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Postnatal development of the pig kidney: Ultrastructure of the glomerulus and the proximal tubule

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INTRODUCTION

The mammalian kidney develops from the union of the ureteric bud with the metanephric blastema (Potter, 1972). Briefly the ureteric bud forms the ureter, pelvis and collecting tubules and induces the formation of nephrons within the metanephric blastema. The first nephron anlage, the renal vesicle, is transformed to the S-shaped body which connects to the collecting tubule and further differentiates into the mature nephron. The formation of nephrons proceeds in a centrifugal pattern so that the oldest and most mature nephrons are located in the juxtamedullary area and the youngest in the superficial cortex. In most mammals, nephrogenesis is not completed at birth resulting in a relatively lower efficiency of the kidney in newborn animals than in adults (Nash & Edelmann, 1973, Loggie, Kleinman & Van Maanen, 1975).

At the ultrastructural level the development of the single nephron segments has been studied to varying extents. Thus the glomerular development has been extensively described in mice (Clark, 1957), rats (Suzuki, 1959), humans (Vernier & Birch-Andersen, 1962, 1963; Aoki, 1966), pigs (Kazimierczak, 1971) and dogs (Hay & Evan, 1979), whereas the ultrastructure of the developing proximal tubule has been investigated systematically in rats only (Yoshimura & Nakamura, 1965; Larsson, 1975*a*, *c*; Larsson & Maunsbach, 1975).

The aim of the present study was to obtain further ultrastructural information about the postnatal development of the glomerular filtration barrier and in addition to describe the concomitant development of the proximal tubule in the pig. The changes observed are compared with the functional development which takes place in newborn piglets (Friis, 1979).

MATERIALS AND METHODS

The experiments were performed on two newborn (1 day old), four 3 weeks, one 6 weeks and two 35 weeks old female pigs (Danish Landrace). The animals of the first three groups were anaesthetized by intraperitoneal injections of pentobarbital sodium, 20 mg/kg body weight, while the animals of the last group were pre-treated with azaperonum (Sedaperone® vet.), 2·8 mg/kg body weight intramuscularly and about 30 minutes later anaesthetized by intravenous administration of pentobarbital sodium, 10–15 mg/kg body weight.

The kidneys were fixed by retrograde perfusion with glutaraldehyde-containing fixatives through the aorta, in the newborn pigs by a procedure for the rat reported by Maunsbach (1966) and in the remaining pigs by a procedure described by Elling,

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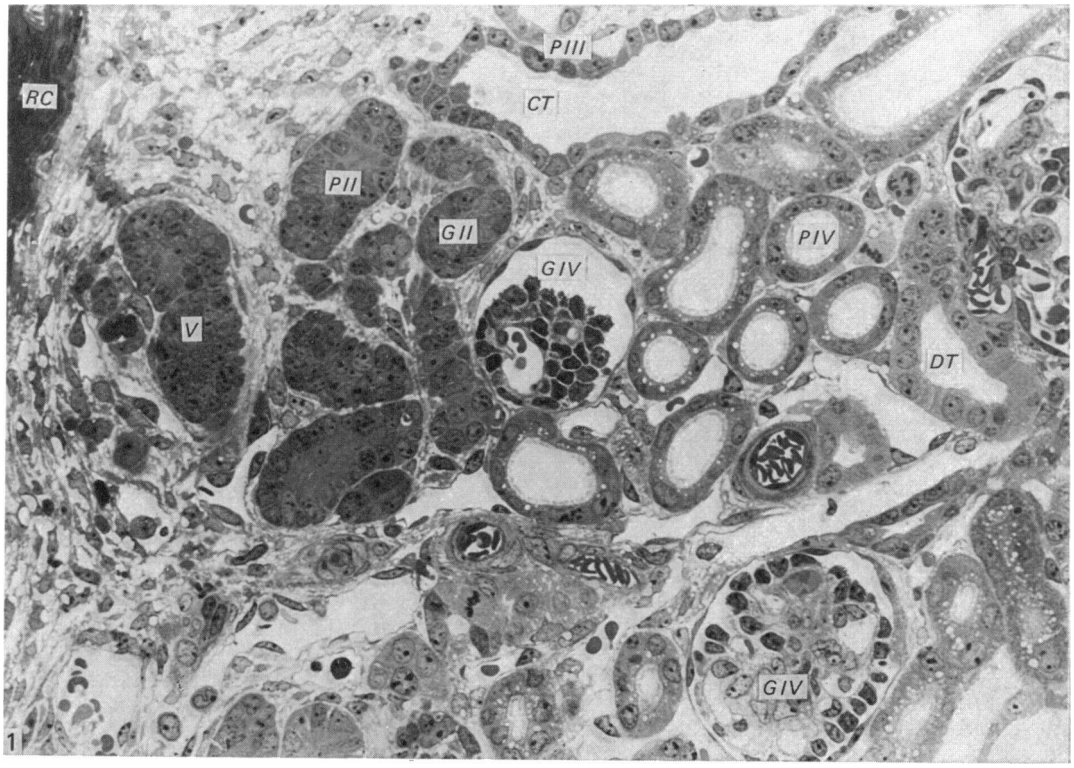
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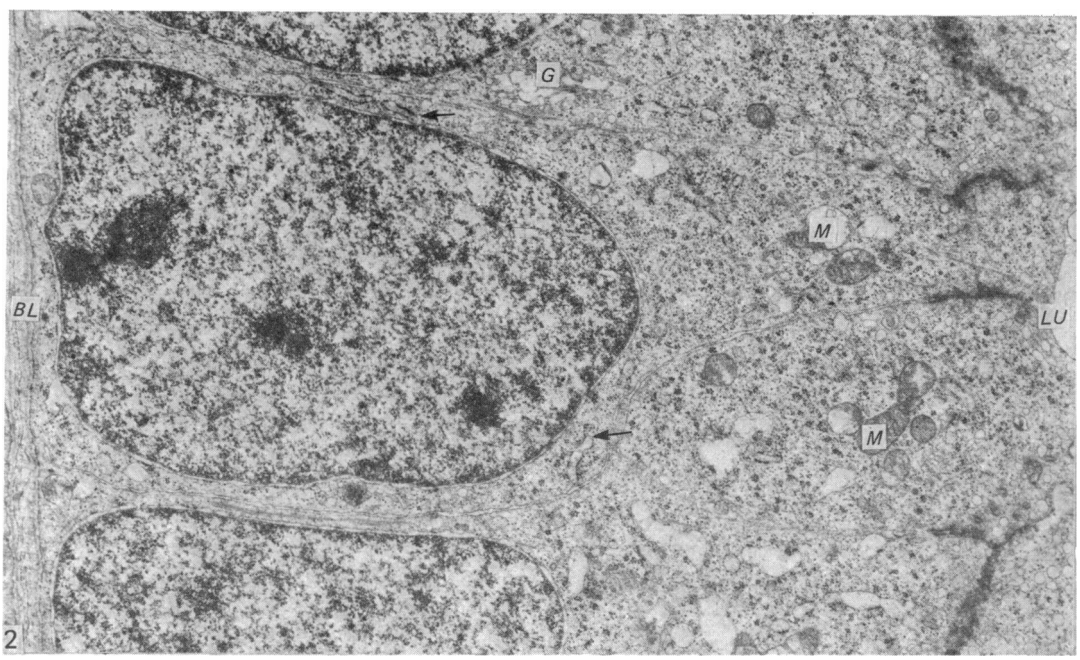
G
M
BL
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2

