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DIFFERENTIATION OF THE CHICK MESONEPHROS

by

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A thesis  
presented to the University of Ottawa  
in partial fulfillment of the  
requirements for the degree of  
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## ABSTRACT

We have conducted a histological and electron microscopical study of the chick embryo mesonephros with emphasis on the early aspects of its differentiation.

Chick embryos were staged according to the method of Hamburger and Hamilton. The mesonephroi of the staged embryos were dissected, fixed and processed for both light microscopy (Hematoxylin/Eosin and Periodic Acid Schiff stains) and electron microscopy.

Fully differentiated mesonephroi were found in all embryos at stage 27 or older. In these mesonephroi, nephrons were found to possess the general characteristics found in typical vertebrate nephrons. The glomeruli possessed capillaries with a fenestrated endothelium, the basal lamina presented 3 layers and podocytes had a large number of interdigitating pedicels. Proximal tubules had a typical brush border, micropinocytotic vesicles and numerous lateral interdigitating finger-like processes; distal tubules presented few apical microvilli and a fair amount of basolateral infoldings. These findings confirm previous observations by other authors conducted on mesonephroi of chick embryos of older ages.

In all embryos younger than those in stage 27, the mesonephroi contained a mixture of fully differentiated nephrons and others in the process of differentiating. The degree of nephron differentiation was determined according to the degree of development of the filtration

barrier in the glomerulus, the size and number of microvilli and pinocytotic vesicles in the proximal tubules. Fully differentiated nephrons were found only after stage 24; the most differentiated nephrons in embryos at stages 20, 21, 22 and 23 had only a partially differentiated filtration barrier. Since urine formation is known to occur after stage 20, it is suggested that the immature nephrons found between stages 20 and 24 are probably functional. However, physiological evidence is still required to confirm their functional status.

These morphological observations will serve as a guidance for experiments in our laboratory in which mesonephric function will be challenged through various experimental procedures and pharmacological agents.

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## Chapter I

### LITERATURE REVIEW

#### 1.1     DEFINITIONS

In all vertebrates, the kidneys are formed by multiple units usually called nephrons. These units are generally (although not always)\* made up of a vascular corpuscle (malpighian corpuscle, glomerulus) and a more or less complex tubule. All nephrons are formed from a specialized part of the mesoderm known as the intermediate mesoderm or nephrotome, which rapidly grows forming two lateral ridges known as urogenital crests.

Embryologists have classified the types of kidney according to which part of the nephrogenic ridges they originate from. Thus, the pronephros originates from the cephalic part of the ridge; the mesonephros from the middle part of the ridge and the metanephros from the caudal part of the ridge.

Comparative anatomists have established that, in addition to having a different embryological location, the pronephros, mesonephros and metanephros differ in their morphological and functional characteristics (see Torrey, 1965 for review). The pronephros is formed by tubules which open into the coelomic cavity through the nephrostomes as well as

■ \*There exist within the vertebrate group some agglomerular forms, for example, certain species of marine fishes do not have a vascular glomerulus as is the case of most vertebrates. It is believed that the absence of this structure is a secondarily acquired adaptive characteristic of these forms during the course of their evolution.

into the pronephric duct. In addition, it consists of glomeruli which are devoid of Bowman's capsules so that the filtrate drains directly into the coelomic cavity. Several glomeruli are fused in some, vertebrates into a single body known as the glomus (Krause, et al, 1979).

The mesonephros is formed by nephrons which in groups of 1 to 4 end in a pair of longitudinal ducts known as mesonephric ducts or Wolffian ducts. The mesonephric nephrons have usually closed malpighian bodies which drain urine directly into the tubules as opposed to the open pronephric glomeruli in which the Bowman's capsule is absent, the visceral epithelium being continuous with that of the coelomic epithelium. The mesonephros secretes a hypotonic urine.

The metanephros is formed by a very large number of nephrons secreting into an excretory system of repeatedly branching ducts known as collecting ducts. The duct receiving all the urine secreted by each kidney is known as ureter. In addition to this complex excretory system, the metanephros may develop a specialized segment of the nephron known as the loop of Henle which is a water-saving device. In the avian metanephros there are two populations of nephrons: a predominant population (70-90%) of "reptilian type" (RT) nephrons located in the cortex of the kidney without loops of Henle and a smaller population (10-30%) "mammalian type" (MT) nephrons located in the medulla with loops of Henle (Johnson and Mugaas, 1970). In avian medullary nephrons, the loop of Henle has been demonstrated to form the anatomical basis for urine concentration through a countercurrent mechanism (Poulson, 1965; Braun and Dantzler, 1972). Blood to the avian kidney comes from two

sources. The renal arteries supply oxygenated blood to kidneys, in addition there is a renal portal system carrying venous blood from the external iliac veins into the renal tissue. The renal portal system supplies peritubular capillary network playing a major role in the elimination of uric acid and urea through the tubules (Siller, 1971). The portal vein has a valve which regulates the amount of afferent venous blood flowing into the kidney. A direct relationship has been suggested to exist between the number of medullary nephrons and urine osmolarity in different species of birds; the larger the proportion of medullary nephrons (with loops of Henle) in relation to the number of cortical nephrons the higher the urine concentrating ability of the species (McNabb, 1969).

Recent micropuncture studies of avian RT nephrons (Lavery and Dantzler, 1982) indicate that there is secretion and reabsorption of phosphate in the proximal tubules. Sodium and water are reabsorbed in all segments of the nephron. Potassium is reabsorbed by the proximal tubules, but is also secreted by the distal segments.

Arginine vasotocin (AVT), the avian equivalent of the mammalian antidiuretic hormone arginine vasopressin (AVP), has been demonstrated to have a direct effect on renal function in birds. Administration of AVT diminishes filtration by the RT nephrons presumably by causing constriction of the afferent arterioles. Although no direct experimental evidence exists, the possibility that AVT increases water permeability of the collecting ducts of MT nephrons has been suggested (Braun and Dantzler, 1984). Injection of 40ng/Kg of AVT decreases glomerular filtration rate by 41%, reduces the excreted fractions of

sodium and chloride ions by 66 and 70% respectively (Ames and Skadhauge, 1971). In birds, parathyroid hormone (PTH) regulates renal transport of calcium and phosphate ions. In micropuncture studies of RT nephrons, 45% of filtered calcium was found to be reabsorbed in the proximal tubules (up to the point of puncture). Since less than 2% of filtered calcium is normally present in urine, it has been suggested that the loops of Henle in avian MT nephrons reabsorb calcium as occurs in the mammalian metanephros (Braun and Dantzler, 1984). There is usually a net reabsorption of phosphate by the proximal tubule, however, net secretion can also occur (Lavery and Dantzler, 1982; Braun and Dantzler, 1984). Parathyroidectomy leads to net tubular reabsorption of phosphate and reduced reabsorption of calcium (Clark and Sasayama, 1981). It does appear that calcium and phosphate excretion are under the influence of PTH which regulates the net excretion or net absorption of these ions depending on the animals need of these ions.

From a comparative point of view, it may be added that the pronephros is usually found only in embryonic stages and larval forms of lower vertebrates. Mesonephroi are found in embryonic and adult fishes, adult amphibians as well as in embryonic reptiles, birds and mammals. Metanephroi are present in the late embryonic and the adult forms of reptiles, birds and mammals.

Other terms used with less frequency are opistonephros and holonephros. Opistonephros is used to designate the kidneys of many fishes which morphologically look like mesonephroi but are derived embryologically from both the middle and caudal parts of the nephrogenic ridge. On the other hand, Holonephros is the term used by some

investigators to designate a very infrequent type of kidney characterized by having each somitic segment give rise to a single nephron instead of several; other investigators (Gibley and Chang, 1967) used the term Holonephros to describe the concept that all kidneys should be considered as variations of a single organ, i.e. that the pronephros, mesonephros and metanephros are all varieties of the same organ derived from the nephrogenic ridge. These two terms (Holonephros and Opisthonephros) will not be used henceforth in this thesis.

## 1.2 DEVELOPMENT OF THE MESONEPHROS IN THE CHICK.

### 1.2.1 Origin of the Nephrogenic Ridge

At the onset of segmentation in the chick embryo, the zone of mesodermal cells which are destined to differentiate into the urogenital system of the embryo and adult are already well demarcated (Hamilton, 1952). The mesoderm is usually at this time divided into a medial somitic mesoderm and an intermediate mesoderm or nephrotome. While the somitic mesoderm forms segments, this segmentation does not extend laterally enough to include the intermediate mesoderm which remains as thin plates of mesodermal tissue interposed between the somitic mesoderm and the more lateral mesoderm. These plates grow to form the nephrogenic ridges which will give rise to a greater part of the urogenital system of the vertebrates (Abdel-Malek, 1950).

In the chick and other avian species, the intermediate mesoderm extends along most of the length of the developing embryo from the cervical to the caudal somitic segments. It is from these nephrogenic ridges as was discussed above that the succession of the various

"kidneys" (pronephros, mesonephros and metanephros) arises. The relationship of the pronephros and the pronephric duct with the subsequent ontogeny of the mesonephros has been studied extensively in the chick (Sedgwick, 1880, 1881; Gruenwald, 1942, 1943; Abdel-Malek, 1950). The pronephric ducts are developed from the fusion of several aggregates of mesenchymal cells located in the nephrogenic ridges opposite to the 10th to 14th segments. These aggregates develop into cords which later acquire lumina thus becoming the pronephric ducts (Abdel-Malek, 1950). These pronephric ducts grow in a caudal direction giving rise to the Wolffian ducts or mesonephric ducts. The importance of the pronephric ducts resides in the fact that their caudal continuation (the Wolffian ducts) appear to induce the formation of mesonephric and later metanephric tubules (Boyden, 1927; Waddington, 1938; Gruenwald, 1942; Bishop-Calame, 1966). Seven pairs of pronephric primordia appear as early as 30 hours of incubation (11 somites) extending from the fifth to the eleventh somitic segments. Four more pairs are added, and in all eleven pairs of pronephric tubules are finally developed. These primordia do acquire lumina but their degeneration ensues soon after this (at forty hours of incubation = 20 somites) (Abdel-Malek, 1950). The pronephric tubules are therefore considered to be non functional in the chick since their degeneration is virtually completed by sixty hours of incubation i.e. at about the 30 somite stage (Abdel-Malek, 1950).

### 1.2.2      Differentiation of Mesonephric Nephrons

The mesonephros is made up of nephric units (nephrons) which consist of a glomerulus attached to a proximal tubule by a neck. The former continues into the distal tubule which is in turn connected to the Wolffian duct by a 'collecting' segment (Stampfli, 1950). The differentiation of the nephric mesenchyme into functional nephric units has been studied in histological sections by a number of workers (Boyden, 1924, 1927; Abdel-Malek, 1950; Stampfli, 1950).

The earliest indication of formation of mesonephric nephrons occurs by aggregation of mesenchymal cells of the nephrogenic ridge into solid cellular spheres (Riedel, 1874); these spheres (also known as renal vesicles) become enlarged as a result of proliferation of their cells. Subsequently, they become transformed into S-shaped tubes. The end of the S-shaped tube which is located distal to the mesonephric (Wolffian) duct will differentiate into the Bowman's capsule of the future mesonephric corpuscle (Malpighian body). The rest of the "S" tubule, will become the various segments of the mesonephric tubule (proximal, distal and intermediate). As the tubule grows and differentiates, the two contralateral curves of the "S" persist. They are responsible for the formation of the two flexures observed in isolated mature nephrons: one distal (in a juxta position relative to the glomerulus) and the other proximal, this is located within the organ as a loop (Stampfli, 1950; Friebova, 1981). The two flexures serve as reference points for determination of the tubule segments in the mature nephron. The proximal tubule is represented by the segment commencing from the glomerulus to the secondary flexure. The intermediate (transition)

segment is a short tubule interposed between the end of the proximal tubule and the beginning of the distal tubule; the latter eventually connects with the terminal distal segment (Stampfli, 1950; Gibley and Chang, 1967).

It is generally accepted that the collecting duct originates from the distal segment of the initial S-shaped tubule and later establishes connection with the mesonephric duct (Croissille, et al, 1971, 1975). This was demonstrated by Martin (1971) using grafts from quail into chicks. In these experiments quail cells can be distinguished by their nuclear characteristics. When Wolffian ducts of quail embryos were grafted into chick mesonephric ridges, either very few or not a single quail cell was found in the resulting ducts.

### 1.2.3 Chronological and Topographical Distribution of Nephrons

The primordia of the functioning mesonephroi are located between the 20th and the 30th somites (Abdel-Malek, 1950; Romanoff, 1960). The first nephrons to be formed at the level of the 20th somite appear at about 3 days. The last ones to be formed appear between the 5th and the 6th days (Stampfli, 1950; Friebova and Goncharevskaya, 1982). From then onwards, growth of the mesonephros takes place only by growth of the existing nephrons. Classical works of Stampfli (1950) and Abdel-Malek (1950) had suggested the existence of a definite pattern of nephron organization and chronological differentiation within the mesonephros. They showed that differentiation progresses in a cephalocaudal direction and that several generations of nephrons appear at successive stages. This became evident in cross sections of embryos between the 4th and 6th days in which nephrons at different stages of maturation co-existed at any given level.



More than any other worker, Friebova (1981, 1982) studied extensively the pattern of distribution of nephron populations within the mesonephros. These studies involved detailed morphometric analyses as well as microdissection techniques (Friebova, 1982). In summary, her findings: a) confirm the existence of two distinct gradients of nephron differentiation, one being cephalocaudal and the other ventrodorsal and b) demonstrate that five populations of nephrons occur in total in the organ (Friebova, 1982). These populations were defined by the topographical positions of their glomeruli. Three of the populations were located ventrally and named V1, V2 and V3. V1 are the first nephrons to be formed and extend all along the mesonephric ridge, V2 and V3 were formed later and were restricted to the caudal two thirds of the ridges.

Two more populations were dorsally located (D3 and D4) and were also restricted to the caudal two thirds of the ridges. After defining the populations, the author made accurate determinations of the number of nephrons in each group. She found an average of 76 to 131 nephrons in each mesonephros of the 6-day chick embryo.

### 1.3 MORPHOLOGY OF THE FULLY DIFFERENTIATED MESONEPHROS

#### 1.3.1 Histology

The histological structure of the chick mesonephros has been the subject of study of many embryologists since the second half of the last century. In a very extensive publication on the mesonephros of the chick embryo as well as those of other avian embryos Stampfli (1950) summarized existing knowledge and included numerous additional

observations. His description of the mesonephros in the chick embryo has been extensively quoted by subsequent authors (Hamilton, 1952; Romanoff, 1960). According to Stampfli, the mesonephric nephron consists of the glomerulus, proximal tubule, intermediate tubule, distal tubule and the collecting tubule which terminates in the Wolffian duct. The glomerulus is spherical and consists of a capillary tuft surrounded by a double-walled, cup-shaped Bowman's capsule with an inner visceral and an outer parietal epithelium. The cells of the two epithelia have different histological appearances. The parietal epithelium is made up of squamous cells; it reflects over the glomerular capillaries as the visceral epithelium. The visceral epithelium consists of columnar cells that are arranged around the glomerular capillaries, separated from the endothelial cells by a basement membrane.

The proximal tubule is continuous with the outer parietal epithelium and its lumen with that of the glomerular urinary space. The cells are columnar with an average diameter of  $45\mu\text{m}$  and a height of 14 to  $18\mu\text{m}$  (Romanoff, 1960). Their nuclei are located towards the base of the cells and their apical parts present a brush border which is absent from other segments of the nephron. The cells lie on a basement membrane. The intermediate (transition) segment follows directly the proximal tubule. The characteristics of the epithelium changes abruptly. The cells in the intermediate portion of the nephron are only  $10\mu\text{m}$  high with a less granular cytoplasm and centrally placed nuclei. The distal tubule follows the intermediate segment. Its cells are high cuboidal,  $8\mu\text{m}$  high and bulge slightly into the lumen. The cytoplasm is more granular than in cells of the preceding segment. The last segment (the

collecting segment) is short, with cells averaging 10 $\mu$ m in height and the tubule having a slightly larger diameter (60 $\mu$ m) than other segments of the nephron. The transition from distal to collecting segments is sometimes gradual and at other times abrupt (Romanoff, 1960).

The course of the nephron in the organ was first described by Chambers and Kempton (1933) and following that by Stampfli (1950) and more recently by Friebova (1981). These studies indicate that the proximal tubule after originating from the medially situated glomerulus travels laterally and then forms a loop returning to the parent glomerulus where it gives rise to the intermediate segment. This segment is in turn followed by the distal segment which returns towards the lateral aspect of the mesonephros and continues into the short collecting duct which in turn joins the Wolffian duct.

#### 1.3.2 Histochemistry

In the chick mesonephros the presence of periodic acid-Schiff (PAS) positive material was investigated by Moog and Wenger (1952) and Gibley (1967). In these studies, PAS reaction product was confined to the brush border of proximal tubule cells, the basement membranes of the glomeruli and those of the tubules. Gibley (1967) reported no PAS-positive granules in the cytoplasm of the tubule epithelium. Moog (1944) demonstrated alkaline phosphatase activity localized principally on the brush border of the proximal tubule epithelium; other nephric segments were negative. This observation was later confirmed by Junqueira (1952) who showed the same pattern of alkaline phosphatase activity only in the proximal tubules of both the chick mesonephros and metanephros. Subsequently, Gibley (1964, 1967) demonstrated the enzyme

to be clearly confine to the columnar cells of the proximal tubule and not in the distal, collecting and Wolffian ducts.

