THE HISTOCHEMICAL LOCALISATION OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN ADULT AND NEWBORN RAT KIDNEYS AT THE ELECTRON MICROSCOPICAL LEVEL

by

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ABSTRACT

The histochemical localisation of ATPase enzymatic activity at the level of the electron microscope was carried out on adult and newborn kidney tissue pre-fixed in 5% glutaraldehyde buffered with 0.1 M sodium cacodylate. Both the lead method at pH 7.2 and the calcium method at pH 9.4 were used. The effects of the modifiers PHMB and L-cysteine were also studied.

In the adult rat kidney, the observations of other investigators on kidney ATPase activity were substantiated. Reaction precipitate was localised at the brush border of the proximal tubules, the membranes of the basal and lateral interdigitations of the proximal and distal tubules, and the plasma membranes of the podocytic foot processes. PHMB exerted an inhibitory effect on distal tubular activity at both pH 7.2 and pH 9.4, while cysteine was inhibitory only at pH 9.4. Glomerular ATPase activity was inhibited by PHMB and L-cysteine at pH 9.4.

In the newborn rat kidney, ATPase enzymatic activity was observed in the tubular elements as well as in the glomeruli. In the undifferentiated tubules, reaction product was abundant on the lateral membranes between individual cells. The luminal and basal plasma membranes, which were simple in contour, showed little or no accumulation of precipitate. However, as the microvilli became long and slender in the early stages of the differentiation of the brush border, there was a concomitant increase in the intensity of the ATPase enzymatic reaction. Similarly, reaction product became associated with the developing basal interdigitations. In the immature glomerulus, reaction precipitate was most often observed where two sets of membranes were in apposition. With differentiation, enzymatic activity was localised primarily on the podocytic foot processes. The localisation of ATPase activity at pH 9.4 was found to be influenced by the time of pre-fixation in glutaraldehyde

while ATPase activity at pH 7.2 was not affected. At pH 7.2, neither tubular nor glomerular ATPase enzymatic activity responded to PHMB or L-cysteine.

For both adult and newborn kidneys, the correlation between structure and function was briefly considered. The adult kidney is an important and efficient homeostatic organ. In urine formation various substances are transported across the cell membranes of the glomeruli and tubules. Ultrastructurally, the glomeruli and tubules show modifications characteristic of cells engaged in active transport processes. There is a large increase in plasma membrane surface area, as exemplified by the intricate interdigitations of the podocytic foot processes, the elaborate basal and lateral infoldings, and the brush border of the proximal tubules. Much ATPase activity was found associated with these plasma membranes. The newborn kidney is not as efficient as the adult kidney in maintaining body homeostasis. It is not only functionally but also morphologically immature. Most of the tubules and glomeruli are undifferentiated and do not show specialisations of the plasma membranes as seen in the adult kidney. There is also a relatively smaller amount of ATPase present in the newborn kidney. For both adult and newborn kidneys, it was postulated that at least two types of ATPases with different pH optima are present on the plasma membranes of the tubules and glomeruli.

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I

INTRODUCTION

The localisation of enzymatic activity using biochemical methods on homogenised tissue fractions is a means of assaying quantitatively the various properties of enzymes under conditions approaching their natural state. These fractionation studies, though useful and indispensable, have an inherent limitation. They show the association of enzymes with certain types of cellular components, but give no indication of the actual distribution of the enzymes within individual cells or within groups of cells. At the present time, a histochemical or cytochemical technique for enzyme localisation represents the only way of determining the sites of enzymatic activity under *in situ* or near *in situ* conditions (Marchesi, 1968). The main advantage of using a histochemical technique for enzyme localisation is that cellular relationships are maintained in an intact tissue section. It is therefore possible to visualise the products of an enzymatic reaction in relation to specific cellular components, for example, mitochondria, nuclei or membranes. This has only been made feasible by vast improvements in the field of electron microscopy. Histochemistry at the level of the electron microscope, opens up large areas in cell ultrastructural research. We must however, bear in mind the limitations of existing methods for the ultrastructural localisation of enzymatic activity. There needs to be adequate preservation of both the ultrastructure and the enzymatic activities of cells and tissues. Ideally, fresh tissue should be used, but this is not possible owing to extremely poor preservation of cell ultrastructure. A compromise is made by a brief pre-fixation in formaldehyde (Kaplan and Novikoff, 1959; Holt and Hicks, 1961), or more commonly, in glutaraldehyde (Sabatini et al., 1963; 1964), prior to incubation. The effect of fixation on the characteristics of enzymes, whether quantitative or qualitative or

both, is not known at the present time. Besides fixation, the tissue undergoes rigorous conditions of dehydration, infiltration, embedding and polymerisation in an oven, in preparation for its examination under the electron beam (Sjostrand, 1967). How these processes affect the tissue are poorly, if at all, understood. Nevertheless, if these limitations are borne in mind, much information can be gleaned from electron microscopical histochemical data.

Glenner (1968) defines the histochemical system as "that system in which intact tissue sections are incubated in a solution containing a substrate, and the enzyme-catalised formation of the final reaction product (a precipitate) can be demonstrated in situ by means of a variety of techniques including light, fluorescent, electron and interference microscopy." Many histochemical methods, to date, are based on simple metal-salt precipitation reactions. The two common procedures for the histochemical localisation of adenosine triphosphatase (ATPase) in tissue sections use lead ions (Wachstein and Meisel, 1957) or calcium ions (Padykula and Herman, 1955a, 1955b; Padykula and Gauthier, 1963) to form a precipitate with the inorganic phosphate which is released by the enzymatic hydrolysis of the substrate in the incubation media, that is, adenosine triphosphate (ATP). The reaction product, which is electron dense and can be viewed directly under the electron microscope, is presumably deposited at, or very near to, the actual site of the enzymatic activity. This issue of whether the site of deposition of the reaction precipitate is the actual site of enzymatic activity, has not been solved yet, as many investigators stress (Otero-Vilardebo et al., 1963; Wachstein and Besen, 1964; Mao and Nakao, 1966).

Most cells store energy in the form of high energy phosphate bonds in ATP (Stumpf, 1953). This energy can be released for use in performing various cellular activities by the enzyme ATPase. Therefore, the localisation of the enzyme ultrastructurally would perhaps lead to some understanding of the mechanisms underlying cellular function.

Since the introduction of the lead (Wachstein and Meisel, 1957) and calcium (Padykula and Herman, 1955a, 1955b; Padykula and Gauthier, 1963) methods for the histochemical localisation of ATPase, many sites in various tissues have been demonstrated to possess this enzyme. This has been shown both with light microscopy (Padykula and Herman, 1955a, 1955b; Wachstein, 1955; Wachstein and Meisel, 1957; Wachstein et al., 1960, 1962; Padykula and Gauthier, 1963; Tewari and Bourne, 1963a, 1963b; McClurkin, 1964; Wachstein and Besen, 1964; Farquhar and Palade, 1966; Moses et al., 1966; Gauthier, 1967; Jacobsen et al., 1967; Brooke and Kaiser, 1969) and electron microscopy (Essner et al., 1958; Kaplan and Novikoff, 1959; Persijn et al., 1961; Lazarus and Barden, 1962; Ashworth et al., 1963; Otero-Vilardebo et al., 1963; Torack and Barrnett, 1963; Wachstein and Besen, 1963; Goldfischer et al., 1964; Lazarus and Barden, 1964; Wachstein and Besen, 1964; Wachstein and Fernandez, 1964; Gauthier and Padykula, 1965; Farquhar and Palade, 1966; Hoff and Graf, 1966; Mao and Nakao, 1966; Rechardt and Kokko, 1967; Wills, 1967; Anderson, 1968).

Many and diverse tissues have been studied concerning the histochemical localisation of their ATPase content and speculations as to the possible or probable roles in the function of their respective tissues have sometimes been put forward. A few examples from this vast area of research are presented. The literature cited is by no means extensive nor complete.

In striated muscles there is a constant turnover of energy associated with the processes of contraction and relaxation. Many of these cellular functions require the splitting of ATP with the concomitant release of energy. Following this line of thinking, it is expected that ATPase activity will be demonstrable histochemically in muscular tissue. In fact, histochemical data on muscle ATPase is available (Padykula and Herman, 1955a, 1955b; Padykula and Gauthier, 1963; Barden and Lazarus, 1964; Gauthier and Padykula, 1965; Klein, 1966; Gauthier, 1967; Grossman and Heitkamp, 1968; Brooke

and Kaiser, 1969; Ogawa and Mayahara, 1969). As many as four different ATPases have been localised in skeletal muscle fibres; a mitochondrial and a myofibrillar ATPase, and two sarcotubular ATPases (Padykula and Gauthier, 1963; Gauthier and Padykula, 1965; Gauthier, 1967). It was possible to differentiate among these four enzymes in terms of their pH optima and their response to activators and inhibitors. Of particular interest in the muscle are the two sites of sarcotubular activity, one at the region of the triad and the other at the region of the H band (Gauthier and Padykula, 1965; Gauthier, 1967). It is suggested that the ATPase at the triad may be involved in the accumulation of calcium ions during the period of relaxation, as well as in the release of calcium ions following a stimulus for muscular contraction. The ATPase at the H band is more specifically associated with the rebinding of calcium ions during relaxation (Gauthier and Padykula, 1965; Gauthier, 1967).

The liver is an important and a functionally active organ whose diverse activities contribute to the structural and functional stability of the whole organism. Morphologically it is a simple organ but metabolically it is not so. Many investigators have applied the histochemical methods for the localisation of ATPase to the liver (Padykula and Herman, 1955a, 1955b; Essner et al., 1958; Novikoff et al., 1958; Holt and Hicks, 1961; Novikoff et al., 1961; Persijn et al., 1961; Wachstein and Bradshaw, 1962; Ashworth et al., 1963; Wachstein and Fernandez, 1964; Moses et al., 1966; Wills, 1967). The products of the enzymatic reaction have been found in the membranes of the endoplasmic reticulum (Wachstein and Fernandez, 1964) but more commonly on the membranes of the microvilli of the bile canaliculi (Essner et al., 1958). The possible participation of these ATPases in molecular transport and/or pinocytosis is suggested (Essner et al., 1958).

From biochemical studies it was found that nervous tissues possess a large amount of the enzyme ATPase (Bonting et al., 1962). However, biochemical data is only statistical and does not indicate the sites of enzymatic activity. Histo-

chemical methods are therefore employed to localise the ATPase activity in various components of the nervous system (Novikoff et al., 1961; Tewari and Bourne, 1963a, 1963b; Torack and Barrnett, 1963; Torack and Markey, 1964; Rechardt and Kokko, 1967). Torack and Barrnett (1963) found reaction product associated with plasma membranes of neurons and neuroglial dendrites adjacent to the cell body, synaptic terminals and glial foot processes adjacent to capillary walls. They put forward the idea that the ATPase present on the membranes of the glial processes may be related to the supposed function of the astrocytes in transporting materials between blood vessels and neurons. At the synapses the enzyme may participate in the synthesis of acetylcholine. Interestingly enough, there is no enzymatic activity in the endothelial cells of the cerebral capillaries, that is, those of the blood-brain barrier, while enzymatic activity is present in other endothelia. Involvement in transfer mechanisms across the walls of small blood vessels is implied. Water, salts and nutrients cross the capillary endothelium to the surrounding cells by active transport and pinocytosis. Simultaneously, waste products from the cells are removed in the blood by similar processes. Probably the enzyme ATPase is necessary for these processes to occur. Perhaps one of the causes for the failure of an interchange of materials between the endothelial cells of the cerebral capillaries and the brain cells, is the lack of the enzyme ATPase leading to an inability of the endothelial cells to carry out transport processes.

ATPase activity has been localised histochemically in practically every system in the vertebrate body (Novikoff et al., 1961; Wachstein and Bradshaw, 1962; Ashworth et al., 1963; Barden and Lazarus, 1963; Bradshaw et al., 1963; Otero-Vilardebo et al., 1963; Goldfischer et al., 1964; Lazarus and Barden, 1964; Wachstein and Fernandez, 1964; Wheeler and Whittam, 1964; Essner et al., 1965; Farquhar and Palade, 1966; Hoff and Graf, 1966; Mao and Nakao, 1966; Tormey, 1966; Vethamany and Lazarus, 1967; Anderson, 1968; Marchesi, 1968; Abel,

1969). The urinary system, with particular emphasis on the kidney, is no exception (Padykula and Herman, 1955b; Spater et al., 1958; Freiman and Kaplan, 1959; Kaplan and Novikoff, 1959; Freiman and Kaplan, 1960; Novikoff et al., 1961; Persijn et al., 1961; Pinkstaff et al., 1962; Wachstein and Bradshaw, 1962; Ashworth et al., 1963; Otero-Vilardebo et al., 1963; Wachstein and Besen, 1963, 1964; Wheeler and Whittam, 1964; Wachstein and Bradshaw, 1965; Jacobsen et al., 1967; Abel, 1969; Jacobsen and Jorgensen, 1969). Most of the research on kidney ATPase, both with the light microscope and the electron microscope, has been on adult kidneys. Few studies have been carried out on newborn or developing kidneys (Pinkstaff et al., 1962; Wachstein and Bradshaw, 1965). These have only been at the level of the light microscope. Up to date, there is no description of ATPase activity in newborn kidneys at the level of the electron microscope, to the knowledge of the author.

The present study is an attempt to confirm the observations of other investigators on the localisation of the reaction product for ATPase in adult kidneys using both the lead (Wachstein and Meisel, 1957) and calcium (Padykula and Herman, 1955a, 1955b; Padykula and Gauthier, 1963) methods at the electron microscopical level. These methods are extended to include newborn kidneys. These are known to have different transport capacity and might therefore show differences in ATPase activity, if the two processes are related. Generally, the newborn kidney is functionally inefficient and cannot withstand changes in acid and base intake (Wacker et al., 1961; Moog, 1965), or changes in hydration (Pinkstaff et al., 1962; Wachstein and Bradshaw, 1965). Rates of glomerular filtration and tubular absorption are low when compared with those of the adult kidney. The modifiers of enzymatic activity, p-hydroxymercuribenzoate (PHMB) and L-cysteine, are used in an initial effort towards determining the specificity of the enzymatic activity demonstrated. Although the specificity of the enzyme system is not fully and thoroughly investigated, it will be designated as ATPase in the following report.

II

MATERIALS AND METHODS

A. MATERIALS

Kidneys were obtained from adult, 3 day-old, 24 hour-old, 12 hour-old, 2 hour-old and 1 hour-old rats. Adult rats were killed by an intra-peritoneal injection of sodium pentobarbitol (0.6 cc of 330 mg. sodium pentobarbitol/10 ml. water). Newborn rats were killed by decapitation. The kidneys were removed immediately and placed in a dish containing 5% glutaraldehyde buffered with 0.1M sodium cacodylate (Sabatini et al., 1963). Cortical tissue was cut with sharp razor blades into 1 mm. cubes and immersed in the fixative.

(All the procedures were carried out at room temperature unless otherwise stated.)

B. PRE-FIXATION IN GLUTARALDEHYDE

To combine histochemistry with electron microscopy, it was found that pre-fixation was often necessary to adequately preserve the ultrastructural features of the tissue (Barrnett, 1959; Holt and Hicks, 1961). Without pre-fixation there was a loss of fine details and therefore it was difficult to determine with which structures the products of the histochemical reactions were associated. Osmium tetroxide, the most common and useful fixative then, gave excellent cytological fixation but seriously reduced or destroyed the activity of many enzymes (Holt and Hicks, 1961; Sabatini et al., 1963). There was need for a fixative which would retain satisfactory ultrastructure as well as preserve enough enzymatic activity to be demonstrable with histochemical techniques. Buffered formaldehyde was used by Holt and Hicks (1961). Sabatini et al., (1963, 1964) introduced glutaraldehyde and other dialdehydes as suitable fixatives for ultrastructural and cytochemical studies. Glutaraldehyde is the most widely used at the present time.

Commercial glutaraldehyde preparations are rather crude and have to be purified by various means (Fahimi and Drochmans, 1968). In these experiments, the stock solution of 25% glutaraldehyde (Eastman Organic Chemicals, Distillation Products Industries, Rochester 3, New York) was always filtered through activated charcoal (Fahimi and Drochmans, 1968) just prior to use.

As will be discussed later, the duration of prefixation in 5% glutaraldehyde-cacodylate (0.1M) affected ATPase activity at pH 9.4 especially in the kidneys of newborn rats. The times of fixation were varied as indicated in Table I.

TABLE I

AGE OF THE ANIMALS AND THE LENGTH OF FIXATION OF THE RESPECTIVE KIDNEYS

Age of animals	Length of fixation (Hours)
Adult	5 3
3 days	5
24 hours	5 3
12 hours	4 2
2 hours	2
1 hour	1½ 1 ½ ¼

Following fixation in glutaraldehyde, the tissue blocks were washed in the cacodylate buffer for at least 1 hour or stored in buffer overnight (4° C) prior to incubation.

C. INCUBATION FOR HISTOCHEMISTRY

Two general procedures were followed for the demonstration of ATPase activity in the kidney. At pH 7.2 the lead method (Wachstein and Meisel, 1957) was used while at pH 9.4 the calcium method (Padykula and Herman, 1955a, 1955b; Padykula and Gauthier, 1963) was used.

(a) Preparation of incubating medium at pH 7.2

12 ml.	distilled water
20 ml.	Tris maleate buffer (pH 7.2)
3 ml.	2% lead nitrate (It was added gradually with constant stirring to avoid precipitation.)
5 ml.	0.1 M magnesium sulfate
25 mg.	ATP (Sigma)

The pH of the solution was adjusted to 7.2 if necessary. It was filtered and made up to 50 ml.

(b) Preparation of incubating medium at pH 9.4

10 ml.	2% sodium barbitol
5 ml.	2% calcium chloride
15 ml.	distilled water

0.15 mg. ATP (Sigma) was added to the above mixture, the pH adjusted to 9.4, filtered and filled up to 50 ml. with distilled water.

The solutions were always made up fresh and used immediately.

Routinely, the tissue blocks were incubated in the respective media at 37°C for 2 hours. Following incubation at pH 7.2, the tissue was rinsed with and stored in buffer overnight at 4°C. Following incubation at pH 9.4, the tissue was washed in several changes of 1% calcium chloride for ½ hour and several changes of 2% cobalt nitrate for 15 mins. before being kept in buffer overnight at 4°C.

D. EFFECT OF MODIFIERS

The response to modifiers (activators and inhibitors) was tested using 2.5×10^{-3} M L-cysteine (Sigma) as a source of sulfhydryl groups, and 2.5×10^{-3} M PHMB (Sigma) as a mercurial compound (Padykula and Herman, 1955b; Gauthier, 1967). PHMB is a mercaptide forming agent and inhibits enzymes having sulfhydryl groups at their active centres (Glenner, 1968). L-cysteine, an SH- compound, would activate an SH-dependent enzyme, while at the same time strongly inhibit any alkaline phosphatase activity (Padykula and Herman, 1955b). It is believed that alkaline phosphatase could act on ATP as a substrate (Padykula and Herman, 1955b; Freiman and Kaplan, 1959; Persijn et al., 1961; Hori and Chang, 1963).

It has been shown that any modifier should be used both before and during incubation to overcome the effects of "differential diffusion of modifier and substrate" (Glenner, 1968). A pre-incubation exposure was effected by adding either L-cysteine or PHMB to the glutaraldehyde-cacodylate fixative for the last hour of fixation. For comparison some tissue blocks were not pre-incubated with the modifiers in the fixative.

PHMB is highly insoluble if it is added directly to the incubating media, as has been suggested (Gauthier, 1967). Therefore it was first dissolved in a dilute alkaline solution of sodium hydroxide before being incorporated into the incubating media or the fixative.

The pH's of the respective solutions were then checked and adjusted.

E. CONTROLS

Control specimens were run simultaneously with the experimental specimens. In the control preparations, all the above procedures were followed with the exception that ATP was omitted from the incubation mixtures.

F. POST-FIXATION IN OSMIUM

The tissue was post-fixed, following incubation and storage overnight in buffer, for 1 hour in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate. This increased the contrast of the membrane systems and stabilised the fine structure of the cells maintained by glutaraldehyde for Epon embedding (Sabatini et al., 1963, 1964).