Hasselager & Friis (1977). In order to obtain an adequate preservation of the first nephron stages, the concentration of glutaraldehyde for the developing renal cortex must be raised in comparison to that suitable for the adult kidney (Larsson, 1975*b*). Accordingly, the following fixatives were used for the different age groups.

Newborn: 6 % glutaraldehyde (Larsson, 1975*b*) and 2·5 % polyvinylpyrrolidone (PVP) in Tyrode solution, pH 7·3, 960 mOsm/kg H_2O (Hi-precision osmometer, Advanced Instruments).

3 *weeks*: 2·5 % glutaraldehyde and 2·5 % PVP in Tyrode solution, pH 7·3, 590 mOsm/kg H_2O.

6 *weeks and* 35 *weeks*: 1 % glutaraldehyde (Maunsbach, 1966) and 2·5 % PVP in Tyrode solution, pH 7·3, 420 mOsm/kg H_2O.

When fixatives of 2·5 % and 6 % glutaraldehyde were used, the perfusion was started with a pre-rinse of Tyrode solution containing 0·4 % lidocaine chloride (pH 7·3, 300 mOsm/kg H_2O) (Larsson, 1975*b*) for about half a minute. The pre-rinse of Tyrode solution was followed by the fixative without interruption of the flow and continued for 5–10 minutes.

Following perfusion, small tissue pieces were obtained from the subcapsular layer of the cortex, the middle area of the cortex and the juxtamedullary area. After additional fixation by immersion for 1–2 hours in the same fixative as used for perfusion, the tissue was rinsed in Tyrode solution and post-fixed for 1 hour in 1 % osmium tetroxide in 0·1 M cacodylate buffer, dehydrated in increasing concentrations of ethanol, and embedded in Epon. Sections 1 μm thick were cut and stained with toluidine blue for light microscopy. Ultrathin sections were cut on an LKB Ultrotome, stained with uranyl acetate and lead citrate and examined at an accelerating voltage of 80 kV in a Siemens 101 electron microscope.

RESULTS

Newborn piglets

In the newborn piglet a thin nephrogenic zone was present in the outer one fourth of the cortex. The nephrons showed a gradient in the degree of development with the mature nephrons in the juxtamedullary area and the immature nephrons toward the capsule (Fig. 1). The development of the nephron could be divided into five stages.

Stage I

Stage I corresponds to the renal vesicle. At this stage of nephron development, the glomerular anlage could not be distinguished from the tubular anlage. The

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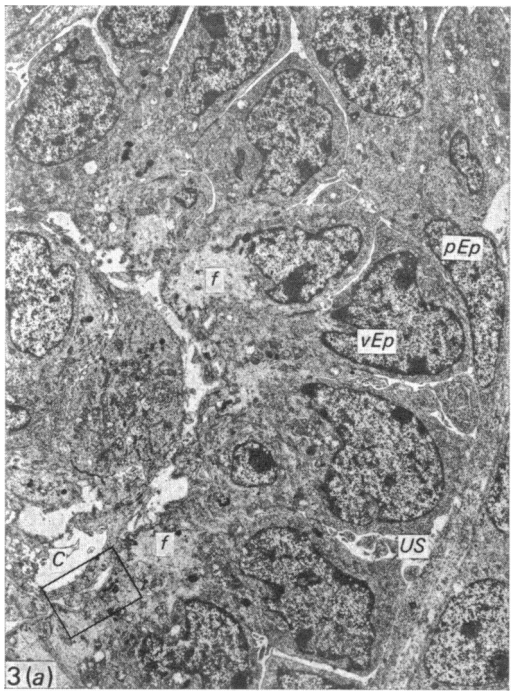
All the figures are from 1 day old piglets. Fig. 1 is a light micrograph, the remaining figures are electron micrographs.

Fig. 1. Section through the superficial renal cortex of a newborn pig. A renal vesicle (*V*) during transformation to an S-shaped body and an S-shaped body with its glomerular anlage (*GII*) and proximal tubular anlage (*PII*) are located below the renal capsule (*RC*). Glomeruli in Stage IV (*GIV*) and proximal tubules in Stage III (*PIII*) and Stage IV (*PIV*) are seen deeper in the cortex. CT, collecting tubule; DT, distal tubule. ×400.

Fig. 2. Cells from the renal vesicle, Stage I, which is surrounded by a thin basal lamina (*BL*). Most of the mitochondria (*M*) are located above the nucleus. Several ribosomes are free in the cytoplasm and only a few cisterns of rough endoplasmic reticulum (arrows) are present. *G*, Golgi complex; *LU*, lumen. ×12000.