The mesonephros undergoes degenerative changes and ceases to function before hatching, this degenerative process has been termed regression or involution. The chick mesonephros has been used extensively as a model to study enzymatic and biochemical changes that occur in regressing embryonic tissues. It is well known that this process in the chick mesonephros begins during the second half of incubation and proceeds until hatching and is accompanied by increased activity of acid hydrolases (Tilney, 1964; Russo-Caia and Hassan, 1965; Salzgeber and Weber, 1966; Patel, 1967; Morris, 1967; Wang, 1972).

### 1.3.3 Ultrastructural Studies

Electron microscopic studies of the chick mesonephros are rare in the literature. Gibley and Chang (1967) studied the fine structure of the functional 8-day chick mesonephros. Their study indicates that the mesonephric nephron has ultrastructural characteristics similar to those found in the kidneys of most vertebrates. The glomerular visceral epithelial cells (podocytes) were shown to have pedicels resting on the capillary basement membrane. The three layers of the basement membrane were similar to those described in avian metanephric glomeruli (Pak Poy and Robertson, 1957). They also described the ultrastructure of the proximal, distal, conducting(intermediate) and collecting segments: (Gibley and Chang, 1967). In the proximal tubules they found microvillous projections on the apical plasma membrane which constitute the brush border as well as subapical pinocytotic vesicles; both indications of active reabsorption.

#### 1.4 MESONEPHRIC FUNCTION

The mesonephros is the principal organ of excretion for the first half of incubation before the onset of metanephric function at about the eleventh day of incubation. One of its functions is to pump large volumes of water and ions into the allantois. For this reason, mesonephric function cannot be understood without a knowledge of water and ion balance in the embryo.

##### 1.4.1 Water and Mineral Metabolism

When laid, the egg's water represents 66% of the total egg mass. This water is distributed in the two fluid compartments present at this time, namely the albumen and the yolk. About 75% of the total water is contained in the albumen and 25% in the yolk. During the 21 day incubation period, 16% of the total water content of the egg is lost through evaporation (Hoyt, 1979), although there is considerable variation in this figure depending on the relative humidity of the incubating environment. Simkiss (1980) reported that a water loss of upto 30% (produced by removing shell overlying the air-chamber) has no apparent harmful effect on the embryo. To the water already present in the egg, metabolic water is later added as the embryo develops. This addition becomes significant only after the fifth day when lipids start to be used predominantly by the embryo as its energy source (Needham, 1931). The albumen is a solution containing large amounts of proteins (10.5%), water (87.84%) and ions (Iseki, 1930; Romanoff, 1967). The concentrations of the various ions in the albumen are different from those found later in the embryonic blood plasma. Thus, the albumen is relatively poor in calcium (0.01%) but rich in potassium (0.12%, Na/K:

1.1). It is hypotonic with regards to plasma (Howard, 1957; Dawes, 1975). Starting about the second day of incubation large amounts of water are transferred from the albumen to the yolk. This water does not mix completely with the yolk but most of it remains accumulated on the upper pole of the yolk sac under the blastoderm forming the subembryonic fluid (New, 1956). The subembryonic fluid reaches a maximum volume of 13ml on the 7th day of incubation (Romanoff, 1967). The transfer of fluid from the albumen to the yolk appears to be an active process. Using cultures of whole embryos, New (1956) showed that the blastoderm is capable of pumping actively, fluid from its dorsal to its ventral (inner) surface. It is thus possible that the blastoderm also mediates in vivo the transport of ions and water from albumen to yolk. Stern and Mackenzie (1983) studied the transport of Na through the epiblast using electrophysiological techniques as well as autoradiography after 3H ouabain injection and cytoimmunological techniques with antibody against rabbit Na-K-ATPase. They concluded that the main activity of the epiblast consists of active Na transport from its dorsal (apical) to its ventral (basal) aspects and that the cells on the lower surface are responsible for this transport.

These experiments tend to indicate that transport from albumen to subembryonic fluid is active with respect to Na and that water and other components follow Na. The subembryonic fluid is, like albumen, hypotonic with respect to blood and also relatively rich in potassium (Howard, 1957). During the first half of incubation the embryo absorbs the subembryonic fluid rapidly; it is thus faced with the entrance of large amounts of hypotonic fluid and must get rid of the excess water.

It does so by eliminating hypotonic urine through the mesonephros and into the allantoic sac (Simkiss, 1980).

Water, Na<sup>+</sup> and Cl<sup>-</sup> are known to be resorbed from the allantoic fluid (Hoyt, 1979). This transport takes place across the endodermic epithelial lining of the allantoic sac (Terepka, et al, 1969; Saleuddin and Kyriakides, 1976). The embryo can probably regulate this resorption process and recuperate in later stages as needed the water temporarily stored as urine in the allantoic sac. It has thus been shown that the volume of allantoic fluid increases steadily from the 5th to the 13th day of incubation but starts to decrease from that age until hatching (Romanoff, 1967; Freeman and Vince, 1974).

In addition to the allantois, another compartment is formed by the embryo: the amniotic sac. The amniotic fluid is isotonic with respect to blood (Howard, 1957; Faber et al, 1973). It serves protective purposes and appears to influence the water and ionic metabolism of the embryo only indirectly. During the second half of incubation, when the subembryonic fluid is exhausted, the supply of fluid from the yolk sac to the embryo diminishes. This is in part compensated by a) metabolic water, and b) resorption of water and proteins of the albumen via the amniotic fluid 'swallowed' by the embryo (Romanoff, 1960; Stewart and Terepka, 1969).

#### 1.4.2 Elimination of Nitrogen Wastes

Deamination of amino acids takes place in all vertebrates in the liver and results in the production of ammonia. The liver of ureotelic animals transforms most of this ammonia into the less toxic urea, which is then excreted through the kidneys. As in other uricotelic groups,

birds lack the enzyme carbamyl phosphate synthetase (Tamir and Ratner, 1963; Griminger, 1976) and cannot make the above mentioned conversion from ammonia to urea; most of the ammonia is converted instead by the liver into hypoxanthine. Hypoxanthine is then converted into uric acid either by the liver, the kidneys or both. In the chick, uric acid is also the main excretory product for purines (Goldie, 1959). In addition to uric acid, uricotelic animals also produce small amounts of urea through the conversion of ornithine and arginine from ingested proteins. During the first half of incubation the chick embryo starts producing ammonia and urea (Clark and Fischer, 1957; Fisher and Eakin, 1957) and then increasing amounts of uric acid (Freeman and Vince, 1974). While both urea and hypoxanthine are produced by the liver, the conversion of hypoxanthine into uric acid takes place mainly in the kidneys as indicated by the presence of hypoxanthine dehydrogenase in the mesonephros and its absence in the embryonic liver (Chaube, 1962). As development progresses, the uric acid formed accumulates in the allantoic sac where it precipitates in the form of urates which combine with mucoproteins secreted by the allantois to form large complexes (Porter, 1963).

#### 1.4.3 Evidence of Mesonephric Function.

Physiological studies were conducted by early embryologists on the chick mesonephros primarily to determine whether or not the organ functions (Boyden, 1927; Chambers and Kempton, 1933). Bakounine (1895), injected indigo carmine into the vitelline vessels of the chick embryo and was able to demonstrate the presence of this dye in the cells of the proximal tubules of the mesonephros. Although the results were not



interpreted correctly at the time, we now know that the dye was eliminated through the glomeruli and concentrated by the proximal tubules; there are thus indications of both filtration by the glomeruli and reabsorption by the proximal tubules. Other workers injected trypan blue and trypan red into the air chamber of the egg (Zaretsky, 1910; Hanan, 1927), and showed the dye to be present in the amniotic fluid. Similar experiments were conducted by Chambers and Kempton (1933); they injected phenol red into the amniotic cavity, yolk sac and extraembryonic veins of the nine day old embryo and in all cases a light pink coloration was observed in the allantois. The cells of the proximal tubules of the mesonephros were distinctly colored. They also showed in cultured mesonephros that proximal tubule cells can transport and secrete phenol red from the culture medium into the lumen.

Boyden (1924, 1927) determined the functional capability of the mesonephros by destroying the growing ends of the Wolffian duct and noted that the mesonephros became hydronephrotic in the regions preceeding the destroyed zone, and that the allantois remained rudimentary. Where the Wolffian duct was destroyed on one side, there was an enlargement of the opposite mesonephros indicating compensatory hypertrophy.

#### 1.4.4 Onset of Mesonephric Function

Most of the evidence for function of the chick mesonephros is based mainly on histochemical and electron microscopical studies as described earlier (sections 1.3.2 and 1.3.3.) as well as on studies on the composition of allantoic fluid (section 1.3.4).

Embryologists have been interested in establishing the exact age at which the mesonephros starts functioning using various dyes (indigo carmine, trypan blue, trypan red, phenol red). Most authors have established the onset of function between the 4th and 5th day of incubation (Boyden, 1924; Hanan, 1927; Hurd, 1928; Chambers and Kempton, 1933). In most cases, the detection of the dye in the urine depended not only on the presence of a functional mesonephros but on effective absorption of the dye from the site of injection. However, these classical authors did not follow uniform procedures to establish the ages of the embryos. This probably explains the disagreement between their conclusions and the fact that urine is present in the allantois as early as three and half days (Hamburger and Hamilton, 1952). On the other hand, electron microscopical studies of the mesonephros before the 8th day of incubation are not available in the literature.

#### 1.4.5 Rationale For the Present Study.

The laboratory for Experimental Embryology at the Department of Anatomy, University of Ottawa has been engaged for several years in the study of chick embryos cultured without shell. They constitute an excellent model for the study of calcium regulation in the chick embryo (Narbaitz and Jande, 1978; Narbaitz, 1979). Burke et al (1979) found, however, that the blood of cultured embryos presented among other changes hypoproteinemia and low haematocrit. Some of the embryos presented edema. Numerous experiments have been conducted in order to establish the cause(s) of these changes. Modifications in the composition of the gas phase, increase or decrease in the relative

humidity and many other variables have not succeeded in eliminating the disequilibrium in fluid metabolism. A modification of the culture medium consisting in addition of pure albumen to the culture medium resulted in tripling the volume of urine (Narbaitz, et al, 1983).

The fact that one can modify the volume and composition of the urine by changing the medium makes this system ideal for the analysis of mesonephric function. Establishing the exact age of the onset of mesonephric function becomes important because in these experiments embryos can be explanted only on the 3rd and 4th days of incubation. One would like to introduce changes in the culture medium only at a time in which the mesonephros is functioning. The present report is a first step in an extensive study of mesonephric function in vivo and in vitro. It consists of a) confirming the electron microscopical characteristics of the fully differentiated mesonephros and b) studying the electron microscopical characteristics of the differentiating mesonephros in order to establish the stage at which it acquires ultrastructural characteristics compatible with function.

## Chapter II

### MATERIAL AND METHODS

Eggs from White Leghorn hens were obtained from commercial sources. They were incubated in a forced air circulation incubator (Petersime Incubator Co.) at 37 °C and 60% relative humidity with automatic turning every 2 hours. The eggs were candled from the third day of incubation onwards.

Staging of the chick embryos was accomplished using the Hamburger-Hamilton method (Hamburger and Hamilton, 1951). Their staging procedure is based on a number of parameters including the state of development of pharyngeal arches, eye pigmentation, size of the allantois, the length of the fore limb and the hind limb buds, the number of somites and the extent of the primary dorsal curvature. Because of the variability of incubation conditions in different laboratories, a given stage is reached at different chronological ages. In our laboratory the chronological ages (counted from the time at which eggs were put in the incubator + 4 hours) required to obtain a given stage were 6 to 12 hours older than the one indicated by Hamburger and Hamilton (1951) and are expressed in brackets below.

## 2.1 Staging Procedure

### Stage 19 ( 3 days)

The number of somites is 37-40; the leg buds are slightly larger than the wing buds; the contour of the posterior part of the trunk is straight; the tip of the tail curved forward. The maxillary and mandibular processes form distinct swellings having approximately the same length. The allantois is a small pocket and not yet vesicular. The eyes are unpigmented.

### Stage 20 (3 1/2 to 4 days)

The somitic segments are 40-43 and extend along the length of the embryo except for the tip of the tail. The leg buds are slightly larger than the wing buds. The maxillary process is distinct, and equal to or longer than the mandibular process. The allantois is now vesicular and averages the size of the mid-brain. A faint grey pigmentation of the eyes is present. 5 embryos of stage 20 were studied with the EM and 3 for light microscopy

### Stage 21(3 1/2 to 4 days).

Somitic segmentation of the embryo is complete with somites numbering 43-44. The dorsal curvature of the embryo includes the lumbosacral region, the contour of the trunk is straight or slightly bent. The maxillary process is longer than the mandibular process and extends to the middle of the eye. The allantois is variable in size but larger than in stage 20. Eye pigmentation is still faint. 6 embryos of this stage were used for light microscopic study and 6 were examined with the EM.

### Stage 22 (4 days)

Somites, limb buds and posterior flexure are similar to stage 21 but the allantois has grown considerably and may overlap the fore-brain. The eye pigmentation is distinct. 3 embryos were examined at light microscopic level and 5 embryos with the electron microscope.

Stage 23 (4 to 4 1/2 days)

Both limbs are as long as they are wide. The maxillary process is lengthened further. The dorsal curvature forms a continuous line from the hind-brain to the tip of the tail. 10 embryos were sectioned for EM study and 4 for light microscopy.

Stage 24 (4 1/2 to 5 days)

Both limb buds are longer than they are wide. The toe plate of the leg bud is distinct but the toes are not yet demarcated. 4 embryos were studied with the EM.

Stage 25 (5 to 5 1/2 days)

The elbow and knee joints are distinct in both dorsal and ventral views. A faint groove demarcating the third toe is present. The maxillary process is fused with the wall of the nasal groove. 3 embryos were sectioned for EM study.

Stage 26, (5 1/2 to 6 days)

Demarcations of the first three toes are distinct. The contour of maxillary process forms a broken line. The mandibular process is lengthened ventrally.

Stage 27 (6 days)

The beak rudiment is visible.

For stages 26 and 27, a total of 12 embryos (8 for electron microscopy and 4 for light microscopy) were examined as mature (fully differentiated) mesonephroi.

## 2.2 Microdissection.

At various times of incubation eggs were periodically removed and staged according to the Hamburger and Hamilton method. A total of 59 embryos were dissected at all developmental stages. In the younger stages, the whole embryo was transferred with the aid of a glass dropper into a dissecting Petri-dish containing the appropriate fixative. The lateral body walls were separated (where union of opposite body walls had occurred) by an incision made in the mid-longitudinal plane along the line of fusion of the abdominal walls. The heart, mesentery and the developing gut were removed, thus exposing the urogenital ridge. Removal of the urogenital ridge was accomplished by passing a fine dissecting needle between the urogenital ridge and the dorsal body wall. Both ridges were transferred into specimen tubes and identification labels affixed. In some of the embryos at stages 20 and 21 the urogenital ridges were not dissected; rather, the caudal portion of the embryo was fixed so that the position of the ridges relative to the somites could be determined.

## 2.3 Light Microscopy.

Specimens for light microscopical study were fixed for 6 to 12 hours in Bouin's fluid. They were then dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, infiltrated in paraffin and embedded in plastic moulds and left to solidify overnight. Serial sections (5um thick) were cut and processed for light microscopy.

### 2.3.1 Staining

The periodic acid-Schiff reagent was employed to distinguish between the different segments of the nephron. Previous studies by Gibley (1967) have shown that this procedure specifically stains the luminal brush border of differentiated proximal tubules and the basement membranes of the chick mesonephros.

The sections were divided into two pools, one stained with hematoxylin and eosin, the other pool stained with the periodic acid-Schiff method.

#### Periodic Acid-Schiff Reagent.

Three solutions were prepared for this reaction.

SOLUTION 1: A fresh solution of 0.5% periodic acid (5.0g in 1000ml distilled water).

SOLUTION II (Schiff Reagent): 1.0g basic fuchsin dissolved in 200ml boiling distilled water, cooled to 50°C and to which 1.0ml of concentrated HCl and 2.0g sodium bisulfite and 0.5g absorbent charcoal were added.

SOLUTION III: A 2% sodium bisulfite solution. Sections were de-paraffinized with xylene and taken to distilled water passing through decreasing concentrations of alcohol. Sections were then oxidized with solution I for 10 minutes, stained with solution II for 12 minutes and treated with solution III for 1 minute and then mounted. Suitable sections were photographed using a Zeiss Ultraphoto II microscope.



## 2.4 Electron Microscopy.

### 2.4.1 Fixative:

Karnovsky's fixative (Karnovsky, 1965) was used as the fixative of choice throughout these studies. It was prepared by dissolving 2.8g paraformaldehyde in 35ml distilled water. The mixture was then warmed to 60°C while adding drops of 1N NaOH (3 drops) from a medicine dropper until a clear solution was obtained; to this were added 5.0ml of 70% glutaraldehyde and 30.0ml of 0.2M cacodylate buffer at pH 7.4. The strength of the solution was halved by adding 35.0ml of 0.2M cacodylate buffer, 25.0ml of distilled water and 10.5ml of 1% calcium chloride.

