural alteration in any of the components of the renal papilla could be detected'. However, it seems unlikely on developmental grounds that the cortical tubules studied by Ganote et al (1968)

should respond to vasopressin in a way different to those in the medulla of the same species studied by Johnson and Darnton (1967). Johnson and Darnton (1967) took no care to cut the papillae at any particular level, nor indeed do their sections appear always to have been cut in comparable planes: It is thus possible that they might have overlooked quite gross changes which were obscured by the gradual increase in height of the collecting tubule cells as the tip of the papilla is approached (eg. cf. Fig. 3/5).

The basic morphology of the cells in the collecting tubules, including junctional complexes, vesicles and the basement membranes; loops of Henle; vasa recta; and the interstitium as reported by Johnson and Darnton (1967) for the rabbit is similar to that recorded in the foregoing paragraphs for the guinea pig.

<u>Interstitium; Light Microscopy.</u> Prenant and Bouin (1911) first drew attention in their famous text-book to two components in the renal papillary interstitium: There was a delicate network of fibres the spaces within which were filled with a optically clear material in their stained preparations.

The fibrillar material was later found not to take up acid dyes, but to be 'singularly covetous of colloidal silver ' (Cajal, 1933): It thus satisfies the histological definition of 'reticulin'.

It is possible that hyaluronic acid determines the normal argyrophilia of reticulin (Picken, 1960) and it is thus important to determine in what way the fibrillar material differs from the clear substance in the interstices. Since the clear material observed by Prenant and Bouin (1911) did not take up any of the protein stains then available there must have been strong presumptive evidence to assume that it was lipoid or polysaccharide in nature.

The introduction by McManus (1946) of the periodic acid - Schiff technique for the demonstration of carbohydrates with vicinal glycol groupings led to the confirmation of the presence of carbohydrate in the papillary interstitium. However, the method did not entirely clarify the naure of the ground substance, since it was found also to stain reticulin (Lillie, 1947; Robb-Smith, 1952). Furthermore, while the reticulin component (Figs. 5/14 to 5/16, silver impregnated) was found in the present study to stain positively by the PAS technique (Fig. 4/9), hyaluronic acid itself was found not to be positively stained (Fig. 4/3). Indeed, the situation is further complicated by the observation made in this study that there is also a fibrillar component in the papilla of the guinea pig which contains sulphate groups (Fig. 4/7) which are not normally found either on hyaluronic acid or PAS positive compounds.

On the basis of toluidine blue metachromasia Ginetzinsky (1958) claimed that what he called hyaluronic acid was to be found in the mucopolysaccharide cement between collecting tubule cells. At the same time

he described a non-metachromatic intertubular mucopolysaccharide net. Ivanova and Vinogradov (1963) used the more specific Alcian Blue dye to demonstrate that there was a hyaluronidase labile acid mucopolysaccharide in the interstitium in addition to another acid mucopolysaccharide structural component. It is of interest to note that, while Ginetzinsky (1958) claimed that vasopressin acted through the release of a urinary hyaluronidase which then digested the collecting tubule intercellular cement, Ivanova and Vinogradov (1963) reported 'swelling and elimination' of the mucopolysaccharide from the interstitium in antidiuresis.

Figure 3/25 (a & b) is a light micrograph of an Araldite 'thick section' to show the relationship between a collecting tubule and a vasa recta in the guinea pig. Water leaving the tubule to enter the vasa recta has to traverse either the collecting tubule cell cytoplasm or the lateral intercellular space and then the space between the base of the tubule and the vessel. The acid mucopolysaccharides that the Russian workers between them have claimed to be depolymerized in antidiuresis occupy the lateral intercellular spaces and the space between the tubule and the vasa recta.

An interesting short report on the result of his histochemical studies of papillary acid mucopolysaccharides was given by Morard (1967), who also compared the physiology of the rat with the rabbit. He found that there was a greater number of anionic charges in the extracellular material of the interstitium during antidiuresis; and that these could be stained by

Alcian Blue in the presence of sodium chloride concentrations that would greatly reduce the staining in water diuresis. Further, he noted that the animals which could secrete the most concentrated urine had the highest sodium chloride concentrations in their papillae in vivo, and that the staining of the acid mucopolysaccharides in sections of these papillae was not swamped by similar concentrations of sodium chloride in vitro. The interesting implications of this are more fully discussed in the histochemical section of this thesis.

Interstitium; Electron Microscopy.

Abrahams and Pirani (1966) undertook histochemical and electron microscopical studies to find out if there was any variation in the interstitial tissue at different levels in the papilla of the rat. Unfortunately, they failed to look for any differences that might accompany altered function. They gave a description of an interstitial cell identical to that found in the guinea pig, and compared it to a fibroblast and mast cell, neither of which it greatly resembles. However they did observe that the formation of ground substance and basement membrane - like material in the renal papilla was dependent upon the maturation of the interstitial cells: In new born rats the interstitial cells were rich in endoplasmic reticulum which became attenuated with age.

A review of the work done by his group on the morphology of papillary basement membranes by Robson appeared in 1963. It was noted that in kidneys

which were unable to respond to vasopressin,either because the animal had been given a water load or was potassium deficient,the basement membranes, particularly around the thin limbs of the loop of Henle, were fibrillar and had become thickened some five times. It was suggested that such an appearance could be associated with impairment of the diffusion of water out of the descending limb and / or the diffusion of salt into it and that either would tend to reduce the efficiency of the counter current system. The changes noted, however, seem to have been due to effect rather than cause since the open fibrillar appearance of the basement membranes seen in water diuresis could not be reversed by vasopressin ~~a~~ until the excess water had first been excreted.

In low power electron micrographs Robson (1963) observed some lateral separation of the collecting tubule cells in hydration while no inter-cellular channels were seen in a rat deprived of water for 48 hours. He considered that this was directly contrary to the light microscopical findings of Ginetzinsky (1958). However, the electron micro-scopical techniques used did not show acid mucopoly-saccharides, and Robson does not seem to have considered what effect this might have. If acid mucopolysaccharide were present in vivo, but was invisible by electron microscopy, it might be expected that the cells would ~~be separated to~~ give an appearance of 'some lateral separation'. If, on the other hand, this material had been digested away in vivo, then there would be little to prevent adjacent cells from coming together during the

processing for electron microscopy so that cells would appear adherent with no lateral channels. Thus it can still be held that Robson's results are consistent with the hypothesis of Ginetzinsky (1958).

Finally, a preliminary report has appeared which suggests that the vasa rectae are permeable to molecules at least as large as albumin, (Moffat, 1967). Moffat conjugated serum protein to Lissamine-Rhodamine B 200 and injected it intravenously into rats. After three minutes circulation time the animals were sacrificed, the renal vascular system washed out, and cryostat sections made of the papilla. When examined by flourescence microscopy the dye in the sections was seen to be distributed throughout the inner medulla and also to be present around the vascular bundles of the outer medulla. Ferritin has also been injected intravenously and its distribution observed by electron microscopy (Moffat, 1968, personal communication).

These reports are therefore all compatible with the view that the peculiar nature of the papillary interstitium could be concerned in the control of the flow of water from the collecting tubule lumen to that of the vasa recta. Before a decision can be reached on this aspect more has to be known about the acid mucopolysaccharides which occupy the 'clear spaces' in the light and electron micrographs. To this end, the histochemistry of the material in sections and extracts of papilla is examined in the next chapter.

CHAPTER 4: THE HISTOCHEMISTRY OF THE ACID MUCOPOLYSACCHARIDES IN THE PAPILLA OF THE GUINEA PIG.

'The ultimate aim of histochemistry is to describe the dynamiv organization of cells and tissues in terms of their structure, composition and function' (Casselman, 1959). The practical aspect was stated by Lison (1936) some thirty years ago: 'La condition morphologique essentielle de l'Histochemie est une localisation topographique exacte et précise de la substance à rechercher; et comme cette localisation ne peut être reconnue que si les rapports avec les éléments cytologiques ambiants sont parfaitment conservés, cette condition entraîne comme corollaire l'intégrité structurale du tissue étudié'.

In the case of the guinea pig papilla attention was directed towards the following three aspects:

1) Water soluble acid mucopolysaccharides had to be preserved within the tissue in a form which was representative of their state in vivo. For example, ethanol is an excellent precipitant for acid mucopolysaccharides in vitro, but its use in histology is limited by virtue of the gross shrinkage of cells and distortion of tissues that accompany its use as a fixative. Again, methods found to give excellent results with epithelial mucins, eg those employing phospho-molybdic acid (Heatley, Jerrome, Jennings and

Florey, 1956), or for mesodermal connective tissues (Curran, 1964), were found unsuitable either because the penetration of the precipitant into the interstitium was too slow or because the high cation concentration at this site interferred with the mechanism of acid mucopolysaccharide precipitation.

2) Most of the basic dyes in routine use, eg. Alcian Blue, or the thiazines, stain acidic mucins blue. This gives rise to sections of inherently low visual contrast and this colour tends to fall into the low sensitivity range of the photographic emulsions most suitable for photomicrography. Thus ways were sought of increasing the sensitivity of staining while retaining a reasonable specificity.

3) Assessment of histochemical specificity had to rely on the following three criteria:

(a) A comparison between the staining behaviour of the material in sections and that of model systems using samples of purified heparin, chondroitin sulphate, hyaluronic acid and glycogen;

(b) A comparison made between the staining behaviour of fractions separated by electrophoresis of material extracted from papillae and that of chemically pure samples; and

(c) Observation of the effects of enzyme preparations on sections and the extracted material.

The experimental methods used are discussed under the separate headings of histology and biochemistry, although there was some overlap in the design of the experiments.

FIXATION EXPERIMENTS.

Demonstration of 'Neutral Polysaccharides' by PAS:

Sections floated out on:	Structure	Precipitant Added to Formol-Cellosolve			
		Aminoacridine HCl, 0.4%	Acridine Orange 1.0%	CPCl 0.5%	Pb. Acetate sat. soln.
Dist. water.	CTg	Two populations evident: One glycogen, other larger granules	Glycogen positve. Nuclei also PAS positive.	Gly. swollen.	Basement membrs. v. strong. Some mast cell grans positive.
	CTb	Occasional large grans. seen	Glycocalyx positive, no additional intercell. grans.	Glycocalyx po. no grans.	No grans.
	I	Reticulin strongly stained.	Good staining of reticulin.	Fair	Fair
70% EtOH		No improvement noticed.	Nuclei now PAS negative.	Colour generally more intense.	Great improvemt. Incr. CTg.
CPCl	No discernable effect on PAS staining.				
Acridine Orange.	Leaves nuclei stained orange & often PAS pos. crystals between section and slide.				
Amino-acridine.	Staining more 'blue' in appearance but generally more intense.				

Table 4/1

Abbreviations in Tables 4/1 and 4/2:

CPCl	Cetylpyridinium chloride.	EtOH	Ethanol
CTg	Collecting tubule granules.	CTb	Collecting tubule cell borders.
I	Interstitium	CTl	Coll. tubule lumenal material.

FIXATION EXPERIMENTS.

Demonstration of Acid Mucopolysaccharides by Colloidal Iron Staining:

Sections floated out on:	Structure	Precipitant Added to Formol-Cellosolve			
		Aminoacridine HCl, 0.4 %	Acridine Orange 1.0%	CPCl 0.5%	Pb Acetate sat. soln.
Dist. water.	Gen.fixn.	Good, but tissue brittle.	Fair, softer tissues; but crystals of Ao deposited.	Good	Shrunken, uneven.
	CT_g	Well presd.	Poor presvn.	Present	Coarse
	CT_l	Intense, & fine granular appearance en face.	Present but homog.	As AO	Fair
	CT_b	Fine granular	Grans. not evident but material present between cells.	Very Poor.	Fair
	Mast Cells	Granules demonstrated.	Good for discrete granules; but makes	Good	Good
	Interstm.	Very dense.	nuclei pos.	Fair	'Stringy'
70% EtOH		No great improvemt.	Nuclei no longer stain pos.	Same	Less strong staining.
CPCl		Staining generally mote intense, but nuclei now positive.	Interstm. more intense.	Mast cell grans. v. coarse.	Interm. much more intense.
Amino-acridine HCl	Neither better nor worse than cetyl pyridinium chloride.				

Table 4/2

Histology.

1. Aqueous Fixation: The acid mucopolysaccharide precipitants recommended by Pearse (1960) and by Curran (1964) were dissolved in formol - cellosolve (Appendix 4/1) since this had been found preferable to formol - alcohol as a general fixative. The use of aminoacridine hydrochloride as a precipitant for histochemical purposes was also investigated. The results appear in Tables 4/1 and 4/2.

Aminoacridine hydrochloride dissolved in formol - cellosolve was found to give the optimal precipitation of acid mucopolysaccharides with satisfactory cytological preservation of the guinea pig kidney. Goodford and Leach (1966) found this fixative also to be suitable for preserving the water and hyaluronidase labile material between smooth muscle cells in the guinea pig taenia coli. It has the disadvantage of producing brittle blocks with skin for example, which contains much fibrous tissue, or when fixation is unduly prolonged.

The degree of shrinkage that this fixative causes was estimated by placing a test slice of guinea pig kidney on graph paper after each step in the processing and measuring the area it covered. After embedding in ester wax (Chesterman and Leach, 1956) the final shrinkage

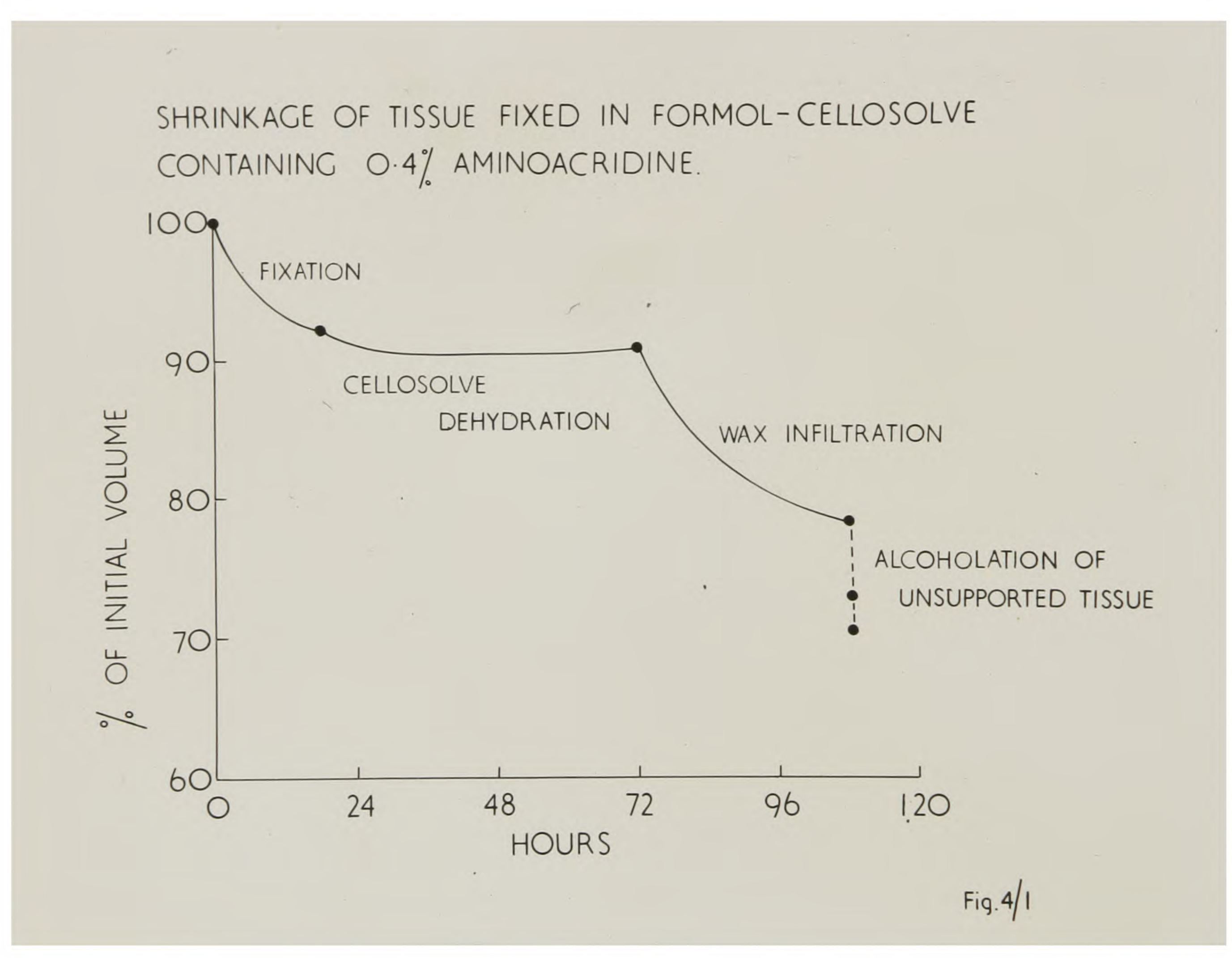


Fig. 4/1

Fig. 4/1.

was 22% by volume/ This compares favourably with results using other recognised fixatives, eg. saturated aqueous mercuric chloride, 30%; 4% formaldehyde, 32%; 96% ethanol, 45% (Baker, 1958). N.B. Baker's figures refer to tissue embedded in paraffin wax which itself produces more shrinkage than ester wax.

Shrinkage would matter little were it usually not uneven with consequent distortion of the tissue. When papillae are fixed in 0.4% aminoacridine hydrochloride dissolved in formol-cellosolve the collecting tubules were found to have appearances easily related to those of unfixed cells in the tissue homogenates (Figs. 3/6 and 3/7). This fixative was therefore adopted for the present study and used as outlined in Appendix 2/1.

Carleton and Leach (1939) used diacetin to flatten paraffin wax sections onto slides thereby avoiding contact between the cut surface of the tissue and water. This was found to lead to improved preservation and staining of mucin and mast cell granules. However, this protein precipitant was found not to enhance the retention of mucins in ester wax sections produced by the technique in Appendix 2/1. Nonetheless it gave rise to the notion that acid mucopolysaccharide precipitants might usefully be added to the warm floating out fluid. The results from this proceedure are included in Tables 4/1 and 4/2.

The considerable improvement observed when a cationic detergent (eg. cetyl pyridinium chloride, or aminoacridine hydrochloride) was used in a very dilute

concentration in the floating out fluid could have arisen in one or both of two ways. First, ester wax sections flatten more readily and at lower temperature if detergent is added to the floating out bath: This tends to lessen the leaching out of soluble substances by the warm water acting on this sections. The second reason is more complicated. Aminoacridine hydrochloride is soluble in cellosolve, and a small amount is leached from the tissue during the dehydration in this agent. When sections are subsequently floated out on water there is less than an optimal amount of precipitant associated with mucins in the tissue, and these are now exposed to a relatively vast volume of warm water in which they may dissolve. Added to this is the possibility that some acid mucopolysaccharides may be masked by lipid in vivo and may thus not be fully precipitated during fixation. The lipid is subsequently removed during clearing and this now leaves the acid mucopolysaccharide free to dissolve in water unless a precipitant is added at this stage.

2. Fresh Frozen Sections: Attempts to retain the maximum amount of acid mucopolysaccharide within the tissue by the use of fresh frozen sections proved unsatisfactory. The use of the (Pearse-Slee) cryostat did not yield sections that approached Lison's morphological criterion, although it was of considerable use for enzyme histochemistry (eg. aryl sulphatase, Fig. 3/24).

The main disadvantage of this technique in the histology of the papilla is that the tip in those animals

producing concentrated urine freezes at a lower temperature than does the base. (Indeed it was this fact which led to the discovery of the osmotic gradient within the papilla.) Thus the apex would be soft and nearly molten, while the base would be hard and brittle, making it impossible to obtain the longitudinal sections which were required if comparisons at the appropriate levels were to be made. Further, while ester wax offered the chance of cutting serial sections at 3 μ, the cryostat employed could only be made to yield irregular sections at upwards of double this thickness. Such sections were useless for the demonstration of inter-cellular boundaries, Golgi regions or interstitial cells. It was also found that fixation was subsequently required to prevent leaching of acid mucopolysaccharides from fresh frozen sections in the staining bath. Tissue preservation was considerably improved if this was done before freezing the block, but in this case it became illogical not to proceed with wax embedding.

3. Freeze Drying and Freeze Substitution:

Dried unfixed tissues have long been used in histology: " 'Leeuwenhock in 1720 describes how he gave orders to the captain of a whaler to procure a piece of whale muscle. . . He carried such a piece of dried muscle about with him, and whenever he wanted a little pleasant relaxation, slices cut off with a knife were examined under the microscope'. His method is still practised at the present day." (Mann, 1902).

In the present study, Altmann's suggestion of freezing the tissue before drying was followed by the now standard proceedure of quenching small pieces of tissue in iso-pentane cooled by liquid nitrogen. The tissue was dehydrated by sublimation of water under reduced pressure after which the block was directly infiltrated with ester wax.

In such preparations a hollow sphere some six cells thick was excellently preserved, but the crust was damaged by handling and the core by the slow growth of ice crystals. In addition, the smallness of the pieces of tissue made it difficult to record the level in the papilla from which the block had been taken. 'Freeze-drying' is synonymous with the biochemist's term 'lyophilization' andd it was quickly appreciated that, whatever the merits of the method in preserving acid mucopolysaccharides in the block, most of the water soluble material was dissolved out of the sections with extreme rapidity during the staining. The method was thus abandoned.

Freeze-substitution on the other hand was found to give excellent preservation of the collecting tubule glycogen in a non polarized form. By this method however the precipitants used for acid mucopolysaccharides were insoluble at the lowest temperatures. This meant that they diffused into the tissue to precipitate the outward diffusing acid mucopolysaccharides only as the system was warmed up. The more rapid penetration and precipitation offered by fixation at room temperature was considered to be preferable.

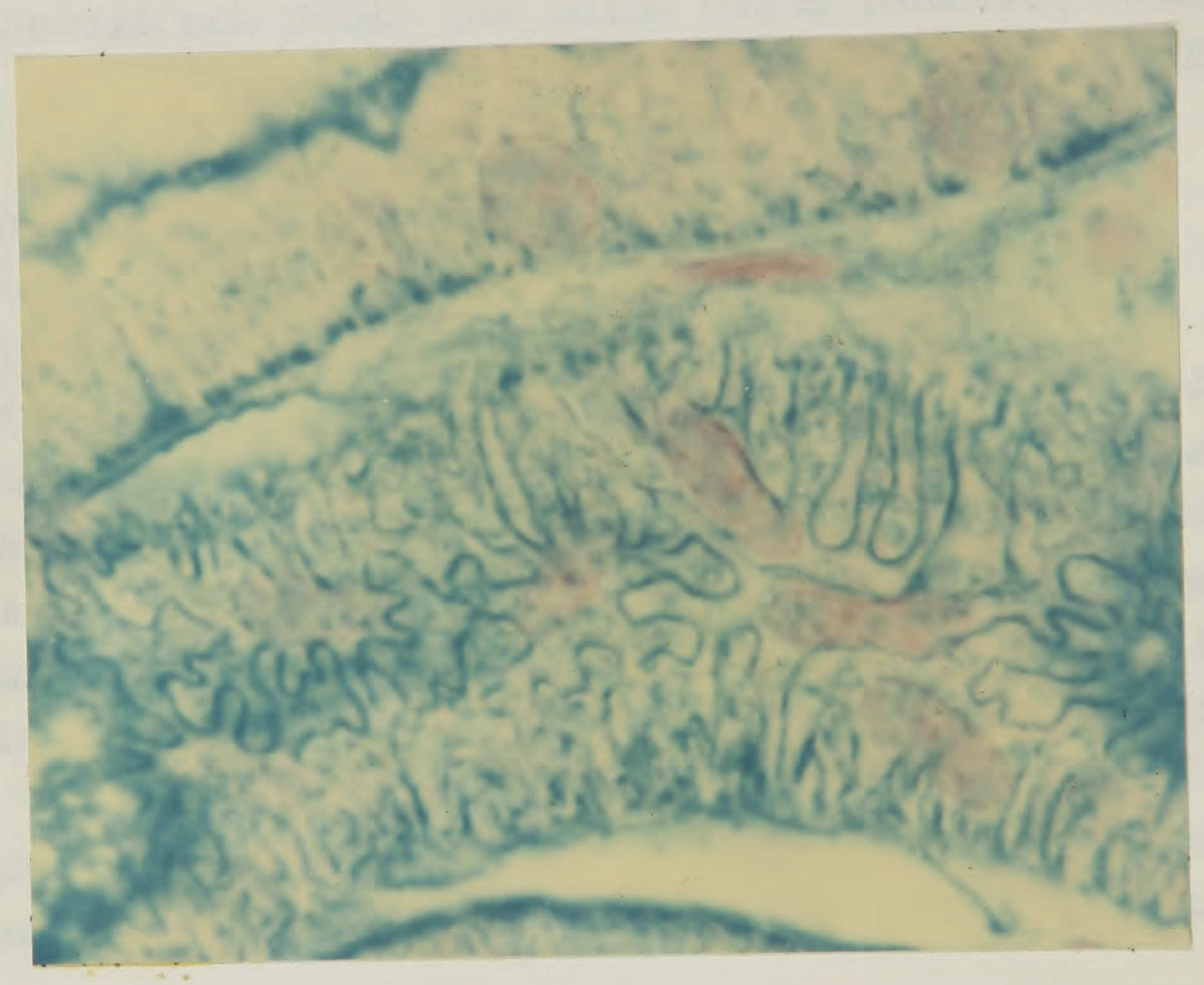


Fig. 4/2. Tangential Section of Cortical Collecting Tubule. Stained with Colloidal Iron; Nuclei counterstained by Mayer's Carmalum. Mag. 1,500. In the centre of the field the cells have been cut near their bases, and the interdigitations are well demonstrated by staining the material between the cells. Basal infoldings are apparant in the distal convoluted tubule at top.

4. Embedding: When sections of tissues which had been embedded in paraffin wax were floated out onto dilute Azur A, acid mucopolysaccharides were found to be intensely stained. On the other hand, if the sections were floated out on warm distilled water, then dried, dewaxed, and stained, the acid mucopolysaccharides were poorly shown. Warm water thus appeared to penetrate the paraffin and to dissolve out the mucins.

The ester wax embedding of Chesterman and Leach (1956) was found to give better morphological preservation after aminoacridine hydrochloride / formol-cellosolve fixation than did paraffin wax, and it is not wetted by water in the same way. Ester wax sections retained the acid mucopolysaccharide granules in the Golgi regions of the collecting tubule cells and permitted the demonstration of the cellular glycocalyces (Fig. 4/2) to an extent never matched by paraffin sections. It was equalled only by the much thicker sections of material embedded in low viscosity nitrocellulose, but these were unsuitable for examination by high power objectives on account of the thickness of the tissue.

Sections were attached to gelatinized slides to which they adhered more firmly than they were found to do to albumin-glycerol treated slides. Background staining was less and the slides were at the same time suitably subbed for autoradiography using stripping film. Coating the sections with celloidin after they were dewaxed did not appear greatly to aid the retention of acid mucopoly-

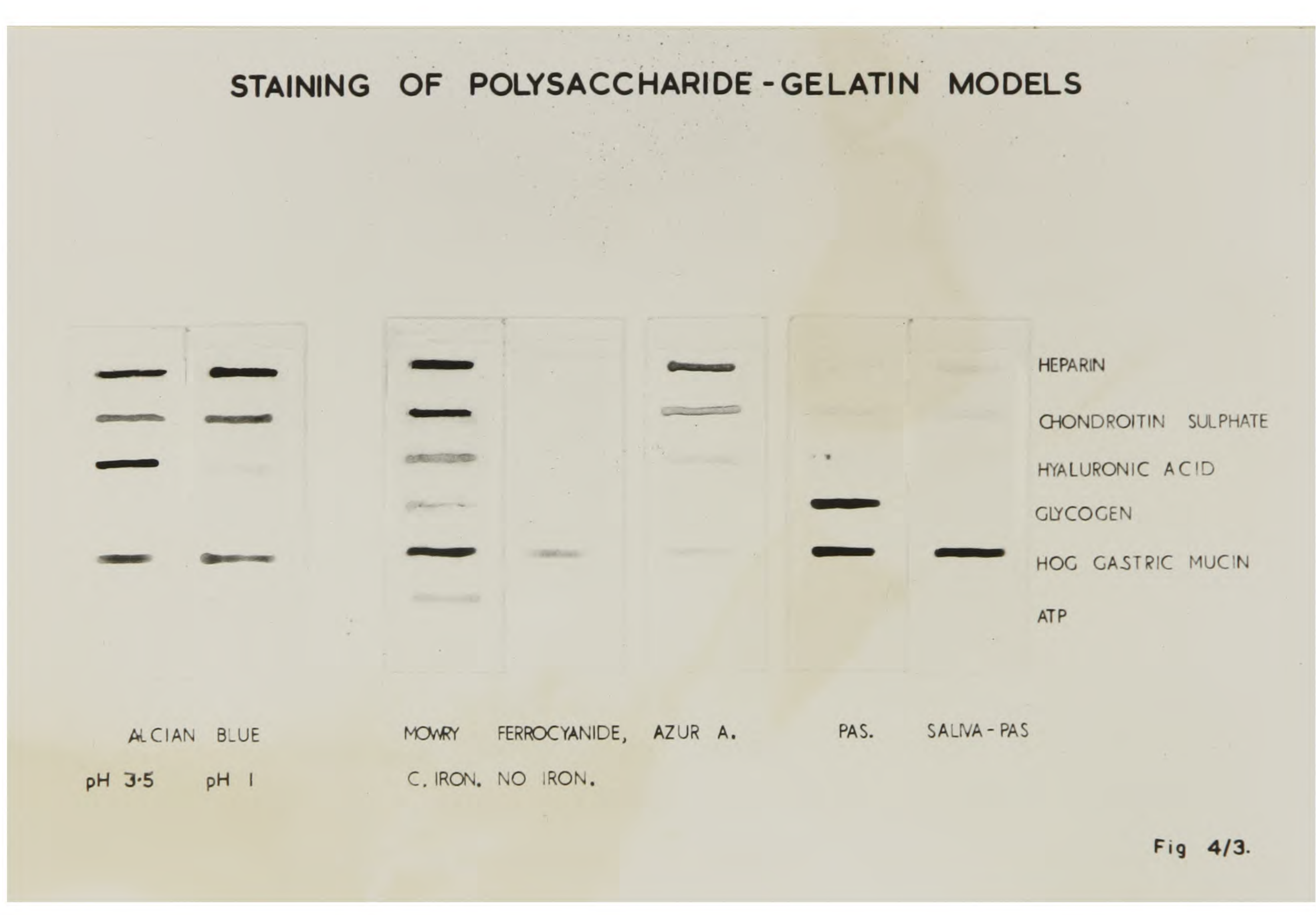


Fig 4/3.

saccharides in the staining bath. The celloidin was rendered insoluble and took up iron to give a granular background in the staining procedures which involved colloidal iron, hence this step was routinely omitted.

5. Specificity of Staining: In order to satisfy Lison's second criterion, that of achieving known specificity, damp gelatinized slides had samples of commercial heparin, chondroitin sulphate, hyaluronic acid, glycogen, hog gastric mucin and ATP (adenosine triphosphate) streaked across them. These slides were subjected to the same histochemical routines that were used on the tissue sections. The results are shown in Fig. 4/3.

(a) Periodic Acid - Schiff Staining: The positive staining by the PAS procedure of glycogen, glycoprotein and 'neutral' polysaccharides in the renal papilla was investigated along the lines suggested by Pearse (1960). The details and results are summarized in Table 4/3. The nature of the PAS positive fibrils was discussed in the previous chapter (cf. Figs. 3/8, 3/29 & 4/9).

(b) Acid Mucopolysaccharide Staining: The object of staining for acid mucopolysaccharides was to show both quantitative and qualitative changes: The latter requires that a distinction be made between carboxyl and sulphate groupings.

INVESTIGATION of PAS POSITIVITY

Proceedure	Demonstrating	Collecting Tubules	Interstitium	Other.
HIO_4 diss. in methanol: Oxidation for 1 hr. Schiff's etc.	Maximal demonstration of PAS pos. material in sections. (McManus & Mowry, 1964)	V. small grans. uniformly distributed. Even borders. Larger grans. in Golgi region & apex of cell.	Fine fibrils & basement membranes. I. cells negative. -	L. of H. show basal infoldings near cortex -
Acetylation, then PAS Staining.	Blocks positivity of 1:2 glycols.	No granules. Borders & cuticle faint.	Basement membrs. still pos.	-
Acetylation, De-acetylation, then PAS Routine.	Unblocks 1:2 glycols & then amino alcohols.	Granules pos, borders strong.	Basement membrs. more dense.	-
Saliva, (Amylase), PAS.	Glycogenolysis	V. small grans. removed. Large grans. unaffected.	No change	-
HIO_4, then Dimedone, then Schiff's.	Blocks connective tissue polysacchs.	V. small grans. unaffected. Large grans. not stained. Lumenal border faint.	-	-
Conclusions		V. small grans. are glycogen. Larger grans. associated with Golgi synthesis of acid mucopoly-saccharide.	Fine fibrils associated with carbo-hydrate. No 'free polysacch-aride' PAS positive.	

Table 4/3.

In an attempt to demonstrate sulphate groups alone the methylation of carboxyl groups was carried out by placing sections in 0.1 N hydrochloric acid in methanol at 37°C for 4 hours. The blocking was then reversed by placing the sections in 0.1% potassium hydroxide in 70% ethanol at 25°C for 30 minutes. These schedules were based on those found most suitable by Yamada (1964) in his investigation into the behaviour of acid mucopolysaccharide - casein films. The steps employed in the present study were thus:

a) A section was stained with Alcian Blue, 1% aqueous, for one hour at 25°C. This stained all the acidic mucinous material with little staining of nuclear material.

b) All the stain was washed from the section in several changes of acetate buffer at pH 3.5, when the acid mucopolysaccharide sulphate and carboxyl groups could be expected to be nearly fully ionized.

c) The section was methylated as outlined and then restained as in (a) except the 1% Alcian Blue solution was buffered to pH 1. Since it is only the sulphate group that should remain ionized at this low pH, and most of the carboxyl groups should have been blocked chemically, it could be expected that only sulphate groups would now be shown. The difference (a) - (c) should provide a measure of the carboxyl group contribution to the basiphilia of the renal papilla.

It was found that staining was reduced in every site in the papilla after methylation. The structures

positively stained after this proceedure, and which may thus be presumed to contain sulphate groups, were the collecting tubule intracellular granules, the collecting tubule outer membrane (glycocalyx), and a fibrillar component in the interstitium. The staining of the amorphous extracellular matrix of the interstitium was abolished by methylation, which indicated that this material was anionic by virtue of the carboxyl groups it contained.

However, there are two lines of evidence that suggest that such a conclusion is an oversimplification. When the control step of demethylation and restaining was performed it was observed that the level of staining could never be made to appreach that initially present. This suggests that some acid mucopolysaccharide was removed altogether from the sections during these manipulations. Pearse (1960) also raised theoretical objections to the method. He investigated the metachromatic staining of tissues known to contain high concentrations of sulphate esters, and found that there was a reduction in the metachromatic staining in addition to abolition of the orthochromatic staining following methylation. He used this oberavtion to argue that sulphate groups were also methylated. However in the present study it was verified that material containing only carboxyl groups (eg. pure carboxymethylcellulose) could be made to stain metachromatically with thiazine dyes. Thus there is an alterantive to the interpretation put upon the situation by Pearse (1960), namely that some of the contribution to the metachromasy came from a favourable arrangement of carboxyl groups within the tissue.

c) <u>Alcian Blue at Controlled pH.</u> More reliable estimates of relative sulphate and carboxyl group content were obtained by varying the pH of the dye bath. Szirmais (1963) showed that at pH 1 condroitin sulphate bound one molecule of a basic dye (Azur A) per disaccharide unit while hyaluronic acid bound none. If the pH was raised to 3.5, then chondroitin sulphate was found to bind two molecules of dye per disaccharide while hyaluronic acid now bound one. Szirmais argued that in the first case only sulphate groups were ionized; carboxyl groups in addition were ionized in the second.