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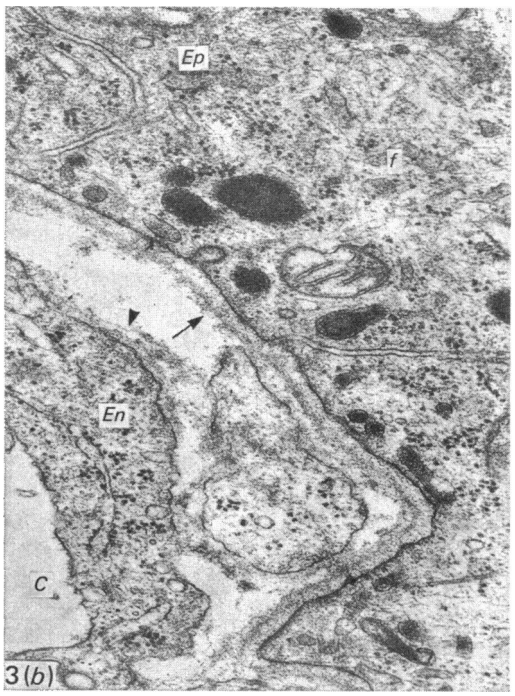

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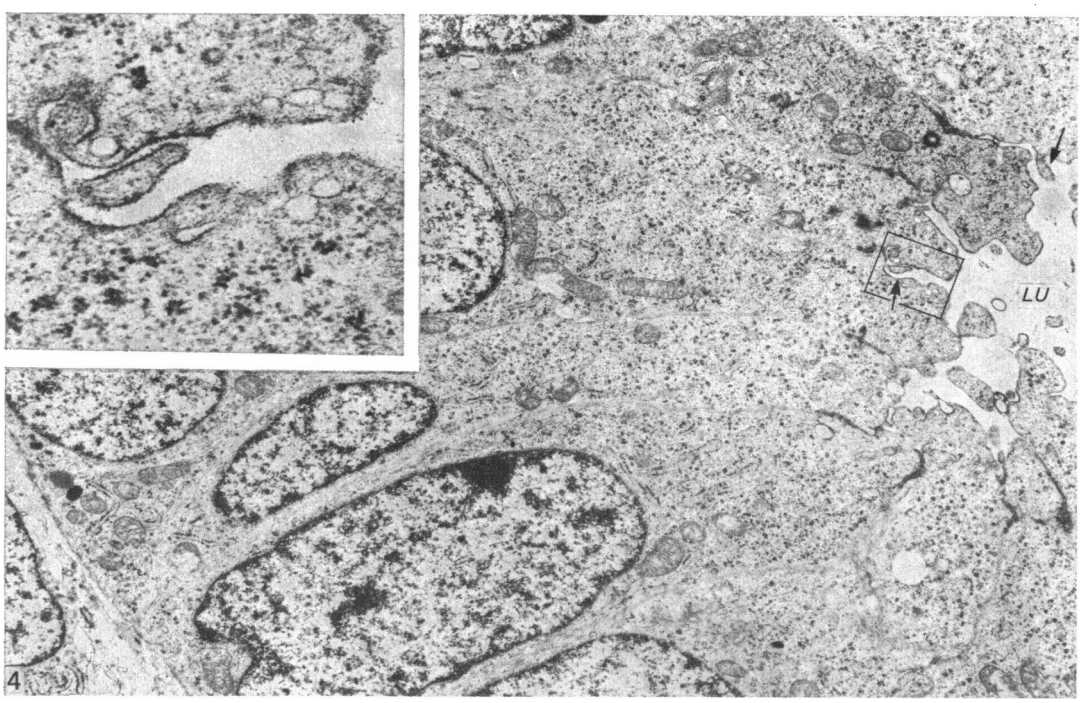

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vesicle (Fig. 2) was round or oval with the cells radially oriented around the narrow lumen. A thin basal lamina separated the vesicle from the surrounding tissue. The vesicle cells were apically sealed by occluding junctions. The large nuclei lay basally and the cytoplasm contained a few randomly distributed mitochondria, a small Golgi complex, sparse rough endoplasmic reticulum, a few small apical vesicles and many polyribosomes.

Stage II

This stage corresponds to the S-shaped body. The upper part of the S-shaped body (the prospective distal tubule) was connected to the collecting tubule, while the lower part formed the glomerular anlage. The *glomerular anlage* (Figs. 3*a*, *b*) was a double-walled bowl as judged from serial sections, the outer wall forming the parietal epithelium of the renal corpuscle and the inner wall, the visceral epithelium. The visceral epithelial cells were separated by narrow intercellular spaces and occluding junctions were seen at different levels down the lateral cell membranes between the cells. The large nuclei were located apically and in the perinuclear areas several mitochondria, a small Golgi complex, many membranes of the rough endoplasmic reticulum and many polyribosomes were present. Dense aggregates of filaments about 5 nm in diameter filled most of the basal part of the cells (Figs. 3*a*, *b*). The filaments were randomly oriented in a light ground substance and between them ribosomes, polyribosomes and a few lysosome-like bodies occurred.

Small glomerular capillaries were seen close to the concave side of the visceral epithelium. The endothelial cells contained abundant organelles, and the endothelial cytoplasm was continuous without fenestrae (Fig. 3*b*).

The basal laminae of the visceral epithelium and the endothelium were usually separated (Fig. 3*b*). The epithelial basal lamina was about 90 nm in thickness and appeared with an outer electron-dense layer and an inner less electron-dense layer. The endothelial basal lamina was a loose layer with indistinct margins.

The *proximal tubule anlage* (Fig. 4) of the S-shaped body was circular or oval in cross section and had a narrow lumen. Generally, the tubule cells had the same ultrastructural appearance as the renal vesicle cells, but from the irregular apical cell surface, small prospective microvilli protruded into the lumen; they contained centrally located thin filaments, as seen in microvilli of mature proximal tubule cells (Fig. 4, inset). Single cilia were occasionally present in this and subsequent stages.