### 2.4.2 Buffers

Cacodylate buffer was prepared by dissolving 32.0g of sodium cacodylate in 800ml of distilled water, the pH was adjusted to 7.4 by adding drops of 1N HCl and monitoring the pH with a pH meter. The volume was brought up to 1000ml so that the working concentration was 0.2M. The washing buffer was made in a similar manner but with the addition of 68.4g of sucrose into 500ml of 0.2M cacodylate buffer and the molarity halved by addition of 500ml of distilled water to yield a final washing solution of 0.1M cacodylate and 0.2M sucrose.

### 2.4.3 Tissue Processing

Pieces of tissues for electron microscopy were fixed for 6 hours in 50% (diluted with 0.2M cacodylate buffer) Karnovsky's fixative (Karnovsky, 1965) at 4°C, washed overnight in 0.1M cacodylate buffer (pH 7.4) with 0.2M sucrose added. Post fixation was done in 1% osmium tetroxide buffered with 0.1M cacodylate buffer at pH 7.4 for 3 hours and

then dehydrated through a series of graded alcohols (30%, 50%, 70%, 90%, 95% , 2 changes of absolute ethanol and 3 changes of propylene oxide). The tissues were transferred into a 1:1 mixture of propylene oxide and Araldite embedding medium (Ladd Research Industries). The medium was prepared by mixing Araldite Resin 502, 20.0g; Dodecenyl succinic anhydride (DDSA), 13.6g and Tri (dimethyl amino methyl) Phenol (DMP-30), 0.46ml. Tissues were kept in this mixture for 12 to 24 hours and in pure Araldite for 4 hours. Embedding was done in freshly prepared Araldite in plastic moulds and cured in the oven at 35°C for 24 hours, 50°C for 24 hours and 60°C for another 24 hours. Sectioning was done on the Reichert OM U2 Ultramicrotome using glass knives. Sections that were suitable for electron microscopy (silver to gray - 90 to 160nm thick) were collected on water, in a plastic trough and picked up with uncoated 150 mesh size copper grids. Sections were first stained with Uranyl Acetate (50% alcohol solution) for 30 minutes and then with Lead Citrate according to (Reynolds, 1963). Grids were examined with a Philips 300 Electron Microscope. Relevant areas of the sections were photographed, and the plates developed with Kodak D-19 developer and fixed in Kodak Rapid Fixer. Positive prints were made on Ilford (Ilfospeed) 3.1M, 4.1M and 5.1M photographic paper using Durst (Laborator S-45 special) Enlarger.

## Chapter III

### RESULTS

#### 3.1 The Mature mesonephros

##### 3.1.1 Gross Characteristics

The fully developed chick mesonephros consists of paired, elongated structures located dorsal to the body cavity on either side of the mesentery. The mesonephros bulges into the body cavity forming, together with other urogenital organs, the so called "urogenital crests". There are apparently no differences in size between the left and the right mesonephroi; the sex of the individual becomes evident after the seventh day of incubation due to the asymmetry of the right and left gonads which appear on the ventral part of the crests (in the case of females, the left ovary being notably larger than the right one). The histological structure of the mesonephros becomes evident in cross and longitudinal sections (Figs. 1 and 2). The mesonephros is formed by functional units called nephrons. Each nephron includes a mesonephric corpuscle, proximal, intermediate, distal tubules, and terminal distal segments. All functional units open into the Wolffian duct which runs longitudinally. The different parts of the nephron appear both in Figure 1 and 2. The histological and ultrastructural characteristics of these parts are described in the following account.

### 3.2 The Mesonephric corpuscle.

#### 3.2.1 Light Microscopy.

The characteristics of the mesonephric corpuscle are illustrated in Figure 3. The corpuscle consists of a tuft of capillaries, the glomerulus, surrounded by a double-walled, cup-shaped capsule (Bowman's capsule) with a visceral layer apposed to the glomerular tuft and a parietal layer of flattened cells which continues distally as the epithelium of the neck segment of the proximal tubule.

The parietal epithelium consists of a single row of flattened squamous epithelial cells that is reflected over the glomerulus as the visceral epithelium and continues at the urinary pole distally as the proximal tubule (Fig. 3). The cells are 3 to 4 $\mu$ m high on the average at their thinnest part, and gradually increase in height distally as they approach the urinary pole (Fig. 3).

The visceral epithelium surrounds the numerous capillaries and is formed by a single row of cuboidal cells widely separated from each other. The lumen of the glomerular capillaries often contain numerous erythrocytes with rounded to oval nuclei. Surrounding the lumen are the endothelial cells which form a single row around the lumen of the capillaries (Figures 3 and 4).

The two epithelia (visceral and parietal) are separated by a patent space, the urinary space, which opens into the proximal tubule and continues as the lumen of the proximal segment of the nephron. The blood vessels forming the glomerulus are arranged around a central core. This central core is made up of a mass of cells, separated by a basement membrane from the glomerular capillaries (Figs. 3 and 4). Siller (1971)

is of the opinion that the cells of the avian central mass phagocytose macromolecules like those of the intercapillary mesangial cells of mammals based on the presence of dark lysosome-like bodies sometimes seen within these cells.

### 3.2.2 Electron Microscopy of the Mesonephric Corpuscle

#### 3.2.2.1 The parietal Epithelium

The electron microscopic observations showed that the apex of the flat cells of the parietal epithelium which are located close to the urinary pole of the mesonephric corpuscle have more microvilli than those in the vicinity of the vascular pole (Fig. 16); these microvilli are distinctly shorter than those found at the surface of the proximal tubules. The lateral surfaces of adjacent cells are joined by junctional complexes; their basal surfaces rest on a distinct basement membrane (Fig. 15 and 16). The cytoplasm contains the usual organelles, ribosomes, smooth and rough endoplasmic reticulum and a Golgi apparatus. A few membrane-enclosed granules and lipid droplets are dispersed throughout the cytoplasm. Cilia are occasionally found on the apical surface of the parietal cells.

#### 3.2.2.2 The Visceral Epithelium

The cells of the visceral epithelium are also referred to as podocytes due to the many foot-like processes (pedicels) they possess (Figs. 5 and 6). Unlike the parietal epithelium, the podocytes do not form a uniform row of cells but follow the irregular contour of the capillaries; their cytoplasmic projections (pedicels) are separated by a

basal lamina from the capillary endothelium (Fig. 5). Podocytes are relatively large cells. The main body of the cell is ovoid and has a large centrally placed nucleus containing one or two nucleoli (Fig. 5). The cytoplasm contains a moderate number of mitochondria and numerous cisternae of rough and smooth endoplasmic reticulum, a well developed Golgi apparatus with flattened cisternae and a few membrane-bound vesicles associated with them (Fig. 5). Pedicels were found to originate from the surface of the cell facing the capillary or from cytoplasmic extensions of podocytes which branch producing primary, secondary and sometimes tertiary processes. Some pedicels after arising from the parent podocyte abut directly on the basal lamina and are consequently very short, having to travel only a short distance from the parent podocyte (Fig. 6). Secondary and tertiary branches are known to interdigitate with those from other branches from neighbouring podocytes. Adjacent pedicels appear to be separated by very small spaces but quite often one finds that these spaces are bridged by what appears to be a fuzzy membrane (Fig. 6). These small diaphragms are, however, visible only in some of the spaces. Microtubules and microfilaments are constantly present in the cytoplasm of podocytes and are especially numerous in the cytoplasm of the primary branches (Fig. 6). Small pedicels contain few cytoplasmic organelles including small vesicles (Fig. 6). The large primary branches sometimes contain one or two mitochondria (Fig. 6).

### 3.2.2.3 · Glomerular Endothelium

This consists of flat cells with nuclei bulging into the capillary lumen (Fig. 5) usually containing two nucleoli. The cytoplasm of the endothelial cell contains the usual cytoplasmic organelles - free ribosomes, mitochondria, rough endoplasmic reticulum and Golgi cisternae. The endothelial cells in the glomerular capillaries are fenestrated (Fig. 5). The fenestrations are more numerous along the peripheral cytoplasmic extensions (Fig. 5). These fenestrations have variable diameters ranging from 500Å to 1000Å.

### 3.2.2.4 · Basement Membrane

The basement membrane (basal lamina) is interposed between the visceral epithelium (podocytes) and the capillary endothelium. Under the low magnification of the electron microscope (Fig. 5), it appears as a thin continuous layer, but with higher power magnifications (fig. 6), three distinct layers are apparent; a lamina rara interna, a lamina densa and a lamina rara externa. The central cell mass of the glomerulus is separated by a basement membrane from the capillary endothelium (Fig. 7). These cells do not appear to have special distinguishing features except for their large nuclei.

### 3.2.3 · The Proximal Tubule

### 3.2.3.1 Light Microscopy of the proximal tubule

As described earlier the parietal epithelium of the mesonephric corpuscle is continuous with the epithelium of the proximal tubule. The characteristic squamous epithelium increases its height and becomes the epithelium of the neck segment of the proximal tubule. The brush border, characteristic of the proximal tubule is initiated at this segment. In the neck region the cells are columnar but do not attain the height of those in the main portion of the proximal tubule. These cells range in height from 8 to 10 $\mu$ m, and have centrally placed nuclei. Their apical surface shows a light PAS positive reaction<sup>3</sup> (Fig. 2); this is also true of their basement membrane.

The proximal tubule proper has a taller epithelium (16 to 18  $\mu$ m high) and a brush border of about 2 $\mu$ m high. The brush border in these cells shows a strong positive reaction with the PAS stain as does the basement membrane.

### 3.2.3.2 Electron Microscopy of the Proximal Tubule

The neck region and the proximal tubule proper are ultrastructurally similar, the only difference consists of the height of the apical microvilli which is shorter in the neck segment. The epithelium of the proximal tubule proper beyond the neck segments is high columnar. The cells lie on a basement membrane which separates them from the endothelial lining of the intertubular capillaries (sinusoidal endothelium). The apical surfaces of the cells present numerous microvilli that project into the lumen of the tubule (Fig. 9). Large cytoplasmic projections into the lumen of the proximal tubule were often seen. Where these appear, the microvilli disappear or their



numbers are greatly reduced, the apical plasma membrane becoming a continuous loop over these projections. The cytoplasm immediately below the microvilli has a denser appearance than in other parts of the cell. This electron density is due mainly to the extensive amount of microfilaments; these are often found projecting into the microvilli. Mitochondria are uniformly distributed throughout the cytoplasm but are not usually found in the sub-luminal zone of high electron density below the attachment of the microvilli. Numerous pinocytotic vesicles are seen in the apical cytoplasm and some are in the process of being formed as indicated by the invagination of the apical plasma membrane in between the microvilli (Fig. 9). Some membrane bound granules were encountered in the luminal cytoplasm. Distributed mostly at the basal part of the cytoplasm are large dark staining granules (which presumably are lysosomes and lipid droplets) (Fig. 10). The Golgi apparatus is located in the supranuclear position while associated with its cisternae are secretory granules. A few profiles of rough endoplasmic reticulum are distributed throughout the cytoplasm. The basal plasma membrane does not show any infoldings. The large intercellular spaces separating adjacent cells contain numerous microvilli (Fig. 9).

#### 3.2.4 Intermediate Segment

This zone corresponds to the short thin segment located between the proximal and distal segments (Fig. 2). The transition between proximal and intermediate segments is abrupt (Figs. 2 and 11). The epithelium which in the proximal segment has a height of 16 to 18 $\mu$ m transforms into one of 6 to 8 $\mu$ m in the intermediate segment. In addition, the brush border of the proximal tubule ends abruptly, evidenced by the abrupt

disappearance of the apical PAS stain (Figs. 2 and 11). The position of the intermediate segment is usually located close to the parent glomerulus (Fig. 11), deep within the mesonephros and since it is short, it is not frequently encountered in sections.

The epithelium of the intermediate segment differs markedly from that of the proximal tubule ultrastructurally due to the conspicuous absence of apical microvilli (Fig. 12). The apical surface of the epithelium is smooth and presents a few short, blunt microvilli. Lateral plasma membranes infoldings are always present (Fig. 12). The basal membranes show a few infoldings. Cytoplasmic organelles are uniformly distributed within the cytoplasm except for a small subapical zone that is devoid of mitochondria, other organelles include rough ER, free ribosomes and Golgi apparatus (which is always supranuclear). The apices of adjacent cells are joined by tight junctions. Cytoplasmic vacuoles are small and not as many as in the other segments of the nephron.

### 3.2.5 The Distal Segment.

#### 3.2.5.1 Light Microscopy.

The distal tubule proper follows the intermediate segment, and is located mostly in a lateral position within the mesonephros (Figs. 1 and 2). The epithelium of the distal tubules is cuboidal ranging in height between 10 to 12 $\mu$ m. In sections the tubules of the distal epithelium do not show a positive PAS reaction on their apical surfaces but their basement membranes were PAS positive (Fig. 2). The nuclei are large and centrally placed. The distal tubule ends in a terminal segment with

histological characteristics identical to that of the distal tubule proper. This segment was identified solely by the continuity of its lumen with that of the mesonephric duct. The histological characteristics of both distal and collecting segments were identical. They are negative to the PAS stain and have the same epithelial height.

#### 3.2.5.2 Ultrastructure of the Distal Segments

##### Distal segment proper :

The distal segment is readily distinguished from the proximal segment because of the paucity of microvilli on the luminal surfaces of its cells (Fig. 13). Doming of the apical plasma membrane is also characteristic. Cytoplasmic projections of the apical domes are frequently encountered (Fig. 13), and occasionally these were found in the lumen, appearing as pinched off parts of the apical cytoplasm. Cytoplasmic organelles were never found in the pinched off parts. Artefactual apical cytoplasmic projections were not present on all cells of the distal segment (Fig. 14).

The basolateral plasma membranes are thrown into folds which are more extensive laterally on adjoining cells and the intercellular spaces than on the basal surfaces (Fig. 13 and 14). The nuclei are oval, centrally located and have one or two nucleoli. Mitochondria are numerous and more frequent in the lateral and basal cytoplasmic areas than in the apical zone of these cells. The distal segments are always in close contact with the endothelium of the intertubular blood sinuses (Fig. 15).

The portion of the distal tubule which is in close proximity to the glomerulus has an epithelium which does not differ from the epithelium

of other areas along this segment. Special attention was paid to this observation since in avian metanephroi the region of the distal which is located close to the glomerulus forms the very specialized macula densa. The interval between the two (glomeruli and distal tubule) occasionally contained cells that were identical to those of the intercellular interstitium but for the most part this interval is devoid of cells. Adult avian kidneys have a juxtaglomerular apparatus that consist of macula densa cells in the distal tubule epithelium and lacis cells between glomerulus and the distal tubule (Christensen et al, 1982). Earlier work by Taylor et al (1970) has demonstrated an increase in renin activity in the kidney of chickens following sodium depletion.

The Terminal Distal Segment.: There are no ultrastructural differences that distinguish this segment from the one preceeding it (the distal segment). Light and dark cells were frequently encountered in this segment but they were by no means peculiar to it, since these cells types have been observed in all segments of the nephrons particularly at younger developmental stages of the embryo (Fig. 18). The cytological details of the collecting segment are identical to those of the distal segment (Fig. 17). They were recognised only on the basis of their continuity with the Wolffian duct (Fig. 17).

## Figure 1

Oblique (close to longitudinal) section of an eight-day chick embryo mesonephros. The general features of the organ are illustrated. The Wolffian duct lies laterally draining the terminal distal segments. Most of the glomeruli are located medially within the organ: these represent the primary generation (the oldest population of nephrons) while in the middle of the organ is the youngest generation of nephrons.

w, Wolffian duct; G, glomerulus.