This observation was substantiated in the present study by separating a mixture of heparin, chondroitin sulphate and hyaluronic acid by electrophoresis on a cellulose acteate membrane, which was cut longitudinally down the middle after the separation. One half was stained in Alcian Blue buffered to pH 1 and the other in Alcian Blue at pH 3.5. The strip from the bath at pH 1 showed heparin to be strongly stained, chondroitin sulphate moderately so, while the position occupied by hyaluronic acid was not stained. That from the bath at pH 3.5 had all three fractions strongly stained.

(The faint staining of the hyaluronic atid at pH 1 on the gelatin/polysaccharide model, Fig. 4/3, was explained when this sample of hyaluronic acid was subjected to electrophoresis. It was found to be contaminated by a small amount of chondroitin sulphate. This fact served to illustrate how dependent the interpretation of results from such model systems is upon the integrity of the pure samples.)

Alcian Blue at pH 1

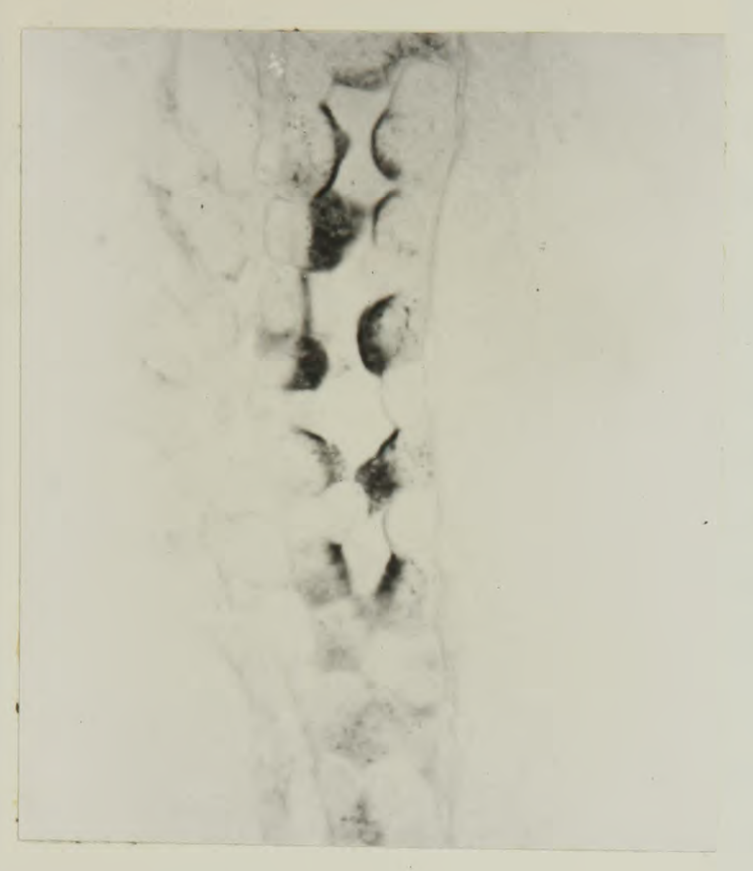


Fig. 4/4. Mag. 700.
Origin of Collecting Tubule in Cortex: Not all cells stain positively, other types of tubule negative.

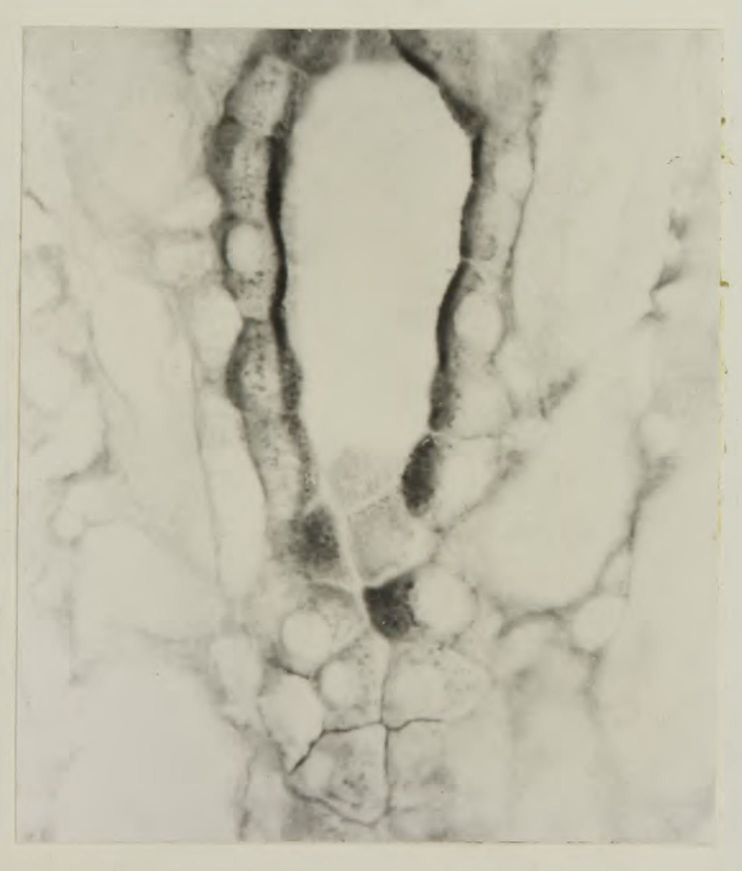


Fig. 4/5. Mag. 700.
Collecting Tubule at Cortico-medullary junctn. All cells alike: Golgi Regions, Intercellular space and apex positive. (Region of most active sulphate incorporation, cf. Fig. 4/14.)

Alcian Blue at pH 1.

Fig. 4/6. Mag. 1,700.
High Power view of
Collecting Tubule
Cells from same region
as in Fig. 4/5 (Cortico-
medullary Junction.)
Note appearance of
'granules' between cells.

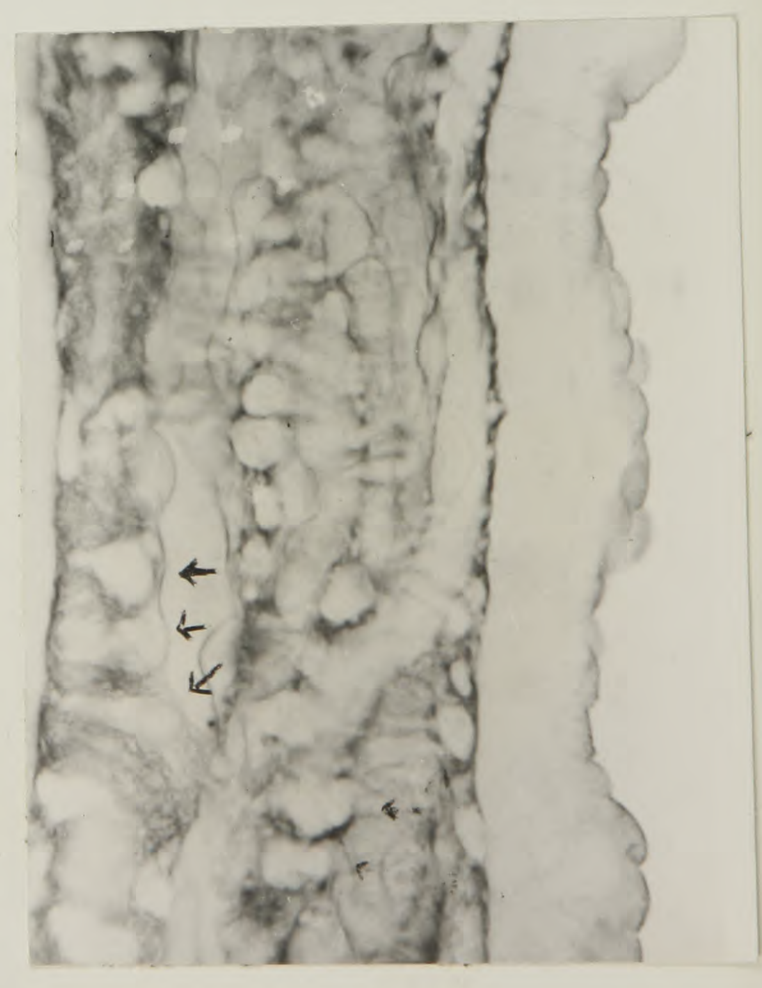


Fig. 4/7. Mag.700.
Collecting Tubule at
tip of Papilla (right
hand side): Lumenal
cuticle positive, no
'granules'. Inter-
stitial cells negat-
ive; but fibrillar
component positive.

Illustrations of sulphate group staining at pH 1 appear in Figs. 4/4 to 4/7. The collecting tubules are readily picked out in the cortex by this method on account of the positive staining of granules within some of the cells that line them. In the region of the medulla all the collecting tubule cells have an identical complement of positively stained granules: These are seen in the Golgi region (Fig 4/5); along the lateral apect of the cells (Fig 4/6) and at the apical border where, in low power micrographs, they give the impression of a cuticle. There is no staining of the extracellular material in the cortex. In the medulla the interstitium becomes more strongly stained as the papillary tip is approached. Fig 4/7 shows well the fibrillar nature of the material between interstitial cells. As the papillary tip is approached the collecting tubule cells contain fewer and fewer granules and the 'cuticle' becomes attenuated (Fig 4/7). The region of maximal staining for sulphated acid mucopolysaccharides corresponds to the region of earliest and most active sulphate uptake (Fig. 4/14).

The staining in the papilla of both sulphate and carboxyl groups by Alcian Blue at pH 3.5 is illustrated in Fig. 4/8. The staining of the collecting tubule intracellular granules is not enhanced, but an amorphous component to the interstitium is now apparent. Since this contains carboxyl groups alone it is likely to be hyaluronic acid. In order to check this point a section was intensely stained by the Mowry Colloidal Iron Method

Mucopolysaccharides of the Interstitium.

Fig. 4/8 Mag. 1,000
Alcian Blue at pH 3.5, same region as in Fig. 4/7. Note amorphous material containing carboxyl groups in addition to fibrillar component stained.

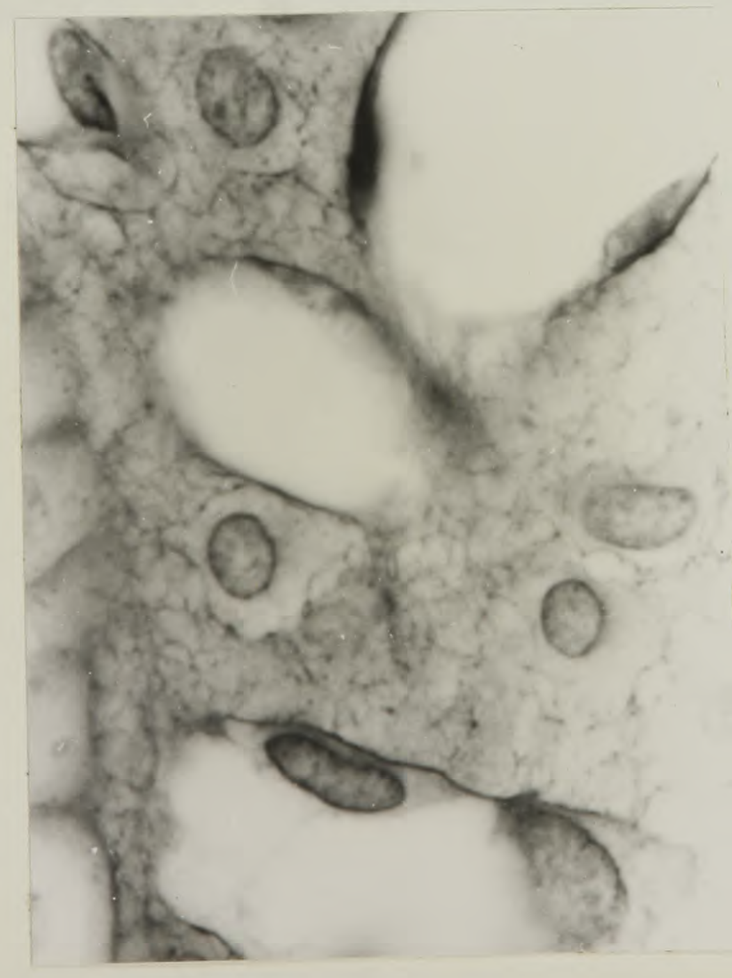


Fig. 4/9. Mag. 1,000
PAS & Haematoxylin. Fibrillar component PAS positive in similar region to Figs. 4/7 & 4/8. Hyaluronic acid not stained.

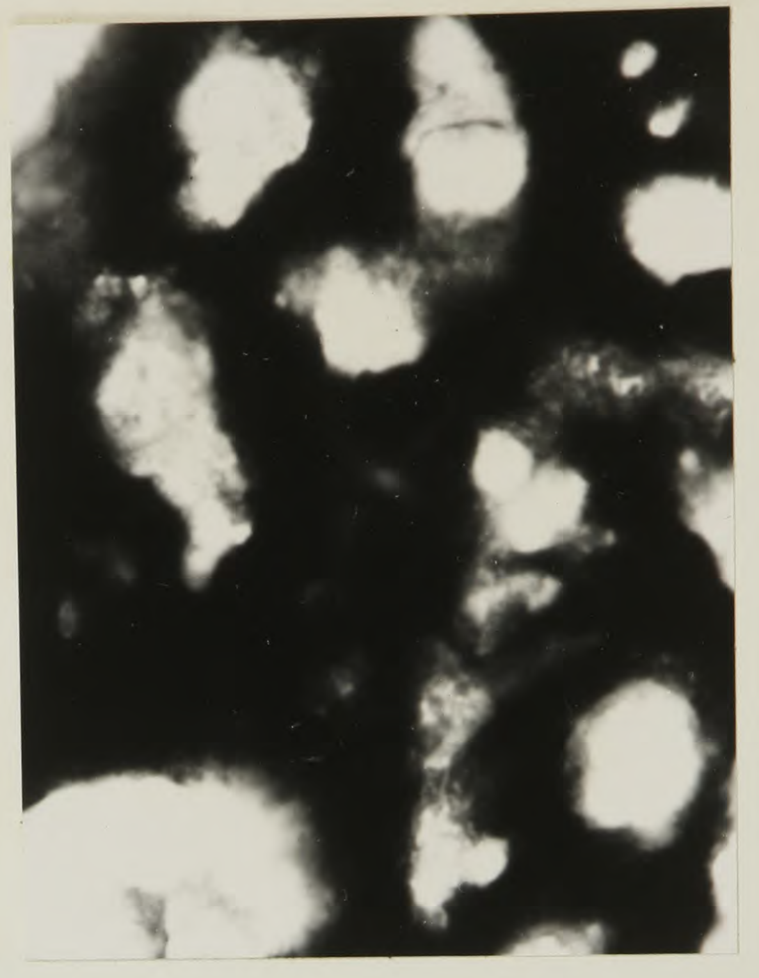


Fig. 4/10 Mag. 700.
Mowry Colloidal Iron
Method to demonstrate
acid mucopolysaccharides.
Similar region as Figs.
4/7 to 4/9.

Fig. 4/11 Mag. 700.
Adjacent Section to
that in Fig. 4/10 but
treated with Hyaluronidase
before Mowry Colloidal
Iron Staining. Hyal -
uronic acid digested,
leaving fibrils (which
appear coarse because of
the ferriferrocyanide
precipitate.).

(Fig. 4/10) to demonstrate to the maximum the carboxyl and sulphate groups that were shown in Fig. 4/8. The two sections were of the same thickness, but it will be seen that the interstitium in Fig. 4/10 is nearly opaque. A section adjacent to that used for Fig. 4/10 was exposed to the action of testicular hyaluronidase ('Hyalase') and then stained in the same baths at the same time as the section in Fig. 4/10. The result appears in Fig. 4/11. It will be seen that the amorphous material has been digested away by the hyaluronidase to leave the fibrillar component. The fibrils appear to be more coarse than those in Fig.4/7. This is probably due to the deposition of fairly large particles of Prussian Blue precipitate around the fibril. The fine fibrils which stained positively for sulphate groups also stained positively by the PAS technique (Fig. 4/9). Hyaluronic acid is not PAS positive, cf. Fig. 4/3.

The results from the use of basic dyes at pH values can be summarized: Collecting tubule cells in the medulla contain granules which stain positively only for sulphate groups - there is no evidence that the material contains an appreciable number of carboxyl groups. These granules appear to be synthesized in the Golgi region of the cells and are to be found most profusely at the apex of the cell, but they are also arranged along the lateral border of the cell. The interstitium has two acid mucopolysaccharide components: One is fibrillar and contains sulphate groups, and the other is hyaluronic acid.

CHARACTERIZATION of GROUPS in TISSUE SECTIONS OF RENAL PAPILLA.

Stain	Coll. T. Cells.	Interstitium.	Interstitial Cells.	L. of H.
Alcian Blue at pH 3.5.	Lumenal cuticle. Golgi region grans. Intercell. grans. Most active in outer medulla.	Dense homog. staining.	Sharply outlined but no intracell. staining.	Thin lumenal lining at tip of papilla.
Alcian Blue at pH 1.	As at pH 3.5: Not visibly less dense.	Fine fibrillar component stained.	-	Thin lumenal lining at tip pos.
Radio-sulphate at 1 hr.	In Golgi regn. & at lumenal surface.	-	-	-
Azur A in 70% EtOH	Meta. grans. Cuticle & borders not visible.	Metachromatic near papillary tip. Ortho-chromatic for main part of medulla.	Cells at tip have v. fine metachromatic granules by this method.	Some meta-chromatic grans. in cytoplasm.
Methyl-ation & then Alcian Blue at pH 3.5.	No granules stained, but thin positive cell boundaries.	Much reeuced: Like Alcian Blue at pH 1.	-	-
Demethyl-ation & Alcian Blue at pH 3.5.	Boundaries & lumenal cuticle strong. No granules positive.	Fibrillar component plus faint homog. material.	-	-
Hyalase Digestion Colloidal Iron Stain.	Grans. intra-cell & lumenal cuticle positive No intercell. grans.	Fibrillar component only stained.	-	-
Inter-pretat-ion.	Carbohydrate with sulphate grps. made by cells.	Hyaluronic acid plus fibrils with sulphate grps.	-	-

Table 4/4.

d) Metachromasia. Ginetzinsky stated in 1958 that, in the case of the rat, the 'epithelial elements are cemented by intercellular layers stained red (by toluidine blue) which shows that they contain hyaluronic acid'. Since, in the present study, hyaluronic acid was found not to stain metachromatically with the thiazine dyes (Fig. 4/3) the question of metachromasia in the guinea pig papilla was investigated more closely.

Renal papillae were found to stain weakly with Azur A if sections were dewaxed and brought down to water in the normal way. If, however, sections still in ester wax were floated out onto dilute (0.05%) Azur A in distilled water, then a considerable amount of dye was taken up. The excess dye could be washed away by several changes of 70% alcohol run in between the section and the slide without a great reduction in the intensity of staining, before the slide was left to dry on the hot plate. When completely dry the sections were dewaxed in xylol and mounted in (Gurr's) neutral synthetic mountant.

By this technique the collecting tubule cells were found to contain metachromatic granules (Fig. 4/13), which were arranged in a manner identical to those which stained positively with Alcian Blue at pH 1. This result supports the conclusion that the granules contain sulphate and cannot thus be composed of hyaluronic acid. When sections were dehydrated and mounted in a medium having a higher refractive index than water it was possible to use high resolution lenses to show additional 'granules' between the cells. When the results from histochemistry

Metachromatic Staining.

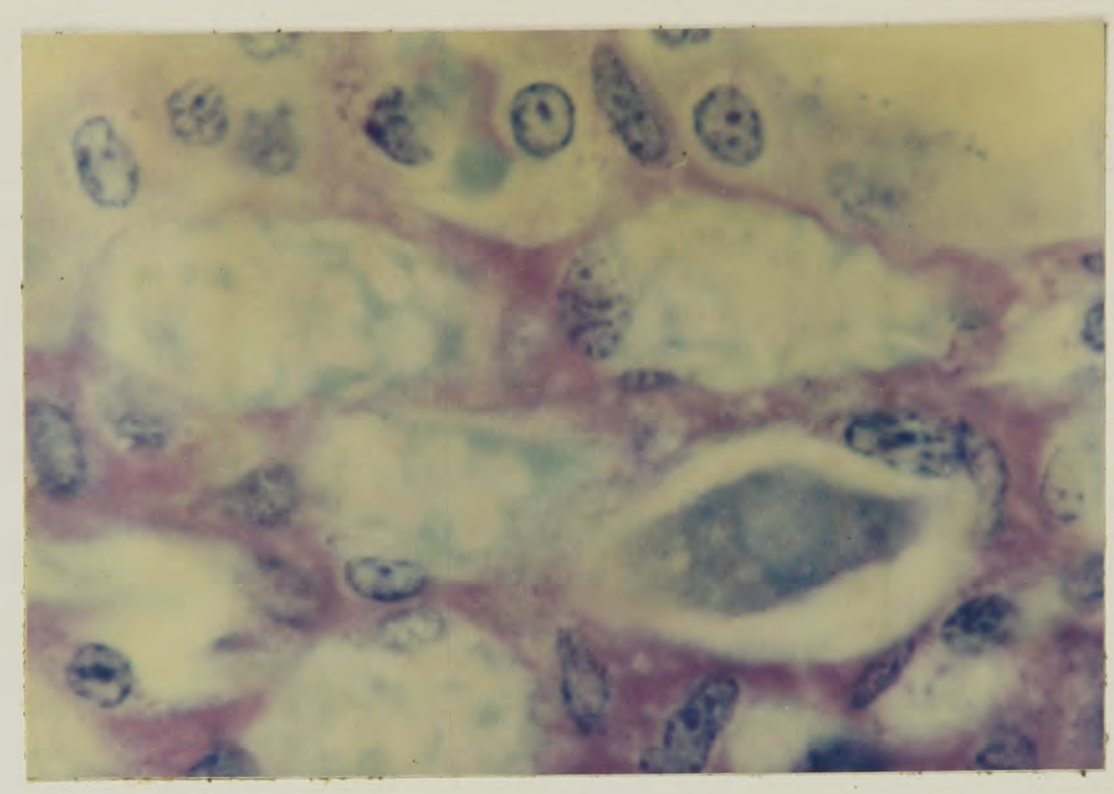


Fig. 4/12. Azur A (Stained in Wax). Mag. 500. Metachromatic extracellular material and collecting tubule granules (top, left & right) near tip of papilla.

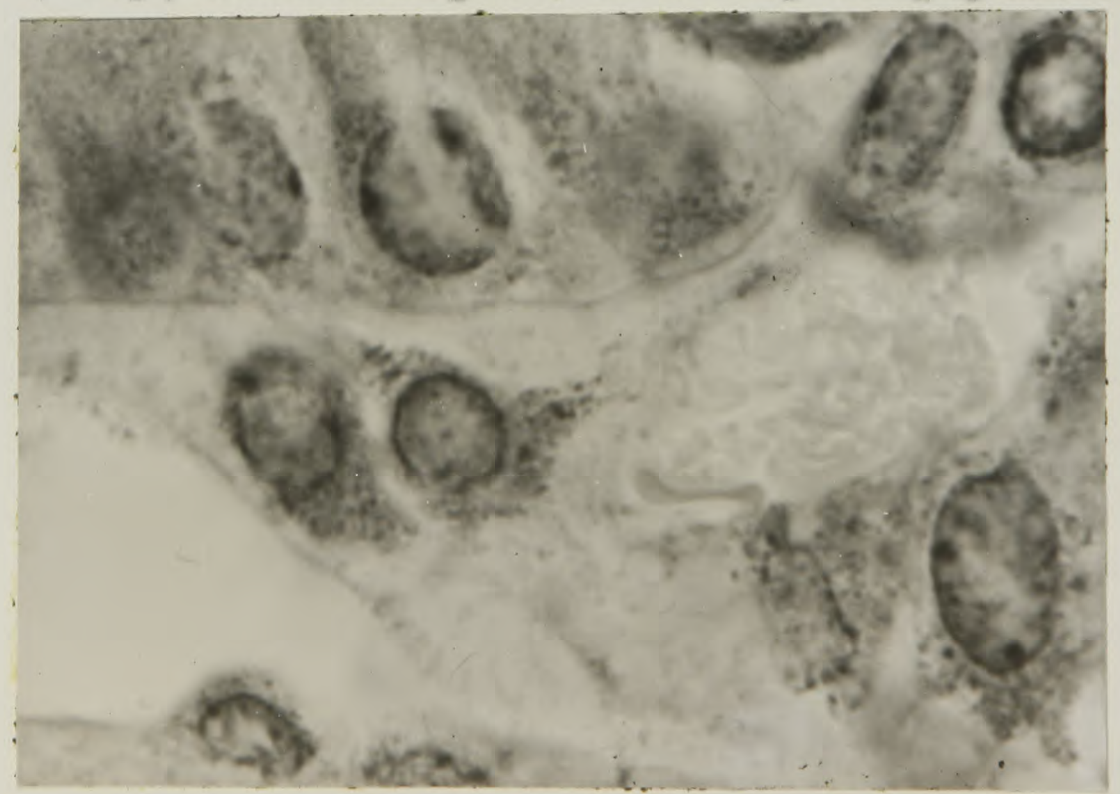


Fig. 4/13. Azur A (Stained in Wax). Mag. 2,000. Metachromatic granules in Interstitial cells near tip of papilla.

are considered in conjunction with those from electron microscopy (eg. Figs. 3/12, 3/15 and 3/17), it is found that true granules do not exist external to the cell membrane. The 'granular' appearance in, for example, Fig. 4/6 can be explained by the sectioning of the lateral intercellular interdigitations.

The interstitium exhibited a gradation from orthochromatic staining at its base to quite strong metachromasia at the tip. The extent of the latter was increased by depriving the animal of water for 48 hours or by injection of vasopressin. Fig. 4/12 demonstrates the metachromatic nature of the extracellular material at the papillary tip in a dehydrated guinea pig. While nuclei have stained orthochromatically the concentrated protein in the four vasa recta is bathochrome (ie. it exhibits negative metachromasy). At the tip of the papilla, the interstitial cells always contained metachromatic granules (Fig. 4/13), which were smaller than those found in, eg., the capsular mast cells but were of about the same size as the granules seen within the collecting tubule cells. The granules within the interstitial cells nearer the base of the papilla were orthochromatically stained.

e) Autoradiography: Further evidence for the presence of sulphate groups within the collecting tubule cell was obtained from the incorporation of injected sulphate. One hour after injection of $S^{35}O_4^{=}$ the label was confined

Radioactive Sulphate.

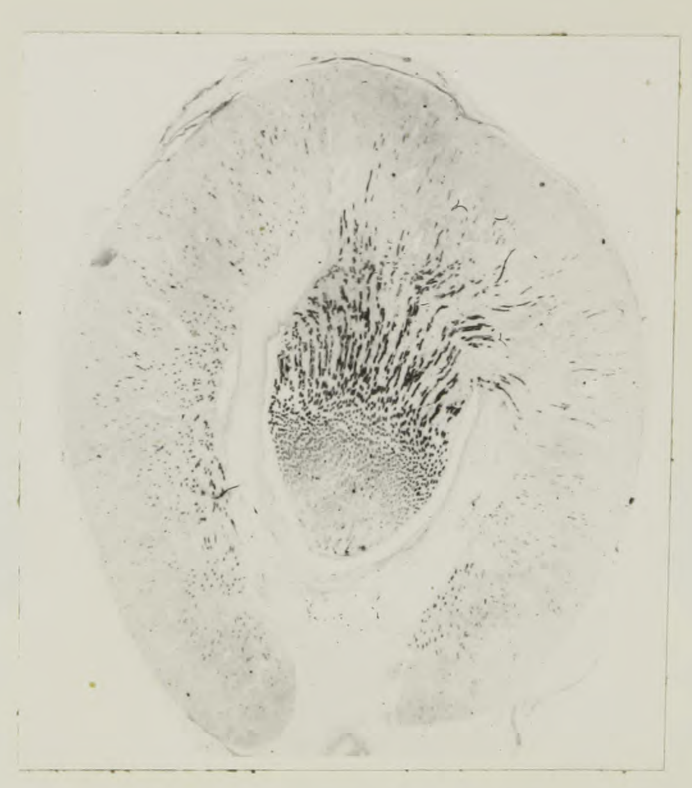


Fig. 4/14. Mag. 7.

This low power, unstained autoradiograph of guinea pig kidney 1 hour after injection of 1 mCi of $S^{35}O_4^{=}$ shows that the label is confined to the collecting tubules alone. The activity is maximal at the junction between cortex and medulla: It is low at the papillary tip.

Sulphate Incorporation.

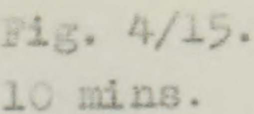


Fig. 4/15.
10 mins.

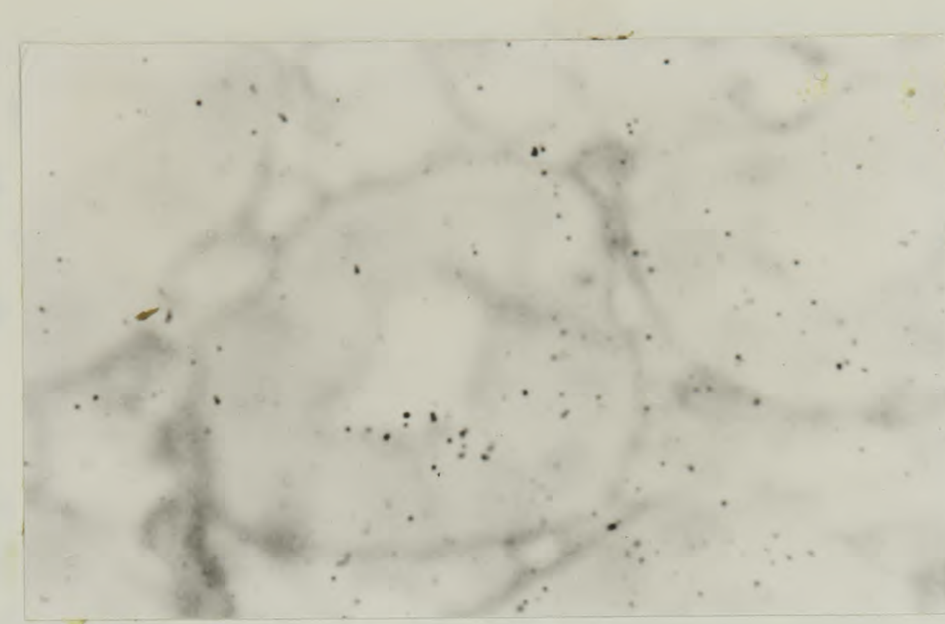


Fig. 4/16
20 mins.

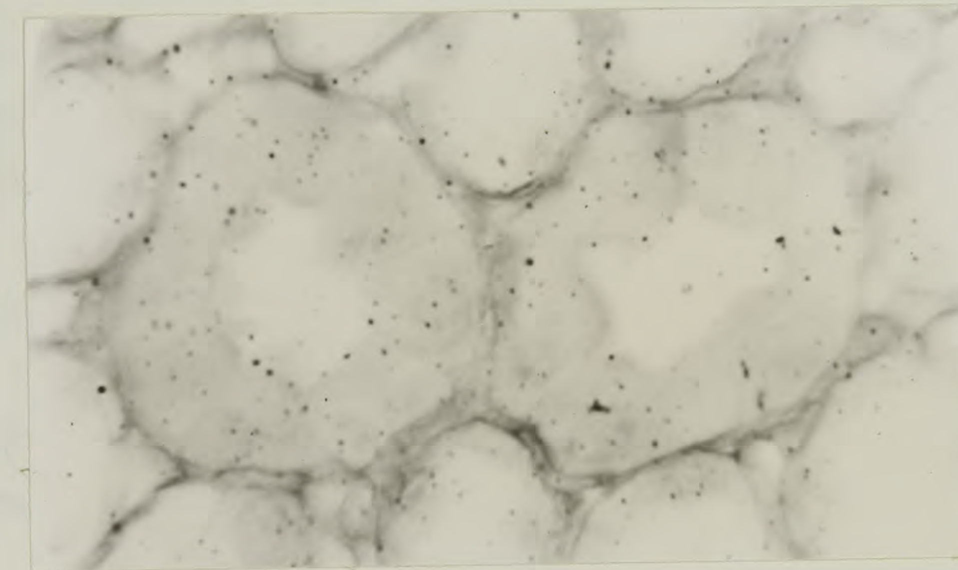


Fig. 4/17.
40 mins.

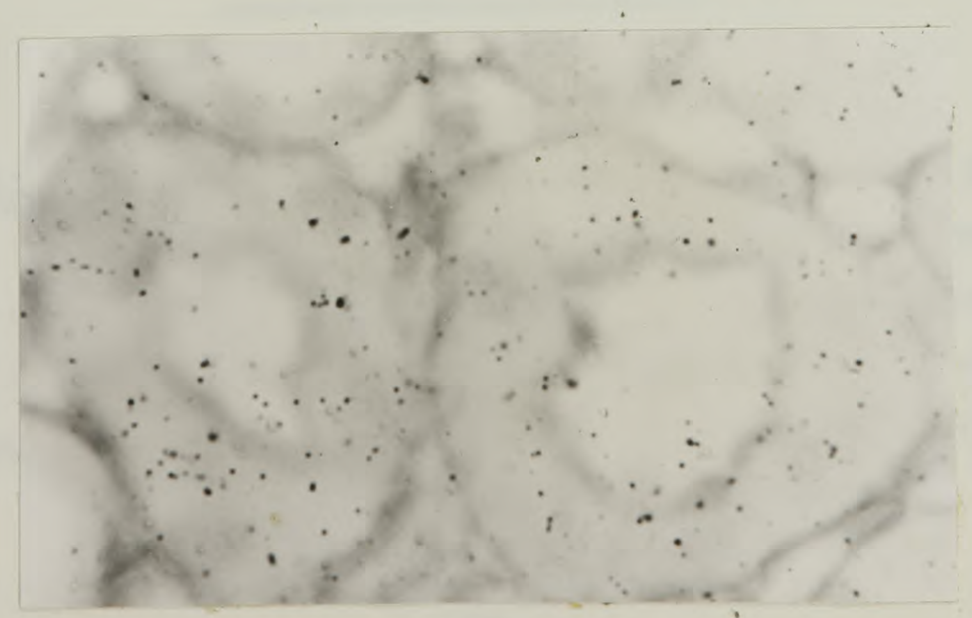


Fig. 4/18.
1½ Hours.

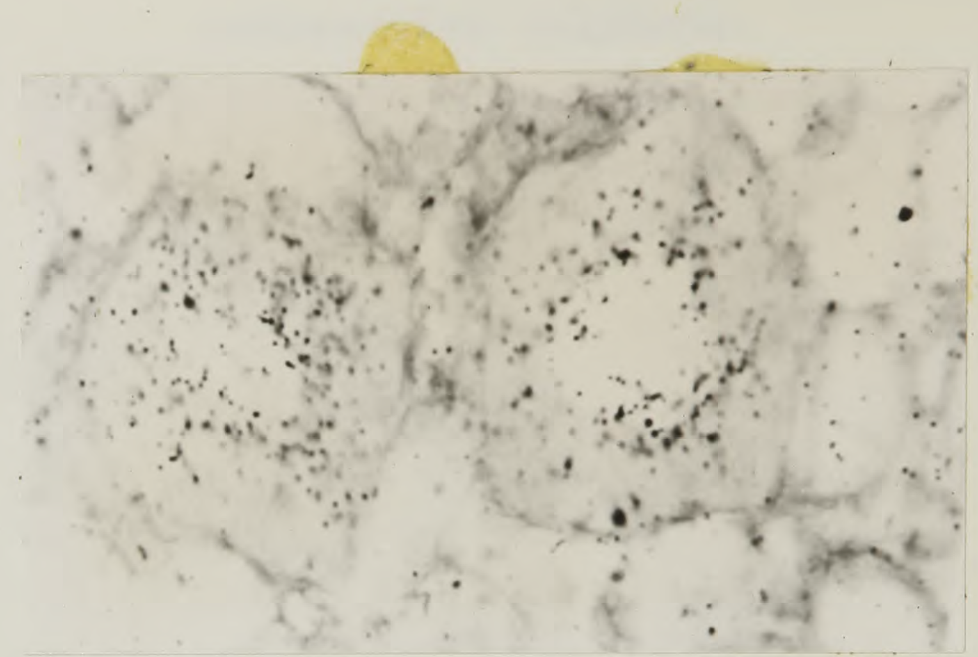


Fig. 4/19.
3 Hours.