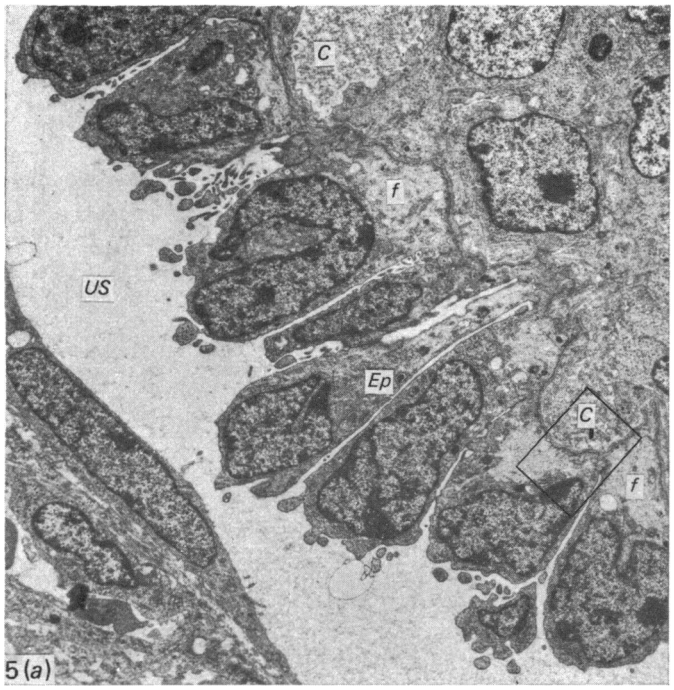
Stage III

The *glomerulus* (Figs. 5*a*, *b*) was spherical or oval and a distinct Bowman's space and a few capillary loops were present. The visceral epithelial cells were still separated by narrow intercellular spaces, in the basal third of which occluding junctions of

Fig. 3. (*a*) Survey picture of a glomerular anlage of the S-shaped body, Stage II. The parietal (*pEp*) and visceral epithelia (*vEp*) are located close together in a semicircular formation. Capillaries (*C*) are seen close to the visceral epithelium. Note the light areas of aggregated filaments (*f*) in the visceral epithelial cells. *US*, urinary space. × 3000.

Fig. 3. (*b*) Part of 3(*a*). Capillary (*C*) in close proximity to the visceral epithelium (*Ep*). Aggregates of filaments (*f*) are located in the basal part of the visceral epithelial cells. The endothelial cells (*En*) are thick without fenestrations. The endothelial (arrowhead) and epithelial (arrow) basal laminae are separated. × 32000.

Fig. 4. Part of proximal tubule anlage in Stage II. The nuclei are located in the basal part of the cells and almost all the organelles in the apical part. A few small prospective microvilli (arrows) protrude into the lumen (*LU*). × 8000. Inset shows the marked area of one prospective microvillus containing thin filaments centrally. × 38500.



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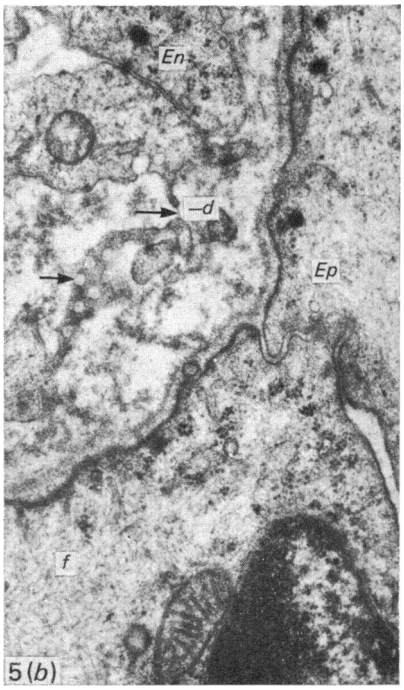

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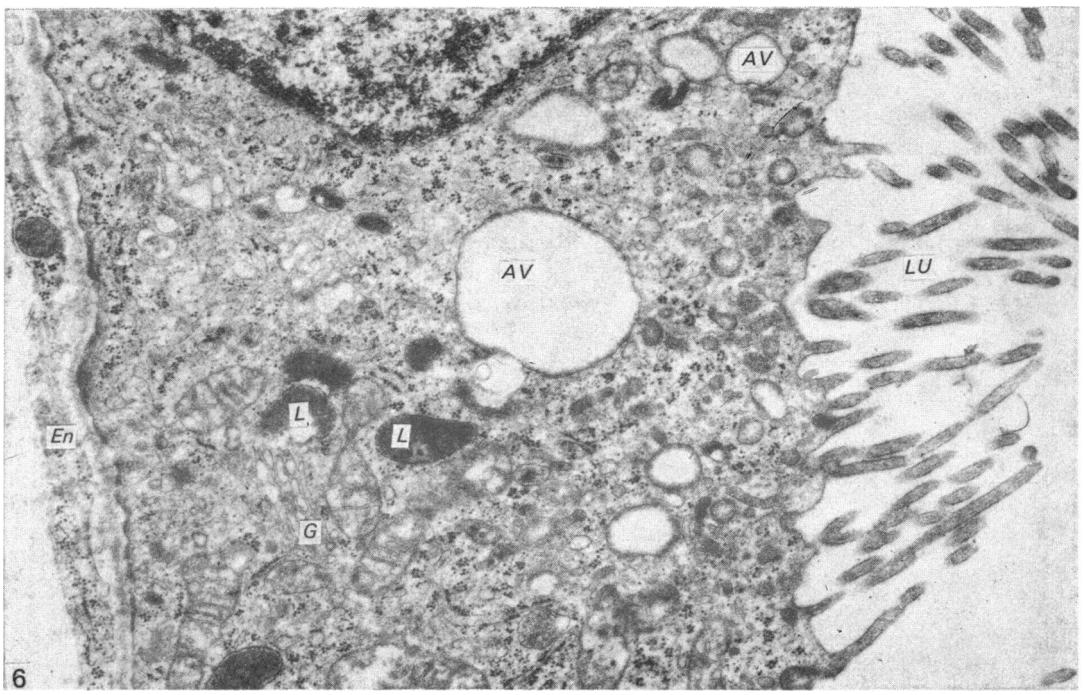

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different lengths were seen. The nuclei were located apically in the cells. The mitochondria, rough endoplasmic reticulum and large aggregates of filaments showed the same appearance and distribution as in the previous stage. The Golgi complex, however, was more prominent and composed of several large cisterns.

The cytoplasmic part of the endothelial cells was more elongated than in Stage II and in thin portions of the cells, small pores or fenestrae closed by thin diaphragms could be observed (Fig. 5*b*). The fenestrae were about 60 nm in diameter.

The epithelial and endothelial basal laminae were separated, as seen in Stage II, but that of the endothelium was more distinct and appeared with an outer electron-dense layer. The thickness of the epithelial basal lamina was about 100 nm (dense layer 50 nm) and of the endothelial basal lamina about 60 nm (dense layer 30 nm).