Haematoxylin-Eosin

75X

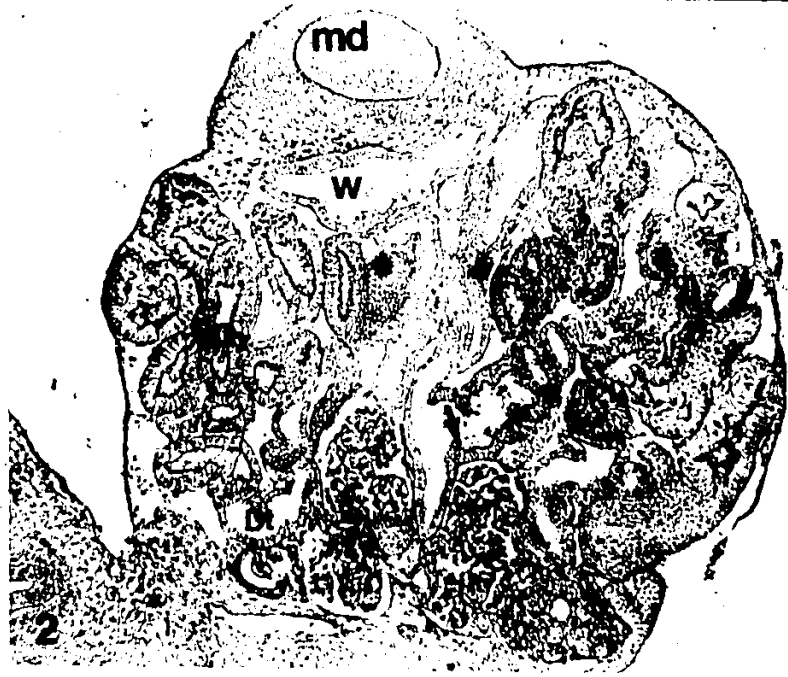
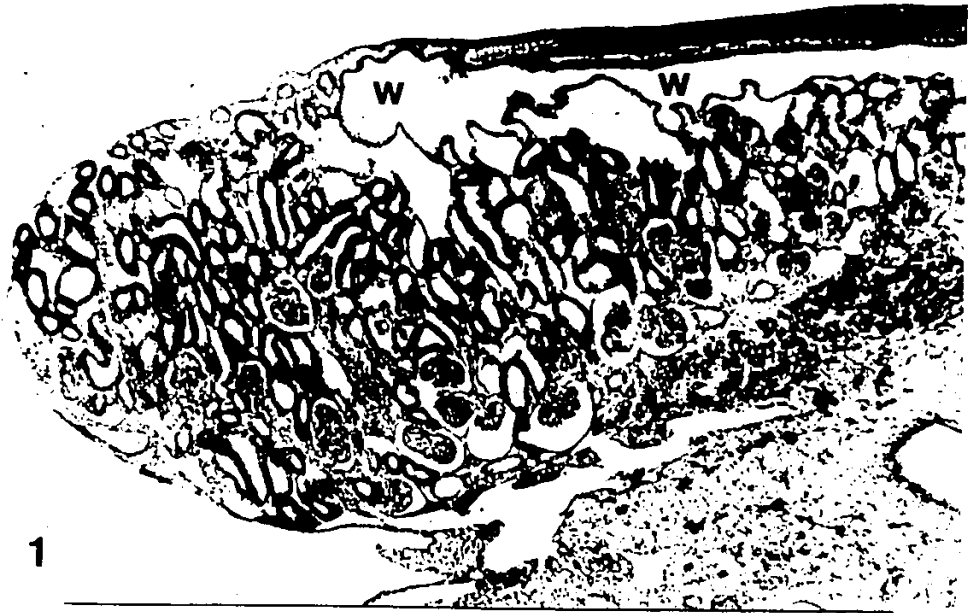
## Figure 2

Transverse section of an eight-day chick embryo mesonephros. The constituents of the mature mesonephric nephron are illustrated. The Wolffian duct lies in close proximity to the Mullerian duct (laterally); the glomeruli are located medially. The brush border of proximal tubules is PAS positive along with the basement membranes of the Wolffian duct, distal tubules and glomeruli.

md, Mullerian duct; w, Wolffian duct; Px, proximal tubule; Dt, distal tubule; G, glomerulus. Arrow indicates the transition from proximal to intermediate segment.

Periodic Acid-Schiff

100X



## Figure 3

A corpuscle of the mesonephros from a stage 27 chick embryo. The glomerular capillaries form a row around a central core of cells (the central cell mass). The proximal tubule begins at the urinary pole as a continuation of the parietal epithelium of Bowman's capsule. The visceral epithelium consist of a row of cells surrounding the glomerular capillaries.

CM, Central cell mass; Px, proximal tubule; Dt, distal tubule;  
U.S., urinary space; L, lumen of proximal tubule at the neck segment.  
cap, glomerular capillary

Toluidine blue

800X

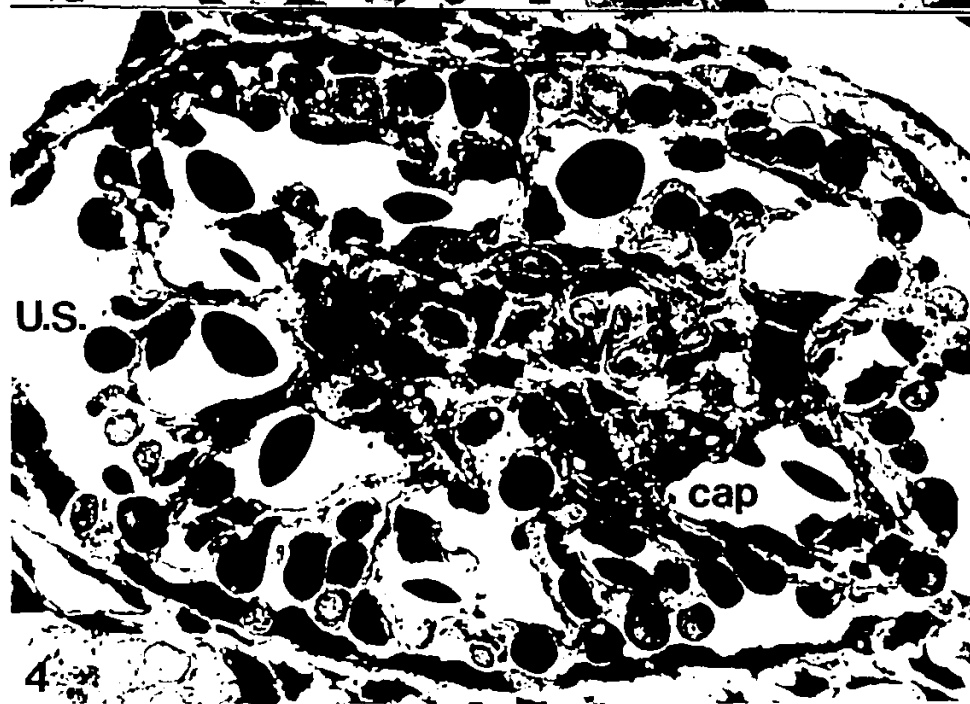
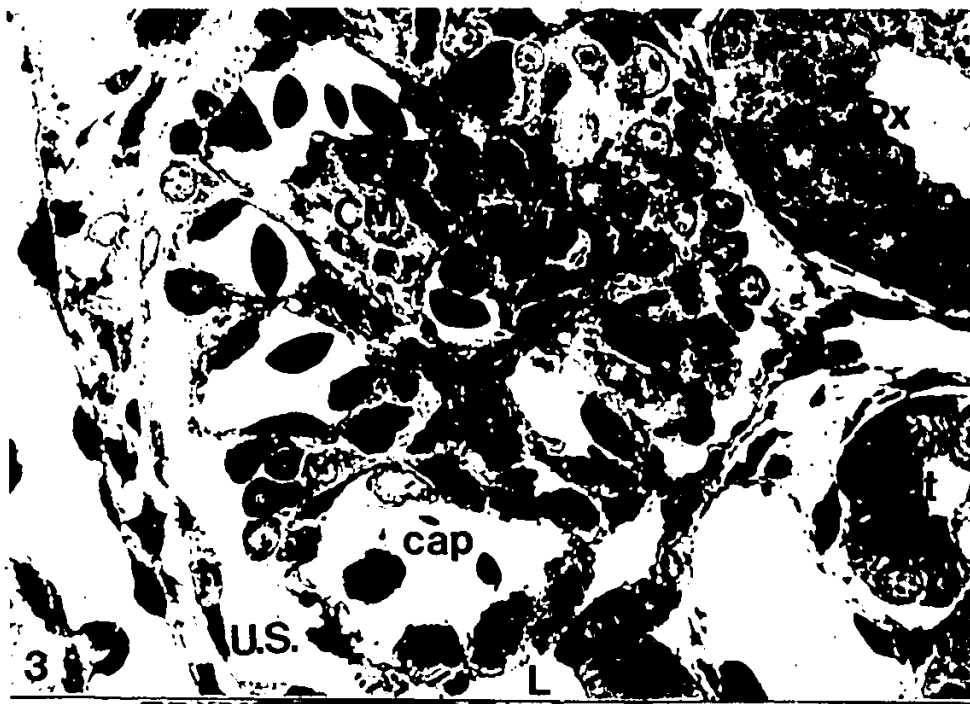
## Figure 4

Renal corpuscle of mesonephros from stage 27 showing the central cell mass.

CM, central cell mass; U S , urinary space; cap, glomerular capillary.

Toluidine blue

800X





## Figure 5

Electron micrograph of two adjacent glomerular capillaries in a mature mesonephros (stage 26). Endothelial cells surrounding the capillary lumina are perforated by minute pores. Endothelial cells are supported by a basal lamina that appears as a single layered structure in this figure. The visceral epithelium (podocytes) is formed by large cells with numerous cytoplasmic processes terminating on the basal lamina, these foot processes (pedicels) form a continuous row along the entire length of the basal lamina. The endothelial pores, basal lamina and the pedicels constitute the glomerular filtration barrier.

En, endothelial cell; lu, lumen of capillary; Pe, pedicel; Po, podocyte. Arrows indicate pores in endothelium; bl, basal lamina.

6400X



## Figure 6

High magnification electron micrograph of the filtration components of the mesonephric glomerulus in a stage 26 chick embryo. The ultrastructure of the different constituents of the filtration barrier is evident only at high magnifications. The primary pedicels contain numerous cytoplasmic organelles including some mitochondria, microfilaments, rough and smooth endoplasmic reticula. Adjacent pedicels appear to be separated by small spaces. The basal lamina consists of three layers; a dense middle layer and inner and outer less electron dense layers.

L, capillary lumen; En, endothelium; bl, basal lamina (a, b, c) lamina rara interna, lamina densa and lamina rara externa respectively; Pe, pedicel (primary); U S; Urinary space.

25000X



## Figure 7

Electron micrograph of the central cell mass in a mature renal corpuscle. At the EM level the central cell mass appears as small aggregates of 3 to 4 cells each surrounded by the basal lamina of the capillary endothelium. The cells do not have any special ultrastructural features.

En, endothelium; CM, central cell mass; L, lumen of capillary.

6400X

## Figure 8

Electron micrograph of proximal tubule epithelium close to the neck region. The cells have nuclei located toward their basal portions. Intercellular spaces are prominent with microvilli projecting from adjacent cells into the spaces. The apical parts of the cells have numerous micropinocytotic vesicles in addition to the numerous microvilli.

MV, microvilli; L, lumen of tubule; Is, intercellular space; V, apical vacuole; pv, pinocytotic vesicles.

1000X



## Figure 9

Electron micrograph of the apical pole of the epithelium of the proximal tubule in a stage 27 embryo. The microvillous projections of the epithelium are characteristic of the proximal tubule in the mature mesonephros. Note the sub apical dense zone with vesicles and vacuoles indicating an extensive reabsorptive function.

mv, microvilli; Pv, pinocytotic vesicles; V, sub apical vacuoles.

25000X





Figure 10

Electron micrograph of a tangentially sectioned proximal tubule of a stage 27 embryo. This Figure shows the extensive lateral intercellular spaces and the large number of lateral microvilli-like processes. The large dark staining granules are for the most part lysosomes and lipid droplets. These granules are not found in all cells of the same segment.

g, Golgi complex; lys, lysosome; mv, lateral microvilli-like processes; Arrow shows lipid droplet.

7300X



## Figure 11

Light micrograph of the transition zone (intermediate segment) of a stage 27 chick mesonephros. The transition from proximal to intermediate segment is abrupt with a change in epithelial height and microvilli. Note that this zone is located close to the glomerulus.

L, lumen; px, proximal tubule; I S; intermediate segment; Dt, distal; G, glomerulus. Arrow indicate transition zone. Araldite embedded, thick section, toluidine blue.

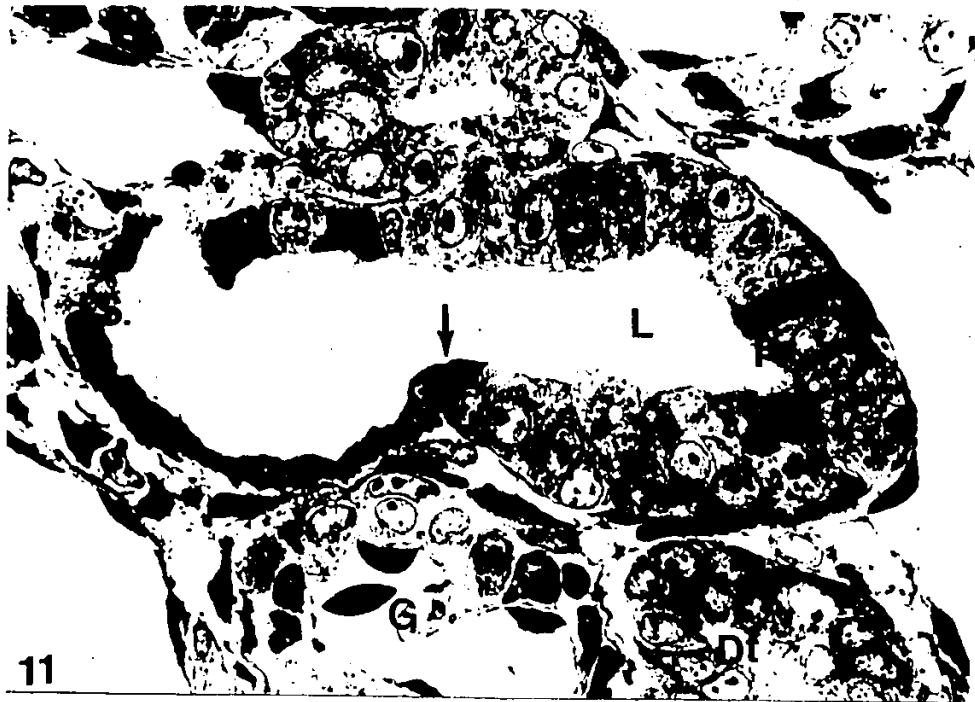
630X.

## Figure 12

Electron micrograph of the intermediate segment. The apices of the cells of this segment present few microvilli. The lateral plasma membranes show extensive interdigitations between adjacent cells. Infoldings can also be seen at the basal surface of the epithelium.

L, lumen; Pl, plasmalemma infoldings.

9100X




The image shows a section of a distal tubule with several cells. Some cells have small, finger-like protrusions extending into the lumen. The cytoplasm of these protruding cells is relatively clear of organelles. The basal membrane shows some slight infoldings.

Figure 13

Electron micrograph of the distal tubule cells showing apical protrusions. These luminal projections of the cytoplasm are restricted only to some cells of this segment. Note that there are no cytoplasmic organelles in the protruded portion of the cells. As is characteristic of this segment of the nephron, the basolateral infoldings of plasma membrane are developed only to a slight degree.

L, lumen; AP, apical protrusions; P1, plasma membrane infoldings.

13000X



13

## Figure 14

Electron micrograph of a typical distal segment in the fully differentiated nephron. The apices of the cells show a slight doming; cytoplasmic organelles are uniformly distributed in the cytoplasm. Golgi complexes are shown. Note the moderate development of the basolateral folds.

L, lumen; Pl, basolateral folds of the plasma membrane; g, Golgi complex; tj, tight junctions.

15000X





## Figure 15

Electron micrograph of the distal tubule located close to a glomerulus. Intertubular blood capillaries are seen in the interval between the glomerulus and the distal tubule. The Golgi apparatus is located in the supranuclear position in the distal tubules in this region.

U S; urinary space; Dt, distal tubule cell; Po, podocyte (visceral epithelium)

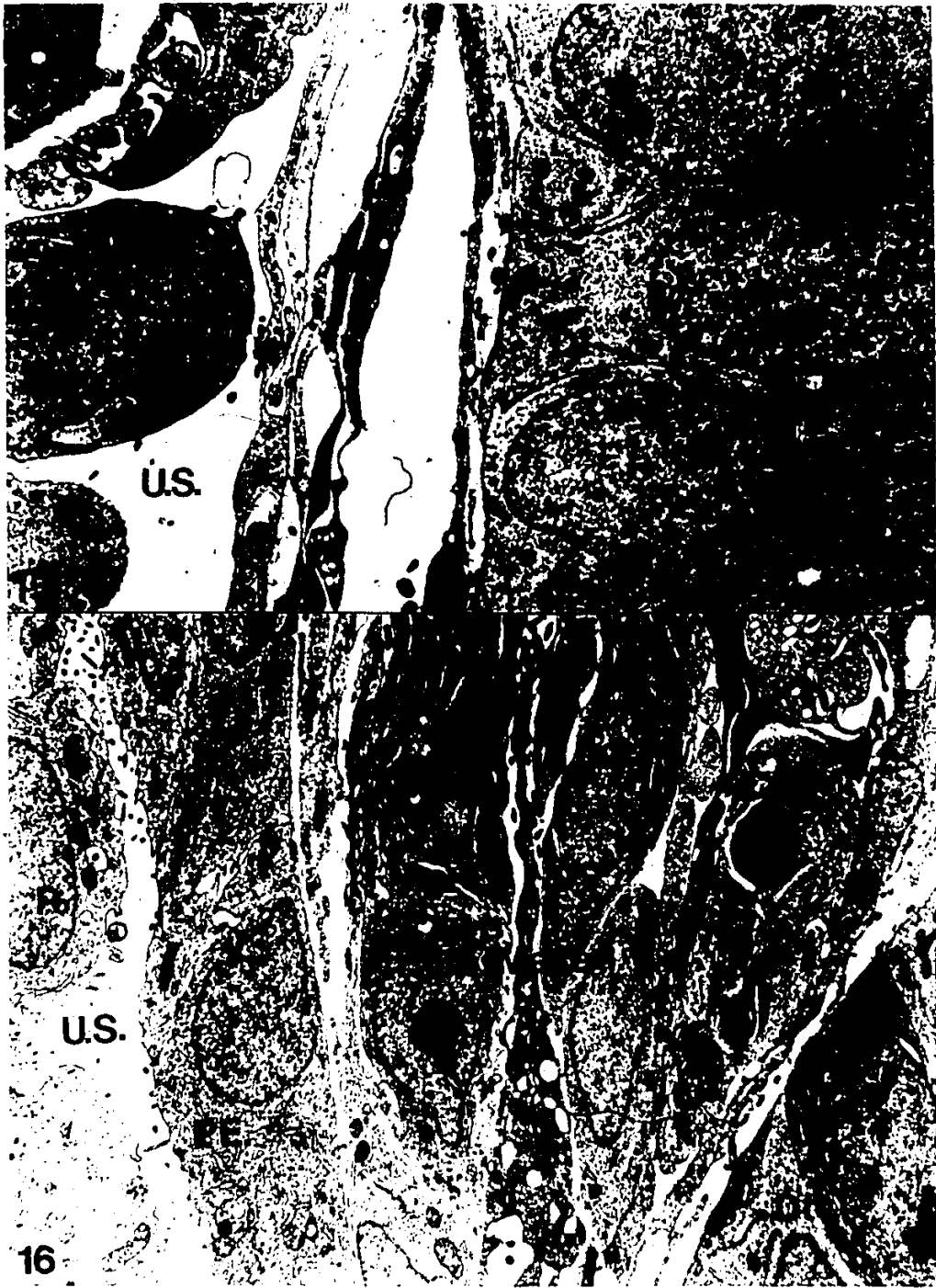
6700X

## Figure 16

Electron micrograph of a glomerulus and distal tubule. The parietal epithelium has only a few microvilli. The cells between the tubules and the glomerulus are intertubular connective tissue cells (Int. Mesen.).