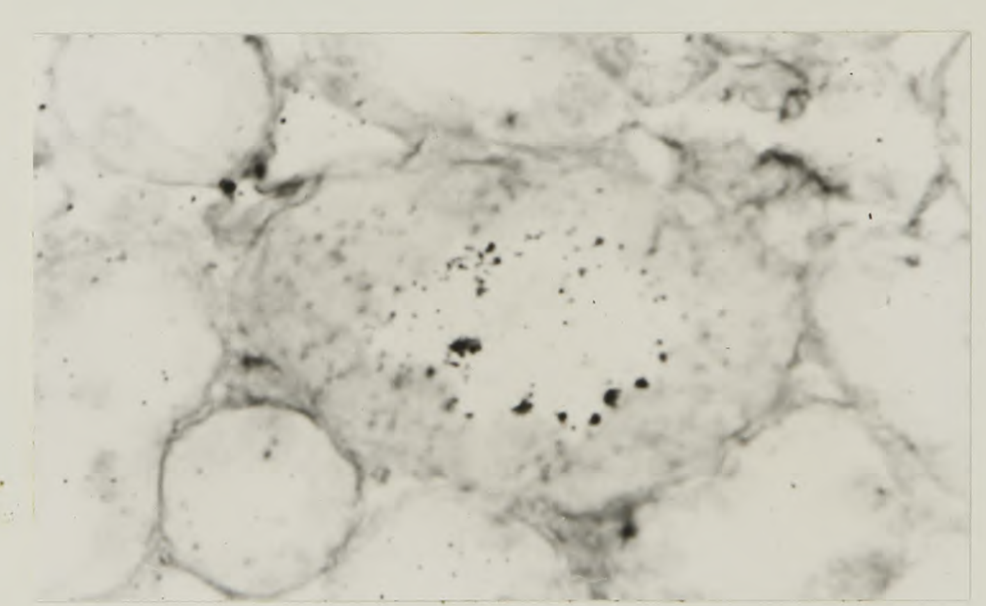
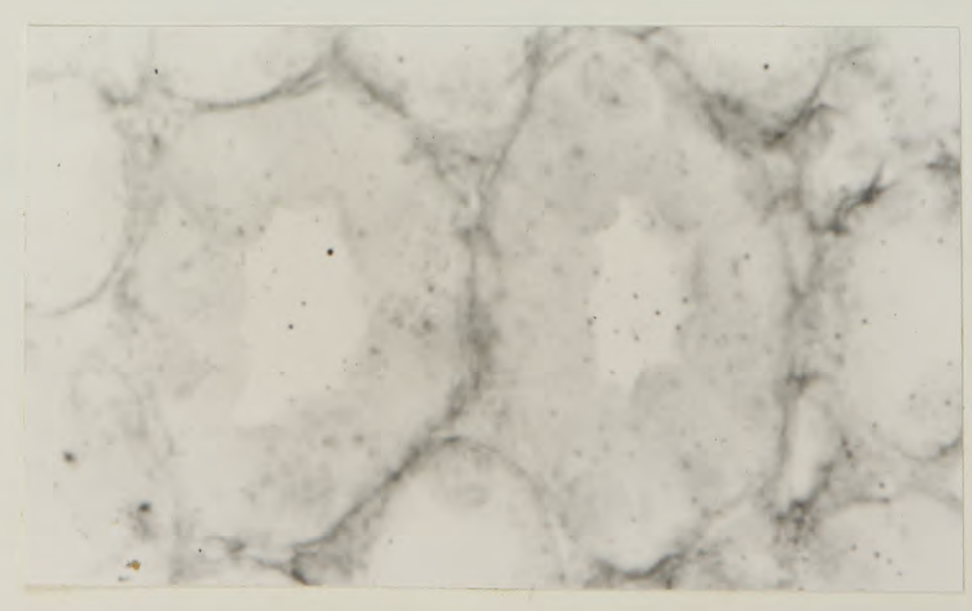


Fig. 4/20.
6 Hours.



exclusively to the collecting tubule cells, especially those in the cortico-medullary region (Fig. 4/14). The label first appears over the cytoplasm of the cells and then becomes concentrated at the lumenal border (Figures 4/15 to 4/20).

The histochemical staining of the intercellular region is identical with that at the cell apex, and, from the position of the Golgi region in the electron micrographs (Fig. 3/12 and 3/16), it is probable that the material in the lateral intercellular space becomes similarly labelled. However, the resolution of the stripping film technique (Appendix 4/2) is such that activity in this region could be represented by developed silver grains within 2 to 4 μ of the source, and thus the intercellular regions would not show up as such in the autoradiographs. The evidence for attributing the radioactivity to synthesised acid mucopolysaccharide and not to, for example, excretion of the un-incorporated ion is brought forward in the biochemical section.

B. Biochemistry.

1. Qualitative Electrophoresis. An electrophoretic separation of the acid mucopolysaccharides extracted from the papilla was used to substantiate the histochemical findings, and to determine the relative proportions of the reactive groups.

From published reports which appeared during the early stages of this investigation (Castor, Greene and Prince, 1965; Dicker and Franklin, 1966) it appeared that the two major components of the acid mucopolysaccharides within the medulla were chondroitin sulphate and hyaluronic acid. These two compounds had been shown to be rapidly and sharply separated by low voltage electrophoresis on cellulose acetate membrane (Manley, 1965). Thus the method of Manley (1965) was adopted with few modifications (Appendix 4/3).

Aqueous fixation of tissue for histology was found to be accompanied by the formation of a viscous gel at the bottom of the container, with little acid mucopolysaccharide being preserved in situ. This indicated that an aqueous extraction would probably secure most of the papillary acid mucopolysaccharides with ~~the minimum of~~ less than that damage ~~that~~ which would accompany alkali or proteolytic extraction. This simple extraction also had the advantage that small volumes could be used and that few steps had to be taken to prepare the final sample for electrophoresis. In this way it was possible to compare the biochemical behaviour of the material extracted from the papilla of one kidney with the histochemical findings in the papilla of the other kidney from the same animal.

Distilled water with a quarter of its volume of n-butanol added, as suggested by Morton (1950) for enzyme extraction and by Maddy (1964) for the isolation of the erythrocyte glycocalyx, was found to be most satisfactory as the medium in which homogenization was performed (Appendix 4/4).

Homogenization was achieved by the use of a teflon pestle rotating in a tube cooled by an ice-salt mixture. The lower aqueous layer obtained by centrifugation of the brei was drawn off and freeze-dried. The addition of salts, eg. calcium, was found neither to increase nor to alter the relative proportions of the acid mucopolysaccharides in the yield: This was possibly due to the high concentration of cations existing initially within the papilla. However, as a check on the results obtained by this simple aqueous extraction, proteolytic extractions were performed on papillae from four kidneys: The combined sample had a wet weight of about 1½ grams. Papain was used dissolved in (a) a phosphate buffer (Manley, 1965) and (b) in an EDTA buffer(Hakkinen, Hartiala, and Terho, 1965), as in Appendix 4/5. The Manley method was used without modification. The proceedure of Hakkinen et al required a short dialysis step to be inserted after the enzymatic digestion since the cetyl pyridinium chloride was found not to precipitate fully the acid mucopolysaccharides from the salt laden buffer. Both methods yielded qualitatively similar results. The acid mucopolysaccharides isolated by the Manley method were separated by electrophoresis and found to bind Alcian Blue at pH 5.5 in the ratio of 4 chondroitin sulphate to 1 hyaluronic acid; while the ratio from the Hakkinen extraction was more nearly 3 to 1. The latter ratio tended to unity with prolonged dialysis which suggested that chondroitin sulphate was lost through the dialysis membrane. If the assumption is made that chondroitin sulphate binds twice the number of dye molecules per disaccharide unit as does hyaluronic acid (Szirmais,

1963), then the ratio of chondroitin sulphate to hyaluronic acid was between 1.5:1 and 2:1 in the extracts obtained by proteolysis.

This is not in good agreement with the results of Dicker and Franklin (1966) who carried out pepsin - trypsin digestion of the renal medulla. They found equal amounts of chondroitin sulphate and hyaluronic acid in that 510 mg. of acid mucopolysaccharides yielded 160 mg of non sulphated, presumably hyaluronic acid, fraction and 160 mg of a sulphated fraction. The nature of the other 200 mg of acid mucopolysaccharide is not made clear.

In the absence of a full chemical analysis of the kidney it would be wrong to assume that an acid mucopolysaccharide which had carboxyl groups only need ipse facto be hyaluronic acid, or that one with sulphate groups need be chondroitin sulphate. Further, the material examined by Dicker and Franklin (1966) had been obtained by proteolysis and it was unjustified to use reasonably well defined terms, such as have been used for well characterized substances in cartilage, unless the molecules in the kidney extract had been shown to have comparable molecular weights and physical properties. Evidence of any possible covalent link with protein was, of course, destroyed by their method of isolation.

<u>Results from Electrophoresis.</u> An electrophoretic separation of the material which is extracted by homogenization of guinea pig papillae in the butanol/water system

Separation by Electrophoresis.

Fig. 4/21. Separation of the acid mucopolysaccharides from guinea pig papilla: Top, densitometer tracing; Arrow, direction of migration; Cleared strip; and, bottom, the relative percentages of dye bound by each peak.

(Appendix 4/4) is illustrated in Fig. 4/21. In addition to bands having the mobility of chondroitin sulphate and hyaluronic acid, two further fractions were stained by Alcian Blue at pH 3.5. A quantitatively small component of this additional material moved more quickly than chondroitin sulphate, while the bulk moved more slowly than hyaluronic acid (Table 4/5).

Alcian Blue at pH 3.5 acts as a cation and forms ionic linkages with polyanions ~~such as~~ in addition to the acid mucopolysaccharides (Pearse, 1960; Scott, Quintarelli, Delloro, 1964). It is thus important to be certain that the material stained in the electrophoretic extracts is acid mucopolysaccharide and not acidic protein, lipoprotein or nucleic acid. That the fastest band does not contain protein is evident by the lack of staining by Nigrosine, one of the most sensitive protein stains for this purpose. The slowest moving band, on the other hand, is positively stained by Nigrosine and thus the material within it probably contains covalently linked protein. None of the bands was likely to be lipid or lipoprotein in nature since most materials of this class would probably have been in the butanol layer which was discarded.

The exclusion of nucleic acid in the fastest band is less certain. The primary phosphate groups of these molecules have a pK of about 2, and thus partially degraded molecules of nucleic acid could be expected to enter the pores of the cellulose acetate membrane, to move during the electrophoretic separation, and to stain by union with a cationic dye.

Densitometry.

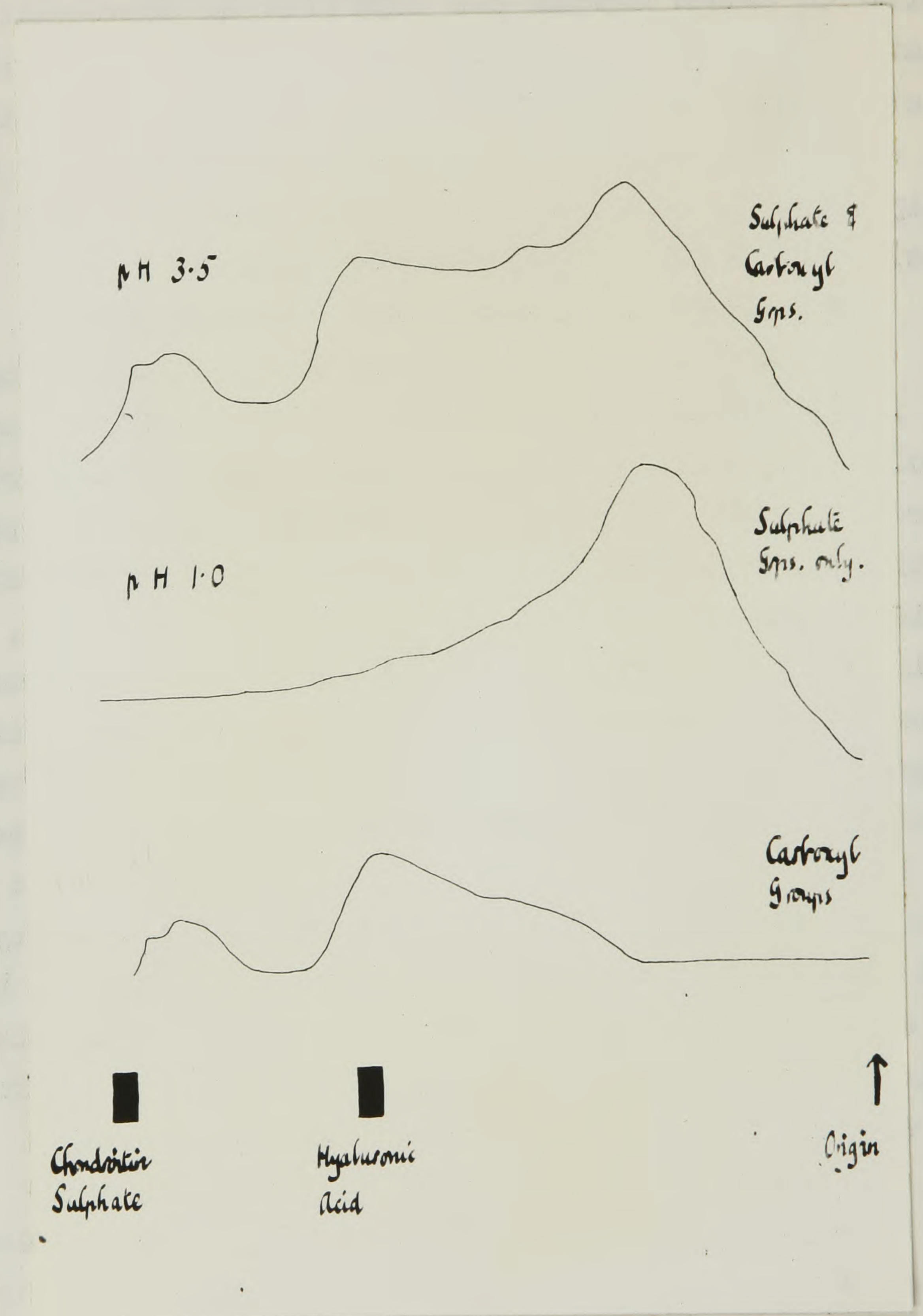


Fig. 4/23. 'Chromoscan' Tracings of Strips,
Top: Stained at pH 3.5 (Carboxyl + Sulphate);
Centre: Stained at pH 1.0 (Sulphate); and
Bottom: The difference between the upper two traces (Distribution of carboxyl groups).

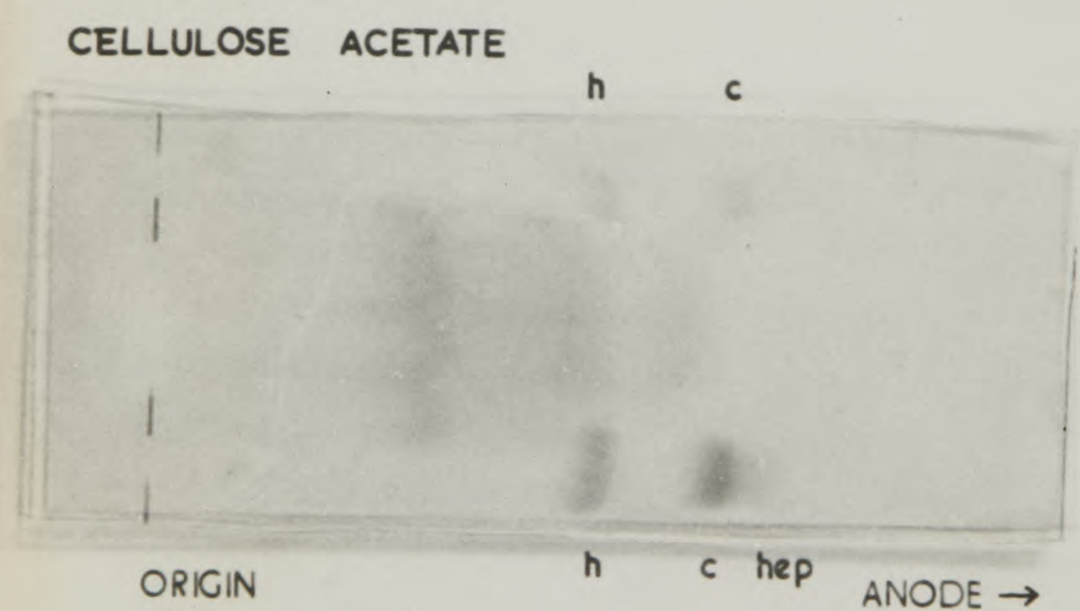
However, a sample of 'yeast nucleic acid' (B.D.H.) subjected to the electrophoretic and staining proceedures was shown to have a mobility less than that of any of the bands in the kidney material. Staining the papilla for RNA (by the Unna-Pappenheim Methyl Green / Pyronin technique) showed very little to be present, and this agreed with the paucity of ribosomes seen in this region by the electron microscope. The richest source of RNA in the guinea pig papilla was probably in the collecting tubule cell nucleoli. Since none of the bands in the electrophoretic separation stained positively with the Feulgen technique DNA was not present in the soluble aqueous extract. The main evidence against the faster bands containing fragments of nucleic acid molecules lay in the incorporation of radioactive sulphate in material with exactly the same electrophoretic mobility (Fig. 4/25). Sulphur is not found in either nucleic acid.

The slowest moving band in the electrophoretic separation was found to be the only one that was PAS positive, and thus to contain material with vicinal glycol groupings (ie. -CH(OH) - CH(OH)-). In order to investigate the nature of the anionic groups on this fraction, strips were cut longitudinally after the separation, one half being stained in Alcian Blue at pH 1 to show sulphate groups and the other in Alcian Blue at pH 3.5 to show both sulphate and carboxyl groups. The two strips were then scanned in a densitometer to give the upper two traces in Fig. 4/23. Subtraction of the tracing from the strip stained at pH 1 from that at pH 3.5 gave the distribution of carboxyl groups, which is shown by the bottom line.

SEPARATION OF BUTANOL-WATER EXTRACT

5 Cms.

X-RAY FILM



I mC Radiosulphate given I hour before death.

Electrophoresis: 200 v for 80 mins at pH 5·6. Stain: Alcian Blue.

Markers: h, Hyaluronic acid ; c, Chondroitin sulphuric acid; hep, Heparin.

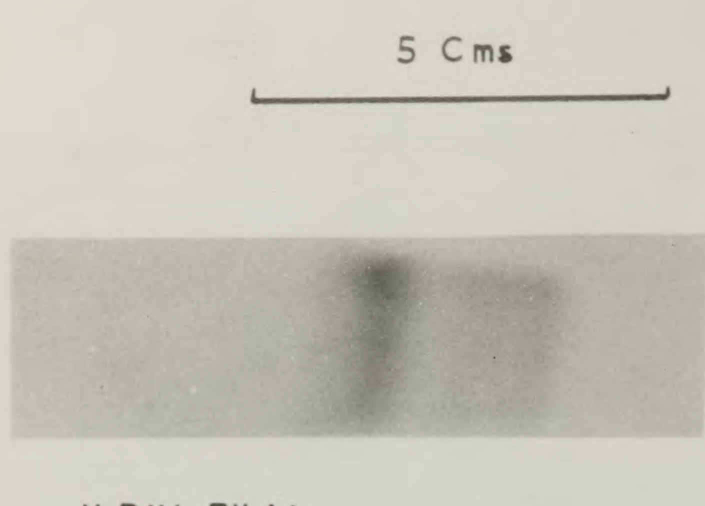
Fig. 4/24

By this means it was shown that the slowest moving band, which to some extent overlaps the hyaluronic acid band, contained only sulphate groups.

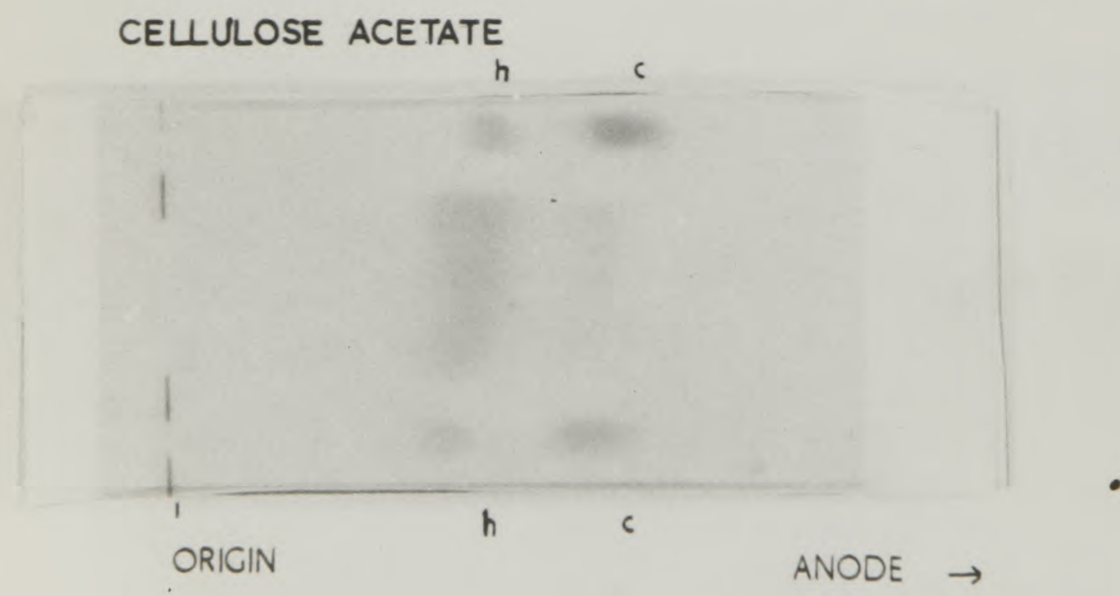
It was stated that the histochemical results showed that radioactive sulphate incorporation at the end of an hour after injection is confined to the material within the collecting tubule cells,(Figs. 4/14 and 4/18). Accordingly that fraction in the extract that contains the label after this interval can be associated with the collecting tubule material.

The result from a butanol-water extract of papilla made one hour after injecting a guinea pig with $S^{35}O_4^{=}$ is depicted in Fig. 4/24; while fig. 4/25 shows the result from a papain digest. The butanol-water extracts showed sulphate incorporation into the protein-polysaccharide (slowest) band, but not into any of the other bands. In the material extracted by proteolysis, the label appeared in a fraction with a mobility very close to that of hyaluronic acid and also in the two faster bands (Fig. 4/25). These results can be interpreted in the light of Fig 4/23: There are essentailly four fractions of acid mucopolysaccharide in the papilla. The slowest moving band contains a sulphated acid mucopolysaccharide which is covalently bound to protein. The next faster band is hyaluronic acid. The two fastest bands were poorly characterised: They stain strongly at pH 3.5 and not so strongly at pH 1, but incorporate sulphate, hence probably contain both sulphate and carboxyl groups. If the protein in the slowest band is digested by papain then three fractions are

SEPARATION OF PAPAIN DIGEST OF PAPILLA



X RAY FILM



I mC Radiosulphate given I hour before death.

Electrophoresis: 200 v for 80 mins at pH 5·6. Stain: Alcian Blue pH 3·5.

Markers: h, Hyaluronic acid ; c, Chondroitin sulphuric acid.

Fig. 4/25

released: The largest quantitatively has a mobility close to that of hyaluronic acid and the other two fall into the faster two bands seen in both types of extraction. Some suggestions as to the constitution of the material in the slowest band are advanced in Fig. 4/26.

It is of interest to note that there is some chondroitin sulphate free from protein in the papilla and that this does not incorporate radioactive sulphate until well after one hour following injection, ie. there was no activity over the chondroitin sulphate band in Fig. 4/24. It appears that some new chondroitin sulphate has been synthesised during this period, but that it is firmly bound to protein (ie. in the slowest band in Fig. 4/24) from which it may be released by proteolysis to give activity over the faster bands in Fig. 4/25.

The demonstration thus of 'free' chondroitin sulphate in the butanol-water extracts of papilla suggests the existence of an enzyme im vivo which cleaves the chondroitin sulphate from the protein and PAS positive parts of the granule produced by the collecting tubule cell. The time lag between synthesis and degradation (ie. of the order of at least an hour) suggests that the access of the enzyme to the substrate is limited.

To explore this possibility further, the effects of commercial enzyme preparations on the extracted material were investigated. Extracts from the papillae of 16 animals were combined and aliquots were subjected to (a)

CHARACTERIZATION of FRACTIONS from BUTANOL - WATER EXTRACTS of PAPILLA

	Bands in the Electrophoretic Separation			
	Slow migration		High mobility	
Front relative to Hyaluronic acid.	0.63	1	1.4	1.54
% Alcian Blue bound at pH 3.5.	25	33	22	19
Binding of Alcian Blue at pH 1.	Pos.	-	faint	faint
Incorporation of Radiosulphate at 1 hour.	Pos.	-	-	-
Azur A	Ortho-	Ortho-	weak meta-	meta-
PAS	Pos.	-	-	-
Nigrosine.	Pos.	-	-	-
Dialysis before Separation.	Retained	Retained	Lost	Lost
Hyaluronidase (testicular) Digestion.	Undigested	Digested	-	-
Papain Digestion.	Digested	Undigested	-	-
Interpretation.	Protein with carbohydzate & sulphate.	Hyaluronic Acid.	Acid polysaccharide chains of low molec. weight.	

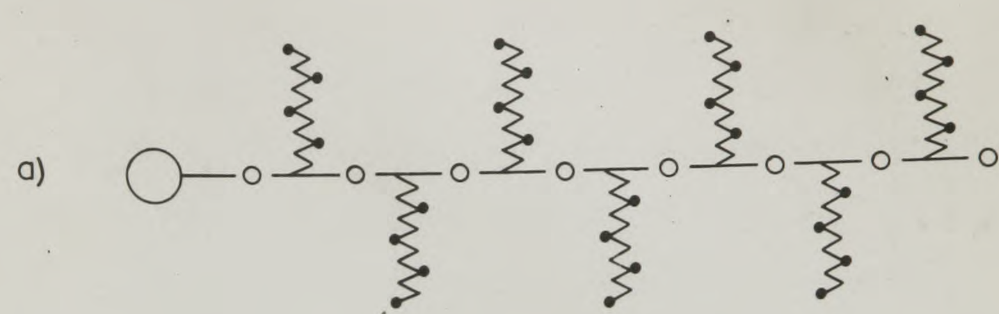
Table 4/5.

dialysis against distilled water; (b) digestion with hyaluronidase; and (c) papain digestion. The enzyme digestions in (b) and (c) were performed by incubation of enzyme and extract within a dialysis sack suspended in the appropriate buffer. The external buffer solution was changed several times to remove the degraded material which had passed through the dialysis membrane. The samples remaining within the dialysis sacks were concentrated by freeze-drying and then subjected to electrophoresis. The results are given in Table 4/5.

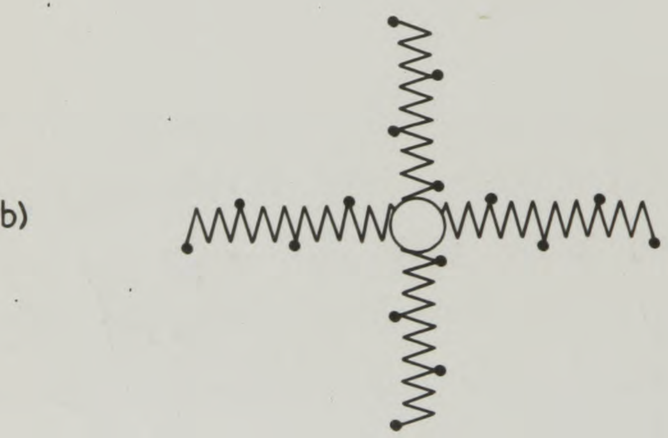
The material in the two fastest bands passed through the dialysis membrane, and thus probably had molecular weights less than 24,000. It was thus not possible by this method to see if hyaluronidase further degraded this material. The hyaluronic acid fraction was found to be digested by hyaluronidase into fragments small enough to pass through the pores of the dialysis membrane, while the slowest moving fraction was partially digested by this enzyme. Papain digestion of the protein in this slowest fraction resulted in the total loss of the acid polysaccharide component through the dialysis membrane. Papain digestion did not affect the hyaluronic acid which was retained within the dialysis sack. These results support the contention that the slowest moving band contains material with a protein - neutral polysaccharide backbone to which partially esterified sulphated polysaccharides are attached (Fig. 4/26). To call such a molecule 'chondroitin sulphate' would be entirely misleading.

POSSIBLE INTERPRETATION OF THE STAINING REACTIONS OF THE SLOWEST BAND

a)



b)



c)

SYMBOL	STAINED BY	NATURE
	Alcian Blue at pH1	$-OSO_3^-$
	Alcian Blue at pH1	Fully Esterified Chondroitin Sulphate
	Alcian Blue at pH1 + PAS	Partially Esterified Chondroitin Sulphate
	PAS alone	"Neutral Polysaccharide"
	Nigrosine	Protein

Fig.4/26

Limitations of the Method. The results described were obtained from material which was rendered soluble after homogenization of papillae in a butanol / water mixture. It could be argued that by this method a protein fraction might be missed if it were not first made water soluble by proteolysis. However, the close correspondence between the appearance of the autoradiographic label in the butanol / water extracts and of the auto-radiographs of tissue sections suggests that the only component shown to be associated with protein was extracted.

The aim of the procedure was to be able to correlate any changes in the extracted material with the appearances in the tissue sections which accompanied an aleterd level of hydration in the animal, and it was thus important to be certain that the method itself produced minimal alteration in the material studied. For example,

(a) The method of homogenization would be expected to rupture lysosomes, since this was the method chosen by Morton (1950) for his biochemical study of hydrolases. In the present case a relatively large volume of ice cold water was used to minimize the chances of an enzyme encountering a suitable substrate. To test the effect of hydrolysis on the electrophoretic pattern in one instance, half the initial material was subjected to the action of testicular hyaluronidase which was added just prior to homogenization, and the remaining material processed in the normal way. No qualitative or quantitative differences could be detected between the electrophoretic pattern produced by each.

Thus it would appear that depolymerised molecules of acid mucopolysaccharide have the same effective ratio of charge to size as the parent compound, so that the effects of depolymerisation are not evident in the electrophoretic separation provided there is no actual loss of material.

(b) Another possible source of error could arise from the prolonged storage of concentrated solutions of the lyophilized extracts. It is possible in theory that glycosidases present could synthesize heterogeneous chains of polysaccharide. It was found, however, that extracts uncontaminated by bacteria could be stored for at least a month at 0°C. and then subjected to electrophoresis without any quantitative change in the pattern produced. In general, the separations accompanying physiological experiments were performed within half an hour of preparing the final solution.

Similarly, the Rutenberg, Cohen and Seligman (1952) method for aryl sulphatase had been shown to give a weakly positive reaction in the collecting tubule cells (Fig. 3/24), and it was thus neccessary to test for the activity of this enzyme in the extracts. In order to do this 225 µCi of radioactive sulphate was added to the water used for homogenization. An extract from papilla was made in the usual way, (but using the solution containing labelled sulphate), except that the time between homogenization and centrifugation was deliberately prolonged to one hour.

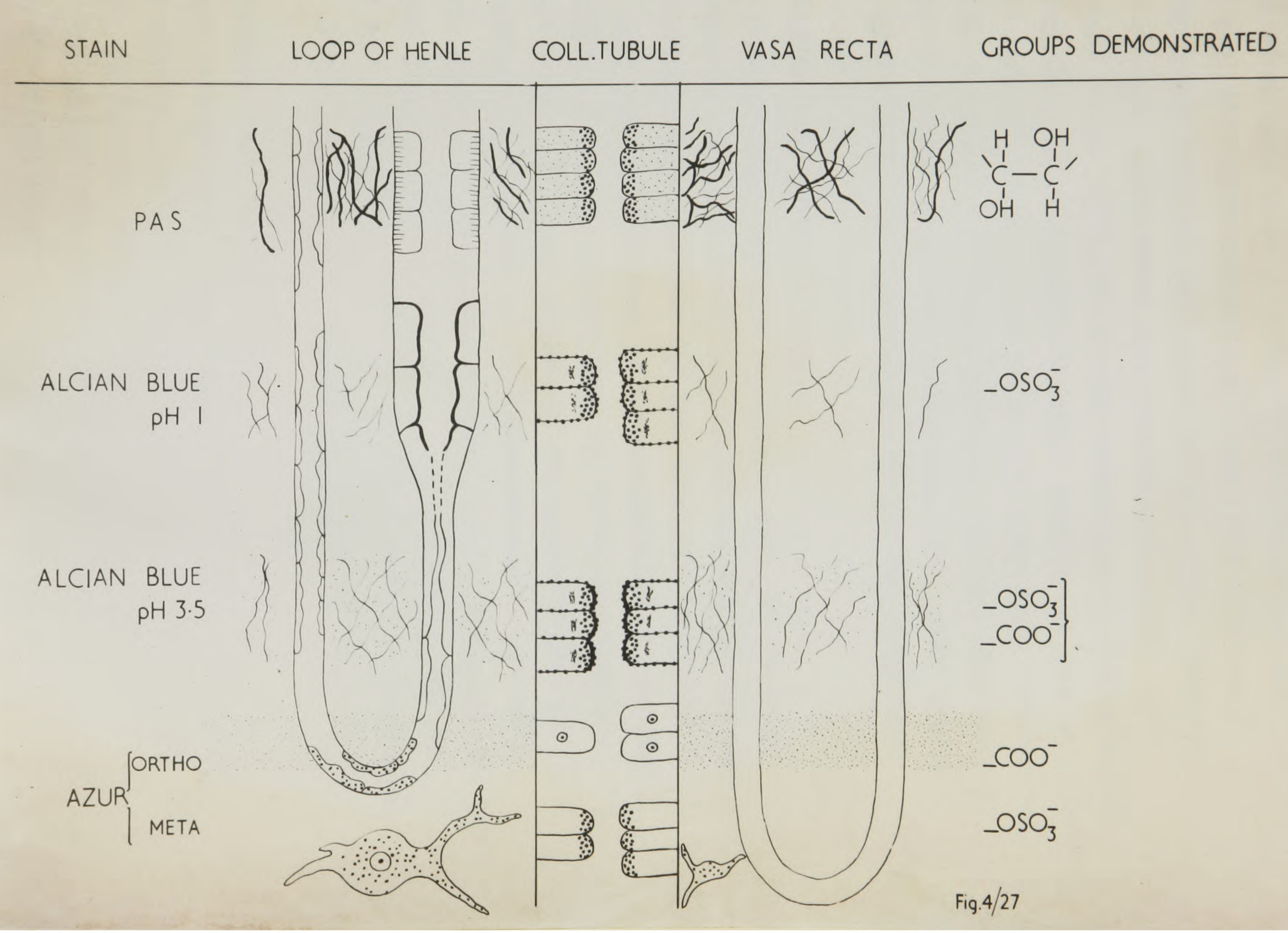


Fig.4/27

The material was subjected to electrophoresis and autoradiographs prepared by laying the strips onto X-ray film after drying and before staining. On staining the strip with Alcian Blue the usual four bands appeared, but the label was not found in any of them. There was, however, a wide dense band on the autoradiograph over the anodal end of the strip which did not stain with Alcian Blue. It was concluded that this represented unincorporated sulphate ions which diffuse from the strip during staining. As a corollary to this, any staining on the experimental strips must have represented molecules sufficiently latge not to diffuse from the cellulose acetate membrane before they were precipitated by the Alcian Blue.

