

**NEPHROGENESIS IN
THE CHICK EMBRYO**

PHD THESIS

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ABSTRACT

The chick embryo has been used to address questions concerning morphogenesis of the urinary tissues (nephrogenesis) of vertebrates. Although of considerable biological and clinical significance, the early nephrogenic tissues in particular have been underutilised in modern developmental analyses. The overall aims of the present study have been to integrate previous nephrogenic analyses and help to provide a framework for utilising and developing techniques and concepts in future studies. The chick embryo is particularly versatile for these purposes.

In amniotes, two types of nephrogenic tissue develop in succession, the nephric and metanephric. The nephric tissues are transient but essential to metanephric development. The nephric tissues also are unusual in undergoing or even completing all stages of morphogenesis (formation, epithelial differentiation, growth and degeneration) during embryonic life.

I have investigated morphological aspects of induction, migration, mitosis and apoptosis, epithelialisation and possible underlying, adhesive interactions during early nephrogenesis, i.e. from initial formation of the nephric tissues until early stages of metanephric development.

While induction of the later nephric and the metanephric nephrons is generally accepted, I have shown that the patterns of formation and expression of the sialylated form of the carbohydrate antigen FC10.2 in the nephric duct and its earliest nephrons are consistent with their induction. Furthermore, there are changes in the expression of S-FC10.2 associated with epithelialisation in both the nephric and metanephric tissues.

Extension of the nephric, though apparently not the metanephric, duct from its area of initial formation has been the subject of a small number of recent studies and I have (re-) examined the relative importance of migration, mitosis and apoptosis in extension of the nephric and metanephric ducts. The putative roles of adhesion in these processes are discussed.

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Table 3

ABBREVIATIONS USED IN TEXT

BSA	Bovine serum albumen
BFS	Buffered formal saline
BUDR	Bromodeoxyuridine
CMF	Calcium- and magnesium-free Tyrode's saline
DAB	Diaminobenzidine
DMSO	Dimethylsulphoxide
H&H	Hamburger and Hamilton (1951) stage
IMS	Industrial methylated spirits
LM	Light microscopy
P&C	Pannett and Compton (1924) saline
PBS	Phosphate-buffered saline
PCV	Posterior cardinal vein
SEM	Scanning-electron microscopy
TEM	Transmission-electron microscopy

For abbreviations used on photographs, see Table 12,
pp. 106-108.

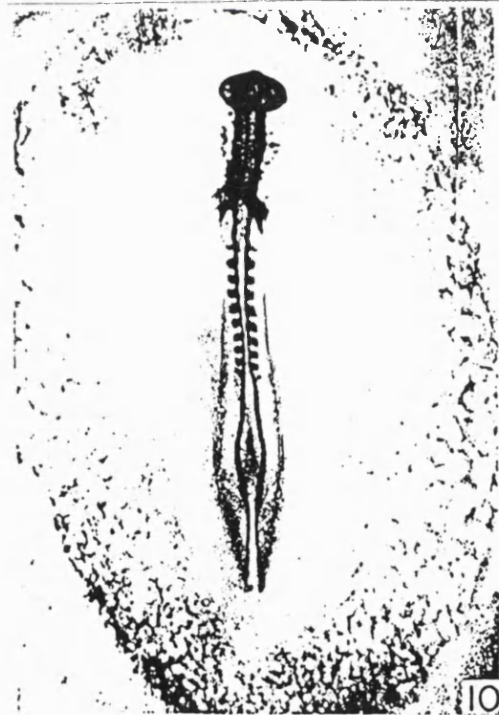
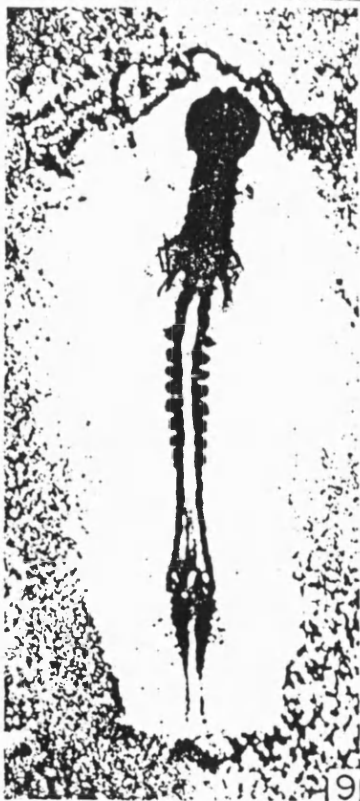
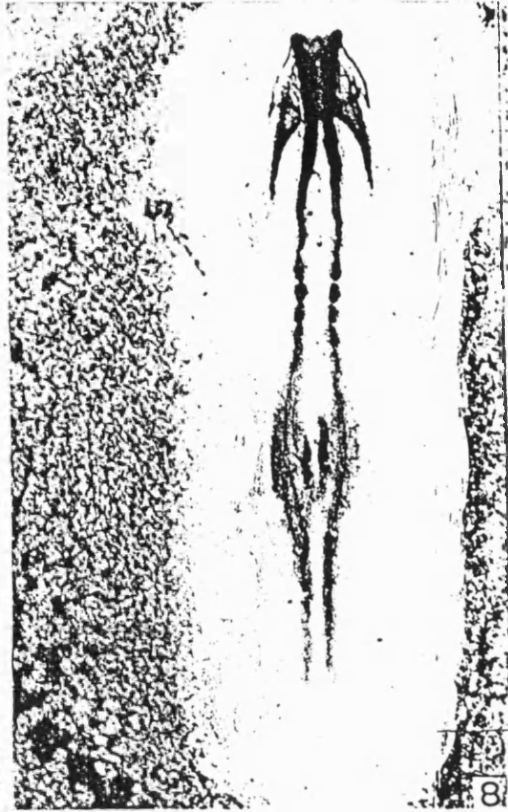
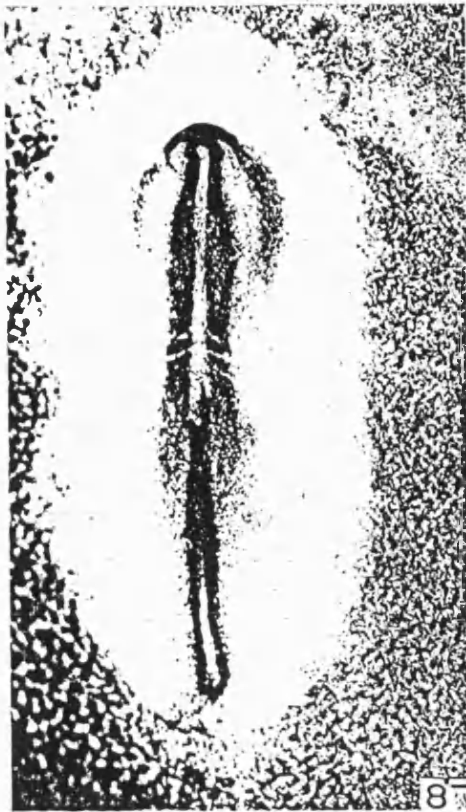
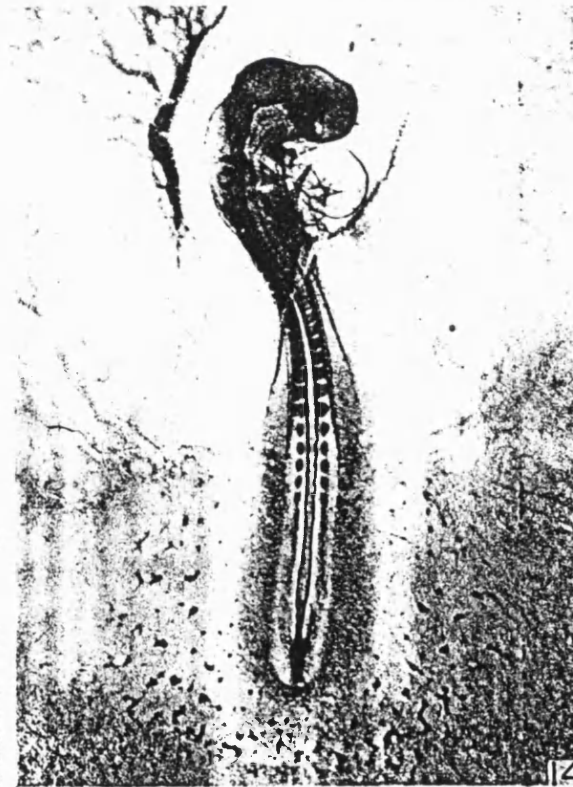
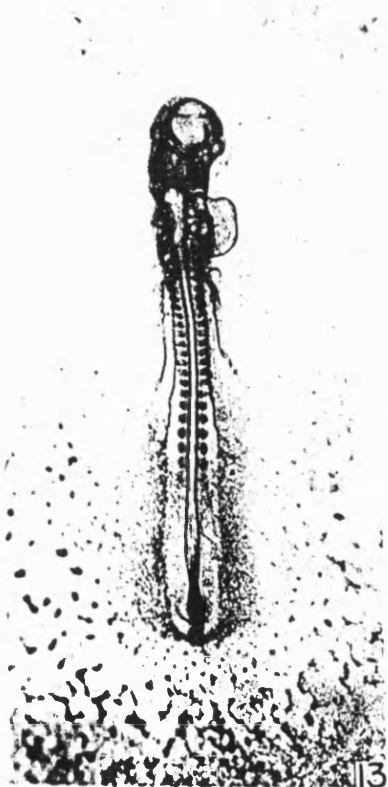
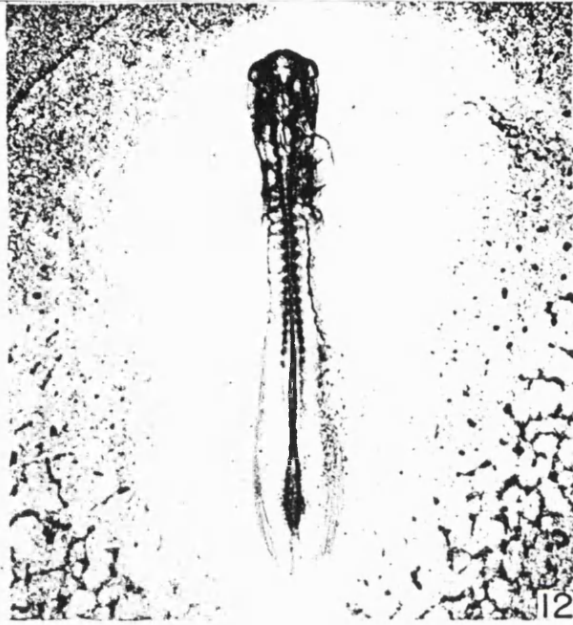
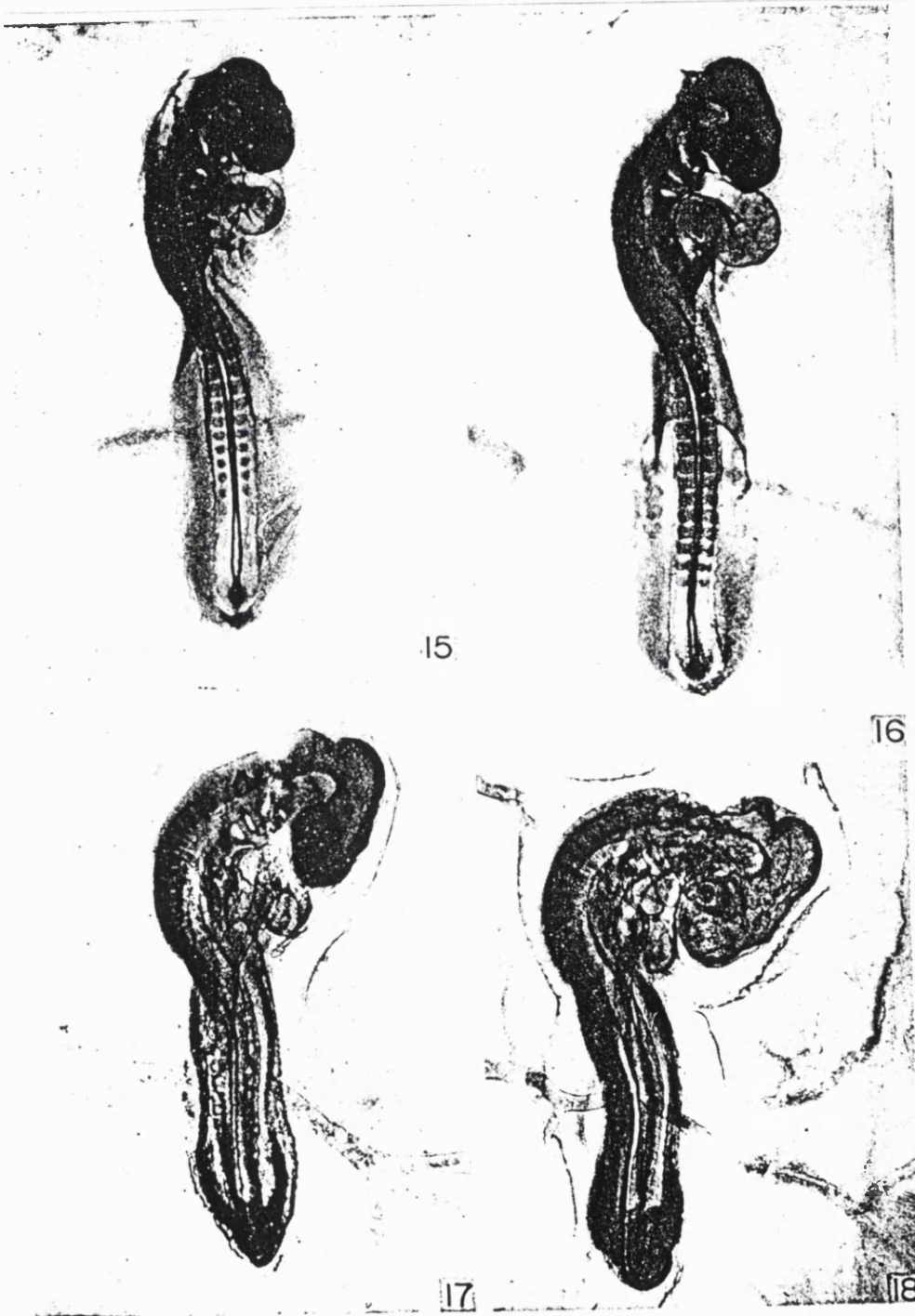
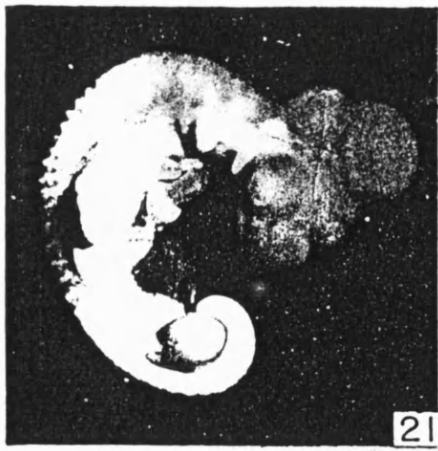
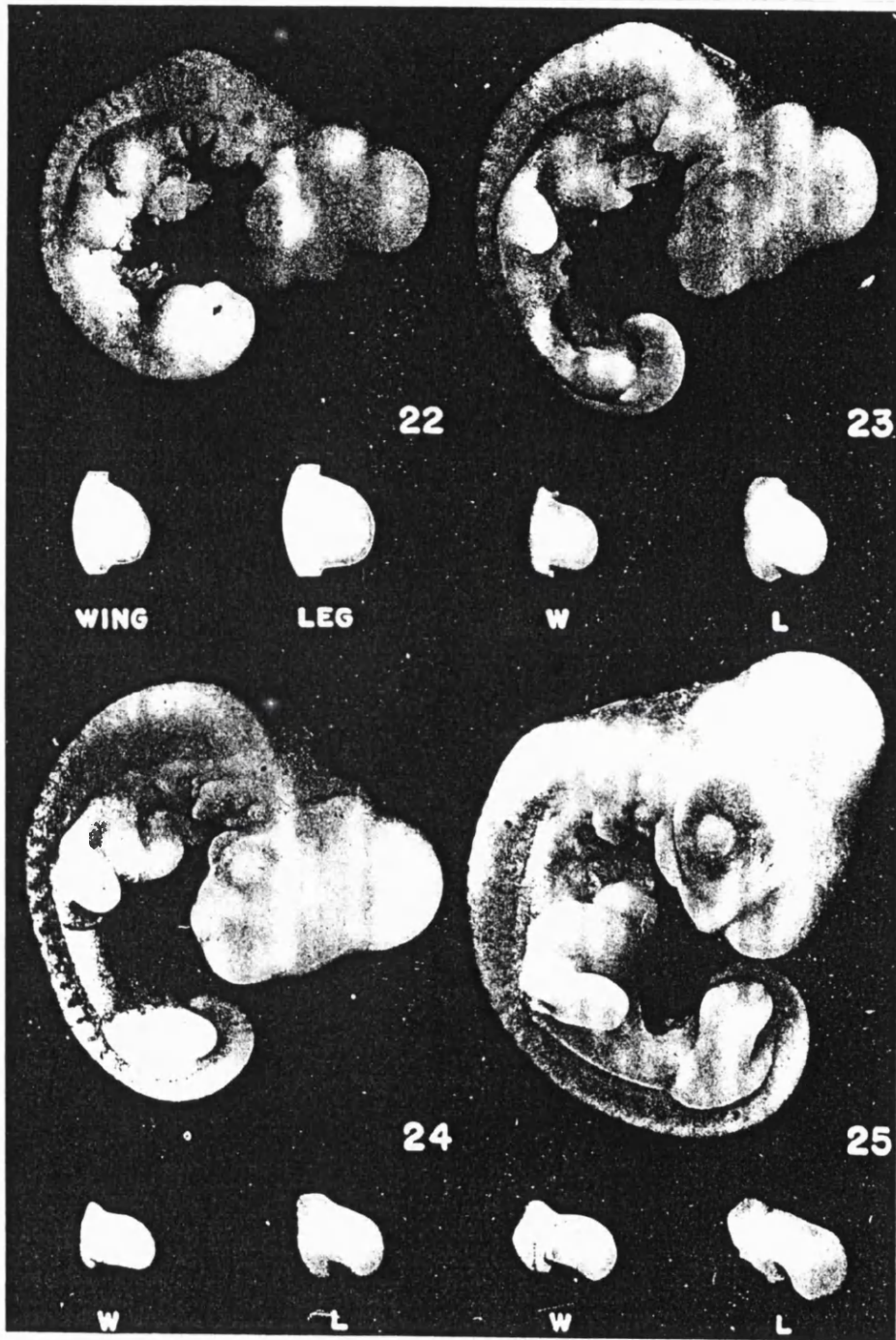


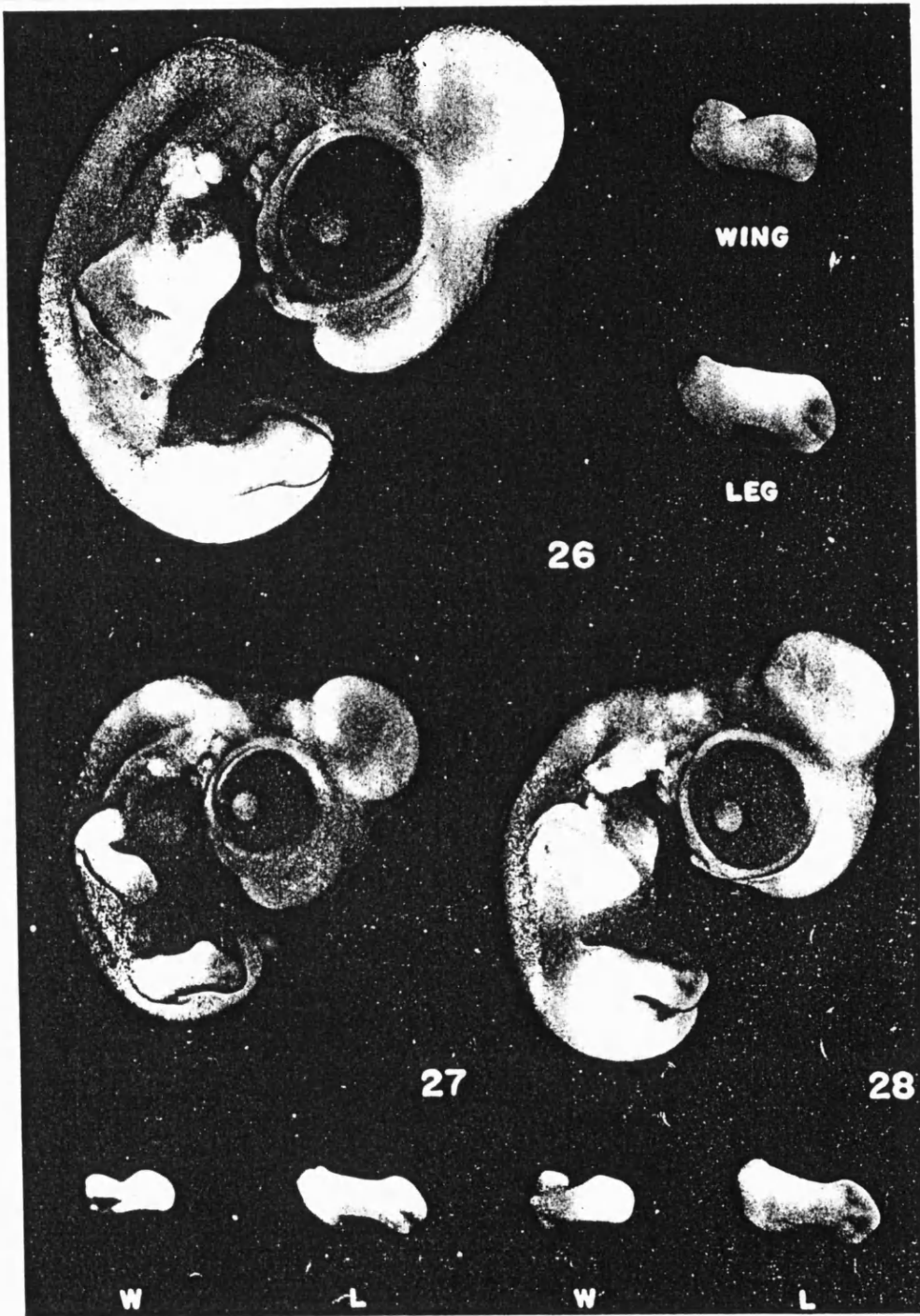
Table 4 Normal table of *Gallus domesticus*, from stage 8 to stage 28. (From Hamburger & Hamilton 1951; continued overleaf). All magnifications approx. X 12.











CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The developing urinary (nephrogenic) tissues of many vertebrates, e.g. the domestic fowl (Gallus domesticus), mouse (Mus mus), three-clawed toad (Xenopus laevis), axolotl (Ambystoma mexicanum) and trout (Salmo trutta), provide suitable models for the analysis of common morphogenetic processes and are of intrinsic interest to nephrologists. However, since the development of specific cell-marking and genetic recombinant techniques (pp. 51-53), the early nephrogenic tissues in particular have received little attention and many nephrogenic analyses which have been carried out have been un-coordinated.

Analytical approaches to morphogenesis may either be 'morphological', to observe normal developmental events, or 'manipulative', to investigate an underlying process by disrupting these events in a controlled manner. Morphological analyses logically lead and are essential to manipulative ones, in providing both indirect evidence of mechanisms for investigation and the means by which the results of manipulations are assessed. It must also be a long-term aim of any visual science to demonstrate both events and processes morphologically and dynamically. Analyses may also be at different levels of resolution, i.e. tissue, cellular or sub-cellular.

The present study aims to form a basis for future analyses of nephrogenesis (morphogenesis of the urinary tissues), by reviewing previous work and using morphological techniques to re-address the main questions to emerge. An attempt has been made to provide a framework by arranging the present study according to the analytical concepts of level, approach, morphogenetic stage and process, technique and model, which are introduced in the present chapter. Terms are defined in Table 5 and an analytical framework is presented in Table 6.

Table 5

TERMINOLOGY

Phylogenesis: development of an organism (evolution)

Embryogenesis: development of the embryo (ontogenesis)

Morphogenesis: development of tissue form/ morphology

Nephrogenesis: morphogenesis of urinary tissues

Embryonic ('normal') stages: developmental stages of the
embryo as a whole

Morphogenetic stages: formation
epithelial differentiation
growth
degeneration

Nephrogenic tissues: nephric/ metanephric ducts
nephric/ meta- nephrons
nephrons on each side comprise a
nephric/ meta- nephros (pl. -oi)

Early nephrogenesis: until appearance of the earliest
metanephrons

Other mesodermal tissues: axial (notochord)
paraxial (somites)
lateral (includes some
gastrointestinal and
reproductive tissue)

cont'd.

Levels of analysis: tissue (multi-cellular: adjacent cells
of similar phenotype)
cellular (whole cells)
sub-cellular (extracellular, cell-
membranous, cytoplasmic, or nuclear)

Approaches to analysis: morphological (non-disruptive)
manipulative (experimental)

Primary morphogenetic processes: induction
migration
proliferation (mitosis)
degeneration (apoptosis)

Analysis: addressing a question, using one or more
techniques

Analytical systems: in ovo
in vitro (cell-, tissue-, or organ-
culture)
(whole-) embryo culture

Model: a species, tissue and system,
e.g. chick nephric, in ovo

Amniote: vertebrate possessing an amnion during
embryogenesis (cf. anamniote)

Table 6

ANALYTICAL FRAMEWORK FOR NEPHROGENESIS

The categories below are numbered according to the order in which they are used in the present study. Their integration forms an analytical framework. See Table 5 for terminology.

- 1 Level: tissue
cellular
sub-cellular

- 2 Approach: morphological
manipulative

- 3 Morphogenetic stage: formation
epithelial differentiation
growth
degeneration

& primary process: induction
migration
mitosis
apoptosis

- 4 Analysis: question and technique
& model: species, tissue and/or system

- 5 Timing
& regions of nephrogenetic changes

In amniote (avian, mammalian & reptilian) embryos, there appear to be two main types of urinary tissue. The nephric tissues, which are transient urinary tissues, undergo much of their morphogenesis at earlier embryonic stages, in an anteroposterior direction; whereas the definitive, metanephric tissues develop later, more posteriorly and in a predominantly radial pattern. The tissues also have at least slightly different morphologies and physiologies. However, their basic morphologies and homeostatic functions are similar, at least at certain levels of resolution and developmental stages, while in anamniotes (fish & amphibians), the nephric tissues are definitive (figs. 1-4).

Furthermore, the metanephric tissues are thought to develop as a result of a process (of induction, Saxén 1987) beginning much earlier than their formation. It appears not only that the nephric and metanephric regions are closely apposed at some stages, but that the nephric tissues are essential to formation of the metanephric tissues. Hence their broad similarities, finer differences and a likelihood of spatial and/ or temporal interactions, show the importance of co-ordinated analyses of nephric and metanephric development.

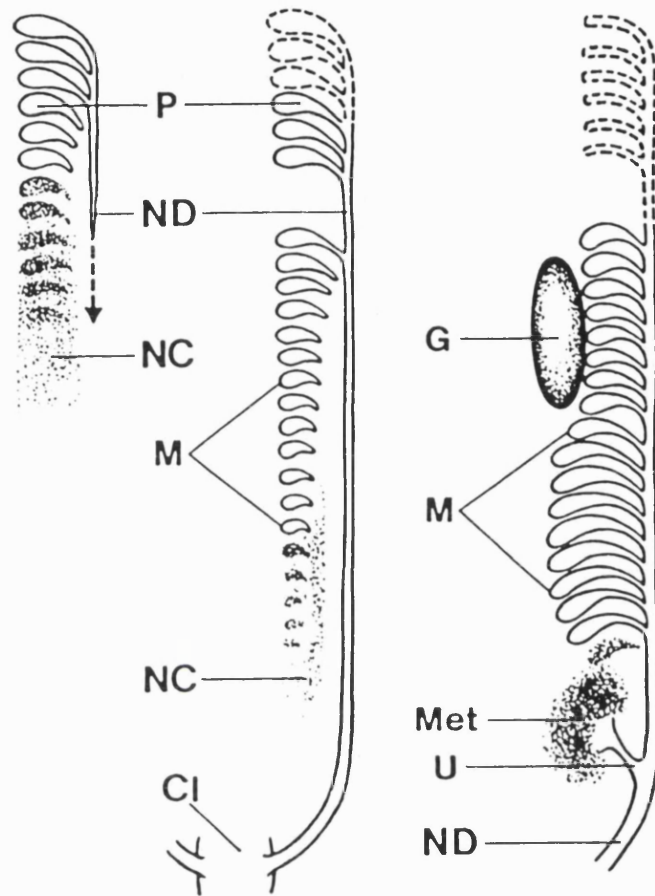


Fig. 1 The nephrogenic tissues of vertebrates, in longitudinal section, according to previous analyses. P pronephros; ND nephric duct; NC nephrogenic cord (presumptive nephrogenic tissue); G gonad; M mesonephros; Met metanephros; U ureter or metanephric duct; Cl cloaca. (From Saxén 1987.)

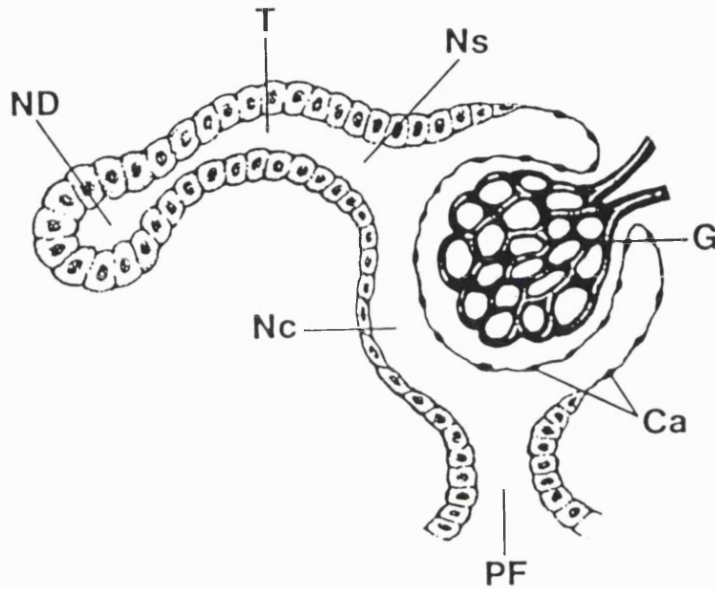


Fig. 2a The vertebrate nephron, in transverse section, showing its relationship to the urinary duct throughout development. ND nephric or metanephric duct; T nephron tubule; Ns nephrostome; G glomerulus; Ca (Bowman's) capsule; Nc nephrocoel; PF peritoneal funnel. (From Saxén 1987.)

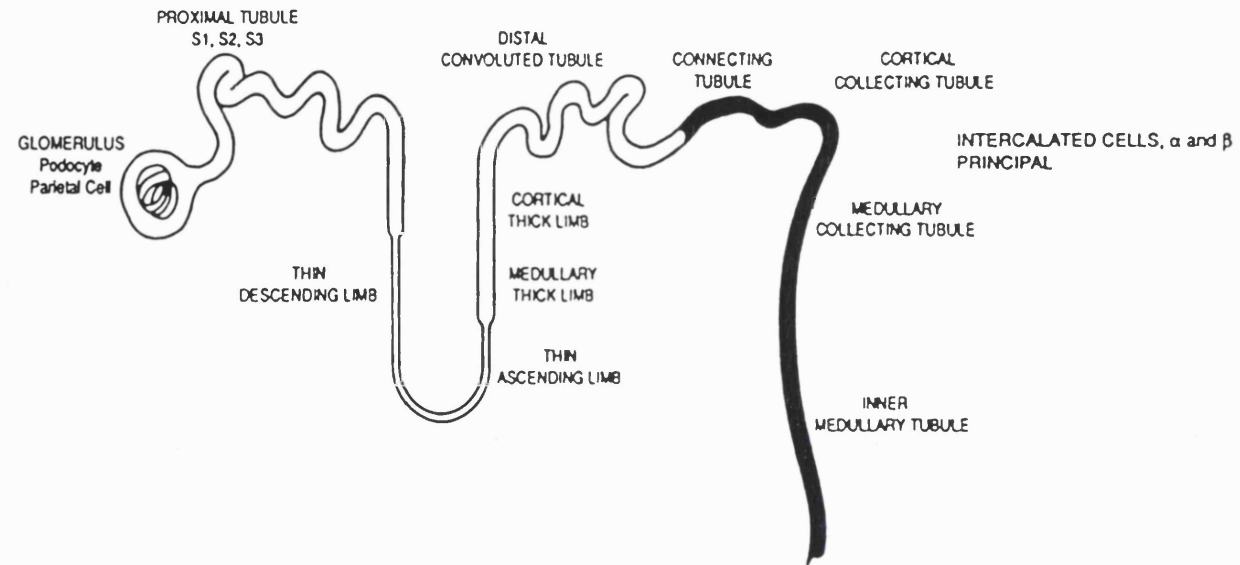


Fig. 2b The mature vertebrate nephron, showing its known segments and indicating some of the different epithelial cell-types. (From Herzlinger et al 1992).