G. ELECTRON MICROSCOPY

The tissue blocks were dehydrated through a graded series of alcohol, half and half of absolute alcohol and propylene oxide, and propylene oxide alone. The tissue was then infiltrated with a 1:1 mixture of propylene oxide and Epon for 2 to 3 hours. Each tissue block was embedded in a gelatin capsule containing fresh Epon, and the Epon allowed to polymerise overnight in a 65°C oven.

Sections were cut with glass knives on a Porter Blum MT-2 ultramicrotome. Silver or gold sections were picked up on uncoated copper grids and examined with a Philips EM 200. All the sections were unstained.

III

OBSERVATIONS

A. MORPHOLOGICAL BASIS FOR HISTOCHEMICAL INTERPRETATIONS

(a) Adult kidney

The structure of the adult kidney has been studied extensively by morphologists and anatomists with the naked eye and under the light microscope (Maunsbach, 1966a, 1966b; Tisher et al., 1966; Rouiller, 1969 - a review). With the introduction of the electron microscope, some of the older and classical descriptions of renal structure have been confirmed and also much extended (Pease, 1955; Yamada, 1955; Suzuki, 1958; Maunsbach et al., 1962; Porter and Bonneville, 1964; Bulger, 1965; Maunsbach, 1966a, 1966b; Tisher et al., 1966; Griffith et al., 1967; Latta et al., 1967; Ericsson and Trump, 1969; Simon and Chatelanat, 1969). Only a brief description of the more prominent ultrastructural features of the proximal tubule, distal tubule and glomerulus will be presented here. These characteristics were the criteria used for assigning the enzymatic reaction for ATPase to a given portion of the nephron.

To the naked eye, the kidney which has been freshly removed, is a glistening, reddish-coloured, bean-shaped structure. It is firm to the touch. A section through the kidney reveals a clear demarcation between the reddish-brown cortex and the paler medulla. Only the cortical structures were studied in this present investigation.

The proximal tubule (Pease, 1955; Porter and Bonneville, 1964; Bulger, 1965; Trump and Ericsson, 1965; Maunsbach, 1966a, 1966b; Tisher et al., 1966; Latta et al., 1967; Ericsson and Trump, 1969) constitutes most of the cortex. The epithelium of the tubule consists of a single layer of truncated pyramidal cells. In the apical portion of the cells bordering the lumen, the plasma membrane is thrown into folds to form numerous closely packed microvilli. These constitute the brush border (Figures 1, 18-19, 22, 24, 37, 40). An

electron-opaque, PAS positive layer of material, the glycocalyx, covers the plasma membrane of the microvilli (Trump and Ericsson, 1965; Latta et al., 1967; Ericsson and Trump, 1969). Within the microvilli, a core of electron-dense material is sometimes present. (Figure 19) Small tubular invaginations from the bases of the microvilli, vesicles and vacuoles of various sizes, are abundant in the apical cytoplasm (Figures 1, 18-19, 22, 24, 40). It is postulated that these invaginations, vesicles and vacuoles represent one pathway for tubular reabsorption of larger molecules (Porter and Bonneville, 1964; Latta et al., 1967; Ericsson and Trump, 1969).

In the basilar zone of the proximal tubule cells, deep infoldings of the plasma membrane divide the cytoplasm into numerous slender compartments within which mitochondria are enclosed. The processes from one cell interdigitate extensively with processes from adjacent cells (Figures 2, 18, 22-23, 40). As expected of these functionally active cells, a great number of mitochondria, closely associated with the infolded membranes of the basilar processes, are present. These mitochondria are large, elongated and possess numerous cristae. They are oriented perpendicular to the basement membrane (Figures 1, 23). At the lateral cell surface, there is also extensive interdigitating cytoplasmic processes. Some are small and are confined to the apical or basilar regions of the cell, whereas others extend from the apex to the base (Bulger, 1965). The brush border, basal and lateral interdigitations greatly increase the surface area of the cells and therefore, also the number of sites where enzymatic reactions can occur. A nucleus, organelles and inclusions are present within the cytoplasm. The basement membrane forms a continuous layer around the proximal tubule.

The epithelium of the distal tubule (Latta et al., 1967; Ericsson and Trump, 1969) is lower than that of the proximal tubule. The cells are cuboidal in shape. In the region of the nucleus, the cells may bulge into the lumen. The apical plasma membrane is not so highly differentiated structurally

as in the proximal tubule. There is no distinct brush border although frequently there are some small, short microvilli (Figure 38). A large number of vesicles may be seen in the apical cytoplasm. A prominent characteristic are the numerous basilar processes containing large and slender mitochondria, which may extend almost to the luminal surface (Figures 3, 38).

The fine structure of the glomerulus as seen under the electron microscope was described as early as 1953 by Hall. Since then there have been many more studies on the ultrastructure of the normal (Pease, 1955; Yamada, 1955; Hall, 1957; Porter and Bonneville, 1964; Jones, 1969; Simon and Chatelanat, 1969) and pathological (Simon and Chatelanat, 1969) glomerulus.

The three components of the glomerulus are the basement membrane, bounded on one side by the capillary endothelium and on the other by the visceral epithelium of Bowman's capsule (Figures 4, 20-21, 25, 39). The cytoplasm of the endothelium is extremely attenuated. These cytoplasmic prolongations possess round pores or fenestrae. It is only in the region of the nucleus that the endothelial cell projects into the capillary lumen.

The basement membrane, interposed between the endothelium and the epithelium, forms a continuous barrier in the filtration process. It is composed of three layers, the lamina rara externa, the lamina densa and the lamina rara interna. The dimensions of the three layers vary with the species. In the adult rat the lamina densa is quite thick and prominent (Figures 4, 21, 25, 39).

The visceral epithelial cells of Bowman's capsule, or better known as the podocytes, are highly specialised. The cells send out small cytoplasmic prolongations, the foot processes, which are apposed on the lamina rara externa of the basement membrane. The foot processes from one podocytic cell interdigitate extensively with those of adjacent cells thus giving rise to an intricate network. The foot processes from one podocyte may rest on the basement membrane of several capillaries (Figure 4). Conversely, each capillary may receive contributions from more than one podocytic cell. Recently, it

has been demonstrated that a coat of neutral and acid mucosubstances invests the plasma membranes of the foot processes as well as the podocytic cell bodies (Jones, 1969).

(b) Newborn kidney

The fresh, unfixed kidney of a newborn rat is small, pale and translucent. It is soft to the touch and its shape is easily deformed by applying pressure. During the first few weeks of life, the rat kidney grows through the formation of new nephrons in the nephrogenic zone of the cortex. Its weight increases seven-fold within the first ten days (Wachstein and Bradshaw, 1965).

In sections of the kidney cortex, the tubules and glomeruli are not closely packed together as in the adult kidney. Instead an abundant stroma of mesenchymal elements separates the tubules from each other and from the glomeruli. Tubules in various stages of differentiation are always present within the same specimen at any one time (Clark, 1957; Suzuki, 1958). This was found to be so even in a 1 hour-old kidney.

Some cortical tubules appear relatively undifferentiated (Figures 5-7, 11-12, 26-28, 34-35, 41). The cells are low and a large nucleus occupies most of the cell volume. The cytoplasm looks simple under the electron microscope. There are few organelles or membranous structures, with the exception of some small, round mitochondria randomly distributed throughout the cytoplasm. The mitochondria possess only a few cristae. The plasma membrane at the luminal surface is simple in contour or may be folded to form some short microvilli (Figures 6-7, 11-12, 27-28). An occasional cilium is present (Figure 35). The basilar membrane too, is not as complex as in the adult with few, if any, interdigitations. The lateral membrane between two adjacent cells is simple, or the beginnings of cytoplasmic interdigitations may be observed (Figures 5, 26-27). At this stage, it does not seem possible to distinguish between the various types of tubules characteristic of each segment of the nephron.

In the process of renal cellular differentiation, the simple and primitive epithelial cells are transformed into highly differentiated functional cells. The successive stages of this process has not been fully worked out yet (Du Bois, 1969). An essential component of renal cellular differentiation is the elaboration of cell membrane for the formation of the brush border in the proximal tubules, and the basal and lateral interdigitations in both the proximal and distal tubules. Among the differentiating tubules, the developing proximal tubule (Figures 8, 13-15, 30-32, 41, 44) is most easily identifiable by the presence of long, slender microvilli which may still be sparse or may be closely packed together. The brush border is acquired through the progressive accumulation of apical microvilli (Clark, 1957; Suzuki, 1958; Du Bois, 1969). It was observed that where a brush border was prominent, apical vesicles and vacuoles as well as a number of electron-dense granules were also present (Figures 8, 14-15, 30-32). There is an increase in the number of basal and lateral cytoplasmic interdigitations (Figures 8, 31-32). Simultaneously the mitochondria become regularly aligned perpendicular to the basement membrane within the basilar cytoplasmic compartments. Clark (1957) views the formation of the basilar infoldings as a result of progressive flutings of the cell membrane. Suzuki (1958) however, hypothesises that vesicles gather around the basilar portions of the cells and around the mitochondria. The vesicles coalesce to form small, flattened sacs which in turn come together as larger sacs wrapping around the mitochondria. By this process, the basilar cytoplasmic compartments are formed.

As with the cortical tubules, glomeruli in many stages of development are present within the same specimen (Figures 9-10, 16-17, 33, 36, 43). Some glomeruli are structurally immature (Figures 9, 16-17, 36)while others have the form of adult glomeruli although smaller in size (Figures 10, 33, 43). Du Bois (1969) describes the progressive stages of development of the glomerulus in the embryonic kidney. In its earliest form, a mass of podocytic cells, prismatic in shape

(Figures 9, 16, 36), denotes the region of the glomerulus. Further along in development, the apical pole of the podocytic cell containing the nucleus bulges out into Bowman's space, while the basal pole sends out cytoplasmic prolongations which differentiate into the foot processes (Figures 17, 33, 43). These increase in length and complexity and come to lie on the trilaminar basement membrane separating the podocytes from the endothelium. Concomitant with the development of the foot processes, the endothelial cytoplasm becomes attenuated and fenestrated, thus increasing the diameter of the capillaries (Figure 10).

B. EFFECT OF FIXATION ON ULTRASTRUCTURE

(a) Adult kidney

As early as 1955, Pease observed that small differences in the preparation techniques when applied to the kidney, could cause large variations in the morphology of the kidney tubular elements. Since then his observations have been substantiated (Maunsbach et al., 1962; Trump and Ericsson, 1965; Maunsbach, 1966a). The apical ends of the cells are especially labile. The proximal tubule cells show great sensitivity to fixation conditions and are affected both by the character of the fixative and by the method of application of the fixative solution.

In the present study, cortical tissue from the excised kidney was fixed by the immersion of small blocks in the fixative solution consisting of 5% glutaraldehyde buffered with 0.1 M sodium cacodylate. In general, the fixative used gave adequate preservation of the organelles in the cytoplasm. However, by this method of immersion in the fixative, some artifacts are present in the proximal tubules (Maunsbach et al., 1962; Maunsbach, 1966a; Tisher et al., 1966). The lumens of the proximal tubules were more often than not closed resulting in a region of closely packed microvilli (Figures 1, 18-19, 22, 37). In vivo, the lumens are found to be open

with a regular brush border, so that such collapsed tubules were artifactual resulting from excessive swelling of the cells during fixation (Maunsbach, 1966a). There may be some cellular debris in the lumens of the tubules. These take the form of cytoplasmic bits and pieces, or even whole, less osmiophilic cells that seem to be extruded into the lumen. An occasional tubule was not collapsed but possessed a patent lumen. Tisher et al. (1966) interprets this as due to dehydration in the preparative techniques for electron microscopy following cell swelling during fixation. Occasionally the membranes in the basilar part of the cells were separated giving rise to extracellular compartments of different sizes. The extent of the presence of extracellular compartments was not consistent (Figures 2-3, 18, 22-23, 38, 40). Variations between cells in the same tubule as well as variations between different tubules were observed. These extracellular compartments are probably indications of sensitive cellular reactions to physiological and pathological (in this case, fixation) changes in the environment.

The distal tubules and the glomeruli were more resistant to the effects of fixation and were always morphologically well preserved. The lumens of the distal tubules were open. There could have been some cell swelling resulting in a reduction in size of the lumen but this was not so obvious as in the proximal tubules where the brush border accentuated the effects.

(b) Newborn kidney

The same concentration of fixative and a similar method of fixation, that is, by the immersion of small tissue blocks, was applied to the newborn kidney. The effects of fixation varied depending on the stage of differentiation of the tubules.

The morphologically undifferentiated cortical tubular cells consisted of a nucleus, some cytoplasm and a few organelles, primarily small, round mitochondria with

sparse cristae, scattered throughout the cytoplasm. The mitochondria in the immature cells seemed more susceptible to fixation artifacts as compared with the adult. Quite a number of the mitochondria were "exploded". (Figures 27-28, 30-31, 38) Otherwise, the preservation of ultrastructure was generally good. The tubules had wide open lumens often filled with cellular debris. Debris was also found in the extratubular stroma. There appeared to be more cellular debris associated with the newborn kidney than with the adult kidney. In cross-sections of some undifferentiated tubules, all the cells but one, were well preserved. This one cell was completely disintegrated with a nucleus that was swollen to immense proportions (Figure 29). Such a phenomenon was not observed in adult kidney tissue.

Developing proximal tubules were recognised by the presence of long, slender microvilli in the apical surface membrane (Figures 8, 13-15, 30-32, 38). They may be few in number or may be closely packed to form a distinct brush border. In the tubules with few microvilli, the lumens were open but contained some cellular debris. In more advanced proximal tubules, that is, those having a well-developed brush border and a number of vesicles and vacuoles in the apical cytoplasm, the lumens were closed (Figures 8, 15, 31-32). Less osmiophilic cells were often seen being extruded into the lumen (Figure 8). The response of these tubules to fixation was very similar to that of the adult. The mitochondria were generally still small and showed some artifacts of fixation.

The glomeruli were always adequately preserved ultrastructurally independent of the stage of development.

C. EFFECTS OF FIXATION ON ENZYMATIC ACTIVITY

The histochemical localisation of ATPase enzymatic activity has most often been carried out on the adult kidney using the lead method at pH 7.2 proposed by Wachstein and Meisel (1957). Those investigators who used both the lead method at pH 7.2 (Wachstein and Meisel, 1957) and the calcium method at pH 9.4 (Padykula and Herman, 1955a, 1955b; Padykula and Gauth-

TABLE II

THE EFFECT OF FIXATION ON ENZYMATIC ACTIVITY

Age of animals	Time of fixation (hours)	Lead method pH 7.2			Calcium method pH 9.4		
		p	d	g	p	d	g
Adult	5	+	+	+	+	+	+
Adult	3	+	+	+			
3 days	5	+	+	+	+	+	-
24 hours	5	+	+	+	-	-	-
24 hours	3				+	+	+
12 hours	4				+	-	-
12 hours	2				+	+	+
2 hours	2	+	+	+	+	-	-
1 hour	1½				-	-	-
1 hour	1				-	-	-
1 hour	½				-	-	-
1 hour	¼				+	+	+

Fixative: 5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2.

\+ reaction precipitate present
\- reaction precipitate absent

p proximal tubules
d distal tubules
g glomeruli

NB. In newborn tissue, wherever it was not possible to differentiate between a developing proximal tubule and a developing distal tubule, it was assumed that the undifferentiated tubules represented both types.

ier, 1963) reported that similar results were obtained with either method (Novikoff et al., 1961). In the adult kidney, these observations on plasma membrane ATPase activity was found to be so. However, with immature newborn kidneys it was observed that the length of fixation affected the localisation of membrane ATPase activity differently depending on the method that was employed (See Table II).

The time of fixation was not important when dealing with adult kidney tissue. Even up to 5 hours fixation time there was much reaction precipitate associated with the membranes, using either the lead method at pH 7.2, or the calcium method at pH 9.4.

The time of fixation however, was more critical in the case of immature newborn kidneys, if the localisation for enzymatic activity was carried out at pH 9.4. A 2 hour-old kidney after 2 hours in the fixative solution still showed reaction product for the enzyme reaction at pH 7.2. But little or no precipitate was present at pH 9.4 with a similar time of fixation. It seemed that the more immature the kidney in terms of age postnatally, the shorter the time of fixation needed to preserve enough enzymatic activity to be demonstrable at pH 9.4 with the calcium method. Adequate amounts of enzymatic activity were always present to result in a positive reaction at pH 7.2. With the kidney of a 1 hour-old rat, even a ½ hour of fixation was sufficient to inhibit any enzymatic activity at pH 9.4 that might have been present in the tissue. With the fixative used (5% glutaraldehyde in 0.1M sodium cacodylate) good preservation of ultrastructure had to be sacrificed to prevent complete inhibition of any ATP-hydrolysing enzymes at pH 9.4.

D. ENZYMATIC ACTIVITY OF THE ADULT KIDNEY

(a) pH 7.2 - lead method

Under the electron beam, the final product of the ATPase enzymatic reaction was visualised as electron-dense

particles, which may be in the form of fine granules or in the form of larger aggregates. The sites of deposition of the reaction product would indicate areas of enzymatic activity.

The proximal tubules showed two distinct regions of enzymatic activity, the microvilli of the brush border and the interdigitations of the basal and lateral membranes (Figures 1-2). The precipitate was most often located on the outer surface of the plasma membranes of the microvilli. At times, but rather rarely, precipitate was associated with the inner cytoplasmic surfaces of the microvilli membranes. Precipitate could sometimes be demonstrated within some of the tubular invaginations arising from the bases of the microvilli, and within some of the small apical vesicles and large apical vacuoles. Some of the membrane interdigitations showed such an abundant deposition of reaction product that the space between the membranes was completely filled with precipitate. Where the reaction was less intense, the precipitate was found to be on the membrane itself and not free in the extracellular space (Figure 2). However, the reaction precipitate may be found on the cytoplasmic aspect of the membranes as well as within the cytoplasm (Figure 1). The basement membrane of some proximal tubules accumulated precipitate (Figure 2). Variations in staining intensity of the cells within the same tubule, as well as between different tubules was encountered. At times two tubules adjacent to each other, reacted differently. One tubule showed reaction precipitate in the brush border and basilar infoldings while the other showed little or no reaction precipitate.

In the distal tubules the reaction product was confined mainly to the cell membranes of the basilar interdigitating cytoplasmic processes and the lateral membranes separating two adjacent cells (Figure 3). As in the case of the proximal tubules, precipitate may be found on both the extracellular and cytoplasmic aspects of the interdigitating membranes and within the cytoplasm (Figure 3). Occasionally, the few short microvilli reacted positively and precipitate

was seen on the microvilli membranes.

The glomeruli were sites of active enzymatic activity (Figure 4). Abundant precipitate was present both on the membranes and within the cytoplasm of the foot processes, in the trilaminar basement membrane but not in the endothelium (Figure 4).

(b) pH 9.4 - calcium method

The results from the localisation of ATPase activity with the calcium method at pH 9.4 were similar to those obtained with the lead method at pH 7.2. Enzymatic activity was demonstrated in the brush border of the proximal tubules, in the lateral and basal interdigitations of the proximal and distal tubules, and in the glomerular epithelial cells.

E. ENZYMATIC ACTIVITY OF THE NEWBORN KIDNEY

(a) pH 7.2 - lead method (Figures 5-10)

With this method it was possible to localise enzymatic activity in the kidneys of newborn rats of various ages, ranging from a 2 hour-old to a 3 day-old. The time of prefixation in 5% glutaraldehyde-cacodylate did not appear to affect the level of enzymatic activity.

In a very immature kidney, for example from a 2 hour-old rat, most of the tubules were still undifferentiated and it was not possible to distinguish between a proximal tubule and a distal tubule (Figures 5-7). A large number of these undifferentiated tubules reacted intensely when incubated in the lead-ATP medium. The lateral membranes between neighbouring cells, which were more often simple in contour, were filled with the reaction product. In fact, the boundaries between individual cells in the tubules were clearly outlined by the deposition of the precipitate in the lateral membranes (Figures 5-7). Some precipitate was found on the plasma membranes lining the lumens of the tubules (Figure 6). Some of the microvilli that were present reacted positively while others reacted negatively in the incubating medium. At this early

stage of differentiation, the basal membranes did not show any elaborate infoldings as seen in the adult kidney (Figures 5-7). There was usually no precipitate on the basal membranes (Figures 5-6). Sometimes, however, a single cell within a tubule showed abundant precipitate on the basal membranes (Figures 5, 7). This was an exception.