C. Summary.

The information given by the histochemical and biochemical approaches is displayed in a composite diagram (Fig. 4/27). The acid mucopolysaccharides within the guinea pig papilla are discussed within the morphological framework presented in the last chapter.

<u>The Collecting Tubule Cell.</u> The collecting tubule cells contain glycogen granules which may be digested by salivary amylase. The lumenal border and the region between cells is seen in the light microscope to be PAS positive, but both are resistent to amylase.

The staining of these regions is also more readily blocked by dimedone than is the staining of glycogen, which indicates that they have a different basic structure (Pearse, 1960).

There are also PAS positive granules within the cytoplasm of these cells, which are larger than the glycogen granules and which cannot be digested by amylase. Their perinuclear distribution corresponds with the Golgi region as demonstrated by the classical silver impregnation methods and the electron microscope. The granules which stain positively for sulphate groups occupy the same region and are the same size in the light microscope. Since this is the region in the cell where radioactivity is first evident after the injection of $S^{35}O_4^{=}$, it is concluded that the larger class of PAS positive granule probably represents 'neutral' polysaccharide undergoing esterification by sulphate.

From the coincidence of radioactivity in these granules and the appearance of radioactivity over the slowest moving band in the electrophoretic separation it was concluded that the material in this band originated from the granules. Thus it was shown that these granules had an acid mucopolysaccharide component, the anionic group of which was principally sulphate; (a neutral polysaccharide part); and that they were associated with protein by a link that was stronger than ionic and capable of withstanding electrophoresis. The protein could be digested by papain, and the acid polysaccharide was partially hydrolysed by hyaluronidase (testicular).

Similarly sized 'granules', which possessed identical staining reactions, were seen in the light microscope to be concentrated at the apical ends of the cells and to lie between the collecting tubule cells. It is suggested that these 'granules' in fact represent cellular interdigitations. The arrangement of the Golgi region between the cell nucleus and the lateral intercellular space was taken to indicate that material is actively secreted into this space. The presence of tight junctions at the lumenal ends of these intercellular spaces was taken to mean that any secretion moving into this space must leave it by the basal opening and must pass into the interstitium.

The lumenal borders of the collecting tubule cells were strikingly specialized in their extensive 'cuticle' of sulphated mucopolysaccharide and PAS positive material. The distribution of this material corresponds closely to that of the fuzz seen in electron micrographs and to the regions which bind colloidal iron.

<u>The Interstitium.</u> The interstitial space was found to be much more extensive in the papilla than in other parts of the kidney.

The space is traversed by reticulin fibrils which can be impregnated by silver and which are PAS positive. Still smaller fibrils are seen which stain positively for sulphate groups: These do not appear to be demonstrable by silver methods, but it is possible that the silver precipitates make the fine fibrils

appear more coarse than they do in the Alcian Blue staining. Alternatively the finest fibrils may be of reticulin which has had sulphated mucopolysaccharide precipitated around them either in vivo or during the histological processing. However, the sections may be repeatedly washed and the fibrillar component continues to stain with constant vigour for sulphate groups, which indicates that little can be 'washed off' after fixation. It is therefore possible that a sulphated mucopolysaccharide is a structural component of the fibrils in the renal papilla.

Hyaluronic acid was found to fill the spaces within the mesh of fibrils. It could readily be washed from sections and was labile to testicular hyaluronidase. The amorphous appearance of this component in sections suggested that it probably existed in vivo as a gel. If it had existed as a sol an irregular precipitation of its components would have been expected to occurr during fixation.

Interstitial Cells. The finest processes of the interstitial cells could not be distinguished by the light microscope from the fibrillar components of the interstitium with which they were closely associated. With the electron microscope only a small part of one cell or its processes could be seen in a particular field.

It was found histochemically that the cells nearest to the papillary tip, where the cation concentration is highest in vivo, contained metachromatic granules. These

granules were smaller than those found within the mast cells of the renal capsule. The fineness of the interstitial cell cytoplasmic processes made it impossible to study the turnover of acid mucopolysaccharide within these cells by light microscopic autoradiography, and it was thus not possible to conclude whether these cells manufactured or destroyed the extracellular matrix.

<u>The Vasa Recta and Loop of Henle.</u> The basement membranes and endothelium of the vasa recta and loop of Henle are too fine to permit histochemical observation of these structures at the papillary tip. From the anatomical point of view it was noted that the vasa recta did not always form simple hairpin loops in the guinea pig papilla. One section would usually furnish unequivocal evidence of a plexus of blood vessels at the papillary tip (~~eg. Fig. 4/8~~). Throughout the rest of the papilla the vessels run in parallel through the interstitium as described by Trueta, Barclay, Daniel, Franklin and Prichard (1947).

The relatively small Golgi regions seen in the cells of the vasa recta or the loop of Henle with the electron microscope suggest that these cells probably play only a small part in the synthesis of carbohydrates in the papilla of the guinea pig.

As the cortex is approached the cells lining the thick limbs of the loop of Henle can be seen to have a PAS positive glycocalyx which shows up their basal

infoldings well in the light microscope. They have a thin lumenal rim of acid mucopolysaccharide which binds colloidal iron and a few metachromatic intracellular granules. However, a thick portion of the ascending limb of Henle's loop was seen infrequently in the papillae investigated in this study, and it was not possible to estimate the contribution it makes, if any, to the formation of the papillary acid mucopolysaccharides.

CHAPTER 5: APPEARANCE OF PAPILLARY ACID MUCOPOLYSACCHARIDES IN DIFFERENT PHYSIOLOGICAL STATES.

A. Changes Induced in Adult Guinea Pigs:

Ginetzinsky (1958) stated that when a rat was dehydrated or given pituitrin the collecting tubule cells lost most of their cytoplasm, which made the nuclei project into the tubule lumen. He argued that this loss of cytoplasm appeared to be identical with the apocrine secretion of the collecting duct epithelium due to antidiuretic hormone: At the same time the 'hyaluronic acid' (sic) intercellular layers disappeared completely.

No evidence to substantiate the morphological conclusions was offered other than two photomicrographs of sections stained with toluidine blue. Unfortunately in view of the observation made in the third chapter of this thesis, and which is expanded in the present chapter, that the collecting tubules have cells which are columnar at the tip of the papilla but cuboidal in the medulla, Ginetzinsky's sections were taken from levels which were not comparable. While a comment that the intercellular cement was everywhere reduced following ADH injection could be valid, the evidence that apocrine secretion does take place must be

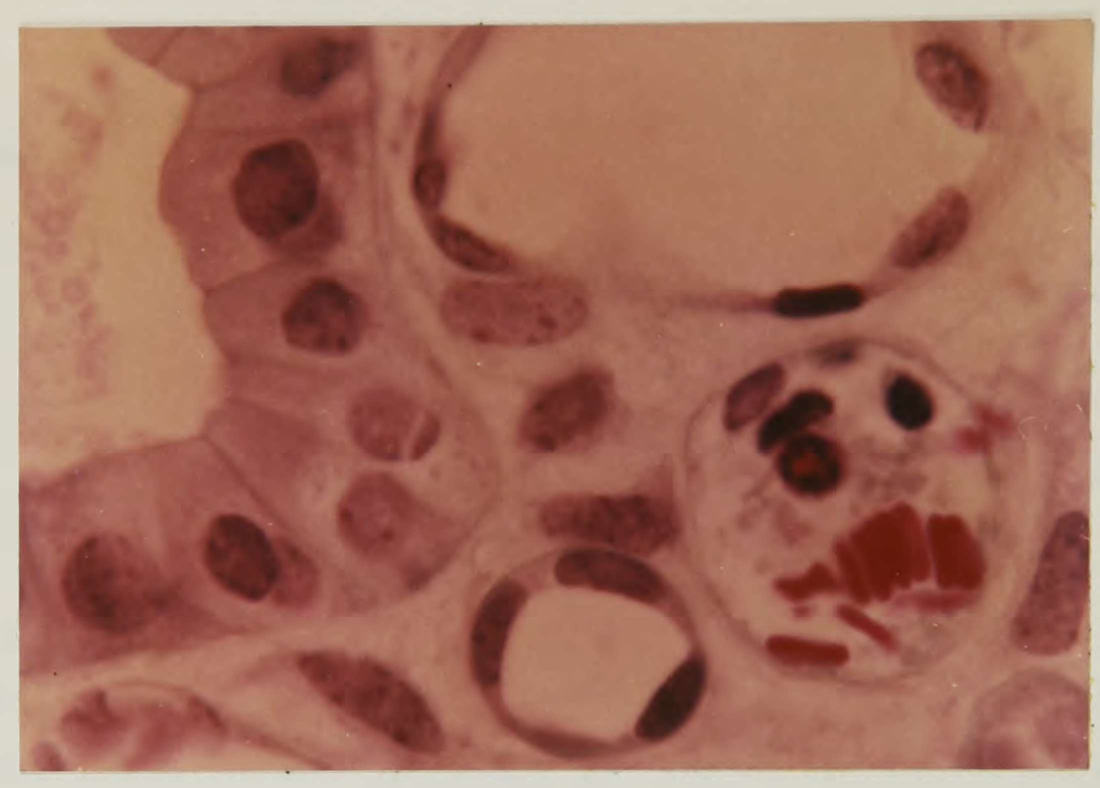


Fig. 5/1. WATER LADEN. Masson Trichrome. Transverse section 3 mm from tip of papilla; Columnar collecting tubule cells, diffuse interstitium and normal blood cells. Mag 500.

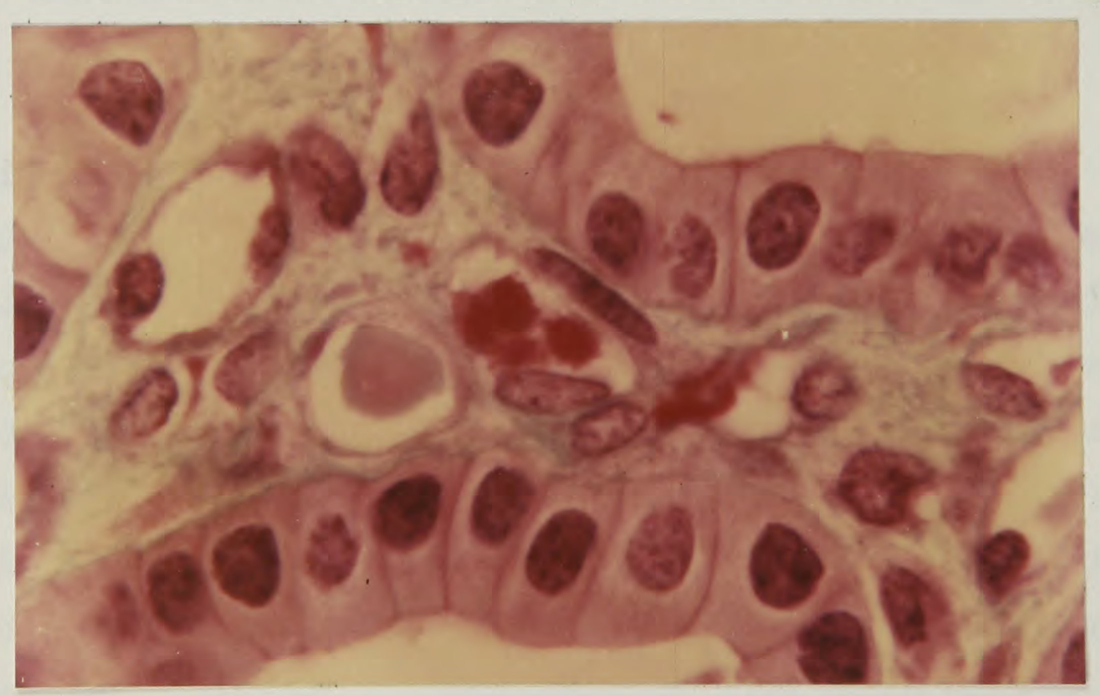


Fig. 5/2. ADH INJECTED. Masson Trichrome. Transverse section 3 mm from tip of papilla; Cuboidal collecting tubule cells, compact interstitium and crenated blood cells. Mag 500 (Comp. vasa recta with that in Fig. 3/25).

considered inconclusive.

Further, when seeking evidence of apocrine secretion, care should have been taken to chose animals in which the kidneys were initially as similar as possible: Ginetzinsky does not state whether this was considered. In the present study littermate guinea pigs of a similar weight were selected for those experiments where actual measurement of the papillary components was made: This study was confined to male animals. Thus differences arising from the effects of age, sex, nutrition, environment and disease were minimized. Four criteria had to be established before it was possible to evaluate the results of Ginetzinsky (1958):

1) There had to de a demonstrable reduction in collecting tubule cell height following ADH injection: This had to take into account the natural variation in cell height within the papilla at different levels.
2) Any such reduction must be due to the direct action of ADH, and must not simply be an expression of, for example, the increased papillary osmotic pressure found in antidiuresis. In other words the possibility that collecting tubule cells might act as simple osmometers in the way that red blood cells do (Figs. 5/1 and 5/2) had to be excluded.
3) Hyaluronidase introduced into the collecting tubule lumen should produce similar depolymerization of the intercellular cement if ADH acts by releasing this enzyme.
4) In the absence for a histochemical method for the demonstration of hyaluronidase activity, its

presumed activity in vivo must be correlated with reduced amounts, or altered partition, of its substrates in the tissues.

These aspects have been examined in the following section of this thesis. The standardised procedures were adopted for the reasons outlined in the relevent section.

Methods.

a) Water Loading. The standard water load for an adult guinea pig was chosen to be 20 ml of distilled water, which corresponds to about 4 ml/100 grams of body weight. (This is equivalent to about four pints consumed by a human subject.) It compares with 4ml/100 grams given to rats by Zain-ul-Abedin (1967); 2 to 8 ml/100 grams given to rats by Robson (1963); and 2ml/100 grams given to rabbits by Darnton (1967), all of whom were studying the morphological effects in the kidney of hydration.

Distress was observed when the load was injected intraperitoneally in a single dose. Thus a soft polythene oesophageal tube was passed through a device holding open the mouth of the animal. Most of a 10 ml dose administered by this route was retained. A further 10 ml was then injected intraperitoneally. Both doses were heated to approximately 37°C before administration. The animal was killed one hour later, by which time a copious diuresis had always been established.

b) Water Deprivation. In experiments involving water deprivation, guinea pigs were allowed free access to dry food pellets, but greenstuffs and drinking water were witheld for periods of 12, 24, 48 and 72 hours prior to slaughter.

c) Antidiuretic Hormone. Pitressin (Parke, Davis) containing 5 pressor units was injected intraperitoneally one hour before death. Zein-ul-Abedin (1967) reported that in the rat single doses of ADH were followed 30 to 60 minutes later by the minimal flow of the most concentrated urine, and the morphological changes described for the guinea pig in the present context were found consistently to be maximal after one hour.

The dose used was large: It represents about fifteen times the dose found satisfactory in man for the control of diabetes insipidus. However, the activity of 'Pitressin' depends upon the balance of two components, lysine-8-vasopressin and arginine-8-vasopressin. Arginine-8-vasopressin is about four times more potent in the guinea pig than is lysine-8-vasopressin (Dicker and Eggleton, 1961). However, 'Pitressin' is a preparation of hog posterior pituitary in which lysine-8-vasopressin predominates. Further, any arginine-8-vasopressin that was present in vivo is less stable and rapidly loses its potency with storage (Goodman and Gilman, 1965). Thus the activity of the hormone preparation used in this study probably lay mostly in the 'non-physiological' lysine-8-vasopressin.

The large dose used in the present study was chosen to give the maximum physiological response with the minimum visible histological damage. With still larger doses the animal became lethargic: In the kidney, larger doses ~~would~~ produced casts of collecting tubule cells, and often blood cells would appear in the collecting ducts. Excessive doses were sometimes associated with the production of an increased quantity of urine, either as a result of renal damage or because of a raised systemic blood pressure elevating the glomerular filtration rate. The results could not be considered meaningful under these circumstances.

d) Simultaneous Administration of Water and ADH. In these experiments the standard 10 ml of water was given down the oesophageal tube followed by 10 ml of water containing 5 pressor units of 'Pitressin' which was injected intraperitoneally. The animals were killed one hour later.

e) Hyaluronidase Injection. Testicular hyaluronidase (Benger's 'Hyalase', 1,000 units) in 1 ml of distilled water was injected intraperitoneally. This was one quarter of the intravenous dose used by Thorn, Knudsen and Koefoed (1961) to produce an antidiuresis in water laden rats. In one experiment this was combined with 5 pressor units of ADH.

f) Radioactive Sulphate. In experiments where radioactive sulphate was used as an indicator of sulphated acid mucopolysaccharide synthesis, 1 mCi $S^{35}O_4^{=}$ was injected

% RELATIVE AREAS OF PAPILLARY COMPONENTS IN WATER LADEN & ADH ANIMALS.

(a) Water laden animal (Guinea pig J, 20 ml)						
Dist. from tip of papilla. mm	Area/π	Coll. T. Lumen.	Coll. T. Cells.	L.of H. & V.R.	Intitm.	I.Cells.
0.5	0.56	13.9	33.5	20.8	27.1	4.7
1.0	1.25	14.9	34.5	19.4	27.6	3.6
1.5	1.56	15.3	34.7	21.1	26.5	2.4
2.0	1.88	15.3	35.6	20.2	23.9	5.0
2.5	3.06	14.4	32.7	20.9	28.6	3.4
3.0	3.24	14.6	30.4	21.2	30.2	3.8
(b) ADH Injected Animal (Guinea Pig K, 5 Units)						
0.5	0.25	13.7	21.1	18.9	38.5	7.8
1.0	1.00	15.5	27.0	19.3	33.9	4.4
1.5	1.56	16.1	21.3	20.4	38.1	4.2
2.0	1.82	16.6	29.2	11.0	39.0	4.0
2.5	2.25	21.4	26.6	10.2	37.6	4.2
3.0	2.56	21.2	25.4	15.2	33.8	4.4
3.5	2.89	23.6	26.8	13.8	32.0	4.0

immediately before carrying out the other procedures. The precise volume of the injection depended upon the calculated radioactivity in the sample supplied by the Radiochemical Centre, Amersham. The volume was usually of the order of 1 ml. The technique of autoradiography is described in Appendix 4/2.

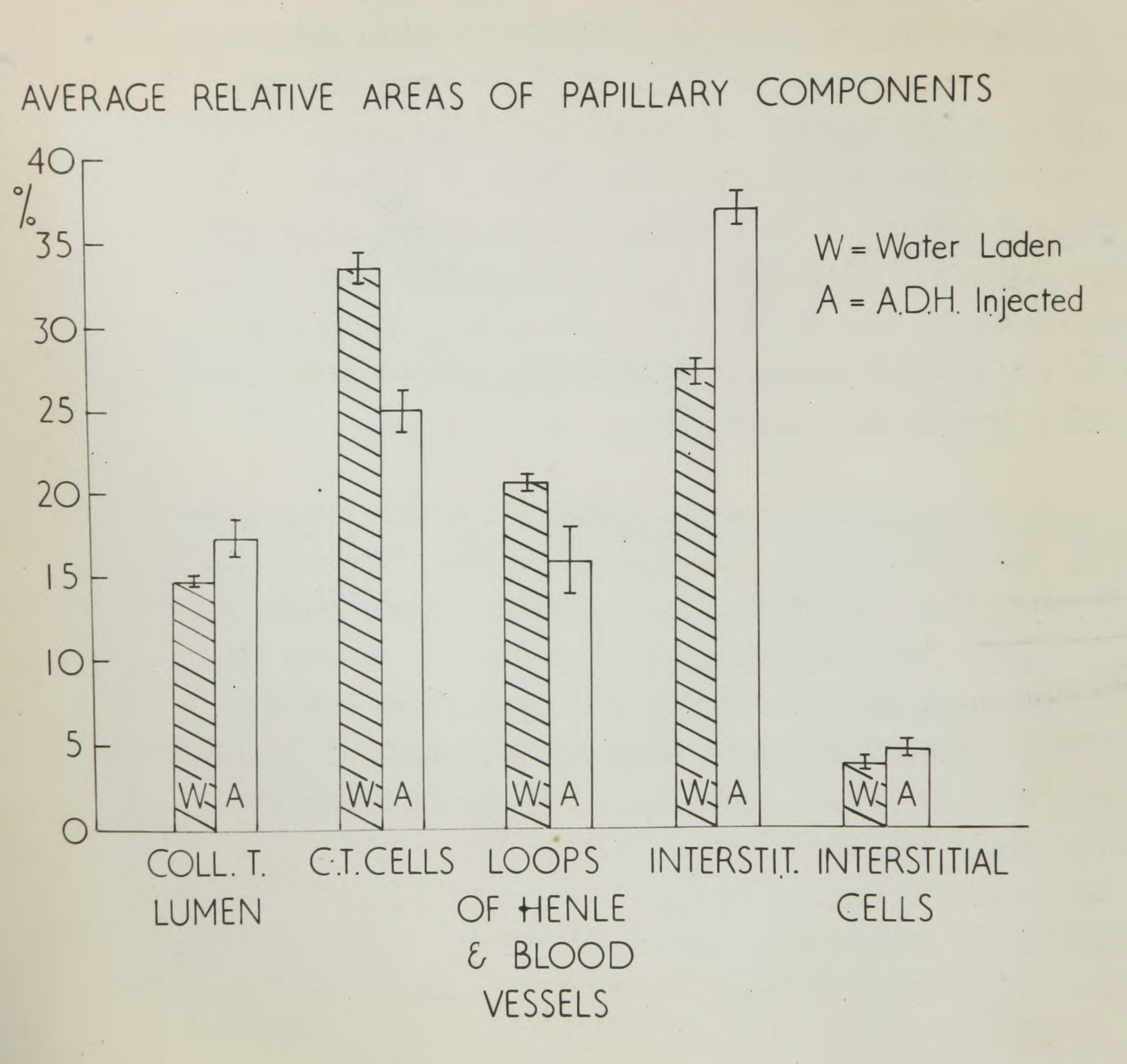
g) Morphometry. The techniques employed for the preparation of sections suitable for the measurement of areas by point sampling, and the estimation of the height of the collecting tubule cells by the use of an eyepiece graticule, are set out in Appendix 2/3.

h) Quantitative Electrophoresis. The extraction of the papillary acid mucopolysaccharides in a mixture of butanol and water, the separation of the fractions by electrophoresis on cellulose acetate membrane, and the assessment of the relative amounts of each by densitometry were the same as those elaborated in Chapter 4. The techniques are given in Appendices 4/3 and 4/4.

Results.

a) Alteration in Height of Collecting Tubule Cells.

The length of collecting tubule cells was measured against an eyepiece graticule. The distance from the base of the cell to the apex at the lumen was


AVERAGE RELATIVE AREAS OF PAPILLARY COMPONENTS
40
%
35
30
25
20
15
10
5
0
W = Water Laden
A = A.D.H. Injected
W A
W A
W A
W A
W A
COLL. T. LUMEN
C.T. CELLS
LOOPS OF HENLE & BLOOD VESSELS
INTERSTIT.
INTERSTITIAL CELLS

found to be less in antidiuresis or following ADH injection than it was in animals given a water load. In a representative pair of littermate male guinea pigs, analysed in Figs. 5/3 to 5/5, at 3 mm from the tip of the papilla the height of these cells in sections from the water laden animal was 13.0 μ (S.D. 1.16 μ) but only 10.05 μ (S.D. 1.21 μ) in those from the animal injected with ADH. In both cases the basal width averaged 9.4 μ (S.D. 1.14 μ). Thus the change from a columnar to a cuboidal shape decreased the cell volume by about 23%.

The relative area occupied by the collecting tubule cells throughout the medulla was measured by the point sampling technique. The results from this appear in Fig. 5/3, and the averaged figures are shown as a histogram in Fig. 5/4, which has also been related to the total cross sectional area of the papilla at given levels in Fig. 5/5. This last representation shows clearly that the collecting tubule cells have a lesser volume in antidiuresis than they do in water diuresis. It should be emphasised again, however, that the height of the collecting tubule cells is not constant along the length of the collecting tubule, those near the medulla always being shorter than those at the tip of the papilla.

While the present results are in agreement with Ginetzinsky's conclusion that the cells decreased in height in the rat following ADH injection, there was no evidence from the results on the guinea pig that a

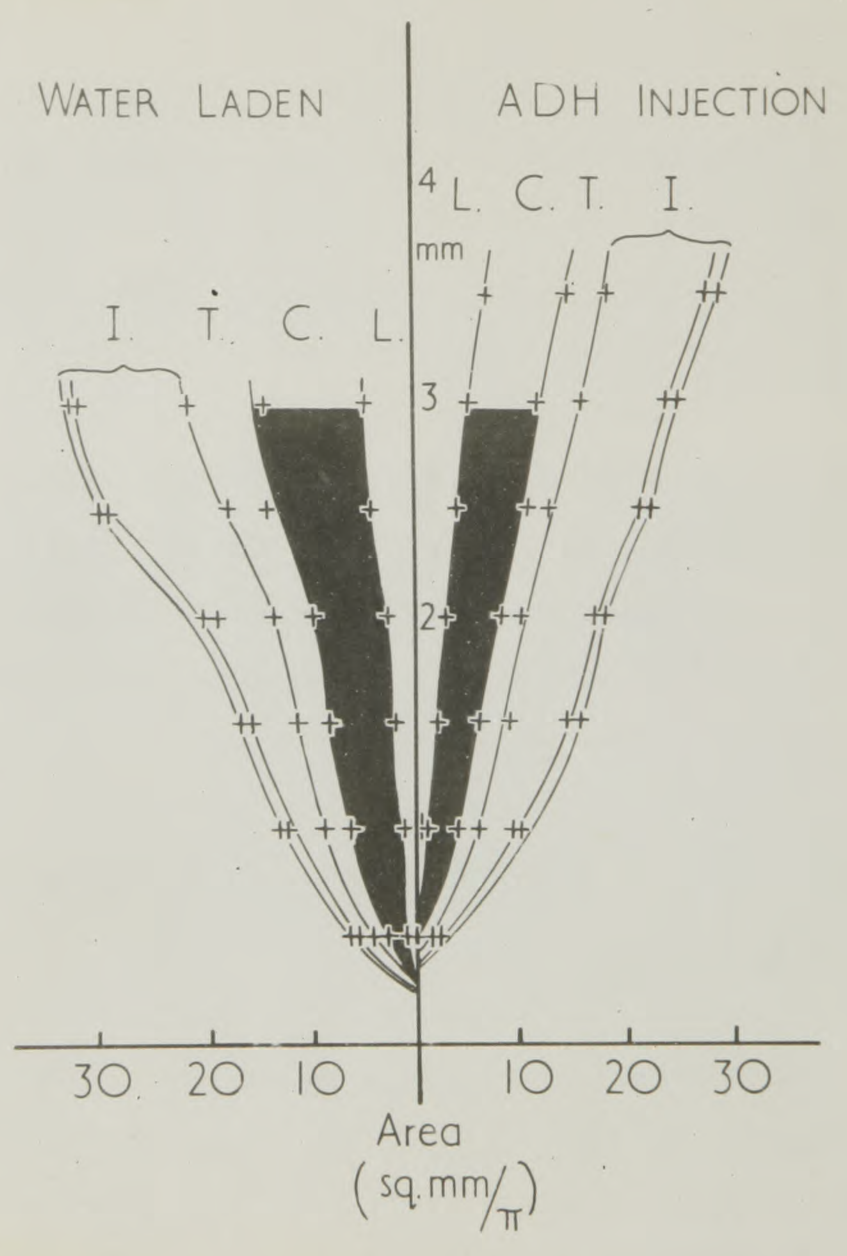


Fig. 5/5.

'complete dissolution of the collecting tubule apical cytoplasm' had ever followed the effective doses of ADH used in the present study. Nuclei were never found to 'bulge into the lumen' in the guinea pig, and the lumenal cell border remained sharply defined.

It was not possible to observe the effect of combining a water load with ADH injection in the morphometric studies. This should be undertaken to discover whether the decrease in cell size is the result of a direct effect of ADH on the apical region of the cell, in which case it would be seen when ADH was present at the same time as a water load. If the effect were purely osmotic, then the presence of a water load might be expected to prevent a build up of osmotic pressure following ADH injection, in which case the collecting tubule cells would remain columnar.

<u>b) The Relationship Between Cell Volume and Adjacent Osmotic Pressures.</u>

The crenated appearance of the red blood cells at the 3 mm level in the concentrating kidney (Fig. 5/2) but not in the water laden kidney (Fig. 5/1) suggests that a higher tonicity exists in the interstitium in antidiuresis.

The papilla along which the osmotic gradient became established in antidiuresis was 5 mm long, in which case the tonicity of the 3 mm level would be in the region of 1 Osm/l if that at the tip is assumed to be 2 Osm/l.

If the further assumption is made that the uncrenated red blood cells seen in the kidney from the water laden animal were exposed to the usual plasma tonicity of about 300 mOsm/l, then it would appear that the collecting tubule cells at this level are subjected to more than a threefold change in the osmotic pressure of their environment. Consequently, if these cells were simple osmometers, the observed decrease of 23% in volume following ADH injection would seem to be small. Several explanations might be advanced:

1) The cells might maintain a fairly constant volume by being relatively permeable to, say, sodium which would tend to enter the cell down an increased concentration gradient in antidiuresis. This would counter the tendency for water to be abstracted from the cytoplasm.

2) The cells might shrink to the full extent if both the lumenal and basal surfaces were bathed by equally hypertonic solutions. In antidiuresis, however, the lumenal fluid is slightly hypotonic to the interstitium, while both are hypertonic to the cell cytoplasm. Hence fluid in transit through the cell might augment the 'true' cytoplasmic volume.

3) The cells might have some barrier or coating to isolate the cytoplasm from the surrounding osmotic conditions. This coating may be a defence against the effects of the hypotonic solution which bathes

the apex of the collecting tubule cells in diuresis. It may thus be that the emphasis should lie more on an increase in cell height in water diuresis than on some destructive process arising in antidiuresis.

Measurement of the actual solute concentration within the cells could be expected to distinguish between these alternatives since in antidiuresis it would be elevated if (1) was true, less so in (2) where partition effects might also be evident, while in case (3) the solute concentration would be expected to be unaffected by the level of diuresis.

In living collecting tubule cells the solute concentration is related to refractive index by the equation:

$$n = n_o + aC,$$

where n, the refractive index of the cytoplasm, represents the refractive index of a mixture containing a concentration C of solute in a pure solvent of refractive index n_o. The constant of proportionality,(a), is of the order of 0.002 for biological solutes (Barer, 1956). Attempts to estimate refractive indices by measurement of optical path differences using a Baker-Smith Double Focus Interference microscope produced inconclusive results due to the difficulty experienced in obtaining perfectly flat preparations of a constant thickness of living collecting tubule cells. (If it had proved possible to obtain satisfactory interference images with the light passing through the thick glass of a haemocytometer this

Phase Contrast.

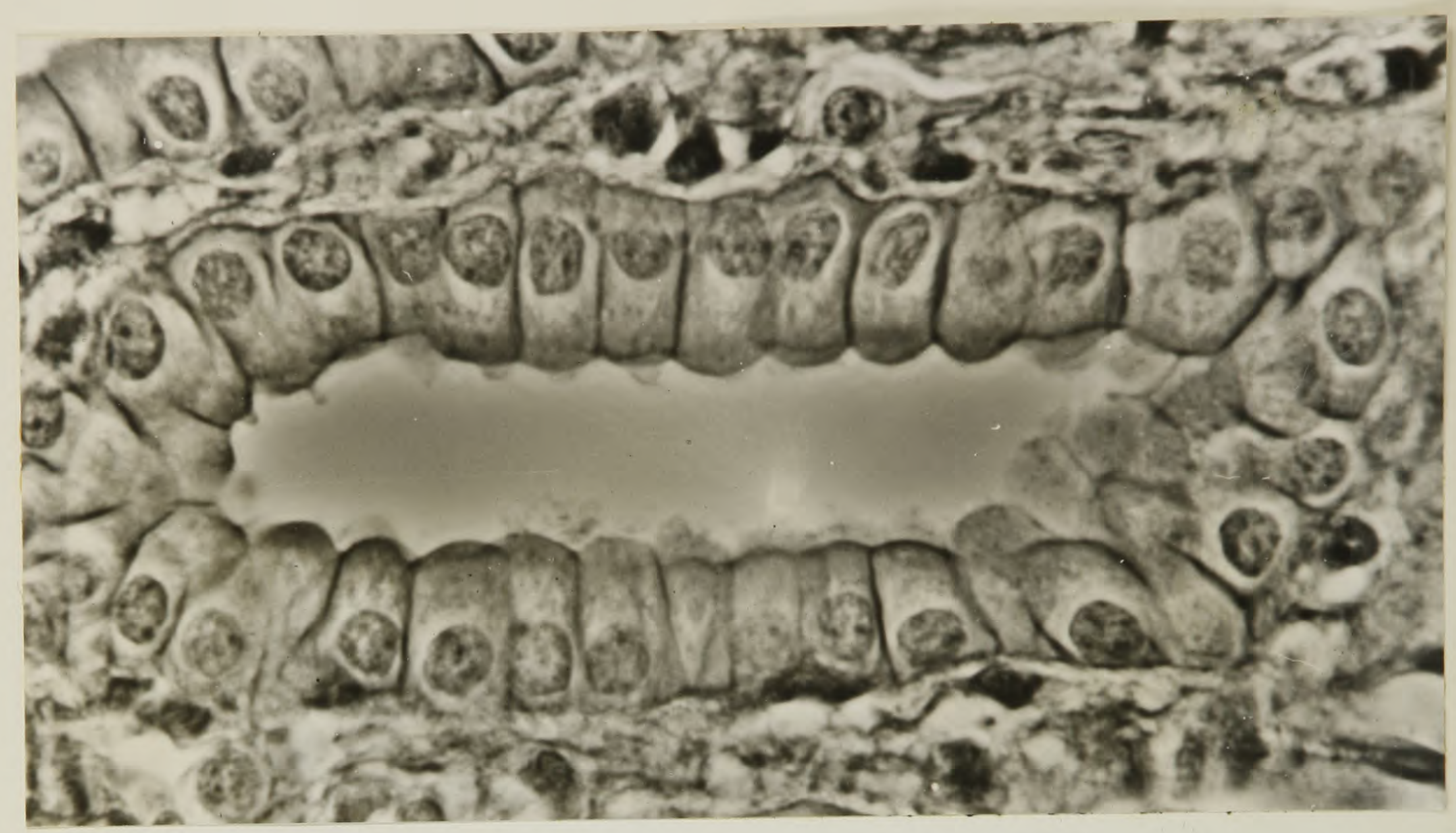


Fig. 5/6. WATER LADEN. Section 3 mm from tip of papilla. Mag. 500. Columnar cells with apical material evident.