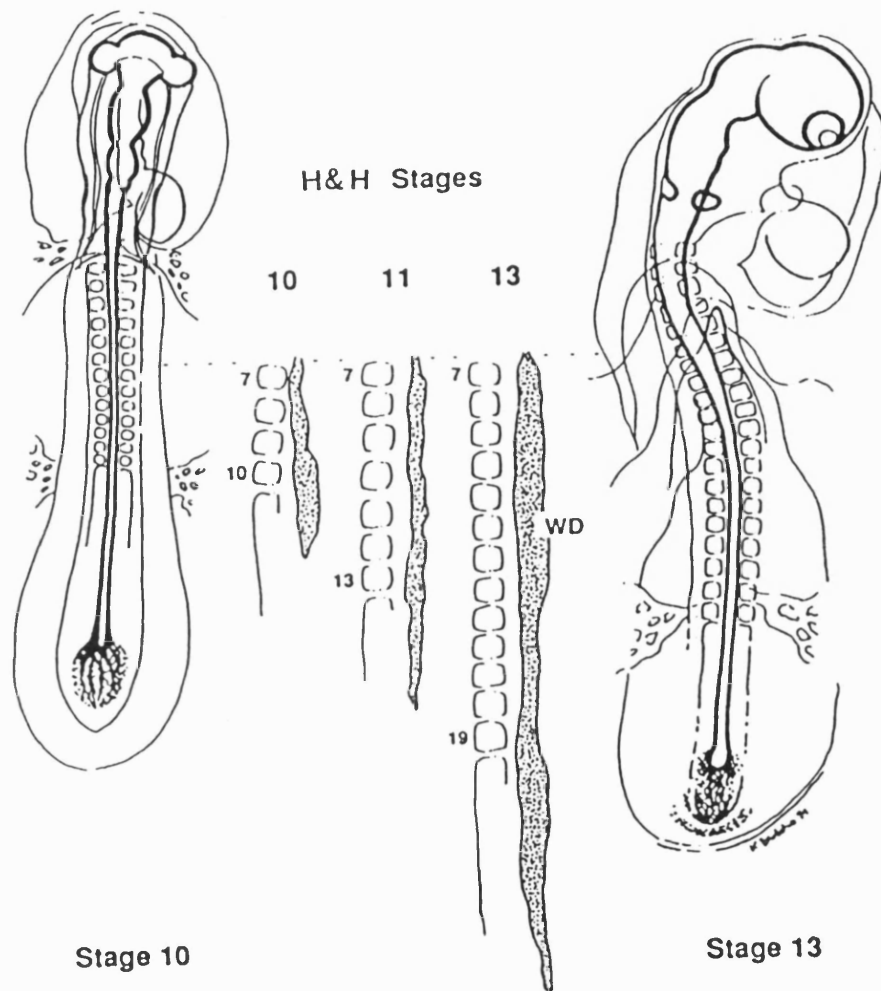


Fig. 3 Early nephric development in the chick, showing the extending nephric (or Wolffian) duct (WD) and its relation to the extending somite file (the most posterior somite is numbered). (From Jacob et al 1992.)

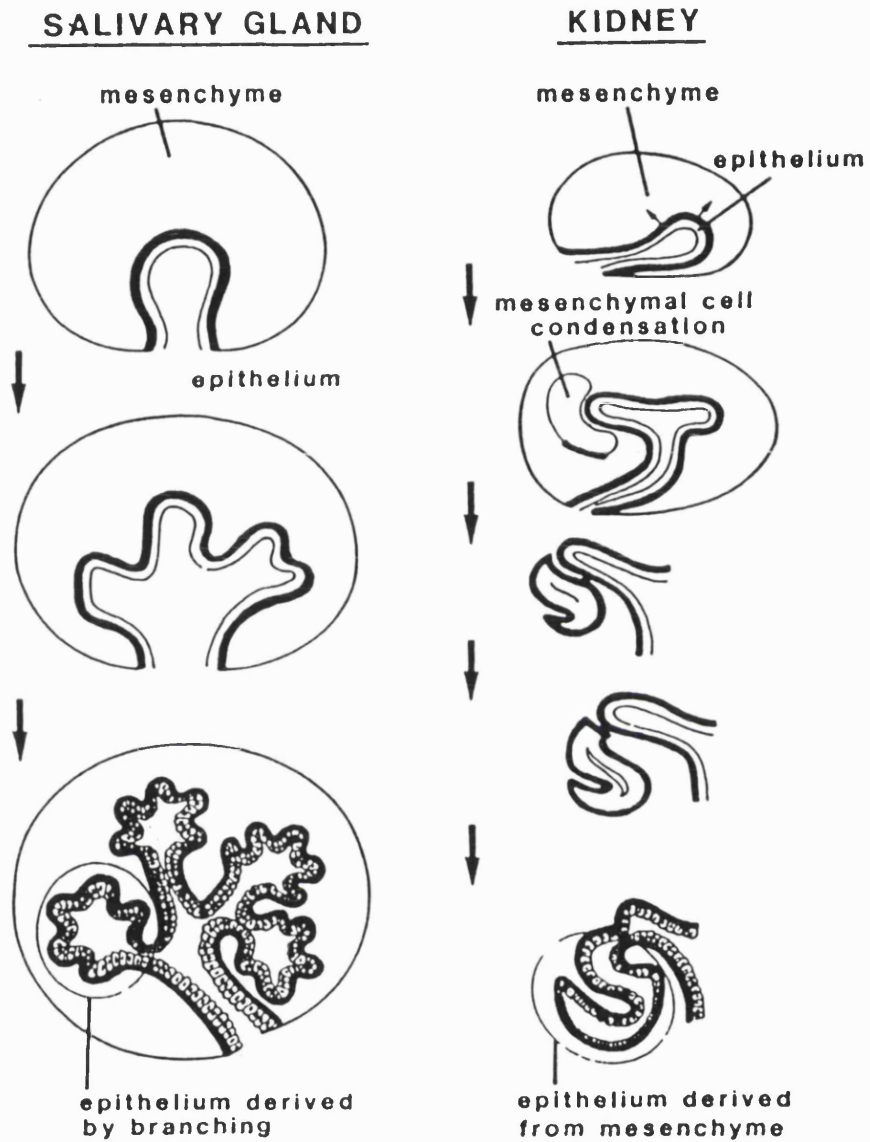


Fig. 4a 'Branching morphogenesis' of two epithelial tissues which are induced to branch by the mesenchyme. In the kidney (metanephros), but not in the salivary gland, the mesenchyme also becomes epithelial. (From Ekblom et al 1987).

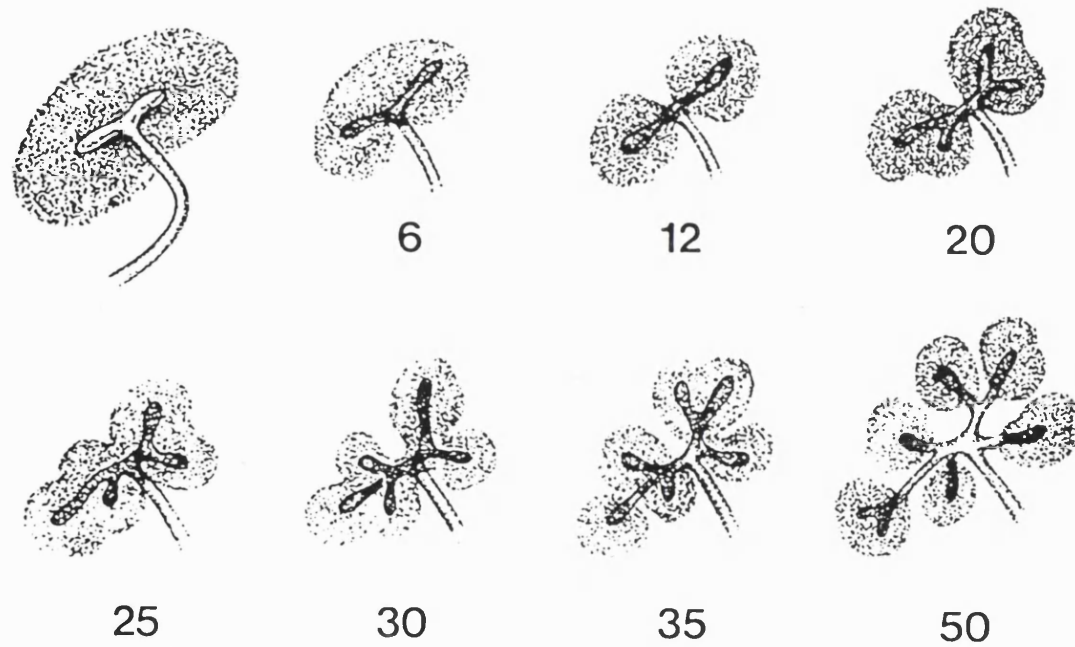


Fig. 4b Early metanephric development in the mouse *in vitro*, showing repeated bifurcation of the metanephric duct (U) and condensation of the metanephric mesenchyme (Mc). The numbers indicate hours after invasion of the mesenchyme by the duct. (From Saxén 1987.)

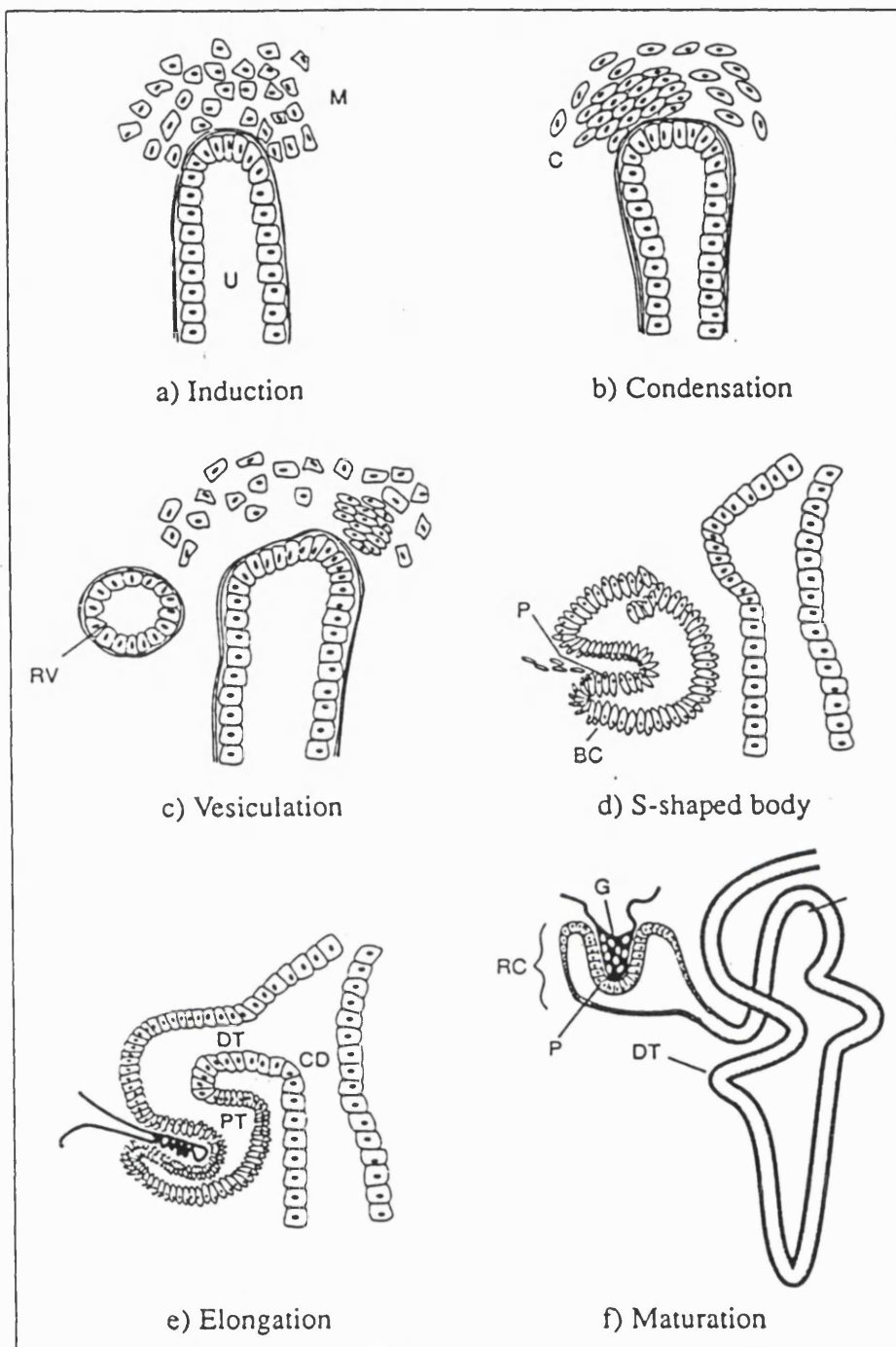


Fig. 4c Early stages of development of the vertebrate metanephron. M metanephric mesenchyme; U ureteric (or metanephric) duct; C condensed mesenchyme; RV renal vesicle; BC Bowman's capsule; P podocytes; PT proximal tubule; DT distal tubule; CD collecting duct; G glomerulus; RC renal corpuscle. (From van Heyningen & Hastie 1992).

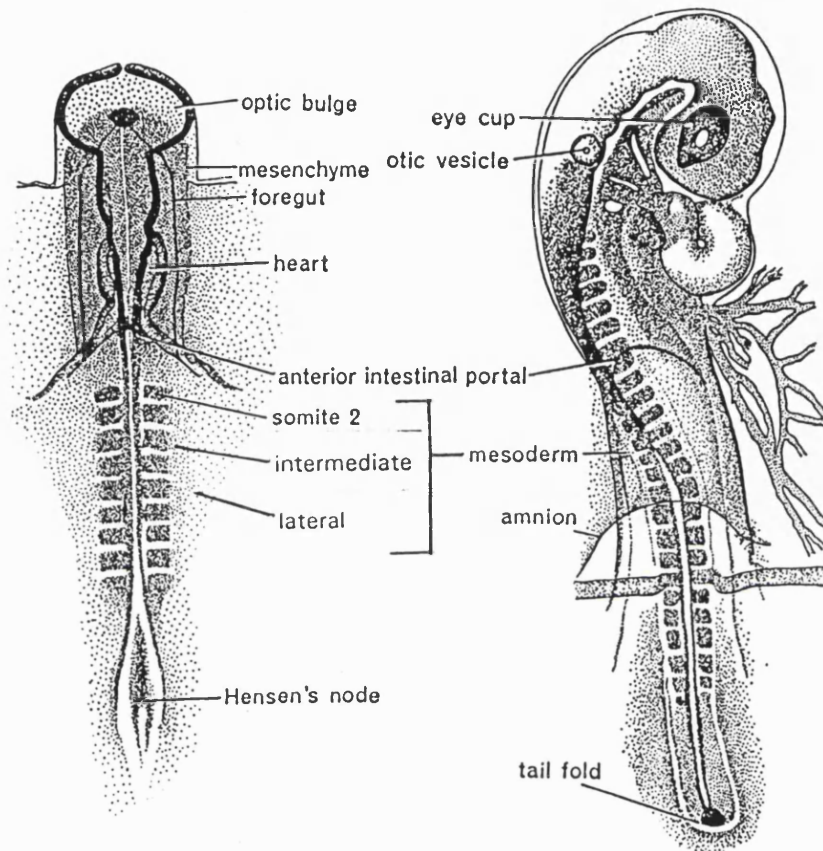


Fig. 5a The chick embryo at about H&H 10 (left) and H&H 15 (right), viewed from the dorsal side, and showing different regions of mesoderm and the amnion. (From Bellairs 1971).

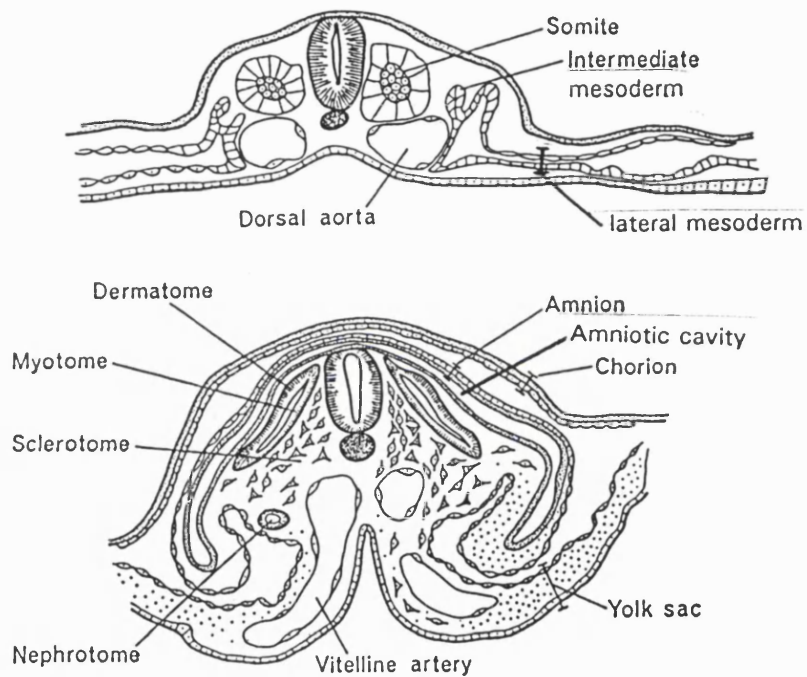


Fig. 5b Transverse section of the chick embryo, showing different regions of mesoderm (top) and the amniotic cavity (bottom). (From Bellairs 1971).

The nephrogenic tissues are at least predominantly mesodermal (fig. 5) and form simple epithelia from mesenchyme. Although in the nephric tissues only a small number of analyses in the chick and Xenopus appear to have addressed epithelial-mesenchymal conversion, it has been shown that the epithelial metanephric duct induces the mesenchyme to form rudimentary metanephrons in the mouse (Saxén 1987). Such formation is common in secretory/excretory epithelia, e.g. respiratory, mammary and salivary alveoli, and parts of the gastrointestinal tract and integument, but metanephric tissue is unusual because the presumptive metanephrons reciprocally induce the duct (fig. 4a; Ekblom et al 1987).

The nephric tissues may also be useful models for morphogenesis in other tissues, although they have not been used as such. For example, while successive morphogenetic stages - phenotypic differentiation, rudiment formation, epithelial differentiation, growth and degeneration - may occur simultaneously in different regions of many tissues, they may be followed more easily in the nephric tissues where morphogenesis is predominantly linear and the tissue as a whole may degenerate.

Nephric tissue degeneration has been regarded as evolutionary (phylogenetic), uncommon (abnormal) in comparison to other embryonic tissues and contradictory to embryonic development (ontogenesis). To some extent this seems to have made the nephric tissues seem less relevant than the metanephric tissues to nephrogenetic analyses and less relevant than other tissues (e.g. precardiac & somitic) for analyses of early mesodermal development. However, the widespread occurrence, significance and genetic basis (e.g. Ellis et al 1991) of morphogenetic cellular death is becoming recognised. It has therefore been termed 'apoptosis', to indicate it is a normal, active process, distinct from pathological necrosis (see Altman 1992).

The nephric tissues are also an interesting morphogenetic model because their morphology is more similar to the metanephric tissues, e.g. in the appearances of the nephrons, than other early mesodermal tissues to their derivatives. The somites, for example, bear little similarity to their muscle and bone derivatives. By investigating their interactions, it may be shown to what extent the nephric and metanephric tissues are the same and

how differences reflect degrees of commitment and environmental influences. For example, it is conceivable that nephric degeneration might be partly caused by transdifferentiation and/ or migration to a metanephric site.

It is also important to analyse apparently similar morphogenetic processes in different tissues, where they may be found to differ. For example,

a great variety of processes have been lumped together under the term "induction" A search for a common determinative factor, or "organizer", failed in the 1930s and today we may fall into the same trap in searching for mechanisms common to the various types of inductive interactions (Saxén 1987, p.52).

It is also important to integrate analyses of a particular tissue between different vertebrates, principally to show what is common or variant and hence the suitability of a particular model for a particular investigation. Although the mouse, as a mammal, might be assumed to bear more similarities than the chick (Gallus domesticus) to human nephrogenesis, this is poorly substantiated and indeed the chick bears many similarities to humans in embryonic development.

Furthermore, while the mouse metanephric tissues comprise a well-established, in vitro system (fig. 6), there are several disadvantages in using the mouse, which may be overcome by the chick. For example, mammalian embryos are difficult to maintain in situ or in culture at post-implantation stages - although Lawson and her colleagues have maintained primitive-streak mouse embryos in culture for about 24 hours, e.g. Lawson et al (1991) - and hence development can not be investigated continually or over a time-interval, such as required for morphogenetic experiments. Also, while there may be an advantage of greater control over influences acting on isolated cells and tissues, localised manipulations in whole embryos are being developed (fig. 7; Tickle 1992; Davies 1992; Murphy & Carter 1992), normal interactions between and within tissues and the development of three-dimensional tissues are only partially replicable in isolation.

The chick embryo would seem particularly suitable for integrated analyses because of its availability and accessibility to a wide range of investigations in ovo, in whole-embryo culture (figs. 8-10), or in isolation, with the nephric and metanephric tissues readily distinguishable from

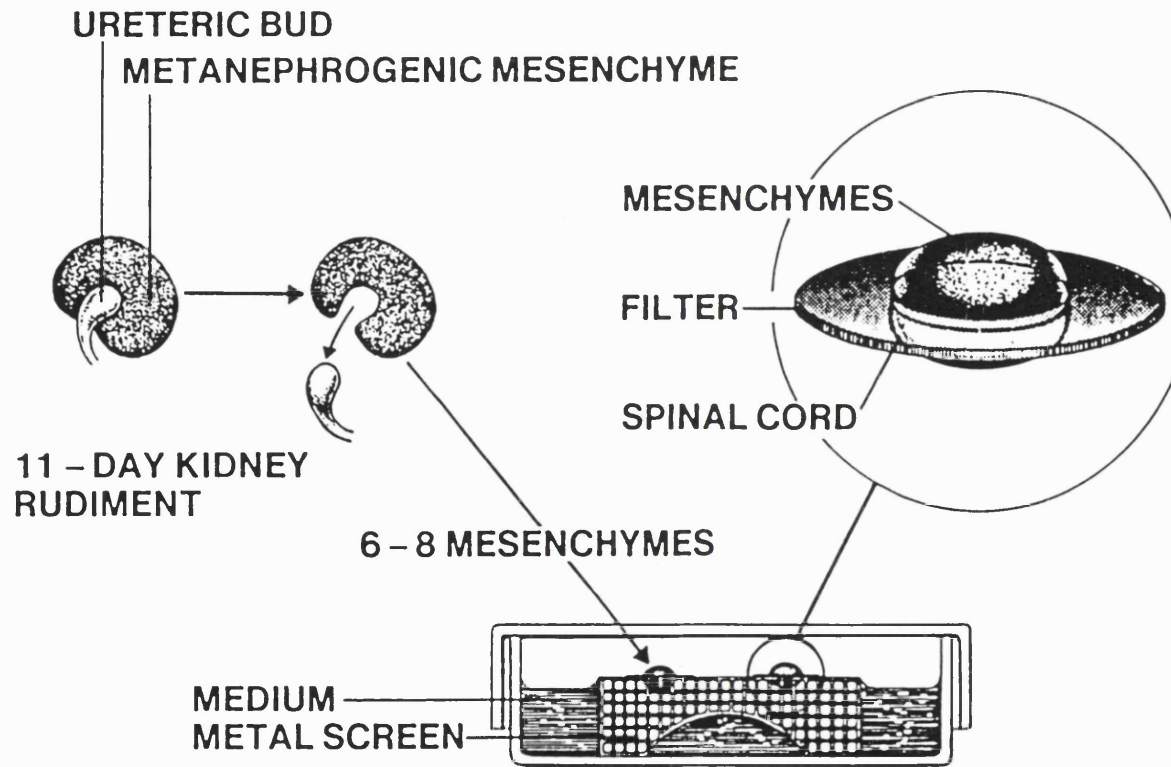
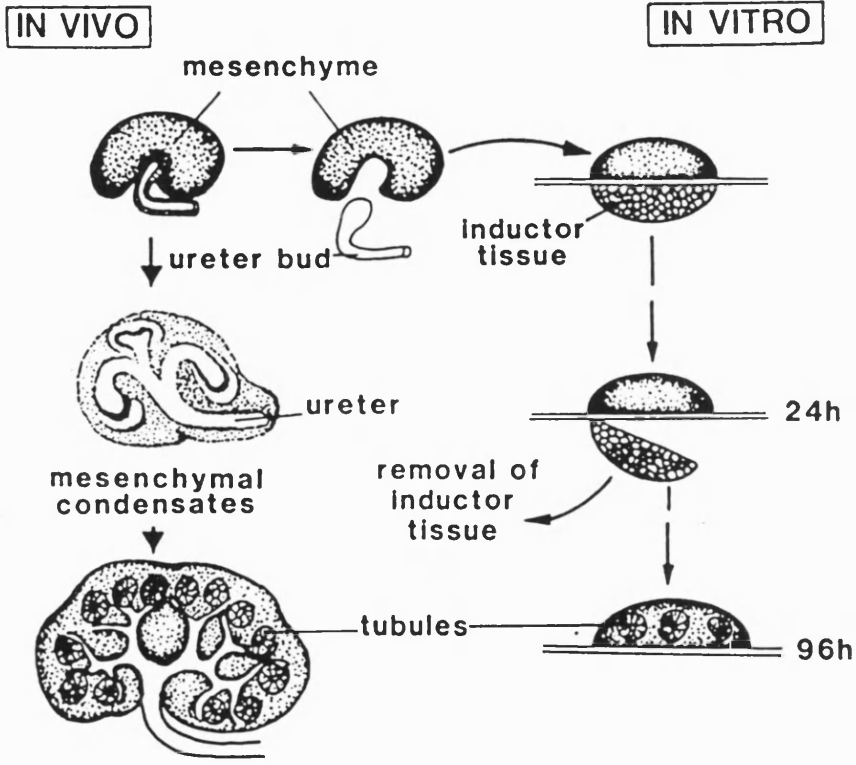
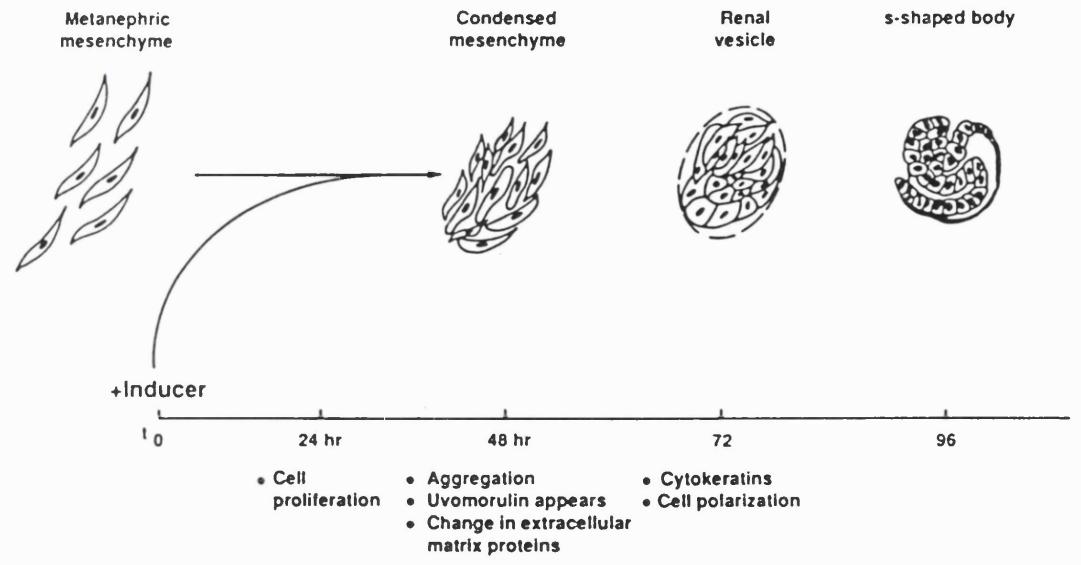


Fig. 6a Grobstein's (1956) transfilter technique, for analysing metanephric induction *in vitro*.
(Redrawn by Saxén 1987.)



b



c

Fig. 6b (left) Comparison of metanephric differentiation *in vivo* (far left) and using Grobstein's (1956) transfilter technique (centre left). (From Ekblom et al 1987).

Fig. 6c (right) The time-course of induction of the metanephric mesenchyme using Grobstein's (1956) transfilter technique. (From Herzlinger et al 1992).

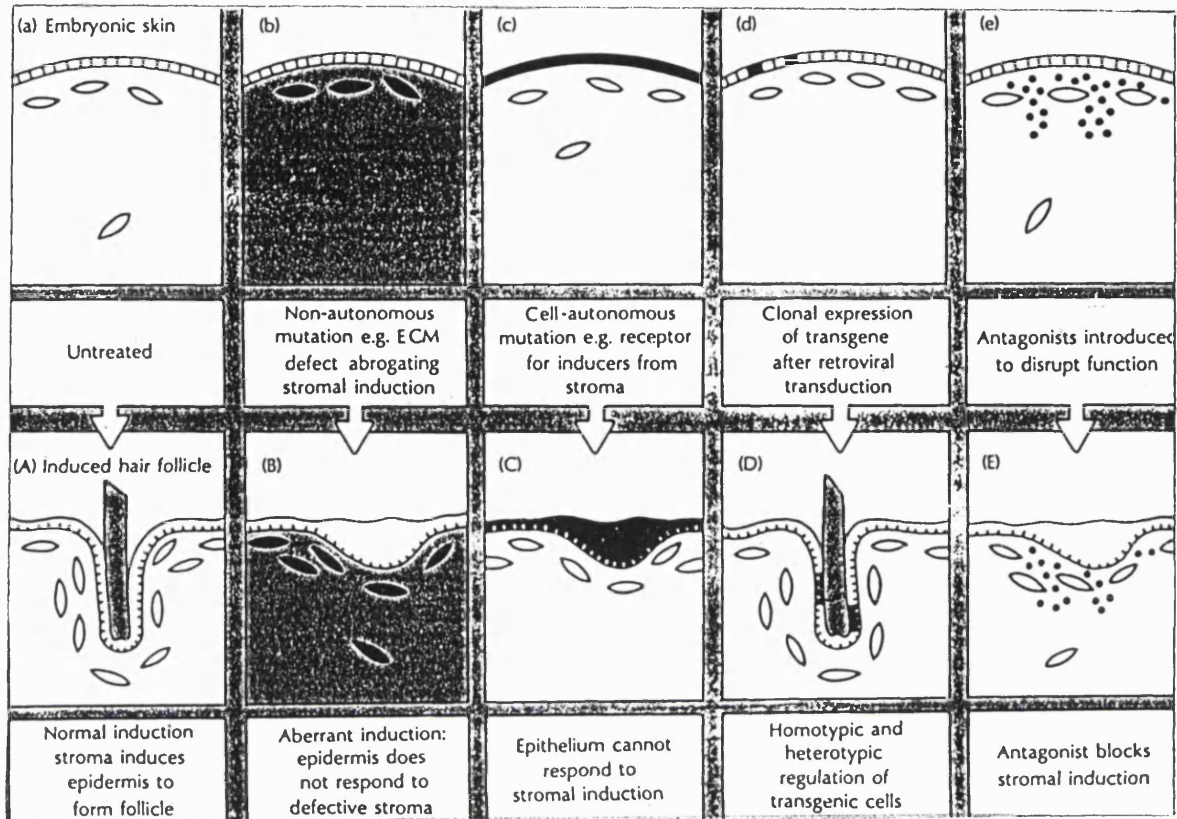


Fig. 7 Local manipulations in the intact embryo, using induction of the hair follicle as a developmental model and genetic or antagonistic techniques. **a-e** embryonic skin before induction; **A-E** after normal or aberrant induction. In **b** and **c** the dark cells have been genetically altered, either using germ-line transgenic approaches or naturally. In **d** the dark cells have been individually transduced with retroviral vectors, which expand into clones in **D**. In **e** the spots represent antagonists of local environmental function (in this case stroma-epithelial interactions), e.g. antibodies, synthetic peptides or proteases. (From Stoker et al 1990b.)