Besides the undifferentiated tubules, tubules in various stages of differentiation were also present within the newborn kidney. A developing proximal tubule possessed a fairly well-developed brush border and many more interdigitations of the basal and lateral membranes (Figure 8). These interdigitations were still by no means as extensive as in the adult. Reaction precipitate was found encrusting the plasma membranes of the microvilli in the brush border (Figure 8). Occasionally some precipitate was present within the microvilli. The basal infoldings had fine precipitate adhering to the membranes. Usually the space between the membranes was not filled up with precipitate as was the case with the undifferentiated tubules (Figure 8).

Developing distal tubules showed an increase in the number of short, irregularly shaped microvilli. The reaction precipitate generally coated the membranes of the microvilli but was also seen within the cytoplasm of the microvilli. The lateral and basal interdigitations were frequently so packed with precipitate that it was impossible to tell if the precipitate was just confined to the outer surface of the membranes, or was also present on the inner cytoplasmic surface as well.

As with the tubular elements in the kidney, immature undifferentiated glomeruli were present together with more well-developed glomeruli within the same specimen. In the undifferentiated glomerulus, the podocytic cells were closely packed so that their limiting membranes were quite often in apposition (Figure 9). Some podocytes showed the beginnings of cytoplasmic prolongations which would eventually develop to form the foot processes. It was observed that re-

action product was almost always present where two sets of membranes were apposed.(Figure 9). Portions of the membranes of the podocytic cell which was not close to the membranes of another podocytic cell sometimes showed precipitate but this was not usually so. Not all the cells in the same glomerulus were reactive. Some cells showed no detectable enzymatic activity.

In a glomerulus further along the course of differentiation, the foot processes of the podocytic cells had increased in number and formed a complex network along the trilaminar basement membrane. The cytoplasm of the endothelial cell was becoming attenuated and fenestrated but not to such a great extent as in the adult (Figure 10) Reaction precipitate was found on the membranes as well as within the cytoplasm of the foot processes and podocytic cell body, which was still situated quite near to the capillaries. The endothelial cells were usually non-reactive. If precipitate were present, it was found mainly on the cytoplasmic surface of the membranes and in the cytoplasm (Figure 10).

(b) <u>pH 9.4 - calcium method</u> (Figures 11-17)

For ATPase enzymatic activity in a newborn kidney to be demonstrable with this method, the time of pre-fixation in glutaraldehyde was found to be critical (Refer to Table II).

A 3 day-old kidney fixed for 5 hours prior to incubation showed enzymatic activity in the proximal and distal tubules but not in the glomerulus. In the proximal tubule, precipitate was found all along the infoldings and also coating the microvilli. Quite a number of the apical tubular invaginations from the bases of the microvilli were lined with the fine precipitate (Figure 15). The lateral and basal interdigitations of the distal tubule gave positive results.

A 24 hour-old kidney pre-fixed for 5 hours showed no reaction precipitate. But if the time of fixation was shortened to 3 hours, there were indications of enzymatic activity in the tubules. In the glomerulus, reaction product

was sometimes present and sometimes not. If present, it was localised on the plasma membranes of the podocytic processes.

Thus by controlling the times of fixation it was possible to localise ATPase enzymatic activity in the tubules and glomeruli of the newborn kidney. In cases where there was just minimal activity, for example, a 12 hour-old kidney with 4 hours pre-fixation, or a 2 hour-old kidney with 2 hours pre-fixation, or sometimes a 1 hour-old kidney with 1 hour pre-fixation, reaction precipitate was observed mostly in the tubular elements. The precipitate, in the form of discrete electron-dense particles, were found on the membranes of the few microvilli and sometimes on the lateral membranes (Figures 11-12). Where the reaction was more intense, for example, a 12 hour-old kidney with 2 hours pre-fixation, or a 1 hour-old kidney with $\frac{1}{4}$ hour pre-fixation, the sites for the deposition of the reaction product for ATPase at pH 9.4 were similar to those at pH 7.2 (Figures 13-14). Some tubules reacted positively while others reacted negatively. This was not dependent on the degree of maturation of the tubules. In the immature tubules, the precipitate in the lateral membranes clearly separated one cell from the next. Some precipitate was also present on the relatively simple plasma membrane lining the luminal surface. The brush border of the developing proximal tubules showed varying amounts of precipitate. Some of the apical vesicles and vacuoles had reaction product on their limiting membranes (Figures 14-15). In developing distal tubules, reaction precipitate was observed mainly in the lateral membranes even though some basal infoldings and some microvilli were present. Some of the tubules showed precipitate in the region of the basement membrane but this did not appear to be associated with the basal membranes. Observations on the enzymatic activity of the glomerulus at pH 9.4 were similar to those at pH 7.2 (Figures 16-17). Glomerular enzymatic activity appeared to be more susceptible to fixation effects. With longer fixation times, enzymatic activity in the glomerulus was inhibited the most.

F. EFFECT OF THE MODIFIERS, PHMB AND L-CYSTEINE ON ENZYMATIC ACTIVITY

(a) Adult kidney

TABLE III

THE EFFECT OF THE MODIFIERS, PHMB AND L-CYSTEINE ON ENZYMATIC ACTIVITY OF THE ADULT KIDNEY

	PHMB						L-cysteine					
	No			Yes			No			Yes		
	p	d	g	p	d	g	p	d	g	p	d	g
pH 7.2	+	-	+	+	-	+	+	+	+	+	+	+
pH 9.4	+	-	+	+	-	-	+	+	+	+	-	-

\+ reaction precipitate present
\- reaction precipitate absent

p proximal tubules
d distal tubules
g glomeruli

No no pre-incubation exposure
Yes with pre-incubation exposure

At pH 7.2, with or without a pre-incubation exposure to PHMB, enzymatic activity was localised mainly at the proximal tubules (Figures 18-19) and glomeruli (Figures 20-21). Within the proximal tubules, the precipitate was

present in the brush border as well as in the interdigitations (Figure 18). In the brush border the precipitate was usually found uniformly distributed over the membranes of all the microvilli, but were at times clumped together in certain regions within the brush border (Figure 19). This was interpreted as an artifact rather than as an indication of heterogeneity of sensitivity to the effects of PHMB among the microvilli. Variations in the intensity of reaction at the infoldings occured between individual cells and between different tubules (Figure 18). Only the membranes of the podocytic foot processes showed an accumulation of the reaction product. There was no precipitate in the cytoplasm of these cells as was often noticed with tissue incubated without PHMB. There was also no precipitate in the basement membrane or endothelium (Figures 20-21).

At pH 9.4, enzymatic activity was demonstrable in the proximal tubules whether there was a pre-incubation exposure to PHMB or not (Figures 22-24). In either experimental conditions, the distal tubules showed no reaction product. There was an occasional distal tubule with precipitate in the interdigitations. However, in the glomerulus a difference was observed between tissue that had been pre-incubated with PHMB and those that had not. Without pre-incubation, some of the glomeruli showed precipitate in the cytoplasm and on the membranes of the podocytic foot processes but not in the basement membrane or endothelium (Figure 25). With pre-incubation, most of the glomeruli showed no reaction product. This observation substantiated Glenner's (1968) suggestion that any modifier should be used both before and during incubation. This would ensure that the modifier had reached the site of enzymatic activity prior to incubation in the substrate medium.

L-cysteine had no effect on the localisation of enzymatic activity at pH 7.2. But at pH 9.4, a pre-incubation exposure to L-cysteine inhibited practically all enzymatic activity in the glomeruli and in most of the distal tubules.

(b) Newborn kidney

TABLE IV

THE EFFECT OF THE MODIFIERS, PHMB AND L-CYSTEINE, ON ENZYMATIC ACTIVITY OF THE NEWBORN KIDNEY

	PHMB				L-cysteine			
	No		Yes		No		Yes	
	t	g	t	g	t	g	t	g
pH 7.2	+	+	+	+	+	+	+	+
pH 9.4	-	-	-	-	-	-	-	-

\+ reaction precipitate present
\- reaction precipitate absent

t tubules
g glomeruli

No no pre-incubation exposure
Yes with pre-incubation exposure

At pH 7.2, neither PHMB nor L-cysteine had any effects on the enzymatic activity of the differentiating tubules (Figures 26-32, 34-35) and glomeruli (Figures 33, 36). Most of the tubules showed intense reaction in the lateral membranes between cells (Figures 26-27, 30-32, 34-35). The luminal surface membrane and microvilli, if developed, showed variations in the amount of precipitate (Figures 30-32).

In developing proximal tubules, the brush border was a distinctly active enzymatic site (Figures 30-32). The glomerulus had precipitate associated especially with membranes which were in apposition (Figures 33, 36).

At pH 9.4, no enzymatic activity was detected in the newborn kidney when PHMB and L-cysteine were used. However, these negative results cannot be considered too significant at the present time. With a newborn kidney a couple of hours old, the inhibitory effects of the fixative used were found to be so overwhelming that it was impossible to assess the effects of the modifiers.

G. CONTROLS

In all the experiments, controls were run simultaneously with the experimental specimens. The controls differed only in that ATP was omitted from the incubating media.

With the calcium method at pH 9.4, no precipitate was found in either adult or newborn tissue.

With the lead method at pH 7.2, there was a tendency for some precipitation in some cells (Figures 40, 44) but not in others (Figures 37-39, 41-43). In adult kidney tissue, this took the form of fine stippling of the cytoplasm, or association of the precipitate with the basement membrane or microvilli of the brush border (Figure 40). In the newborn kidney, a fine diffuse precipitate was sometimes present. If the microvilli were long and slender, as in the developing proximal tubule, there was some precipitate on the membranes (Figure 44).

These observations were not consistently present in all the specimens. If precipitate was present in the control specimens, it was always less than in the experimental situation.

H. NUCLEAR STAINING

In both experimental and control specimens, nuclear staining was found to be erratic. Variations occured between

individual cells in the tubules and in the glomeruli. Some nuclei were perfectly free of precipitate while others showed a large amount. Nuclei from two adjacent cells often behaved differently, with precipitate present in the one but not in the other. Wherever precipitate was observed, it was mainly associated with the nucleolus and with the denser heterochromatin (Figures 10, 27). Even then there were differences in intensity. Nuclear staining did not appear to follow any consistent pattern.

IV

DISCUSSION

A. GENERAL

Both the lead method at pH 7.2 (Wachstein and Meisel, 1957) and the calcium method at pH 9.4 (Padykula and Herman, 1955a, 1955b; Padykula and Gauthier, 1963) for the histochemical localisation of ATPase activity have been used widely to demonstrate enzymatic activity in various tissues where active transport is known to occur. Recently, the specificity of the histochemical localisation of ATPase enzymatic activity with the Wachstein-Meisel procedure has been seriously questioned (Moses et al., 1966; Rosenthal et al., 1966; Moses and Rosenthal, 1967, 1968; Rosenthal et al., 1969). The possibility that there is non-enzymatic hydrolysis of the substrate ATP by the lead in the incubating medium was proposed. It is thought that this non-enzymatic hydrolysis of ATP by lead could account for the deposition and localisation of precipitate on plasma membranes, the most often observed sites of intense reaction with the lead method. Moses and Rosenthal (1968) suggest that there is "a selective affinity of certain tissue-reactive groups at the sites of localisation for the complexes formed by the interaction of lead and ATP." At the present time it is difficult to envision how this tissue factor with a selective affinity for the products of the non-enzymatic hydrolysis of ATP could account for the substrate specificities of many plasma membranes (Novikoff, 1967), the effects of modifiers only on some membranes (Novikoff, 1967) and the differing patterns of sites of localisation of precipitate obtained with the same tissue under varying conditions. Marchesi (1968) in his investigations of ATPase activity on red blood cell membranes found that there was non-enzymatic hydrolysis of ATP in the incubating medium but that it did not account for the deposition of the lead phosphate on the red blood cell membranes. Jacobsen and Jorgensen (1969) found

the staining of the plasma membranes of the kidney characteristic of enzymatic hydrolysis rather than non-enzymatic hydrolysis, while Grossman and Heitkamp (1968) found no measurable non-enzymatic hydrolysis of ATP by lead in chemical assays of the media used for the histochemical localisation of ATPase activity.

We are far from an understanding of the complexities of the reactions involved in the reaction mixtures used in histochemistry, especially where a heavy metal like lead is present. It is probable that the various constituents of the incubating media interact with each other. There is a suggestion that lead in the incubating media may not be in the form of free ions but may form chelates (Tetas and Lowenstein, 1963; Berg, 1964; Tormey, 1966; Rechardt and Kokko, 1967; Moses and Rosenthal, 1968; Tice, 1969). This would then imply that the reaction precipitate on tissue sections may not be just simple lead phosphate but may be a more complex compound (Marchesi, 1968; Moses and Rosenthal, 1968). It is essential that variables of the reaction be carefully controlled. In the present study of ATPase enzymatic activity in both the adult and newborn kidney tissue, the composition of the incubating media were kept constant so that the significance of any differences in the sites of deposition of the reaction precipitate could be assessed. Also a method not involving the use of lead salts was deemed desirable as a comparison (Goldfischer et al., 1964). Therefore both the lead and calcium methods were applied in the investigation of ATPase enzymatic activity in adult and newborn kidney tissue.

Under the conditions employed in this present study, only plasma membrane ATPase was demonstrable. Mitochondrial ATPase was not detected although there would be an occasional mitochondrion with reaction precipitate associated with it. Fixation in glutaraldehyde has been found to have an inhibitory effect on mitochondrial ATPase (Torack and Barrnett, 1963; Lazarus and Barden, 1964; Wachstein and Besen, 1964; Essner et al., 1965; Vethamany and Lazarus, 1967; Anderson, 1968).

1968). Fresh unfixed tissues are of course ideal for demonstrating any ATPase activity, including mitochondrial ATPase, but this is not always possible at the level of the electron microscope. Fixation in formalin preserves mitochondrial ATPase enzymatic activity in some tissues (Lazarus and Barden, 1962; Wachstein and Bradshaw, 1962; Ashworth et al., 1963; Bradshaw et al., 1963; Otero-Vilardebo et al., 1963; Essner et al., 1965; Lazarus and Vethamany, 1965; Rechardt and Kokko, 1967; Vethamany and Lazarus, 1967; Ogawa and Mayahara, 1969) but not in others (Wachstein et al., 1960; Wachstein and Bradshaw, 1962; Barden and Lazarus, 1963; Otero-Vilardebo et al., 1963; Wachstein and Besen, 1964; Gauthier, 1967). It appears that mitochondria from different tissues show different susceptibilities towards fixation. Where mitochondrial ATPase has been localised, it is still not fully agreed upon whether the precipitate is on the inner cristal membranes (Ashworth et al., 1963; Otero-Vilardebo et al., 1963; Anderson, 1968; Marchesi, 1968) or within the matrix (Lazarus and Barden, 1962, 1964; Lazarus and Vethamany, 1965; Rechardt and Kokko, 1967; Vethamany and Lazarus, 1967; Grossman and Heitkamp, 1968; Ogawa and Mayahara, 1969) of the mitochondria. To further complicate the issue of mitochondrial ATPase, a recent paper suggests that mitochondria from different tissues may have different affinities for lead salts (Wilson, 1969).

There are three possible explanations to bear in mind when considering the localisation of reaction precipitate at the plasma membranes of kidney tubular cells and glomerular cells. Firstly, fixation of the kidney tissue in glutaraldehyde could alter the cell membranes in such a way that they act as barriers to substances entering the cells. This being the case, enzymes present within the cytoplasm and in the cell organelles would diffuse towards the membranes and react with the substrate in the incubation medium, that is ATP, thereby releasing the reaction precipitate at the extracellular aspect of the cell membranes. This seems unlikely in view of the experimental results presented here. Although reaction

precipitate is most frequently observed on the extracellular aspects of the plasma membranes (Figures 2, 5-9, 11-18, 20-23, 26-27, 30-36), it is also present within the cytoplasm (Figures 1, 3, 4, 10, 25, 36), in the nuclei (Figures 10, 27) and occasionally within the mitochondria. It appears that substances from the substrate medium can enter the cells. It is entirely possible that fixation has altered the membranes in some way.

Secondly, in undifferentiated tubules of the newborn kidney intense reaction precipitate is present on the lateral membranes between individual cells. In the glomeruli, apposed membranes show an accumulation of reaction product. It is suggested that the enzymes on the luminal surface membranes and basal membranes of the tubular cells, and on the free membranes of the podocytic cells may not be so firmly attached to the membranes and are therefore "washed" towards the apposed membranes. If this were the case, then one would expect gradients in the intensity of the deposition of the reaction product. This was not observed. Occasionally, there would be intense reaction precipitate on the luminal surface membranes (Figure 27) or on the basal membranes (Figure 5) or on the free membranes of the podocytes (Figures 16, 36).

Thirdly, the localisation of reaction precipitate on the membranes could indicate ATPase enzymatic activity at the membranes themselves, as suggested here. In view of the above, it seems most likely that actual plasma membrane enzymatic activity was demonstrated in the present experiments.

B. PLASMA MEMBRANE ENZYMATIC ACTIVITY

(a) Adult kidney

With both the lead and calcium methods for the histochemical localisation of ATPase enzymatic activity, the reaction precipitate was deposited on the plasma membranes of the proximal and distal tubules and glomeruli. Variations in intensity of staining were present. Besides, some tubules and glomeruli showed the presence of the react-

ion product while others did not. These similar observations had been made previously by Wachstein and Besen (1964). This variability has been interpreted as artifactual (Goldfischer et al., 1964) although other possible explanations could also be offered. At the present stage of development of histochemical techniques, quantitation of enzymatic activity can only be based on the density of the final reaction product deposited (Glenner, 1965). Therefore variations in staining intensity found in the adult kidney when incubated for an ATPase reaction would indicate various degrees of enzymatic activity. Different nephrons at any one time may be in different functional states (Caulfield and Trump, 1962). In the proximal tubules, differences in the intensity of the ATPase reaction could indicate differences in enzymatic activity associated with the various segments. It has been found that proximal tubules show segmentation in terms of their ultrastructure, function and histochemical reactions (Kissane, 1961; Maunsbach, 1966b; Tisher et al., 1966; Jacobsen et al., 1967; Latta et al., 1967; Ericsson and Trump, 1969). In the present study, the differences in the deposition of the reaction product were not correlated with the various segments of the proximal tubule.

The main purpose, of course, of applying histochemical methods for the demonstration of enzymatic activity to the kidney, is not only an attempt to localise the sites of enzymatic activity in terms of the ultrastructure of the tissue, but also to try to correlate specific sites of enzymatic activity with the known functions of the tissue. Unfortunately for most enzymes in the kidney, including ATPase, we have no specific idea of the role they play in kidney function. Most of the suggestions that have been put forward for the roles of ATPase in kidney function are highly speculative. Such speculations, based on available data, are useful and may lead to further experiments which might help elucidate some of the complexities of kidney function.

Proximal tubules show localisation of reaction product to the membranes of the brush border, the basal and

the lateral interdigitations. The ATPase reaction in these sites was not eliminated by the addition of L-cysteine or PHMB to the incubating media indicating that the enzyme demonstrated was not sulfhydryl-dependent (Padykula and Herman, 1955a, 1955b). L-cysteine served the dual purpose of being a source of sulfhydryl groups as well as an inhibitor of alkaline phosphatase, which enzyme has been found to be also present in the brush border. In this investigation L-cysteine had no effect on brush border enzymatic activity, in agreement with the findings of Padykula and Herman (1955b) but not with the findings of Freiman and Kaplan (1959) where brush border activity was abolished by L-cysteine. This discrepancy is probably due to species differences (Wachstein and Besen, 1964) in enzymatic activity of the kidney. Padykula and Herman (1955b) examined kidneys of rats, while Freiman and Kaplan (1959) used those of dogs. In all probability, both alkaline phosphatase and ATPase are present in the brush border of the rat kidneys studied here.