The *proximal tubules* in this and the following stages were identified in serial sections on the basis of continuity with a glomerulus. At Stage III the cells of the proximal tubules (Fig. 6) had decreased in height and the luminal diameter had increased compared to the previous stage. The number and length of microvilli varied considerably between tubules and between cells within the same tubule. Only a few lateral and basal interdigitations of the cell membrane were seen. The round or oval nuclei occupied a large part of the cell volume. The cytoplasm contained slightly more, randomly distributed mitochondria than in Stage II, a well developed Golgi complex with long and narrow cisterns, rough endoplasmic reticulum and many polyribosomes. A few small and large endocytic vacuoles were present in the apical part of the cell, while lysosome-like bodies and a few lipid droplets were located basally.

Stage IV

The *glomerulus* was larger and contained more capillary loops than in Stage III. The visceral epithelial cells (Fig. 7) were separated by wide spaces and adjacent cells were only connected by occluding junctions at their bases. In this stage, formation of foot processes was initiated. The young foot processes were separated from neighbouring cellular bodies by narrow semicircular spaces. The distribution pattern of the cytoplasmic filaments changed as the formation of foot processes proceeded. The light areas of aggregated filaments disappeared while bundles of filaments could be recognized in the foot processes. The extension of the Golgi complex was further increased, while the other organelles appeared as in Stage III.

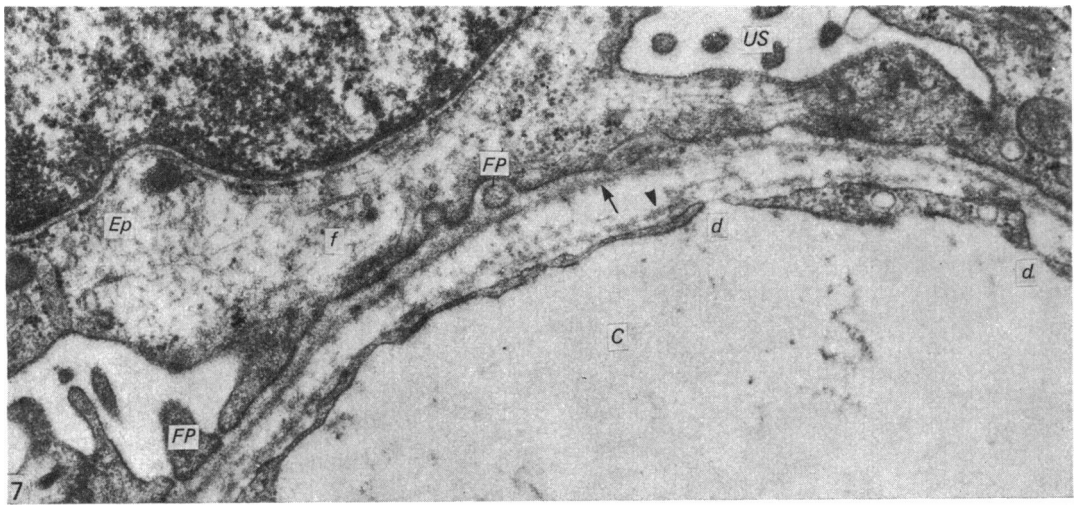
The cytoplasmic layer of the endothelial cells was thin and the fenestration was more frequent than in the previous stage. The fenestrae were still closed by thin diaphragms (Fig. 7).

The epithelial and endothelial basal laminae were separate in some regions while in others, where the epithelium and endothelium appeared close to each other, they formed the typical mature basal lamina with a central electron-dense layer lined by two less electron-dense layers. In the slit between well developed foot processes the

Fig. 5(*a*). Part of glomerulus in Stage III. The glomerulus contains few capillaries (*C*) and the intercellular spaces of the visceral epithelial cells (*Ep*) are narrow. Light areas containing filaments (*f*) are recognized basally in the visceral epithelial cells. *US*, urinary space. × 3800.

Fig. 5 (*b*). Part of 5 (*a*), which shows the connection between the epithelium (*Ep*) and endothelium (*En*). The endothelium has small fenestrae (arrows), which are closed by thin diaphragms (*d*). *f*, filaments. × 22500.

Fig. 6. Part of proximal tubule in Stage III. A few microvilli protrude into the lumen (*LU*). Small and large apical endocytic vacuoles (*AV*) appear first in this stage. *L*, lysosome-like bodies; *G*, Golgi complex; *En*, endothelium. × 16000.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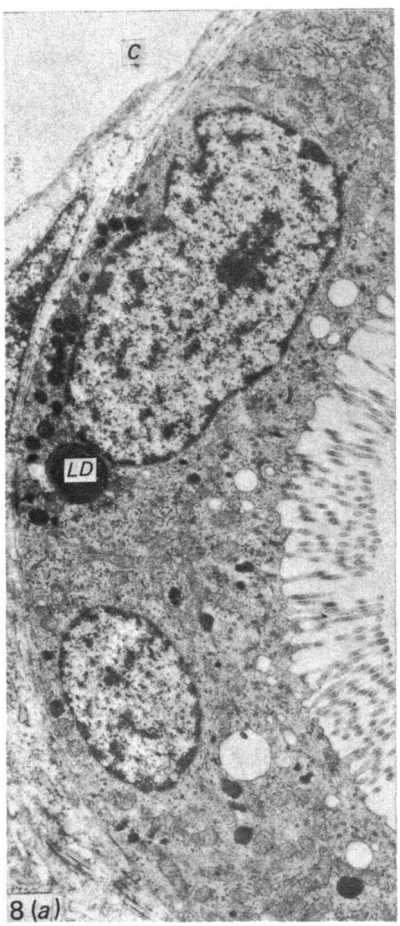
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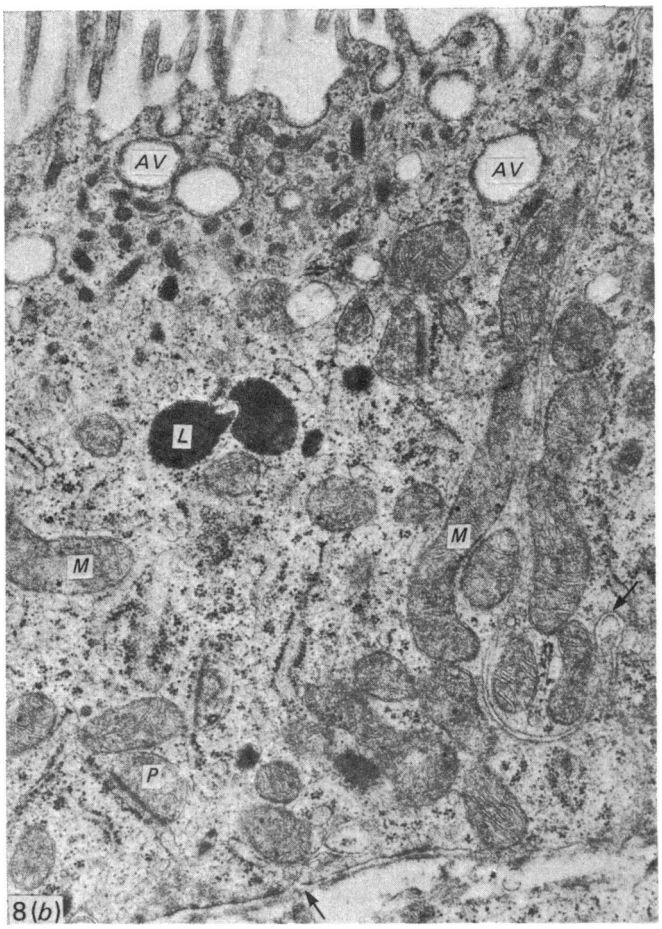
AV
AV
L
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P
8 (b)