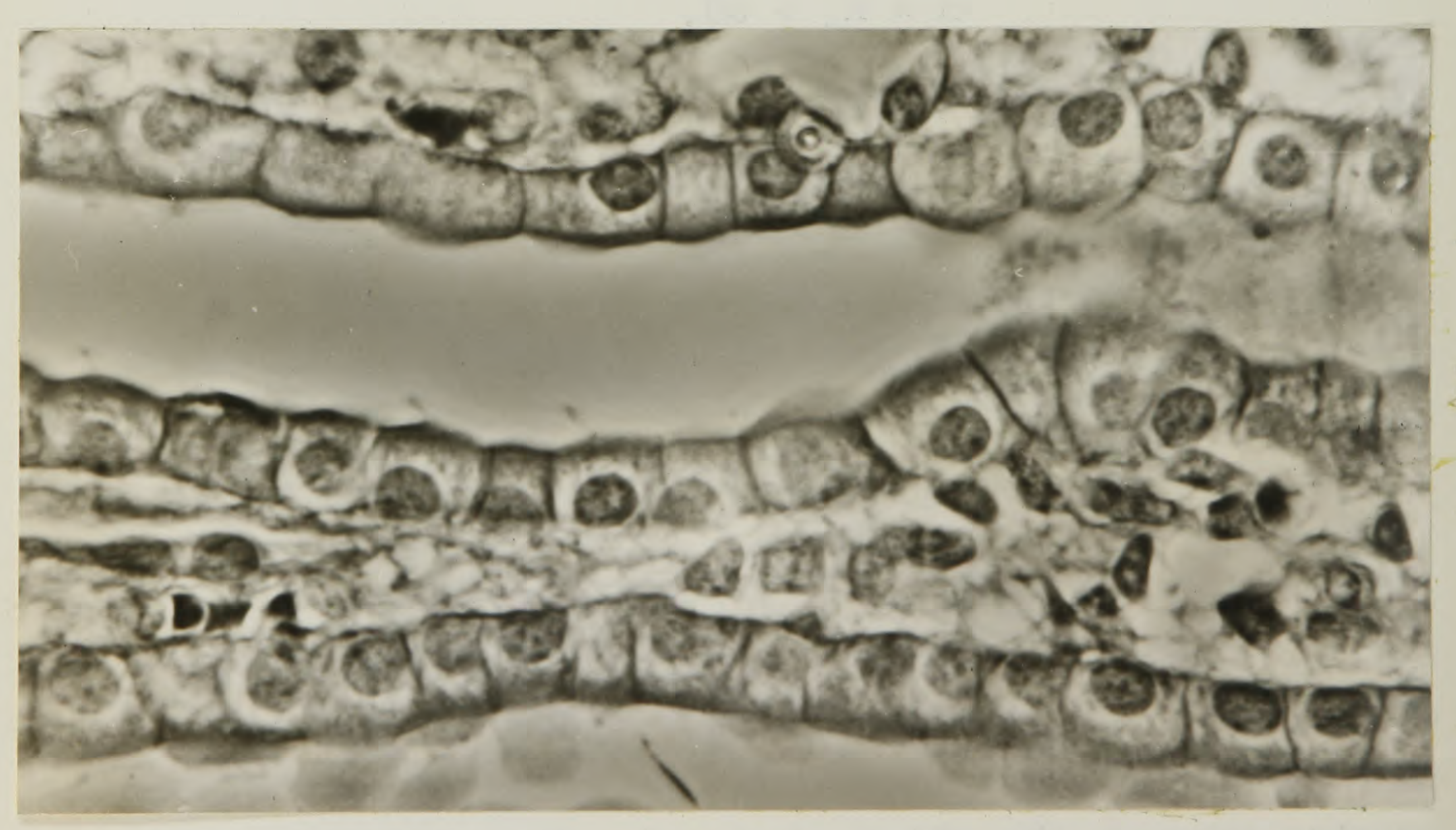


Fig. 5/7. WATER DEPRIVATION (48 hours). 3 mm from tip of papilla. Mag. 500. Cuboidal cells.

problem might well have been overcome.)

Nonetheless it was noted that the apical region of the cells in collecting tubule fragments from concentrating kidneys appeared to be more dense optically in relation to the cytoplasm at the base of the cell than it did in those from the water loaded animals. The lateral and basal regions of the cytoplasm did not appear greatly to alter. This result suggests that in antidiuresis the lumenal border of the collecting tubule cell becomes sensitive to the raised osmolarity of the lumenal contents.

The examination of formol-cellosolve fixed unstained sections of tissue by the Zernike phase contrast method gave results which were visually very similar. The curved apical regions of cells from water laden animals contained evenly distributed fine granules (Fig. 5/6), while in the smaller cells from animals deprived of water or given ADH, the apex was almost flat and the granules were more densely packed (Fig.5/7.) In addition the apical layer of 'fuzz' is well demonstrated in the lower row of columnar cells in the water laden kidney, Fig. 5/6: It is not evident on the cells from the animal injected with ADH (Fig 5/7).

The conclusion drawn from these observations was that the lumenal border of the collecting tubule cell appears to be a less effective osmotic barrier following ADH injection. If the base of the cell has no such barrier and there is rapid equilibration between

between the cytoplasm and the interstitium across this border, then the appearance of these cells in antidiuresis is compatible with the transport of water through them. Alterantively, water could pass through the apex of the cell and leave through the lateral intercellular spaces in antidiuresis, leaving the basal cytoplasm unaffected. The changes described, however, are certainly not correlated with apocrine secretion in the usual sense of the term.

If apocrine secretion does take place in response to ADH, then it might be argued that the collecting tubule cell should have the same appearance when ADH is given together with water as it does when the hormone acts alone. This might be thought to be an unphysiological state, but it is one that arises clinically in patients who have tumours which secrete ADH (Schwartz, Bennett, Curelop and Barter, 1957; Azzopardi, 1966). In the experimental condition the tonicity of the interstitium and tubular fluid would be expected to be low, and thus the shrinkage of collecting tubule cells due to osmosis should not arise. This condition was not simulated in any of the experiments where living tissue was examined directly; but it was investigated in connection with radioactive sulphate and acid mucopolysaccharide staining, since it had been shown (Chapter 4) that the ~~region~~ apex of the cells ~~involved~~ had a very high concentration of sulphated acid mucopolysaccharide.

Sulphate Incorporation.

Fig. 5/8.
WATER
LADEN.

Fig. 5/9.
ADH.

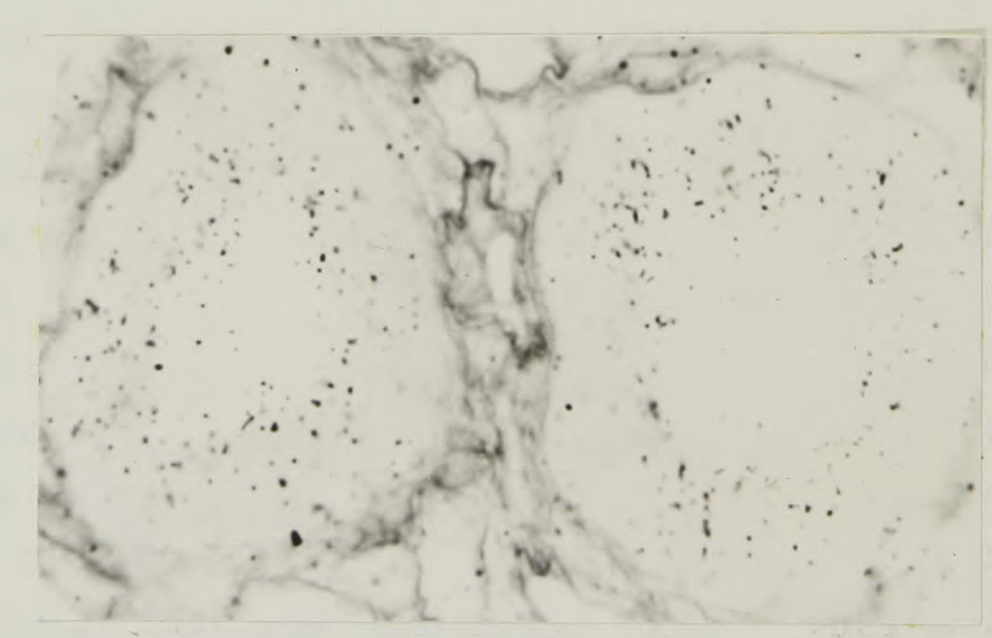
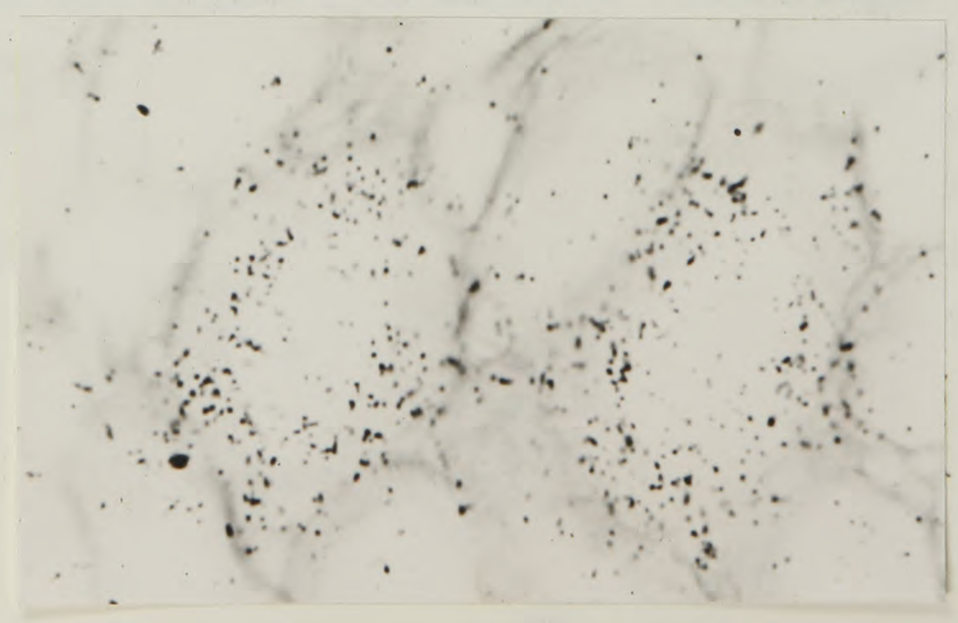


Fig. 5/10.
WATER
LOAD &
ADH.



All sections PAS-stained before coating.

c) The Effect of ADH on Acid Mucopolysaccharide Histochemistry.

The effect of combining a water load with ADH injection was observed in four experiments. Longitudinal sections were taken through the apex to base of the papillae from guinea pigs which had been injected with 1 mCi of $S^{35}O_4^{=}$ before the start of the experiment. It was possible with such sections to use the microscope stage verniers to measure distances from the tip of the papilla and thus to compare similar regions.

Little difference could be found between water laden and control animals in the distribution and density of the label: In both cases the collecting tubules were lined by high columnar cells with the label most dense at the apical ends after one hour (Fig. 5/8). The collecting tubules of animals injected with ADH alone had smaller cells with a lesser density of labelling. The grains in this condition were also more spread out (Fig. 5/9). The appearance of the collecting tubule cells in animals given both water and ADH fell between these extremes (Fig. 5/10).

Sections adjacent to those taken for autoradiography were stained by the colloidal iron method for acid mucopolysaccharides. This method demonstrated definite intercellular material and thick apical cuticle associated with the collecting tubule cell in the control and water laden animals, Fig. 5/11. The lumenal lining was reduced both in animals receiving ADH alone and in those receiving water in addition (Figs 5/12 & 5/13).

Acid Mucopolysaccharide Staining.

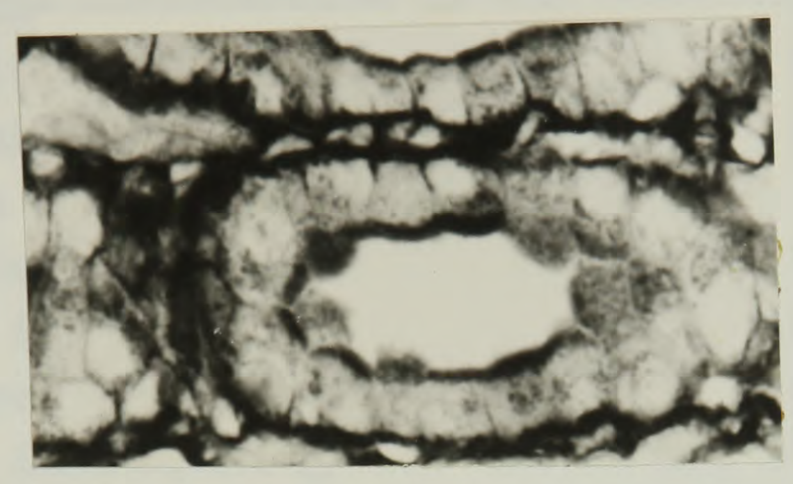


Fig. 5/11. WATER LADEN. Mag. 200.

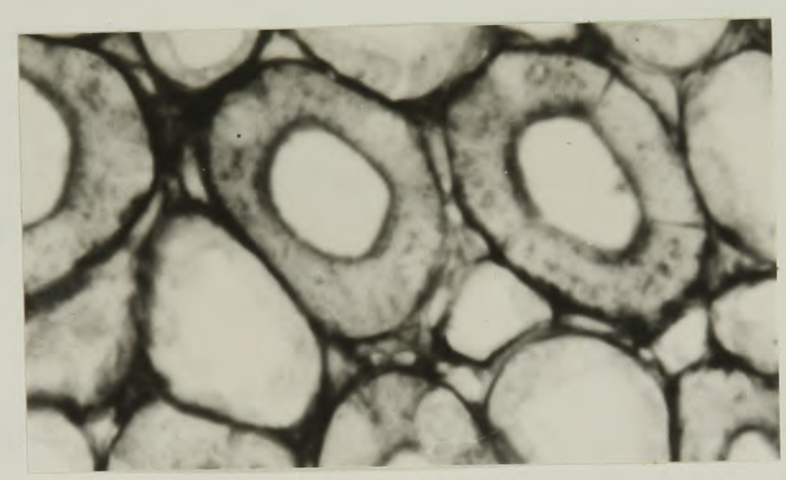


Fig. 5/12. ADH INJECTION. Mag. 200.

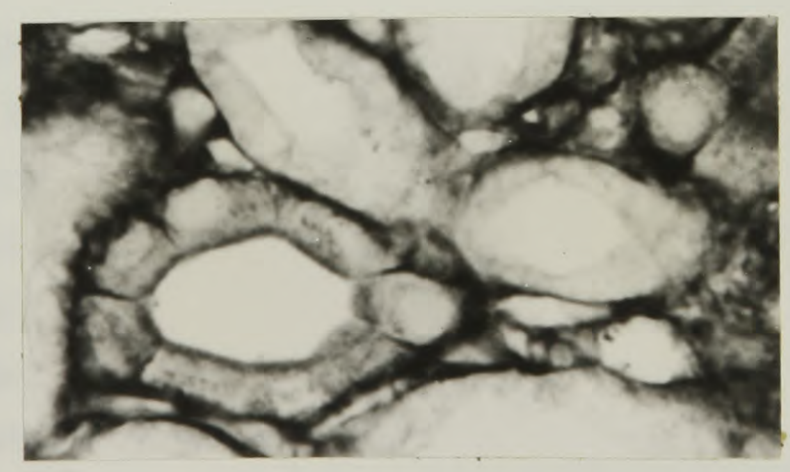


Fig. 5/13. ADH & WATER LOAD. Mag. 200.

Silver Impregnation of Reticulin.

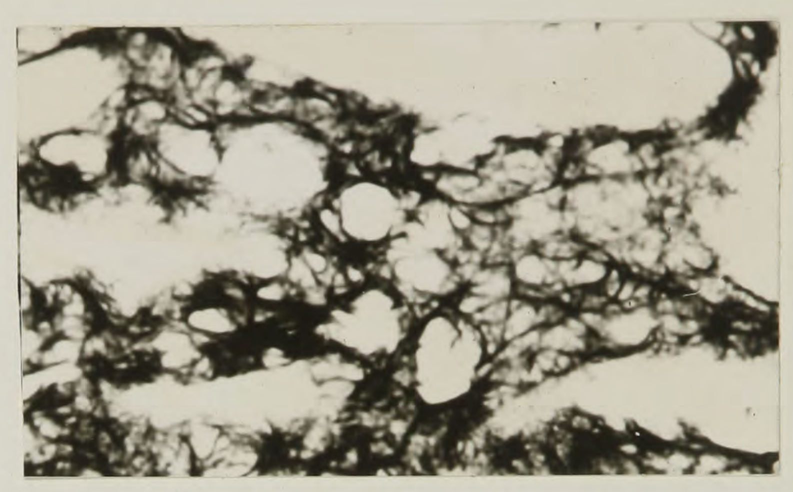


Fig. 5/14. WATER LADEN. Mag. 200.

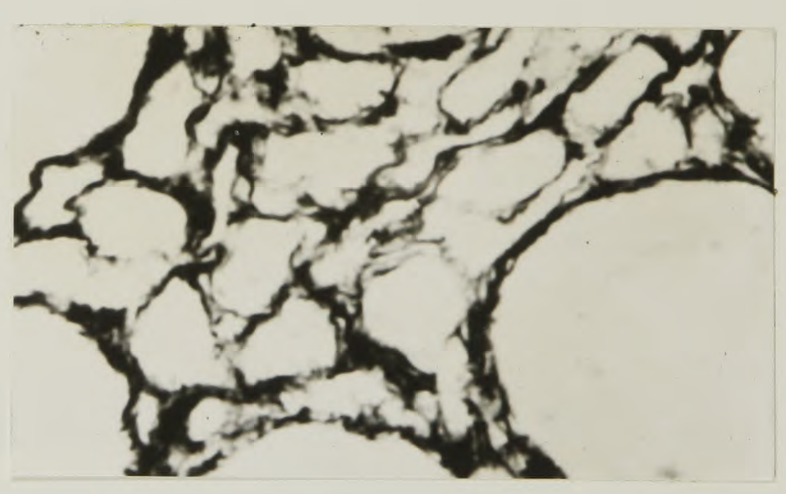


Fig. 5/15. ADH INJECTION. Mag. 200.

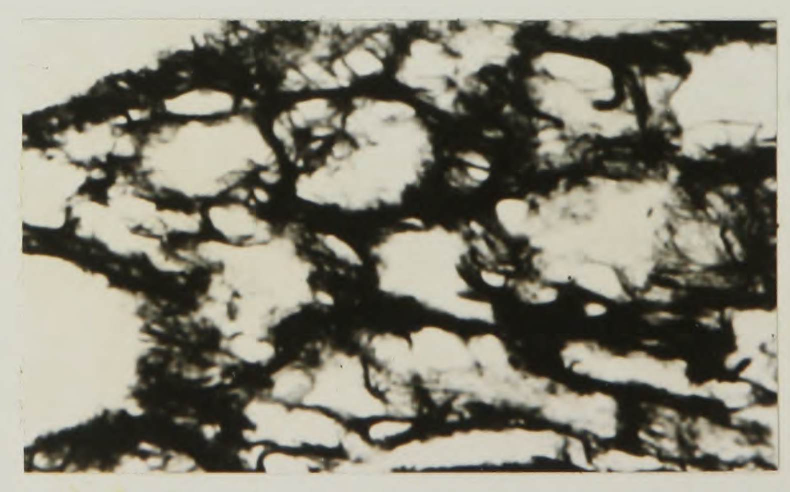


Fig. 5/16. ADH & WATER LOAD. Mag. 200.

The interstitium in the papillae from animals injected with ADH was optically dense after staining for acid mucopolysaccharides: It became diffuse when water, or water in conjunction with ADH, was administered. Labelling was never seen over the interstitium one hour after injection of radioactive sulphate even at either extreme of water loading or deprivation.
The change in morphology was strikingly shown in sections stained for reticulin (Figs. 5/14 to 5/16). Water loading caused the interstitial reticulin network to take on an expanded appearance. At the same time some thin walled vessels collapsed, which made them difficult to see in light microscopical sections. The thin limbs of the loop of Henle, and certainly some blood vessels, remained patent.

d) Possibility of ADH Action Through Hyaluronidase Released Elsewhere.

The thick lumenal layer of acid mucopolysaccharide found in well hydrated animals (Fig. 3/13) accounts for some 2 μ of the cell length as vizualised by light microscopy. It appeared feasible from the histochemical results that ADH could somehow be acting to cause the removal of this material, ~~which~~ It would then reduce the apparent cell volume by about 20%, which is in good agreement ~~from~~ with the results from morphometry.

Darmady, Durant, Matthews and Stranack (1960) pointed out that, in the rabbit, iodinated vasopressin was bound only by the more distal part of the distal convoluted tubule and the cortical part of the collecting tubule, but not by that part of the collecting tubule which ran through the papilla. This can be accepted as further evidence against the hypothesis of Ginetzinsky (1958) that ADH directly causes apocrine secretion by the papillary collecting tubules. It is possible, however, that the hormone could act in the cortex, rather than over the whole length of the collecting tubule, to release hyaluronidase into the urine. This might then depolymerise the lumenal acid mucopolysaccharide as it was carried further down the tubule.

The possibility that hyaluronidase might act in this way was investigated in the rat by Thorn, Knudsen and Koefoed (1961). These workers found that doses in excess of 35,000 I.U./kg. of bovine testicular hyaluronidase infused into a jugular vein consistently produced an antidiuresis in water laden animals. Their criteria for antidiuresis were that there should be a reduction in the rate of urine formation with a rise in osmolarity of that secreted.

This aspect was explored in four male littermate guinea pigs by the following experiment: All the animals were injected with 1 mCi of $S^{35}O_4^{=}$ followed by:

Hyaluronidase Injection.

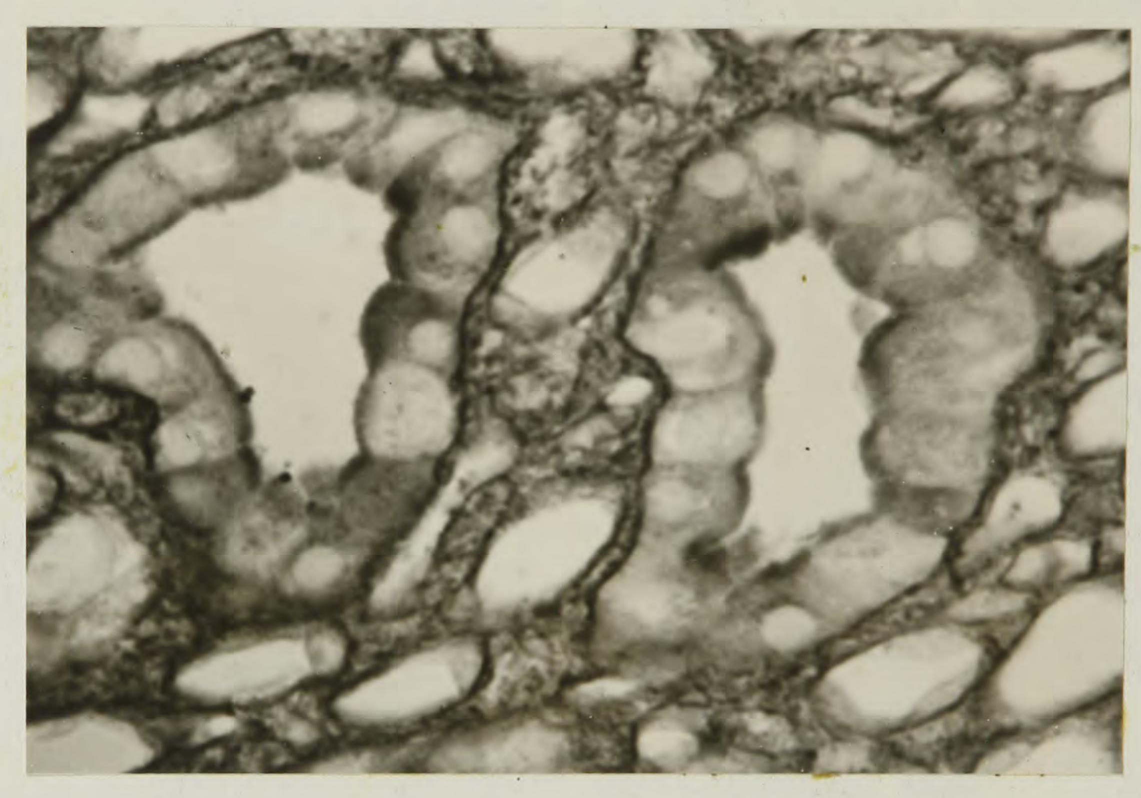


Fig. 5/17. Alcian Blue at pH 3.5. Mag. 500.
1,000 Units of 'Hyalase' (Bengers) injected 1 hour before death. The interstitial hyaluronic acid has been digested, and no granules are to be found between collecting tubule cells.

Hyaluronidase Injection.

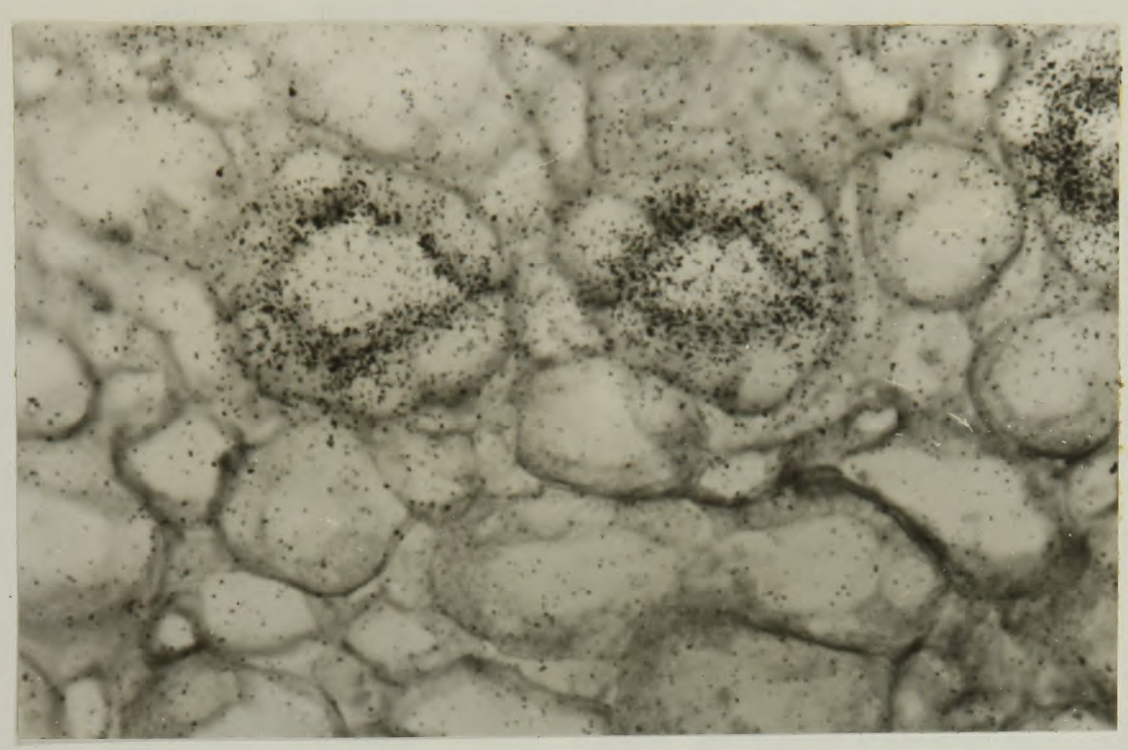


Fig. 5/18. CONTROL.

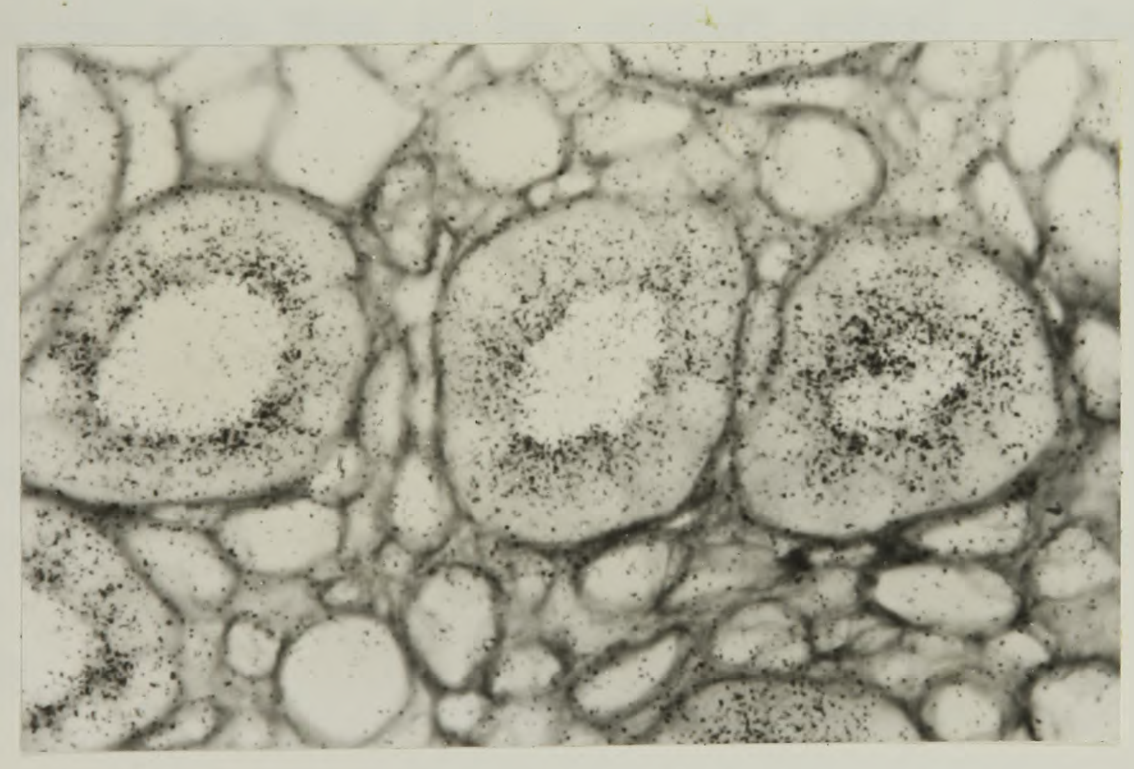


Fig. 5/19. HYALURONIDASE INJECTED.

Both sections mag. 200; PAS stained before being coated.

Hyaluronidase Injection.

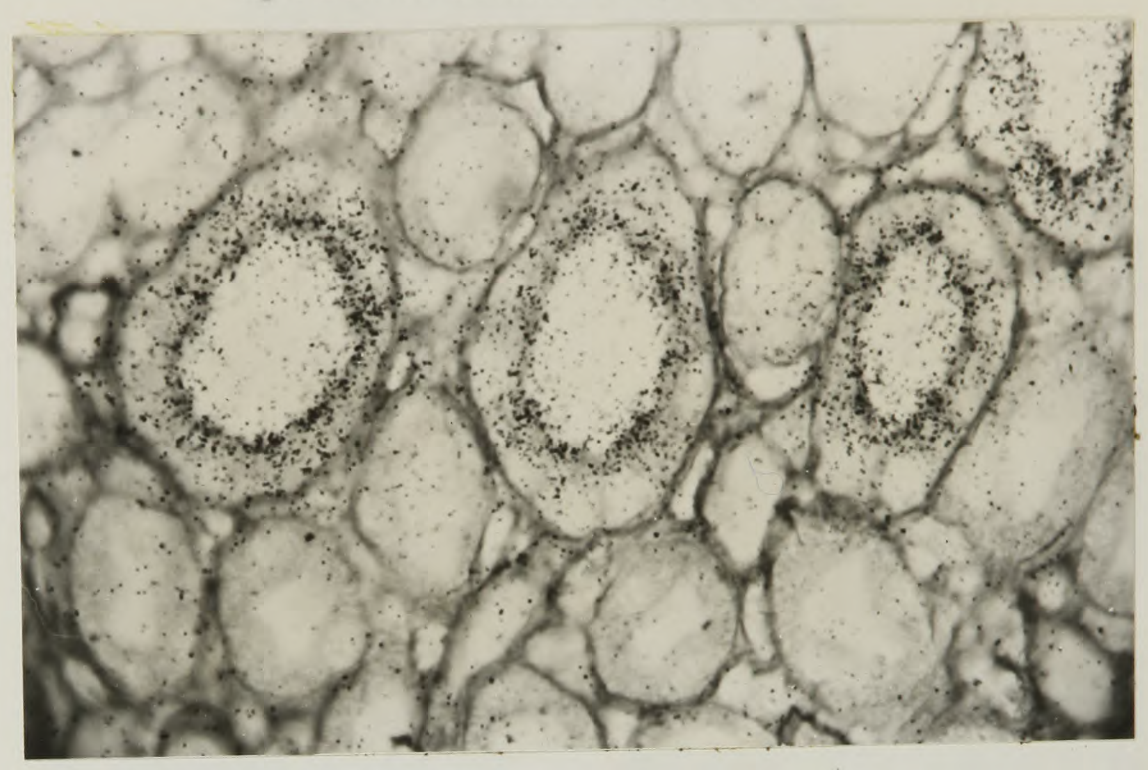


Fig. 5/20. ADH INJECTION.

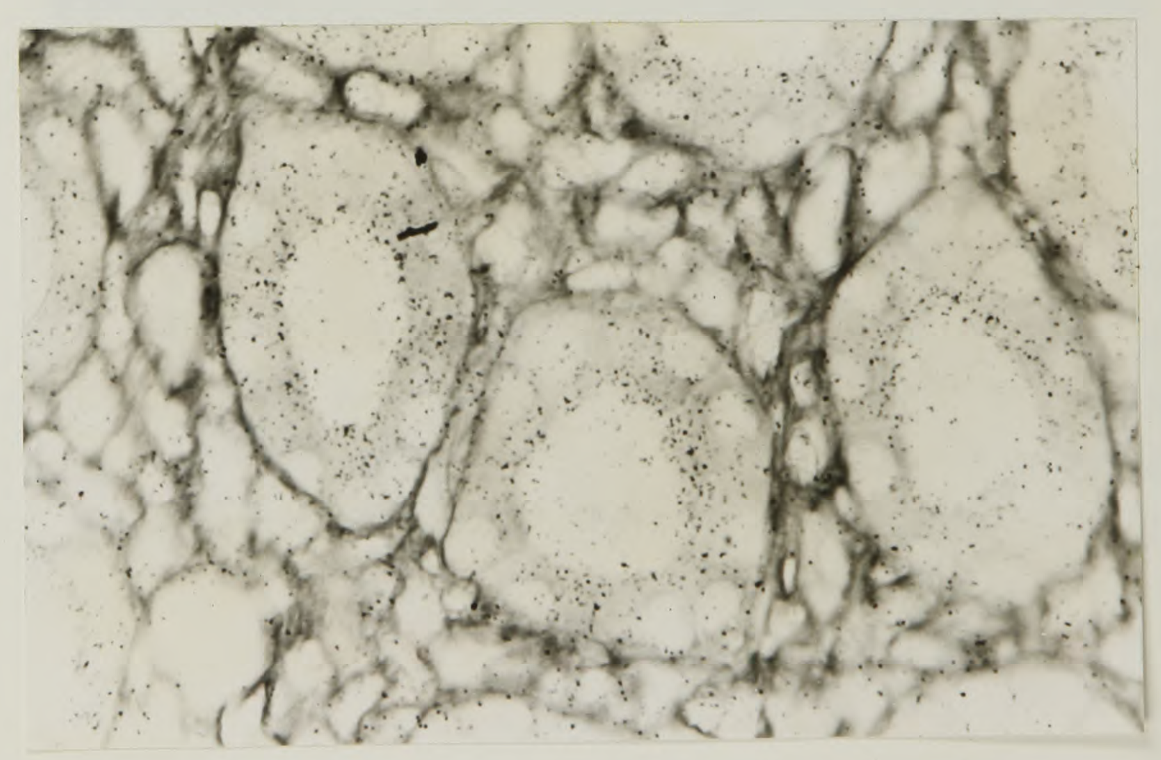


Fig. 5/21. ADH & HYALURONIDASE INJECTED.

Both mag. 200; PAS stained before being coated.

a) No further treatment: The control animal.
b) Hyaluronidase: Benger's 'Hyalase', 10^3 units.
c) Pitressin, 5 units; and
d) Hyalase (10^3 Units) and Pitressin (5 Units).

It was expected that in the last case, (d), there would be evidence of summation if ADH had an effect to reduce the rate of synthesis of acid mucopolysaccharide, rather than to release an enzyme which depolymerized it.