The precise function of the ATPase associated with the brush border is not entirely clear but it is most likely involved in the transport of substances from the tubular lumens into the cells and vice versa in the process of urine formation from the glomerular filtrate. It is an energy-requiring process involving the movement of substances up an electrochemical potential gradient. This, by definition, is "active transport" (Solomon, 1962). Not only ATPase (Spater et al., 1958; Wheeler and Whittam, 1964; Ericsson and Trump, 1969) but also alkaline phosphatase (Wilmer, 1944; Rosenberg and Wilbrandt, 1952; Kissane, 1961; Matthiessen, 1966) have been implicated to take part in active transport across membranes. In much of the literature on the histochemical localisation of ATPase in various tissues, there is a tendency to consider the ATPase so localised as associated only with the sodium pump (Solomon, 1962; Post and Sen, 1965). This ATPase is sodium-potassium activated and ouabain-sensitive. There is much controversy as to whether the sodium-potassium activ-

ated ATPase is demonstrable at all with the present available histochemical techniques (Bonting et al., 1962; McClurkin, 1964; Tormey, 1966). The present author takes a more general view as to the nature of the ATPase or ATPases shown by the lead and calcium methods, especially with respect to the kidney. Besides participating in the active transport of cations like sodium and potassium, it perhaps also participates in the active transport of anions like phosphate and sulfate, sugars, amino-acids and fatty acids (Loewy and Siekevitz, 1963). It is not known whether there is a common mechanism underlying the active transport of all these substances and therefore involving one common ATPase, or whether a number of ATPases are working in concert. The latter suggestion seems more plausible.

In the brush border of the proximal tubules, small molecules presumably enter the cells by active transport across the plasma membranes. Larger molecules, colloidal materials and proteins probably enter by way of pinocytosis. The substances which are to be absorbed stream towards the bases of the microvilli where vesicles and vacuoles of various sizes are pinched off. This form of membrane flow probably requires energy (Loewy and Siekevitz, 1963) and therefore an ATP-dephosphorylating enzyme to make the energy available. Ericsson (1965a, 1965b) in his studies of the transport and digestion of hemoglobin, found that the hemoglobin was rapidly pinocytosed and metabolised. Droplets containing hemoglobin appeared all below the brush border. Four hours after the intravenous injection of hemoglobin, the brush border of the proximal tubules was abolished. Ericsson postulated that the brush border membrane was being used up for absorption droplets and that membrane renewal was not keeping pace with membrane loss. Under physiological conditions, membrane renewal and loss would be balanced. Renewal of the plasma membrane of the brush border is conceivably an energy-requiring process with an associated ATPase.

The basilar and lateral interdigitating membranes tremendously increase the surface area of the cell in contact

with the extracellular space. There is a large number of mitochondria closely apposed to these membranes where transport processes can occur. These membranes show an intense ATPase reaction. It has been observed that the extracellular spaces widen in response to conditions of fixation and also to various solutions injected intravenously (Caulfield and Trump, 1962) by increased water fluxes. The ATPase in the basilar regions of the proximal tubule cells presumably transport large amounts of salts and water.

Where little correlation between structure, function and enzymatic activity in the proximal tubules is possible, there is an even lesser possibility in the distal tubules. The ATPase in the distal tubules appears to be sensitive to PHMB at pH 7.2 and pH 9.4 but also sensitive to L-cysteine at pH 9.4. The significance of this is not known. The functions postulated for the distal tubules include active transport of sodium, potassium secretion, acidification of the urine and ammonia secretion (Ericsson and Trump, 1969). A precise correlation is not possible at the present time.

In the glomerulus, enzymatic activity was localised primarily on the plasma membranes of the podocytic foot processes at pH 7.2 and pH 9.4. Other investigators have found reaction on the endothelium alone (Kaplan and Novikoff, 1959), or on both the endothelium and epithelium (Ashworth et al., 1963; Wachstein and Besen, 1964). The ATPase in the glomerulus responded to both PHMB and L-cysteine. This could just be an indication of the glomerulus' sensitivity to any foreign substances. It was once thought that the foot processes had only a supportive function in the glomerulus and that substances once past the trilaminar basement membrane, flowed in between the filtration slits set up by the interdigitating foot processes. There is now a suggestion that transport processes do occur across the glomerular epithelial membranes especially in relation to resorption of proteins that may have filtered across the endothelial pores and basement membrane (Jones, 1969).

The author postulates that in the adult rat kidney, at least two types of ATPases are localised with the lead and calcium methods. One enzyme has a pH optimum at 7.2 and the other has an optimum at 9.4. These two types of enzymes show differences in response to the modifiers PHMB and L-cysteine. The enzyme with an optimum at pH 9.4 appears to be more sensitive to the effects of modifiers. Unlike the four types of ATPases in the muscle which can be spatially separated in terms of their pH optima and their response to various activators and inhibitors (Gauthier, 1967), those in the kidney are both localised at the plasma membranes. This postulate, of course, is highly speculative.

(b) Newborn kidney

In the rat, the newborn kidney is not only morphologically but also functionally immature, as compared to the adult. The newborn kidney is an inefficient homeostatic organ and cannot tolerate changes in acid and base intake (Wacker et al., 1961; Moog, 1965). The newborn rats cannot handle changes in hydration (Pinkstaff et al., 1962) which is especially evident if they are overloaded with water (Wachstein and Bradshaw, 1965). Rates of glomerular filtration, urea clearance, absorption of glucose and accumulation of vital dyes are low when compared with those of the adult (Wachstein and Bradshaw, 1965). This functional inefficiency can generally be correlated with a less than full complement of enzymes. With morphological development, the enzymes for physiological function appear and/or increase in amount. Enzyme accumulation can be taken as one aspect of functional differentiation (Pinkstaff et al., 1962).

That there is morphological immaturity in the newborn kidney is quite obvious from a study of a tissue section from a newborn kidney. Most of the tubular elements are undifferentiated. The extent of the plasma membrane surface area available for the transport of substances from the glomerular filtrate is small. There is no brush border, or if

present, only in a relatively undeveloped form. The interdigitations of the basal and lateral membranes are scarce. The glomeruli too, are mostly morphologically undifferentiated. The endothelium possess few fenestrations and the glomerular epithelial cells have few foot processes. Concomitant with the observations of morphological immaturity is the observation that the enzyme ATPase is relatively less abundant. In the tubules, enzymatic activity was noted mainly on the lateral membranes in between individual cells. There was no great accumulation of reaction precipitate on the basal membranes or luminal surface plasma membranes, undifferentiated morphologically though they may be, as in the adult. Only with the differentiation of these membranes, as in a developing proximal tubule, is there accumulation of reaction product, and therefore an indication of increased enzymatic activity. The weak reaction for ATPase enzymatic activity on the luminal surface membrane and few microvilli of the undifferentiated tubules would indicate some transport of substances from the tubular lumens into the cells. The shortest possible route for substances out into the extracellular space would be via the lateral membranes, where thereis intense reaction for ATPase enzymatic activity. These observations do not agree with those of Wachstein and Bradshaw (1965) who found no tubular ATPase in the newborn kidney, even in the more mature developing proximal tubules. However, Pinkstaff et al. (1962) showed ATPase activity in all fetal and post-natal stages. The endothelium in the immature glomerulus often showed deposition of reaction product. Possibly active transport occurs across the endothelium of the newborn glomerulus to compensate for the paucity of fenestrae through which glomerular filtrate can pass. The glomerular epithelial cells showed much reaction precipitate associated with the plasma membranes which were in apposition with other membranes, thus probably delineating the pathway for glomerular filtrate from the capillaries to Bowman's space.

As with the adult, it is postulated that there are

probably at least two types of enzymes present in the newborn kidney with pH optima at 7.2 and 9.4. At birth, the ATPase active at pH 7.2 is probably abundant and stable and therefore more resistant to fixation effects. At pH 9.4, the effects of fixation on enzymatic activity of the newborn kidney were very apparent. We can only speculate as to why this is so. This enzyme with a pH optimum at 9.4 could be only present in small amounts, or was unstable, or was in an inactivated form, or a combination of all these three possibilities. With a diminished total plasma membrane surface area, it is conceivable to have a diminished amount of enzymes associated with it. During development enzymes may change from the inactive form to the active form as well as change in stability (Moog, 1965). The increase in the quantity of enzymes demonstrable histochemically could result from protein synthesis (Priestly and Malt, 1968) or an activation of enzymes previously in an inactive form.

C. SUMMARY OF ENZYMATIC AVTIVITY OF THE ADULT AND NEWBORN KIDNEY

The functional capacity of both the adult and newborn kidney can be correlated with the extent of its morphological differentiation and with the amounts of enzymes present. The adult kidney maintains body homeostasis efficiently. The tubular elements in the cortex and the glomeruli are ultrastructurally complex. The amount of ATPase enzymatic activity demonstrable histochemically is considerable. The enzymes appear to be highly stable and are not affected by long periods of pre-fixation in glutaraldehyde prior to incubation in the ATP substrate medium. In the proximal tubules, the reaction precipitate is localised on the membranes of the brush border microvilli, the limiting membranes of some of the apical vesicles and vacuoles, and the membranes of the basal interdigitations. The brush border ATPase is probably related to the active transport across plasma membranes of small molecules from the tubular lumens into the

cells and vice versa (Loewy and Siekevitz, 1963). Larger molecules, colloidal materials and proteins perhaps enter the cells by pinocytosis. Some of the tubular invaginations from the bases of the microvilli and some of the apical vesicles and vacuoles sometimes show enzymatic activity. These cellular components associated with pinocytosis might represent a form of membrane flow within the cell which is energy-requiring (Loewy and Siekevitz, 1963). The basal infoldings divide the cytoplasm into numerous compartments within which are contained large, elongated mitochondria. These basal interdigitations are highly sensitive to conditions of fixation and to solutions injected intravenously (Caulfield and Trump, 1962). It is suggested that the ATPases in the basilar parts of the proximal tubule cells are associated with the transport of salts and water. In the distal tubules, it is difficult to correlate the ATPase enzymatic activity present on the basilar infolding membranes with some of the postulated functions of the distal tubules, for example, transport of sodium, secretion of potassium and ammonia, and acidification of the urine (Ericsson and Trump, 1969). In the glomerulus, reaction precipitate is observed on the podocytic foot processes. Besides having a mechanical supportive function, the podocytic cells probably actively resorb proteins that have filtered through the endothelial fenestrae and basement membrane (Jones, 1969).

The newborn kidney, as compared with the adult kidney, is functionally immature, morphologically undifferentiated and enzymatically less adequately endowed (Wacker et al., 1961; Pinkstaff et al., 1962; Moog, 1965; Wachstein and Bradshaw, 1965). Enzymatic activity that is demonstrable histochemically is localised on the lateral membranes between individual cells of the undifferentiated tubules, on the apposed membranes of podocytic cells and sometimes in the endothelium. The presence of these enzymes would show that the newborn kidney, though still immature, is capable of carrying out many of the functions essential for maintaining homeo-

stasis. In the undifferentiated tubules there is no brush border with its complement of enzymes. This would perhaps explain, partially, the low rate of glucose absorption in the newborn kidney (Wachstein and Bradshaw, 1965). Newborn rats cannot eliminate excess water from their bodies as efficiently as the adults (Pinkstaff et al., 1962; Wachstein and Bradshaw, 1965). If water is mainly transported across the membranes of the basilar interdigitations of the proximal tubules cells, then the lack or paucity of basilar infoldings with the corresponding absence of enzymatic activity, would account for the decreased functional capacity in terms of water movement.

For both the adult and the newborn kidneys, it is postulated that there are at least two types of ATPases localised at the plasma membranes with pH optima of 7.2 and 9.4. In the adult, the enzymes appear to be unaffected by prefixation in glutaraldehyde. In the newborn however, the enzyme active at pH 9.4 seems to be sensitive to the length of prefixation.

D. NUCLEAR STAINING

The significance of nuclear staining in tissues incubated for the ATPase enzymatic reaction is still in abeyance. Nuclear staining is frequently observed (Padykula and Herman, 1955a; Novikoff et al., 1958; Holt, 1959; Pinkstaff et al., 1962; Sandler and Bourne, 1962; Wachstein and Bradshaw, 1962; Ashworth et al., 1963; Deane, 1963; Tewari and Bourne, 1963a, 1963b; McClurkin, 1964; Wachstein and Besen, 1964; Klein, 1966; Moses et al., 1966; Yasuzumi and Tsubo, 1966; Jacobsen and Jorgensen, 1969). In most instances, as with the author's observations, nuclear staining is erratic and inconsistent and is considered as artifactual rather than as a manifestation of enzymatic activity. The mechanism of nuclear staining is not clear but the most commonly given reason is an affinity of nuclei for lead and calcium phosphates (Ashworth et al., 1963; Deane, 1963; Moses et al., 1966). In some instances, investigators have found a consistent

pattern of nuclear staining. Pinkstaff et al. (1962) observed nuclear staining in the proximal tubules in all fetal and post-natal stages, and an increasing nuclear staining in distal tubules. Sandler and Bourne (1962) could produce or cause to vanish nuclear staining by varying the magnesium sulfate concentration in the incubating media. Others have found nuclear staining to be dependent on the concentration of ATP and of lead in the medium (Padykula and Herman, 1955a; Novikoff et al., 1958; Moses et al., 1966). Yasuzumi and Tsubo (1966) by modifying the histochemical method used could localise reaction precipitate in the region of the nuclear pores. Some speculations as to the function of this nuclear ATPase have been put forward. This ATPase, if present, would hint at a metabolic interaction between the nuclei and the cytoplasm (Sandler and Bourne, 1962; Tewari and Bourne, 1963a, 1963b; McClurkin, 1964; Yasuzumi and Tsubo, 1966).

E. CONTROLS

In practically all control specimens, no precipitate was observed. But occasionally there was precipitate associated with the brush border of the proximal tubules (Persijn et al., 1961; Wachstein and Besen, 1964) when the method of Wachstein and Meisel (1957) was used. The precipitate is electron-dense but the nature of the composition of the precipitate is not at all clear. In the present study, it was observed that there was a tendency for accumulation of precipitate to occur as the microvilli developed in length and in complexity. Perhaps with differentiation, the microvilli membranes acquire the glycocalyx coat which has an affinity for the precipitates formed as a result of the interaction between the various components of the incubating media. There does not appear to be this selective affinity for precipitates on the glomerular podocytic cell membranes where a coat of mucosubstances is also present (Jones, 1969).

ILLUSTRATIONS

Figures

ABBREVIATIONS

L	lumen
C	capillary
N	nucleus
M	mitochondrion
E	parietal epithelium
Et	erythrocyte
p	podocyte
f	podocytic foot process
a	small apical vesicle
v	large apical vacuole
t	tubular invagination
m	microvillus
b	brush border
e	endothelium
bm	basement membrane

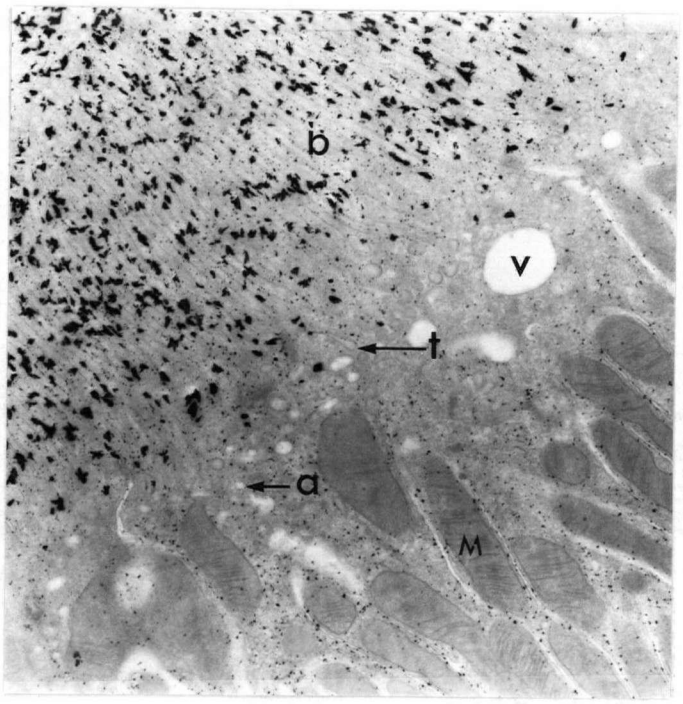

Figure 1

5 hours pre-fixation in glutaraldehyde.
A proximal tubule. The brush border (b) of the proximal tubule from an adult kidney is extensive. In response to the mode of fixation, the microvilli are closely packed together and the tubular lumen is obliterated. In the apical cytoplasm are a number of tubular invaginations (t), small apical vesicles (a) and larger apical vacuoles (v). The cytoplasmic compartments, with associated mitochondria (M), formed by the infoldings of the basal membranes extend almost up to the base of the brush border. Reaction precipitate is present in the brush border, in the cytoplasm and on the cytoplasmic aspect of the infolding membranes.

x 11,800

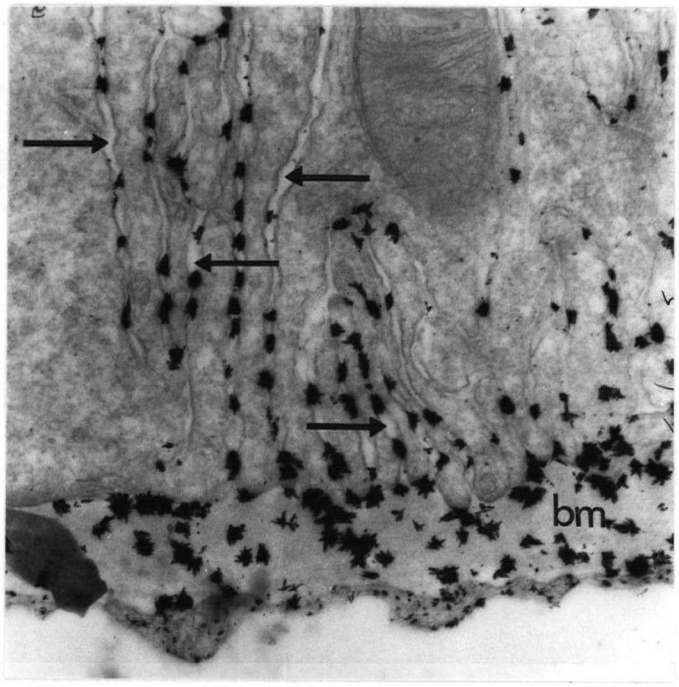

Figure 2

5 hours pre-fixation in glutaraldehyde.
A proximal tubule. Some of the interdigitations of the basal membranes are clearly shown. The extracellular compartments (arrows) formed by the infolding membranes are slightly enlarged in response to the effects of fixation. The reaction precipitate, in the form of clumps, is found adhering to the interdigitating membranes and is also present in the basement membrane (bm).

x 34,300

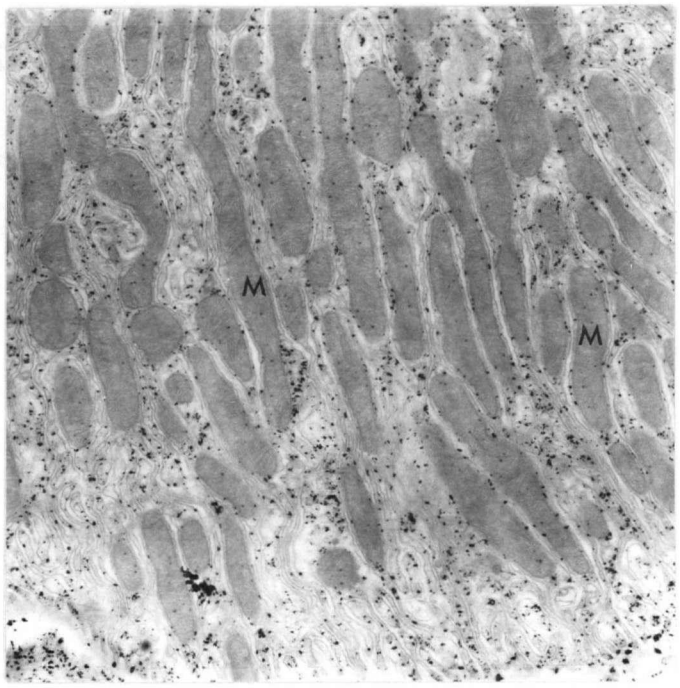

Figure 3

5 hours pre-fixation in glutaraldehyde.
A distal tubule. There is a much more elaborate system of interdigitations of the basal membranes. A large number of elongated mitochondria (M) are closely associated with the membranes. The reaction precipitate is present on the cytoplasmic aspects of the membranes as well as within the cytoplasm.
x 16,300