surface of the epithelial or common basal lamina was usually covered with a slit membrane. The thickness of the separated epithelial and endothelial basal laminae was about 110 nm (dense layer 60 nm) and 80 nm (dense layer 40 nm), respectively. The common basal lamina measured about 160 nm (dense layer 70 nm).

The *proximal tubule* cells (Figs. 8*a*, *b*) had decreased further in height and the number and length of microvilli still varied between the cells. Adjacent cells showed moderate lateral and basal interdigitations. The oval nuclei were located basally and many small and large endocytic vacuoles were present apically. The number and size of mitochondria, the number of dense bodies and the extension of the Golgi complex were increased in comparison to Stage III, while the amount of rough endoplasmic reticulum and the number of polyribosomes were unchanged. Most of the dense bodies were presumed to be lysosomes and lipid droplets, while a few were identified as peroxisomes due to their typical marginal plate. The peroxisomes were in close association with membranes of the endoplasmic reticulum on the surface of which ribosomes occasionally occurred. In some cells several lipid droplets of different size were accumulated close to the basal plasma membrane (Fig. 8*a*).

Stage V

The *glomerulus* contained multiple capillary loops. The visceral epithelial cells (Fig. 9) had achieved their mature flattened configuration with the primary foot processes subdivided into numerous small interdigitating processes. Bundles of filaments filled the foot processes while they were rarely seen in the perikaryon.

The endothelium was largely fenestrated and the diaphragms covering the fenestrae appeared only occasionally. The diameter of the fenestrae ranged from 100 to 140 nm.

A mature basal lamina with three layers was present overall. Its thickness ranged from 140 nm to 200 nm and the central electron-dense layer from 70 to 130 nm.

The *proximal tubules* (Fig. 10) had a brush border of constant width and the cells showed numerous lateral and basal interdigitations. In contrast to previous stages, the cytoplasmic ground substance was relatively electron-dense. The number of randomly distributed mitochondria, apical endocytic vacuoles, lysosome-like bodies and peroxisomes was increased compared with Stage IV. Some of the peroxisomes demonstrated two marginal plates. The Golgi complex and rough endoplasmic reticulum showed the same appearance as in Stage IV, while the number of polyribosomes was decreased. A few large lipid droplets were recognized in the basal part of the cells but they were not as prominent as seen in the previous stage.

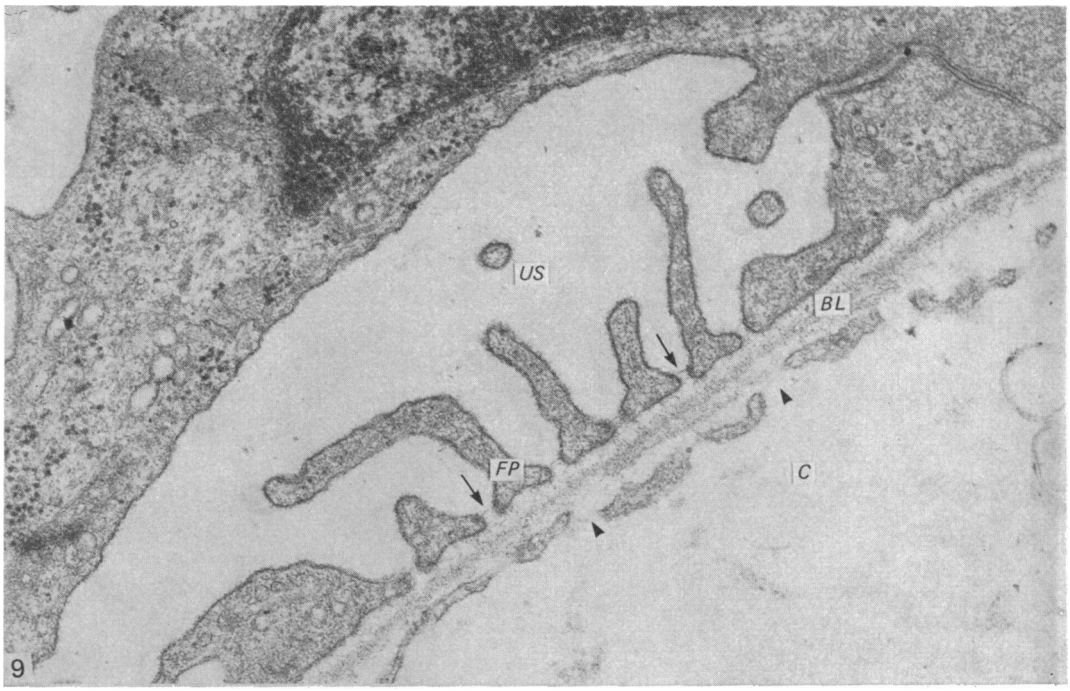
3 weeks, 6 weeks and 35 weeks old pigs

In two 3 weeks old piglets, small nephrogenic areas were present below the renal capsule and all five developmental stages of the nephron could be recognized. In the

Fig. 7. Part of a glomerulus in Stage IV. The visceral epithelial cells (*Ep*) have few foot processes (*FP*). The fenestrae in the endothelium are still closed by thin diaphragms (*d*). The endothelial (arrowhead) and epithelial (arrow) basal laminae are separated. *f*, filaments; *C*, capillary; *US*, urinary space. × 27500.