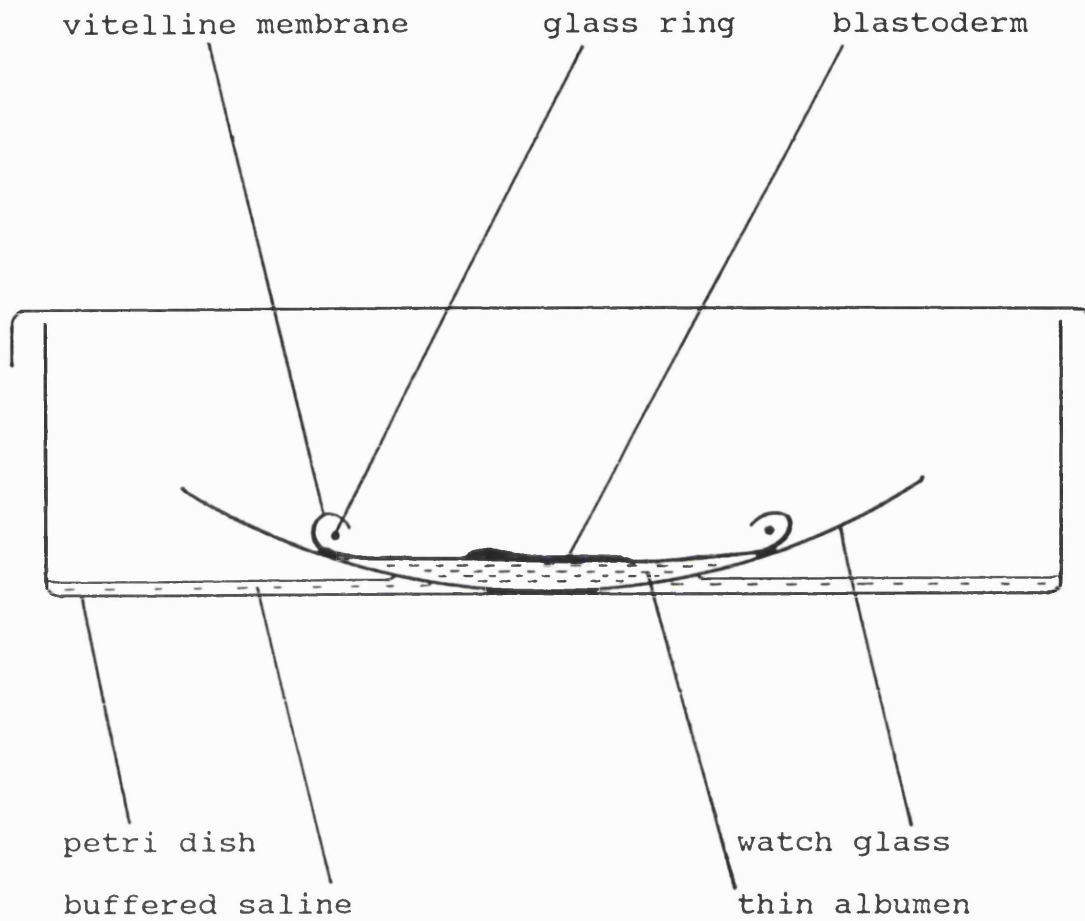


Fig. 8 New's (1955) technique for whole-embryo culture of the chick, from laying to about two days incubation (H&H 17-18). (Re-drawn.)

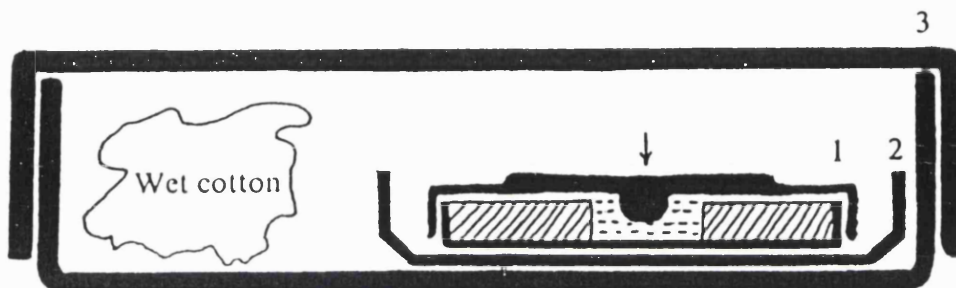


Fig. 9 Augustine's (1977) technique for whole-embryo culture of the chick, from about two to five days incubation (H&H 13-28). Numbers indicate plastic tissue-culture dishes of increasing diameter. The arrow indicates the embryo, lying dorsal side up. At the earlier stages the embryo is suspended by its vitelline membrane and at later stages by its vascular membranes over the edges of dish 1. Dish 1 is filled with semi-solid agar except for a central canal containing tissue-culture medium. (From Augustine 1977.)

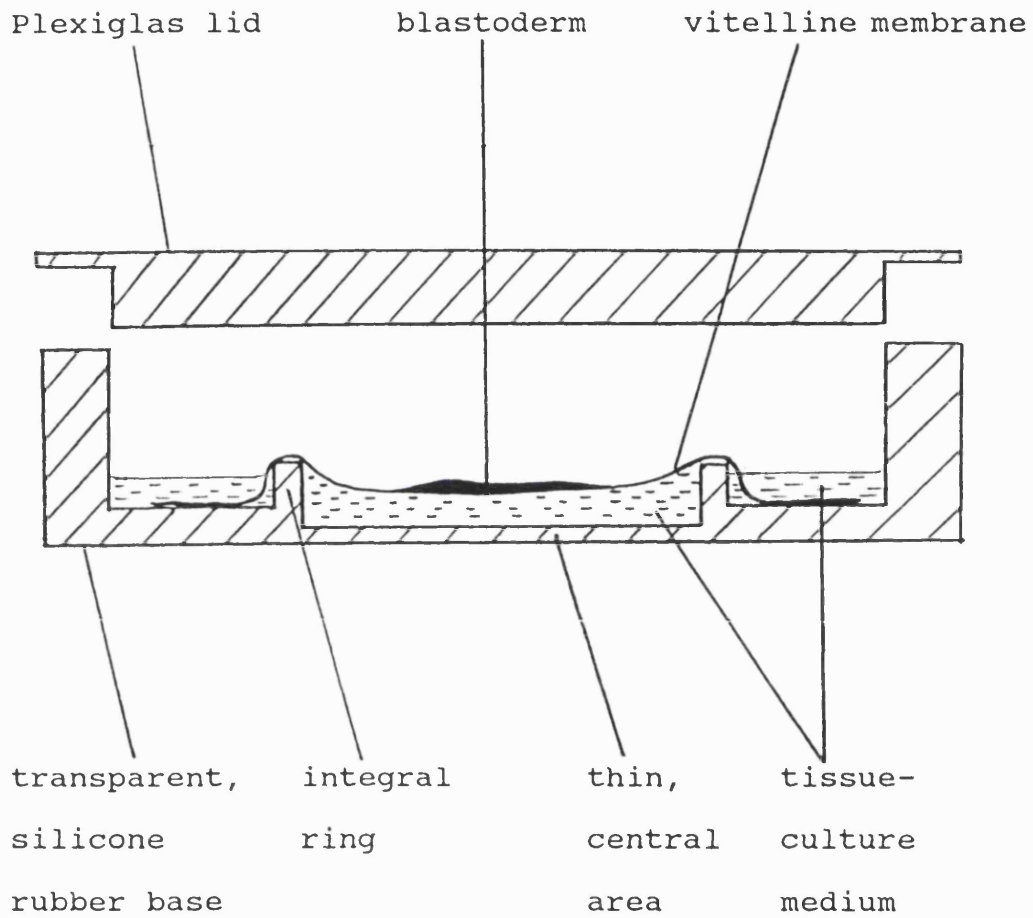


Fig. 10 Kučera & Burnand's (1987) technique for whole-embryo culture of the chick, from about 8 hours to three days incubation (H&H 2-18). The thin, central part of the base allows observation and injections without removing the lid. (Re-drawn.)

each other, at all post-laying stages. Yet it has been considerably under-utilised in analyses of nephrogenesis. Table 4 (pp. 11-16) is a partial Normal Table of chick development.

The early blastocysts of mice and chicks may also be obtained either by fertilisation in vitro or removal from the female (in the chick embryo cleavage is usually completed before laying). Additionally, because they are fertilised and usually develop externally without a hard shell, the early blastocysts of many Amphibia (e.g. axolotl and Xenopus) and fish (e.g. Salmo trutta) are particularly suitable for comparative analyses. Egg-encased reptilian embryos would appear equally suitable to those of birds, while those of viviparous reptiles would seem comparable to mammalian embryos in accessibility.

Recent nephrogenic analyses have fallen almost without exception into two mutually exclusive categories: i. tissue level, of the nephric tissues, in whole embryos, either chicks (e.g. Jacob et al 1992) or amphibians (e.g. Lynch & Fraser 1990); ii. cellular or sub-cellular level, of the metanephric tissues, in vitro, of mice (reviewed in Saxén 1987 and Bard 1992a&b). Analyses of vertebrate

nephrogenesis were last reviewed by Romanoff (1960: chick), Fox (1963: amphibian) and Saxén (1987) and Bard (1992a&b) (mouse). There appear to have been no comparative reviews since Fraser (1950), Burns (1955) and Torrey (1965). Reptilian development has received little attention (Deeming & Ferguson 1991).

The usual means of investigating morphogenetic interactions is through analysis of the 'primary' (Ekblom et al 1987) morphogenetic processes of induction and migration. These appear to be ubiquitous during regulatory development, as of vertebrates. An example of more cell-autonomous, or mosaic-type, morphogenesis is found in the nematode, Caenorhabditis elegans. However, this too is increasingly becoming recognised as interactive (e.g. Bossinger & Schierenberg 1992).

Embryonic induction, demonstrated first by Spemann in 1901, occurs 'whenever two or more tissues of different history and properties become intimately associated and alteration of the developmental course of the interactants results' (Grobstein 1956). Induction has been fairly extensively analysed in the metanephrons, where it is permissive and reciprocal with the metanephric duct (Saxén 1987). There have been few

investigations of induction in the nephric tissues, where it appears that the nephrons may be induced by the duct (see Chapter 2, pp. 73-76). Further investigations are especially necessary because the permissive nature of metanephric inductions suggests that earlier, directive inductions (fig. 11) might take place in the nephric tissues. The nephric cells might then migrate to the presumptive metanephric region; the role of migration in induction has been recognised (Saxén 1987).

Migration may be short- or long-distance. At the cellular level, short-distance migration may result in rearrangement, such as the formation of epithelia from dense mesenchyme, while long-distance migration of whole cells is seen by those of the neural crest and the primordial germ cells. Long-distance migration of parts of cells is seen in one of the neural crest's derivatives, the peripheral sensory neurons, whose axons migrate. At the tissue level, long- and short-distance migration occur in formation of the mesoderm (gastrulation; see Stern 1992a) and its subsequent movements, for example in the precardiac mesoderm (e.g. Linask & Lash 1986), and in wound-healing. A number of analyses of the nephric duct have concluded that it extends antero-

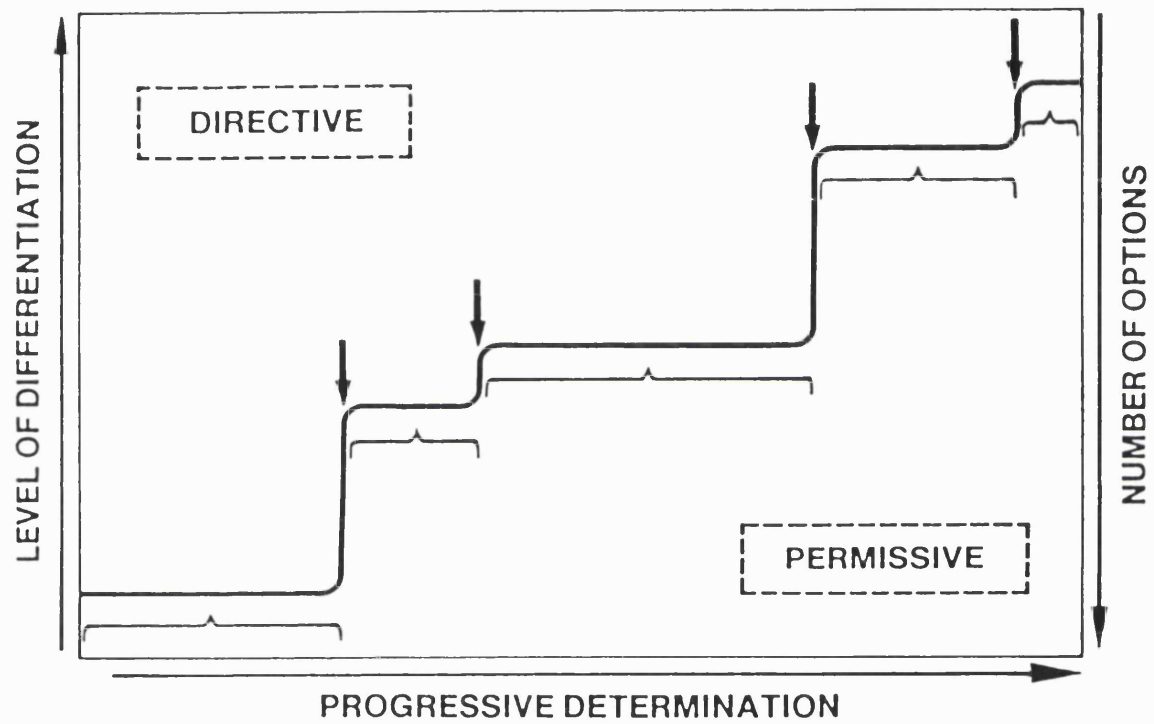


Fig. 11 Summary of the hypothesis of directive and permissive inductions. (From Saxén 1987.)

posteriorly by migration. The evidence is generally unsatisfactory, though the best is that of a single, chimaeric analysis by Martin (1976) (see Chapter 2, pp. 76-84).

Mitosis and apoptosis are the other two 'primary' morphogenetic processes. Before the importance of migration in development was appreciated, it was thought that morphogenesis was brought about by differential rates of mitotic cell division (see Abercrombie 1982). Although mitosis and apoptosis are not necessarily interactive and occur in apparently mosaic development, increasing attention is being focussed on their interactions with other processes in animal development, typified by work on 'growth factors', which appear to play roles in mitosis (see Rozengurt 1992) and other morphogenetic processes. Mitosis is thought to be an early response to induction (Saxén 1987).

The most usual means of growth (an increase in volume) of a tissue (adjacent cells of similar phenotype) appears to be mitosis, although it can be brought about by expansion of the intracellular matrices (cellular volume). Tissue growth should not be confused with changes in shape, e.g. extension, formation of a mesenchymal rudiment and

epithelialisation, in which tissue volume (which excludes epithelial lumina) may remain constant. Changes in shape of a tissue may be caused by localised expansion or contraction of extracellular matrix (ECM), or relative changes in intercellular or cellular-extracellular adhesion (see Chapter 3).

In C4 ('higher') plants, by contrast, morphogenesis appears to be caused by mitosis and tissue-level folding. Each cell is surrounded by an ECM 'wall' which is permeable and contains the carbohydrate cellulose (see Roberts 1990) together with lectins similar to those now known to be widespread in many vertebrate embryos (see p. 172). Somatic plant cells are usually turgid and non-migratory; hence changes in tissue-shape are caused by relative mechanical stresses and growth (fig. 12). Plant pattern-formation has been reviewed by Sachs (1991a&b); Irish & Sussex (1992); Becraft & Freeling (1992); Jürgens (1992); and Nelson & Langdale (1992).

As the nephric and possibly the metanephric tissues develop from the intermediate mesoderm (fig. 5, pp. 31-32), they may be expected to have similarities or interactions with other mesodermal tissues, i.e. notochordal (axial), somitic (paraxial), and some reproductive and

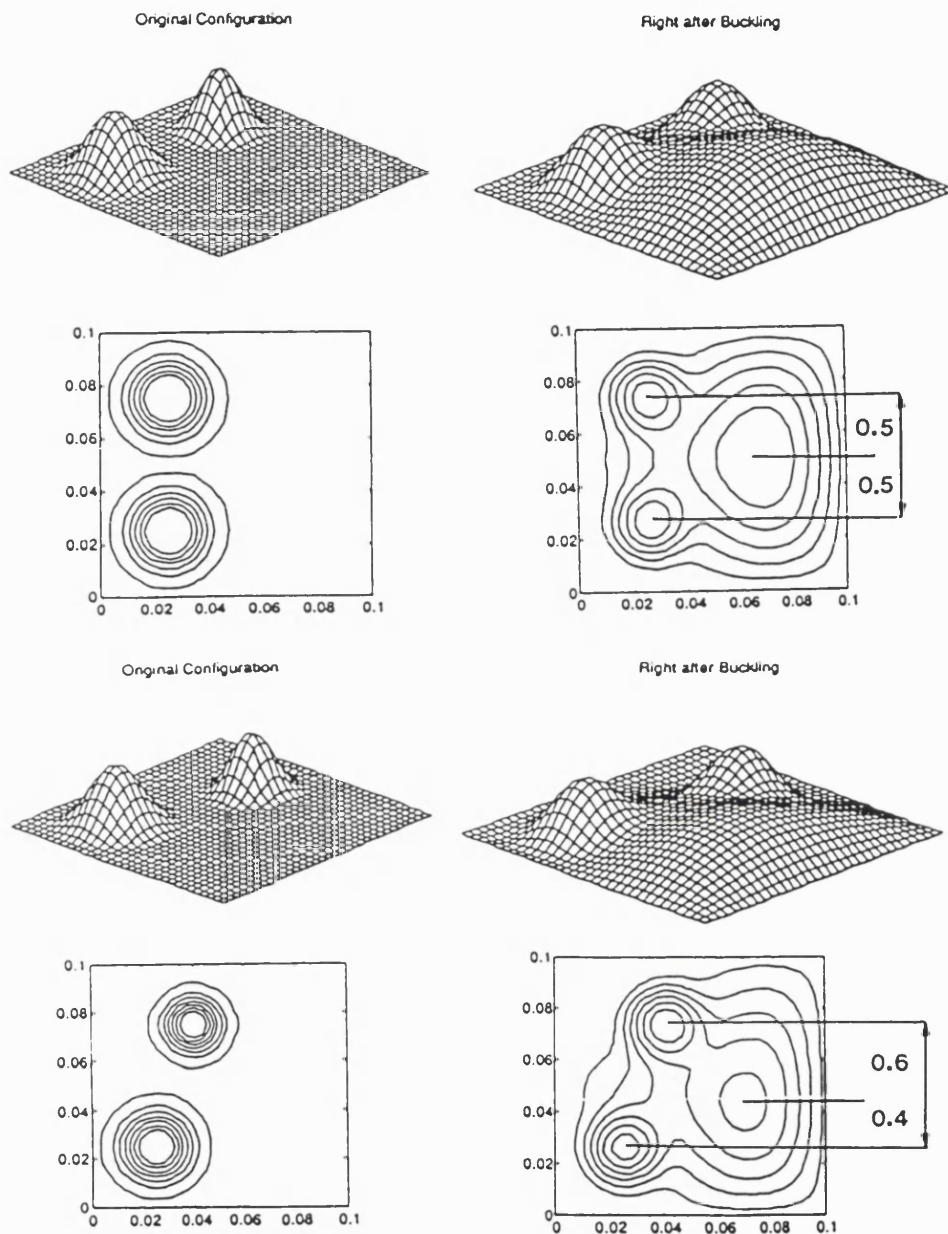


Fig. 12 Computer model of mechanical stresses thought to occur in the upper surface of a growing shoot apex during leaf morphogenesis. The lines represent sine waves of applied force; these may interfere constructively to form a peak or trough, or destructively to form a flat region. (From Selker et al 1992.)

gastrointestinal tissues (lateral), or neighbouring tissues of ectodermal or endodermal derivation.

The developing urinary and reproductive tissues are associated in several ways in amniotes. For example, in the male the nephric ducts become vasa deferentia, while in the female the Mullerian (paramesonephric) ducts extend anteroposteriorly and close to the nephric ducts soon after the latter have reached the cloaca (Abdel-Malek 1950). While both urinary and reproductive tissues form by mesenchymal-epithelial interactions (fig. 4a, p. 28), it is thought that only in the urinary (metanephric) tissues is an epithelium reciprocally induced by mesenchyme (Ekblom et al 1987), whereas only in the reproductive tissues are hormones involved in such interactions; these characteristics distinguish urinary and reproductive tissues both from each other and other mesenchymal-epithelial tissues (Cunha et al 1987). Hormonal involvement does not appear to affect induction fundamentally as, for example, salivary gland epithelium develops normally when combined with mesenchyme from the prostate or seminal vesicle (Cunha et al 1987).

An example of an epithelial tissue adopting a mesenchymal appearance is found in the migration of

individual neural crest cells from the dorsal region of the neural tube (e.g. Bancroft & Bellairs 1976).

The urinary and reproductive systems of amniotes are also associated with the gastrointestinal tract. For example, both urinary and reproductive systems share a common outlet with the gastrointestinal tract, the cloaca, which like parts of the gut is an endodermal derivative. It has also been suggested that the earliest nephric tissues are induced by the endodermal foregut (chick: Croisille et al 1976; Ambystoma: Etheridge 1968). A notable association between the reproductive and gastrointestinal tissues is seen in the migration of the primordial germ cells through the gut mesenteries.

Techniques

Until the development of monoclonal antibodies as molecular probes allowed more precise sub-cellular analyses, morphological questions usually concerned the appearances of tissues and cells in fixed tissues using light microscopy or, from the 1950s, scanning- or transmission- electron

microscopy. Some other investigations used cinemicrography in conjunction with carbon particles or vital dyes as cellular markers, while embryos treated with vital dyes could also be fixed and sectioned to construct fate-maps of the presumptive tissues. Although dynamic, fate-mapping is morphological rather than manipulative.

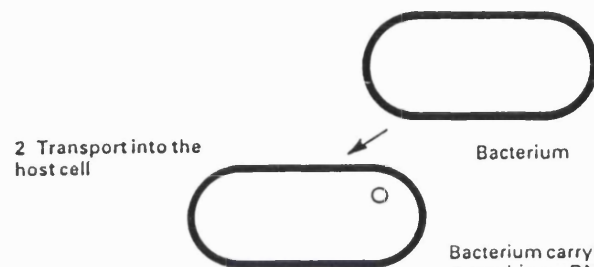
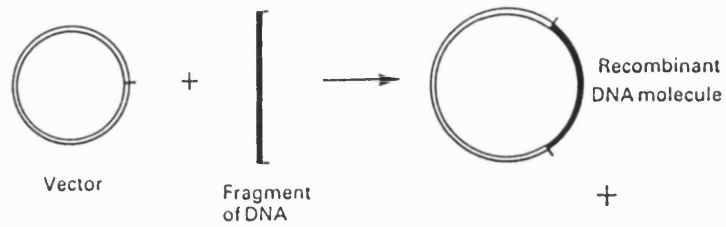
While light- and electron- microscopy still provide the mainstays of morphological analyses of fixed tissues, confocal-laser and image-enhancing (SIT) videomicroscopy have been developed more recently and allow observation of living tissue on tissue and cellular levels. The specificity of cellular markers has also been considerably improved by the development of the lipophilic cell membrane dyes, DiI (red) and DiO (green) (Honig & Hume 1989), which do not become dislodged as carbon particles tend to and are used to label groups of cells, and to LRD (lysine-rhodamine-dextran; Gimlich & Braun 1985), which may be injected intracellularly. In conjunction with the new types of microscopy, these dyes can be used to follow the movements and progeny of cells spatially in three dimensions as well as temporally.

Manipulative questions have been investigated on tissue or cellular levels by means of microsurgical ablations, interceptions or heterotopic transplantations of living tissue, either in the embryo or in isolation, together with cellular or molecular markers. The trans-filter technique, used for analysing metanephric induction in vitro, is shown in fig. 6 (p. 37-38). The chick embryo may be used either in the egg or in whole-embryo culture (figs. 8-10, pp. 40-42). Formation of quail-chick and quail-mouse chimaeras (Le Douarin & Barq 1969; Martin 1990; *) utilises the quail cell nucleolus as a cellular marker; alternatively DiI and DiO may be used. Molecular probes, by contrast, are often monoclonal antibodies to differentiation antigens (phenotypic makers). Other methods of cellular ablation have been reviewed by O'Kane & Moffat (1992).

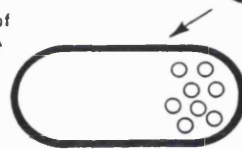
On the sub-cellular level manipulations may be antagonistic, such as in the competitive inhibition of the fibronectin by the synthetic peptide GRGDS (Boucaut et al 1984), or genetic (fig. 7, p. 39; Stoker et al 1990b). Genetic manipulations are based on recombinant and cloning techniques (fig. 13).

* Sariola 1985

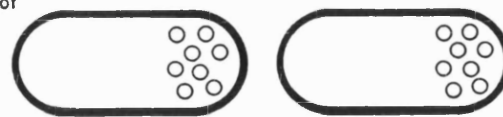
1 Construction of a recombinant DNA molecule



3 Multiplication of recombinant DNA molecule



4 Division of host cell



5 Numerous cell divisions resulting in a clone



Fig. 13 Outline of the procedure for genetic cloning.

(From Brown, 1990).

The present study

The present study has used a morphological approach and LM, SEM and immunohistochemical staining of fixed, normal, tissue, to investigate aspects of formation, epithelial differentiation, growth and degeneration of the nephric tissues and early development of the metanephric tissues of the chick embryo. An overall theme is the relative importance of in situ and distant changes leading to early nephrogenesis (H&H 8-27). Chapters 2 and 3 address this theme at the tissue and sub-cellular levels respectively, examining morphological evidence for induction and migration. Chapter 4, at the cellular level, quantifies mitosis and apoptosis in the nephric duct as it fuses with the cloaca. Some associations between the nephrogenic tissues, primordial germ cells, notochordal, somitic and gastrointestinal tissues are discussed in Chapters 2 and 3.

CHAPTER TWO

**TISSUE LEVEL
NEPHROGENESIS**

CHAPTER TWO

INTRODUCTION

In the nephrogenic tissues, the tissue level is that at which a duct or nephron is analysed as a whole, either collectively or individually. This usually involves whole embryos. Traditionally, the tissue level has been the domain of anatomists, physiologists and embryologists relying on the light microscope and it is also on this level that the great majority of previous nephric morphogenetic analyses, both morphological and manipulative, have been conducted. However, while these have raised a number of important questions, few analyses are recent. There also appear to have been comparatively few - particularly manipulative - morphogenetic analyses of the metanephric tissues at this level.

The tissue level is also where an integrated morphogenetic analysis should begin, because it is the level both on which the effects of the 'primary' morphogenetic processes are evident and on which interactions - and perhaps additional processes - leading to shape change or growth are to be expected. To analyse the latter, spatially three-dimensional, tissue-level models will be required, the dearth of which has recently been emphasised (Saxén 1987; Bard 1992b).

It will be apparent from the following review that the main questions to emerge from previous morphogenetic analyses on the tissue level of the nephric ducts and nephrons are:

- 1 Do the nephric ducts or nephrons form first? (Table 7)
- 2 Does the nephric duct form from the nephrons or do the nephrons form from the duct? (Table 7)
- 3 What processes underly formation of the nephric duct and the nephrons?
- 4 Does nephric duct extension follow in situ and/or distant formation? (Table 8)
- 5 What processes underly nephric duct extension from a distant site? (Table 8)

- 6 Are the nephric ducts and/ or nephrons segmented anteroposteriorly? (Table 9)
- 7 What is the pattern of epithelialisation in the nephric duct?
- 8 How may the nephric tissues be classified? (Table 10)
- 9 What, if any, are the morphogenetic roles of the nephric tissues?

In the remaining part of the present chapter, questions 1-8 will be re-addressed in terms of the timing and regions of formation, epithelial differentiation and growth, using light- and scanning-electron microscopy of fixed, normal tissue. Where possible, I have expanded the questions to include both the nephric ducts and nephrons, at most stages, and the early metanephric tissues, i.e. the metanephric duct and the earliest metanephron rudiments. Degeneration of the nephric duct and possible morphogenetic roles of the nephric tissues will be discussed briefly in Chapters 4 and 5 respectively.

TABLE 7

SEQUENCE OF FORMATION & NEPHRIC DERIVATION,
AS INDICATED BY PREVIOUS ANALYSES

<u>Duct first</u>	<u>Later nephrons</u> <u>due to ducts</u>
(Morphological, chick:)	(Manipulative, chick:)
Balfour&Sedgwick(1879)	Boyden(1924,1927)
Sedgwick(1881)	Gruenwald(1937,1942)
Jacob etal(1986)	Waddington(1938)
Jarzem&Meier(1987)	Bishop-Calame(1965)
<u>Nephrons first</u>	<u>Ducts due to</u> <u>earlier nephrons</u>
(Morphological, chick:)	(Morphological:)
Boyden(1927)	All the adjacent
Waddington(1938)	' <u>nephrons first</u> '
Abdel-Malek(1950)	
Lillie(1953)	
Romanoff(1960)	

(Morphological, amphibian:)

See p. 64

TABLE 8

REGIONS & MEANS OF EXTENSION IN NEPHRIC DUCT FORMATION,
AS INDICATED BY PREVIOUS ANALYSES

REGION	MEANS OF EXTENSION
<u>Anterior only</u>	<u>Migration</u>
(Morphological:)	(Morphological, <u>Xenopus</u> :)
All the adjacent	Lynch&Fraser(1990)
<u>'migration'</u>	
	(Manipulative, chick:)
	Calame(1959,1962)
	Poole&Steinberg(1984)
	(Manipulative, amphibian:)
	Burns(1938)
	van Geertruyden(1942)
	Spofford(1945)
	Cambar(1952a&b)
<u>In situ</u>	<u>Induction</u>
(Morphological:)	(Morphological, chick:)
All the adjacent	Croisille etal(1976)
<u>'induction'</u>	
	(Morphological, amphibian:)
	Field(1891)
	Etheridge(1968)
	Poole(1988)

TABLE 9

ANTEROPOSTERIOR SEGMENTATION IN THE NEPHRIC TISSUES,
AS INDICATED BY PREVIOUS ANALYSES

Intermediate mesoderm

Segmented

(Morphological, chick:)
Meier(1980)

(Morphological, amniotes:)
Torrey(1965)

Not segmented

(Morphological, chick:)
Jacob etal(1986)

(Morphological, amniotes:)
Torrey(1965)

Nephrons

Segmented

(Morphological, amniotes:)
Balfour(1875)
Fraser(1950)

(Morphological, amniotes:)
Torrey(1965)
Price(1897)
Fraser(1950)

Not segmented

(Morphological, amniotes:)
Torrey(1965)

Duct

Initially segmented

Balfour&Sedgwick(1879)
As Table 7, 'Nephrons first'

Not segmented

As Table 7, 'Duct first'

TABLE 10

DIFFERENT TYPES OF NEPHRIC TISSUE,
AS INDICATED BY PREVIOUS ANALYSES

NEPHRONS

<u>Pro- & meso-nephrons</u>	<u>One type or indistinct</u>
(Morphological, chick:) Abdel-Malek (1950)	(Morphological, chick:) Torrey (1965)
(Morphological, fish:) Youson (1981)	(Morphological, amniotes:) Fraser (1950) Burns (1955) Torrey (1965)
(Morphological, anamniotes:) Torrey (1965)	(Morphological: amphibians) Fox (1963)

DUCT

The duct has usually been named in relation to the nephrons at the same anteroposterior level, i.e. pronephric at anterior levels and mesonephric at more posterior levels, even though it has been regarded as continuous.

PREVIOUS MORPHOLOGICAL ANALYSES

Formation of the nephric tissues

Using LM in the chick, Balfour & Sedgwick (1879; Sedgwick 1881) reported that the nephric ducts were formed before any nephrons. Using SEM, Jacob et al (1986) and Jarzem & Meier (1987) also presented such evidence, arguing that the duct can first be seen as a short ridge adjacent to the nascent sixth somite pair, i.e. at Hamburger & Hamilton (1951) (H & H) stage 9⁻. Using vital dyes in salamanders, the duct was shown to form after, posteriorly to, but from separate tissue than, the earliest nephrons (O'Conner 1938; 1939). This is generally agreed for amphibians (Fox 1963). (See Table 7, p. 60 and Gillespie & Armstrong 1985).