P E; parietal epithelium; Po, podocyte; U S; urinary space; Dt, distal tubule.

6700X



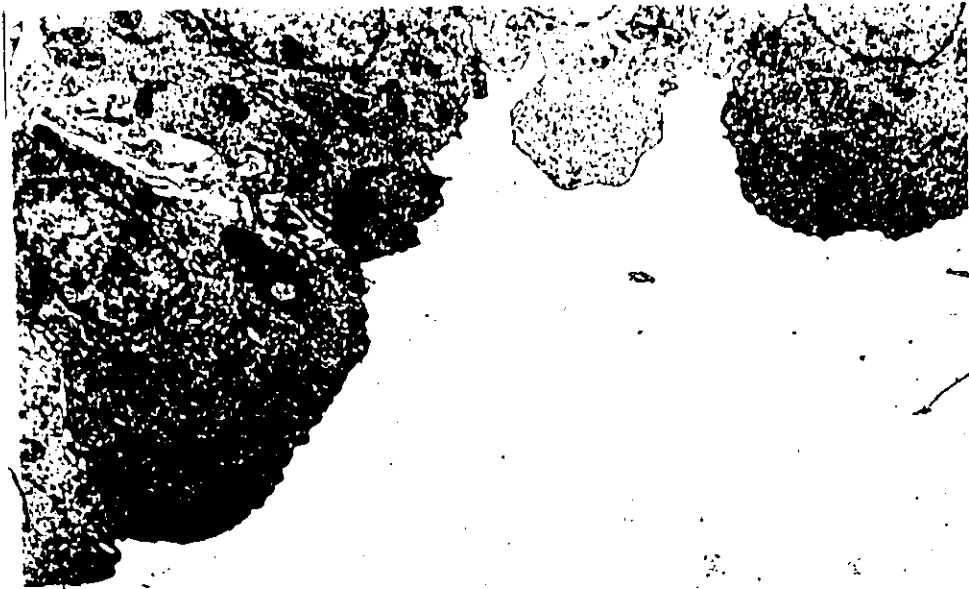
## Figure 17

Electron micrograph showing the entrance of the terminal distal segment into the Wolffian duct in a stage 25 embryo. The terminal distal segment does not differ ultrastructurally from that of the distal epithelium except for the more elaborate ramifications of the lateral intercellular microvillous-like interdigitations.

W, Wolffian duct; TD, terminal distal segment (collector).

6400X

## Figure 18



T.D.

W

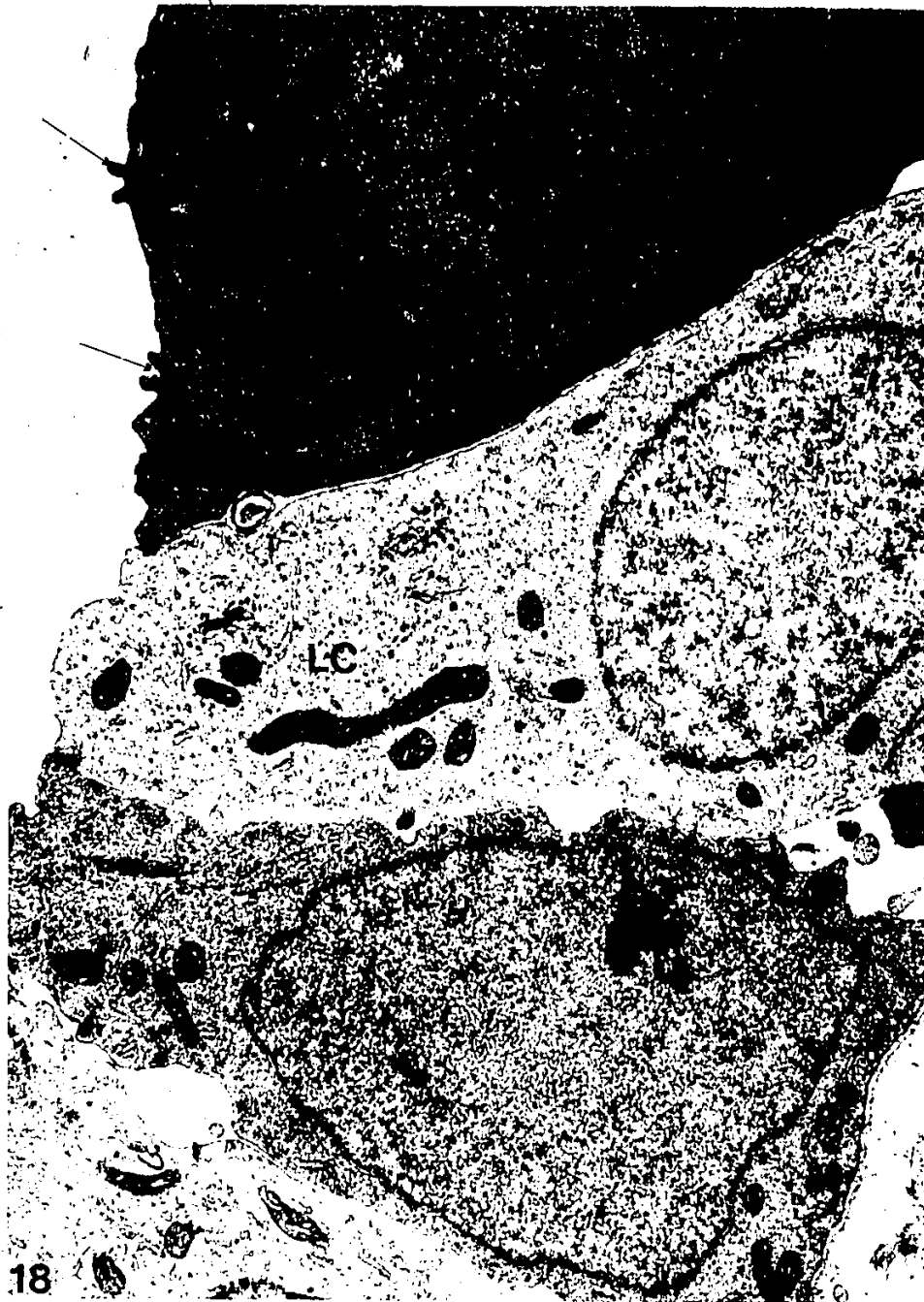


17

Electron micrograph of the terminal distal segment (collecting duct) of a stage 22 chick embryo showing 'light' and 'dark' cells. Arrows indicate microvilli the apical membrane of a 'dark' cell.

DC, dark cell; LC, light cell.

14000X



### 3.3 Differentiation of the Mesonephric nephron.

#### 3.3.1 Light microscopy.

In 2 embryos examined at stage 19 it had been established that nephron differentiation begins prior to stage 20 but all the nephrons were at the earlier stages of differentiation as evidenced by the lack of capillaries in the developing glomeruli. Nephron differentiation within the mesonephros continues up to stage 25 in the 3 embryos examined at this stage. As the first nephrons to appear complete their differentiation, new ones appear caudally and proceed to differentiate. The observations in this study confirm classical descriptions of nephron differentiation. The nephrons appear first as solid masses which then become hollow, transforming into vesicles the original solid mass of cells. As the vesicles lengthen they acquire an S shape. Immediately, the distal portion (with respect to the Wolffian duct) of the S curves forming a hollow U-shaped capsule which is the primordium of the renal corpuscle. The details of this initial stages of differentiation are shown in Figs. 19 to 23. Fig. 19A to D is a schematic representation of the main stages involved in nephron differentiation in the chick embryo according to Patten and Carlson (1974). The first stage of differentiation of the mesonephric nephron is illustrated in Fig. 19E. The mesenchymal cells of the nephrogenic ridge have begun to condense forming a mass medial to the Wolffian duct. The cells in this mass divide actively increasing its size. As the mesenchymal mass grows, a space appears in the mid-vesicular position with the cells arranged radially around the space. The vesicular stage is considered in this study as stage 1 of differentiation (Fig. 24). Lengthwise growth of the

vesicle converts the spherical vesicle into an elongated tubule with an S-shaped configuration (Fig. 20). The S-shaped tubule represents stage II of nephron formation. The cells of the tubule are uniformly columnar throughout its length with no histological distinction between the proximal and distal parts. The proximal end (relative to the Wolffian duct) of this early nephron connects laterally to the Wolffian duct whose epithelium is already histologically differentiated with a patent lumen (Fig. 20). The distal end of the nephron lies medially with its lumen becoming the primitive glomerular urinary space.

Glomeruli are formed at the distal end (relative to the Wolffian duct) of the S-shaped body. The earliest indication of glomerular formation is indicated by the thickening of the inner layer of the glomerular anlage. This thickened portion grows until it forms a solid mass of cells (Fig. 20 and 21). Thus, the distal end of the S-shaped tubule is converted to a U-shaped structure (the primordium of Bowman's capsule) with the inner epithelium transformed into a spherical mass of cells and the outer epithelium becoming greatly flattened (Figs. 20 and 21). A cleft (the urinary space) separates the inner from the outer epithelia (Fig. 22). This cleft is continuous with the lumen of the tubule. The accumulated cells resulting from the proliferation of the inner layer of Bowman's capsule give rise to the glomerular anlage. Nephrons with a solid glomerular anlage are considered stage III nephrons in this report; no capillaries are present at this stage (Fig. 22).

In the final stage of differentiation, (stage IV) capillaries appear in the glomerular anlage (Fig. 23). The cells of the glomerular

5



anlage remain as a layer directly surrounding the capillaries (visceral layer of Bowman's capsule or podocytes). It is not clear if the solid central mass of the renal corpuscle is derived also from the glomerular anlage or from cells that penetrate the corpuscle together with the glomerular blood vessels.

### 3.3.2 Electron Microscopic Observations.

At stage I, the cells forming the vesicle appear undifferentiated with no special characteristics (Fig. 24). In the stage II nephron few cytological characteristics of interest were observed. The epithelium is similar all along the tubule (Fig. 25). The cells of the tubular epithelium are small and are separated by large intercellular spaces. No apical microvilli or pinocytotic vesicles are evident. The cytoplasm contain sparsely distributed mitochondria, endoplasmic reticulum and a few vacuoles. At stage III the glomerular anlage is undifferentiated (Fig. 27), but parts of the nephron have started to differentiate into proximal and distal segments (Fig. 26). The cells of the epithelium in the proximal segment present large oval nuclei that are centrally located and display a uniformly dense cytoplasm with very few mitochondria distributed uniformly within it. An occasional cilium and sometimes few microvilli are found on the luminal membrane of these cells. Microvilli are also found at the lateral surface of the cells, their apical portions are united by tight junctions (Fig. 26).

By stage IV the glomerular anlage now contains blood vessels. Electron microscopical studies show that not all glomeruli classified as stage IV with the light microscope are identical. The differentiation of the filtration barrier is a progressive phenomenon occurring at

different rates in various glomeruli and in different zones of the same glomerulus. In the initial steps of this differentiation (Fig. 28), the visceral epithelium, represented by podocytes, is formed by large elongated cells with irregularly shaped nuclei. Numerous microvillous projections emerge from the free surfaces of these cells in proximity to the urinary pole. Their cytoplasm contains more organelles than the cells in the parietal epithelium (Fig. 28). Except for their apical portions podocytes are closely adherent between themselves so that no space exists through which urine could filter freely towards the urinary space. On the capillary aspect of the visceral epithelium, the capillary endothelial lining is separated from the visceral epithelium by a single layered basal lamina. The capillary endothelial cells are elongated with large nuclei (Fig. 28). No endothelial pores were observed. The cytoplasm contains only a few organelles which are concentrated in the perinuclear area. In the more differentiated glomeruli (Figs. 29 and 30) podocytes are increasingly being separated from each other by spaces through which the filtrate can have access to the urinary space. At the site of their contact with the basal lamina, podocytes are however still in close apposition. A few pedicels are sometimes found in this area (Fig. 30). The more differentiated areas of the stage IV glomeruli are similar ultrastructurally to the mature glomeruli described previously.

Differentiation of the proximal segments occurs simultaneously as the glomeruli in the stage IV nephron. Figs. 31 and 32 show the epithelia of proximal and distal segments respectively in the stage IV nephron. Although they are not fully differentiated, all the

characteristics of the mature tubule are already present. Further differentiation of the tubular epithelium will involve increase in the number and size of apical and lateral processes in proximal segments and increase in complexity of the basolateral folds in the distal segments.

## Figures 19A to D

Schematic representation of the stages of differentiation of the chick mesonephric nephron at the light microscopic level reproduced from Paalen and Carlson (1974). It illustrates the sequence of events leading to the formation of the fully differentiated and functional nephron in the chick embryo and is reproduced here in order to facilitate the comprehension of the following micrographs (Figs. 19E to 23).

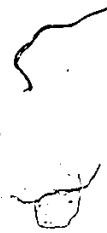
## Figure 19E

Low power light micrograph of a transverse section at the caudal third of a stage 20 chick embryo showing the first stage in nephron formation as aggregates of cells in close proximity and medial to the Wolffian duct.

N T; Neural tube; mp, mesonephric primordium; D A; Dorsal aorta; Noto, Notochord; p c v; posterior cardinal vein; W, Wolffian duct.

Paraffin embedded 5um section.

PAS-Haematoxylin . 250X



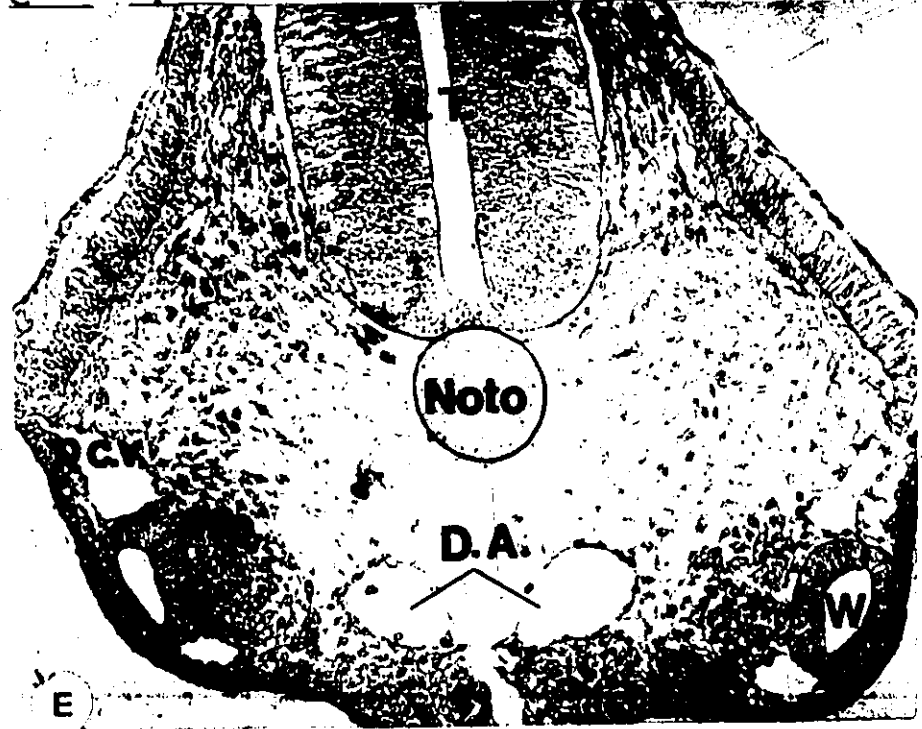
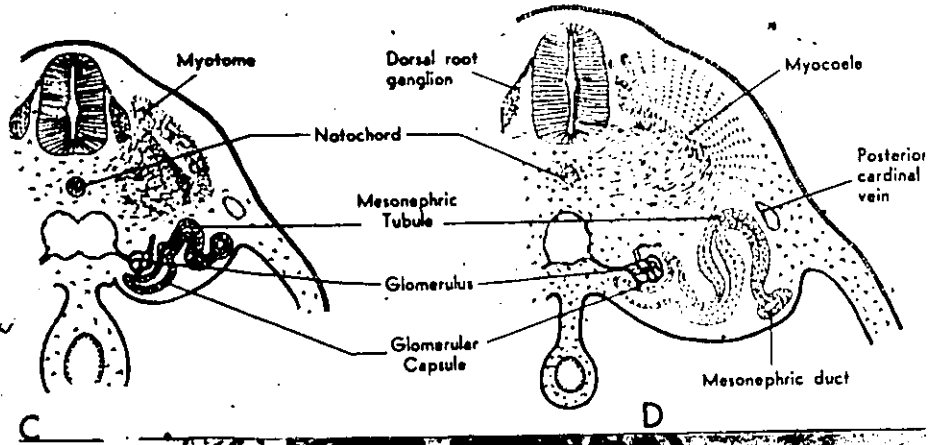
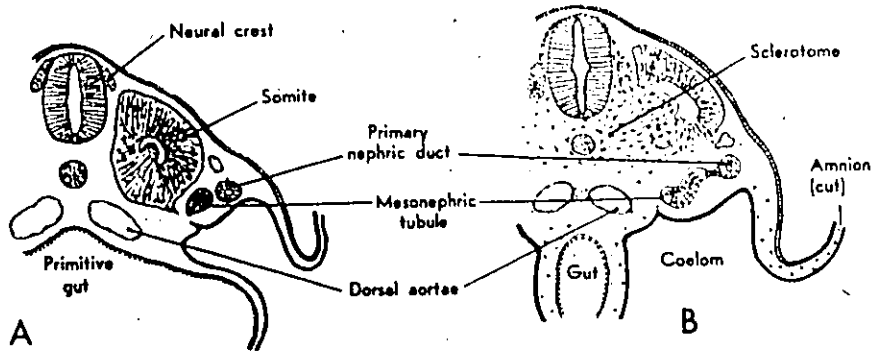


Figure 20

Light micrograph of differentiating mesonephric nephron (stage II) from a stage 21 embryo. The glomerular anlage lies medially. The tubule at this stage has established connection with the wolffian duct and the S-shape configuration is already evident. The epithelium is uniformly high columnar with no histological distinction as to proximal and distal segments. T, tubule; G, corpuscular anlage; W, Wolffian duct; II: second stage of nephron differentiation.