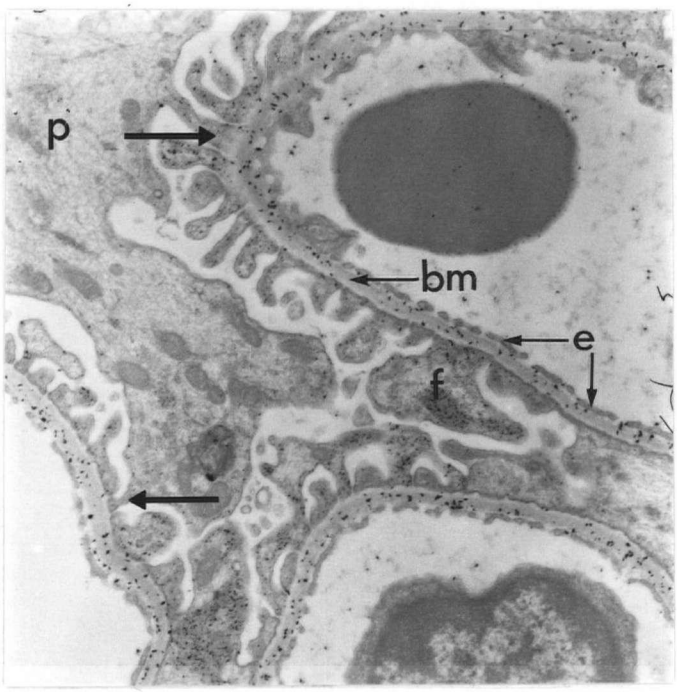

Figure 4

5 hours pre-fixation in glutaraldehyde.
A glomerulus. The basement membrane (bm) separating the fenestrated endothelium (e) from the intricate pattern of podocytic foot processes (f) is prominent. One podocytic cell (p) may send out cytoplasmic processes to more than one capillary (arrows).Fine reaction precipitate is seen on the membranes and in the cytoplasm of the podocytic foot processes, in the basement membrane but not in the endothelium.

x 11,800

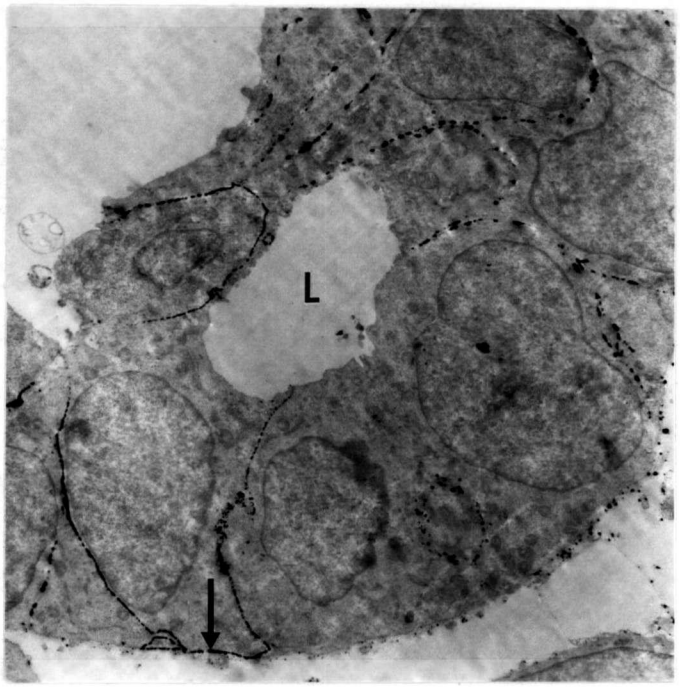

Figure 5

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An undifferentiated tubule. There is intense enzymatic reaction on the lateral membranes between the individual cells. The plasma membrane lining the lumen (L) shows no sign of enzymatic activity. Only one of the tubular cells (arrow) has reaction precipitate associated with the simple basal membrane.

x 4,700

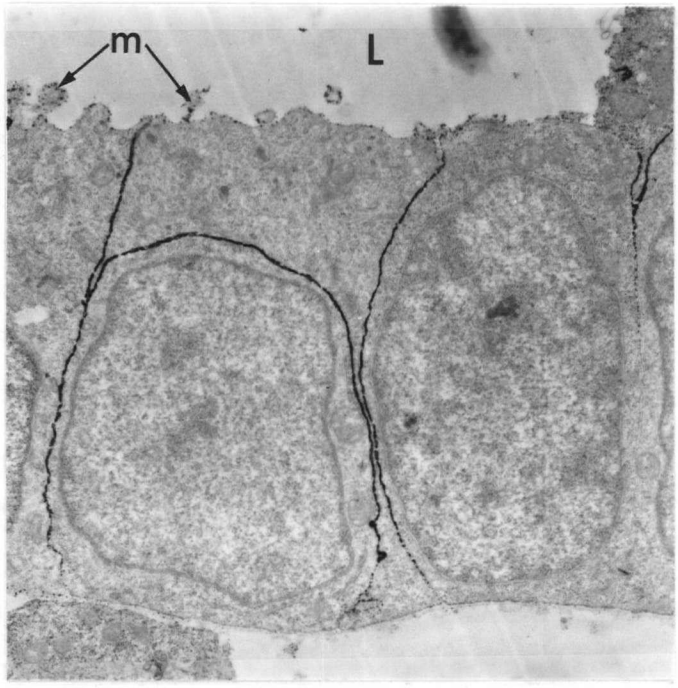

Figure 6

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An undifferentiated tubule. Reaction precipitate is abundant on the lateral membranes. At the luminal surface (L) a small amount of precipitate is present on the plasma membrane of the few short microvilli (m) as well as within the core of the microvilli. There is no reaction on the basal membranes.

x 9,100

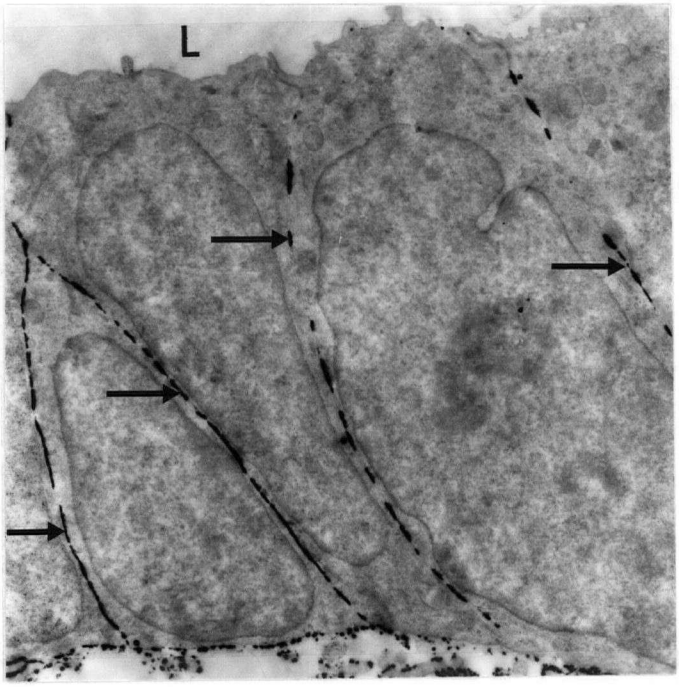

Figure 7

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An undifferentiated tubule. The deposition of the reaction product for ATPase activity on the lateral membranes (arrows) clearly demarcates the lateral boundaries of each cell in the tubule. The basal membranes show some enzymatic activity. However, there is no precipitate on the luminal (L) surface plasma membrane.

x 9,100

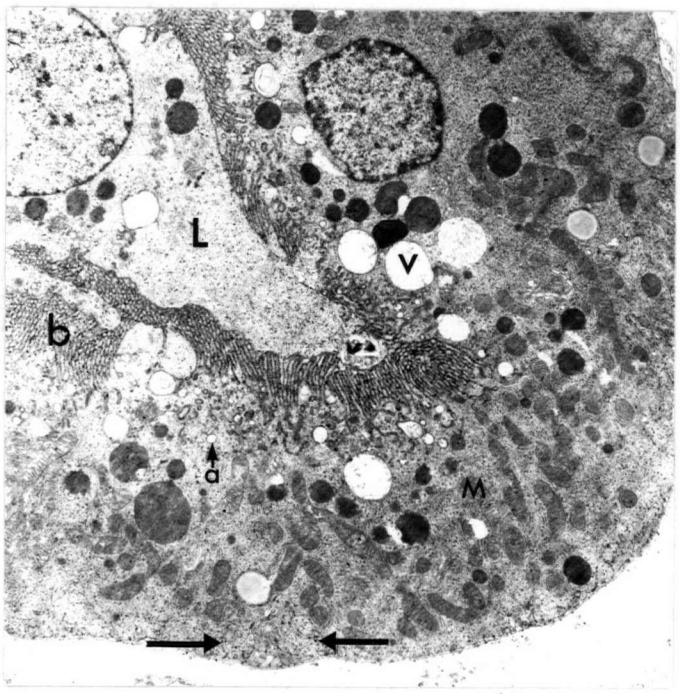

Figure 8

24 hour-old kidney. 5 hours pre-fixation in glutaraldehyde.
A developing proximal tubule. Unlike the undifferentiated tubule, the brush border (b) is relatively well-developed and there are a large number of small apical vesicles (a), large apical vacuoles (v) and mitochondria (M). In the lumen (L) there is a probably degenerating cell. The fine reaction precipitate encrusts the microvilli of the brush border (b). There is some reaction product on the membranes of the developing basal interdigitations (arrows).

x 4,700

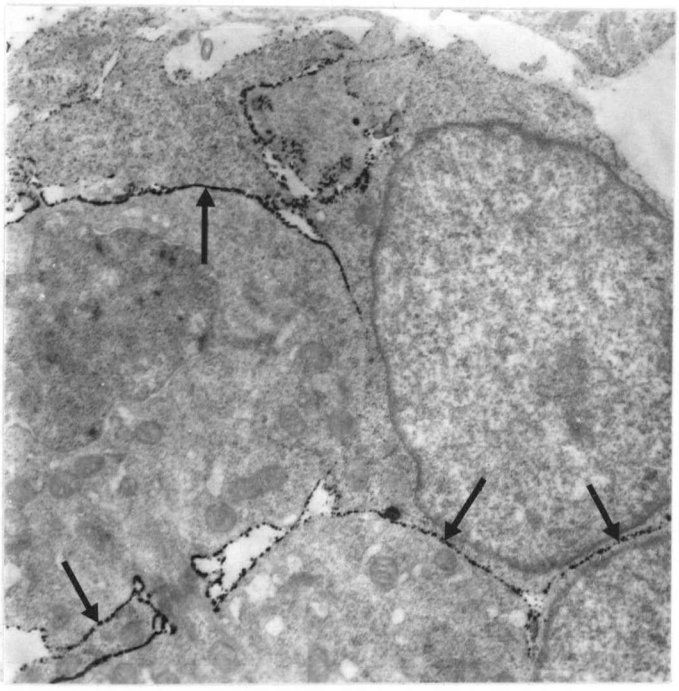

Figure 9

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An immature glomerulus. The podocytic cells are closely packed together. Reaction is most prominently observed where two sets of membranes are in apposition (arrows).

x 9,100

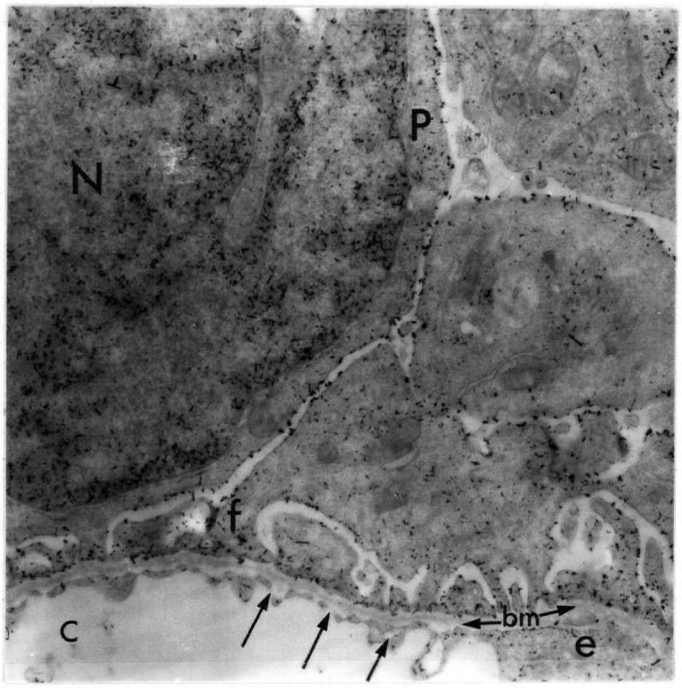

Figure 10

24 hour-old kidney. 5 hours pre-fixation in glutaraldehyde.
A slightly more differentiated glomerulus than in Figure 9. The podocytic cell body (p) is still situated close to the capillary (C). There are a number of podocytic foot processes (f) abutting on the trilaminar basement membrane (bm). The endothelial cell (e) is becoming fenestrated (arrows). The reaction precipitate is found not only on the cell membranes but also within the cytoplasm of the podocytic cell body, the podocytic foot processes and endothelium. There is also quite a bit of precipitate associated with the denser heterochromatin regions of the podocytic cell nucleus (N).

x 11,800

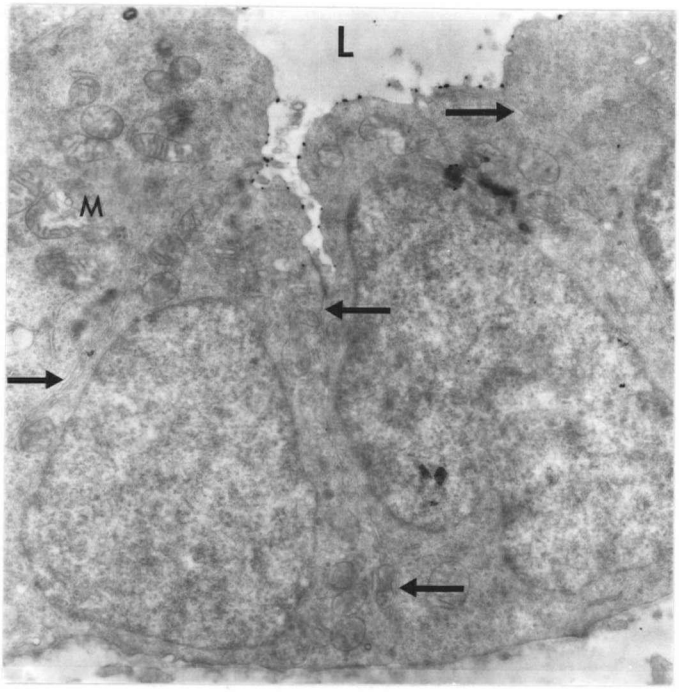

Figure 11

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An undifferentiated tubule. Morphologically the tubular cells are well preserved except for a few "exploded" mitochondria (M). With 2 hours pre-fixation, there is almost always no demonstrable enzymatic reaction. Occasionally a few tubular cells show discrete particles of reaction precipitate on the plasma membrane lining the lumen (L). There is no reaction on the lateral membranes (arrows) or basal membranes.

x 9,100

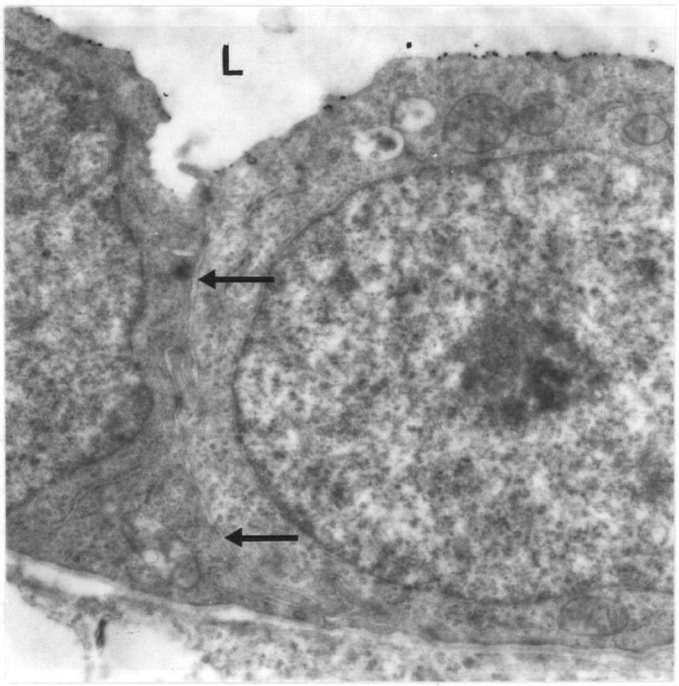

Figure 12

1 hour-old kidney. 1 hour pre-fixation in glutaraldehyde.
An undifferentiated tubule. As in Figure 11 there is only a small amount of precipitate associated with the membrane lining the tubular lumen (L). There is no enzymatic activity on the lateral membranes (arrows) or basal membranes.

x 11,800

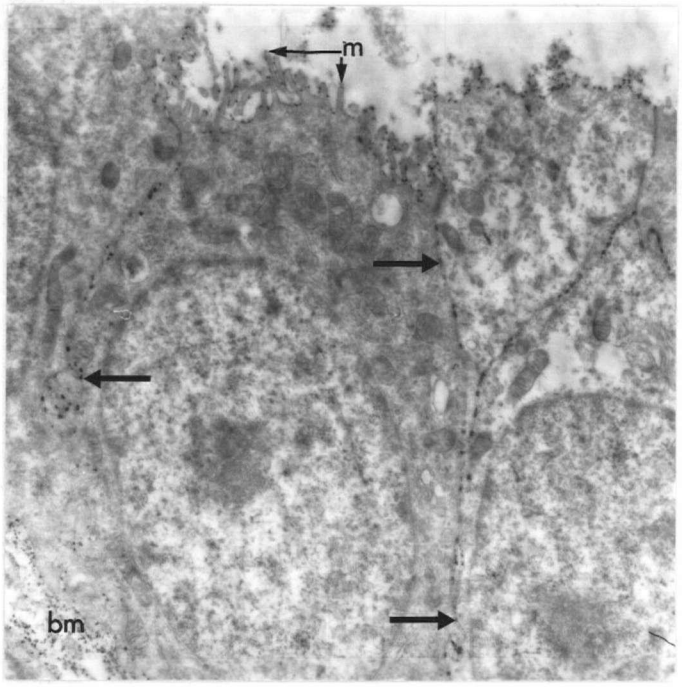

Figure 13

1 hour-old kidney. 15 mins. pre-fixation in glutaraldehyde.
A developing proximal tubule. The microvilli (m), though still few in number, are becoming long and slender. Ultrastructurally the cells do not appear to be so well preserved as in Figures 11-12. However, with a shortened pre-fixation period in glutaraldehyde (15 mins. instead of 1 hour) enzymatic activity is present on the microvilli membranes (m) and on the lateral membranes (arrows). There is some precipitate in the region of the basement membrane (bm).