Between the time of Balfour's and Sedgwick's work and Jacob et al's (1986) SEM study, the common assumption had become that the nephric ducts formed by the posterior growth and fusion of the distal tubules of the earliest ('pro-') nephrons in the chick. Using LM, Price (1897) and Brauer (1902) presented evidence of this in some lower vertebrates, while Toivonen (1945) did so in the

rabbit. However, there appears to be no such evidence in other higher vertebrates, where it is generally thought that the earliest tubules are not continuous with the ducts (e.g. Saxén 1987).

Therefore, either the later belief was unfounded and due to the earlier chick studies being lost, ignored or forgotten while later studies were too superficial to be accurate, or the conclusions of the earlier chick studies were made with insufficient evidence. Abdel-Malek's (1950) morphological analysis appears to be the only one published on the chick in the intervening period and he thought the nephrons formed first.

After originating at the mid-trunk level, the nephric ducts of all vertebrates extend posteriorly along the lateral edges of the somites and fuse with the cloaca (figs. 1 & 3, pp. 24 & 27). Morphological questions about anteroposterior extension on the tissue level have mainly concerned whether the ducts form anteriorly or in situ. (See Table 8, p.61).

In the axolotl (Ambystoma mexicanum) there is 'a critical coupling of duct extension and differentiation of the [axial] mesoderm such that the duct tip lies approximately two somites

behind [posterior to] the last formed somite', while in the sturgeon (Acipenser transmontanus) the tips are significantly more posterior than the somites (Poole 1988). In the chick, this distance is usually three to four somite-lengths, but may vary between individuals and also between the right and left sides of the same embryo; it has been suggested that the right duct leads the left (Abdel-Malek 1950).

In the chick the position of each duct as a whole also varies relative to the somites: in a progressively more posterior direction it becomes enveloped by the lateral mesoderm, thus moving from just beneath the ectoderm to just ventral to the sclerotome, i.e. from lateral to ventrolateral with respect to the somites (Jarzem & Meier 1987).

Anteroposterior segmentation has also been repeatedly associated with formation of the nephric tissues and is itself related to the idea of a 'multi-tubular' duct as described above. Thus the assumption was that a 'pronephric tubule' (nephron) existed for each segment or somite length. Using SEM on H&H stage 15 chicks, Meier (1980) thought that as well as the paraxial (somatic) mesoderm, both the intermediate and lateral mesoderms were segmental.

However Jacob et al's (1986) investigation did not show segmentation in the intermediate mesoderm. According to Torrey (1965), the intermediate mesoderm of lower vertebrates is segmented as 'nephrotomes', each of which develops into a nephron, but that of higher vertebrates is unsegmented. (See Table 9, p. 62).

Anteroposterior level and even mediolateral/dorsoventral position have been used in conjunction with differentiation as the bases of classification of the nephric tissues. Thus, 'pro-' nephrons have been said to develop first, anteriorly, and 'meso-' nephrons later, more posteriorly. Abdel-Malek (1950) reported anteroposterior overlapping between the two types, based on their mediolateral and dorsoventral positions. The duct has conventionally been known as the 'pronephric' duct when first formed, but as it extends posteriorly to the level of the 'meso-' nephrons it has usually, though not always, become known as the 'mesonephric' duct. (See Table 10, p. 63).

Epithelial differentiation of the nephric tissues

Epithelial differentiation of the nephric nephrons has traditionally been another criterion of nomenclature. 'Pronephrons' have been described as structurally relatively simple and probably only forming in 'lower' vertebrates (anamniotes), in which the later, more complex nephrons are those of the definitive, 'back' kidneys ('opisthonephroi') (Torrey 1965). According to Ellis & Youson (1989), the extant agnathans, lampreys and hagfishes all retain some parts of the pronephros which are functional and drain into the duct as adults. From previous studies of amphibians it is not clear whether such 'pronephrons' are retained either phylogenetically or ontogenetically.

Attempts have also been made to use physiological capacity, although this has been recognised as being variable and as a basis of nomenclature it has therefore been used in conjunction with morphological criteria. In lower vertebrates the pronephrons are thought usually to be functional in embryonic or larval stages and also sometimes in the juvenile; in the brown trout, Salmo trutta, for example, the pronephroi function after

hatching until the opisthonephroi are sufficiently differentiated (Tytler 1988).

In the equivalent anteroposterior position to the opisthonephrons of lower vertebrates and also differentiating to a functional capacity in higher vertebrates are the 'mesonephrons'. Using LM, Abdel-Malek (1950) made a clear morphological distinction between 'pro-' and 'meso-' nephrons in the chick. However, the distinguishing criteria are too superficial (Fraser 1950; Burns 1955; Fox 1963; Torrey 1965) and 'in the chick embryo especially, the transition between tubules judged to be pronephric and the first mesonephric tubules is so gradual as to cast doubt on the reality of the pronephric variety' (Torrey 1965). Though retaining the conventional nomenclature for the early nephrons, Burns (1955) and Torrey (1965) therefore adopted the term 'nephric' for the duct. In the present study, no distinction will be made between 'pro-' and 'meso-' nephric nephrons either.

Degeneration of the nephric tissues

In higher vertebrates, it is known that the nephric ducts of the male become vasa deferentia, whereas the nephric ducts of the female and the nephric nephrons of both genders degenerate (e.g. Abdel-Malek 1950). It is thought that this degeneration is achieved by programmed, 'apoptotic' cellular death. However this does not appear to have been substantiated. It is therefore not inconceivable that the tissues re-differentiate, rather than or in addition to dying, in females perhaps into metanephric tissues and in males into either metanephric or reproductive tissues.

Formation and epithelial differentiation of the cloaca

There have been a small number of tissue-level, morphological analyses of the cloaca (chick: Boyden 1922; 1924; amphibians: O'Conner 1940), and chick-quail-chimaera analyses including observations relevant to cloaca formation (Martin 1971; 1976).

Formation of the metanephric tissues

Formation of the metanephric tissues of amniotes begins after the nephric ducts have fused with the cloaca and have begun to degenerate anteriorly, but are still differentiating posteriorly. The metanephric duct (ureter) rudiments have rarely been investigated in chicks or mice. It is usually stated that in chicks (e.g. Romanoff 1960) and mice (e.g. Saxén 1987) the metanephric ducts originate as diverticulae of the nephric ducts, extending anteriorly into undifferentiated intermediate mesoderm. Thus the metanephric ducts follow a slightly different course through similar tissue, but in the opposite direction to that of the nephric ducts. As in the nephric ducts, it is not clear whether extension is of a distantly (in this case, posteriorly) formed rudiment or in situ.

The tip of each metanephric duct rudiment forms repeated bifurcations which enlarge distally and the mesenchyme condenses immediately around these enlargements to form rudiments (or 'blastemae') of the metanephrons (figs. 4b-c, pp. 29-30). There are many classical, morphological studies of formation of the metanephric kidneys of representatives of

several vertebrate classes; a recent study in the chick is Gambaryan's (1992). Most recent metanephric analyses have been in the mouse in vitro model (for reviews see Saxén 1987).

Epithelial differentiation of the metanephric tissues

The distal part of the metanephric duct rudiment is the presumptive renal pelvis and its repeated bifurcations are the presumptive collecting ducts. The other parts of the kidney differentiate from the metanephric mesenchyme (Saxén 1987). See figs. 2b (p. 26), 4b (p.29) and 4c (p. 30).

'Segmentation' along the metanephron into morphologically distinct regions is the basis of differential filtration and ion transport (fig. 2b, p. 26; Herzlinger et al 1992).

The nephric nephrons and metanephrons exist simultaneously for a period of embryonic life, which might have led to the concept of a 'holonephros', to incorporate both nephric and meta- nephrons (see Torrey 1965; Saxén 1987).

PREVIOUS MANIPULATIVE ANALYSES

It should be noted that the term 'experimental' has frequently been applied to non-manipulative as well as manipulative analyses. Hence in the present study, the former are termed 'morphological' (pp. 64-72) and 'experimental' is used interchangeably only with 'manipulative'. It should also be noted that in previous analyses, manipulative approaches have been used to address issues concerning events as well as processes of nephrogenesis.

Formation of the nephric tissues

Induction of all but the earliest of the nephric nephrons and of the cloaca by the nephric ducts has been demonstrated in the chick embryo by ablation or interception of the ducts and, in one case (Gruenwald 1942), heterotopic grafting of non-nephrogenic tissue. Ablation and interception experiments have also been carried out in the chick by Boyden (1924; 1927), Waddington (1938), Gruenwald (1937; 1942) and Bishop-Calame (1965). The experiments by Boyden (1924; 1927) and Gruenwald

(1937; 1942) also pertain to metanephric development (see p. 86).

By ablating or intercepting the ducts at the posterior tips in ovo, Boyden (1924) showed that formation of the cloaca was due to induction by the nephric ducts, with either one of the ducts being sufficient; production of urine by the nephric nephrons was excluded as a necessary stimulus. In 1927 Boyden showed by similar means that the later-forming ('meso-') nephrons only formed in the presence of the nephric ducts, i.e. that the nephrons were induced by the ducts. The latter was confirmed by Gruenwald (1937) and Waddington (1938), who intercepted the ducts posteriorly in ovo.

Bishop-Calame (1965), apparently also in ovo, provided further evidence of induction of the nephrons by the ducts, by interception experiments, and showed that the critical distance of interception posterior to the duct tip behind which nephrons were no longer induced was the equivalent of two to three somite lengths.

By making longitudinal cuts on either side of, i.e. laterally intercepting, the intermediate mesoderm at the early neural-plate stage in embryo

culture, Waddington (1938) showed that formation of the nephrons was unaffected. Though he appears not to have excluded the possibility of the areas of mesoderm subsequently re-joining, Waddington (1938) concluded that the nephrons were not induced by somitic or lateral mesoderm, but that they may have been by neural tissue, with which the intermediate mesoderm overlaps. It has also been suggested that the nephrons are induced by the underlying, gut endoderm (Croisille et al 1976).

However by ablating the duct in ovo, Gruenwald (1942) found, apparently for the first time, that induction by the nephric ducts was not essential for formation of the nephrons and also suggested that in the absence of the duct they may be induced by nervous tissue. Also in ovo, Gruenwald (1942) grafted an area of axial tissue containing notochord, medial somite and neural tube and all three germ layers, i.e. normally non-nephrogenic, onto the duct pathway posteriorly to its growing tip and showed that the duct extended through the tissue without inducing it to form nephrons. However as the tissue had already epithelialised, it would have had to re-differentiate to form nephrons, which would probably have required a stronger or additional

inducing stimulus than normal; mesenchymal tissue appears not to have been grafted.

In various amphibian species, ablations and interceptions of the nephric ducts have been carried out to investigate induction of the nephrons, by O'Conner (1938; 1939; 1940), Nieuwkoop (1948), Bijtel (1948) and Poole & Steinberg (1981; 1982) among others. There has been a greater number of these than in the chick but results have also been inconsistent. Hence Etheridge (1968) suggested that the endoderm was inducing early nephron formation in Triturus; this was subsequently suggested for chicks by Croisille et al (1976).

Migration has been experimentally inferred either by ablation experiments as described above, which showed that extension was not the result of in situ addition of tissue to the duct along its pathway (other ablation experiments are those by Burns [1938], van Geertruyden [1942], Spofford [1945], Cambar [1952a & b] in amphibians and Calame [1959; 1962] and Poole & Steinberg [1984] in the chick), or morphologically by vital staining (in amphibians) or chick-quail chimaera formation (pp. 78, 80, 82-84). More recently, heterotopic grafting has been used to assess directionality and its

tissue of origin (duct or neighbouring tissue) in nephric duct migration.

As in the induction experiments described above, there are some inconsistencies in the evidence for migration versus in situ recruitment. In situ formation was postulated by Field (1891), who also thought the ducts were derivatives of the somitic mesoderm and thus disputed a previous conception that they were directly of ectodermal origin. For example, the mesoderm from which the nephrons form might in certain circumstances induce the duct, causing the latter to form in situ. It would be particularly interesting to find evidence of reciprocal induction between the nephric duct and nephrons, as occurs in the metanephric tissues (pp. 33 & 86-87). For a summary of the conclusions of previous studies addressing the question of in situ versus distant formation of the nephric ducts, see Table 8, p. 61.

Evidence supporting recruitment in situ would also necessitate a re-examination of the hypothesis of the duct's segmental origin, as first put forward for the 'primitive' vertebrates Bdellostoma (Price 1897) and Gymnophiona (Fraser 1950 [Brauer 1902]) and for the chick by Balfour (1875) (see also pp.

66-67). As Balfour & Sedgwick (1879) found that the duct formed before the nephrons, they might appear to have discounted a segmental origin for the presumptive nephrons ('nephrotomes'). Fraser (1950), however, on the basis of comparative morphology, re-iterated the ducts' putatively segmental origins. (See Table 9, p.62).

Until recently it was thought that Xenopus differed from other amphibians with its ducts developing from 'fissures' in situ (Poole 1988); however Lynch & Fraser (1990), in a thorough, morphological investigation using videomicroscopy and fluorescent vital-staining have demonstrated anteroposterior migration more clearly than probably any study.

In Ambystoma, Poole & Steinberg (1981) appear to have been the first to use SEM rather than LM to analyse the results of microsurgery (ablation) because at stages of duct migration yolkeness of amphibian embryos makes paraffin-wax sectioning difficult. Previously, results had been obtained 'only after the duct rudiment had completed its caudal migration, when secondary influences might have deviated the duct from its originally chosen path.'

In chicks, SEM has been used at least since Jacob & Christ's (1978) study, principally for its higher resolution and its capacity for viewing whole mounts rather than sections. SEM has revealed that the posterior duct tip is more extensive than can be seen under the dissecting light-microscope. Thus even Bishop-Calame's (1965) morphometric analysis concerning induction should be re-examined.

Heterotopic grafting experiments to ascertain tissue-level processes of duct migration have comprised either rotations or translocations of the duct (Table 10, p. 63). Most of these have been carried out in amphibians, by Howland (1921; 1926), Mackemer (1929), Maschkowzeff (1936), Holtfreter (1944 - emphasising the probable importance of the substratum), Ti-Chow-Tung & Su-Hwei-Ku (1944), Nieuwkoop (1947 - postulating amoeboid migration of duct cells), Bijtel (1948; 1968), Poole & Steinberg (1982; 1984), Gillespie & Armstrong (1986) and Zackson & Steinberg (1987). Poole & Steinberg and their colleagues have thus exploited SEM on an experimental model about which there had been a considerable debate forty years previously and apparently only a single study since (Bijtel 1968).

In the chick, Martin (1971; 1976) appears to have been the first to carry out grafting experiments to analyse migration, which she did in ovo using light microscopy and the quail nucleolar cell-marker (Le Douarin & Barq 1969) (see also Martin 1990). Jacob & Christ and their colleagues have subsequently utilised SEM in morphological analyses to support chimaeric grafting in ovo, in which the posterior tip of the duct has been the subject of focus (Jacob & Christ 1978; Jacob et al 1984).

In Ambystoma, Holtfreter (1944), Ti-Chow-Tung & Su-Hwei-Ku (1944) and Nieuwkoop (1947) and in Siredon but not Rana, Maschkowzeff (1936) showed that a donor nephric duct rudiment introduced without its own cloaca usually migrated dorsally or medially to join the path of the host rudiment in order to reach the host cloaca. However in Ambystoma (Howland 1921) and Triton (Mackemer 1929), donor ducts had not joined the host duct pathway.

Holtfreter (1944) compared contemporary uncertainty and disagreement over duct extension to that concerning the extension of nerve axons and in the light of Weiss's observations in vitro (1934), which led to the theory of contact guidance (Weiss

1955), postulated that the duct was similarly guided by local, physical forces, exerted possibly by the substratal vasculature. More recently, Harris (1980) has shown that fibroblasts cause mechanical alterations, i.e. stress-lines, on silicone rubber or collagen substata. This phenomenon is known as traction (see also p. 178).

In contrast, Masckowzeff (1936) had observed that the host duct turned slightly towards the approaching donor duct and, moreover, in Triton when the presumptive cloaca of the host was also introduced (Bijtel 1948) the host duct grew towards the cloaca of the host rather than its own. Therefore distant, chemical attractants in the donor duct or cloaca were postulated.

Poole & Steinberg (1982) have attempted to elucidate the process of guidance of the duct on the tissue level by heterotopic grafting of the duct or ablation of a putative source of chemical attraction. Telotaxis, or attraction from a distance (e.g. by chemicals, chemotaxis) is indicated by migration ceasing on removal of the attracting source and is distinct from local forms of guidance, of which there are three types, each reflecting a different substratum. These are either

omnidirectional (i.e. no guidance, indicating a uniform substratum), bidirectional (by an oriented substratum, e.g. physically by contact guidance), or unidirectional (by a polarised substratum, e.g. chemically by haptotaxis), as shown in fig. 54.

Using these criteria, Poole & Steinberg (1982) showed that the duct of Ambystoma mexicanum is guided by a local, polarised mechanism and postulated that this was haptotaxis due to an anteroposterior gradient of increasing adhesion at the level of the duct tip. Evidence in support of an anteroposteriorly travelling, local, polar, guiding mechanism was provided by their demonstration that guidance was stage-dependent at all anteroposterior levels, while an anteroposteriorly travelling wave of adhesion in neighbouring tissues at these stages has been proposed by Bellairs & Portch (1977), Bellairs et al (1978: somites), among others. Gillespie & Armstrong (1986) have attempted to delineate the region of lateral mesoderm able to support duct migration, while more recent work has aimed to elucidate a chemical basis for the putative adhesion (see p. 178).

Martin (1971) grafted the caudal tip of the quail nephric duct onto the lateral mesoderm of the


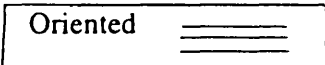
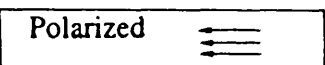
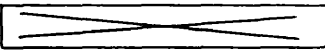
Source of guidance information	Substratum properties	Migration disoriented by removing distant tissue	Orientation of migration
Local	Uniform 	No	Omnidirectional
	Oriented 	No	Bidirectional
	Polarized 	No	Unidirectional
Distant		Yes	Unidirectional

Fig. 54 Cellular or tissue migration, in relation to the system of guidance. (From Steinberg & Poole 1982).

chick host whose own duct had been intercepted. Thus, in contrast to Poole's and Steinberg's experiments the normal pathway was available only to the donor; as the quail duct joined the path and fused normally with the cloaca, it was indicated that in the chick the duct's substratum and/or the cloaca were guiding the donor duct and, furthermore, that although the lateral mesoderm supported its migration, the duct preferentially migrated over the intermediate mesoderm.

Also to prevent the chick host duct extending posteriorly, Jacob et al (1978; 1984) removed the tip, before grafting anterior or posterior parts of a quail duct into its former position. The donor duct portion was either in a normal or reversed (presumably) anteroposterior orientation, but always grew towards the posterior end of the host; this happened regardless of whether the graft was from an anterior (more epithelialised) or a posterior (mesenchymal) part of the donor duct. Thus the importance and oriented rather than polarised nature of the substratum was demonstrated, both before (1978) and in a similar set of experiments after (1984) those of Poole & Steinberg (1982).

Epithelial differentiation of the nephric tissues

The process of epithelial differentiation (including canalisation and differentiation of more than one type of nephric nephron) appear not to have been analysed manipulatively. This belies the possible importance of tissue level constraints.

Degeneration of the nephric tissues

There appear to have been no previous, manipulative investigations of degenerative processes in the nephric tissues. Possibilities which should be investigated on the tissue level are their intrinsic nature and their ability to re-differentiate. For example, is it purely intrinsic that the nephric nephrons but not the nephric ducts of the male higher vertebrate should appear to disintegrate, and do the ducts re-differentiate or are they passively 'annexed' by the developing reproductive tract? What happens to those tissues which appear to disintegrate: might they have a morphogenetic function themselves, such as in inducing other, perhaps metanephric structures?

Formation of the metanephric tissues

Evidence from the ablation and interception experiments in ovo of Boyden (1924; 1927) and Gruenwald (1937; 1942), discussed above in relation to nephric development, was also used to show that the cloaca is necessary for formation of the metanephric duct rudiments (Boyden 1924), while the nephric ducts are necessary for formation of the metanephrons (Boyden 1927; Gruenwald 1937) in the chick.

Gruenwald postulated that it was the metanephric duct rudiments rather than the nephric ducts which induced the metanephrons (1937) and furthermore (1942) showed that, in contrast to 'meso-' nephrons which sometimes formed in the absence of the nephric ducts, absence of the metanephric duct rudiments prevented formation of metanephrons - though the metanephric mesoderm still became more dense.

Grobstein (1956) showed in the in vitro mouse model that the normal inducers of the metanephrons are the metanephric duct rudiments (fig. 6, pp.37-38; see also pp. 177-179 & 181-183). However, it has recently been suggested (Sariola et al 1988,

1989) that it is neurons growing on the metanephric duct rudiments which are the inducers; this is currently under investigation by Sariola and his colleagues. For a review of induction of the mouse metanephros in vitro on the tissue level, see Saxén (1987), Ekblom (1987) and Bard (1992a&b).

The metanephrons have been focussed on at the expense of the metanephric duct rudiments in the most frequently used, mouse, model and there remains the necessity to complement analyses of nephric duct extension with those of the metanephric ducts.

Epithelial differentiation of the metanephric tissues

Manipulative analyses of metanephric epithelial differentiation have been predominantly in vitro, though mainly on the cellular and more recently sub-cellular, levels (see Saxén 1987 and Bard 1992a). Hence the tissue-level, three-dimensional interactions putatively underpinning epithelial differentiation have not been investigated.

CHAPTER TWO

METHODS

Scanning-electron microscopy (SEM) was used to observe the nephric tissues of 16 embryos at Hamburger & Hamilton (1951; H&H) stages 14-18, following either freeze-fracturing (Appendix 2) or dissection. Light microscopy (LM; Appendix 4) was used to observe both the nephric and metanephric tissues from before their earliest appearance (H&H 8) until formation of the metanephrons had begun (H&H 27), in either transverse or longitudinal sections (TS, LS) of 45 embryos. The whole embryos were also photographed, either before fixing (LM or SEM) or after clearing (LM). (See Tables 3 for abbreviations, 4 for H&H stages and 5 for terminology.)

For all procedures, fertile hens' eggs (Gallus domesticus, Ross Broiler; supplied by Ross Poultry plc) were incubated while being rotated in a humid atmosphere at 37.5°C. Eggs incubated for times corresponding to the appropriate embryonic stages were opened at the blunt end and the embryo was removed together with its extra-embryonic membranes. At the earlier, i.e. nephric, stages, these membranes extend over less than a third of the surface of the yolk and can thus be fully exposed without removing the yolk from the egg.

Each embryo was rinsed in Pannett & Compton (1924) saline (P&C, Appendix 1) where the vitelline membrane was removed. In embryos of later, i.e. metanephric, stages, the other extra-embryonic membranes and the head were removed, whereas in the younger embryos the head and area opaca, from which these membranes develop, were retained until after fixing.

SCANNING ELECTRON MICROSCOPY (SEM)

Preparation

Preparation for SEM requires fixing with a cross-linking fixative such as glutaraldehyde, followed by rinsing in a physiological buffer, osmication and dehydration in a ketone or ester, e.g. acetone, as these are miscible with both the preceding solutions and carbon dioxide. Osmic acid contains ions of the heavy metal, osmium, which impede absorption and therefore enhance reflection of electrons during microscopy. Carbon dioxide has a suitable pressure-temperature relationship for its use in a critical-point drying apparatus. Dessication is necessary for the maintenance of a vacuum in which to operate an electron beam. Finally, specimens must be coated finely with a good electrical conductor; atomised gold is used for this (see Appendix 2).

Microscopy & photography

The vacuum tube of the scanning electron microscope (Hitachi) was opened following the introduction of air through a valve. The coated specimens were placed in the numbered holes of the

integral specimen-holder, the SEM was closed and re-evacuated and the specimens observed and photographed, using Ilford FP4 120mm black & white film. Stereo pairs were photographed at a tilt angle of 10°.

LIGHT MICROSCOPY (LM)

Preparation

Embryos were transferred from P&C to buffered formal saline (BFS, Appendix 3). BFS penetrates more quickly and forms weaker bonds than glutaraldehyde, which helps preserve the integrity of the outer cell surface, particularly if tissue is exposed to the fixative for a minimum effective period. Therefore each embryo was fixed for only one to two hours, depending on size, and after rinsing and removal of the area opaca in PBS was further preserved by dehydrating in serial ethanols and clearing in cedar wood oil within a day if possible (see Appendix 4).

Microscopy & photography

A Zeiss 'Universal' microscope with various plan and planapo objectives and an Olympus OM2, 35mm camera attachment were used. Kodak Ektachrome daylight (64 ASA) colour reversal film was used.

Whole-embryo photography

Whole embryos were photographed in cedar wood oil, using an Olympus OM10, 35mm camera with bellows attachment and 20mm macro lens, with direct and /or reflected light from a fibre optic source (Schott Mainz). Ilford Pan F 35mm monochrome film (50 ASA) was used.

FILM PROCESSING

Monochrome negatives were developed with Paterson Acutol developer, fixed with Amfix, and printed using Ilford Multigrade III RC deluxe paper, a Leitz Focomat IIC enlarger and Ilford Ilfospeed developer. Colour films were processed commercially (CPL Laboratories).

CHAPTER TWO

RESULTS

On the tissue level, morphological techniques (SEM of dissected or freeze-fractured embryos, including stereoscopic SEM, and LM of paraffin-wax sections) were used to address questions of morphogenetic changes directly and underlying processes indirectly, at most stages of nephric and early stages of metanephric development, i.e. H&H 8-27. Figures are shown on pages 109-149, with abbreviations in Table 12, pp. 106-108. The LM sections were stained with monoclonal antibodies to S-FC10.2 or S-NB10.3B4 (see Chapter 3). The results of the present chapter are summarised in Table 11 and on pages 151-154.

TABLE 11

MORPHOLOGICAL DEVELOPMENT OF THE NEPHRIC TISSUES

H&H STAGE	NEPHRIC TISSUE	
	DUCT	NEPHRONS
8-10	Single 'nephric' rudiment, mesenchymal, without lumen; anteriorly attached at intervals to somites, posteriorly not readily discernable from segmental plate or lateral mesoderm	
10-12	Mainly a single rudiment, broad, mesenchymal and without lumen, attached at intervals to somites; also separate nephron-shaped rudiment, without central lumina	
12-14	Rudiment narrower, longer than at H&H 10-12; wall compact, with lumen forming intermittently in midline	Rudiment or rudiments, with some degree of separation from duct rudiment; wall compact, no lumen
14-16	Rudiment of similar width to H&H 12-14, but longer; wall thinner, lumen more developed, though still intermittent	Rudiments mostly separate from duct rudiment, with lumina forming; some, non-patent, attachments present
16-18	Duct of similar width to H&H 14-16, but at or near final length, fusing with cloaca; lumen continuous along length, wall thinner	Nephrons rosette-shaped and separate from duct; each with central, initially circular lumen, walls thinner
18-20	Duct of similar width, wall of similar thickness, to H&H 16-18; has fused with cloaca; outer face of wall invaginated to complement nephrons	Nephrons still separate from duct, though extended laterally, as are lumina; lumina of greater diameter and walls thinner than at H&H 16-18
20-22	Duct similar to H&H 18-20, but walls fusing with those of nephrons; some sites of fusion non-patent	Nephrons fusing laterally with duct; lumina becoming curved; glomeruli and capsules forming
22-28	Duct continues to fuse with distal ends of nephric tubules; lumen of duct widens	Nephrons elongating proximally and distally, but within a restricted space which forms the boundaries of the nephros

The questions addressed have been expanded from those listed on pages 58-59, to include both types of nephric tissue and the metanephric tissues until the first appearance of the metanephrons. It should become apparent (see Discussion, pp. 150-163) that in order to address many of these questions, formation, epithelial differentiation and growth may need to be analysed in combination and that, furthermore, they are all related by a general theme of in situ versus distantly controlled morphogenesis. Hence the results are grouped as follows:

- 1 Do the ducts or nephrons form first?
- 2 What is the pattern of epithelial differentiation in the ducts and nephrons?
- 3 Do the ducts and nephrons form in situ or by antero-posterior migration?
- 4 Is development of the tissues segmental?
- 5 Is there morphological evidence of different types of urinary tissue?

Questions concerning underlying morphogenetic processes are addressed indirectly, initially in the present chapter and subsequently in conjunction with the results of Chapter 3.

THE NEPHRIC TISSUES

Do the nephric ducts or nephrons form first?

Using light-microscopy, at H&H 9⁺ a mesenchymal nephric rudiment (NR) could be seen laterally adjacent to somites 5 or 6 and extending to at least the ninth somite as the latter was forming (fig. 14a&b). By H&H 11, this first nephric rudiment had extended posteriorly to approximately the same level as the most posterior (thirteenth) somite (fig. 35).

From H&H 11 to 15 a second longitudinal rudiment (NN) appeared to have separated laterally from the first rudiment (figs. 16-18, 22, 23, 26, 31-33) and almost immediately the second rudiment could be seen to have segregated anteroposteriorly into smaller, mesenchymal rudiments (figs. 17-20), which subsequently segregated further into rosette-shaped, individual-nephron rudiments (Ro) (fig. 34). The tissue remaining in the region of the first nephric rudiment did not segregate anteroposteriorly, but became the nephric duct rudiment (ND). Thus it appeared that the first nephric rudiment (NR) was a common source for the nephric duct and the nephric nephrons.

What is the pattern of nephric epithelialisation?

Epithelialisation together with formation of a central lumen occurred in an anteroposterior direction, both in the duct and the nephron rudiments. In the duct rudiment, the first visible signs were a uniformly thick wall and punctate appearance down the central axis; fig. 16 shows this appearance clearly in an anterior part but progressively less clearly in more posterior parts of the duct. The posterior tip of the duct rudiment became epithelial as it fused with the lateral wall of the epithelial cloaca (figs. 52 & 53).

After the duct had an otherwise epithelial appearance, cellular bridges could sometimes be seen in longitudinal wax sections, apparently linking opposite walls of the nephric duct. Such bridges might indicate the last regions of the duct to epithelialise, while their irregularity might suggest an un-segmented though not uniformly graded anteroposterior pattern of epithelialisation.

The first nephron rudiment formed as the duct rudiment began to epithelialise. It segregated first into groups of presumptive nephrons and then into individual-nephron rudiments (figs. 17-20), but the

overall pattern of epithelialisation in the nephrons was similar to that in the duct rudiment (compare duct rudiments in figs. 15-17 with nephron rudiments in figs. 18 and 20). The presumptive nephrons began epithelialising while in groups of presumptive nephrons (figs 19 & 20). As individual nephrons formed they took on a characteristic rosette-shape (figs. 34, 36). Each nephron rosette (Ro) formed a central lumen (e.g. fig. 39) and elongated (fig. 38), initially with formation of afferent tissue; probably a glomerular capsule (figs. 39-42, 61) and subsequently of efferent tissue; probably the distal nephric tubule (figs. 38, 43-48, 55).

In TS, typical triplets of duct, nephron and vasculature (afferent, efferent or both) soon after fusion of the duct with the cloaca are shown in figs. 36-41. The locations of the nephric tissues relative to other tissues are also shown in oblique sections (figs. 62 & 64).

By H&H 21, the nephrons appeared more similar to teardrops than rosettes and some had fused with the nephric duct (not shown). After H&H 21, nephron formation appeared to be more immediately epithelial, rather than via mesenchymal rudiments. The nephric nephrons continued to increase in size

until at least H&H 27, after the metanephric duct had reached the site of formation of the metanephrons. By H&H 27, the nephric duct had widened and the nephrons appeared to be regionally differentiated into proximal and distal tubules (PT, DT); the glomerulus (G) was clearly recognisable (figs. 55, 57-59).

Is formation of the nephric tissues in situ or by anteroposterior migration?

The nephric rudiment was first clearly visible at H&H 9⁺ (8 somites), lying adjacent to somites 5 or 6 - 8 and extending into the segmental plate (as indicated by expression of S-FC10.2; fig. 14; see Chapter 3). It was of similar mesenchymal appearance throughout. Hence it appeared to have formed in situ, rather than by posterior migration of a shorter rudiment. From H&H 10 the rudiment extended posteriorly. Its caudal tip was mesenchymal (figs. 22 & 25) and joined to or spread medially over the segmental plate (pre-somitic tissue) and/ or laterally over the lateral plate (pre-gastrointestinal or -reproductive tissue) (figs. 22-24, 26, 27, 35). The more anterior parts of this

rudiment became more compact as it epithelialised anteroposteriorly (figs. 16, 18, 22, 26-30).