The results are illustrated in Figures 5/18 to 5/21. It was found that this preparation of hyaluronidase did mimic the effect of ADH to some extent, in that the amount of radioactive sulphate incorporated in either case was lower than that in the control animal. When the enzyme and the hormone were given together less sulphate was incorporated than with either alone, (Fig. 5/21). It was concluded that ADH had an effect in addition to the release of hyaluronidase which might be explained by its known action in constricting the vessels supplying the vasa recta (Fourman and Kennedy, 1966), which would reduce the amount of label available to the cells. It was evident from the result that hyaluronidase could flow down the nephron and depolymerize acid mucopolysaccharide in the collecting tubule.

It was shown in the previous chapter that this prepartion of hyaluronidase digested some but not all of the acid mucopolysaccharide - protein material that was associated with the collecting tubule cells. It is almost certainly not identical with the endogenous enzyme (Cobbin and Dicker, 1962) and, indeed, the enzyme

found in urine appears not to be the same as a more quickly acting principle which can be extracted by homogenizing kidneys in 0.5 M sucrose (Dicker and Elliott, 1962).

Thus, while the present results are compatible with the hypothesis that ADH acts through the release of hyaluronidase, they do not constitute a proof: ' Il ne suffit pas qu'une chose soit possible pour la croire.' For example, it is possible that depolymerization of interstitial hyaluronic acid could have arisen from diffusion of the injected enzyme from the vasa rectae, and this may have produced entirely abnormal conditions of blood flow and osmotic pressure within the papilla, which in turn would influence the metabolism of the collecting tubule cells. Again, no check was made to be certain that the events witnessed in the papilla were not secondary to changes in glomerular filtration possibly caused by the action of the hyaluronidase on the glomerular basement membrane.

e) Evidence for a Hyaluronidase-like Activity in Vivo.

In an attempt to detect any changes in the relative amounts of the papillary acid mucopolysaccharides which might indicate hyaluronidase activity in vivo, the acid mucopolysaccharides were isolated from groups of adult male guine pigs papillae. A total of 32 animals was divided into the three categories:

% BINDING of ALCIAN BLUE at pH 3.5 by FRACTIONS in BUTANOL-WATER EXTRACTS.

Bands		Water Laden	Control	ADH Injected
Slowest	mean	33	25	21
	S.D.	11.2	9.4	10.6
Hyal. a.	mean	34	33	46
	S.D.	8.4	12.2	7.1
Intmd.	mean	20	22	19
	S.D.	6.3	4.2	12
Fast	mean	15	19	13
	S.D.	6.1	4.2	4.9
Ratio of Slow to Hyal.a.		0.97	0.75	0.46

Table 5/1.

a) No treatment other then handling. (10)
b) Standard water load, 20 ml. (8)
c) Antidiuretic hormone, 5 units. (14)

The results are presented in the table (Table 5/1) and as a histogram (Fig. 5/22). It was found that the proportions of the acid mucopolysaccharides isolated from the control and water laden guinea pigs overlapped in every case. On the other hand the extracts from animals injected with ADH contained about half the ratio of ~~collecting tubule~~ sulphated mucopolysaccharide to hyaluronic acid that was observed in the water laden and control animals.

It should be stressed that the results from the electrophoretic separation give only the relative amounts of the acid mucopolysaccharides extracted and give no indication of the total amount present in the papilla. However, the suggestion that there is a loss of the sulphated material in antidiuresis following ADH injection is supported by the three histological observations;

i) that there is less of the collecting tubule sulphated material in sections after ADH injection (Fig.5/12);

ii) that there is less bound radioactive sulphate one hour after the injection of ADH (Fig. 5/20); and

iii) that occasional casts of sulphated material may be seen in the collecting tubule lumen after ADH injection, especially when large doses are used.

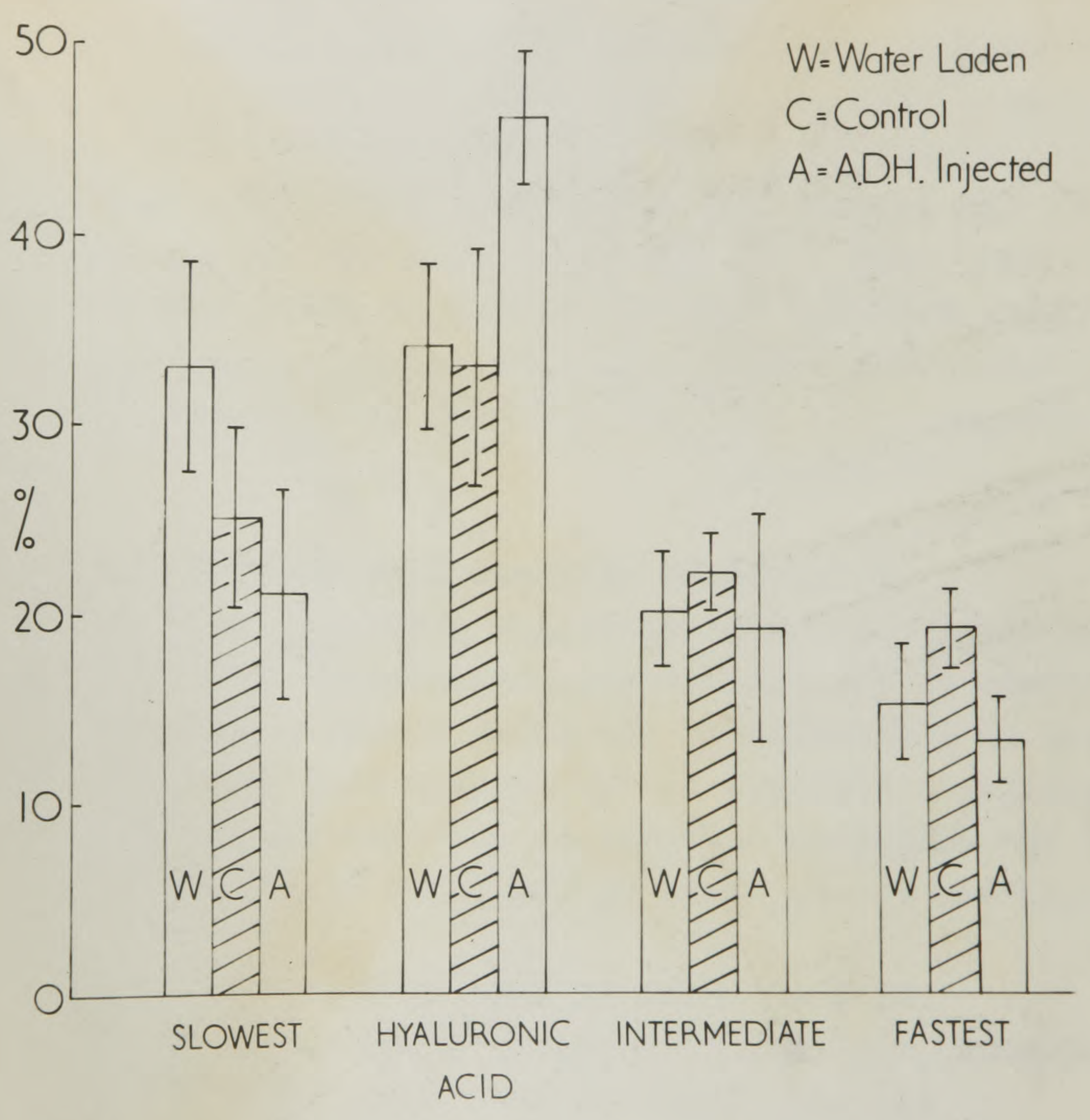


Fig. 5/22.

B. The Appearance of Acid Mucopolysaccharides in the Papilla of the Immature Guinea Pig.

Young animals secrete abundant hypotonic urine up to the time when they are normally weaned; They then begin to respond to antidiuretic hormone (Boss, Křeček and Kraus, 1962). To determine whether any structural or histochemical change accompanied the increasing sensitivity to ADH, kidneys from near full term embryo guinea pigs were processed according to the standard routine (Appendix 2/1), which was found to preserve embryological material reasonably well. The following features were noted in the sections stained for acid mucopolysaccharides:

i) Cortex: In the full term guinea pig embryo all the glomeruli except those at the very periphery of the cortex appeared to be fully developed, with patent capillaries, squamous epithelium around Bowman's capsule and sharply defined basement membranes. The glomeruli were surrounded by convoluted tubules differing only from those in the adult by having relatively small lumens. The more mature the embryo the less interstitial space did it have in its renal cortex.

The more immature glomeruli at the periphery did not appear to have many patent capillaries and the epithelium of Bowman's capsule was cuboidal.

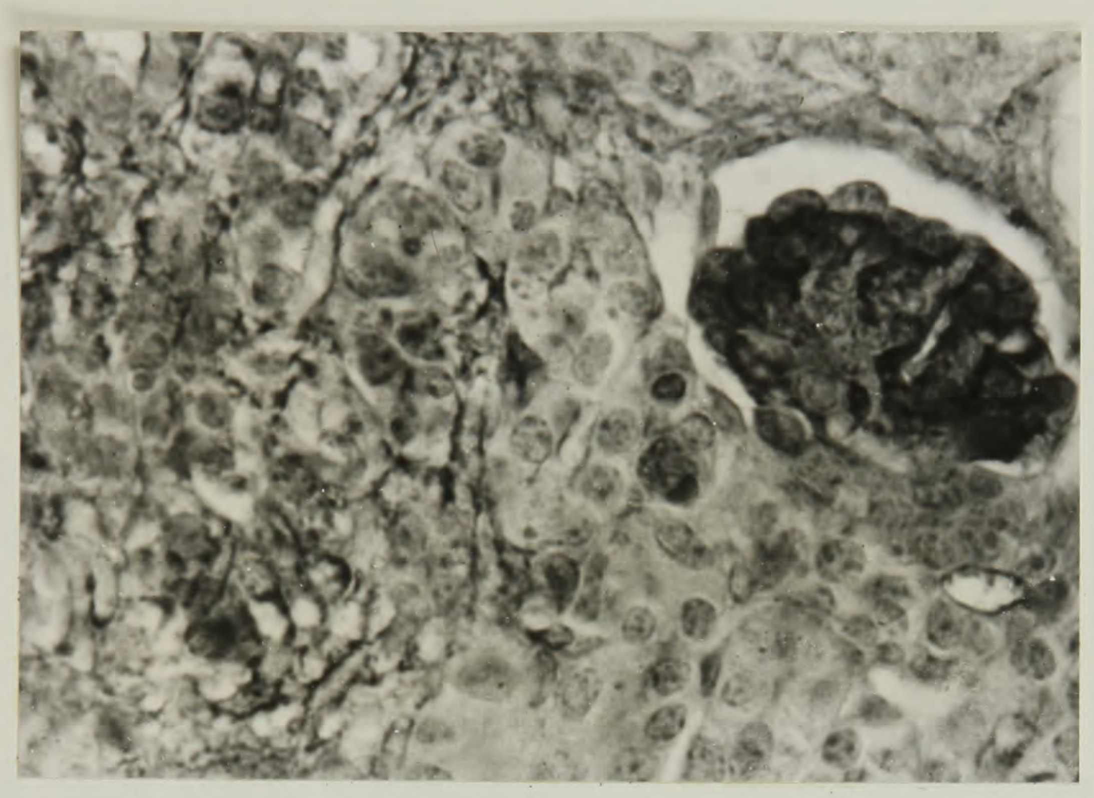


Fig. 5/23. Cortex of Kidney from Immature Guinea Pig. Colloidal Iron & Mayer's Carmalum Stain: Mag. 500. Glomerular capillaries not patent, immature tubules in connective tissue at left.

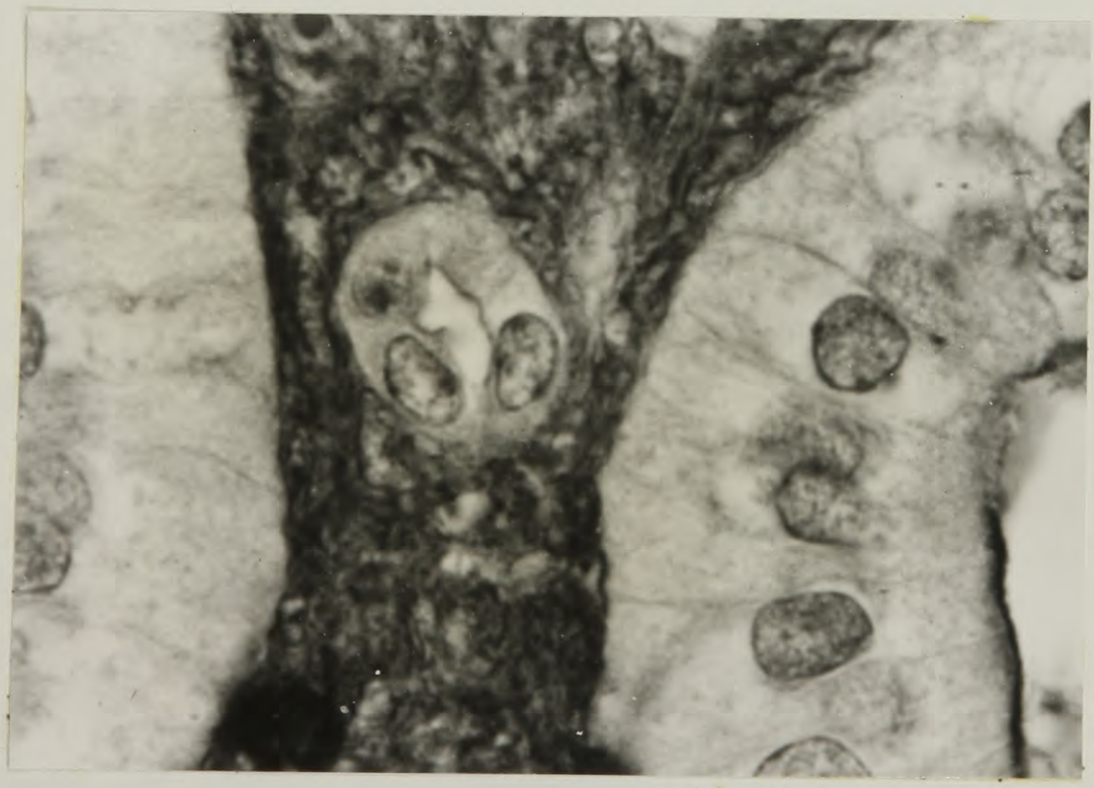


Fig. 5/24. Papilla of same kidney. Collecting tubules developed, but loops of Henle all thick.

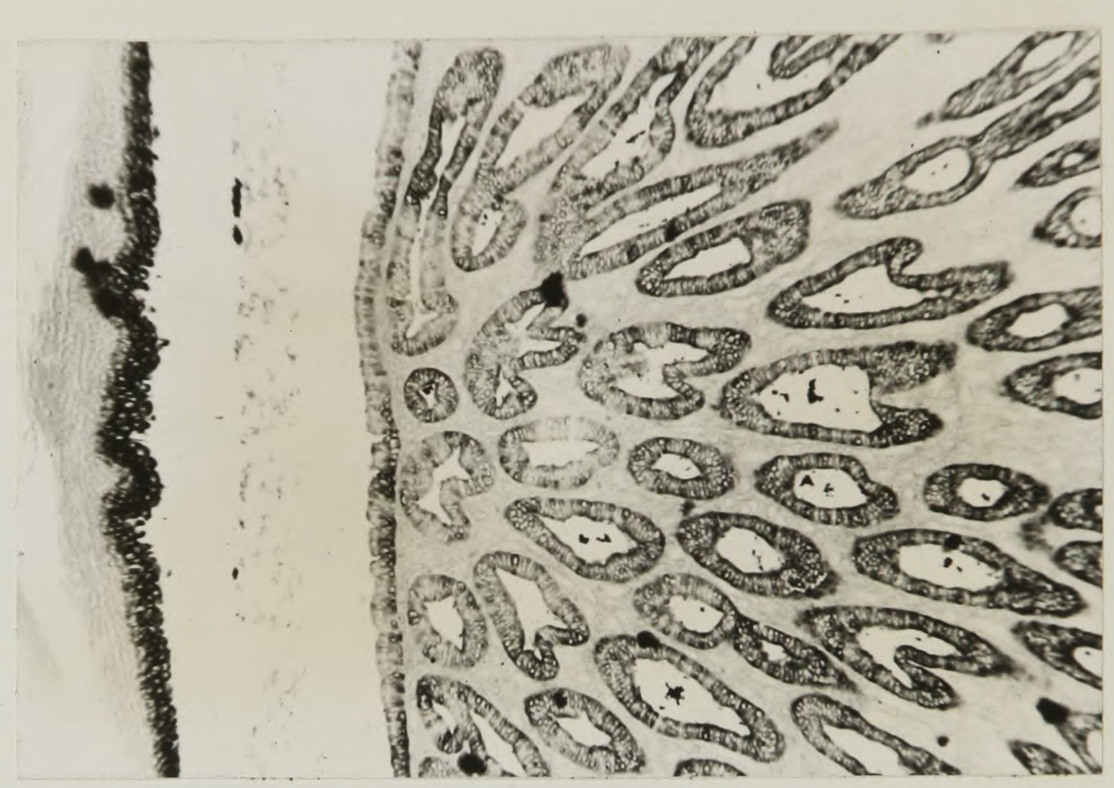


Fig. 5/25. Low power of papilla stained by PAS. There is far more glycogen in the collecting tubules than ever appears in the most hydrated adult.

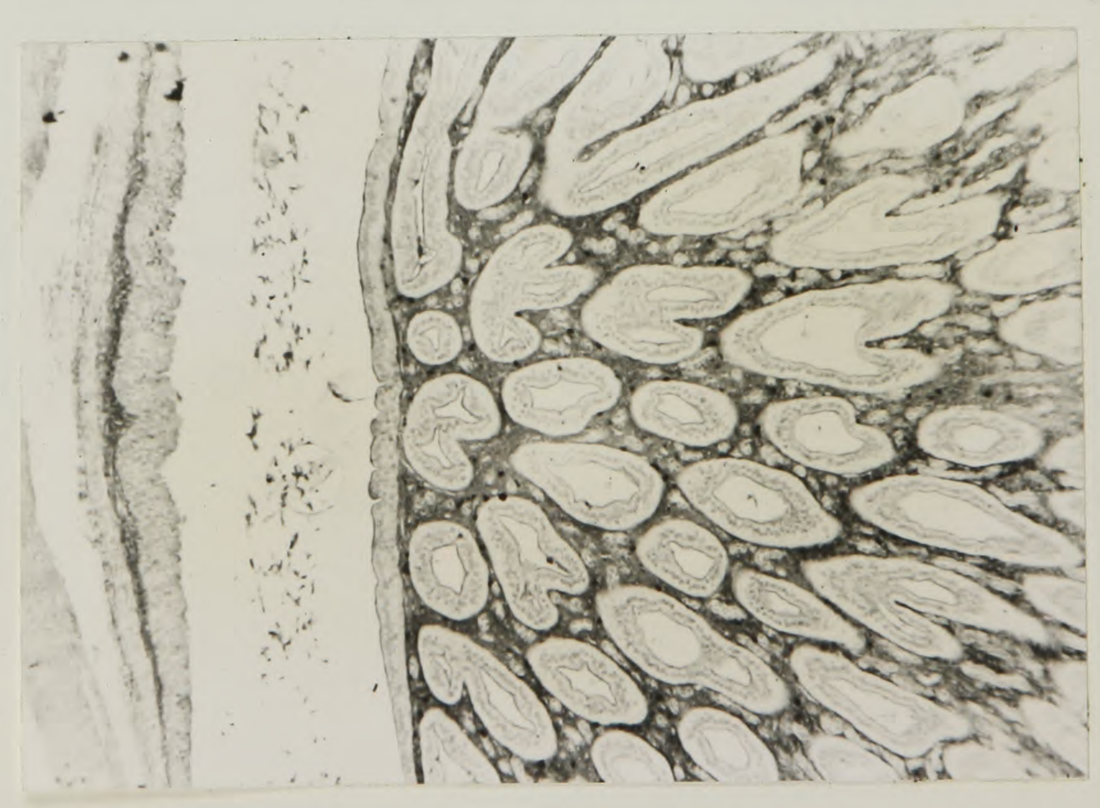


Fig. 5/26. Adjacent section stained by Colloidal Iron & Mayer's Carmalum showing a complementary distribution of acid mucopolysaccharides.

It was common to find considerable amounts of connective tissue between the immature tubules which surrounded such glomeruli (Fig. 5/23). The medullary rays also contained connective tissue which was rich in acid mucopolysaccharide, a feature which is not characteristic of the adult kidney.

ii) Medulla: The medulla of the full term guinea pig embryo was structurally well developed. Vasa recta and loops of Henle were evident right at the tip of the papilla, hence their downgrowth from the cortex had been completed. However, the limbs of Henle's loop were lined throughout by cuboidal cells more akin in size to those found only in the thick portion of the ascending limb in the adult (Fig. 5/24). At the tip of the papilla in the adult both limbs of Henle's loop would be lined with cells having little cytoplasm, and nuclei which projected into the tubule lumen (cf Fig. Fig. 3/2).

The columnar cells of the collecting tubules had a well developed lumenal acid mucopolysaccharide cuticle comparable to that seen in the adult, although the lateral borders stained less strongly for sulphate groups. The cells were replete with glycogen (Fig. 5/25) to an extent never seen even in extreme water loading of the adult guinea pig. The level of collecting tubule glycogen has been shown to correspond to the degree of water diuresis in the rabbit (Darnton, 1967; Johnson and Darnton, 1967), being at its maximum when the flow of urine was at its peak and much reduced in

antidiuresis. On the basis of these results it might be concluded that the collecting tubule cells in the full term guinea pig embryo do little work to conserve water. This is in agreement with the undectable levels of antiduretic hormone at this age.

The papillary interstitium was more diffuse, more reticular and less strongly stained by basic dyes than in the adult (Figs. 5/24 and 5/26). It had the expanded appearance found in the adult in extreme water diuresis.

ii) Conclusion: The full term guinea pig embryo papilla probably provides a sample of tissue which has never been subjected to the influence of antidiuretic hormone. However, it is not clear how far the appearances described for the papilla are due to this and how far they represent structural immaturity.

The appearance of the papilla in general simulated that of the adult in water diuresis. It would thus appear that the absence of circulating ADH, and the formation of hypotonic urine, can be associated with high columnar collecting tubule cells which have a lumenal cuticle of acid mucopolysaccharide. In this condition the papillary interstitium is expanded and takes on a fibrillar appearance. The functional significance of these observations is considered in the next chapter.

CHAPTER 6: DISCUSSION.

1) Species Differences in Papillary Structure.

Sperber (1944) first drew attention to the fact that the development of the renal medulla was related to habitat. Schmidt-Nielsen and O'Dell (1961) qualitatively related the length of the inner zone of the medulla to renal concentrating ability. Black (1965), finding it difficult to distinguish between the inner and outer zones of the medulla, plotted the maximum concentration that could be achieved in the urine against the ratio of total medullary to cortical thickness and obtained the relationship:

$$U_{max} = 1.06 (M/C) - 0.51 \qquad (i)$$

where U_{max} was the maximum concentration of the urine measured in Osmols per litre, M was the length of the medulla and C the length of the cortex.

The relationship had a correlation coefficient of 0.9 for the sixteen species studied but, nonetheless, carries the implication that animals with negligibly short papillae can produce urine having a negative osmolarity. It is suggested that this arises because the whole medulla, rather than the papilla, was chosen to represent the region in the kidney where the final concentration of the urine is achieved.

On the other hand a more plausible relationship emerges if the length of the inner zone, as was suggested by Schmidt-Nielsen and O'Dell (1961), is taken as the reference point. In the present study the data given by Black (1965) for maximum urinary concentration were related to inner zone (ie. papillary) thickness, as demarcated by acid mucoploysaccharide staining. Drawing by eye the best straight line through the points led to the derivation of the empirical relationship:

$$U_{max} = 1.8 \, (M'/C') + 0.5 \qquad \text{(ii)}$$

where M' was the length of the inner zone of the medulla and C' was the length of the outer zone and cortex. Equation (ii) implies that animals with very small papillae are unable to concentrate urine to a tonicity much greater than that of blood. It was unfortunate that values for maximum urinary concentration could be found for only six of the species examined histologically. The correlation coefficient, which has little meaning for such a small sample, was 0.9 (Appendix 2/2).

No clear correlation could be shown between the texture of the papillary interstitium and maximum urinary concentration. As a generalization, the results in Table 2/1 indicate that animals living on a diet with a high water content tend to have papillae with a diffuse open appearance: This is especially true of man, dog and

guinea pig. Animals living on dry foods (eg the jird and mouse) have a compact and tight papillary inter - stitium.

In this respect, the measurements of inter - stitial space support an observation made by Morard (1967) that desert mammals capable of secreting very hypertonic urine have a high density of negatively charged mucins in the interstitial space. However, the extracellular volume is relatively much smaller in the desert species than it is, for example, in the dog or rabbit.

The absence of a significant amount of collagen in the papilla deserves comment. Reticulin is often regarded as a precursor of collagen (Le Gros Clark, 1965) and to be laid down first in growing or healing tissues. However, in the papilla the amount of collagen does not appear to increase with maturity, which suggests that reticulin probably performs a specific function. The function of reticulin in growing tissues is probably to provide physical support while at the same time permitting the movement of cells within the tissue which constitutes the 'tactics' of growth. The changes in morphology of the interstitium in different physiolog- ical states may well depend reticulin to maintain the resilience of the delicate meshwork of fibrils which confine the hyaluronic acid. The physiological significance of the movement of tubules in the papilla is discussed ~~at~~ later in this chapter.

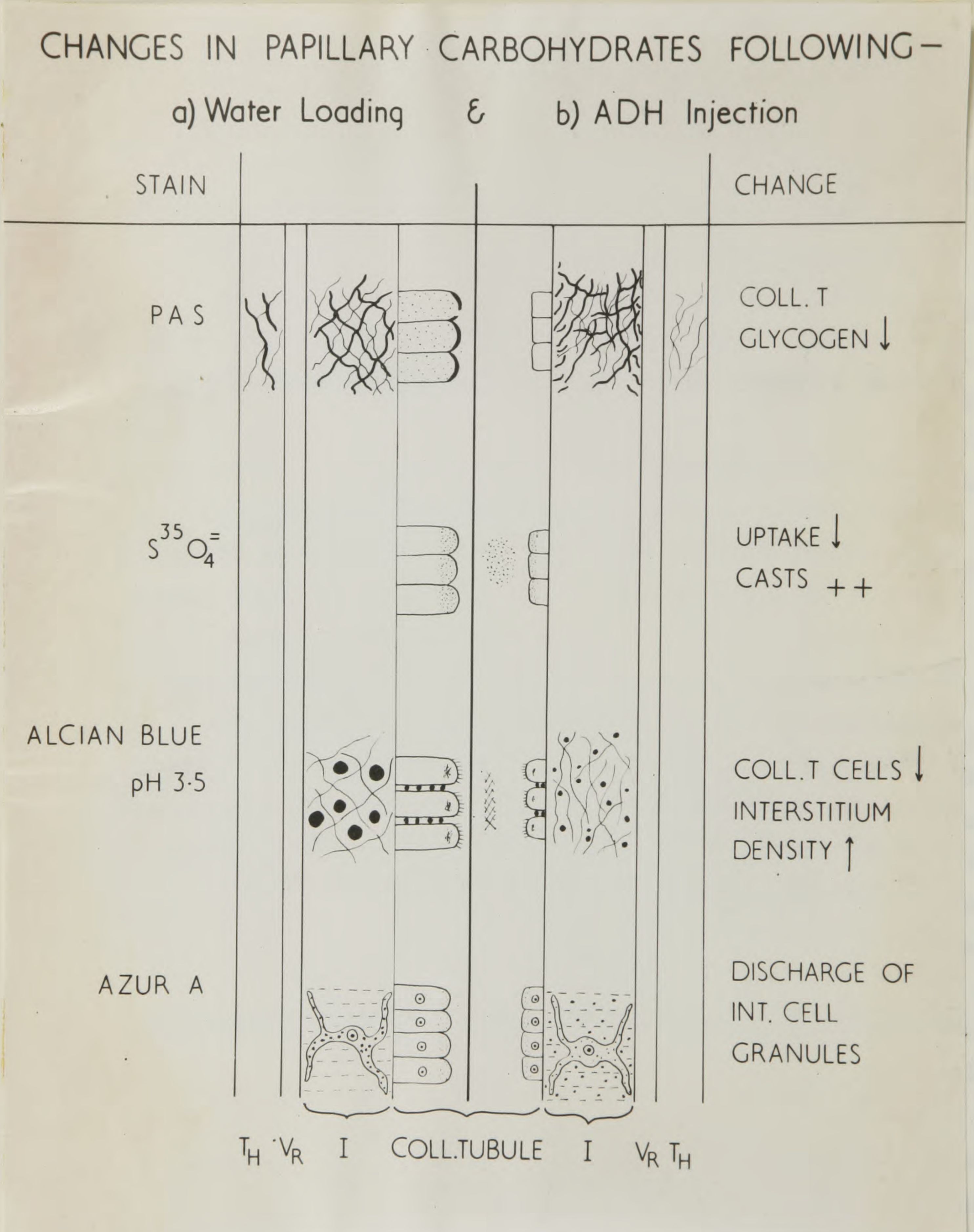


Fig. 6/1 T_H = Thin Limb of Henle's Loop.
V_R = Vasa Recta.
I = Interstitium.

2) Physiological Histology of the Guinea Pig

Collecting Tubule.

The histochemical changes induced in the acid mucopolysaccharides of the papilla of the guinea pig by varying the state of hydration of the animal are summarized as a diagram in Fig. 6/1.

The collecting tubule cells of the guinea pig synthesize a sulphated acid mucopolysaccharide which is covalently linked to protein. The degree of esterification of this acid mucopolysaccharide appears to be variable in that a number of - CH(OH) - CH(OH) - groups are left free and stain with the PAS routine. This material is synthesized within the Golgi complex of the cells and then moves towards the apex and lateral borders. The granules seen along the lumenal border and in the lateral intercellular spaces with the light microscope, and the 'fuzz' seen in the electron micrographs seem to be composed of this acid mucopolysaccharide since they have identical staining properties. Some of the possible arrangements of the polysaccharide chains that would give biochemical and histochemical results of the type described are suggested in Fig. 4/26.

The collecting tubules also contained granules of glycogen: These were more numerous in water diuresis than they were in antidiuresis. This observation is in

agreement with quantitative biochemical and histo - chemical measurements of reduced amounts of collecting tubule glycogen following dehydration in rabbits (Darnton, 1967). The morphology of the collecting tubule cell suggested that more acid mucopolysaccharide was produced in water diuresis than in water deprivation, and that the disappearance of glycogen had therefore nothing to do with acid mucopolysaccharise synthesis. It is possible that medullary blood flow is reduced sufficiently in antidiuresis (Fourman and Kennedy, 1966) to tip the metabolism of these cells from oxidative phosphorylation into anaerobic glycolysis. This possibility could be examined by radioactive labelling of the glycogen to determine whether it was broken down or converted to acid mucopolysaccharides in antidiuresis. It might be pointed out that the synthesis of acid mucopolysaccharides involves the breakdown of high energy phosphate bonds, which are most economically produced by oxidative phosphorylation. It is thus reasonable that synthesis should be maximal in water diuresis when the blood flow through the medulla is at its greatest and the oxygen tension is at its highest.

The synthesis of the sulphated acid mucopolysaccharide was followed by radioactive labelling of the sulphate group. It was found that the larger part of the material was secreted into the tubule lumen. Secretion took place about one hour after esterification in the control animals and continued for at least twenty four hours. The evidence from electron microscopy indicates that there is also secretion into the lateral

intercellular spaces. The mechanism whereby this occurs differs, however, from the release of a discrete granule from, say, a pancreatic acinar cell. When the material reaches the outer surface of the cell it appears to become bound to the glycocalyx. Labelled material within the intracellular granules is readily washed out of sections. When the labelled material has reached the apex of the cell, however, it cannot be washed out, even by quite strong saline solutions. This suggests that the bonds formed between the material and the outer surface of the cell must be stronger than ionic: It is possible that covalent bonds might be formed through the protein part of the molecule.

In antidiuresis, or following ADH injection, free casts of this material could be found within the collecting tubule lumen, and these were very readily washed out of sections. It was also found that the amount of material bound at the lumenal end of the collecting tubule cells was reduced in these conditions, and that this effect could be reproduced in the living animal by injecting testicular hyaluronidase. It was considered that the loss of apical acid mucopolysaccharide went a long way towards explaining the decreased volume of these cells in antidiuresis.

The resolution of the autoradiographic method used in this study was insufficient to demonstrate that the intercellular 'granules' were definitely composed of the same material as that within the cells. However,

their histochemical behaviour was identical. The fact that some of the polysaccharide chain might not be fully esterified could account for the occasional appearance of PAS positive granules between the cells, similar to those described by Boss, Breddy and Cooper (1961). However, if the PAS method is combined with Alcian Blue staining, these granules take on a purple colour which indicates that there are also acid groups associated with the neutral polysaccharide. Boss et al. used only the PAS routine in their studies and reached the conclusion that the number and appearance of these granules did not seem to reflect the state of hydration of the animal. The impression was gained in this study that the appearance of purely PAS positive granules was so rare as probably to indicate a fault in the ester - ification of polysaccharides, possibly by a dying cell; to confine attention to these would be to ignore the vast bulk of the acid mucopolysaccharide produced by the collecting tubule cells. Nonetheless, the point should be made that positive staining by either the PAS technique or by Alcian Blue at pH 1 precludes the material from being hyaluronic acid. The metachromasy of the material between collecting tubule cells can be explained on the basis of sulphate groups.

It should prove possible by microdensitometry to quantitate the reduction in amount of stainable acid mucopolysaccharide associated with the collecting tubule in antidiuresis. The visibly thinner cuticle and reduced quantity of intercellular material found in the animal deprived of water for 48 hours, or injected with ADH,

coupled with a 23% reduction of cell volume in this condition, indicated that there was a loss of sulphated acid mucopolysaccharide. This conclusion was borne out by the results of the electrophoretic separation of extracts. The ratio of sulphated acid mucopoly - saccharide to hyaluronic acid fell from 0.97 in the water laden animal to 0.46 in the one given ADH.

The lesser amount of extracellular acid mucopolysaccharide in antidiuresis could be the result of either decreased synthesis or of enzymic depolymerization as suggested by Ginetzinsky (1958). In this study the development of the Golgi region was taken to indicate the activity of the cell producing acid mucopolysaccharide. This organelle was always found to be extensive between the nucleus and lateral cell wall in well hydrated animals, but it became condensed if the animals were dehydrated or injected with ADH. It was concluded that synthetic activity was greater in the water laden state. The mechanisms controlling the rate of acid mucopolysaccharide synthesis were not investigated, but it is possible that the higher blood flow through the medulla of the animal in water diuresis could be a contributing factor.

There was, however, some evidence that material secreted during antidiuresis was more rapidly destroyed than that secreted in water diuresis. For example, casts appeared in the collecting tubule lumen which suggested that

part of the glycocalyx had been split off in anti - diuresis and was in the process of being carried away in the urine. This observation cannot be taken as proof of the release of hyaluronidase in antidiuresis in the manner envisaged by Ginetzinsky (1958). It is equally possible that there is a constant clearance of the enzyme through the kidney, as was suggested by Berlyne (1960), and that the smaller volume of fluid moving more slowly down the collecting tubule enables the enzyme to have a greater effect than it would have when rapidly washed past its substrate in diuresis. The rate of reduction of viscosity of hyaluronic acid by hyaluronidase is increased in concentrated salt solutions. It is thus possible that the secreted enzyme has an effectively greater activity in the hypertonic urine produced in antidiuresis. This argument substantially weakens the quarrel between Ginetzinsky and Berlyne. The controlling factor in the rate of acid mucopolysaccharide depolymerization in vivo could just as well be salt concentration as enzyme quantity.

There was no evidence in the present results for an increased release by apocrine secretion of hyaluronidase by the collecting tubule cells in anti-diuresis. The smaller volume in this condition can be accounted for by the loss of apical acid mucopolysaccharide and, possibly, a small factor due to osmotic effects. An objection to the theory of apocrine release was that there

were tight junctions between the collecting tubule cells and it was difficult to see how an enzyme, if it were released into the tubule lumen, could pass through these to act on the intercellular material.