x 7,500

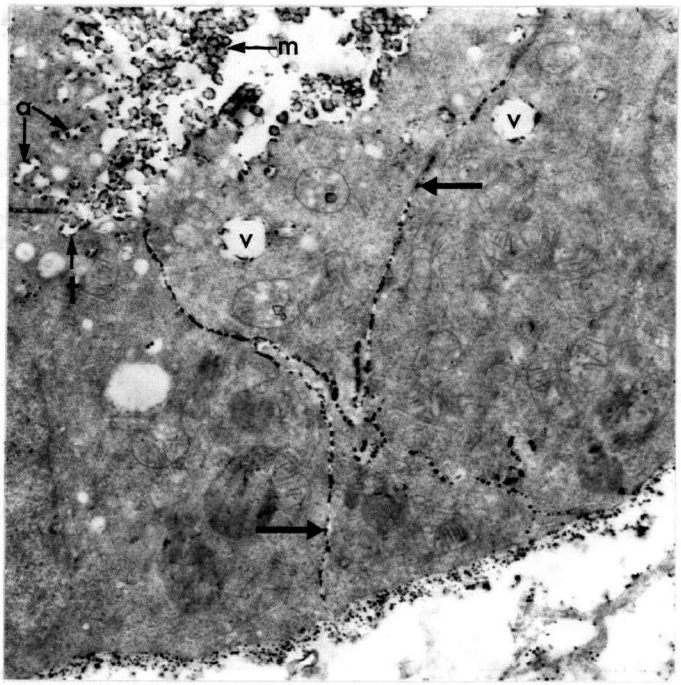

Figure 14

12 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
A developing proximal tubule. The microvilli (m) of the relatively well-developed brush border are coated with reaction precipitate deposits. The tubular invaginations (t) from the bases of the microvilli (m), the small apical vesicles (a) and some of the large apical vacuoles (v) also show the presence of reaction product. There is an intense reaction on the lateral membranes (arrows). At the base of the tubular cells, some precipitate is present but it is not clear whether the precipitate is associated with the basal membranes or the basement membrane.

x 9,100

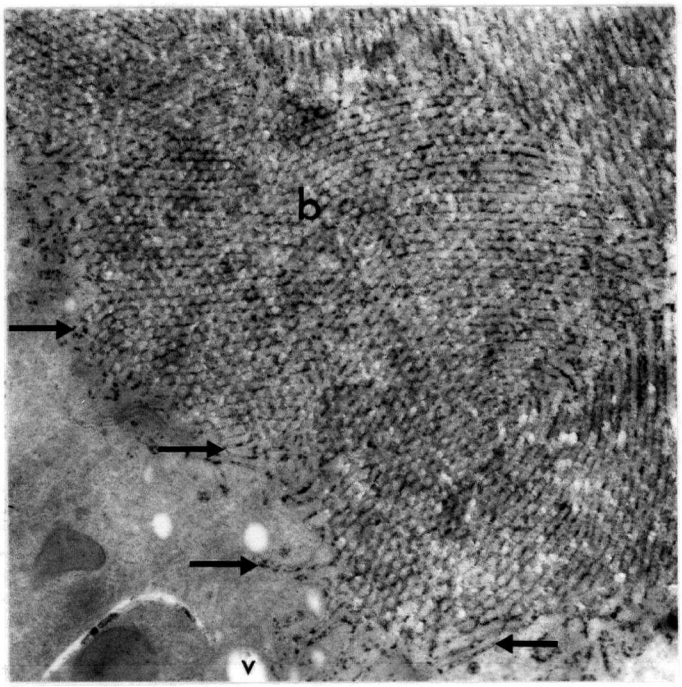

Figure 15

3 day-old kidney. 5 hours pre-fixation in glutaraldehyde.
Brush border of a developing proximal tubule. The brush border (b) is quite extensive. It is in a collapsed state as a result of the immersion method of fixation used. It is clearly seen that the fine precipitate encrusts the membranes of the microvilli. A number of the tubular invaginations from the bases of the microvilli (arrows) show a marked deposition of the reaction precipitate. The few small apical vacuoles (v) that are present have no reaction precipitate.

x 11,800

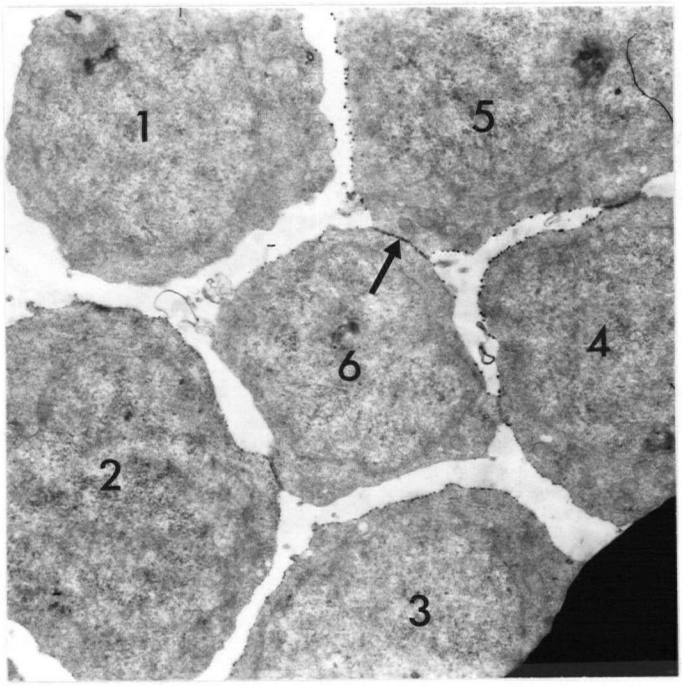

Figure 16

12 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An immature glomerulus. This group of six podocytic cells show variations in response to incubation in the ATP substrate medium. Some cells show a weak reaction (1, 2) while others a more pronounced reaction (3, 4, 5). The podocyte in the centre (6) is not reactive except where its cell membrane is in apposition with the cell membrane of another podocyte (5) (arrow).

x 7,500

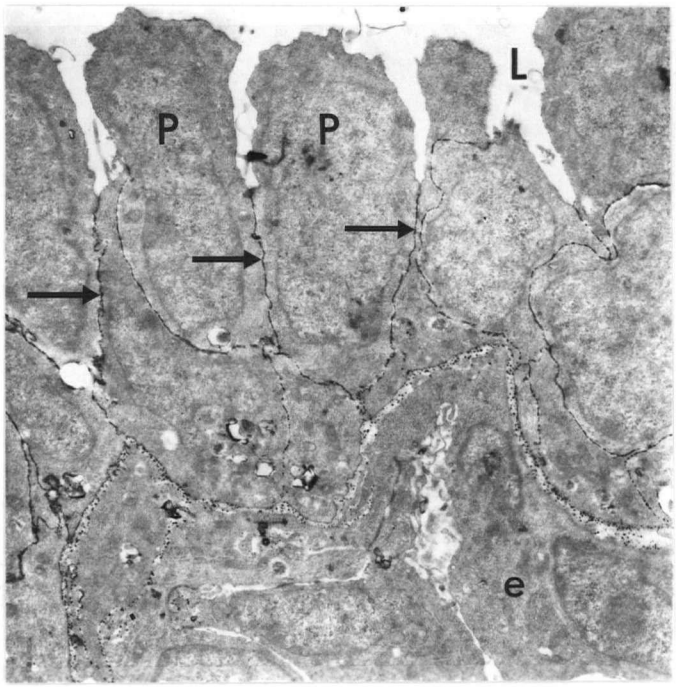

Figure 17

12 hour-old kidney. 2 hours pre-fixation in glutaraldehyde.
An immature glomerulus. The podocytic cells (p) are closely packed together at the basal poles but the apical poles are free in Bowman's space (L). There are no foot processes. The endothelium (e) has not assumed its fenestrated form. It is very apparent that there is an intense ATPase reaction where the membranes of the podocytic cells are in apposition with the membranes of the neighbouring cells (arrows), but not if they are free and exposed in Bowman's space (L). The endothelial cells (e) have no reaction precipitate.

x 4,700

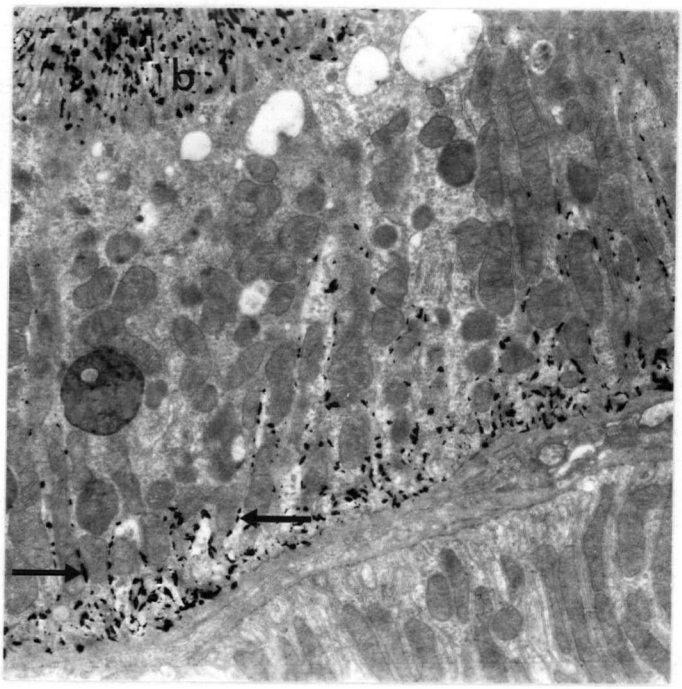

Figure 18

3 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
Two proximal tubules. Though adjacent to one another, the two tubules show variations in enzymatic activity. In one tubule there are two distinct regions of enzymatic activity; at the brush border (b) and basal interdigitations (arrows). The basal infoldings of the other tubule show no deposition of reaction precipitate.

x 7,500

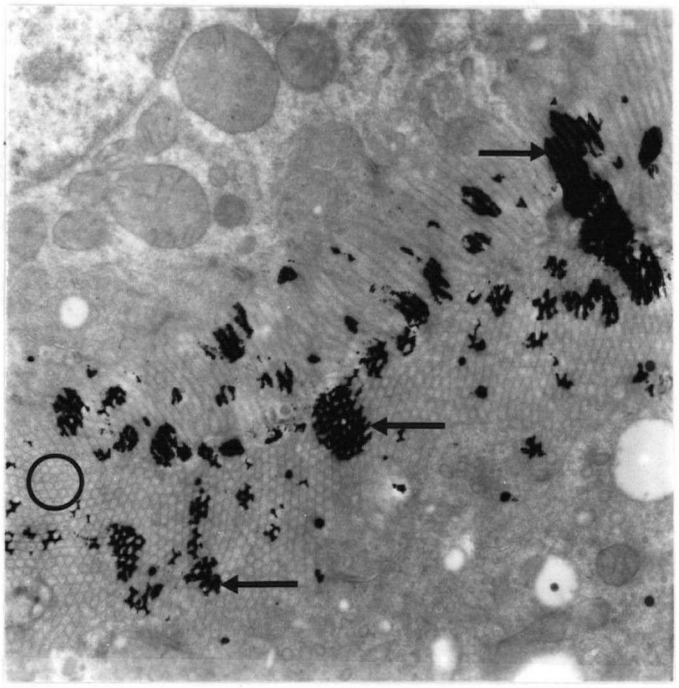

Figure 19

3 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
Brush border of a proximal tubule. There is some indication of an electron-dense material within the core of the microvilli (circle).
The reaction precipitate is not uniformly distributed over the membranes of all the microvilli in the brush border. Instead clumps of precipitate are observed in various regions of the brush border (arrows). This is probably artifactual rather than a result of heterogeneity of response to PHMB.

x 11,800

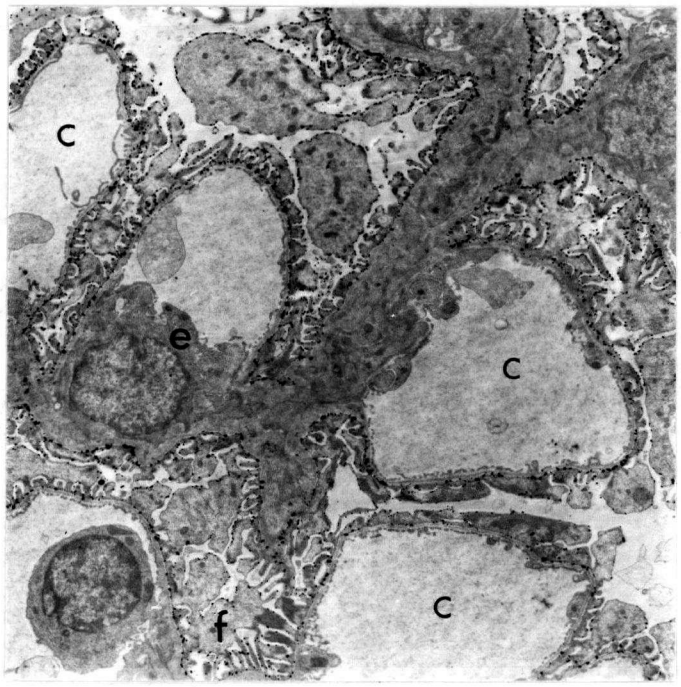

Figure 20

3 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
Glomerulus. This is a low magnification electron micrograph of a glomerulus showing various capillaries (C) and the intricate network of podocytic foot processes (f) on the trilaminar basement membrane separating the endothelium (e) from the visceral epithelium. The reaction precipitate is distributed all along the plasma membranes of the podocytic foot processes but not on the membranes of the endothelial cells.
x 3,700

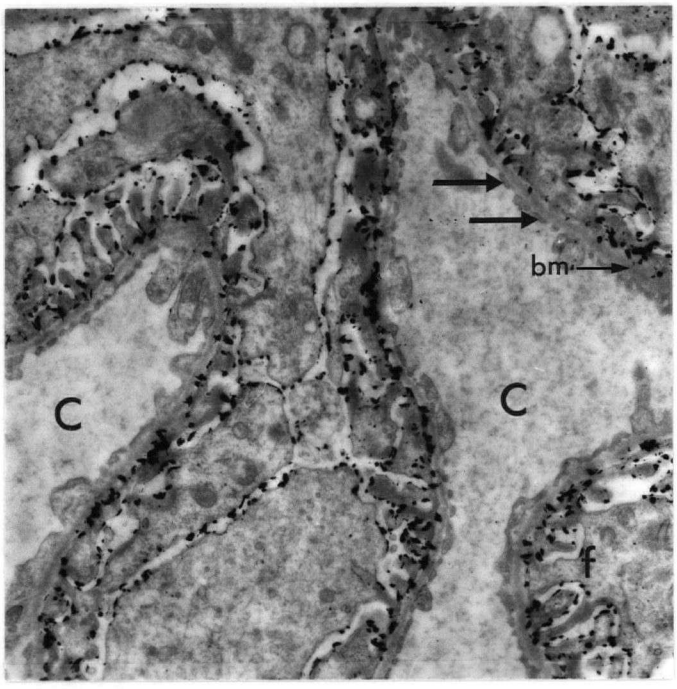

Figure 21

3 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
Glomerulus. The fenestrated endothelium (arrows) of the capillaries (C) is separated from the podocytic foot processes (f) by a distinctly visible basement membrane (bm). There is no reaction precipitate on the endothelium. However reaction precipitate is present all along the plasma membranes of the podocytic foot processes.

x 9,100

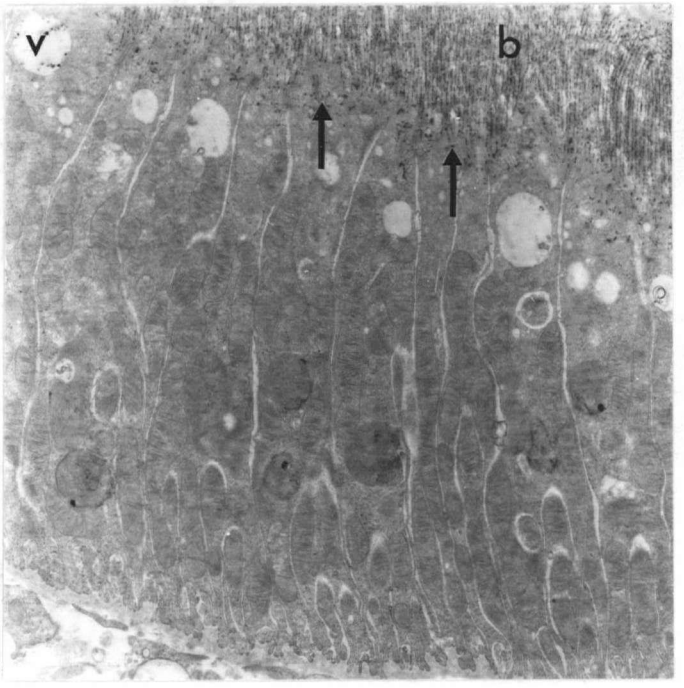

Figure 22

3 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 9.4.
Proximal tubule. The basal interdigitations in the proximal tubule are quite extensive. Some may be confined to the basal portions of the cell while others may extend almost to the bases of the brush border (b). The extracellular compartments formed by the interdigitations are very sensitive to the effects of fixation and are often separated, as exemplified here. There is no ATP-ase activity on these membranes although enzymatic activity is readily observed in the brush border (b) and in the tubular invaginations (arrows) arising from the bases of the microvilli. Only one apical vacuole (v) has some reaction precipitate associated with it.

x 5,700

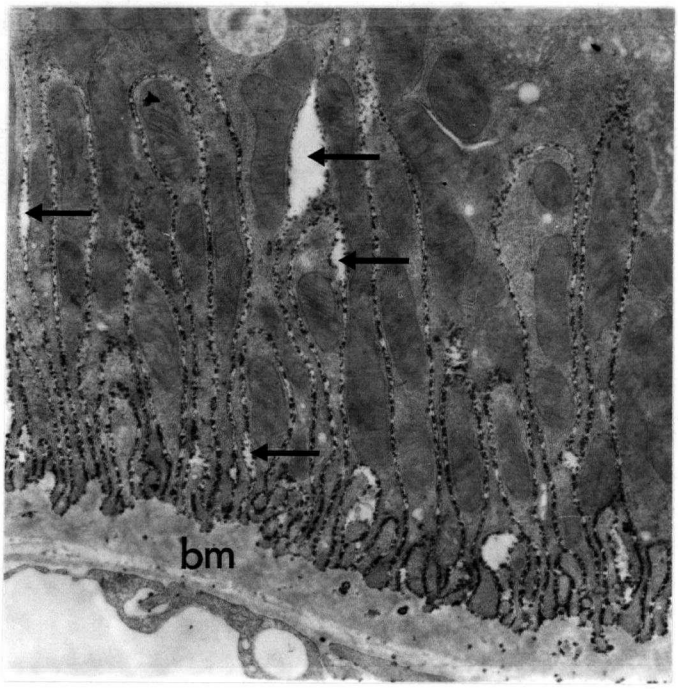

Figure 23

3 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 9.4.
Proximal tubule. The enlarged extracellular compartments (arrows) bound by the infolding basal membranes indicate the sensitivity of the basal regions of the proximal tubules to the effects of fixation. Not all the extracellular compartments are enlarged to the same extent. Unlike Figure 22, the basal interdigitations show an intense enzymatic reaction. The fine reaction precipitate is seen adhering to the membranes and is not free in the extracellular space. The basement membrane (bm) is relatively thick and is generally free of any precipitate.
x 11,800

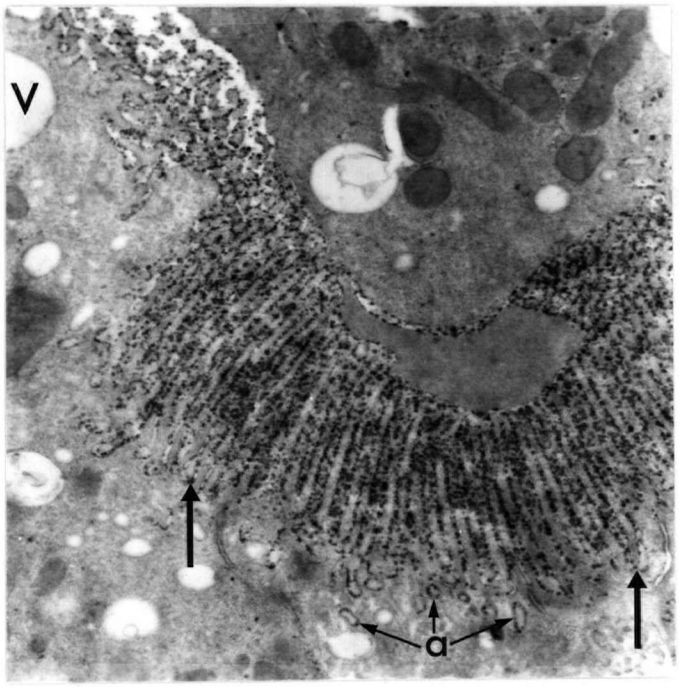

Figure 24

3 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 9.4.
Brush border of a proximal tubule. There is an abundant deposition of reaction precipitate along the membranes of the microvilli making up the brush border. A large number of the tubular invaginations from the bases of the microvilli (arrows) are also coated with the reaction precipitate. Some small apical vesicles (a) have reaction product on their limiting membranes. The large apical vacuoles (v) that are present have no accumulation of reaction precipitate.
x 11,800