Especially noticeable at the caudal end of the duct (ET) were long, multicellular branches (B). These were observed with SEM at H&H 14-16 in at least three embryos, as lateral projections either or in combination, anteriorly or posteriorly, though not ventrally or dorsally (figs. 22-24, 26, 27). Such branches appear not to have been reported previously, but stereoscopic SEM showed clearly that they were branches of the duct and not, for example, vascular rudiments (V), such as of the posterior cardinal vein, which were readily distinguishable (figs. 22 & 23).

The mesenchymal nature of the duct tip is consistent with in situ formation, though the overall shape of the rudiment might appear to be consistent with migration. The branches might conceivably be associated with either type of formation.

The mesenchymal part of the duct appeared to have a variable location with respect to the somites (S) and segmental plate (SP). At H&H 9⁺ the duct mesenchyme could be seen (see Chapter 3) to extend

to the level of the segmental plate (fig.14), while at H&H 11 it appeared to be anterior to the last-formed somite (Sz, fig. 35). By H&H 15, however, it was seen to be either adjacent (fig. 30) or posterior to the last somite (figs. 26, 27). Formation of the nephric duct was taken to be complete by the time it fused with the cloaca (Cl) at H&H 17⁻, though subsequently it was joined by the nephrons and its lumen appeared to have widened (fig. 57).

It was also difficult to ascertain whether the nephrons initially formed in situ or by migrating from the first rudiment, albeit over a comparatively short distance. As the proximal tissue (glomerular capsule) formed, polarity was established in the nephron. The region of the presumptive distal tubule then (re-) joined the duct, while itself becoming epithelial (figs. 38 and 40-49). The re-joining seemed to occur by migration and invasion by the nephron, principally because the distal region of the nephron became somewhat attenuated, while the duct became complementarily indented.

The rounded appearance of the epithelialising, presumptive distal tubules extending towards the duct usually contrasted with the more open

mesenchymal appearance of the tip of the nephric duct extending towards the cloaca (e.g. figs. 21, 22 & 25). However, the open, mesenchymal morphology of the duct tip was itself highly variable at similar embryonic stages (e.g. figs. 22, 24b) and in a few embryos of the same stages, the duct appeared rounded and more compact in SEM (not shown).

Is development of the nephric tissues segmental?

Evidence of anteroposterior segmentation of the nephric duct during its formation was not immediately apparent, using SEM (figs. 22, 27) or LM (figs. 16, 17). However, segmentation of the epithelialising duct could not be excluded (see p. 95) and even seemed likely from the anteroposteriorly punctate appearance of the duct and first-nephron rudiments when stained with S-FC10.2 (see Chapter 3).

The pattern of formation of the nephric nephrons appeared to be more complex than that of the duct, because it involved segregation as well as extension and epithelialisation. The individual nephrons were arranged at regular anteroposterior intervals when recently formed and could therefore

be said to be segmental. Moreover at intermediate anteroposterior levels at H&H 18, approximately four nephrons corresponded to each somite length (fig. 34). It also appeared that the initial anteroposterior subdivision of the nephron rudiment from H&H 11 was consistently into groups of about four, rather than individual, presumptive nephrons (figs. 17, 18, 32). Hence it seems likely that formation of the nephrons is to some degree segmental, with an anteroposterior pattern related to that of the somites.

THE EARLY METANEPHRIC TISSUES

Using LM, the metanephric duct (MD) was first seen at H&H 24 (4 days), as an outgrowth of the cloaca. By H&H 27 (5.5 days), this duct had extended anteroventrally into the metanephric mesenchyme (MMc) (figs. 56, 57, 60), although its site of origin in the anterior wall of the cloaca could still be seen (fig. 60; and as indicated by S-FC10.2, fig 57).

The metanephric duct epithelialised and formed its canal almost as soon as it formed from the

cloaca, while its extending tip (ET) was more similar in appearance to that of a nephric nephron re-joining the nephric duct than of a typical nephric duct. This epithelial, rather than mesenchymal, type of formation of the metanephric duct was also more similar to that of post-rosette nephrons than to the nephric duct or earlier nephron rudiment(s). Furthermore, the greater but variable width of the metanephric duct from its early stages was more similar to that of the nephric duct at the same embryonic stages (e.g. fig. 57) than to the nephric duct when it began to epithelialise.

Also in contrast to the nephric duct, the metanephric duct could be seen to have branched only after it had reached its target mesenchyme and apparently by dividing equally (bifurcating) at its most distal extreme. This was first seen at H&H 27 (5.5 days), when the mesenchyme of the presumptive metanephrons began visibly to condense around the bifurcating tip (figs. 56 & 57). In apparent contrast to formation both of post-rosette nephric nephrons and the metanephric duct, the mesenchymal nature of the early metanephron rudiments was more similar to that of earlier formation of the nephric tissues.

ARE THERE MORPHOLOGICALLY DISTINGUISHABLE TYPES OF NEPHROGENIC TISSUE?

Based on morphology during formation and epithelial differentiation, no evidence of different types of nephric nephron or duct (such as 'pro-' and 'meso-') was found. Moreover, there were considerable similarities between the nephric duct and its nephrons, in their forming apparently from a common rudiment (NR), in their pattern of epithelialisation and putatively in their segmentation. Relative timing and location of differentiation and degeneration at different anteroposterior levels were not investigated sufficiently to compare morphogenetic changes, such as the rate or degree of maturation, within the nephric tissues.

At the stages examined, morphological differences were apparent between the nephric and metanephric tissues and between the metanephric duct and its nephrons, as described in the preceding section on the early metanephric tissues.

TABLE 12

ABBREVIATIONS USED ON PHOTOGRAPHS

a TISSUES

Where applicable, the following are used equally to apply to rudimentary and more epithelial tissues.

Am Amorphous material within lumen

AT Anterior tip of nephric duct

B Branch of nephric duct

Cl Cloaca

CD Collecting duct

DT Distal tubule

ECM Extracellular matrix

Ect Ectoderm

End Endoderm

ET Extending tip of urinary duct

F Site of fusion

G Glomerulus

GC Glomerular capsule

Gh Hindgut

Gm Midgut

GM Gut mesentery

HN Hensen's node

cont'd.

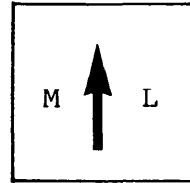
IM Intermediate mesoderm
LB Hind limb bud
Lu Lumen
LM Lateral mesoderm
Mc Mesenchyme
MD Metanephric duct
MN Metanephron
NC Notochord
ND Nephric duct
NN Nephric nephron
NNs Nephric nephros
NR First nephric rudiment
NT Neural tube
PCV Posterior cardinal vein
PGC Primordial germ cells
PM Paraxial mesoderm
PT Proximal tubule
RBC Red blood cells
Ro Rosette (nephric nephron)
S Somite (and number, e.g. S4)
Sz Most posterior somite
SP Segmental plate
V Vasculature

cont'd.

b DIRECTIONS

These are shown in a box, as on the right:

- ↑ Anterior
- D Dorsal
- L Lateral
- M Medial
- V Ventral



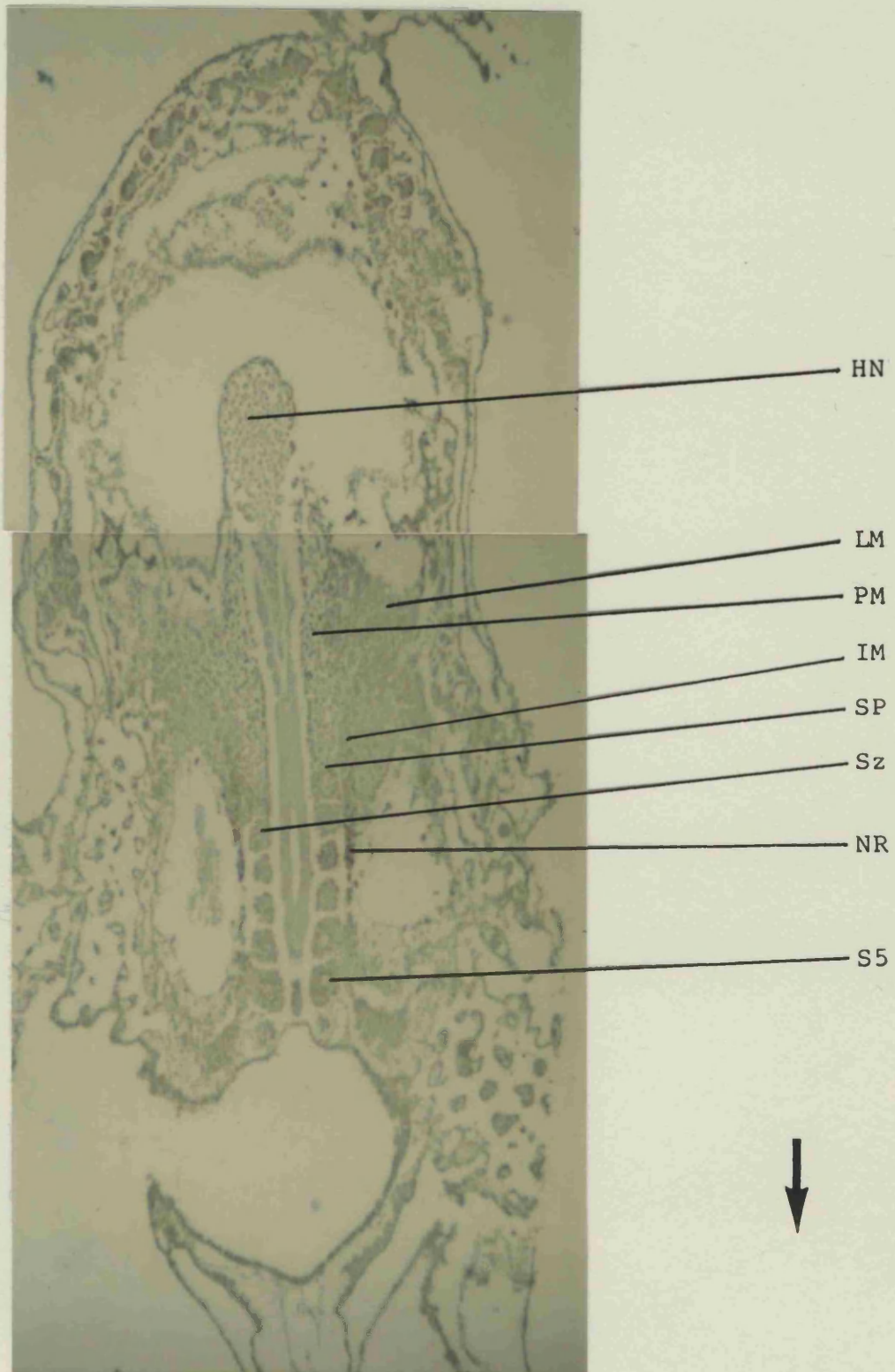


Fig. 14a H&E 9⁺ (9somites), coronal section
stained with S-FC10.2, showing early nephrogenic
tissues bilaterally, X60

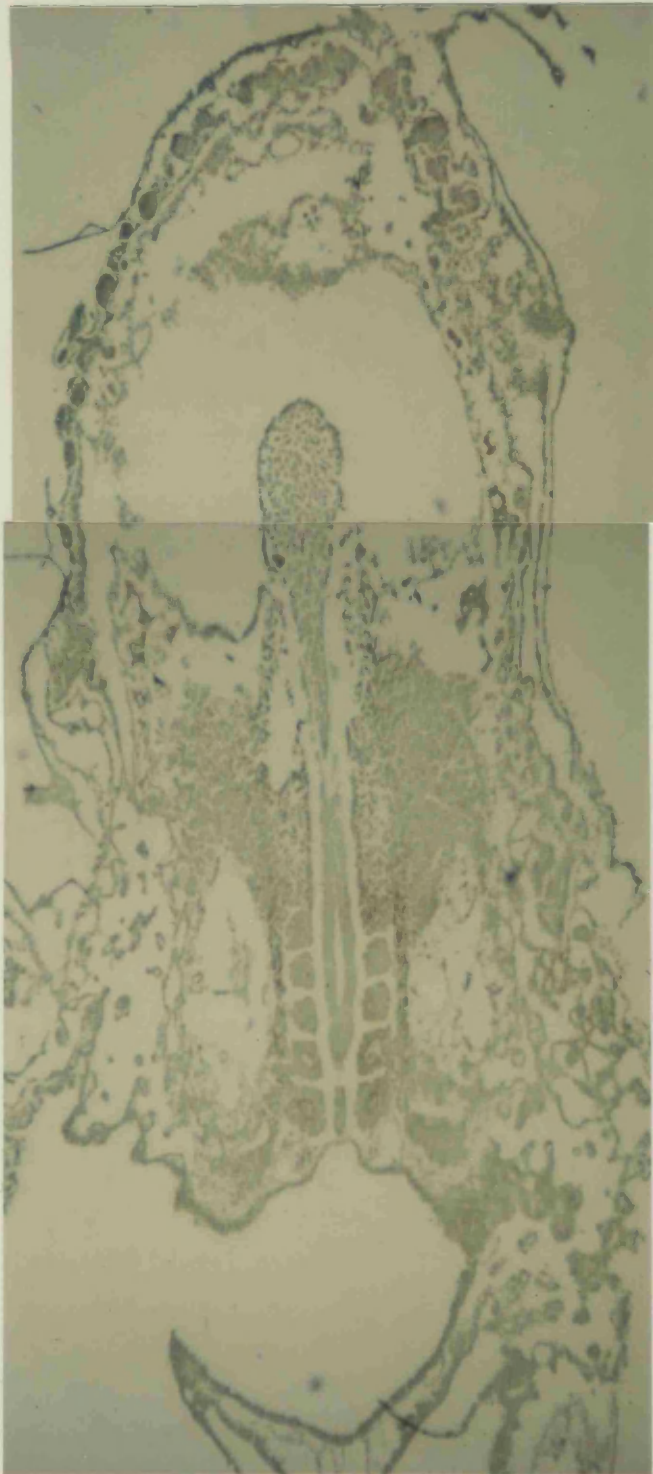


Fig. 14b Adjacent section to fig. 14a
(Labels as in fig. 14a)

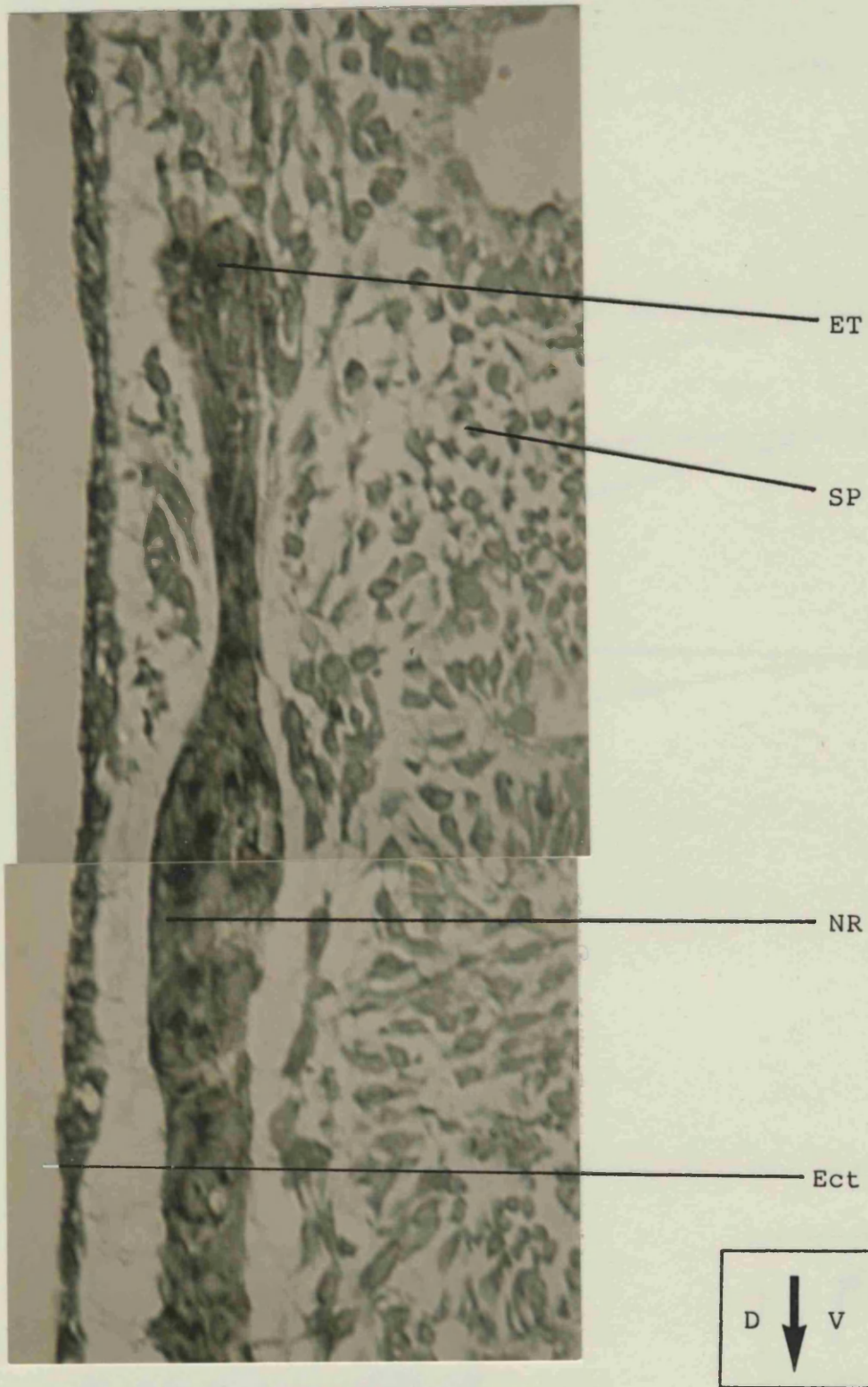


Fig. 15 H&E 11, parasagittal section
stained with S-FC10.2, X760

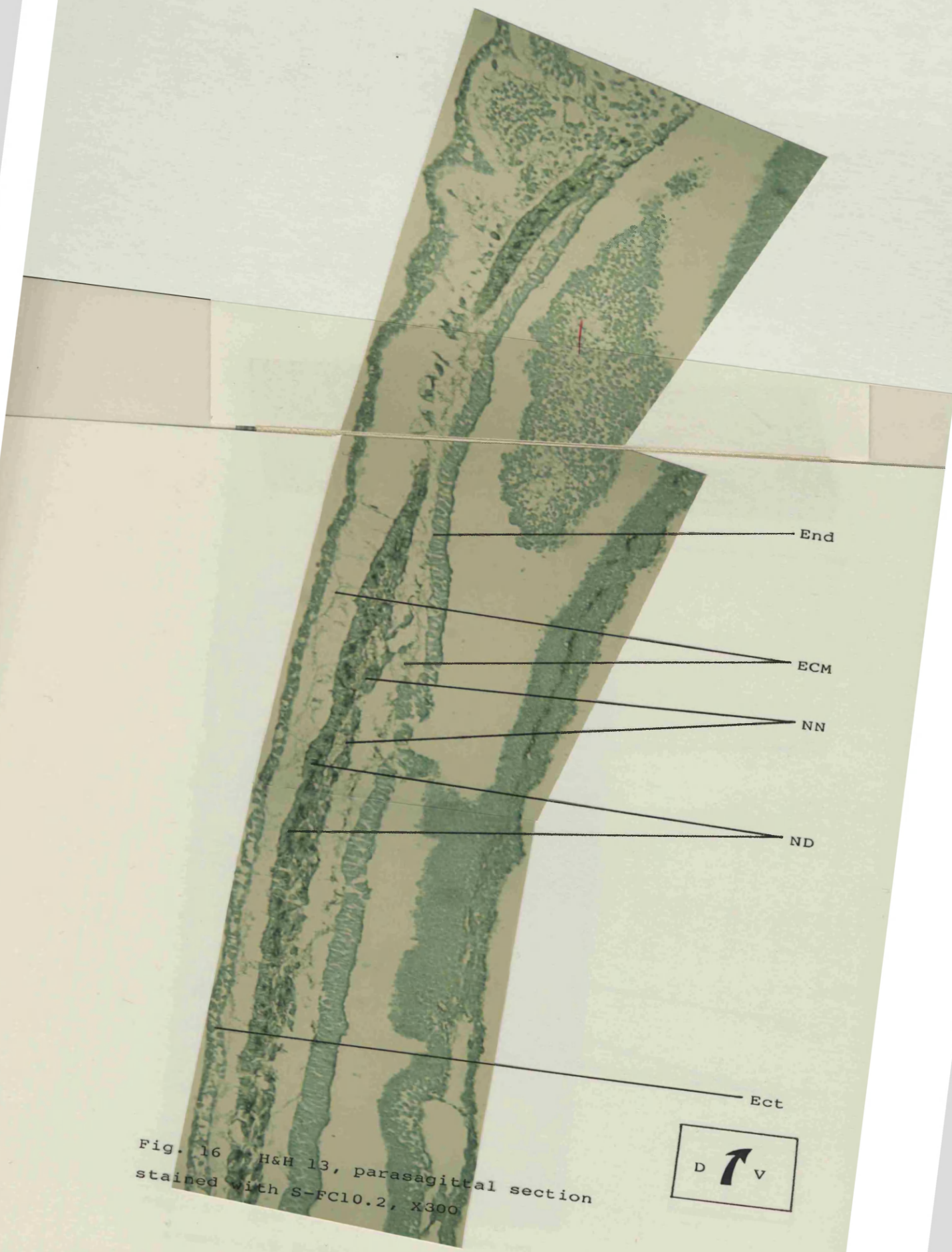


Fig. 16 H&H 13, parasagittal section
stained with S-FC10.2, x300

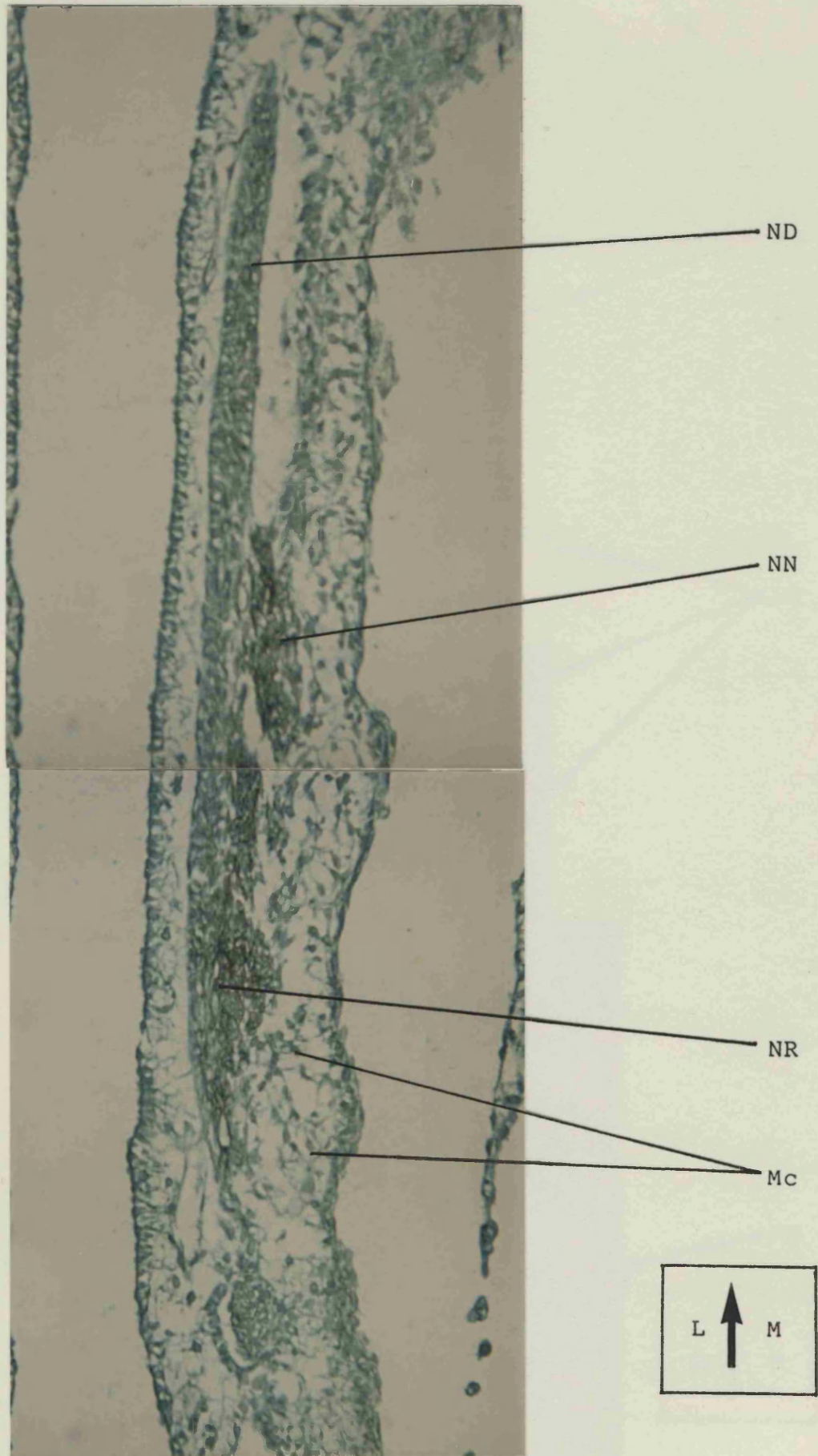


Fig. 17 H&H 15⁻, coronal section
stained with S-FC10.2, X300

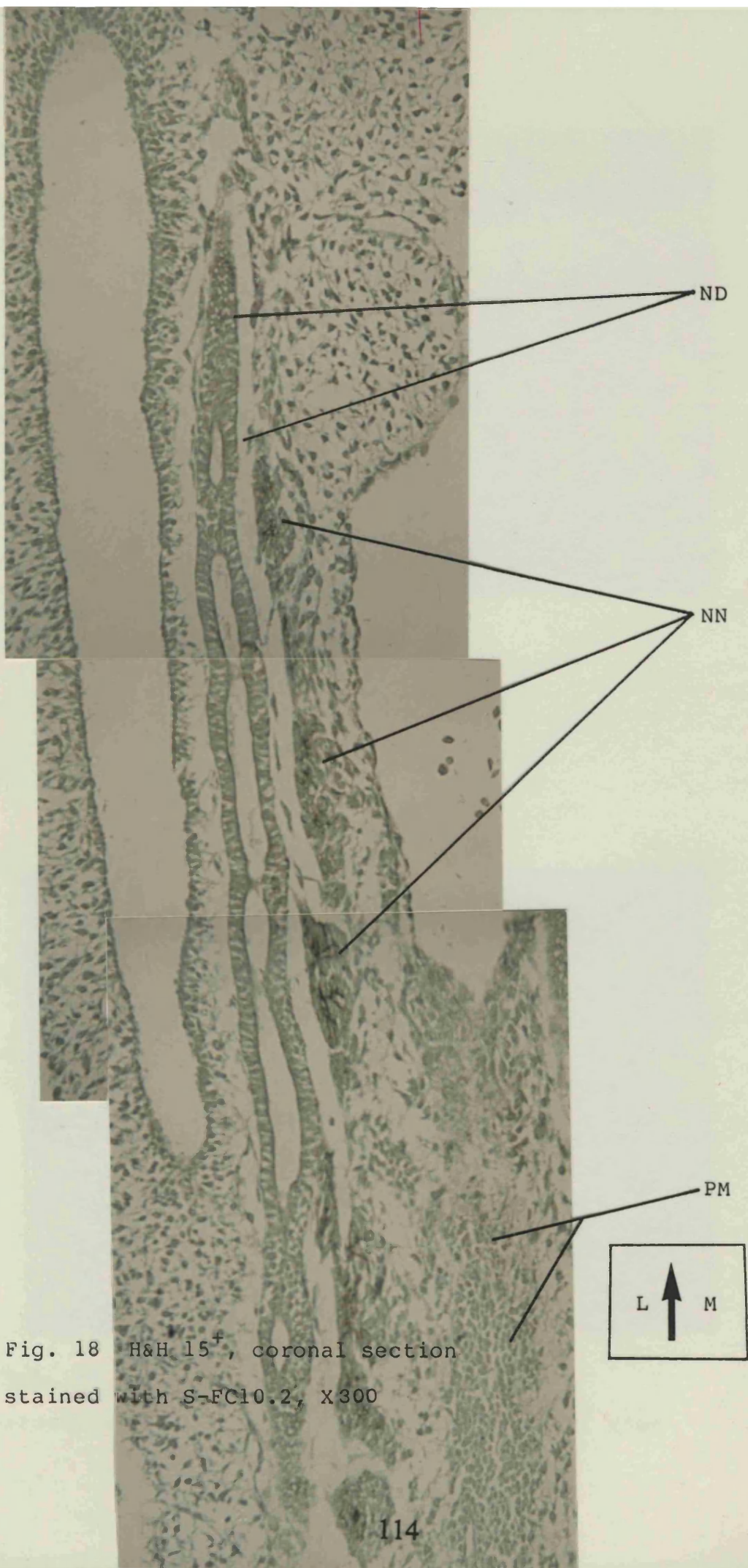
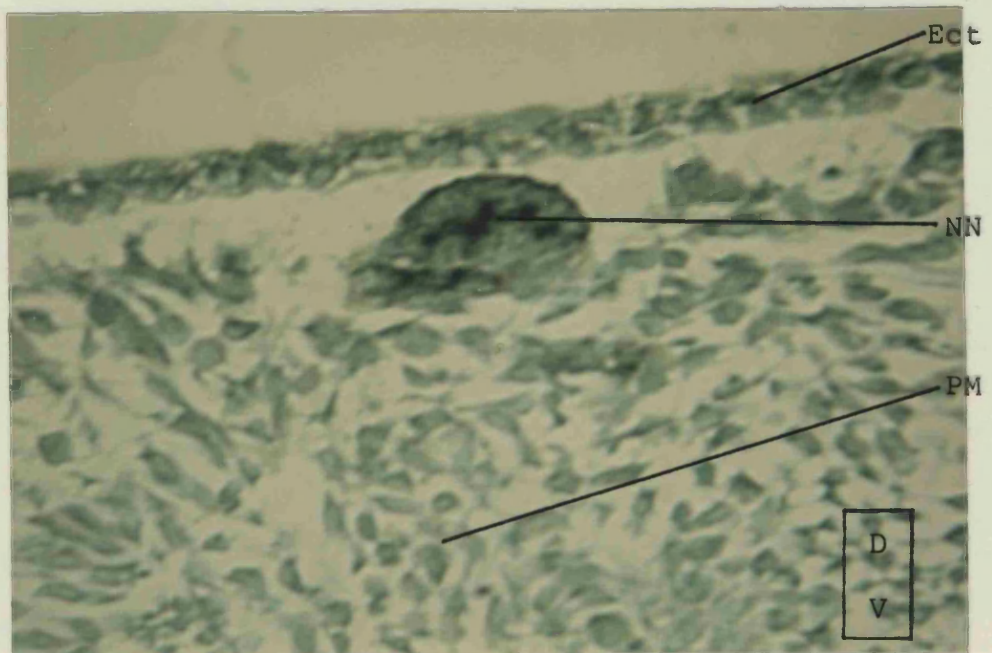
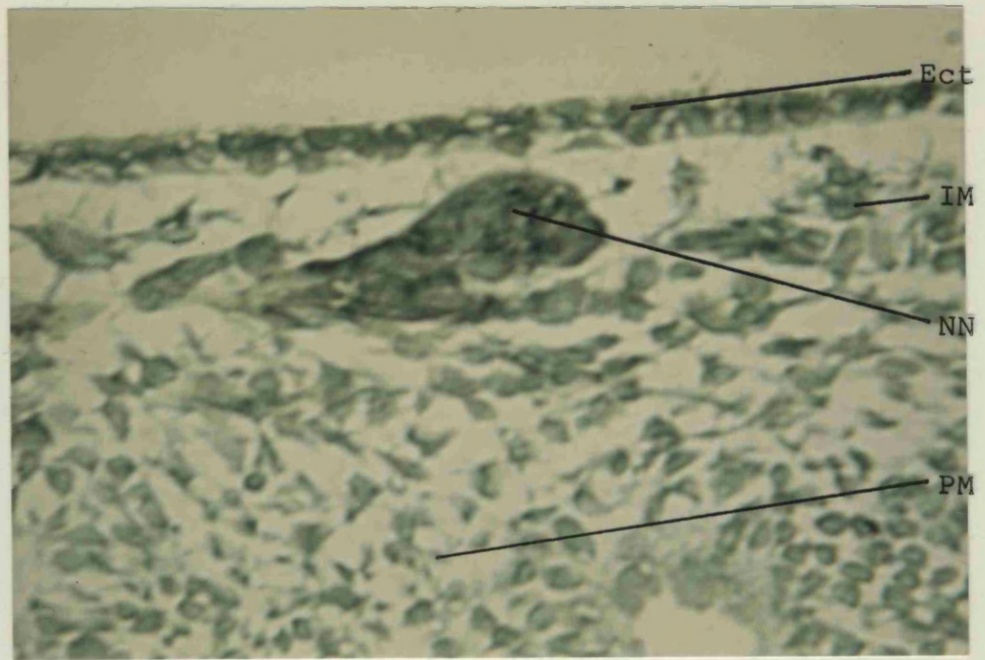