There appear to be no reports by other workers which deal with the incorporation of sulphate by the collecting tubule cell. Of the animals examined, the guinea pig was found to have collecting tubules with the most developed lumenal acid mucopolysaccharide cuticle and extensive Golgi regions. It is argued that, in this respect, the guinea pig may be somewhat atypical. Most terrestial animals have survived evolution by developing mechanisms for the conservation of water. The guinea pig, like other desert species, has the long papilla associated with the production of small volumes of strongly hypertonic urine. However, the guinea pigs used in this study were descended from many generations of the species bred in captivity in a temperate climate with abundant drinking water. (Furthermore, it was standard practice to give the animals a liberal intake of greenstuffs to combat the tendency to scurvey in this species, and this would increase the dietary water intake.) Thus the animals may have spent the whole of their life combatting the opposite to that problem for which evolution had provided a defence. It was thought that this may be the reason why there was consistently no difference to be detected in sulphate incorporation and acid mucopolysaccharide staining between the water laden and control animals.

It may well be that other workers have chosen smaller animals, eg. hamster, rat or mouse, for autoradiographic studies on account of the smaller doses required and the ease of handling. Although it was found in the present study that the collecting tubules in all these species do take up radioactive sulphate, they are far from ideal for the study of quantitative changes. The collecting tubule cells are so small that it is difficult to be certain whether the activity is in the cell or the interstitium and, for some reason, the Golgi region is more difficult to demonstrate consistently by silver impregnation methods.

There have been several morphological studies of collecting tubules subjected to the action of ADH. The earliest electron microscopic results to appear in fact led to doubt being expressed that intercellular channels opened up in antidiuresis. Working with rats that had been deprived of water for 48 hours, Robson (1963) commented that intercellular channels were not to be seen in his electron micrographs of magnifications of up to 1,500. He did, however, note some lateral separation in hydrated animals and commented that ' this would seem to indicate an effect directly contrary to that suggested by Ginetzinsky.'

It should be pointed out that none of the (low power) electron micrographs considered by Robson showed the existence of any extracellular polysaccharides. It might be argued that when these

are present the cells should have been separated by an electron clear space. When the acid mucopoly - saccharide is digested away in vivo, then the cells would become closely apposed during the dehydration of the tissue as it was processed for electron microscopy. This argument id based upon the observation made by Robertson (1959) that there is usually an electron clear gap between adjacent epithelial cells of 100 to 200 Å, which can be reduced to as little as 15 Å by treatment of the tissue with hypertonic sucrose solutions before processing for electron microscopy. In other words, there appeared to be a structural component present at the outer surface of cells which held them apart: This could be made to shrink in volume by removing water from it. Polysaccharides are strong contenders for this material. They do not usually react with the stains used for electron microscopy and thus appear electron translucent. They are known to bind large volumes of water through the formation of hydrogen bonds. In the present study it was shown by colloidal iron staining that there was an anionic polysaccharide component exterior to the collecting tubule cell, although this was by no means clear in as low power fields as those used by Robson. Thus, it cannot be said that Robson used electron microscopy to the full in investigating the changes described by Ginetzinsky from light microscopical studies.

In a recent study, Ganote, Grantham, Moses Burg and Orloff (1968) observed the effects of vasopressin applied to living cortical collecting tubules teased out from rabbit kidneys. These workers

compared the phase contrast appearance of living tissue with that given by the electron microscope of material fixed in glutaraldehyde; a fixative which gives better preservation of tissue than the methods available to Robson (1963). Amplifying the observation of Ginetzinsky (1958) Ganote et al. show electron micrographs of tubules fixed during vasopressin induced periods of high osmotic water transport which have dilated lateral intercellular spaces. However, vasopressin was found not to induce any change in cell structure in the absence of an osmotic difference and significant bulk water flow across the tubule wall.

In his study, Robson (1963) gave vasopressin to rats which had already been water laden and it is possible that the transtubular osmotic gradient is much reduced under these conditions. This would abolish the condition that Ganote et al. consider to be necessary for the dilation of the intercellular spaces. Even so, such an argument cannot be made to account for the separation seen between cells by Robson in rats in diuresis: In this respect the results of Ganote et al. directly contradict those of Robson unless the unseen role of the glycocalyx is accepted.

Interest in the collecting tubule as a possible site of variable water permeability may have led to a misplaced emphasis on the change in cell shape as the animal is dehydrated. In other words, the importance might not be that their volume decreases in antidiuresis but that it should increase in diuresis. The tall

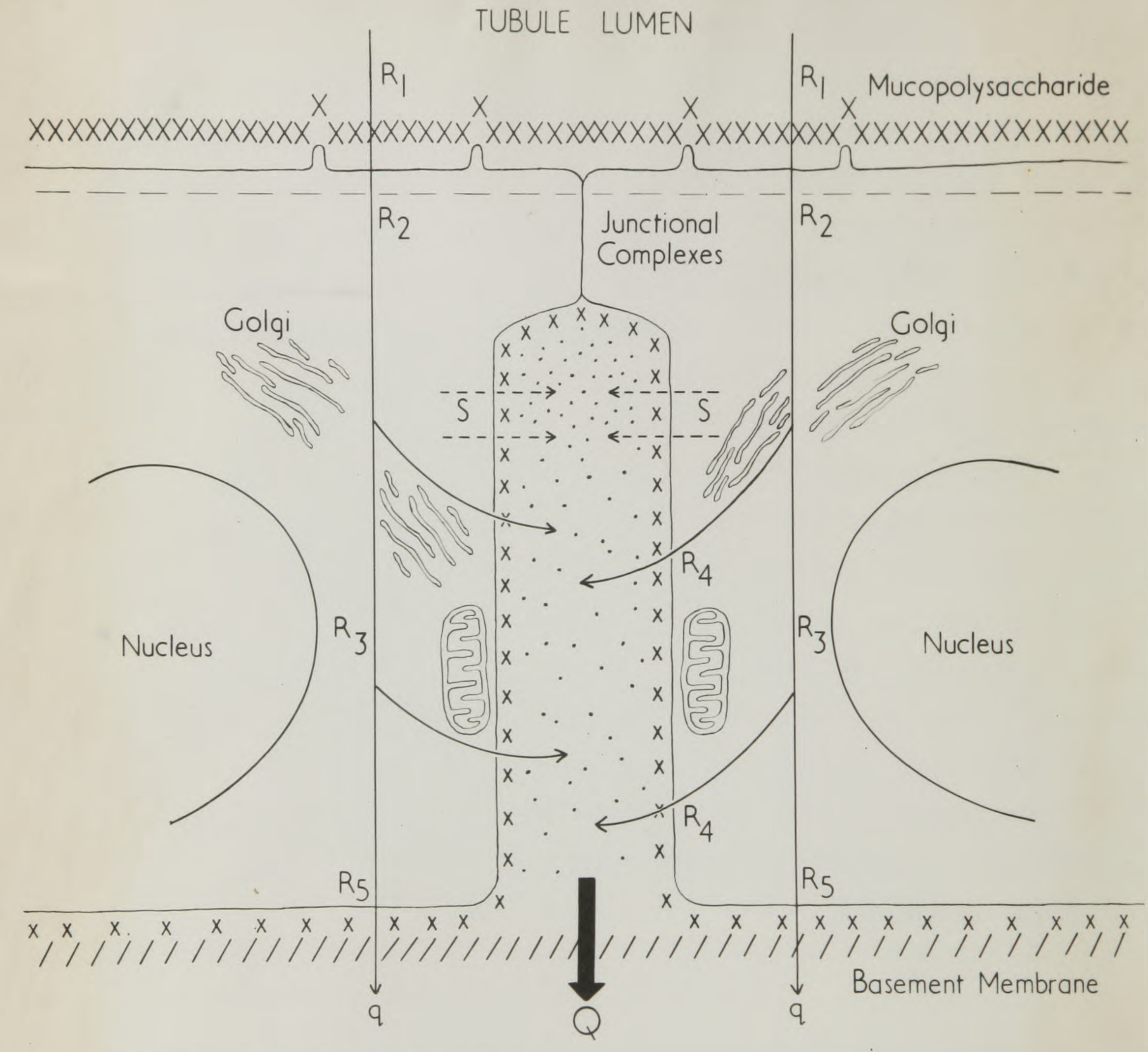
columnar cell has a low apical surface area to cell volume ratio in comparison to a cuboidal cell. It is thus possible that a columnar cell is better able to resist a potential loss of sodium from the interstitium in a water diuresis. This might explain why the tallest collecting tubule cells are to be found at the tip of the papilla where the sodium chloride concentration of the interstitium is at its highest. A similar explanation has been put forward to account for the change from cuboidal to columnar shaped cells in the endoderm of coelenterates when they are taken from 15% sea water and grown in fresh water (Lockwood, 1963): 'This may well be important in the water and salt regulation of the colonies in fresh water'. It may also be important in the conservation of a hypertonic papillary interstitium in the mammal during water diuresis.

3. A Proposed Route for the Flow of Water Through the Collecting Tubule Wall.

The results from the electron microscopic and histochemical studies on the collecting tubule cell of the guinea pig are summarized diagrammatically in Fig.6/2.

The lateral intercellular spaces which are closed at one end are characteristic of a number of types of epithelium known to transport water, eg. the

PASSAGE OF WATER THROUGH THE COLLECTING TUBULE WALL



RESISTANCES TO WATER FLOW

R_1 = Lumenal Fuzz
R_2 = Subterminal Complex
R_3 = Cytoplasm
R_4 = Cell Edge
R_5 = Cell Base

Q = Flow via Lateral Space
q = Transcytoplasmic Flow
x x x = Glycocalyx
XXXX = Lumenal Border Specialization

Fig. 6/2.

gall bladder, salivary gland striated duct cells, intestinal and renal cortical tubular cells. The water transport across the wall of the rabbit gall bladder has been analysed by Diamond and Tormey (1966), Tormey and Diamond (1967) and Diamond and Bossert (1967).

It is suggested that the similar arrangement in the renal papilla could function as follows: Solute (S) is pumped into the lateral intercellular space near its closed end (Fig. 6/2). This has two consequences. First, solute would tend to diffuse down its concentration gradient towards the open end of the space; secondly, the presence of solute in the space would draw water from the adjacent cells. If the walls of the intercellular space cannot be greatly deformed, then this would result in the flow of solution from the open end of the space. If the solute pump were to work at a constant rate then a standing osmotic gradient would be set up along the space, with the most hypertonic fluid near the closed end and the most dilute fluid emerging from the open end. In this case:

a) The tonicity, P, of the fluid emerging from the intercellular gap can be increased by two mechanisms: (i) The rate of solute extrusion, dS/dt, could be increased; or, (ii) the permeability to the flow of water into the intercellular space from the collecting tubule lumen

might be reduced. Water flow could be restricted at the lumenal border of the cell at R_1 (the glycocalyx), at R_2 (the subterminal complex), at R_3 (the cytoplasm), or at R_4 (the glycocalyx at the side of the cell plus the effect of the material in the intercellular space).

(b) The volume of the fluid leaving the lateral intercellular space would be raised by an increase in the rate of solute extrusion, or by increased water permeability as outlined above.

This means that the tonicity of the fluid leaving the space can be represented by the product of a function of the rate of solute extrusion and a function of the total resistence:

$$P \propto f(\ dS/dt\ ).g(\ R_1 + R_2 + R_3 + R_4\ ) \qquad (i)$$

The flow, dV/dt, leaving the gap between the bases of adjacent cells can similarly be given as

$$dV/dt \propto \frac{f(\ dS/dt\ )}{g(\ R_1 + R_2 + R_3 + R_4\ )} \qquad (ii)$$

Thus, for a constant rate of secretion,

$$dV/dt \propto \frac{P}{g\ (\ R_1 + R_2 + R_3 + R_4\ )^2} \qquad (iii)$$

Solute extrusion in the form of active sodium pumping must take place in the collecting tubule since Marsh and Solomon (1965) found that the sodium concentration in the collecting tubule fluid was less than that in the interstitium; at the same time the lumen of the tubule had a negative potential with regard to the exterior, which indicated that sodium was being with - drawn and that chloride ions were lagging behind. The peripheral arrangement of mitochondria along the lateral border of the collecting tubule cell (Fig. 3/18) is similar to that of, eg. the basal infoldings of the convoluted tubules where energy is required for active transport. Although ADH has been shown to increase the transport of sodium across amphibian skin and bladder (Orloff and Handler, 1964) there is no direct evidence that it does so in the mammalian renal collecting tubule. Thus it is not possible at the present time to predict to what extent this rate, ie. dS/dt, may be varied.

It is unlikely that all the resistances R_1, R_2, R_3 and R_4 will have the same value; hence equation (iii) may be written as an approximation:

$$dV/dt \propto \frac{P}{g\,(R')^2} \qquad (iv)$$

where R' is the limiting resistance.

Although collecting tubules are usually looked upon merely as permeability barriers to the flow of water

across them, equation (iv) gives equal emphasis to the possibility that they may play a part in conserving the osmotic pressure of the papilla by regulating the value of the limiting resistance, R'.

The present study has gone some way towards identifying these barriers. For example:

R_1 is the resistance to the passage of water which is offered by the specialized glycocalyx at the lumen. This has been shown to contain neutral polysacch - aride chains and chains of a polysaccharide which contains sulphate groups. It may act as a barrier to the flow of water in the way that hyaluronic acid has been shown to do in fascia (Day, 1952). If it has a structure similar to chondroitin sulphate, it might also act as a cation exchanger favouring the movement of sodium through it, but impeding the movement of potassium (Kulonen, 1952; Joseph, Engel and Catchpole, 1952). The thickness of the glycocalyx is reduced in anti - diuresis and thickened in water diuresis so that some degree of variability is established.

R_2 This region of the cell corresponds to the terminal web in the intestinal columnar epithelial cell (Porter and Bonneville, 1963; Puchtler and Leblond, 1958) across which water is believed to move freely. Neither in the present study, nor that made on isolated tubules by Ganote et al (1968), was any change noted in this region following ADH administration. It is thus unlikely that R_2 can

be the limiting resistance to water flow.

R_3 is the resistance to the flow of water offered by the cytoplasm. However, it was concluded that the actual length of the cytoplasm decreased little, if at all, in antidiuresis. Nonetheless it may be an oversimplification to treat the cytoplasm as a fluid (cf. Wilson and Heilbrunn, 1960) which necessarily has the same physical properties in different physiological states. If the value of R_3 is high, relatively more water would be expected to leave the cytoplasm via the lateral intercellular spaces than it would if R_3 were low, when water would readily traverse the cell to leave across its base.

R_4 The cytochemical results make it seem unlikely that the lateral glycocalyx differs qualitatively from that at the lumenal border of the cell. The lateral disposition of the Golgi complex in hydration suggests that the direction of secretion of the sulphated acid mucopolysaccharide is lateral as well as apical. R_4 is to be treated in the same way as R_1 in that it also is reduced in amount in anti - diuresis and thus possesses variability. It was not found possible to alter the appearance of the material at R_4 independently of that at R_1 either by altering the level of hydration of the animal or by injecting ADH.

R_5 Again, R_5 represents the glycocalyx and is likely to be very similar in nature to R_1 and R_4. However the basal area of the collecting tubule cell is small relative to the area of its lateral surfaces, hence the flow across the base of the cell, q, is likely to be small compared with that leaving the lateral intercellular space, Q. For example, a cell 18 u long and 8 u in dia. has an intercellular space some 36 u long because of the tortuosity. Hence the lateral surface area would be about $2.\pi.4.36$ sq. u, whereas the area of the base of the cell would be $\pi.4^2$. sq. u. Thus, assuming the resistance per unit area is the same for both cases,

$$P_4 : P_5 = 288 : 16,$$

where P_4 and P_5 represent the respective permeabilities. In other words, about twenty times as much water could be expected to leave the cell via the lateral intercellular channels provided that R_3 was low in relation to R_4 and R_5.

If the rate at which sodium is pumped into the intercellular space is increased by the action of ADH, then it can be predicted from equation (i) that the fluid leaving the base of the intercellular space could have a higher osmolarity, provided that the increase in solute pumping was greater than any decrease in limiting resistance. This would explain an otherwise puzzling observation published by Zain-ul-Abedin in 1967. He found

that ADH slowly infused into rats in water diuresis did not cause the tonicity of the papilla to fall, which would have happened if the effect of the hormone was simply to let more water out of the collecting tubule move into the interstitium. In fact, the tonicity of the interstitium was found slowly to rise to the high levels normally encountered in hydropenic animals. The result obtained by Zain-ul-Abedin can be explained if the presence of the water load was the factor which prevented a fall in the limiting resistance while the stimulatory effect of ADH on sodium extrusion was unaffected. In this case it would be expected that fluid leaving the lateral intercellular space would have a high osmotic pressure.

McDonald and de Wardener (1964) have also published observations which can be interpreted to suggest that (dS/dt) and (R') could be under separate control. In their experiment, isolated dog kidneys were perfused with blood from intact dogs receiving continuous intravenous Pitressin (either at a rate of 200 or 400 mU/hour). The urine produced by the perfused kidney varied in osmolarity from 361 mOsm/l to 1338 mOsm/l. It was always iso- or hyper- tonic to plasma. Howver when the plasma volume of the intact donor was increased by a saline infusion in addition to the continued Pitressin, the osmolarity of the urine from the perfused kidney fell to 78 to 234 mOsm/l. It had become hypotonic although vasopressin was present in effectively the same concentration as in the first case.

It was concluded that the production of hypotonic urine under these conditions was due to the presence of a circulating substance which decreased the permeability of the distal convoluted tubule and collecting duct.

It would be of the greatest interest to repeat the experiment of de Wardener and McDonald and to examine the histological appearance of the intercellular and apical material in the two cases. It would be wise to make certain that the perfused kidney was still viable and capable of concentrating urine when the saline load was lifted from the donor.

Ganote et al. (1968) have advanced an argument which would put the limiting resistance in the region of R_1, ie. the lumenal glycocalyx. They found that a decrease in the osmolarity of the solution bathing the blood surface of collecting tubules caused the cells to swell independently of the presence or absence of vasopressin. The peritubular (or blood) border of these cells appeared to be highly permeable to water. In the absence of vasopressin, tubules perfused with a solution hypotonic to the outer medium had an appearance identical to that of tubules perfused with isotonic fluid. Swelling of the apex of the cell was observed only when the tubules were perfused with a hypotonic solution at the same time that vasopressin was applied to the base of the cell. Clearly, the lumenal membrane in the absence of the hormone was relatively impermeable to water, but when vasopressin was added to the blood border of the cells the permeability to water of the lumenal membrane

increased and water entered the cells and intercellular spaces as it traversed the tubule wall. The need to apply vasopressin to the basal region of the cell suggests that it does not have a direct effect on the lumenal barrier, nor does it easily penetrate the cell by this route. Once it has entered the cell across its base, however, it in some way reduces the lumenal barrier to the flow of water.

It is unfortunate that Ganote et al (1968) did not process their material for electron microscopy in a way that would demonstrate the polysaccharide material described in the present study, and hence it is not possible to tell whether their experiments produced morphological changes at the site suggested for R_1. Nonetheless, in view of the reduced staining of the apical mucopolysaccharide and decreased sulphate incorporation in antidiuresis, it is tempting to speculate that ADH acts at some point to regulate the physical properties of the apical region of the cell.

4. Physiological Histology of the Interstitium.

The changes in appearance of the interstitium which follow dehydration, or the injection of ADH, are summarized in Fig 6/1 (opposite page 103). The expanded reticulin network seen in water diuresis becomes more compact so that it is difficult to distinguish individual fibrils. The acid mucopolysaccharide

staining becomes optically more dense. The meta - chromasia at the papillary tip is reinforced and extends further towards the cortex.

This metachromasia survives alcohol dehydration, but this criterion can no longer be accepted as proof of the presence of the sulphate radicle as suggested by Lison (1936): By the use of model systems such as that described in Chapter 4, it is possible to show that some polyanions which contain carboxyl as the only acid group, eg. carboxymethylcelluiose, also exhibit alcohol resistant metachromasy. The explanation in the present case might lie in the fact that the tip of the papilla becomes more dehydrated in antidiuresis, and this might be sufficient to pack the ionic groups of, say, hyal - uronic acid closely enough together to give metachromatic staining. Although pure dye samples dissolved in strong salt solutions exhibit metachromasy, this cannot be the mechanism responsible for the phenomenum in sections of the papilla, since low molecular weight solutes are lost from the tissue in the processing for histology.

In the main body of the papilla the interstitium was never found to exhibit alcohol resistant metachromasy. The amorphous material between the fibrils stained strongly with Alcian Blue at pH 5.5 but only very weakly at pH 1: This was found to be the behaviour of hyaluronic acid in the electrophoretic separation. It was concluded that hyaluronic acid occupied the extracellular space in the papilla, and that it was physically restricted within the reticulin. The role of the very fine fibrils which stained positively for sulphate groups was not

satisfactorily elucidated: They may represent a special class of reticulin fibril, or be composed of early reticulin fibrils around which there has been a condensation of sulphated polysaccharide.

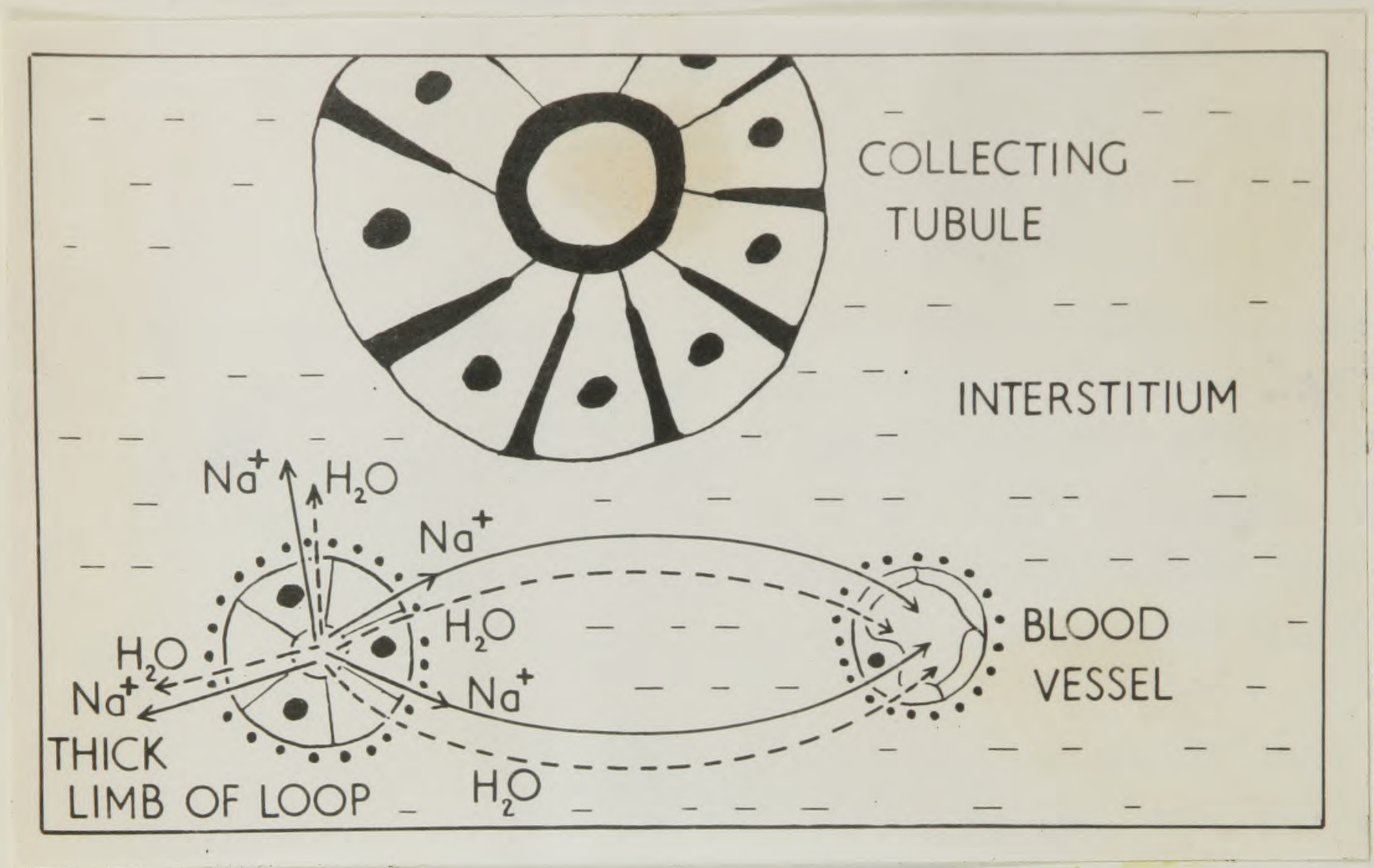
The appearance of the interstitium in well hydrated guinea pigs is similar to that descrided by Robson (1963) for the rat. In Robson's electron microscopic study, failure to concentrate urine was associated with a thickened, fibrillar basement membrane to the thin limb of Henle's loop. The limits of optical resolution do not permit delineation of basement membrane from interstitium, but the open expanded appear - ance of the material around the loop of Henle is confirmed in the present study on the guinea pig given a water load. Also in agreement with Robson's observations was the conclusion that the change in the interstitium was probably brought about by hydration itself, rather than by the absence of circulating hormone, since the change induced was not reversed if vasopressin was given to hydrated animals.

The physiological significance of the specialized extracellular space in the papilla is not readily appreciated from a study of its morphology. However, similar systems in other regions have received considerable attention. For example, Fessler (1960) produced a model of umbilical cord which consisted of hyaluronic acid trapped within a system of fibrils, and he used it to show that such a structure would imbibe water, whence it became rigid and would resist

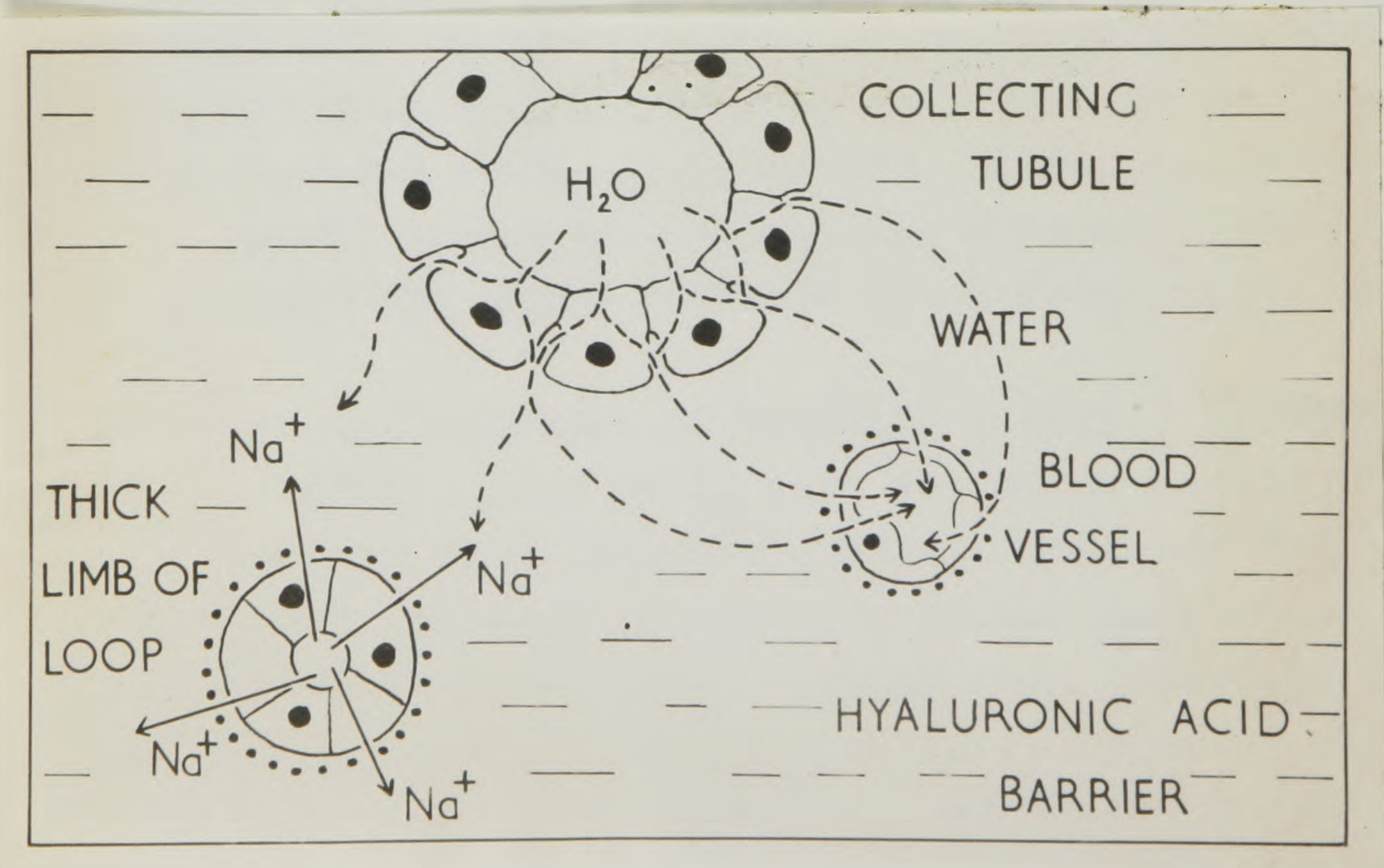
compression of vessels running through it. Again, the anatomy is similar to that described by Florey and Pullinger (1935) when they demonstrated that reticulin held open lymphatics in oedematous tissue. The spiral windings of coarse fibres around some of the structures within the medulla (Figs. 3/27 to 3/29) suggest another mechanism for resisting compression, such as that which might be found reinforcing a garden hose.

That there are changes in physical pressure within the papilla ~~ar~~ is suggested by the results of Gardner (1966), who investigated the density of the papilla in water laden and dehydrated rats. The volume of the papilla was greater in the water laden animal, and this was found to be due primarily to a higher water content. The fall in density of the papilla recorded in hydration can be explained by the expansion of a poly - saccharide network in the interstitium, adjacent poly - saccharide chains being separated by water which forms hydrogen bonds with them.

Gardner (1966) observed an increased gravimetric density in the papillae taken from rats given ADH. This is compatible with the morphological results in the present study: ADH caused the guinea pig interstitium to become compact and optically more dense. This decrease in volume of the extracellular space is of interest in view of the results of experiments performed by Katchalsky (1964) on gels of hydrated anionic macromolecules. Katchalsky found that the volume



WATER DIURESIS



ANTIDIURESIS

Fig. 6/3.

occupied by the gel decreased when the concentration of salt in the fluid surrounding it was increased. In other words, the addition of salt to a constantly ionized gel caused some of the water contained within it to be pumped out. This raises the possibility that, if ADH increases the amount of salt pumped into the papillary interstitium (in the way that it increases the pumping out of the proximal convoluted tubule, Clapp, Watson and Berliner, 1963) then the compact appearance of the interstitium could be a secondary phenomenum.

The manner in which such a squeezing, however achieved, might influence the movement of water from the collecting tubule lumen to that of the vasa recta in antidiuresis is depicted in Fig 6/3. This has been based upon the arrangement of the tubules in the guinea pig papilla, eg, as in Fig. 3/25.

The central feature of the scheme is the close apposition of the ascending limb of the vasa recta and the collecting tubule. Trueta, Barclay, Daniel, Franklin and Prichard wrote in 1947: 'The bundles of vasa recta are supported by a connective tissue stroma . . . grouped amongst the tubules in a pattern which must have a definite significance, but which we have not studied in detail'. On this point Thorburn and Stacy (1965) remarked that, while sheep and dog kidneys have ~~xxx~~ papillae of about much the same size, the sheep can secrete urine one and a half times as concentrated as that of the dog. The only difference in morphology was the greater development of the bundles

of vasa recta within the papilla of the sheep.

It should be pointed out that the arrangement of the vasa recta as described by Trueta et al. makes them inefficient as countercuuuent exchangers (which they are usually considered to be) than if they were distributed uniformly throughout the papilla. On the other hand, the grouping of the ascending limbs of the vasa recta around the collecting tubules provides a route for water to be carried from the papilla without causing the interstitium to become diluted However, no proof has so far been obtained that the bundles of vessels around the collecting tubules do in fact contain more ascending than descending limbs.

'Only if some way could be found to make the water reabsorbed from the more proximal parts of the collecting duct move preferentially out of, rather than more deeply into, the medulla would a rising concentration be achieved in the interstitium ' (Berliner and Bennett, 1967). It is suggested that in antidiuresis the vasa recta and collecting tubules come together either as the result of depolymerization of the acid mucopolysaccharide lying between them, or as a result of increased tension within the reticulin mesh of the interstitium. The tension of the reticulin could be increased either by contraction of the interstitial cells, brought about by the action of ADH, or by the expansion of the hyaluronic gel within it. The intercellular cement between collecting tubule cells was found to be removed in anti diuresis. The morphology of the region suggests that

any enzyme acting between the cells would also gain access to the interstitium, and could act upon the acid mucopolysaccharide there to bring the tubule and vessel more closely together. The application of Occam's razor would seem to dispose of a direct involvement of the interstitial cells, although Vimtrup and Schmidt-Nielsen (1952) suggested that these cells might contract in response to ADH. (The concentration of ADH at the tip of the papilla may be three times greater than that in the general circulation, and fairly low levels of vasopressin can certainly cause other mesodermal tissues, eg. smooth muscle, to contract.)

In water diuresis, a gel of hyaluronic acid between the collecting tubule and the vasa recta would impede the flow of water between the two. The hindrance to the movement of molecules through the gel was found by Ogston and Sherman (1961), who used a model system, to be proportional to the effective size. Thus little restriction is put upon the movement of sodium while water was exceptionally retarded since it formed hydrogen bonds with the polysaccharide hydroxyl groups.

In the papilla the structural fibrils immobilize the hyaluronic acid, which in turn regulates the flow of water, according to its degree of polymerization and ionization, without interfering with the x sequestration of sodium. Indeed,the association

between the water molecules and the polymer leaves less free solvent for the solution of sodium chloride. The solutes in the papilla thus exert a higher osmotic pressure than they would in the absence of hyaluronic acid. There exists an 'excluded volume' of solvent, which simulates the effect of dissolving a given number of osmotically active particles in a smaller volume of water, and produces a system with a high osmotic pressure. It is possible that in antidiuresis the hyaluronic acid of the papilla could counter the effect of water leaving the collecting tubule, which would otherwise lower the osmotic pressure of the papilla.

The action of the hyaluronidases which have been described by Dicker and Elliott,(1962), and Cobbin and Dicker (1963) might be on the interstitial colloid in addition to the intercellular cement of the collecting tubule. Dicker and Eggleton (1960) found that the level of hyaluronidase activity and ADH concentration fell in the urine of man in water diuresis, but that both rose again on restoration of normal flow. Two subjects with an inherited diabetes insipidus of renal origin excreted no hyaluronidase following ADH injection. This was in contrast to normal subjects, or those with diabetes of pituitary origin, in whim large increases of urinary hyaluronidase followed ADH injection. These results probably constitute the strongest evidence to date for a role for hyaluronidase in the mechanism of action of ADH. The tubules in the papilla of man

are separated by a good deal of connective tissue of the type described for the guinea pig, and it is possible that a hyaluronic acid / hyaluronidase system might be well developed. Unfortunately it was not possible during the course of the present study to obtain a post mortem sample of papilla from a patient with nephrogenic diabetes insipidus to see if any derangement could be found in the acid mucopolysaccharides.

If the hyaluronidase that destroys the sulphated acid mucopolysaccharide between collecting tubule cells is lysosomal in origin, then it is possible that cortisol and allied steroids might have their effect on the kidney by inhibiting the release of hydrolases through their action in stabilizing lysosomal membranes. It has been reported that purified hyaluronidase itself is inhibited in vitro by cortisol (Matthews and Dorfman, 1954). As Robinson (1954) put it: 'Adrenal cortical hormones might close the pores which the antidiuretic hormone seems to open. Itseems that adrenal cortical hormones are required to provide an essential background for the regulation of the excretion of water though they do not directly control it'.