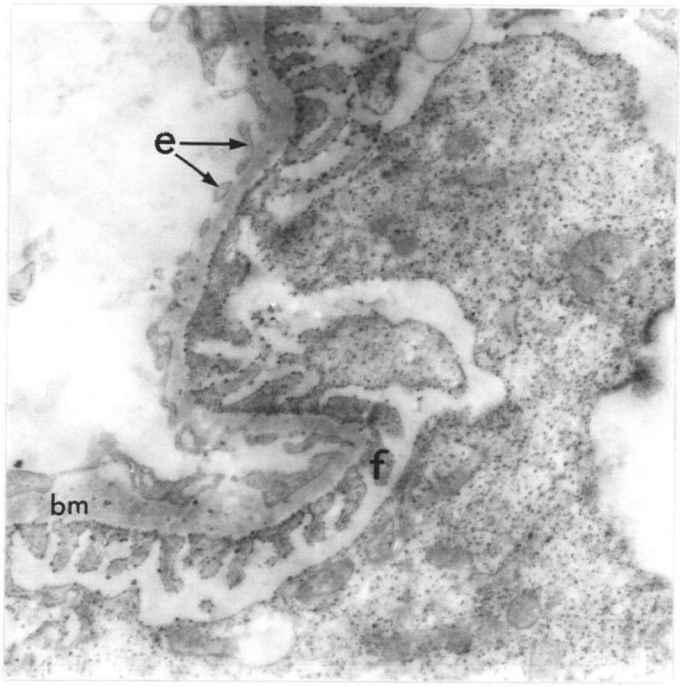

Figure 25

3 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 9.4.
Glomerulus. The fine reaction precipitate is observed not only along the membranes of the podocytic foot processes (f) but also within the cytoplasm (compare with Figures 20, 21). There is some precipitate in the basement membrane (bm) which is probably due to diffusion of reaction precipitate from the adjacent podocytic foot processes. The fenestrated endothelium (e) shows no enzymatic activity.

x 16,300

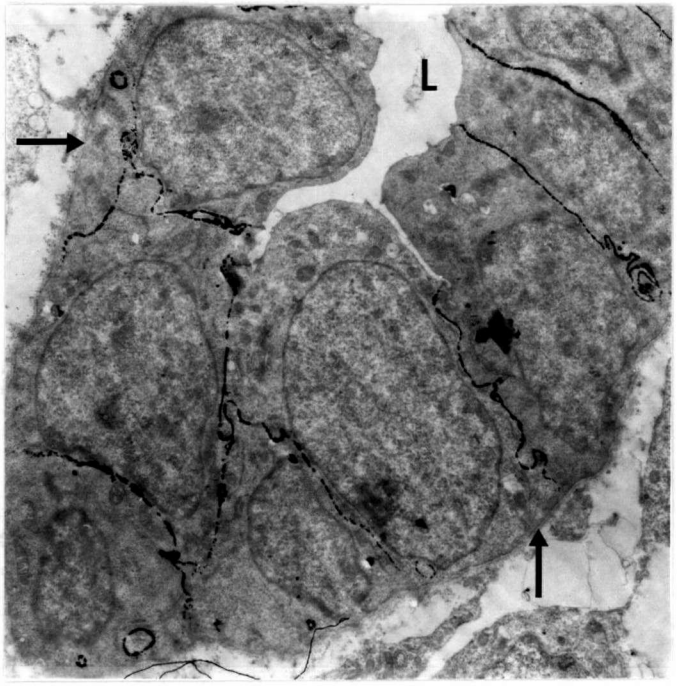

Figure 26

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
An undifferentiated tubule. The plasma membrane lining the lumen (L) as well as the basal membranes (arrows) of the tubular cells are still simple in contour. The lateral membranes show the beginnings of interdigitations which will become much more complex with continuing differentiation. Heavy deposits of reaction precipitate on the lateral membranes accentuate the lateral boundaries between the tubular cells. There is no reaction precipitate on the basal membranes or on the luminal surface membrane.
x 4,700

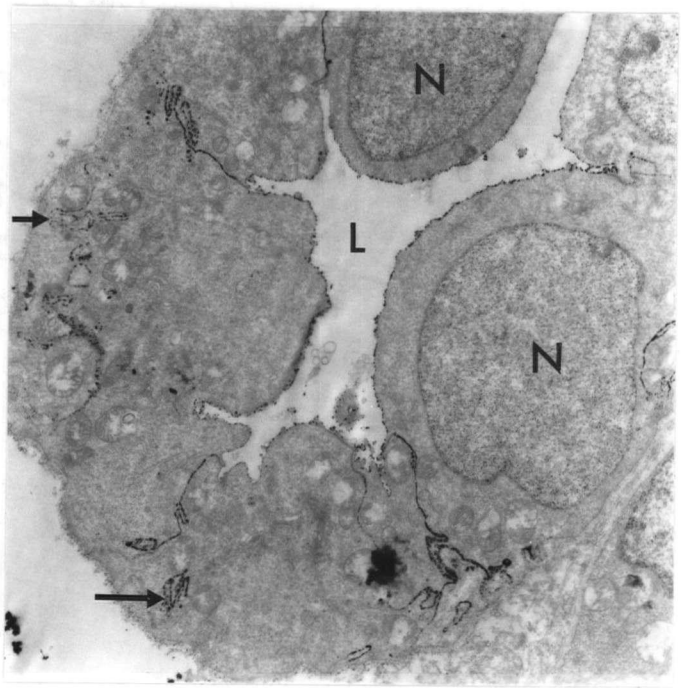

Figure 27

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
An undifferentiated tubule. The outline of the luminal (L) surface membrane is still relatively smooth although a few microvilli are present. The basal membranes of some cells are beginning to infold (arrows). The lateral membranes also show some interdigitations. There is intense enzymatic reaction all along the luminal surface plasma membrane, the interdigitating lateral membranes and the membranes of the basal infoldings, wherever they are present. A few of the nuclei (N) show an accumulation of fine precipitate.

x 5,700

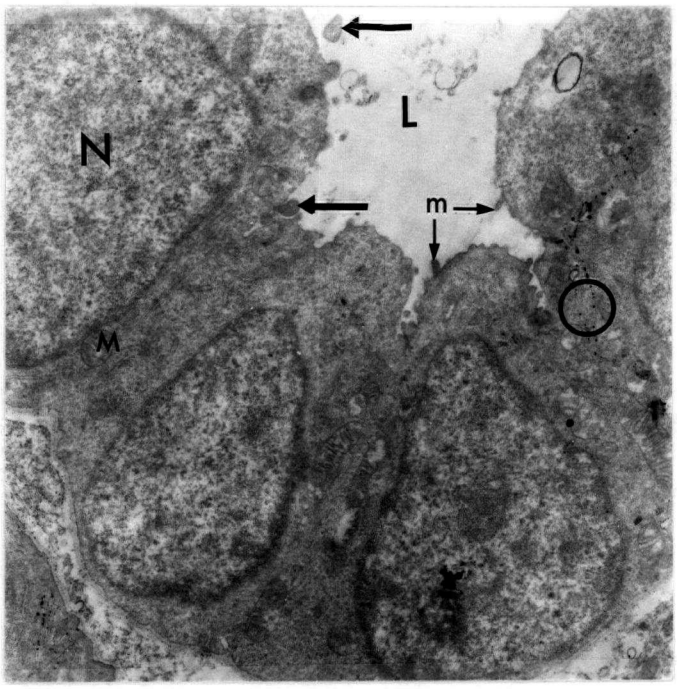

Figure 28

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
An undifferentiated tubule. Ultrastructurally the tubular cells are simple. Each cell consists of a large nucleus (N) and a few mitochondria (M) contained within a small amount of cytoplasm. At the apex of the cell there are a few microvilli (m). Part of the apical cytoplasm appears to be blebbing off (arrows) and discarded into the lumen (L) as cellular debris. The lateral membranes follow a straight path from the lumen to the base of the cell. The basal membranes show no complex interdigitations as in the adult. There is practically no demonstrable enzymatic activity except for part of the lateral membrane. (circle).

x 7,500

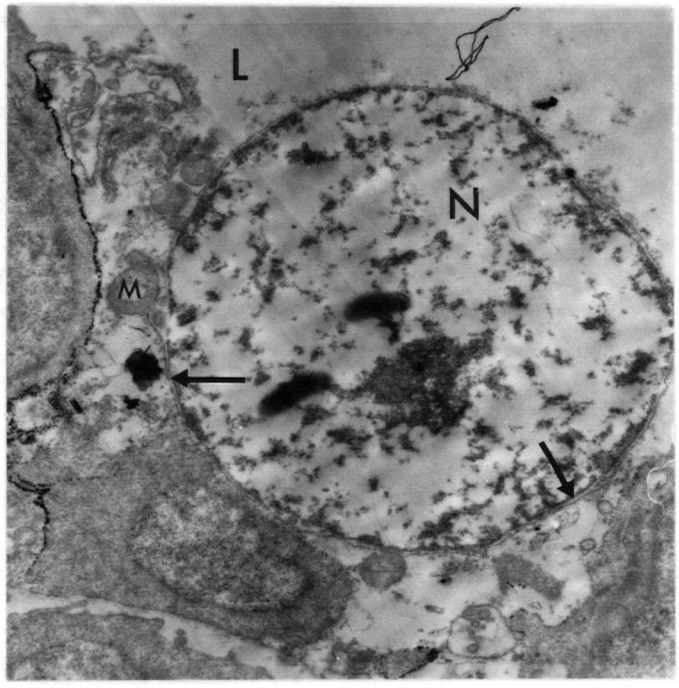

Figure 29

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
An undifferentiated tubule. All, but one, of the cells is normal and show deposition of reaction precipitate on the lateral membranes. This one cell is "exploded." The contents of the cell, that is, the cytoplasm and mitochondria (M) are being spilled out into the lumen (L). The nucleus (N) is swollen to immense proportions. The nuclear membrane (arrows) appears intact. The chromatin material adheres to the nuclear membrane or is suspended in the nucleoplasm. The reason for this particular cell's extreme sensitivity to experimental conditions is not known.

x 7,500

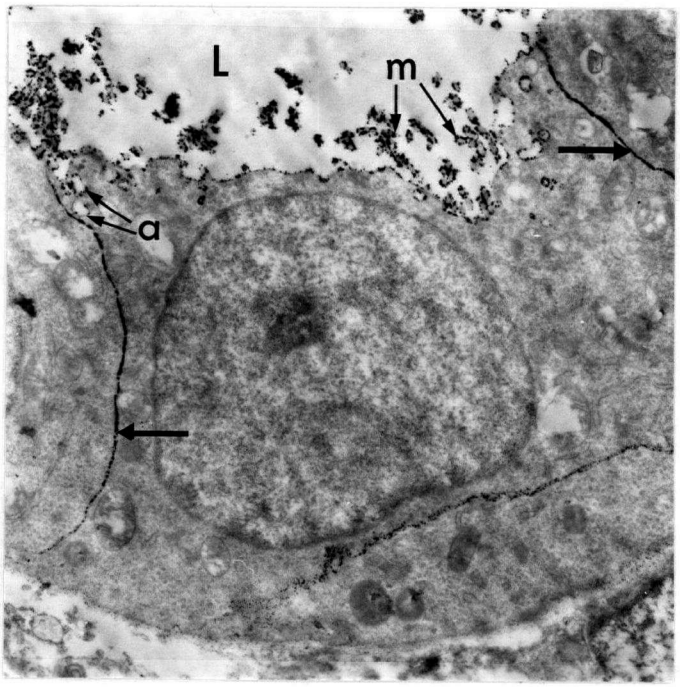

Figure 30

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
A developing proximal tubule. The tubule shows the first definite signs of differentiation into a proximal tubule. The microvilli (m) are still sparse but relatively long and slender. There are a number of apical vesicles (a) in the apical cytoplasm. The lateral and basal membranes are simple in contour. Reaction precipitate is deposited heavily on the lateral membranes (arrows), the luminal (L) surface membrane and the membranes of the microvilli. Some of the small apical vesicles (a) have reaction precipitate all along the limiting membranes.

x 7,500

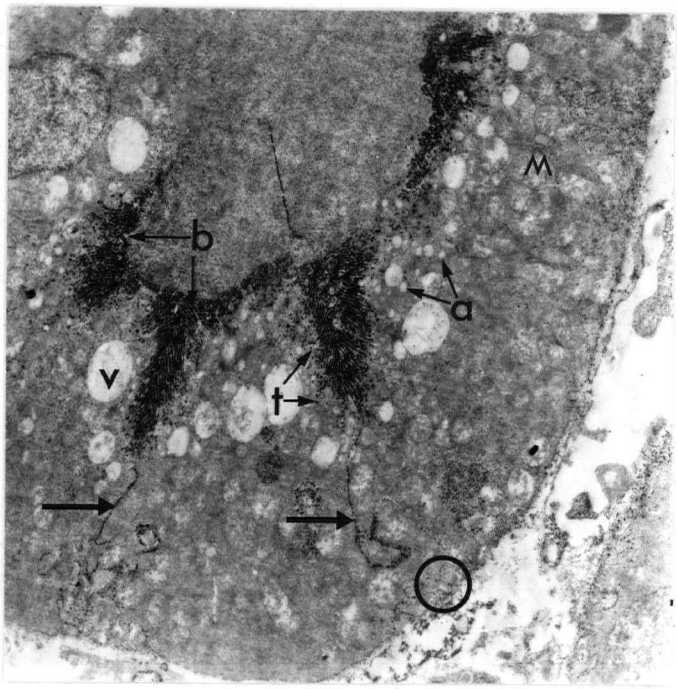

Figure 31

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
A developing proximal tubule. This is a tubule further along in differentiation (compare with Figure 30). The brush border (b) is quite well-developed, the lateral membranes pursue a slightly more tortuous course from the lumens to the base of the cell (arrows) and some interdigitations of the basal membranes are seen (circle). There are a large number of tubular invaginations (t) from the bases of the microvilli, small apical vesicles (a) and large apical vacuoles (v). Some of the small, round mitochondria (M) that are present appear to be sensitive to the effects of fixation. Enzymatic activity is most prominent in the brush border (b), the tubular invaginations (t), and on the lateral and basal interdigitations.
x 3,700

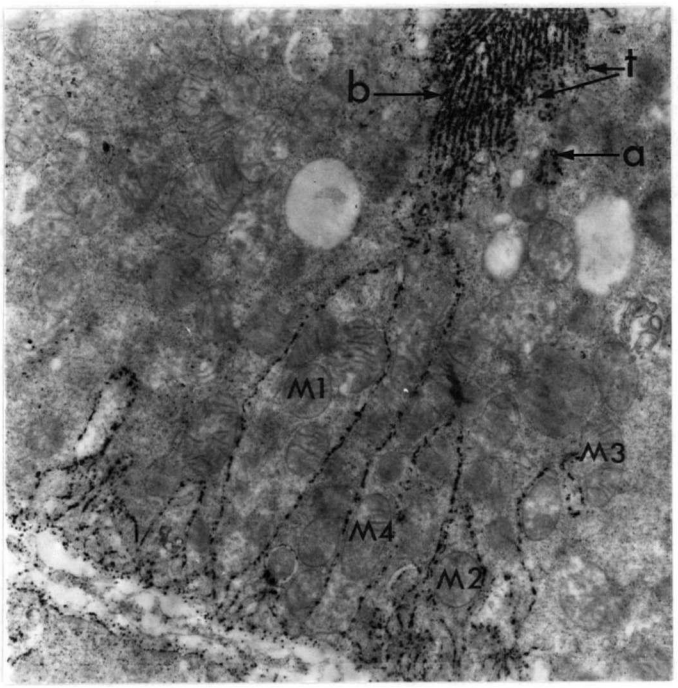

Figure 32

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. Pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
A developing proximal tubule. This tubule is more differentiated than those in Figures 30 and 31. The brush border (b) and the basal interdigitations are quite extensive. Cytoplasmic compartments with associated mitochondria (M) are developing. Most of the mitochondria are still small and round (M1, M2) while others are becoming elongate (M3, M4). The reaction precipitate is observed on the membranes of the basal interdigitations, the brush border, tubular invaginations (t) and small apical vesicles (a).

x 9,100

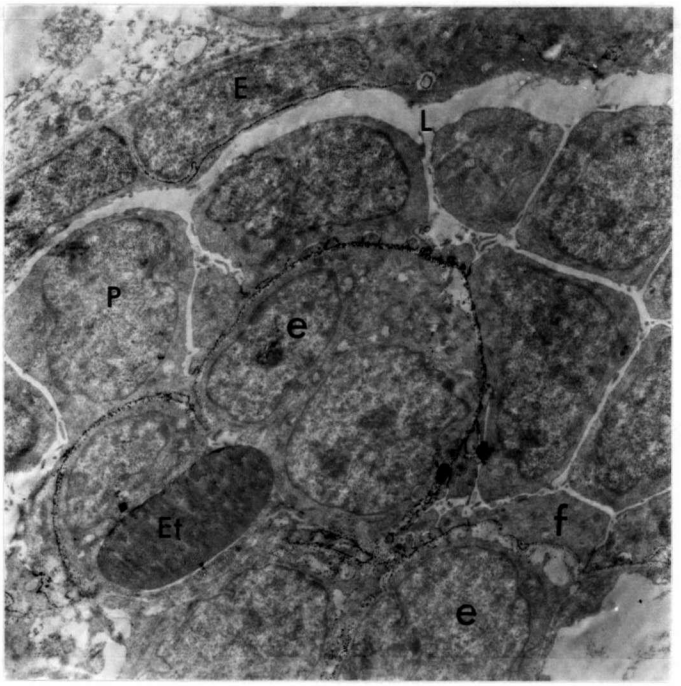

Figure 33

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to PHMB. Incubation with PHMB at pH 7.2.
An immature glomerulus. The parietal epithelium (E) delineates the outermost extent of the glomerulus. The visceral epithelium, made up of the podocytic cell bodies (p) and the beginnings of foot processes (f), surround the endothelium (e) of two capillaries. The capillary lumens are small but can be recognised by the presence of an erythrocyte (Et). The apical poles of the podocytic cells are free in Bowman's space (L) while the foot processes from the basal poles abutt on the basement membrane around the capillaries. Enzymatic activity is only present on the membranes of the foot processes in direct contact with the basement membrane, and also on the lateral membranes of the parietal epithelial cells.

x 3,700

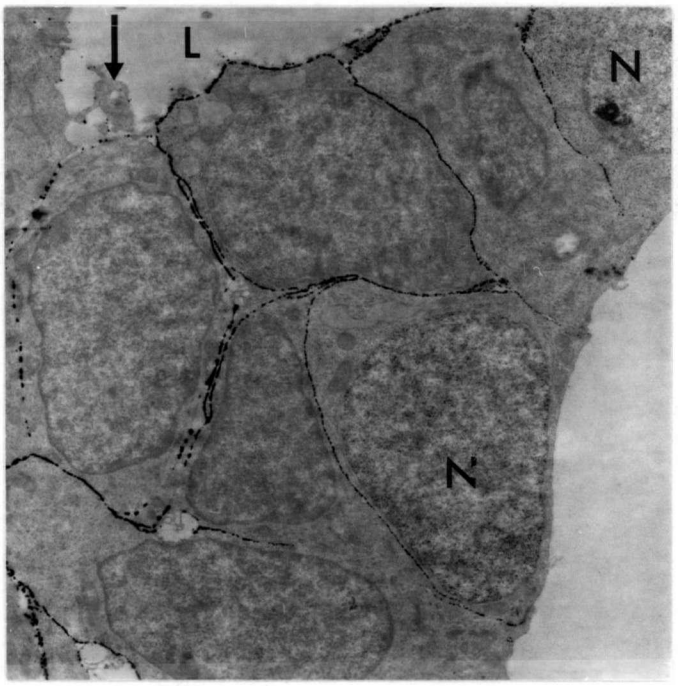

Figure 34

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to L-cysteine. Incubation with L-cysteine at pH 7.2. An undifferentiated tubule. The tubule is in an early stage of development. The lumen (L) containing some cellular debris (arrow) is still small. A tubule consisting of a single layer of cells is probably formed from the growth and movement of such a group of undifferentiated cells. Then these undifferentiated cells acquire the ultrastructural features characteristic of each portion of the nephron. The deposition of reaction precipitate is abundant all along the lateral membranes separating each individual cell. There is also some precipitate on the luminal surface membranes. Only two of the nuclei (N) appear to have fine precipitate associated with them.