Fig. 18 H&E 15⁺, coronal section
stained with S-FC10.2, X300



Figs. 19 (top) & 20 (bottom) H&H 11,
parasagittal sections stained with S-FC10.2, X760

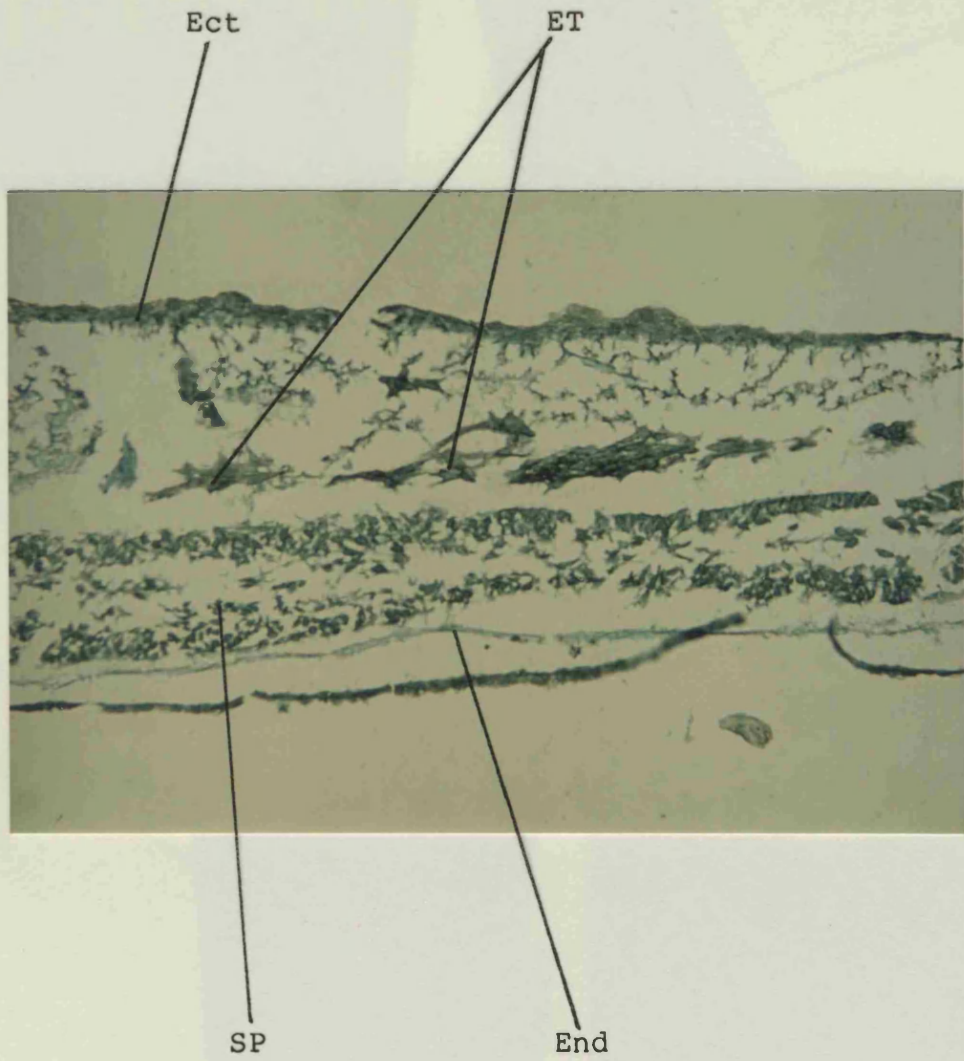


Fig. 21 H&H 11, parasagittal section
stained with S-FC10.2, X235

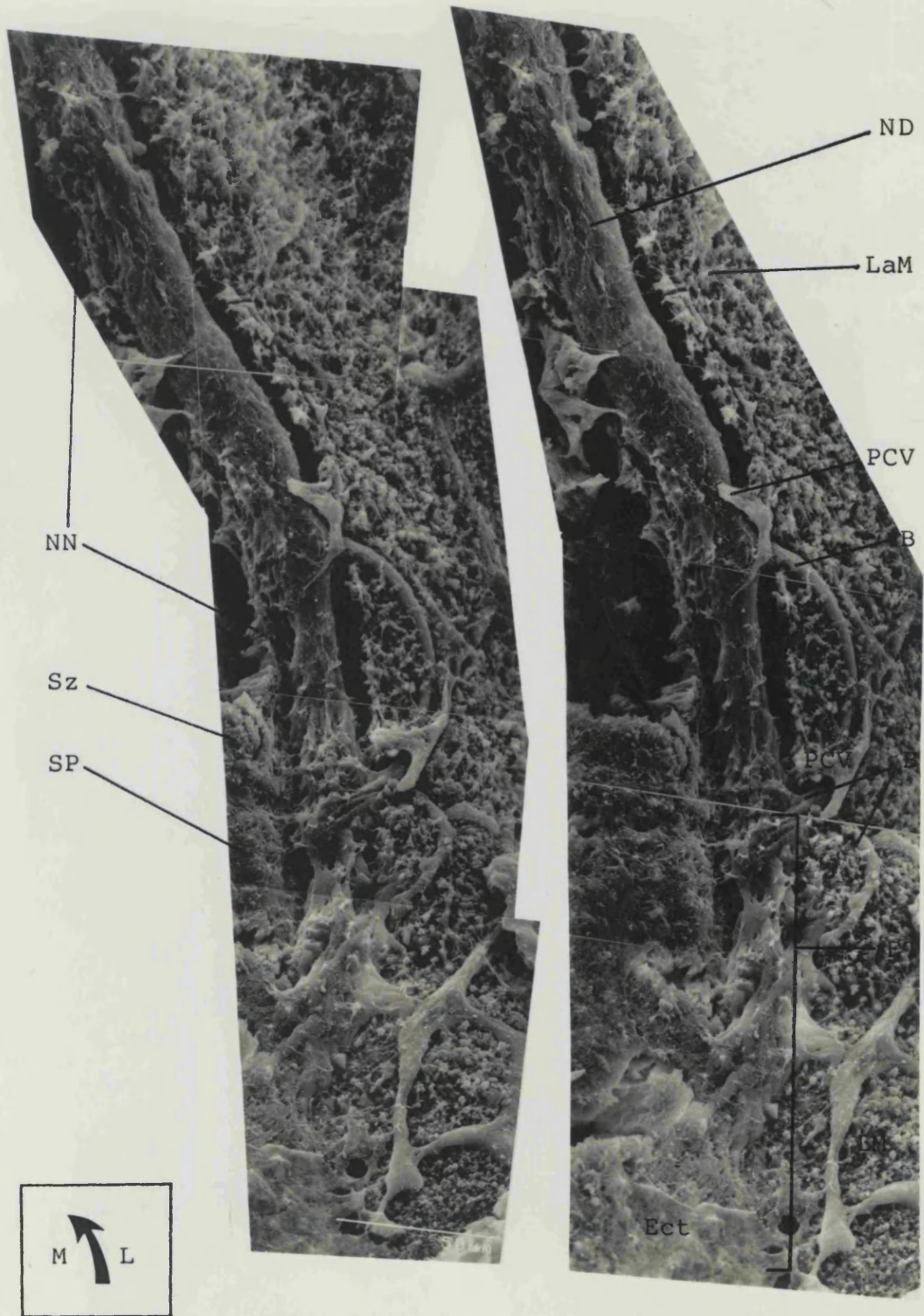


Fig. 22 H&H 14, ectoderm removed, SEM
 showing posterior regions of nephric rudiments;
 stereo pair, X720

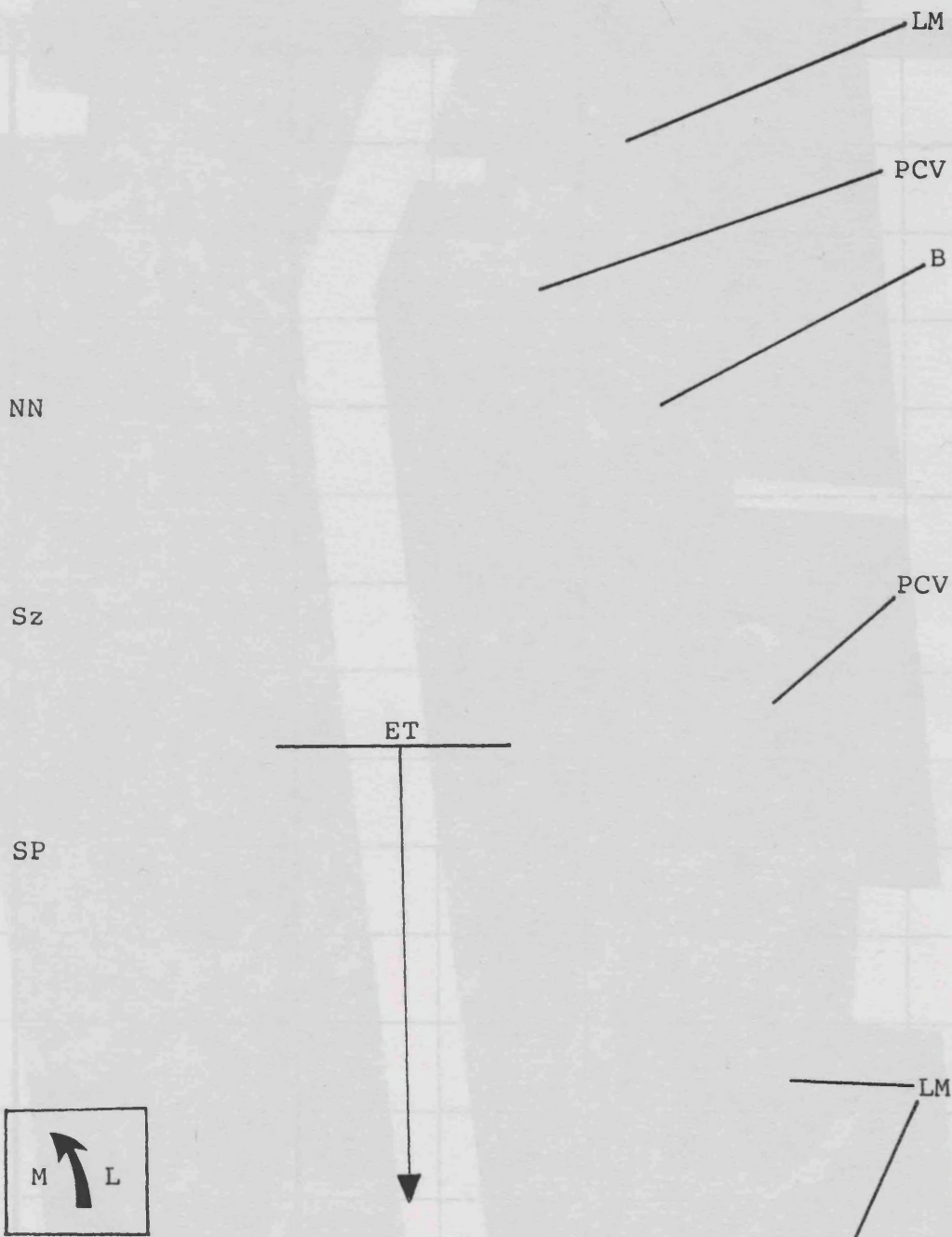
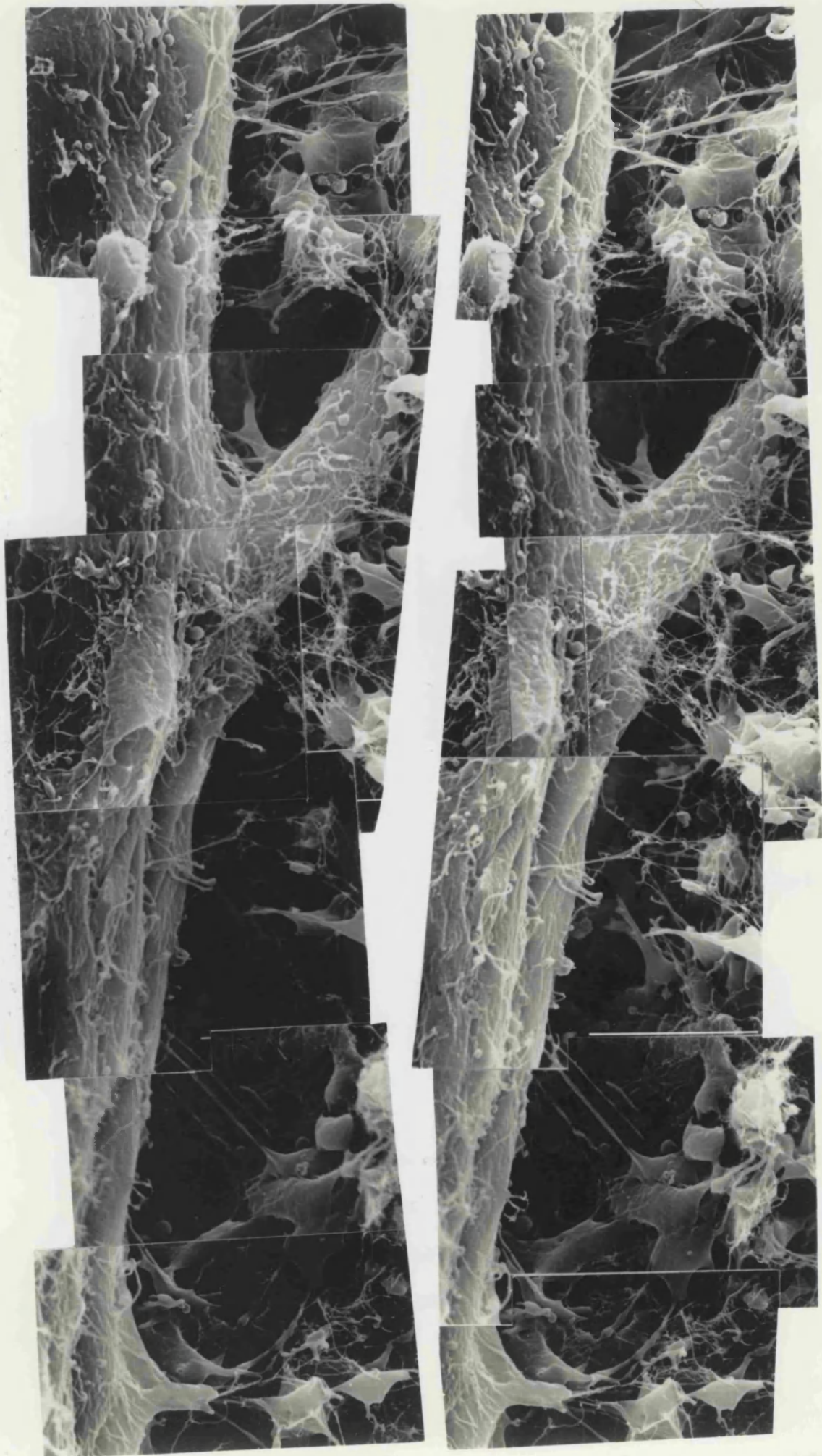


Fig. 23 H&H 14, ectoderm removed, SEM showing most posterior regions of nephric rudiments; stereo pair, X1200





Fig. 24a H&H 14, ectoderm removed, SEM showing a branching point near the posterior end of the extending nephric duct; stereo pair, X3000



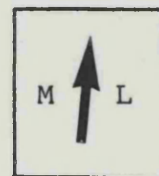
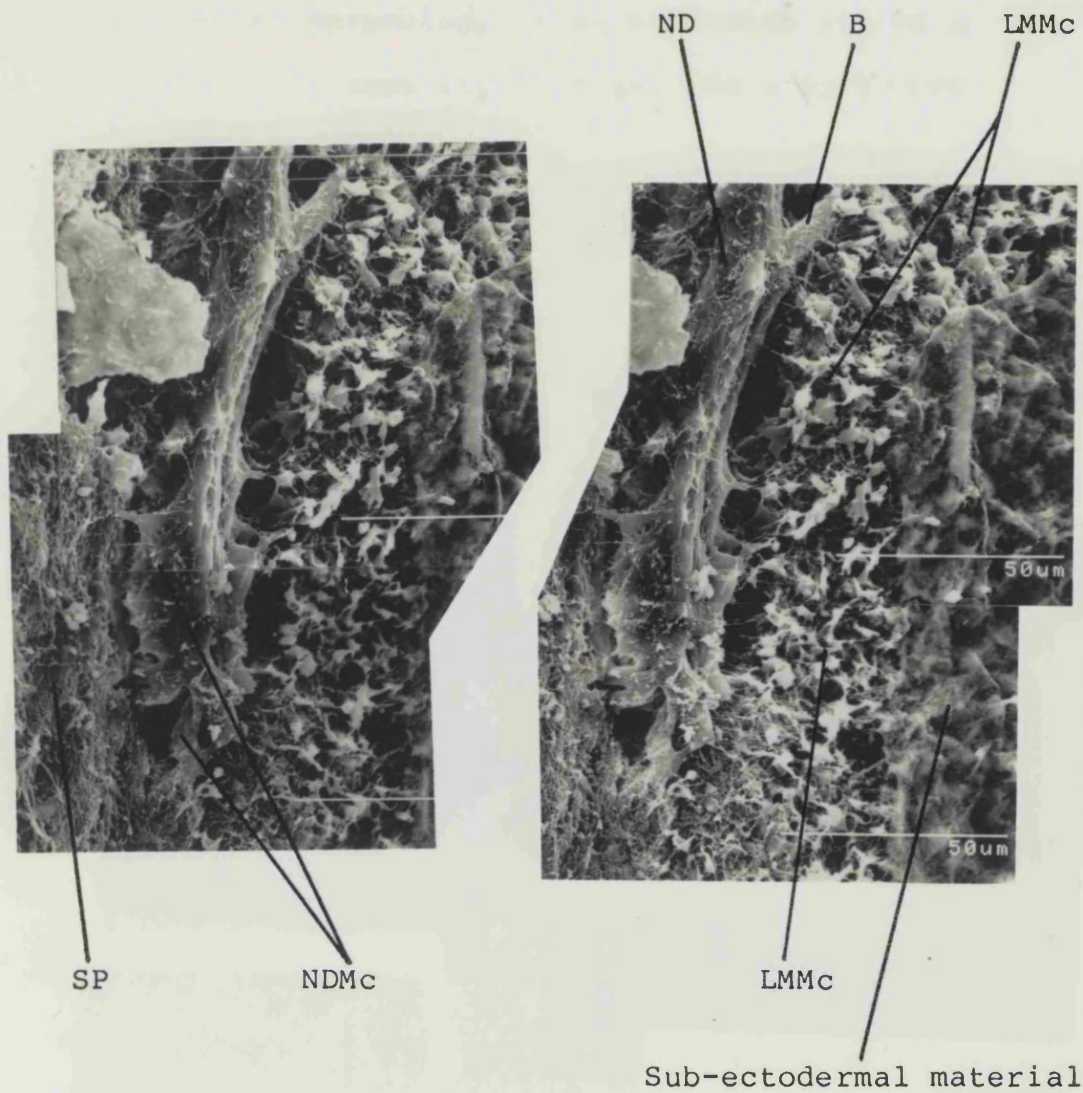
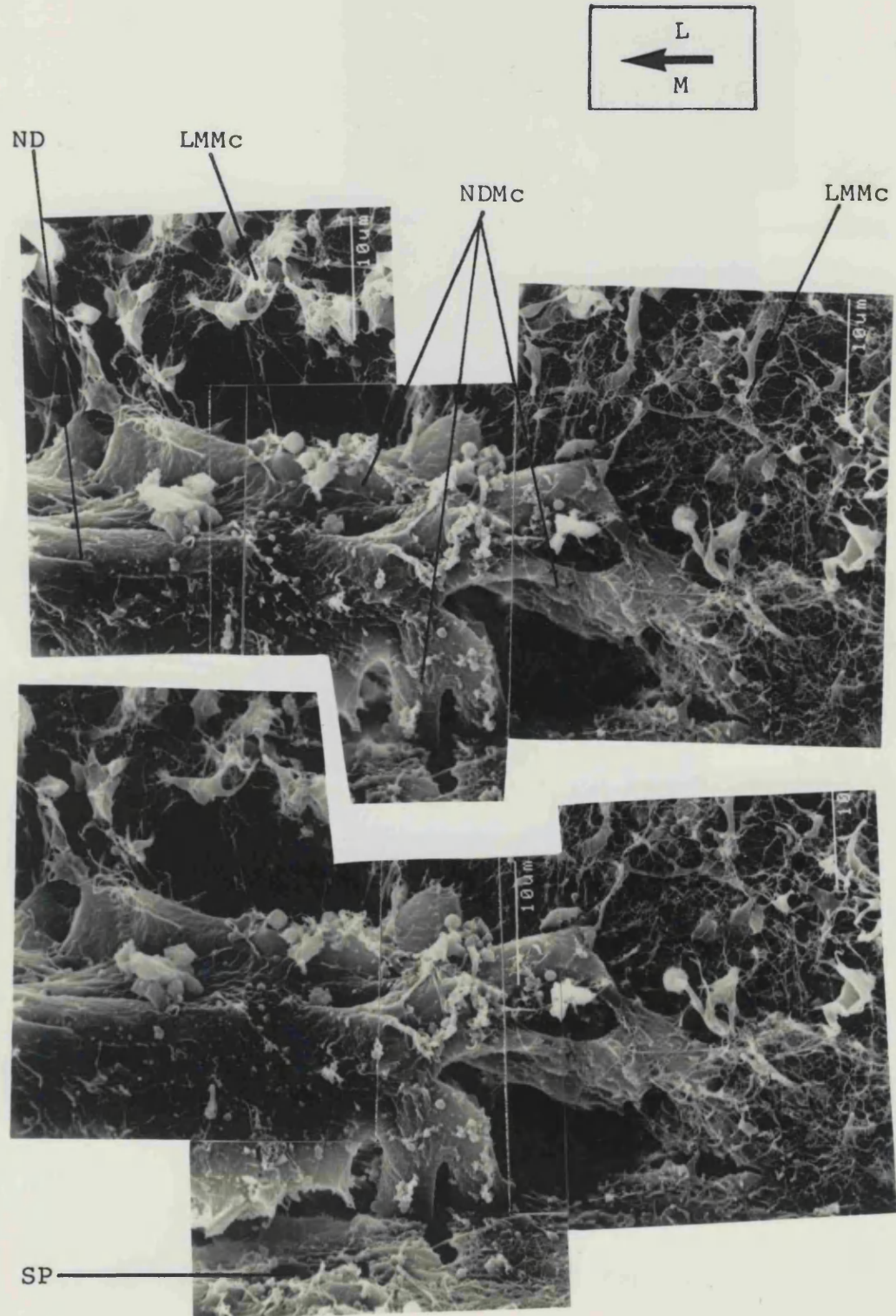


Fig. 24b H&H 14, ectoderm removed, SEM
 showing posterior tip of nephric duct, with
 branching and mesenchymal morphology;
 same branch as fig 24a; stereo pair, X600

Fig. 25 H&H 14, ectoderm removed, SEM to show the mesenchymal morphology of an extending tip of a nephric duct; same tip as figs. 24a & b; X1850



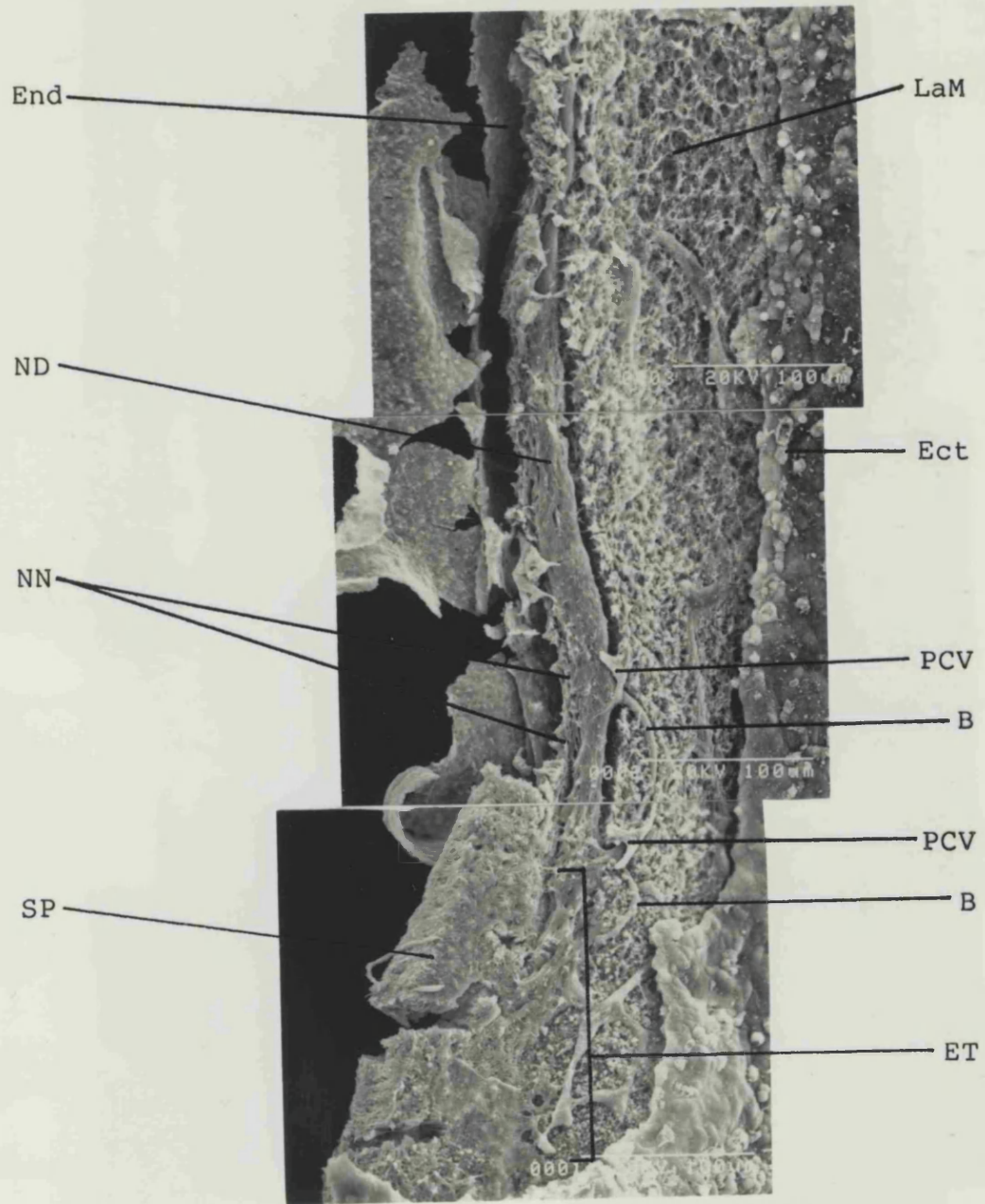
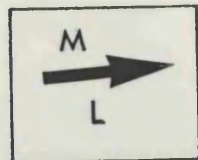
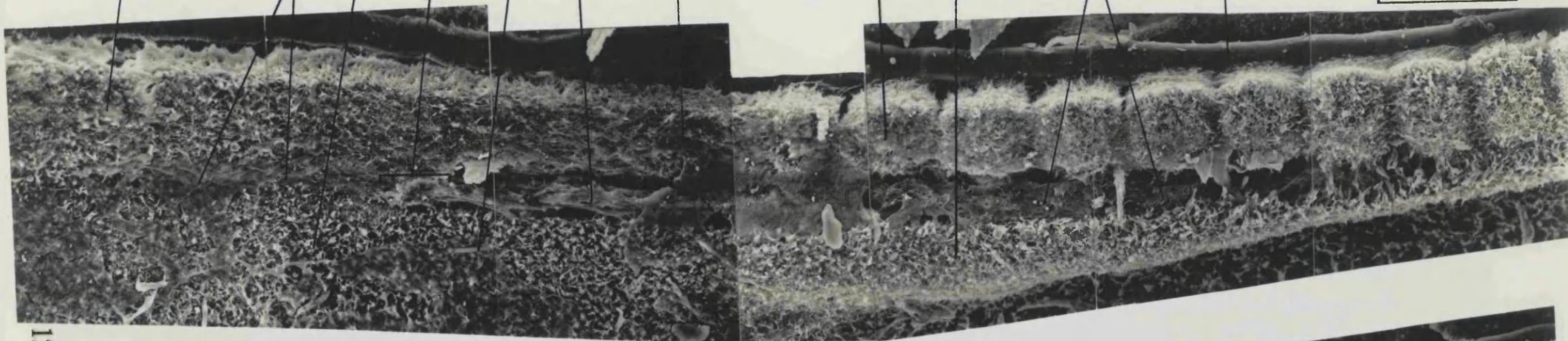


Fig. 26 H&H 14, ectoderm removed, SEM, X400

Fig. 27 H&H 14, ectoderm removed; stereo pair, X 170



PM IM LM ET B ND SP Sz LM ND NC



123



Sub-ectodermal material

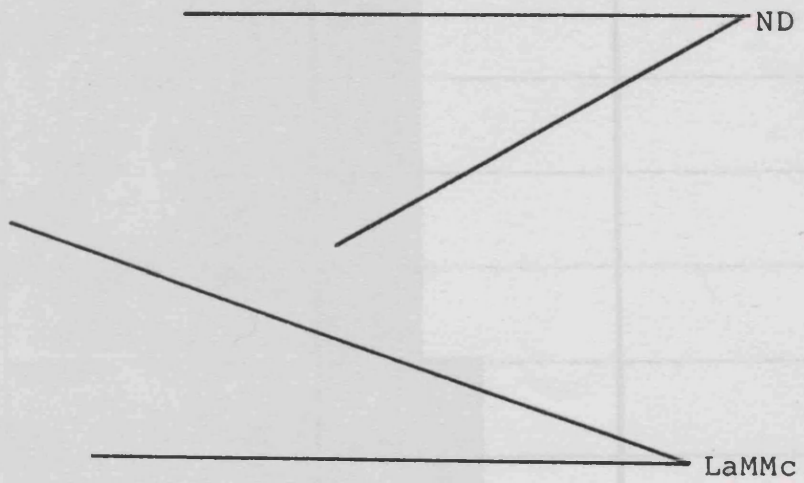
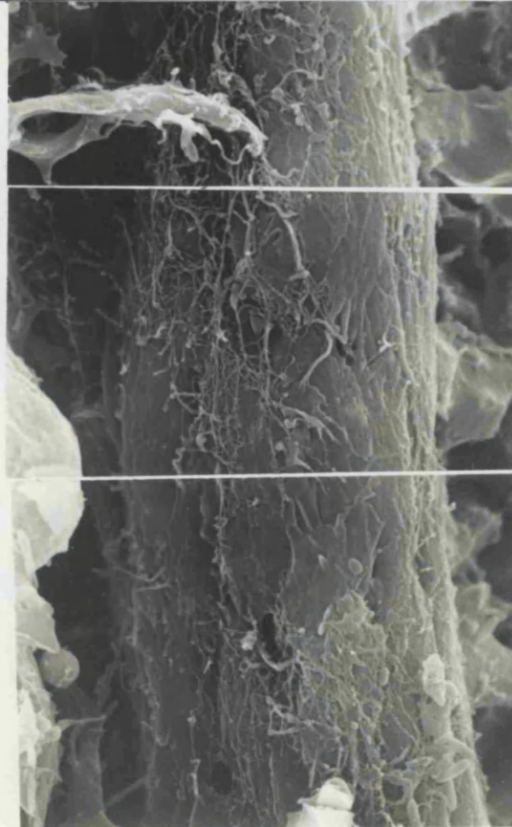
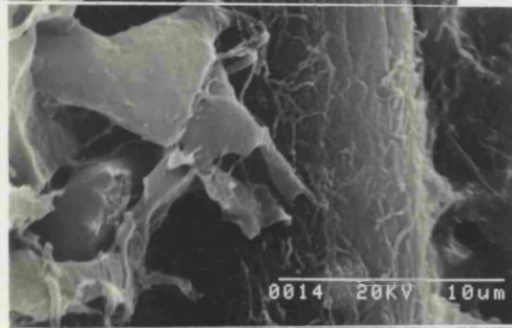
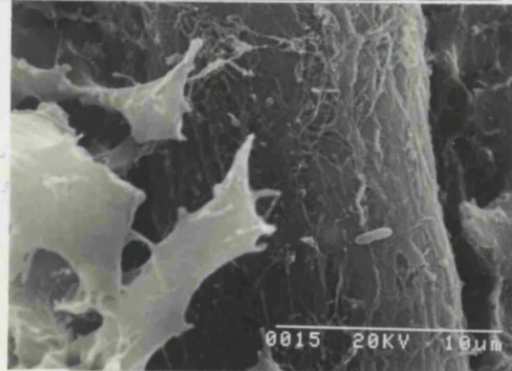
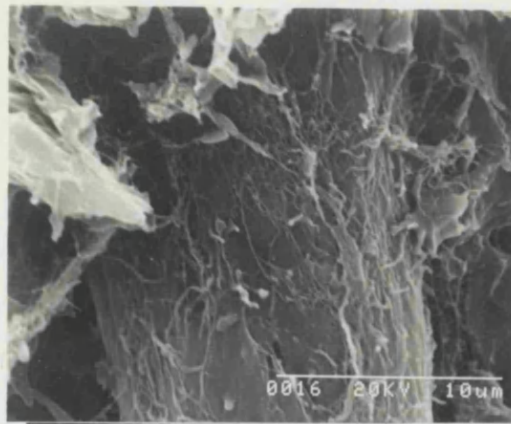