Araldite embedded; Toluidine blue 200X

Figure 21

Corpuscular anlage of a different embryo at stage 21. Similar stage of differentiation as in Fig. 20. Note the degree of differentiation of the parietal epithelium, the size of the urinary space and the lack of glomerular capillaries at this stage of differentiation. The parietal epithelium is reflected over the glomerular anlage cells as the visceral epithelium. Pe, parietal epithelium; us, urinary space; Ga, glomerular anlage cells; II: second stage of nephron differentiation. Araldite embedded; Toluidine blue 350X

Figure 22

Differentiating nephrons (stage III) in a stage 22 embryo. This Figure also shows the relative disposition of the differentiating nephrons within the organ. The glomerular capillaries are yet to make their appearance but the length of the tubule has increased considerably as evidenced by the number of times the tubular part of the nephron is cut in a single histological section. Note the extensive intertubular capillaries present in this zone of the organ when compared to Fig. 20.

G, glomerulus; W, Wolffian duct; T, tubule of nephron. III: third stage of nephron differentiation. Araldite, Toluidine blue 250X

Figure 23

Differentiating nephron (stage IV) from a mesonephros of a stage 23 embryo. The stage IV nephron is the first stage at which glomerular capillaries make their appearance. The tubule have acquired the characteristic epithelium of the mature (fully differentiated) nephron.

IV: nephrons at stage four of differentiation.

Araldite, Toluidine blue.

250X

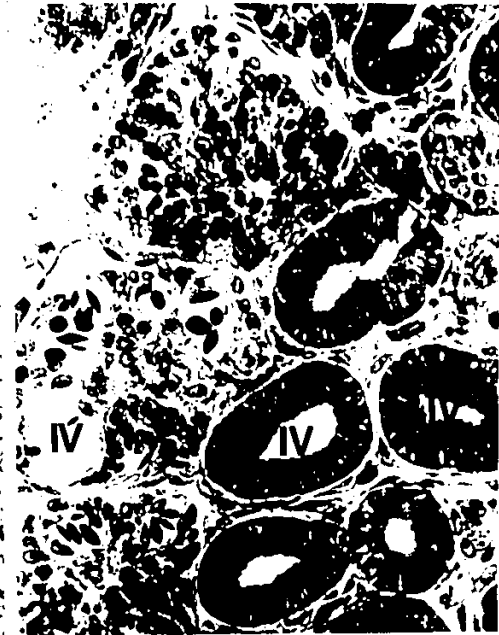
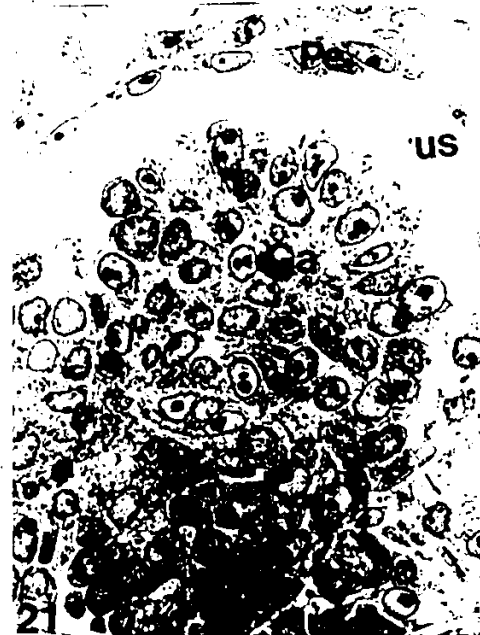




Figure 24

Electron micrograph of differentiating nephron (stage I) from a stage 23 embryo. The micrograph shows the renal vesicle formed from the solid spherical mass with the appearance of a space in the midvesicular position. (this early lumen represents the beginning of the tubular lumen). The cells of the vesicle are arranged radially around the lumen.

L, lumen of vesicle; Mi, mitosis.

6300X.



24

## Figure 25

Electron micrograph of the stage II nephron taken from an embryo of stage 21. The Figure illustrates the ultrastructural characteristics of the tubule at the early S-shaped stage. Note that there are no cytological features that distinguish parts of the tubule into segments. A few minute microvilli are beginning to appear on the apical surface of some cells.

L, lumen; T J; Tight Junction.

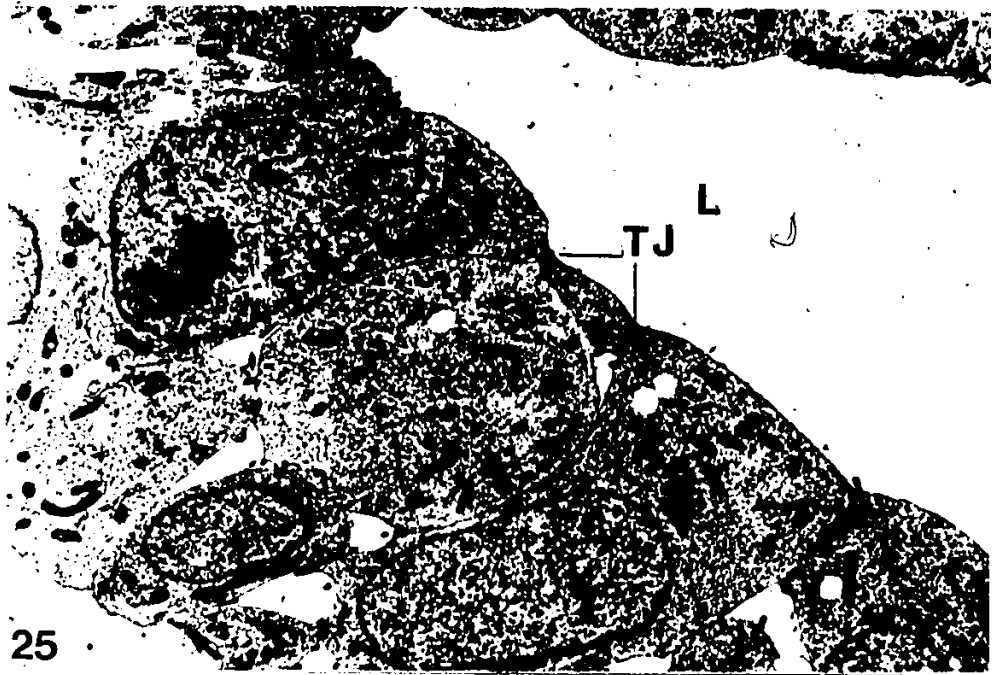
5500X

## Figure 26

Electron micrograph of a proximal tubule from a stage III nephron in a stage 22 embryo. The apical portion of the cells show a considerable number of microvilli. A few pinocytotic vesicles and apical vacuoles can be seen. Lateral intercellular spaces are shown.

V, apical vacuole; PV, pinocytotic vesicle; MV, microvilli; L, lumen.

11700X



25

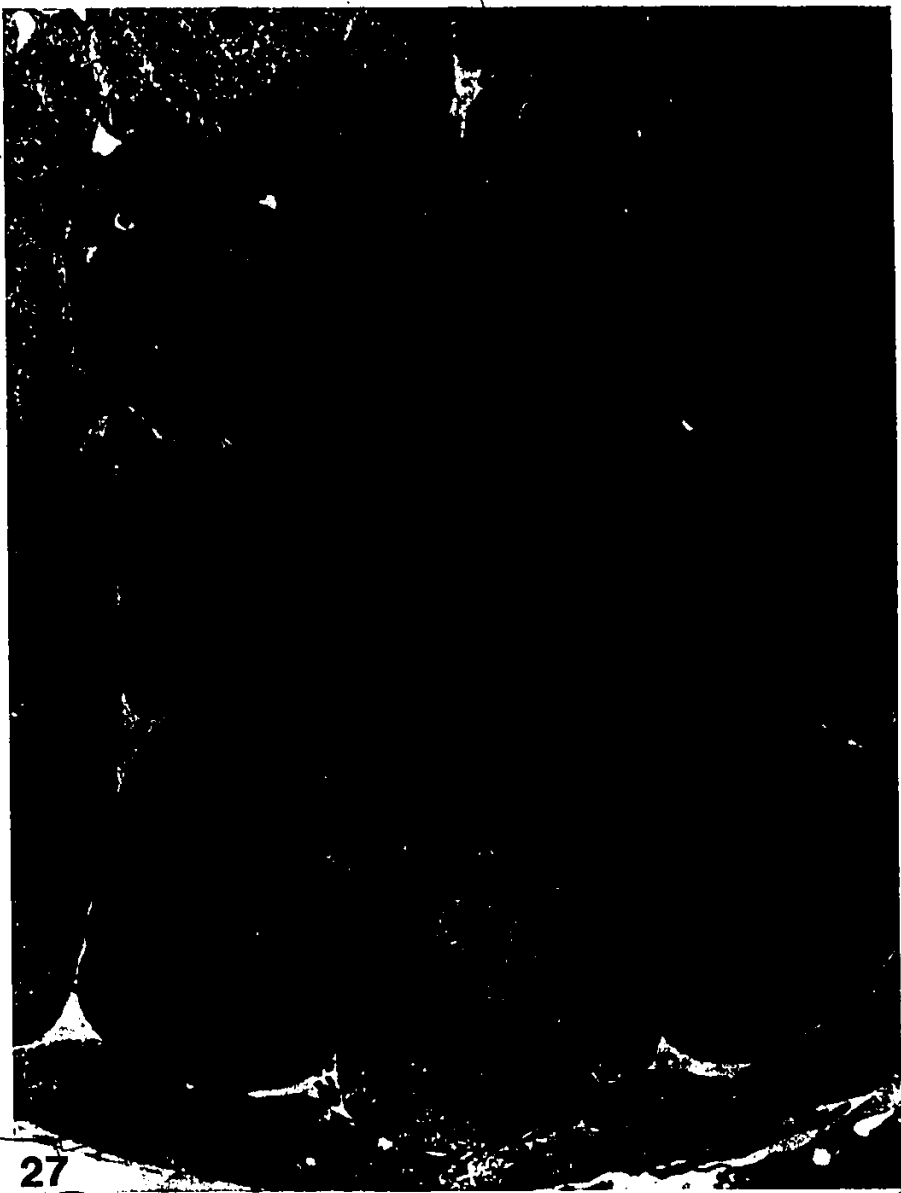


## Figure 27

Electron micrograph of the corpuscular anlage in a stage 23 embryo. Although the glomerular capillaries are not yet present, epithelium of the Bowman's capsule is already identifiable. The parietal epithelium has flattened considerably and is separated from the visceral epithelium by the urinary space. The cells of the visceral epithelium are ovoid, relatively large and have large nuclei located centrally; their cytoplasm contains few organelles.

V E; visceral epithelium; P E; parietal epithelium.

7200X



27

Figure 28

Electron micrograph of the same glomerulus as above (Fig. 28). In this area of the glomerulus the endothelium is considerably more flattened than in the previous figure. A few microvillous projections emanate from the free surfaces of cells of the visceral epithelium.

En, Endothelium; mv, microvilli; P E; parietal epithelium; V E; visceral epithelium.

6700X





## Figure 29

Electron micrograph of stage IV glomerulus in a stage 24 embryo showing the capillary lumen, endothelium, visceral and parietal epithelia. Although the capillaries are present in this glomerulus, the components of the filtration barrier are not developed to a large extent in this region of the glomerulus.

En, endothelial cell; V E; visceral epithelium; mv, microvilli; P E; parietal epithelium. Arrows indicate narrow intercellular channels that separate adjacent visceral epithelial cells.

6600X



## Figure 30

Electron micrograph of stage IV glomerulus from a stage 24 embryo. Compared to the preceding two figures the different parts of the filtration apparatus are better differentiated.

U S; urinary space; cap, endothelial capillary; En, endothelium; V E; visceral epithelium; P E; parietal epithelium.

6600X

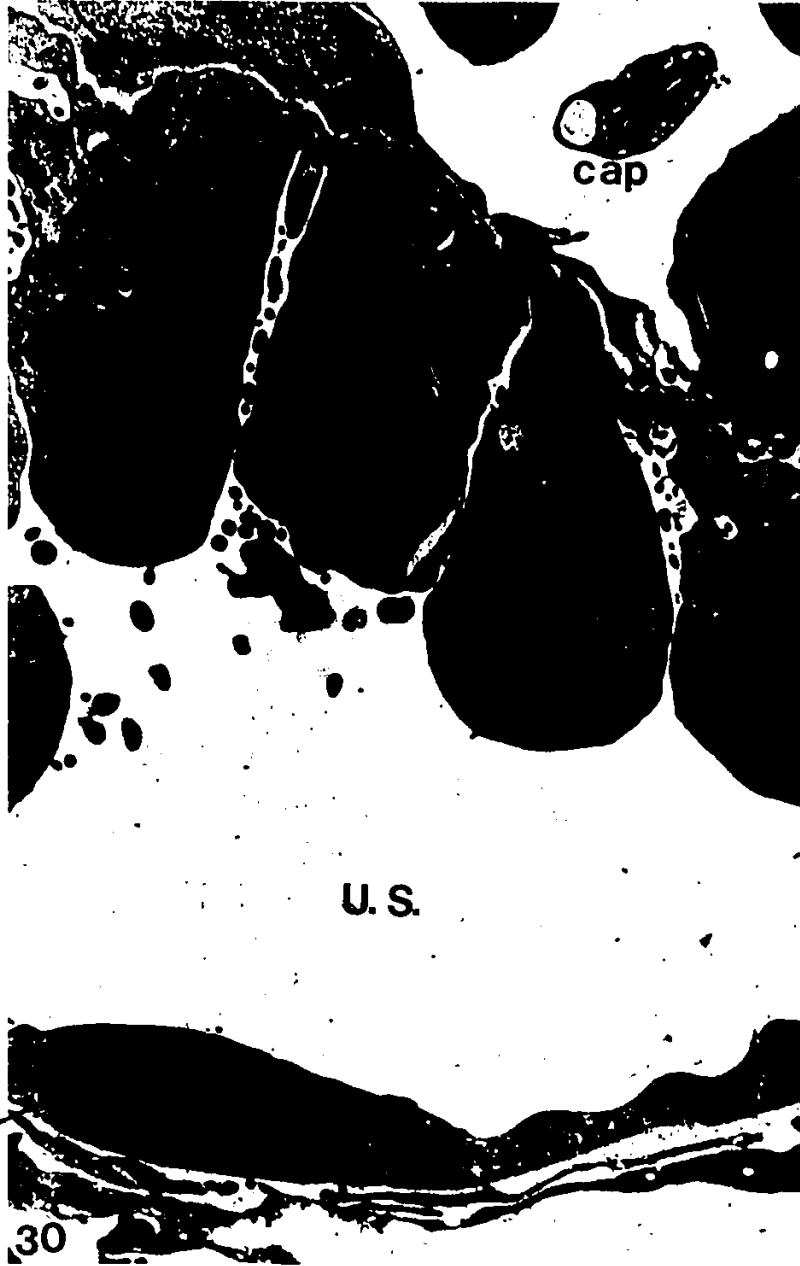


Figure 31

Electron micrograph of part of a proximal tubule of a stage 23 embryo. The characteristics of the proximal epithelium in the fully differentiated nephron are for the most part established in the stage IV nephron, although microvilli are not as numerous as in the more advanced stages.