Fig. 8(*a*). Part of proximal tubule in Stage IV. Note the accumulation of lipid droplets (*LD*) in the basal part of one of the cells. *C*, Capillary. × 6000.

Fig. 8(*b*). Proximal tubule cells in Stage IV showing a few basal and lateral interdigitations (arrows). The mitochondria (*M*) are large, and many apical endocytic vacuoles are present (*AV*). *P*, peroxisome with marginal plate; *L*, lysosome-like body. × 18000.



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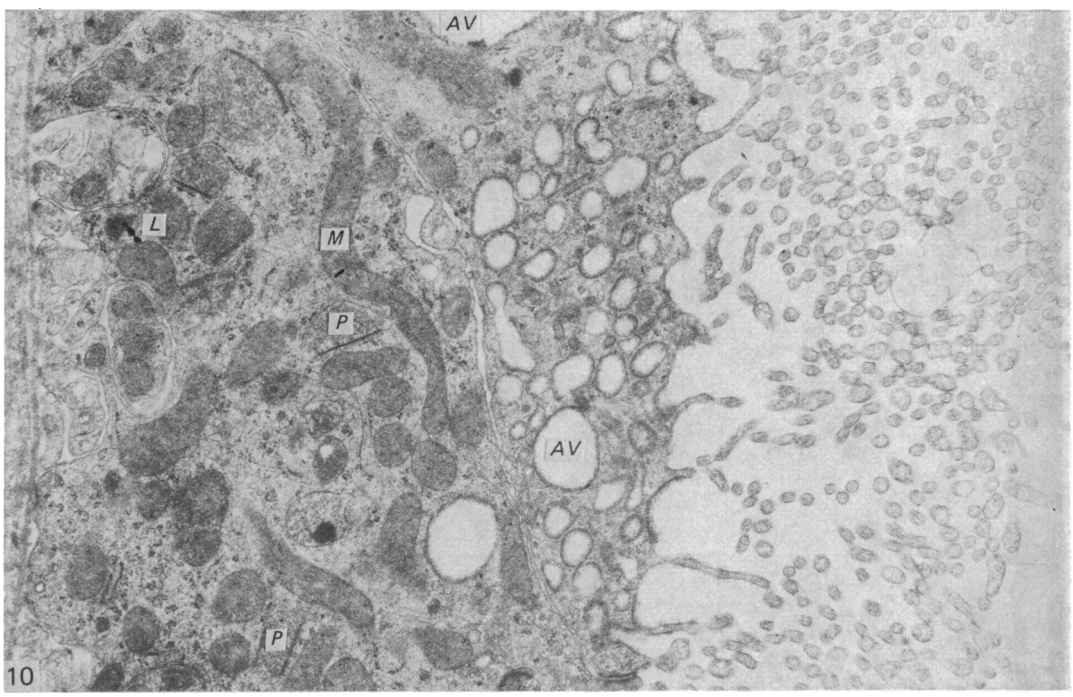

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other two 3 weeks old piglets, the formation of new nephrons was completed; nephrons were identified from Stage IV to V. All the nephrons in 6 weeks and 35 weeks old pigs corresponded to Stage V. However, in the 6 weeks old pig, the superficial glomeruli and tubules were smaller than those in the juxtamedullary region. In both 6 weeks and 35 weeks old pigs, the endothelial fenestrae were without diaphragms.

DISCUSSION

The observations on the general development of the nephron from the renal vesicle to the S-shaped body and further on to the mature nephron confirm earlier light (Potter, 1972) and electron microscopic studies (Jokelainen, 1963; Kazimierczak, 1971; Larsson, 1975*a*). However, at the ultrastructural level this study describes new features of the developing glomerular filtration barrier and proximal tubule in a species not often used as a laboratory animal.

Development of glomerulus

Development of the glomerular filtration barrier includes three processes: Formation of visceral epithelial foot processes, flattening and fenestration of the endothelium and formation of a common epithelial and endothelial basal lamina.

In contrast to previous work dealing with the developing visceral epithelial cells (Kazimierczak, 1971; Miyoshi, Fujita & Tokumaga, 1971; Hay & Evan, 1979) this study reveals large areas of aggregated filaments in these cells. The appearance of well preserved filaments may be explained by the high concentration of glutaraldehyde used for fixation. During formation of foot processes the filamentous areas disappear and the localization of the filaments shifts to the foot processes. Filaments have been found in a variety of cell types and among the many functions attributed to them are axon elongation of neurons (Yamada, Spooner & Wessells, 1971), dendrite elongation of melanocytes (Jimbow & Fitzpatrick, 1975) and cell motility of macrophages (Reaven & Axline, 1973) and of epidermal cancer cells (Malech & Lentz, 1974). The change in distribution pattern of filaments seen in the present study suggests that they are involved in the elongation of the foot processes and subsequently act as a skeleton in these structures.

The present observations demonstrate a concomitant increase in the number and diameter of the endothelial fenestrae from Stage III and onwards, contributing to a facilitation of filtration. Rhodin (1962) has described the endothelial fenestrae as closed by diaphragms in the mouse glomerulus in contrast to that reported for the fenestrae in the glomerulus of the rat and of man (Farquhar, 1975). Endothelial diaphragms have also been recognized in the glomerulus of the newborn piglet (Kazimierczak, 1971; Bergelin & Karlsson, 1975). The present observations show the appearance of endothelial diaphragms in the immature glomerulus which disappear during differentiation of the endothelium.

Fig. 9. Glomerular filtration barrier in Stage V. The visceral epithelial cells have many foot processes (*FP*). In the spaces between the foot processes thin slit membranes are seen (arrows). The fenestrae in the endothelium are without diaphragms (arrowheads). The basal lamina (*BL*) has three layers, one electron-dense middle layer lined by two less electron-dense layers. *C*, capillary; *US*, urinary space. ×40000.