Fig. 28 H&H 14, ectoderm removed, SEM showing anterior, epithelial region of nephric duct becoming enveloped by lateral mesoderm mesenchyme; X5000



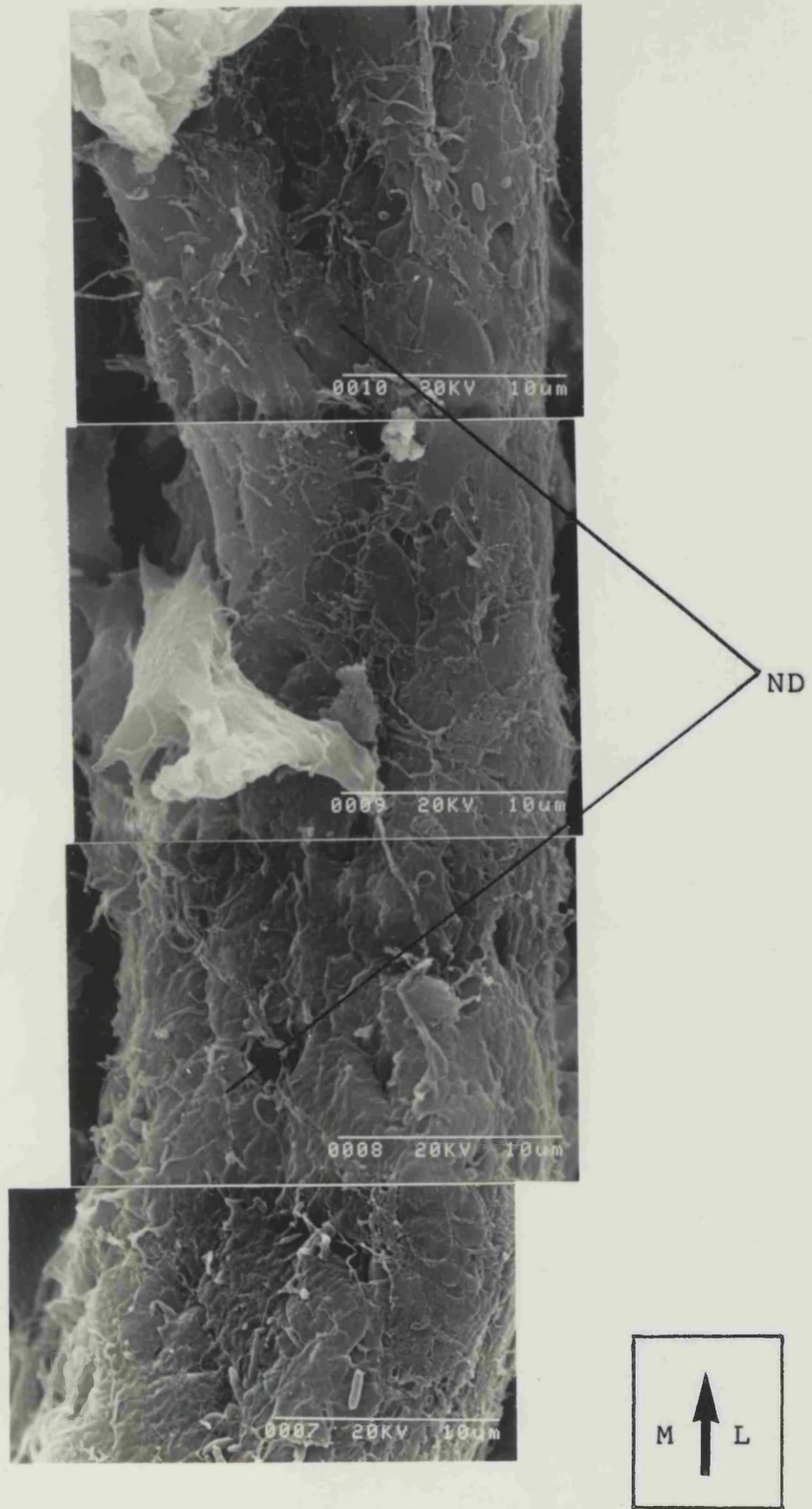


Fig. 29 H&H 14, ectoderm removed, SEM showing epithelial morphology of anterior region of nephric duct; X5000

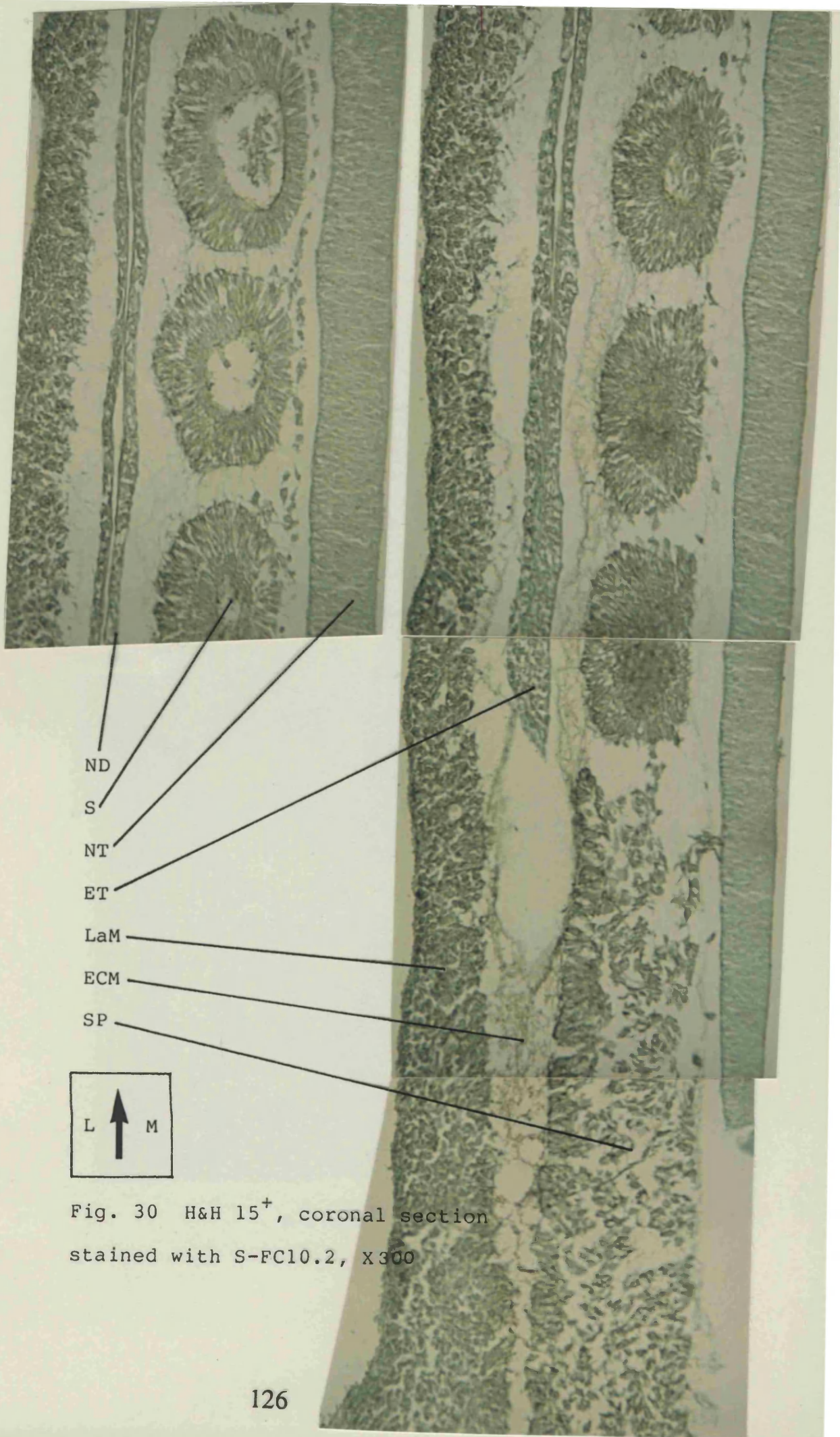


Fig. 30 H&H 15⁺, coronal section
stained with S-FC10.2, X300

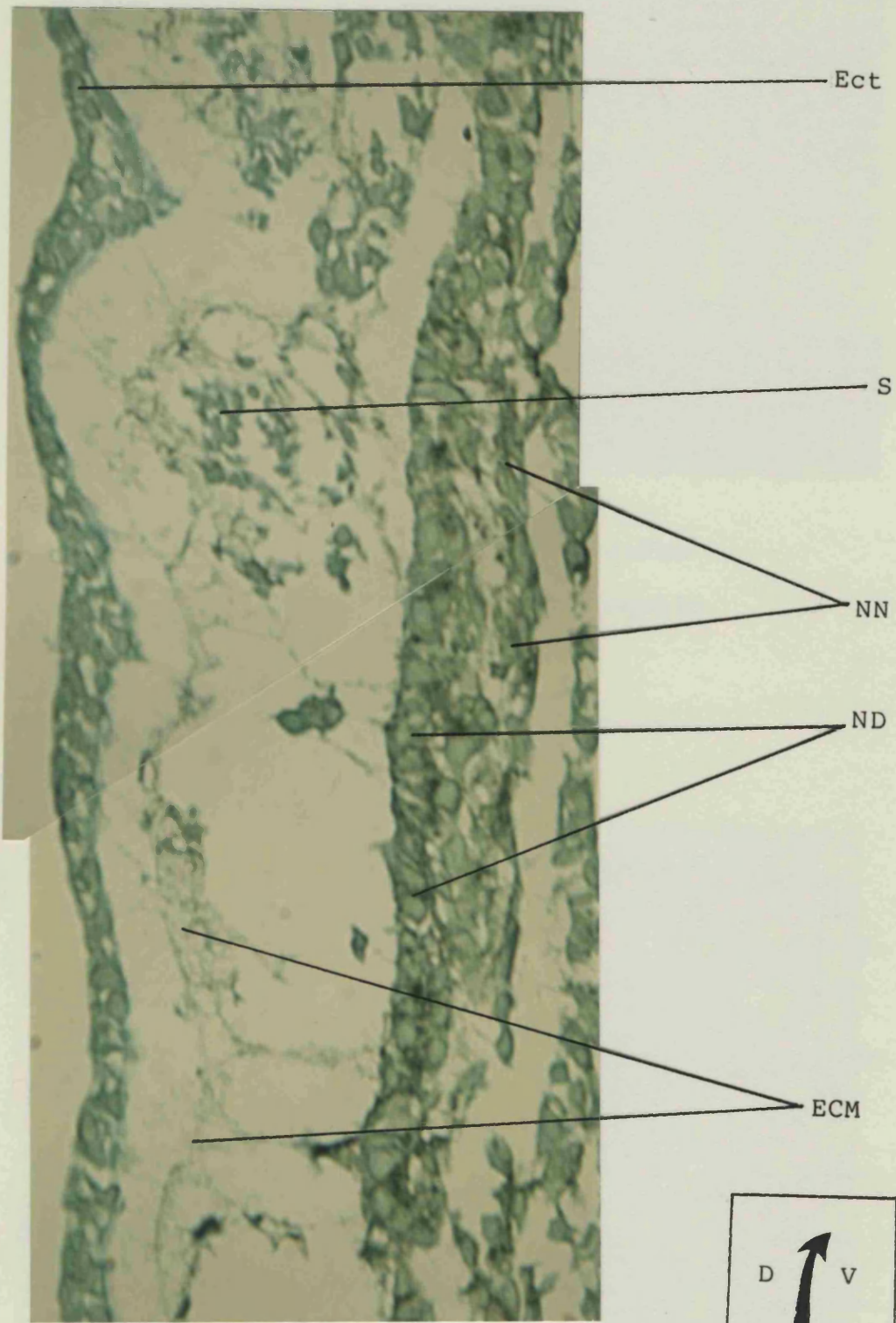


Fig. 31 H&E 13, parasagittal section
stained with S-FC10.2, X760

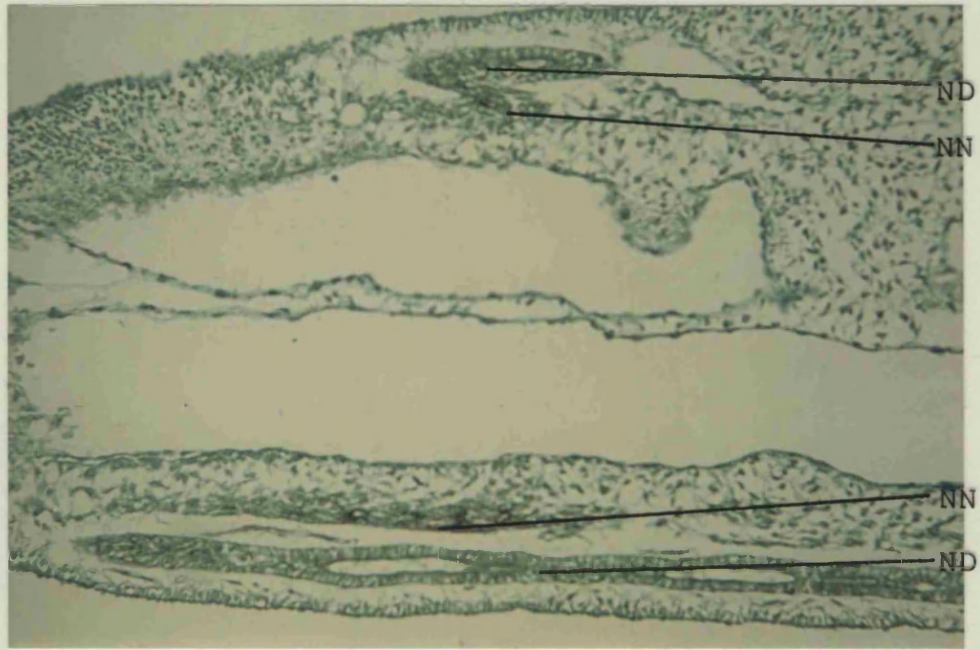


Fig. 32 H&H 15⁺, coronal section stained
stained with S-FC10.2, showing nephrogenic
tissues bilaterally, X185

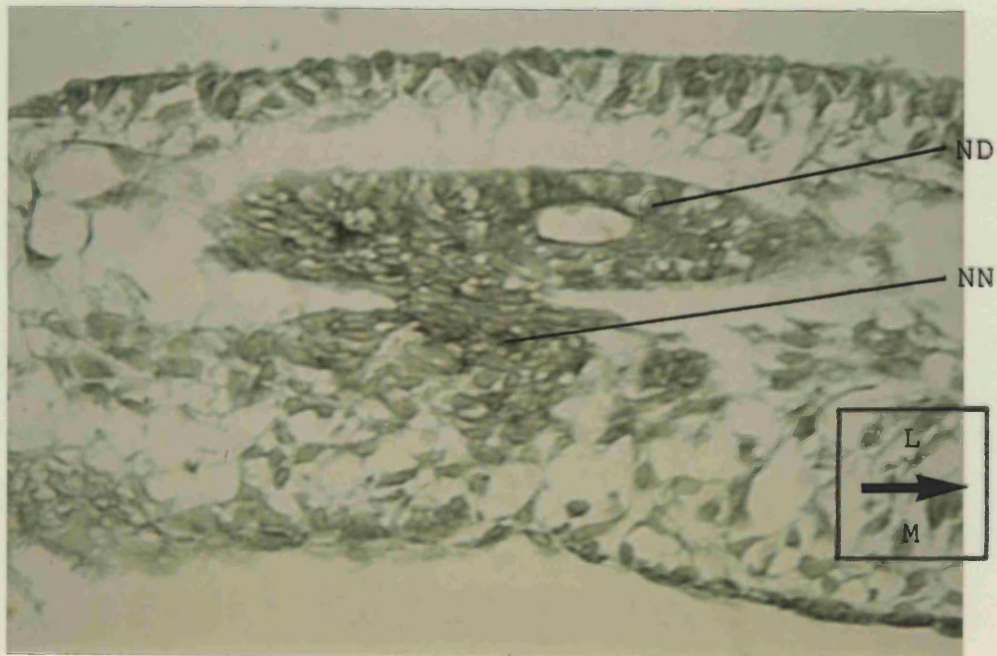


Fig. 33 H&H 15⁺, coronal section
stained with S-FC10.2, X590

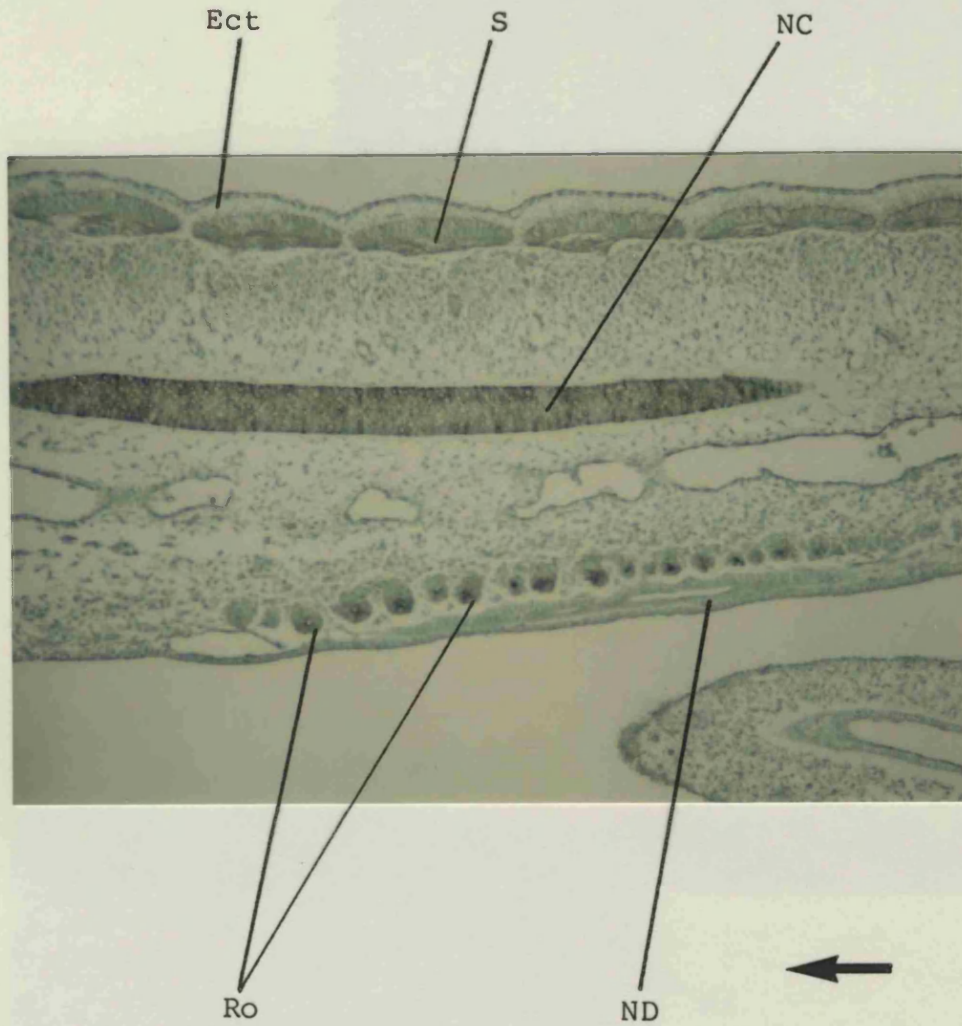


Fig. 34 H&E 18, oblique section stained with S-FC10.2, X120

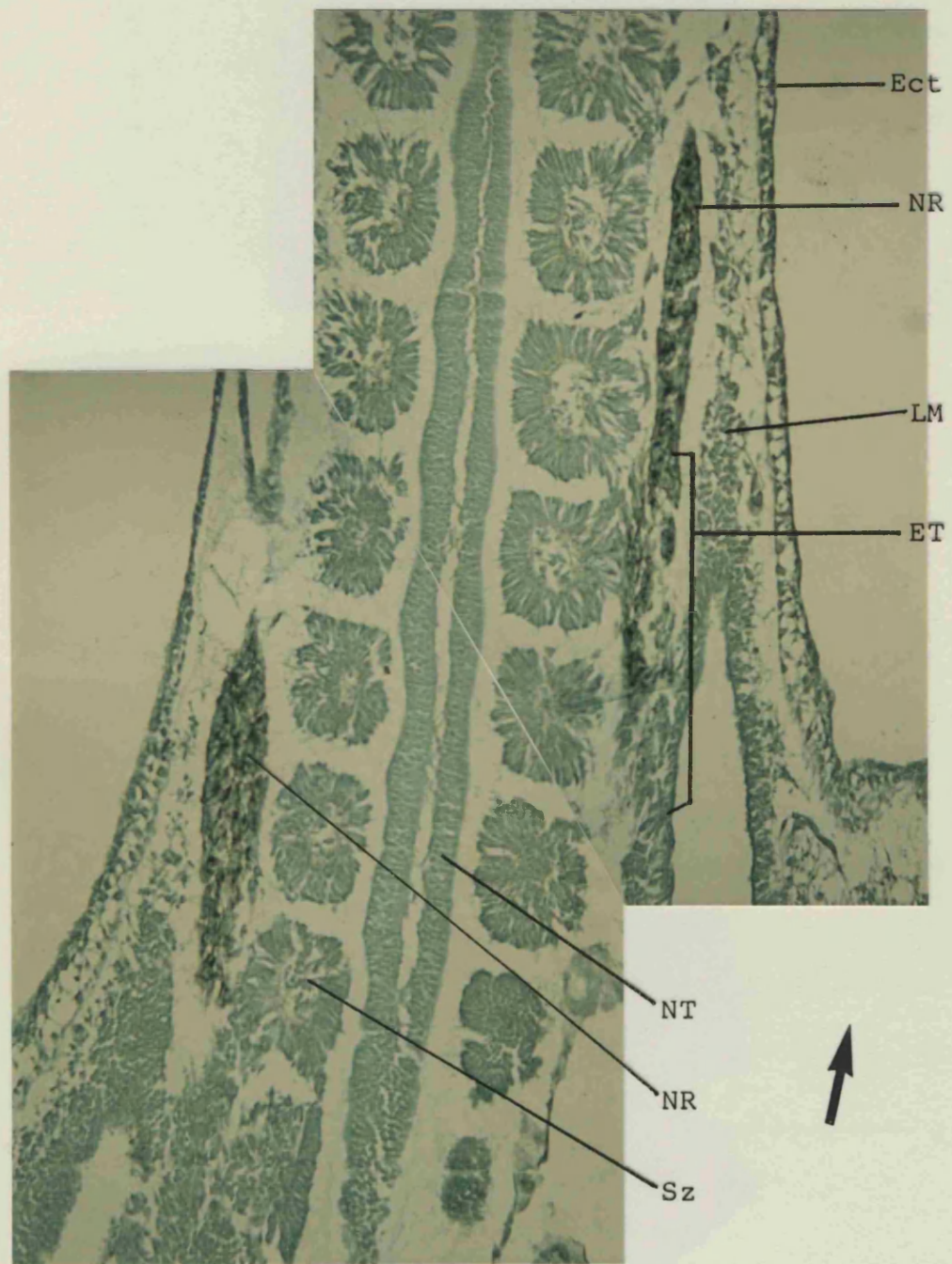
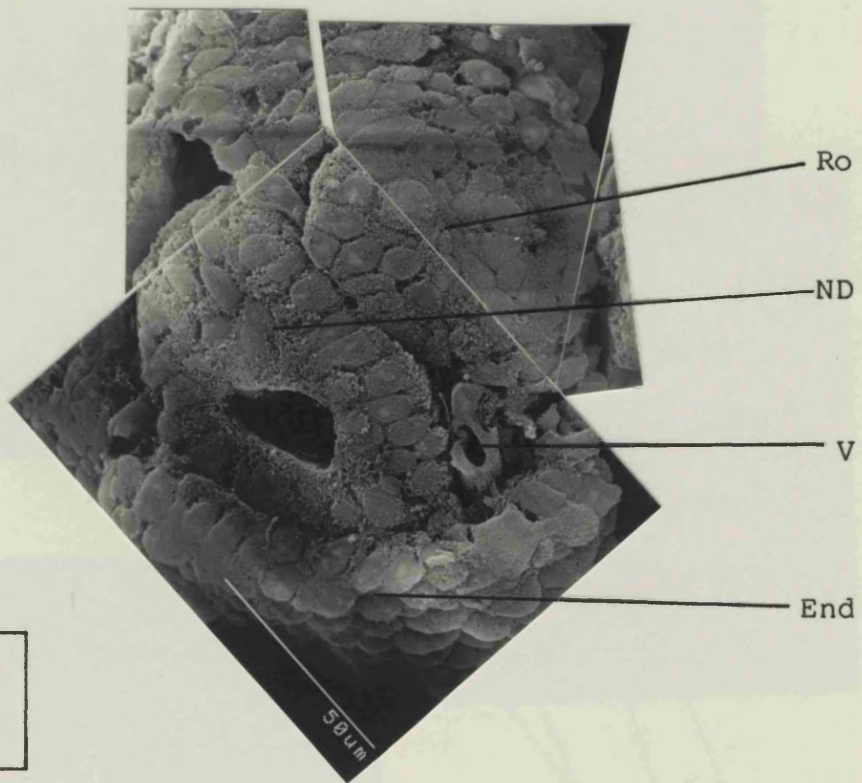
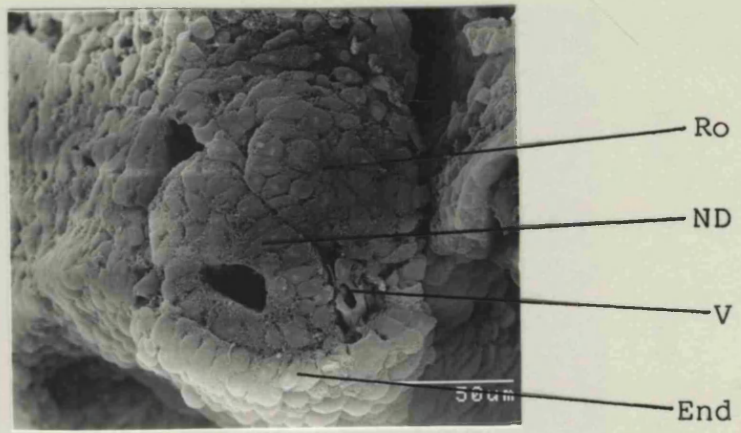


Fig. 35 H&E 11, coronal section stained with S-FC10.2, showing first nephric rudiment bilaterally, X 235



D	M
L	V

Fig. 36 H&H 16-17, mid-A-P level, transverse freeze-fracture SEM; a (top) X600 and b (bottom) X1000

Fig. 37 H&H 18, mid A-P level,
 transverse freeze-fracture;
 a (left) X 100; b (centre) X 1000

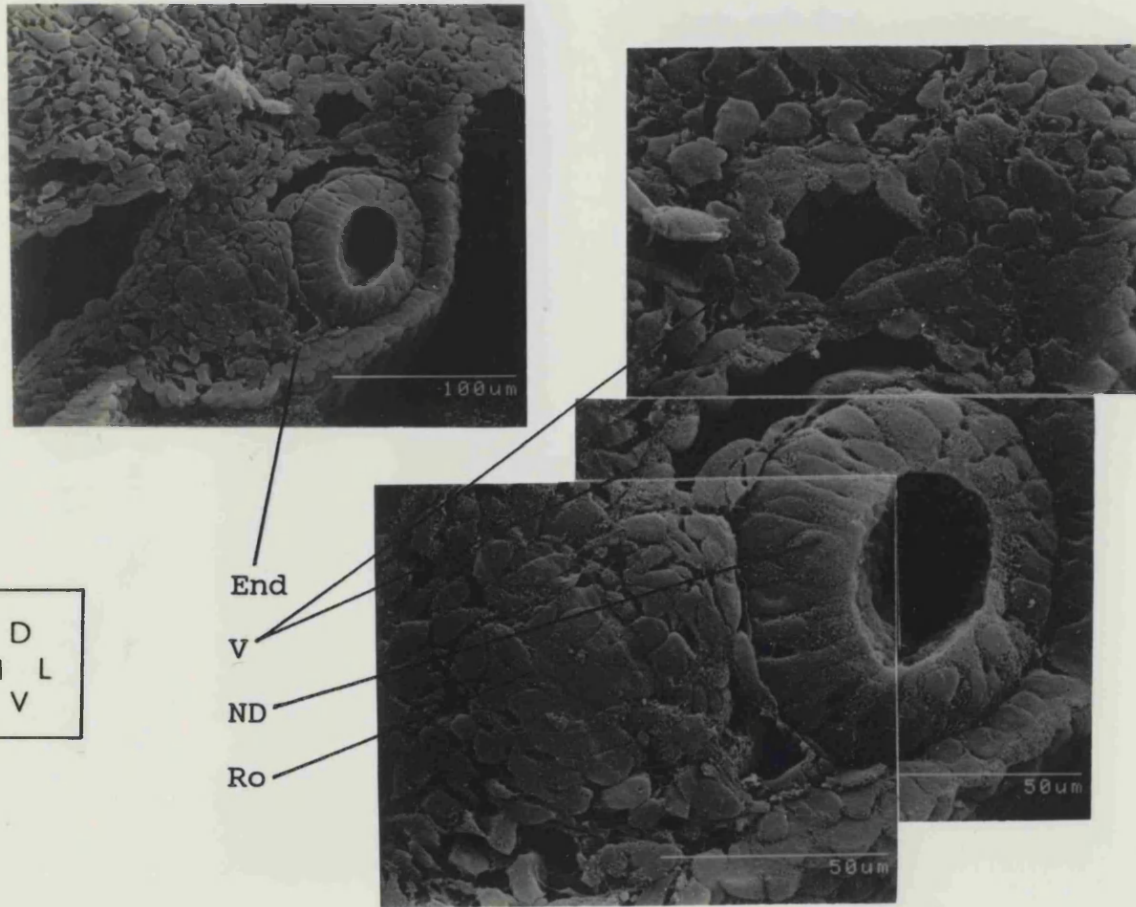
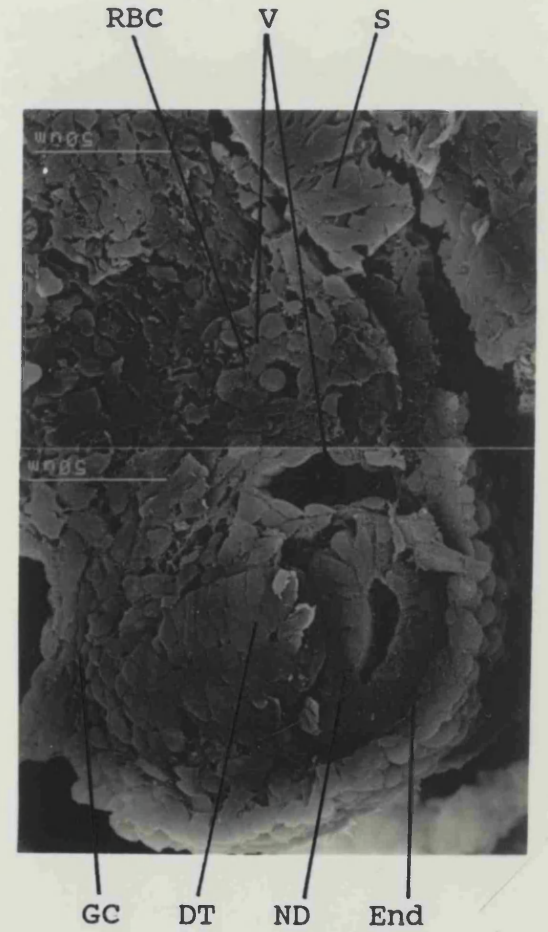
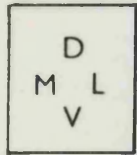
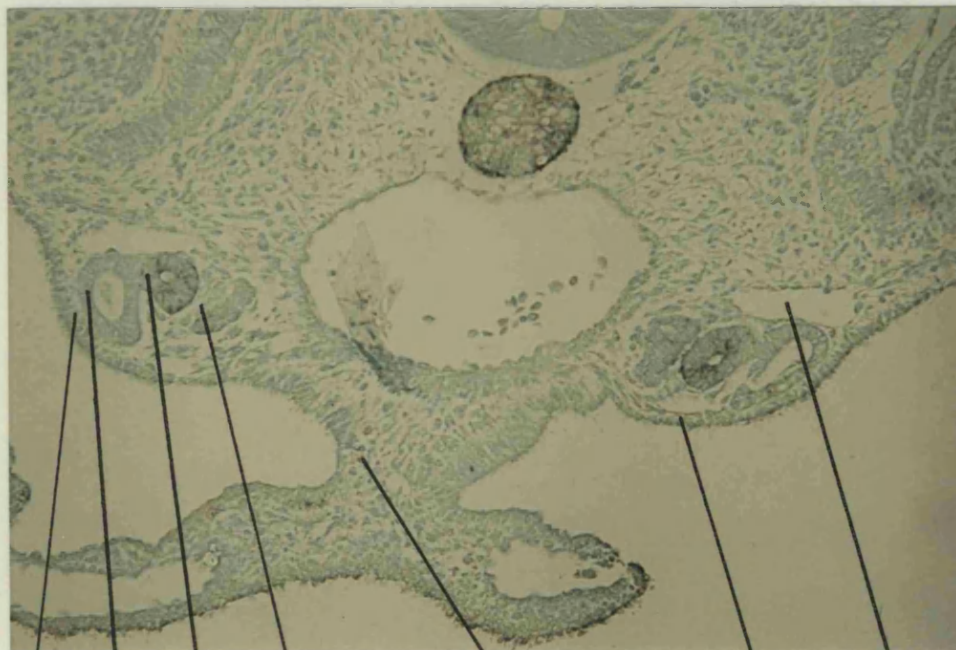