L, lumen of tubule; mv, microvilli. Arrow shows micropinocytotic vesicles.

6300X





## Figure 32

Electron micrograph of the distal tubule of a stage IV nephron,  
(stage 23 embryo).

g, Golgi apparatus; L, lumen.

6900X



32



### 3.4 Initial Differentiation of the mesonephros.

From sections of the mesonephros studied with the light microscope, it had been established that by stage 26 of development mature nephrons are present throughout the mesonephros. However between stages 20 and 22 only the anterior nephrons appear to be at stage IV of differentiation. The purpose of this part of the study was to establish the exact stage of embryonic development at which characteristics of the nephrons are similar to their equivalents in the fully differentiated mesonephros known to be functioning. The protocol consisted of studying cross-sections of the urogenital ridges from embryos at stages 20 to 22 starting at their cephalic ends. Serial sections 2um in thickness were stained with toluidine blue and studied with the light microscope, as soon as these sections reached a renal corpuscle, thin sections were prepared for electron microscopy. By so doing, 2 to 3 glomeruli from each mesonephros were studied in addition to the last pronephric (external) glomeruli.

During the early stages of its development (stages 20 to 22) the mesonephroi appear as two thin cords on both sides of the mesentery; each one extends from the cervical somitic segments anteriorly to the cloacal region posteriorly. It was difficult to identify with the dissecting microscope the cephalic end of the mesonephros, that is, its first glomeruli. At all stages studied the first glomeruli were encountered at about the level of the 19th or 20th somites. Anterior to this the mesonephric ridges were made up of entirely of degenerating pronephric tissue.

### 3.4.1 The Wolffian duct

At all stages the Wolffian ducts were patent at the level of somites 19 and 20 but there was no mesonephric tissue differentiating into nephrons. A feature of the Wolffian duct is the highly differentiated state of its cells even at stage 20 (Fig. 33). The cells show a degree of ultrastructural complexity that parallels that of the distal epithelium in the fully differentiated organ as seen in mesonephros of embryonic stages 25 and beyond. The cells are low columnar and show a slight doming of their apices. A few vesicles are present in the supranuclear cytoplasm, the nuclei are large and centrally placed. The organelles are uniformly distributed but the Golgi is always in the supranuclear position. Intercellular spaces are large, basal in location and show infoldings of the plasma membranes. A thin basal lamina supports the cells of the Wolffian duct.

### 3.4.2 The External (pronephric) Glomeruli.

These glomeruli are remnants of the degenerated pronephric tissue. They usually overlap the level of the most cephalic of the mesonephric nephrons. The external glomeruli have an appearance strikingly similar to the differentiating glomeruli of the mesonephros (Fig. 34). These glomeruli are not, however, surrounded by the external layer of Bowman's capsule but are suspended in the coelomic cavity into which they presumably eliminate their filtrate (Figs. 34 and 35). The visceral epithelium which separates these glomeruli from the coelomic cavity is continuous laterally with the coelomic epithelium (Fig. 35). The glomerular capillaries are surrounded by the visceral epithelium on the outer side (externally). On examination with the electron microscope,

the ultrastructural characteristics of the external glomeruli parallel those of differentiating mesonephric glomeruli with regards to the degree of structural differentiation of the filtration barrier (Fig. 36). The visceral epithelium is made up of cells that are ultrastructurally similar to podocytes of the mesonephric glomerulus. The foot-processes are poorly developed but a distinct unilayered basal lamina is present (Fig. 36). External glomeruli were not encountered in the mesonephric ridges of embryonic stages beyond stage 22.

#### 3.4.3 The Cephalic Corpuscles at stages 20 to 22.

In each of the stage 20 embryos sectioned antero-posteriorly only the first 3 anterior nephrons have mesonephric corpuscles that are in stage IV of differentiation, i.e. have capillaries in them (Fig. 37). Additional ones are found at stage 21 and their number continues to increase in subsequent stages. The filtration barrier is incompletely formed at stage 20; while in some parts podocytes are in close contact with each other, in other parts they are separated by parts of the basal lamina covered only by pedicels. This variability between different zones of the barrier in the same glomerulus is illustrated in a high magnification light micrograph (Fig. 38). Fig. 39 shows a zone of a stage 20 glomerulus in which pedicels are in contact with the basal lamina and podocytes are close together. On the other hand in Fig. 40 the barrier possesses numerous pedicels. At stage 23 differentiation of the first three glomeruli had advanced with most zones of each glomerulus having numerous pedicels in contact with the basal lamina and numerous endothelial pores.

#### 3.4.4 The tubules of the Cephalic Nephrons.

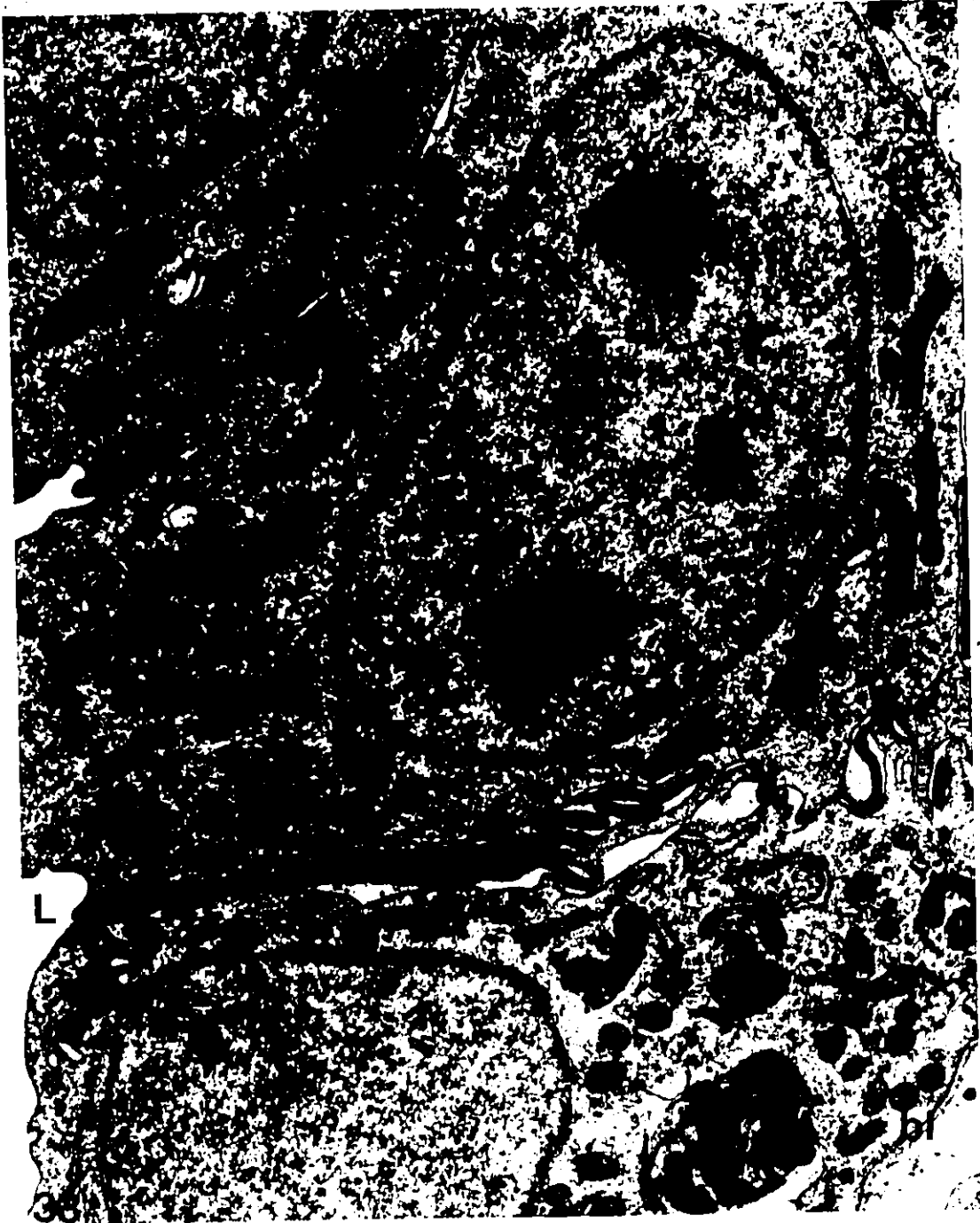
At stage 20, the first three nephrons have short (< 100um) tubular connections to the Wolffian duct; at later stages the tubules elongate rapidly. At the light microscopic level, no histological distinction can be made as to proximal and distal segments. With the electron microscope however, the proximal and the distal segments can be identified. In the proximal segment, pinocytotic vesicles are found in the subapical cytoplasm. A few microvilli are present on the apical surface of the cells. (Fig. 41). The lysosomes found in this segment at later stages of embryonic development are absent at this stage. The degree of differentiation of the proximal segments of the first three nephrons vary in different embryos of the same stage. Differentiation of the distal segments in the first nephrons do not advance much between stages 20 and 23. The cells in the tubular epithelium are cuboidal with nuclei centrally located (Fig. 42). Plasma membrane infoldings at the base of the cells are present in the most rudimentary form.

## Figure 33

Electron micrograph showing part of the Wolffian duct taken from a stage 20 embryo (at the cephalic end of the mesonephric ridge). The Wolffian duct shows a degree of ultrastructural development comparable to that found in the fully differentiated mesonephros. Note the similarity of the micrograph to that of the distal cells in Fig. 14.

L, lumen; bl, basal lamina.

17000X



Handwritten scribble or signature.

## Figure 34

Low magnification light micrograph of the anterior (cephalic) end of the mesonephric ridge in a stage 21 embryo showing the relationship of the external glomerulus and the surrounding structures.

DA, dorsal aorta; Me, mesentery; co, coelom; ce, coelomic epithelium; EG, external glomerulus. Araldite embedded, 2um section. Toluidine blue 250X

## Figure 35

Higher magnification of the same section shown in Fig. 34. The capillaries of the external glomerulus and the visceral epithelium have similar histological appearance to those of the 'normal' mesonephric glomerulus.

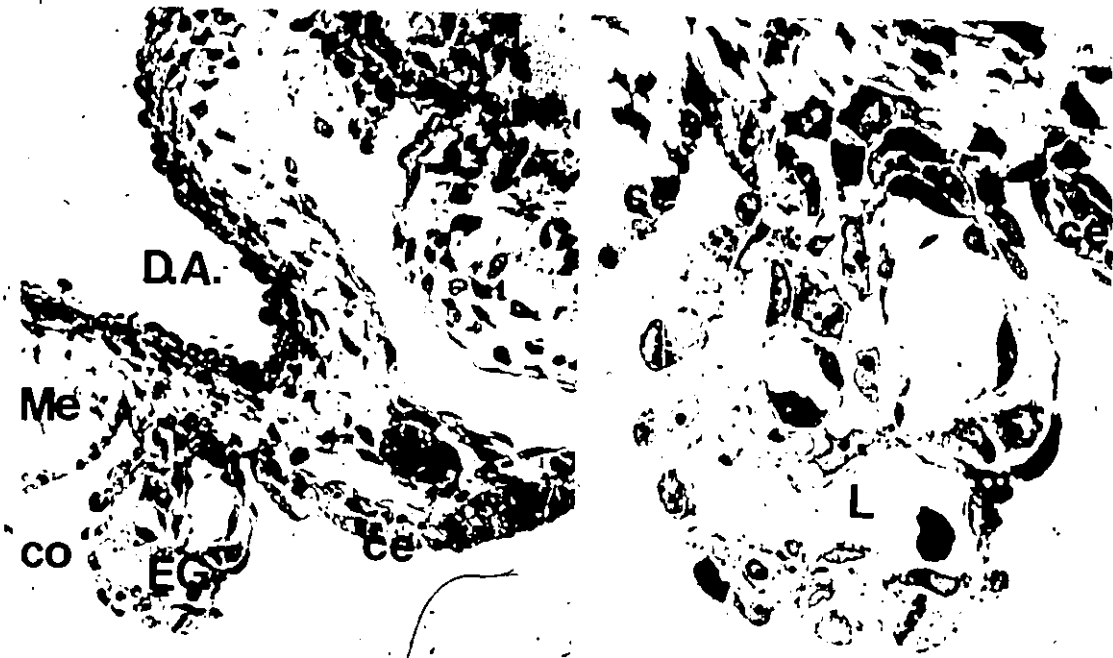
L, capillary lumen; ce, coelomic epithelium. 630X

## Figure 36

Electron micrograph of part of the external glomerulus shown in Fig. 35. The visceral epithelium is well developed and has pedicels. The basal lamina on which the pedicels abut is a single layered structure.

L, capillary lumen; En, endothelium; bl, basal lamina; Pe, pedicel; V E; visceral epithelium. Arrows show small membrane bound granules.

10400X



34

35



36



## Figure 37

Light micrograph showing the first (anterior) glomerulus in a stage 20 embryo. This Figure illustrates the general location of the first (cephalic) nephrons with respect to other embryonic structures; it possesses a number of glomerular capillaries.

D A; dorsal aorta; W, Wolffian duct; co, coelom; Me, mesentery.  
G, glomerulus.

200X

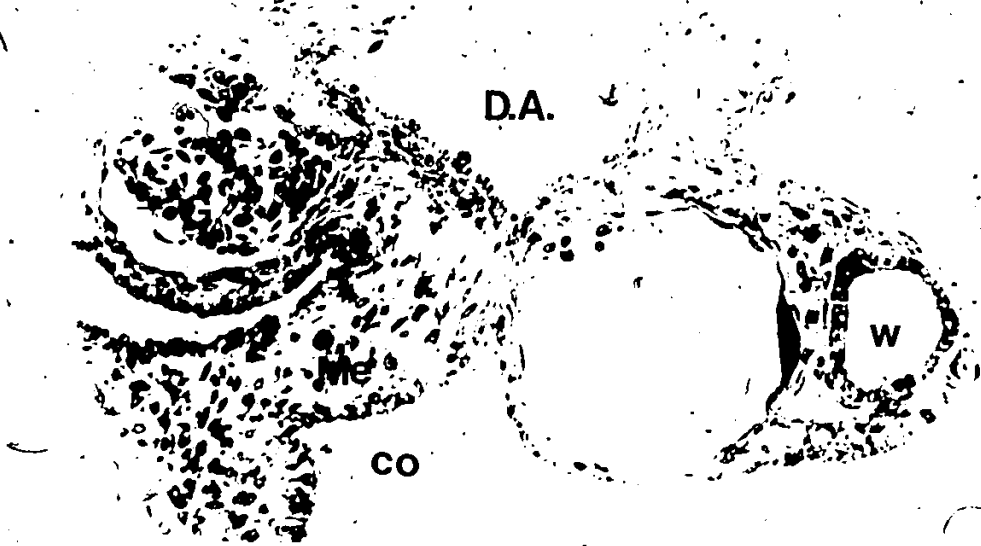
## Figure 38

High magnification light micrograph from another stage 20 embryo showing one side of the mesonephric ridge with the first cephalic glomerulus. Its proximity of the Wolffian duct is indicative of the characteristic shortness of the tubule (not seen in the figure).

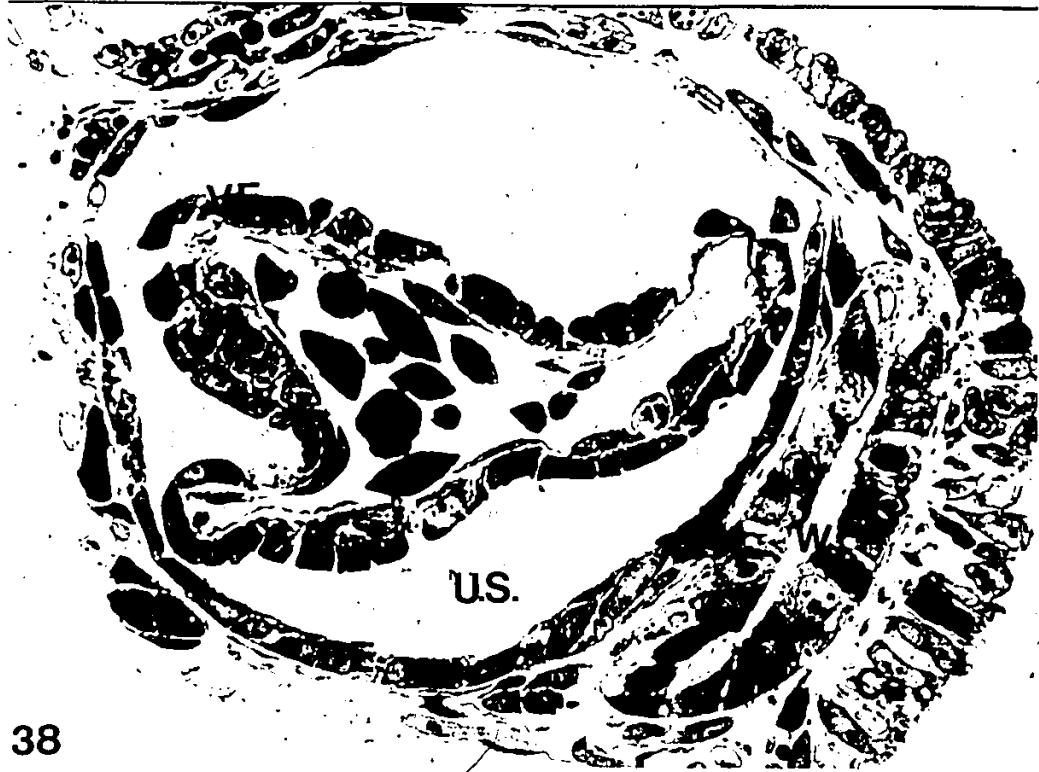
Ce, coelomic epithelium; U S; urinary space; V E; visceral epithelium; P E; parietal epithelium; W, Wolffian duct.