Fig. 10. Part of proximal tubule in Stage V. The basal interdigitations are prominent. Many apical endocytic vacuoles (*AV*), mitochondria (*M*) and peroxisomes (*P*) with one or two marginal plates are present. *L*, lysosome-like body. ×15800.

The observation of separate epithelial and endothelial basal laminae up to Stage IV confirms previous suggestions (Farquhar, Wissig & Palade, 1961; Vernier & Birch-Andersen, 1962) that both epithelial and endothelial cells contribute to glomerular basal lamina synthesis. Since the thinning of the endothelial cells occurs before the lamina is fully formed it is likely that the epithelial cells, rich in organelles, form most of the lamina. The total basal lamina and the electron-dense layer within it show a moderate increase in thickness during development, first by fusion of the two separated basal laminae and subsequently by further growth.

Tracer studies with peroxidase in the newborn rat indicate onset of glomerular filtration in a developing stage corresponding to the present Stage III (Larsson & Maunsbach, 1975). The basal lamina has generally been accepted as the main barrier for passage of molecules in the same range as plasma proteins in the mature glomerulus (Farquhar, 1975) while the endothelium, due to the small number of fenestrae, appears to be an additional barrier in the immature glomerulus (Webber & Blackbourn, 1970). On the basis of tracer studies with ferritin, Vernier & Birch-Andersen (1963) have suggested that the basal lamina of the human glomerulus becomes a more effective filter during maturation, an assumption which agrees with the increasing thickness of the basal lamina seen both in the developing human glomerulus (Vernier & Birch-Andersen, 1962) and in the present study. On the contrary Artursson, Groth & Grotte (1971) measured the renal clearance of dextran of different molecular sizes in infants and adults and concluded from the results of their studies that the pore radii of the glomerular basal lamina of the neonate were relatively small and increased with age.

Development of the proximal tubule

Each developmental stage of the proximal tubule corresponds to a rather well defined developmental stage of the glomerulus. During maturation the apical cell surface of the proximal tubule is enlarged by formation of microvilli, change of cell shape from a tall pyramidal to a short cylindrical form and by an increase in tubule diameter. In the present study single microvilli appear in the proximal tubule anlage of the S-shaped body, Stage II, while Larsson (1975*a*) reported that the microvilli in the rat tubule formed in a developing stage corresponding to the present Stage III.

As previously mentioned, Larsson & Maunsbach (1975), using peroxidase as a tracer, found that glomerular filtration occurs in nephrons of a developing stage corresponding to the present Stage III but not in the S-shaped body, Stage II. The present study agrees with these results based on the following observations: (*a*) The width of the tubule lumen at Stage II is small and increases markedly from Stage II to Stage III; (*b*) the cells of the proximal tubule anlage in Stage II appear with an irregular apical surface, probably due to the fixative having penetrated from the vascular side of the tubule only; and (*c*) the large apical vacuoles occur in the tubule cells no earlier than Stage III, simultaneously with an increase in the amount of lysosome-like bodies indicative of onset of tubular absorption in this stage.

The increase in the number of mitochondria from the first two developmental stages of the proximal tubule to Stage III and onward suggests an enhanced energy requirement for cellular processes which corresponds with the increase in the amount of apical vacuoles and lysosome-like bodies and may reflect a greater tubular absorption. The present study shows the mitochondria to be randomly oriented both in the immature and mature proximal tubule of the pig. This is in contrast to the

mitochondria in the rat tubule, where they gradually become oriented perpendicular to the basal lamina during development (Larsson, 1975*a*).

Dense bodies identified as peroxisomes first appear in cells at Stage IV, i.e. when glomerular filtration is presumably well established. The findings agree with the observations by Larsson & Maunsbach (1975) and these authors have suggested that the peroxisome acts in cellular processes that arise after onset of glomerular filtration. Among other functions, the peroxisomes have been associated with lipid metabolism (Masters & Holmes, 1977). Accordingly, the high content of lipid droplets in the tubule cells at the present Stage IV may be a result of the small number of peroxisomes seen at this stage.

Morphological development as related to functional maturation

The present study demonstrates a continuous formation of new nephrons up to about 3 weeks of age; after this time the morphological development consists of a differentiation of nephrons already present. Thus, in the first weeks of life a heterogeneous pattern of nephron stages is present in the piglets.

In an accompanying functional study in piglets, the glomerular filtration rate (GFR), estimated as inulin clearance, and the active tubular secretion of organic anions, estimated as PAH clearance and renal PAH extraction, were measured from birth to 8 weeks of age (Friis, 1979). During this period GFR and clearance of PAH increase from 0·27 to 0·59 ml/min/g kidney and from 1·02 to 1·72 ml/min/g kidney, respectively. At 8 weeks of age GFR and PAH clearance reach adult values. PAH extraction increases from 0·75 at birth to its adult value, 0·86, at 3 weeks of age. Though clearance data in the developing kidney represent a composite from nephrons at different stages of maturity, the above-mentioned results and the present ultrastructural observations of different age groups demonstrate that functional and structural development are closely related.

SUMMARY

The detailed anatomy of the structures forming the glomerular filtration barrier and the proximal tubule was studied during postnatal development of the pig kidney.

Development of the glomerular filtration barrier included three processes: Formation of visceral epithelial foot processes, flattening and fenestration of the endothelium and formation of a common epithelial and endothelial basal lamina. During formation of foot processes the distribution pattern of aggregated filaments in the epithelial cells was changed, suggesting that the filaments participate in the elongation of the foot processes. In the immature glomerulus the endothelial fenestrae were closed by thin diaphragms which disappeared during differentiation of the endothelium.

Microvilli were observed in the proximal tubule cells in an early developmental stage, presumably before onset of glomerular filtration. Few cellular organelles were seen in the tubule anlage but the number of mitochondria, apical vacuoles and lysosome-like bodies increased markedly during maturation. Dense bodies identified as peroxisomes first appeared in a late developmental stage.

The formation of nephrons continued up to about 3 weeks of age; after this time the morphological development was a differentiation of nephrons already present. The present observations, together with results of an accompanying functional study, demonstrate that changes in functional parameters reflect the structural development.

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