Against this view, Ahmed, George, Gonzalez-Anvert and Dingman (1967) have advanced the argument that glucosteroids act on the central nervous system rather than directly upon the kidney. These workers found high levels of ADH in the plasma of patients with adrenal cortical insufficiency; the levels could be made

to return to normal values by administering glucosteroids. Since the action of ADH is not abnormally prolonged in adrenal cortical insufficiency, and the duration of action is not significantly altered by the administration of steroids, it was argued that the glucosteroids porbably set the level at which ADH was released by the central nervous system.

The settlement of this question awaits the development of a reliable method for the histochemical localization of hyaluronidase activity. However, this thesis has drawn attention to the fact that the junctional complexes between the lateral cellular borders make it unlikely that hyaluronidase released into the collecting tubule lumen could find its way to the interstitium. At the same time it is not clear how an enzyme acting in the interstitium could find its way into the urine. This problem might be resolved if there were two sites of origin, one being the interstitial cell and the other the collecting tubule cell.

The challenge remains to devise new techniques to determine which of the appearances described are cause and which are effect in the physiology of the kidney. The present study has demonstrated a morphological effect of antidiuretic hormone on the collecting tubule cells and draws attention to an effect it appears to have on the extracellular colloid of the papilla, the constancy of the'milieu interieur' of which determines the freedom of its owner.

5) Conclusions.

1) The renal papilla in most species is a well defined region where the tubules are separated by connective tissue which contains hyaluronic acid.

2) The development of the papilla is related to the maximum concentration that can be achieved in the urine by the empirical equation:

$$U_{max} = 1.74 \, (M'/C') + 0.6,$$

where U_{max} is the maximum concentration of the urine in Osmols/litre; M' is the length of the region extending from the tip of the papilla towards the cortex which stains positively for hyaluronic acid; and C' is the continuation of this line to the renal capsule. The correlation coefficient, for the six species studied, was 0.9.

3) The collecting tubule cells synthesize an acid mucopolysaccharide that is not hyaluronic acid. It contains sulphate groups and a limited number of vic - glycol groups and it is bound to protein. This material forms a thick lumenal 'cuticle' at the apical border of the cell, and is to be found in the lateral spaces between cells.

Conclusions.

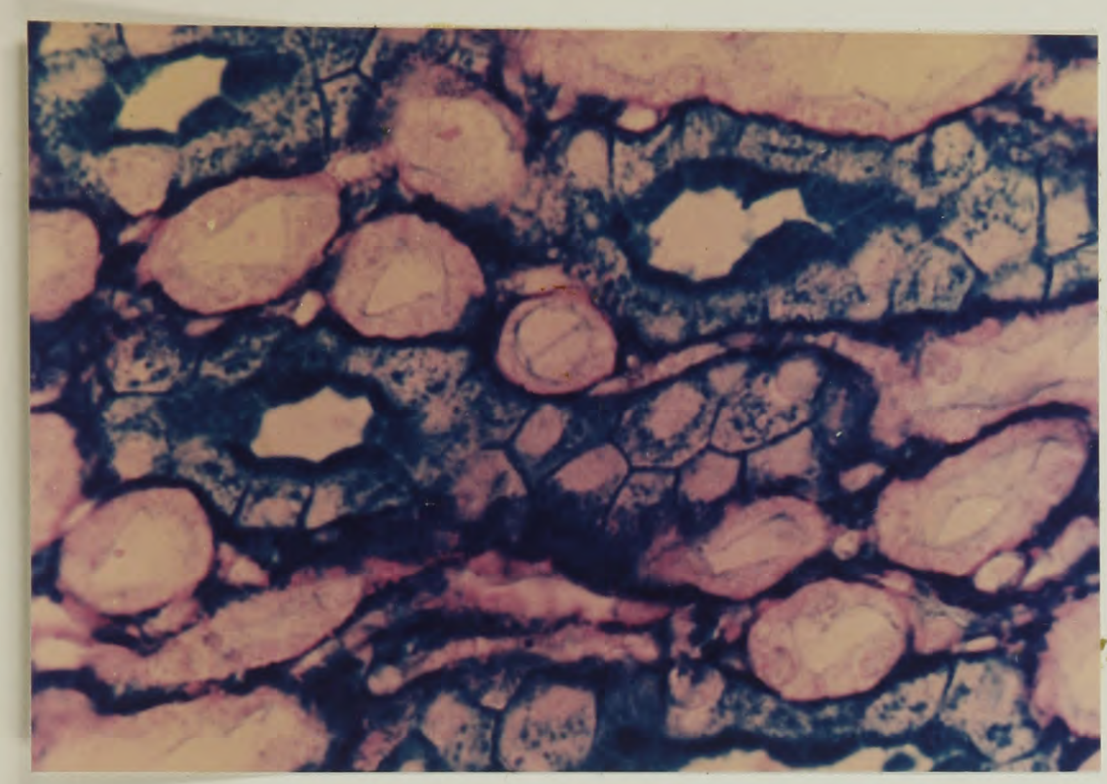


Fig. 6/4. Colloidal Iron & PAS

WATER LADEN: Note extensive Golgi regions, thick lumenal cuticle and acid mucopolysaccharide in the lateral intercellular spaces.

Fig. 6/5. (Over page). Colloidal Iron & Mayer's Carmalum.

ANTIDIURESIS: Note much smaller Golgi regions, thinner lumenal cuticle and no intercellular staining. Compact interstitium except for open vessels near base of collecting tubules.

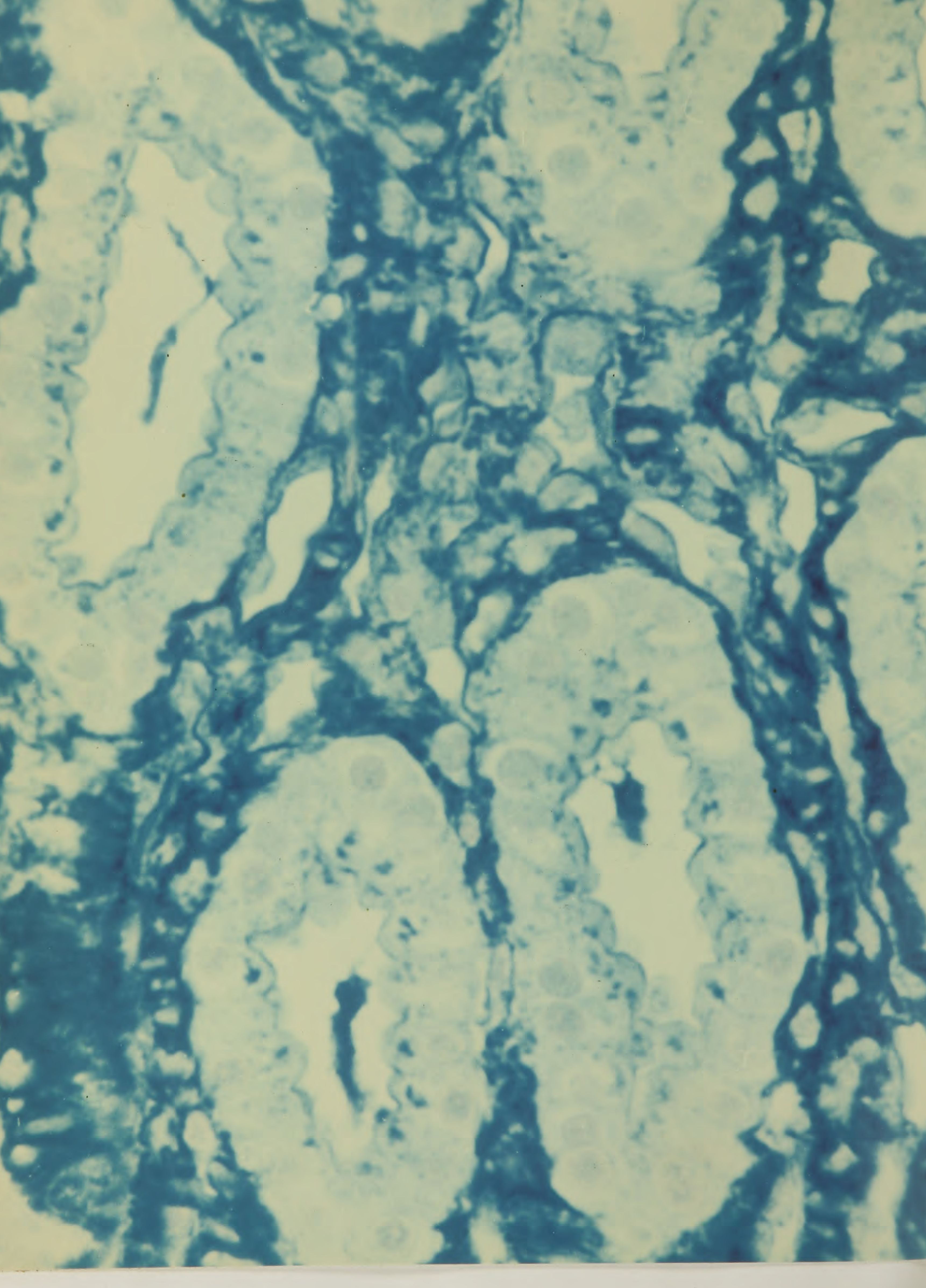


Fig. 6/5. ANTIDIURESIS. Colloidal Iron & Carmalum.

4) In antidiuresis, or following the injection of antidiuretic hormone, the volume of the collecting tubule cells (as measured in the light microscope) falls by approximately 20%.

5) Most of this reduction in volume can be accounted for by the loss of the apical acid mucopolysaccharide in antidiuresis. In this condition there is also depolymerization of the acid mucopoly - saccharide which exists between collecting tubule cells.

6) The interstitium becomes compact in antidiuresis and the intensity of staining of acid mucopolysaccharides is increased. In water diuresis the interstitium appears to expand and the reticulin fibrils become separated as though they were under tension. These changes are probably explained by the increased hydration in diuresis of the hyaluronic acid gel which is trapped by the reticulin. The appearance is due to water loading itself and the change is not reversed if antidiuretic hormone is injected before the water load has been excreted.

APPENDIX.

2/1 The Processing of Tissue and Staining of Sections for the Demonstration of Acid Mucopolysaccharides:

a) Materials Required.

Histological Fixative:

Formol (40% CHO)	100 ml.
Cellosolve, (2-Ethoxy-Ethanol)	255 ml.
Distilled water,	45 ml.
Aminoacridine hydrochloride,	1.6 gms.

Modified Ester wax:

Diethylene glycol monostearate	100 gms.
Diethylene glycol distearate	100 gms.
Castor oil	5 gms.

Gelatin Solution for Subbing Slides:

Gelatin	5 gms.
Chrome Alum	0.5 gms.
Water to make	1 litre.

Colloidal Iron Solution:

To prepare the stock solution 1 litre of distilled water was brought to the boil. When it was boiling vigorously, 6 ml of ferric chloride (39 %) solution (B.D.H.) was added drop by drop and the boiling continued for five minutes after the last drop had been added. This produced a deep red solution of colloidal iron which kept well. It did not improve the specificity of staining if the stock solution was dialysed and this step was usually omitted.

The staining solution was prepared immediately before use by mixing one part of the stock solution with one part of 25% acetic acid.

Potassium Ferrocyanide Solution:

This was prepared immediately before use by mixing equal parts of a solution of 2% freshly dissolved potassium ferrocyanide and a 2% solution of hydrochloric acid.

b) Fixation and Embedding.

i) Small pieces of tissue were fixed in the aminoacridine hydrochloride / formol - cellosolve fixative for at least two, but not more than, 18 hours.

ii) The tissue was then passed directly into pure cellosolve for dehydration. Three changes of about four hours each were employed.

iii) The tissue was then transferred to a mixture of equal parts of cellosolve and toluol overnight.

iv) It was then cleared in pure toluol, two changes within the hour usually being sufficient.

v) The cleared tissue was then embedded in the ester wax at 50°C.

The technique of casting and trimming ester wax blocks was as outlined by Chesterman and Leach (1956). Sections were cut at either 3 μ or 5 μ with a rotary microtome (Reichert). They were floated onto gelatinized slides to which a drop of 0.005% aminoacridine hydrochloride had been added: Excess fluid left after the sections had flattened was sucked off with a Pasteur pipette, and the sections were left to dry for an hour or so on the hot plate.

c) Staining.

i) The sections were dewaxed with xylol.

ii) The xylol was removed by absolute alcohol, after which the slide was passed through 96% alcohol. ~~After~~ There after the staining techniques were as follows:

Alcian Blue at pH 3.5 (Carboxyl & Sulphate Groups):

iii) Rinsed in 3% Acetic acid.
iv) Placed in the dye bath of 1% Alcian Blue dissolved in 3% Acetic acid for 30 mins. at room temperature.
v) Rinsed in 3% Acetic acid, followed by distilled water.

Alcian Blue at pH 1.0 (Sulphate Groups):

iii) Rinsed in 1% Hydrochloric acid.
iv) Placed in the dye bath of 1% Alcian Blue dissolved in 1% Hydrochloric acid for 30 mins. at room temperature.
v) Rinsed in 1% Hydrochloric acid, followed by distilled water.

Sections stained in Alcian Blue were dehydrated in 96% Ethanol which was saturated with barium hydroxide to convert the dye to an insoluble pigment and also to intensify the colour. This was followed by immersion in absolute alcohol, clearing in xylol and mounting in (Gurr's) 'Neutral Synthetic Mountant'.

Colloidal Iron (for all anionic groups):

iii) Rinsed in 12.5% Acetic acid.
iv) Immersed in the Colloidal Iron Reagent for 1 hour at room temperature.

v) Rinsed in 12.5% acetic acid: Three changes, five minutes each.

vi) The sites which had bound colloidal iron were then demonstrated by immersing the sections in the potassium ferrocyanide solution.

vii) After rinsing in distilled water the sections were counterstained by Mayer's carmalum or the Feulgen technique. The colloidal iron staining was often followed by the PAS routine.

viii) Sections were dehydrated, cleared and mounted in the normal manner.

Appendix 2/2: Stastical Treatment of the Data in Table 2/1.

a) Calculation of the Relationship between Papillary Length and Maximum Osmolarity of Urine.

In Fig. 2/18 the best straight line as judged by eye was drawn through the plotted values for M'/C' and U_{max} which gave the relationship:

$$U_{max} = 1.8 \, (M'/C') + 0.5 \qquad (i)$$

The relationship would be more accurate if it were derived statistically, for which the data in Table 2/1 are abridged below:

M'/C' 100 = x	U_{max} = y
46	1.4
66	1.5
68	2.0
124	2.7
125	2.4
125	3.2

If a linear relationship exists between x and y it can be expressed in the form:

$$y = mx + c,$$

where m is the slope of the line and c is a constant. Assuming such a relationship, the values of x and y may be substituted in the six equations:

$$\begin{aligned}
1.4 &= 46m + c \\
1.5 &= 66m + c \\
2.0 &= 68m + c \\
2.7 &= 124m + c \\
2.4 &= 125m + c \\
3.2 &= 125m + c \\
\hline
13.2 &= 554m + 6c
\end{aligned}$$

These may be added,

Each may be multiplied by its coefficient of m, and similarly added:

$$\begin{aligned}
64.4 &= 2116m + 46c \\
99.0 &= 4356m + 66c \\
136.0 &= 4624m + 68c \\
334.8 &= 15376m + 124c \\
300.0 &= 15625m + 125c \\
400.0 &= 15625m + 125c \\
\hline
1334.2 &= 57722m + 554c
\end{aligned}$$

This gives two equations for m and c which may be solved by multiplying the first by 554 and the second by 6 to eliminate c:

$$7312.8 = (554)^2 m + (6 \quad 554) c$$
$$8005.2 = (57722 \quad 6) m + (6 \quad 554) c,$$

Whence, $m = 1.74 \quad 10^{-2}$.

Substituting this value for m gives,

$$c = 0.59.$$

Thus, the equation relating maximum urinary concentration to the ratio of papillary to cortical length is more accurately given as:

$$U_{max} = 1.74 \ (M'/C') + 0.6 \qquad (ii)$$

Since one osmol of any undissociated solute is equal to its molecular weight, the dimensions of U_{max} are $M \ L^{-3}$, and these will thus be the dimensions of the constants m and c.

<u>b) Calculation of the Correlation Coefficient:</u>

Selecting an arbitary value for maximum urinary concentration as 2 Osm/l, and an arbitary value for M'/C'.100 of 70, we may define u and v:

$$u = 10 (y - 2) \quad \text{and}$$
$$v = 10 (x - 70).$$

Thus;

	u	v	uv	uv	u^2	v^2
	-6	-240		1440	36	57600
	-5	- 40		200	25	1600
	0	- 20		0	0	400
	+7	+540		3780	49	291600
	+4	+550		2200	16	302500
	12	+550		6600	144	302500
Sum:	12	1340		14220	270	956200
Mean:	2	223				

The correlation coefficient (r) between x and y is given by the relationship:

$$r = \frac{\frac{1}{N} uv - \bar{u}\bar{v}}{\sigma_u \cdot \sigma_v}$$

where N is the number of pairs of x and y for which values are available, ie. 6; $\bar{u}$ and $\bar{v}$ are the mean values for u and v respectively; and

$$(\sigma_u)^2 = \frac{u^2}{N} - (\bar{u})^2 \quad \text{and} \quad (\sigma_v)^2 = \frac{v^2}{N} - (\bar{v})^2$$

Whence, $r = \frac{2370 - 446.66}{(6.4)(331)}$ Ie. $r = 0.908.$

c) Calculation of the Regression Equation for y on x:

From the definitions of u and v,

$$y = \frac{u + 20}{10} \qquad \text{and} \qquad x = \frac{v + 70}{10}$$

Similarly, the averages are related:

$$\bar{y} = \frac{\bar{u} + 20}{10} \qquad \text{and} \qquad \bar{x} = \frac{\bar{v} + 70}{10}$$

Thus, $\bar{y} = 2.2$ and $\bar{x} = 29.3$

Also O_x and O_y are related to O_v and O_u by

$$O_y = \frac{O_u}{10} \qquad \text{and} \qquad O_x = \frac{O_v}{10}$$

Thus the regression equation for y on x can be written:

$$y - \bar{y} = r.\frac{O_y}{O_x}.(x - \bar{x}),$$

Whence,

$$y = \frac{1.75}{100}. x + 1.68,$$

Ie.

$$U_{max} = 1.75 (M'/C') + 1.68 \qquad \text{(iii)}$$

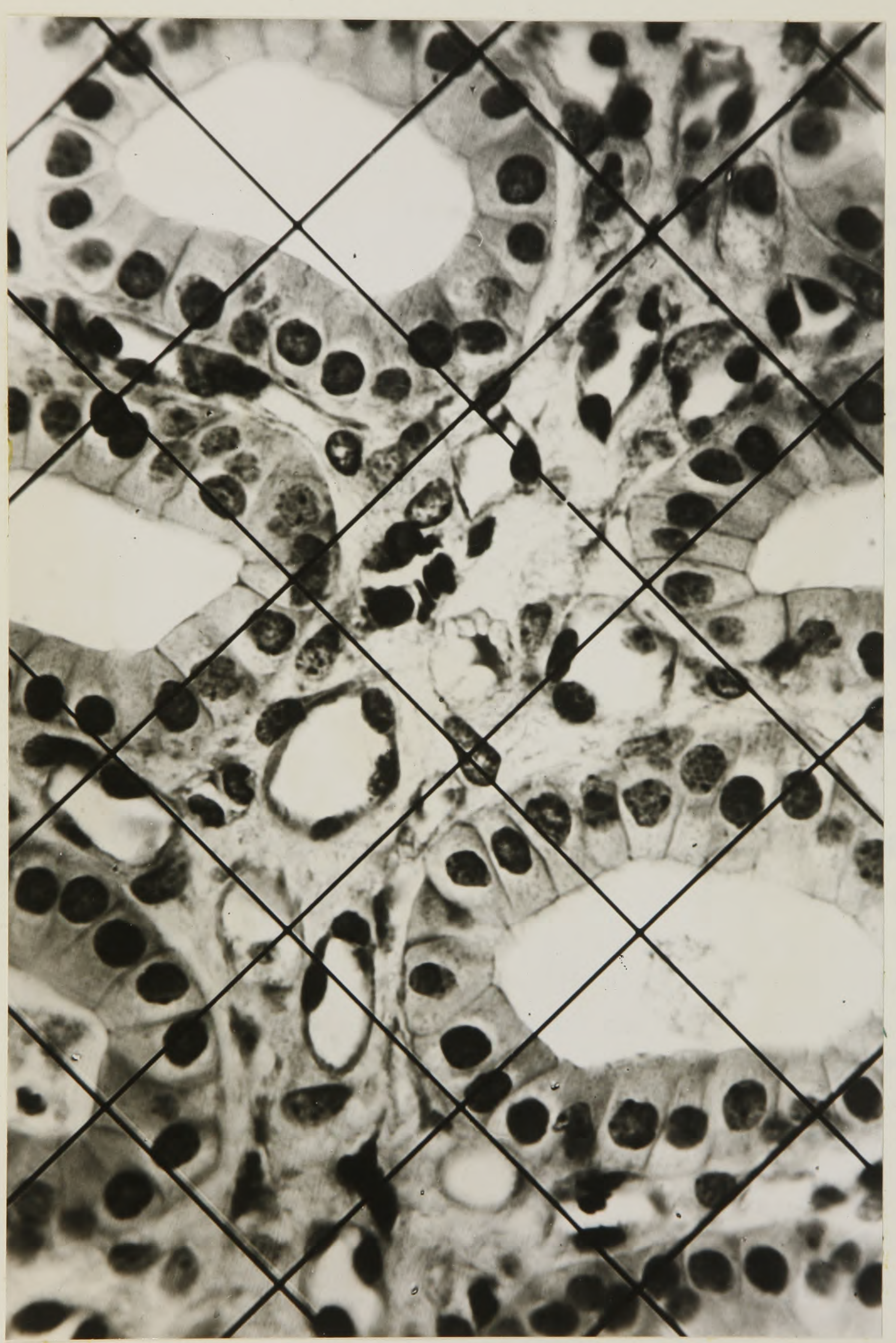


Fig. A/1. Masson Trichrome. Specimen Field.

Appendix 2/3. Morphometry.

For the measurement of relative areas, an eyepiece micrometer ruled in squares was used. With the micrometer resting on the diaphragm of the eyepiece it was possible to superimpose the grid at the point of focus of the section, as in Fig. A/1. The structures lying under 500 intersections were recorded: The frequency of 'hits' upon a given structure is proportional to its area (Mayer, 1963). For example, as regards the field in Fig. A/1, the relative areas recorded would be:

Collecting tubule cells	12	or	60%
Collecting tubule lumen	2		10%
Interstitium	4		20%
Interstitial cells	1		5%
Loop of Henle, Vasa recta	1		5%

The intersections were recorded by pressing the appropriate button on a Clay-Adams Laboratory Counter which made the operation speedy, not tiring, and therefore less subject to operator inaccuracy. In addition to economy and speed, this method has the further advantage over ~~that~~ the photographic recording that would be required for planimetry in that different levels of focus can be used for accurate identification of structures, especially those lying towards the edge of the field (cf. Fig. A/1).

To minimize subjective selection of the fields, the areas counted were presented in strict order along orthogonal axes by movement of the microscope stage. 500 intersections were found to give reproduceable counts varying by not more than 4%. This was considered to be a sufficient level of accuracy for the present study.

Appendix 2/4. Electron Microscopy.

Blocks of tissue of about 1 mm^3 were rapidly excised from papillae and fixed for 30 mins. at $4^{o}C$ in 1% osmium tetroxide buffered to pH 7.4 in veronal acetate. From this they were transferred to 70% alcohol, and then through the alcohol series to absolute alcohol dried over anhydrous sodium sulphate.

The blocks were embedded in Araldite and sectioned on a LKB Ultratome 11 to give sections of a thickness which showed gold interference patterns. The sections were stained with uranyl acetate and lead citrate before being examined on an A.E.I. EM 6B electron microscope.

The cytochemical illustrations in Chapter 3 were obtained from material fixed as per Appendix 2/1, sectioned by hand and immersed in the colloidal iron solution, rinsed in acid and then processed for electron microscopy as above, omitting the stains.

Appendix 4/1. Acid Mucopolysaccharide Precipitation.

The following precipitants were added individually to formol-cellosolve (Appendix 2/1) at the concentration indicated:

Aminoacridine hydrochloride	0.4%
Acridine Orange	0.4%
Cetyl Pyridinum Bromide	0.5%
Lead Acetate	0.4%

Towards the end of the work, hexaminecobaltic chloride, used by Matthews and Dorfman (1953) to precipitate heparin, was used as a saturated solution in formol-cellosolve. This was found to give excellent preservation of acid mucopolysaccharides in tissue which was reasonably preserved. It could not be used in autoradiographic experiments because traces of hexaminecobaltic chloride in sections produced strong autochemograms.

Acid mucopolysaccharide precipitants such as pure ethanol, acetone or pure cellosolve were found not to preserve tissues sufficiently well to merit their use.

Appendix 4/2. Autoradiography.

Before embarking upon radiographic studies the following sources were consulted:

Doniach and Pelc.	Autoradiograph Technique.	1950
Pearse.	Histochemistry.	1960
Beneš.	Fundamentals of AR.	1966
Rogers.	Techniques of Autoradiography.	1967

Sulphur-35 emits beta rays of suitable energy for light microscopic autoradiography and, as $S^{35}O_4^{=}$, has been used by a number of workers to trace the synthesis of sulphated mucins, eg. Heatley, Jerrome, Jennings and Florey (1956), who gave a lucid account of their technique, and Lindner (1965) who reviewed its use in the histochemistry of acid mucopolysaccharides.

Carrier free sodium sulphate solutions were supplied by the Radiochemical Centre, Amersham. The specific activity of the sulphur at reactor unload was thought to be in the region of 20 to 25 curies/mg on the basis of retrospective analyses of previous batches. Thus after one half life the specific activity would be about 13.4 - 17 curies/mg; after two half lives, 8 - 10 curies/mg; and after three half-lives 5 - 6 curies/mg. When used in this study the sulphur was about 2½ half-lives old, and thus had a specific activity of 6 - 7 curies/mg. This corresponds to 0.5 µg of sulphate per millicurie, which was the dose injected.

A human subject weighing 70 kg excretes about 2.5 grams of inorganic sulphate in 24 hours (Eastham, 1963). This corresponds to an excretion of approximately 400 µg per hour by a guinea pig on a purely weight basis.

In the histochemical studies the injected dose was adjusted to give the animal exactly 1 mCi of radioactive sulphate. Since this corresponds to about one eight hundredth of the amount excreted in one hour it would not appear that the experiment is open to the common criticism that a biological system has been grossly overloaded with an injected isotope in order to produce sufficient activity in tissue sections.

A. Autoradiography of Tissue Sections.

Sections were produced according to the scheme in Appendix 2/1. They were dewaxed and, in some experiments, stained before being coated with Kodak AR 10 stripping film. They were dried and exposed in a sealed tin containing sillica gel which was kept in the refrigerator for one to two weeks. The background was kept to a minimum by development for not longer than 5 mins at 20°C in Kodak D 19b. When compared with the more modern nuclear emulsions, AR 10 stripping film has only a modest resolution (2 to 3 μ with isotopes with the energy of Sulphur-35). However this was acceptable in view of the ease of the technique in the hands of an inexperienced worker; and the opportunity it offered of being able to coat a few sections quickly at frequent intervals without wastage of emulsion.

The following controls were performed to test for possible chemical actions of the fixative, tissue

components or dyes on the emulsion:

a) From an animal to which no label had been given, unstained and stained sections were processed in parallel with those from an animal injected with the label. The developed film showed no localized silver grains over the sections from the control animal, and hence artefact due to positive autochemography was excluded.

b) Similar sections were coated but exposed to light before being placed in the refrigerator and developed in the routine manner. On the first occasion this was tried, one of the slides (PAS stained) was uniformly fogged except over the tissue section which was overlaid by clear film. When the experiment was repeated with extra care being taken over the drying stage, and the storage in a dry condition, this phenomenum did not recurr. From this result it was concluded that there were no chemicals coming from within the tissue to cause latent image fading, but that this had arisen in the one case from the diffusion of water from the tissue section into the emulsion (which had permitted silver halide to be formed again).

In view of the satisfactory nature of these controls, it was considered unecessary to coat sections with Celloidin before applying the emulsion. When adopted, this procedure was found to lower the sensitivity of the method to a variable extend depending upon the

thickness of the interposed film. It also very adversely affected the resolution.

Some difficulty was experience initially with lifting of gelatin from parts of the sections when the fixed and washed autoradiographs were dehydrated in alcohol and cleared in xylol. At the suggestion of Mr. Leach, dehydration in cellosolve and mounting directly in balsam dissolved in cellosolve was tried. This was found to be an excellent technique. The sections cleared perfectly when left on a hot plate for about half an hour after mounting, and no further trouble with the separation of the emulsion from sections was experienced. The mounting medium, however, becomes remarkably acid and is thus not suitable for a number of routine histological stains.

B. Autoradiography of the Extracts.

To produce autoradiographs of the material in the electrophoretic separations, thoroughly dried strips were placed in contact with Kodirex X-Ray film, and a weight applied to keep the two in close contact. Contrast was more important than resolution in this case thus development was prolonged for 15 to 20 mins in Kodak D 19b at room temperature, after which the film was fixed in the usual way.

This X-ray film has emulsion on both sides and the prolonged development time gave rise to a good deal of background on the obverse side. The latter

recorded no useful information since the S^{35} beta rays were unable to penetrate the 'Estar' base. The emulsion was therefore removed from the back of the film after processing by placing it front face uppermost on filter paper soaked in 10% NaOH. It was left for ten minutes and then washed in tepid water until all the gelatin had been removed from the back of the film.

Appendix 4/3: The Separation of the Extracts from the Papilla.

'Methods in Zone Electrophoresis' (Sargent, 1966) was consulted before the choice of cellulose acetate as the supportive medium for electrophoresis was made. Manley (1965) has reported successful results using this technique on a similar problem.

The electrophoresis tank supplied by the Shandon Scientific Company was used. It was modified by sticking a layer of plastic sponge to the lid. This sponge was saturated with water and served to keep the atmosphere above the cellulose acetate membrane saturated with water vapour, and thus minimized the chromatographic effects of evaporation from the membranes as they warmed up during the separation. The standard (Vokam) power pack supplied by the Shandon Scientific Company was employed.

A number of buffer systems was tried in the tank (eg. Barbitone, 0.06 Molar at pH 8.6; Michaelis sodium acetate, pH 9.2; Pyridine/acetic acid, pH 5.3) but the most satisfactory separation was achieved with the following buffer (suggested by C. A. Pasternak):

Ammonium Acetate	13.88 grams
Glacial Acetic Acid	1.14 ml
Distilled water to	2.0 litres.

This had a pH of 5.6. A good separation of hyaluronic acid from chondroitin sulphate could be obtained along a 20 cm strip of cellulose acetate by applying 200 volts across it for 1½ hours. This was achieved by setting the current at 0.5 mA/cm strip width. A most satisfactory feature of the buffer was that it was volatile so that when strips were oven dried at 80°C it did not interfere with the subsequent staining at selected pH.

The dried strips were usually stained in 1% Alcian Blue (G.T.Gurr) dissolved in 3% acetic acid (pH 3.5) or in 1% Hydrochloric acid (pH 1) with gentle agitation at room temperature for 30 mins. The strips were then rinsed in either 3% acetic acid or 1% hydrochloric acid after which they were washed in running tap water for ½ hour.

No method was found for permanently clearing which did not either distort the strip or cause the Alcian Blue to fade. (One method which produced permanently clear but shrunken strips was as follows: The strips were immersed in a mixture of 30% glacial acetic acid, 68% ethanol and 2% glycerol to produce softening. They were then carefully layered onto a sheet of glass which was placed in an oven at 80° C.

Some of the Alcian Blue must have been lost from the strips at this stage since the solution became blue. While satisfactory for demonstration and photographic purposes it appeared that the method lacked sufficient accuracy for densitometry.)

Those strips to be used for densitometry were carefully dried and then immersed in liquid paraffin (B.P.) for half an hour. The cleared strips were scanned in a Joyce Chromoscan (a double beam recording and integrating reflectance densitometer) using anoragne filter (No. 5022) and slit No. 5006. The count from each peak was expressed as a percentage of the total count.

Appendix 4/4: Butanol / Water Extraction of Acid Mucopolysaccharides from the Papillae

Tissue was ground in approximately ten times its volume of a mixture, containing 4 parts of distilled water to 1 part of n-butanol, in a 'Quickfit' Potter-Elvehjem homogenizer cooled in ice. The brei was then centrifuged for ten minutes at 2,500 rpm to separate three layers - The top one contained lipid in butanol, the middle was an aqueous layer and there was a small amount of sediment at the bottom. The aqueous layer was aspirated and freeze-dried. Just before the electrophoretic separation, the freeze-dried powder was taken up in a volume of distilled water equal

to one tenth that of the initial tissue volume. This gave a thick opalescent solution, 10 ul of which was streaked across 2.5 cms at the origin on the cellulose acetate.

Appendix 4/5: Proteolytic Extraction.

(a) Extraction after the method of Manley (1965):-

Four papillae, totalling about 1.5 grams, were pooled for each proteolytic extraction. They were homogenized in phosphate buffer at pH 6.5 (0.1 M) and more buffer was added to bring the volume up to 20 ml. Disodium ethylene-diamine- tetra-acetic acid (Sodium edetic acid) was added to give a 0.005 Molar solution. Crystalline papain (B.D.H.) was activated by the addition of cysteine hydrochloride to a final concentration of 0.005 M and added in the proportion of 0.0025 grams to 20 ml. Air was excluded to keep papain in its reduced form and the mixture was incubated with occasional shaking in an oven at 56°C for 18 hours.

After this the digest was dialysed against 4 M sodium chloride solution for six hours to break any secondary protein - polysaccharide bonds, and then against tap water for a further six hours and finally against slowly running distilled water for another twelve hours. After centrifugation of the sample the clear supernatant was freeze-dried and stored.

(b) Extraction after the method of Hakkinen, Hartiala and Terho (1965):-

In each run, material from four papillae was homogenized in 20 ml of freshly prepared buffer. This buffer (recommended by G.A.Pasternak) had the following composition:

Sodium acetate	13.6 grams.
Edetic Acid (E.D.T.A.)	0.6 grams.
Cysteine hydrochloride	1.4 grams.

Four milligrams of crystallized papain (B.D.H.) was added, the air excluded, and the mixture incubated with occasional shaking at 56°C for 18 hours.

The mixture was then filtered under vacuum through a sintered glass funnel. Undigested tissue was washed with a small volume of 4 N sodium chloride which was then filtered and the second filtrate added to the first. 1% Cetyl pyridinium chloride was added drop by drop to the combined filtrates until a froth just remained after shaking. The solution was left to stand for two hours at room temperature, when precipitation of the acid mucopolysaccharides was complete, and then centrifuged to collect the precipitate. This was then dissolved in the smallest volume of warm 2.5 N magnesium chloride, from which the acid mucopolysaccharides were precipitated out by the addition of four volumes of alcohol. The material was then dried in vacuo.

Prior to the electrophoretic separation the material was taken up in a volume of distilled water equal to one tenth of that of the initial tissue volume.

Difficulty was experienced in judging how much cetyl pyridinium chloride should be added to precipitate the acid mucopolysaccharides fully without redissolving first hyaluronic acid and then chondroitin sulphuric acid (Scott, 1955; Scott, 1956). It was also felt that the high salt content of the initial tissue, plus that added in the washing, could lead to selective fractionation at this step. Thus recourse was had to a six hour dialysis of the combined filtrates against distilled water before the cetyl pyridinium precipitation in order to remove most of the low molecular weight solutes.

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