Araldite embedded, sectioned at 2um and stained with toluidene blue.

600X.



37



38

## Figure 39

3 Electron micrograph showing glomerular capillary from a stage 20 embryo comparable to the one shown in Fig. 38. Note the degree of ultrastructural differentiation of the filtration barrier in the first anterior nephrons and although the filtration barrier is fairly well developed, podocytes are still relatively close together. Arrow indicates filtration pore in endothelium;

pe, pedicel; L, lumen; Po, podocyte U S; urinary space.

14400X



## Figure 40

Electron micrograph of glomerular capillary of the first glomerulus from an embryo at stage 22. The basal lamina presents a one layered structure; the endothelium is considerably flattened and pedicels abut on the basal lamina.

En, endothelium; pe, pedicel; Po, podocyte; L, lumen; U S; urinary space.

17000X



L

40

U. S.

## Figure 41

Electron micrograph of apical part of the initial segment of proximal tubule from the first nephron in a stage 20 embryo. Although quite short, this tubule shows a surprising degree of differentiation characterized by many microvilli, MV; pinocytotic vesicles, Pv; and a dense cytoplasm.

20000X

## Figure 42

Electron micrograph of distal part of stage 20 nephron (tubule close to the Wolffian duct). The cells of the distal segment of the nephron have already assumed most of the characteristics of the fully differentiated nephron.

L, Lumen of tubule; EC, Endothelium of intertubular capillary.

17000X





## Chapter IV

### DISCUSSION AND CONCLUSIONS

The results presented in this study show that the basic morphological characteristics of nephrons described in vertebrate metanephric kidneys are also found in the chick mesonephros.

#### 4.1 The glomerulus

IN the glomerulus of the fully differentiated nephron it was confirmed that the filtration barrier comprised of a fenestrated capillary endothelium, a tri-layered basal lamina and pedicels from the visceral epithelial cells similar to Gibley and Chang's (1967) and Jacob, et al (1977a) observations in the chick mesonephros,

The structure of the capillary endothelium in the chick mesonephros was found to be similar to the one described in the literature for glomeruli of other species (Latta, 1970). However, there are disagreements in the literature concerning the nature of the endothelial fenestrations in different vertebrate kidneys. Diaphragms have been reported as closing the endothelial pores of mouse glomeruli (Rhodin, 1962). Later observations in different species including the mouse have shown that most if not all endothelial pores lack diaphragms and thus can only prevent passage of blood cells and platelets (Latta, 1973). The observations in the present study show that capillaries in mesonephric glomeruli have endothelial pores that are not closed by diaphragms.

The basal lamina forms the second component of the filtration barrier. In this study, the description of the tri-layered structure comprising a lamina rara interna (sub-endothelial), a lamina densa and a lamina rara externa agrees with their description by Gibley and Chang (1967). The lamina rara interna makes contact with pedicels from the visceral epithelium. In the mammalian metanephros, the visualization of diaphragms which occlude slit-pores between adjacent pedicels requires special fixation procedures (perfusion with glutaraldehyde and tannic acid). These procedures were not presently followed; for this reason, slit diaphragms were only observed occasionally.

A distinct feature of the avian glomerulus is the presence of a central core of cells surrounded by glomerular capillaries. This core was described in the glomeruli of adult chicken by Pak Poy and Robertson (1957) and later by Suzuki (1959); it was also found in the chick embryo mesonephros by Russo-Caia et al, (1977). Lumb (1973) confirmed its existence with the scanning electron microscope. In the present study the existence of this structure was confirmed.

Most of our understanding of glomerular function is derived from physiological studies on mammalian metanephric glomeruli. Available evidence accumulated over the last three decades indicates that the glomerulus is essentially a passive blood filter, retaining blood cells, platelets and large molecular weight proteins. The precise role of the three components of the filtration barrier has been the subject of controversy (see Farquhar, 1975 for review). Specifically, Farquhar et al, (1961) have shown that the endothelial pores allow passage of ferritin molecules MW 480000 and colloidal gold particles but red blood

cells and platelets are blocked. Graham and Karnovsky (1966) studied the passage of horseradish peroxidase (HRP) (MW 40000) and myeloperoxidase (MPO) (MW 160000-180000) and concluded that HRP crosses all the components of the filtration barrier reaching the urinary space while myeloperoxidase was stopped by the slit diaphragms. These results were partially contradicted by Venkatachalam et al, (1970) who showed that HRP was also partially retained by the basement membrane. Based upon the above experimental evidence, it was concluded that endothelial pores restrict only red blood cells and platelets, the basement membrane restricts passage of particles with molecular weight greater than 160000 and the filtration slits diaphragm between pedicels stops the passage of particles with molecular weights greater than 68000 or a radius 30 to 36<sup>o</sup>Å (Renkin and Gilmore, 1973). Later studies have shown that the passage of different molecules through the different components of the barrier depends also on factors other than molecular weight such as the molecular diameter, electrostatic charge and the velocity and location of the molecules in the blood stream of glomerular capillaries (Blatt et al, 1970; Ryan et al, 1976). Modern views tend to accept in general the concept that large molecules are stopped or retarded by the basement membrane, that intermediate size molecules are retained by slit diaphragms and that only smaller molecules (probably less than 40000 MW) cross the barrier. This would be in agreement with the fact that serum albumin (MW 69000) does not cross the barrier under normal circumstances. The fact that the elements of the filtration barrier described here in the mesonephric glomerulus are ultrastructurally similar to those found in the species in which the above described

physiological experiments were conducted suggests that the filtration mechanisms may be similar in both cases.

#### 4.2 The proximal tubule.

The proximal tubules show very similar structural characteristics in all vertebrate species (Maunsbach, 1973). The primary function of the proximal tubule is the reabsorption of low molecular weight proteins, amino acids, glucose, as well as a significant part of filtered ions and water present in the glomerular ultrafiltrate (Graham and Karnovsky, 1966; Maunsbach, 1966a and 1966b). To this end, there are ultrastructural characteristics of the proximal tubule epithelial cells that correlate with their function as described below.

##### 4.2.1 Microvilli and Apical vacuoles

The brush border of the proximal tubule is known to be due to the presence of numerous microvilli on the luminal aspect of the cells. In the metanephros of mammals the epithelial cells in the proximal tubules have been divided into three zones (Sjostrand and Rhodin, 1953), the apical zone (microvillous layer), intermediate zone (below the base of the brush border and above the nucleus) and the basal zone (from the nucleus to the basement membrane). The microvilli greatly increase the surface area of the cells and are devices characteristic of most absorbing epithelia. It has been suggested that the presence of microvilli at the luminal surface increases the absorptive surface area by a factor of 40 (Maunsbach, 1973). Thuneberg and Rostgaard (1972) indicated the presence of some contractile mechanism within the microvilli that probably assists in cellular transport across the plasma

membrane through movement of microvilli. The structure of microvilli presented here agrees essentially with their description in the kidney tubules of other vertebrates (Suzuki, 1958; Maunsbach, 1973). In some tubules, disappearance of microvilli on the luminal surface was observed. In some tubular cells all or part of the apical surface appears to evaginate forming a smooth-surfaced dome without microvilli. This phenomenon was first described with the light microscope by Stampfli (1950) who suggested that this represented a secretory process. This suggestion was based on the fact that some of the everted portions of the cytoplasm appeared free in the lumen of the tubule. In the case of the chick mesonephros the ultrastructural characteristics of these everted portions of apical membranes do not appear to represent a secretory process but rather fixation artifacts. Preliminary experiments by Narbaitz (personal communication) showed that the number of everted cytoplasmic bodies increases when fixation is conducted at room temperature or delayed for periods of up to one hour. He also found that the protrusions do not exist in mesonephroi fixed by perfusion. Tiedemann and Wettstein (1980) has also suggested that similar structures in the pig mesonephros are artefactual.

The supranuclear zone (intermediate zone of Sjostrand and Rhodin) consists of many pinocytotic vesicles, vacuoles and secondary lysosomes. They represent, collectively, an indication of resorption of molecules from the filtrate. The presence of an active supranuclear zone in the differentiated chick mesonephros is confirmed in the present investigations.

#### 4.2.2 , Lateral Intercellular Spaces and Lateral Microvilli

In mammalian metanephric kidneys the intercellular spaces of the proximal tubule form cone-like clefts between adjacent cells with the apex separated from the tubular lumen by junctional complexes; their bases are limited by the basement membrane of the epithelium (Sjostrand and Rhodin, 1953; Tisher et al, 1966). The lateral plasma membrane of epithelial cells shows numerous infoldings and protrusions into the lateral intercellular spaces; these increase the exchange surface between the epithelial cells and the spaces. It has been suggested that osmotic gradients are formed in these spaces by solutes that induce passive movement of water into the spaces from the cells (Diamond and Tormey, 1966; Diamond, 1971). An increase in the diameter of these spaces has been observed during transepithelial water transport in kidney tubule segments and in gall bladder epithelium (Ganote et al, 1968; Grantham, et al, 1969; Tormey and Diamond, 1967). Lateral interdigitations and infoldings of the plasma membrane also appear to be concerned with the active transport processes involved in osmotic regulation of ions and water as demonstrated by the presence of mitochondria associated with them and the histochemical localization of transport enzymes (Tiedemann and Schluns, 1975; Rostgaard and Moller, 1980). Similarly, in the mammalian metanephros numerous infoldings associated with mitochondria are present in the basal membrane of the proximal epithelium. Thoenes (1973) suggested that epithelia with basal labyrinths, lateral interdigitation of plasma membranes with compartmentalized mitochondria are ion transporting epithelia. Such ultrastructural adaptations (necessary for ion transport) are found in

other epithelia noted for ion and water transport such as gills and rectal glands (Berridge and Oschman, 1972). It is believed that apical microvilli, supranuclear vesicles and vacuoles are indications of resorption from the tubular lumen. Lateral and basal infoldings are evidence of transport from the epithelium to the interstitial spaces (Thoenes, 1973). Proximal tubules in birds have large lateral spaces but their lateral membranes have microvilli rather than infoldings. These microvilli probably represent surface area-increasing devices similar to infoldings. The present observations confirm that in the chick mesonephric proximal tubules' lateral spaces contain numerous microvilli and not infoldings. Basal mitochondria-associated infoldings were not observed. This agrees with similar observations in the chick embryonic metanephros (Narbaitz and Kacew, 1978).

4.3 The distal tubules: Structure and functional correlation.

4.3.1 Basolateral Infoldings In Distal Epithelia.

It is well known that the distal segments and the collecting ducts of adult kidneys in vertebrates are characterised by extensive interdigitations and amplification of the basolateral plasma membranes, (Myers et al 1966; Osvaldo-Decima, 1973), and in the human metanephros a clear association has been observed between the infoldings and the compartmentalization of the basal mitochondria in the cytoplasm.

Of the numerous studies done on vertebrate kidneys a considerable degree of variability has been reported with regard to the presence, number and size of basolateral infoldings. Dorup and Maunsbach (1982) reported an almost complete absence of these structures in the distal

tubules of human embryonic metanephros. However, Tiedemann (1976) showed an extensive labyrinth formed by interdigitations of plasma membranes in the cat mesonephros. His results show that mitochondria are compartmentalized by the infoldings; less extensive infoldings were also found in the sheep mesonephros (Tiedemann, 1976).

Enzyme studies have shown that the basolateral infoldings have a direct correlation with the transport of sodium and potassium ions across the plasma membranes. Rostgaard and Moller (1980) showed that the enzyme  $\text{Na}^+\text{-K}^+\text{-ATPase}$  was localized on the basolateral plasma membrane of epithelial cells in distal tubules in the rabbit metanephros and was not found in luminal plasma membrane above the terminal bars. In the adult avian kidney, Dantzler (1972) has shown that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity in kidney slices is responsive to changes in sodium and potassium concentration in culture media.  $\text{Na}^+\text{-K}^+\text{-ATPase}$  plays a crucial role in transepithelial sodium transport in addition to maintaining the intracellular ion composition, this enzyme has been shown to have the highest level of activity in cells of the distal nephron in the kidneys of different animals species suggesting this segment's high capacity for transepithelial sodium and potassium transport, (Imai and Nakamura, 1982; Katz, Doucet and Morrel, 1979). In the case of birds, similar organization occurs in the distal tubules of the adult metanephros (Siller, 1971). In the chick embryonic metanephros however, basal infoldings in distal tubules are few, localized close to interepithelial spaces and do not compartmentalize mitochondria (Narbaitz, et al, 1982). Similarly, our present observations show that in the distal tubules of the chick mesonephros



basal infoldings although constantly present were never abundant. This probably indicates that ion transport in the embryonic distal tubules, although present, is not as active as the one observed in the metanephros of the adult.

The light and dark cells described here have also been described in the distal and collecting tubule of the starling by Nicholson (1982). His observation that these cells do not appear as morphologically specialised (some have no apical microprocesses) in the starling as in their mammalian counterparts (Imai and Nakamura, 1982) agrees with the observation in this study. Dark cells have been suggested to increase in number along the nephron following potassium depletion (Evan et al, 1980).

Apical protrusions in the distal epithelium similar to the ones observed in the proximal tubule epithelia were also found in some cells of the distal segment. These protrusions are similar to those shown in the micrographs of Tiedemann (1976) in the cat and sheep mesonephroi, Koga's (1972) in human mesonephros and Gibley and Chang's (1967) in the chick mesonephros. Although once thought of as secretions (Wendler, 1965; Tiedemann, 1976), it is now known that apical protrusions are artefactual (Tiedemann, 1980).

#### 4.4 Nephron Differentiation

The process of nephron differentiation in the kidneys of higher vertebrates has been studied at both the light and electron microscopic levels. Most of these studies were done primarily in metanephric kidneys in which nephron differentiation is not completed at birth but

continues post partum. Similar studies on the mesonephric kidney of the chick have not been done at the ultrastructural level but there are light microscopic reports in the literature of nephron differentiation in the chick (Stampfli, 1950; Abdel-Malek, 1950). Essentially the processes involved (from aggregation of mesenchymal cells to the vascularization of nephric glomeruli) are identical to those described for mammalian metanephric kidneys (Jokelainen, 1963; Kazimierczak, 1971; Friss, 1980).

Glomerular differentiation has been described by different authors (Suzuki, 1959; Friss, 1980) and attempts have been made to correlate the onset of function with a specific stage of nephron formation. According to Friss (1980), this occurs at stage III of nephron differentiation which is equivalent to stage IV of differentiation presented here i.e. at a stage when all the ultrastructural components of the filtration barrier are sufficiently differentiated to permit filtration. The results presented here show that the various components of the filtration barrier in the most advanced nephrons complete their differentiation at stage 24. Prior to this stage glomeruli show podocytes with very few and short pedicels and a thin monolayered basal lamina. Since urine is present in the allantois after stage 20 (Hamburger and Hamilton, 1952), it is evident that filtration must occur through the partially differentiated glomeruli present between stages 20 and 24.

#### 4.4.1 Differentiation of proximal tubule epithelium

IN the chick mesonephros differentiation of tubular segments is said to commence at the S-shape stage, and functional maturity of the nephron established on the 5th day of incubation as determined by the positive localization of PAS and alkaline phosphatase activities on the luminal border of proximal tubule epithelium (Mitra 1967, Gibley, 1967). At the ultrastructural level, proximal tubule segments were presently identified at stage 20 of embryonic development. Microvilli were present as short, blunt luminal projections of the plasma membrane. Pinocytotic vesicles indicating absorptive function were also evident at this stage. The ultrastructural criteria (apical and lateral processes, pinocytotic vesicles e.t.c.) are probably more valid parameters at earlier stages of proximal epithelial differentiation than histochemical studies. The fact that nephrons between stages 20 and 24 had proximal tubules with ultrastructural signs of resorptive activity confirms the above conclusions since these nephrons with incompletely differentiated glomeruli are already active.

The external glomeruli of the chick were surprisingly very well differentiated ultrastructurally. The transmission electron microscopic study of these glomeruli presented here agrees with the scanning EM study of Jacob, et al, (1977b). The possibility that external glomeruli are functional in the chick has not been established (Abdel-Malek, 1950), However this was initially suggested by Davies (1950) for the duck mesonephros. Based on the close similarity of their filtration barrier to that in functional mesonephric glomeruli, Jacob, et al (1977b) suggested that they are functional. However, it must be stated

that these ultrastructural studies are in themselves insufficient proof of mesonephric function especially during stages 20 to 25. It is hoped that recent advances in single nephron isolation, micropuncture and perfusion techniques that have been used successively in other vertebrate kidney types will now be applied to the chick mesonephros.

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