x 5,700

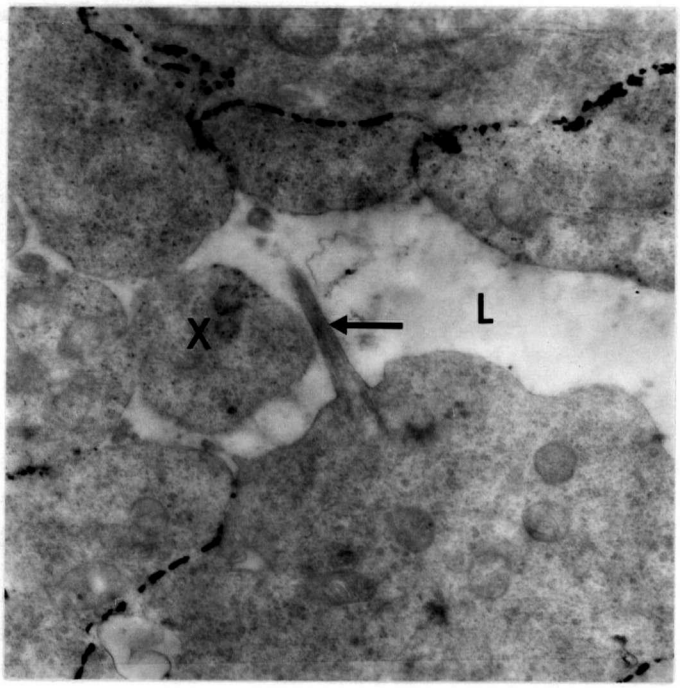

Figure 35

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to L-cysteine. Incubation with L-cysteine at pH 7.2. An undifferentiated tubule. Occasionally a cilium (arrow) is seen arising from the apical plasma membrane. Otherwise the luminal plasma membrane is simple in contour. A piece of cytoplasmic material (X) appears to be extruded into the lumen (L). Only the lateral membranes between various cells show an abundant deposition of reaction precipitate.

x 16,300

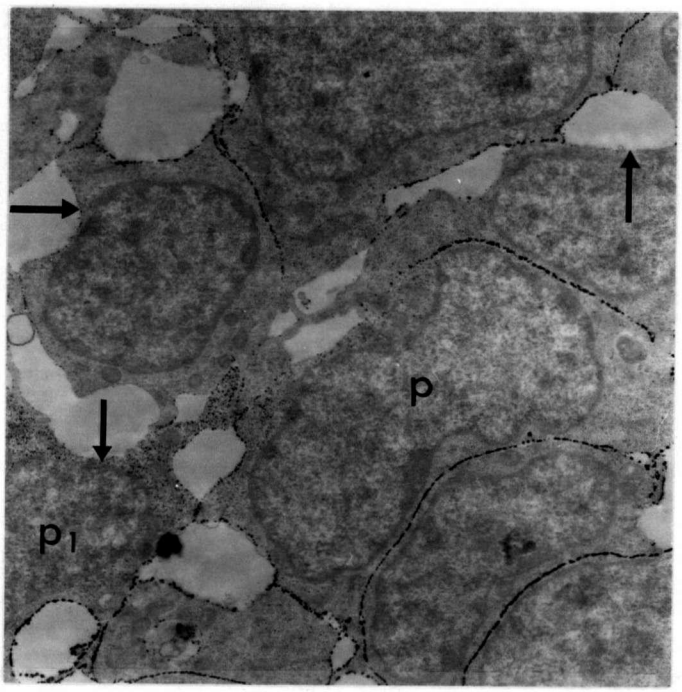

Figure 36

2 hour-old kidney. 2 hours pre-fixation in glutaraldehyde. No pre-incubation exposure to L-cysteine. Incubation with L-cysteine at pH 7.2. An immature glomerulus. The podocytic cells (p) are still closely packed together. The cytoplasm of some of the cells are beginning to develop into foot processes. There is intense enzymatic reaction on practically all the membranes whether they are in apposition or are free. Some of the free membranes show a lesser amount of or no precipitate (arrows). In one podocytic cell (p1) there is also some precipitate in the cytoplasm.

x 7,500

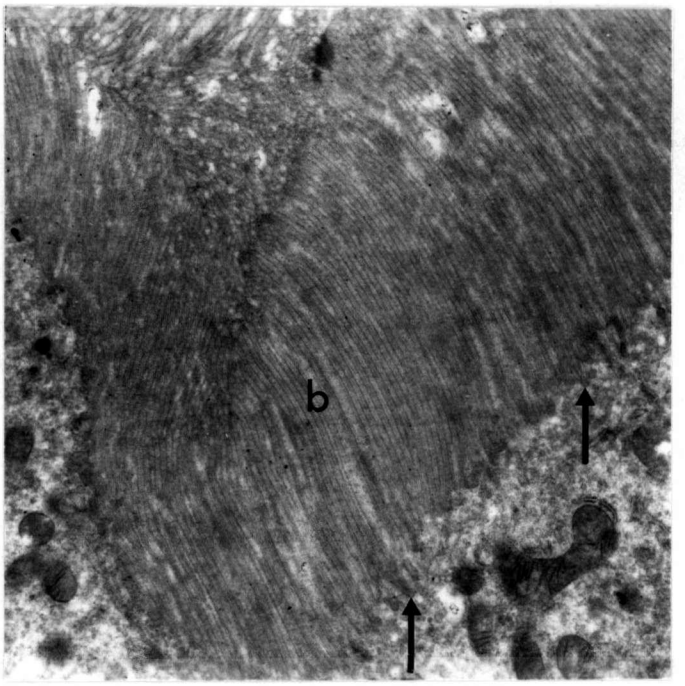

Figure 37

Brush border of a proximal tubule.The brush border (b) is very well-developed. The microvilli are long and slender and are closely packed together. This collapsed condition of the brush border is artifactual and is a result of the mode of fixation used. The tubular invaginations from the bases of the microvilli appear to contain a dense material (arrows). There are no apical vesicles or vacuoles in the apical cytoplasm of this particular cell. There is no accumulation of electron-dense precipitate except for a few specks here and there.

x 9,100

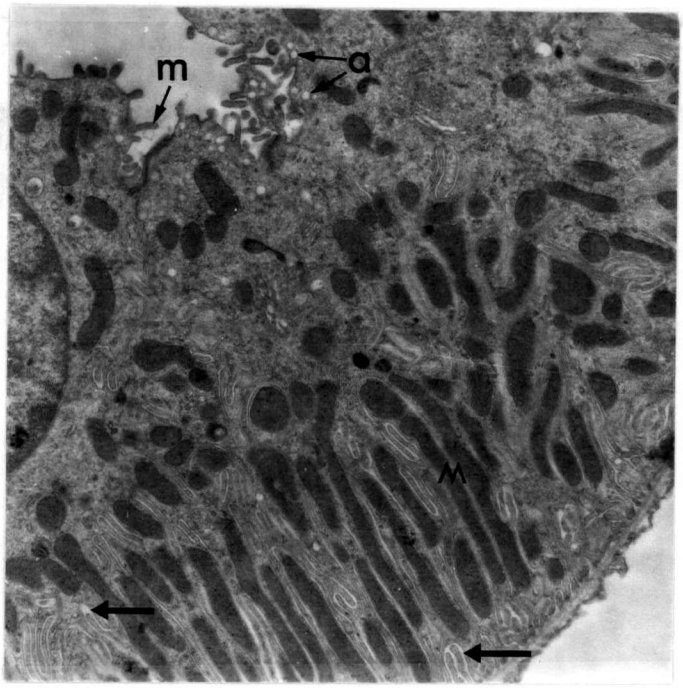

Figure 38

Distal tubule. The basal membranes infold extensively dividing the cytoplasm into numerous compartments within which are contained elongated mitochondria (M). The extracellular compartments are enlarged slightly probably in response to fixation conditions (arrows). On the luminal surface of the cell there are a few short microvilli (m). In the apical cytoplasm there are a few small apical vesicles (a). There is no precipitate on the basal interdigitations, on the microvilli or within the cytoplasm.
x 7,500

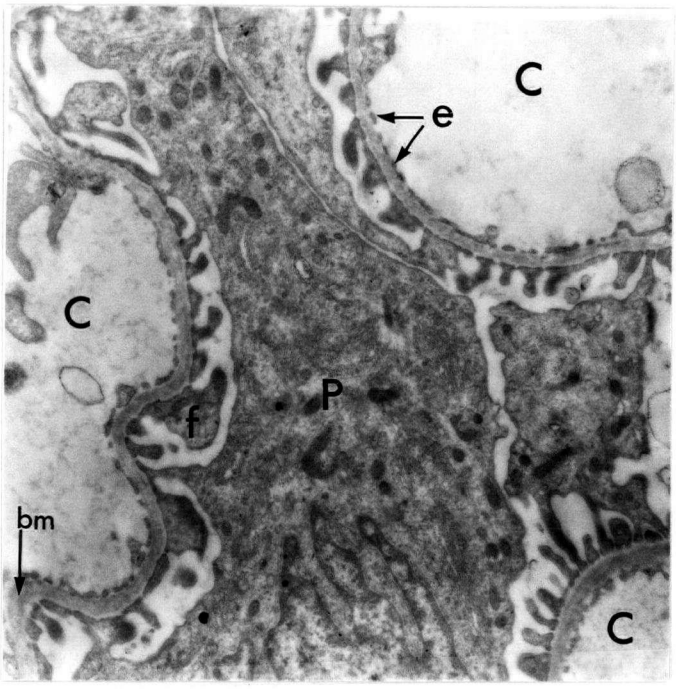

Figure 39.

A glomerulus. The lumens of three capillaries (C) are seen in this electron micrograph. The endothelium (e) lining the capillaries is fenestrated. A thick basement membrane (bm) is interposed between the endothelium and the podocytic foot processes (f), which are cytoplasmic prolongations of the podocytic cells (p). The endothelium, the basement membrane and the podocytic foot processes are the three components of the filtration apparatus. No precipitate is observed within the glomerulus.

x 9,100

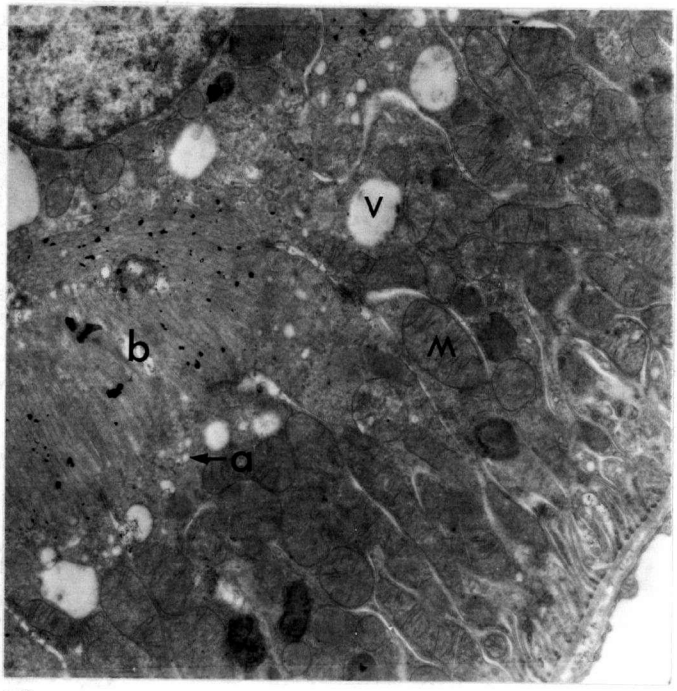

Figure 40

A proximal tubule. The brush border (b) is well-developed. In the apical cytoplasm there are a large number of small apical vesicles (a) and large apical vacuoles (v). Numerous mitochondria (M) are present in the cytoplasmic compartments formed by the elaborate interdigitations of the basal membranes. The extracellular compartments are enlarged in response to the conditions of fixation. Some electron-dense precipitate is observed in the region of the brush border (compare with Figures 1 and 18).

x 7,500

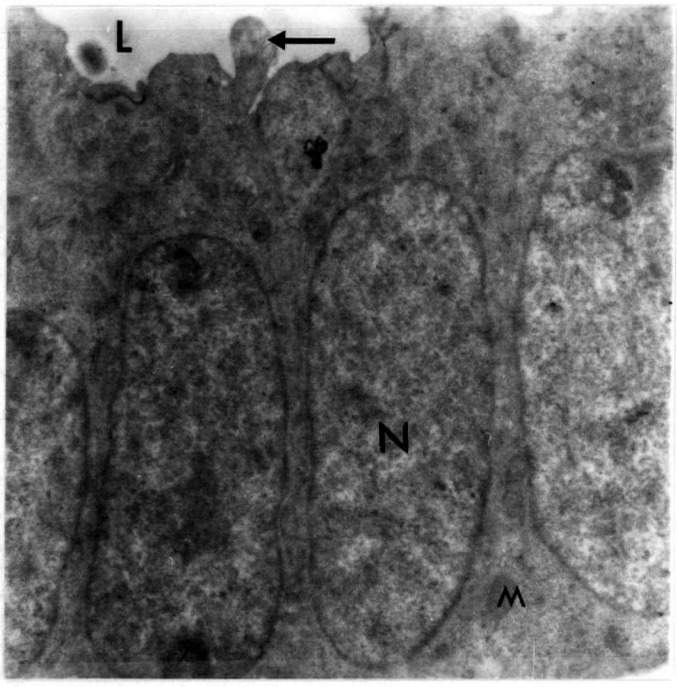

Figure 41

An undifferentiated tubule. The tubular cells are ultrastructurally simple. The nuclei (N) are large. In the cytoplasm there a few small round mitochondria (M). The luminal surface membrane is simple in contour. Part of the apical cytoplasm seems to be blebbing off (arrow) and being discarded into the lumen (L) as debris. No precipitate is present on any of the plasma membranes.

x 9,100

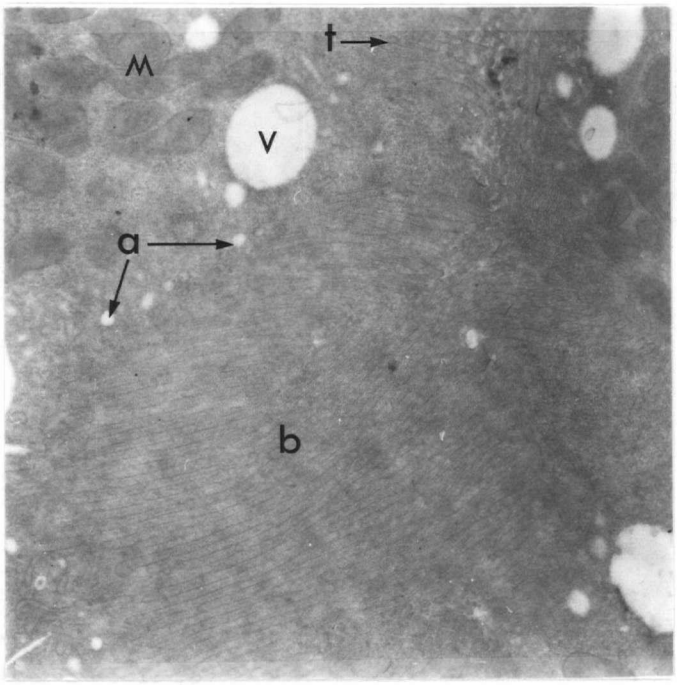

Figure 42

Brush border of a developing proximal tubule. The brush border (b) is relatively well-developed. There are a large number of long slender microvilli. Tubular invaginations (t), small apical vesicles (a) and large apical vacuoles (v) are present in the apical cytoplasm. There are also a few mitochondria (M). There is no precipitate present.

x 11,800

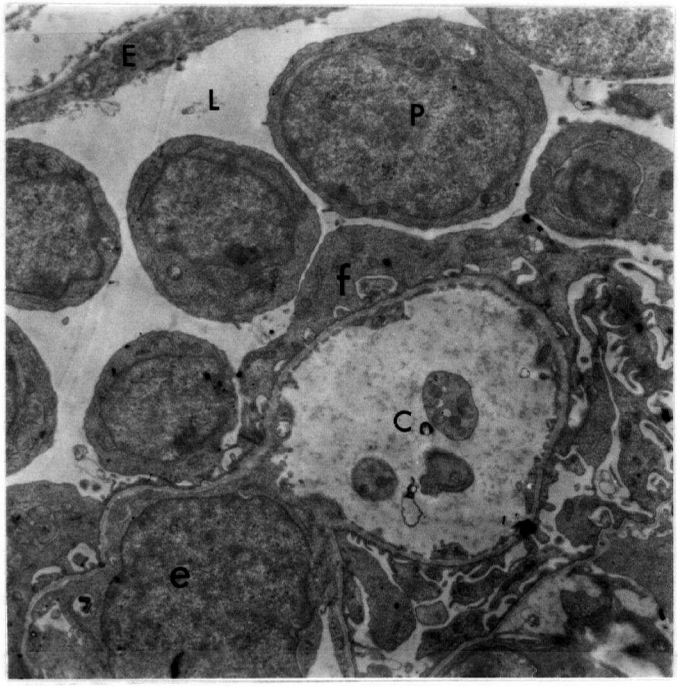

Figure 43

An immature glomerulus. The parietal epithelium (E) is the outermost component of the glomerulus. Within Bowman's space (L) are the podocytic cells (p). A large nucleus is present in the apical poles of the cells. Podocytic foot processes (f) arise from the basal portions of the cells and abutt onto the basement membrane separating the podocytes from the endothelium (e). The capillary lumen (C) is still small. There is no deposition of precipitate in the glomerulus. (The dark chunks are dirt particles).

x 4,700

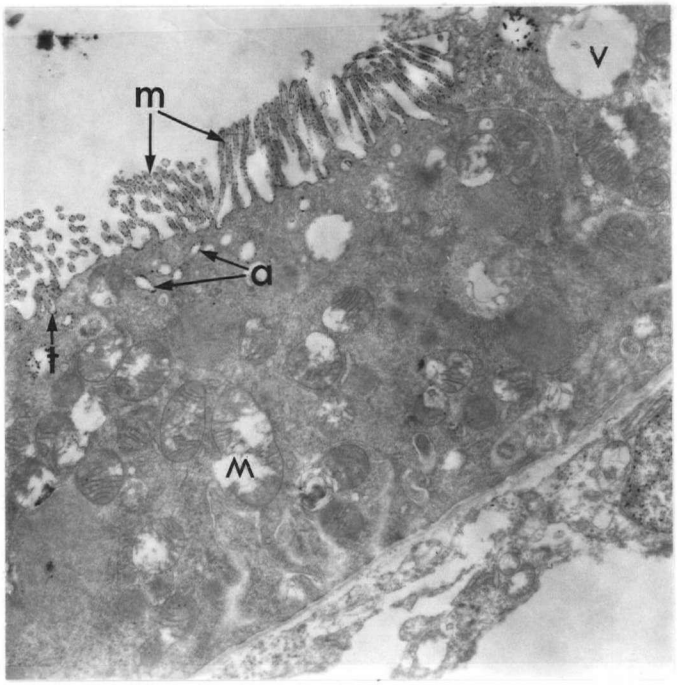

Figure 44

A developing proximal tubule. The microvilli (m), though still few in number, are long and slender. Tubular invaginations (t), small apical vesicles (a) and large apical vacuoles (v) are present in the apical cytoplasm. There are quite a few mitochondria (M), but these appear to be particularly susceptible to the effects of fixation. Electron-dense precipitate is seen adhering to the microvilli membranes and some of the tubular invaginations and apical vesicles. The amount of precipitate is less than in tissues incubated in a medium containing ATP as a substrate. (Compare with Figures 8, 30-32).

x 7,500

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