Fig. 38 H&H 18, mid A-P level,
 transverse freeze-fracture, X 600



132





ND

Am

NN

GC

GM

End

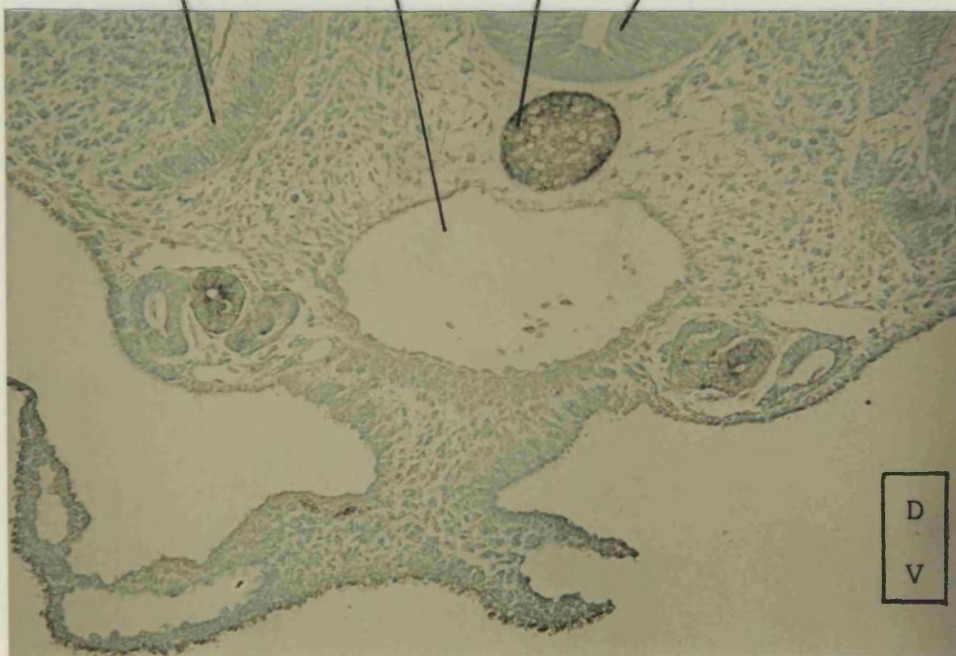
V

S

Ao

NC

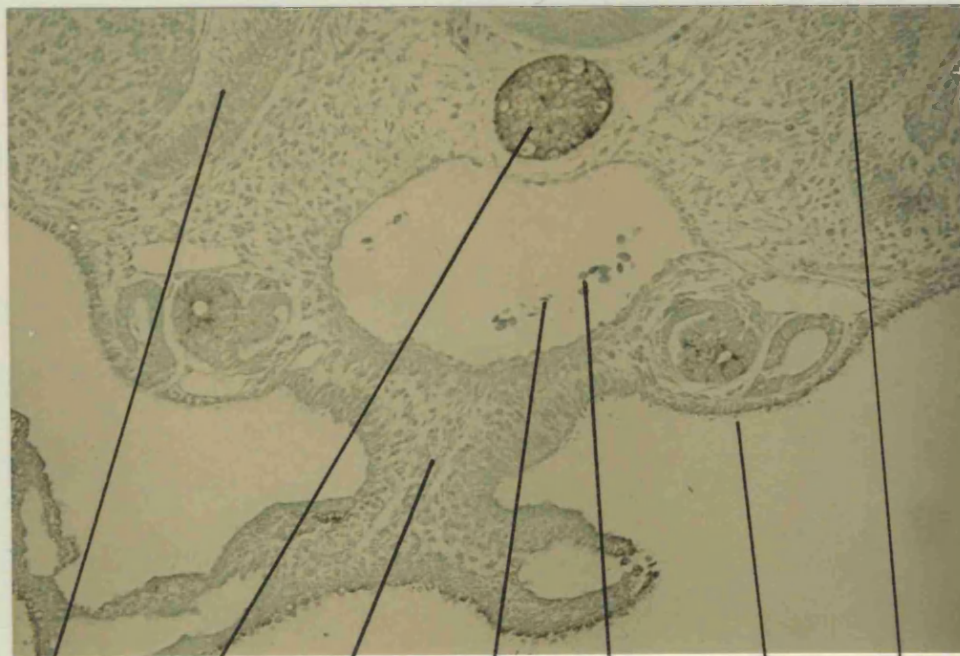
NT



D

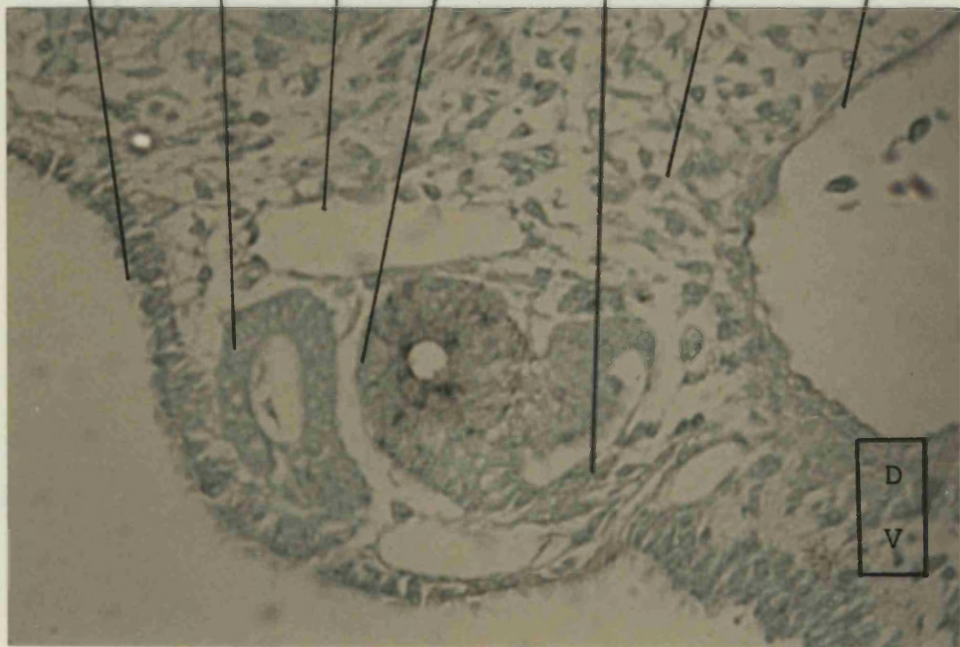
V

Fig. 39a (top) and b (bottom) H&E 18, mid-A-P level, transverse section stained with S-FC10.2, X190



S NC GM Ao RBC End S

End ND V DT GC Mc Ao



D
V

Fig. 40 H&H 18, mid-A-P level, transverse section stained with S-FC10.2, a (top) X190; b (bottom) X480

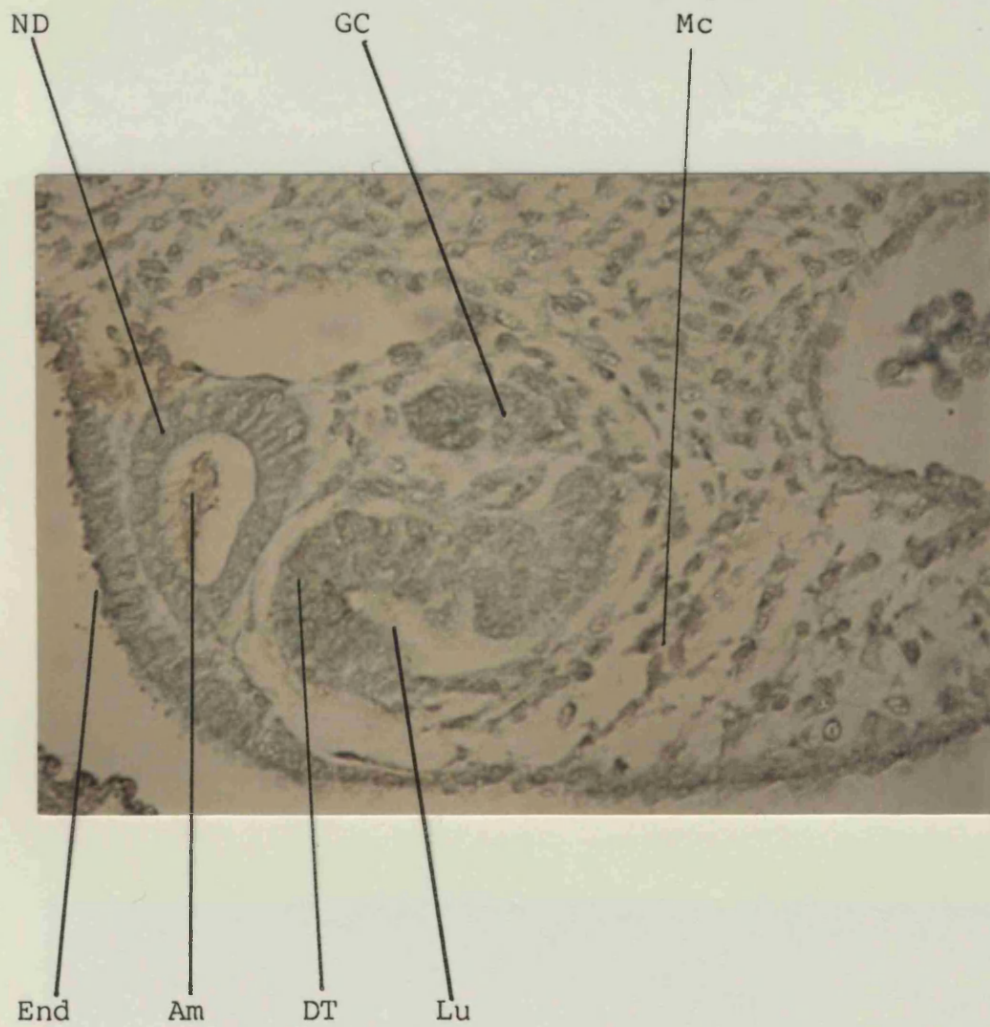


Fig. 41 H&E 18, mid-A-P level, transverse section,
 stained with S-FC10.2, X480

	D	
L	V	M

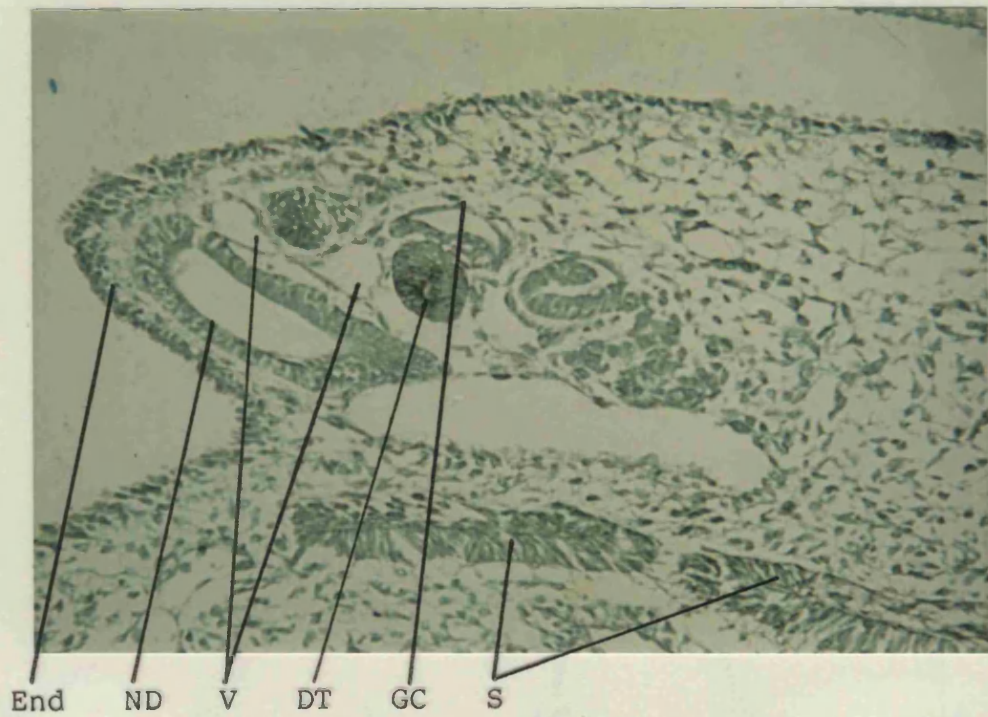


Fig. 42 H&H 20⁻, mid-A-P level, oblique section,
stained with S-FC10.2, X300

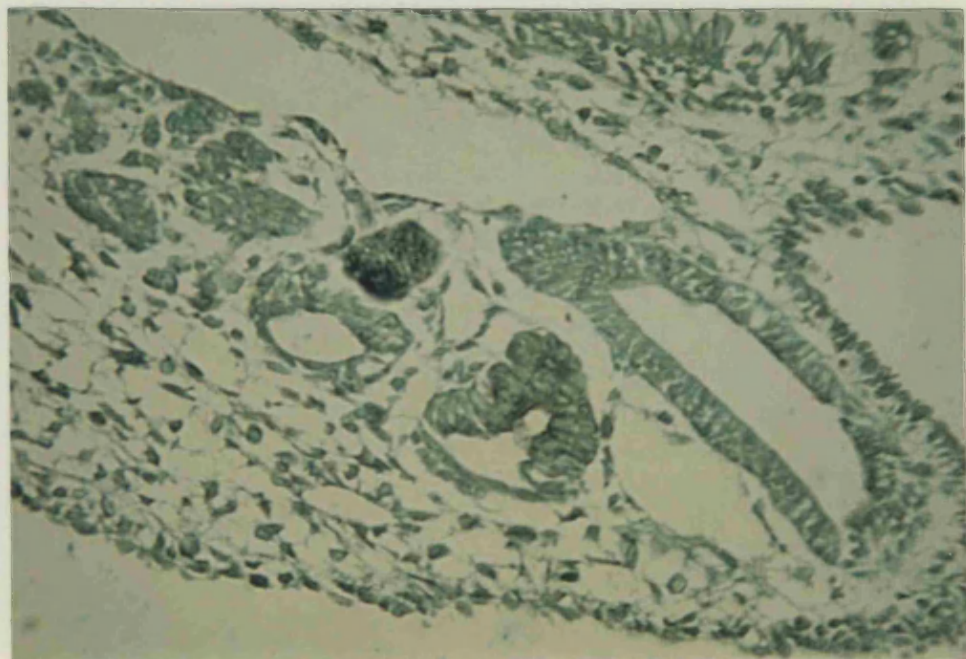
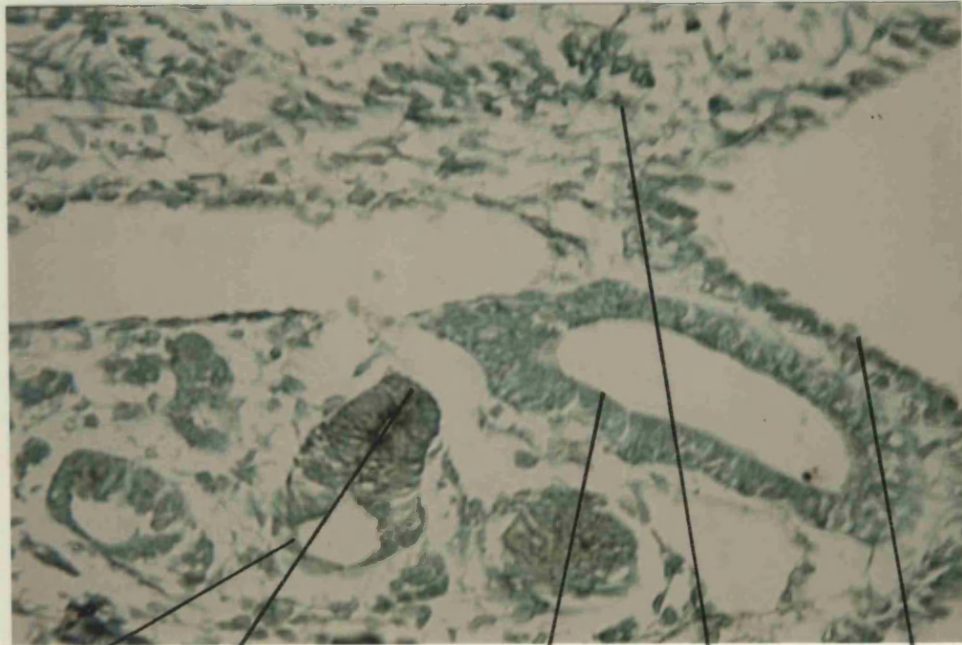


Fig. 43 H&H 20⁻, mid-A-P level, oblique section,
stained with S-FC10.2, X 480; labels as for fig. 42



GC DT V ND PM End

Fig. 44 H&H 20⁻, mid-A-P level, oblique section,
stained with S-FC10.2, X480

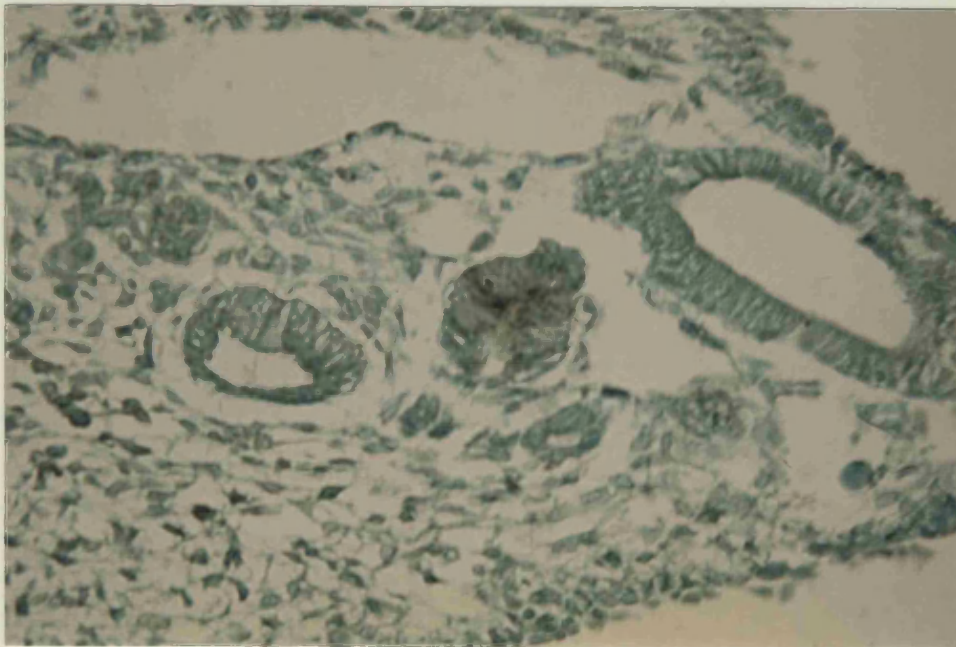
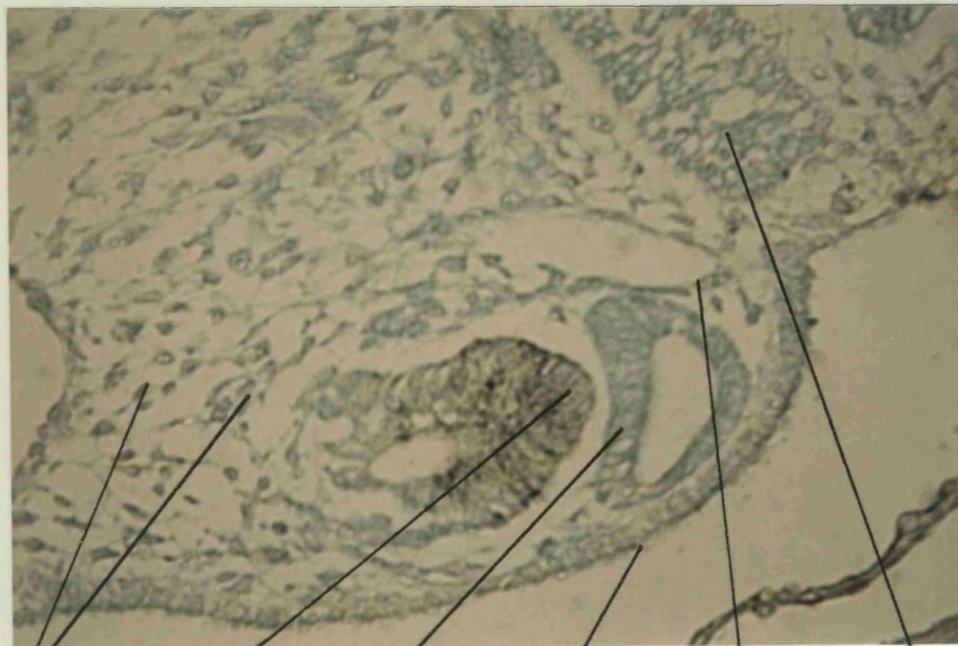


Fig. 45 H&H 20⁻, mid-A-P level, oblique section,
stained with S-FC10.2, X480; labels as for fig. 44



Mc

DT

ND

End

V

S

Fig. 46 H&E 18, mid-A-P level,
transverse section stained with
S-FC10.2, X 480

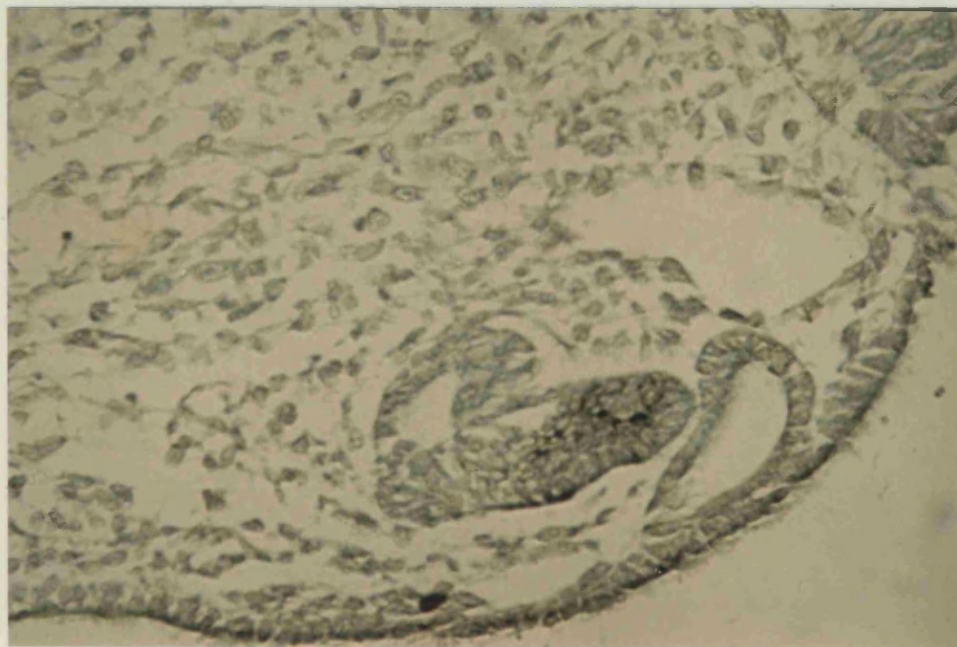
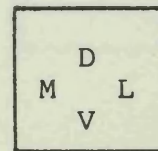


Fig. 47 H&E 18, mid-A-P level, transverse section,
stained with S-FC10.2, X 480; labels as for fig. 46

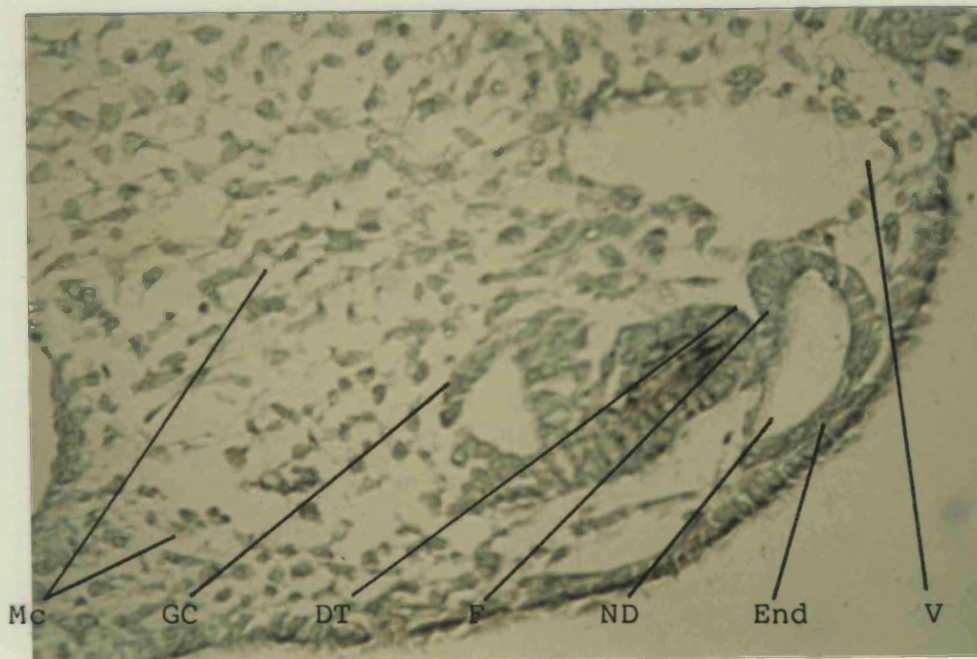


Fig. 48 H&H 18⁺, mid-A-P level,
transverse section stained with
S-FC10.2, X 480

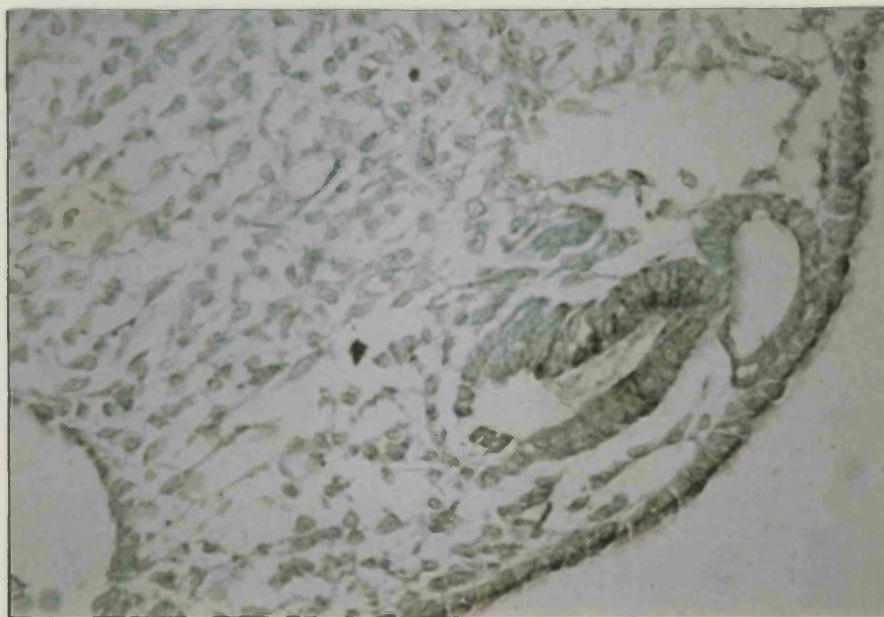
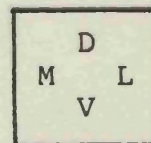


Fig. 49 H&H 18⁺, mid-A-P level, transverse
section stained with S-FC10.2, X 480; labels as
in fig. 48

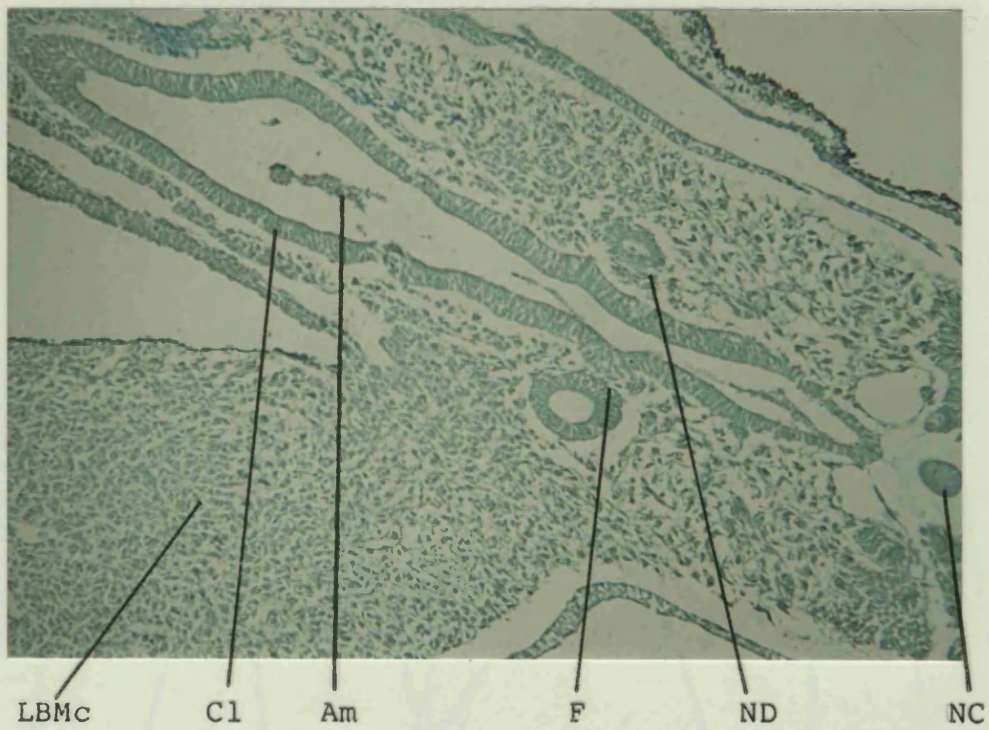


Fig. 50 H&H 18-19, posterior level,
transverse section stained with S-
NB10.3B4, X190

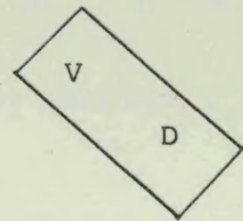


Fig. 51 H&H 18-19, posterior level, transverse
section stained with S-FC10.2, X190; labels as
in fig. 50

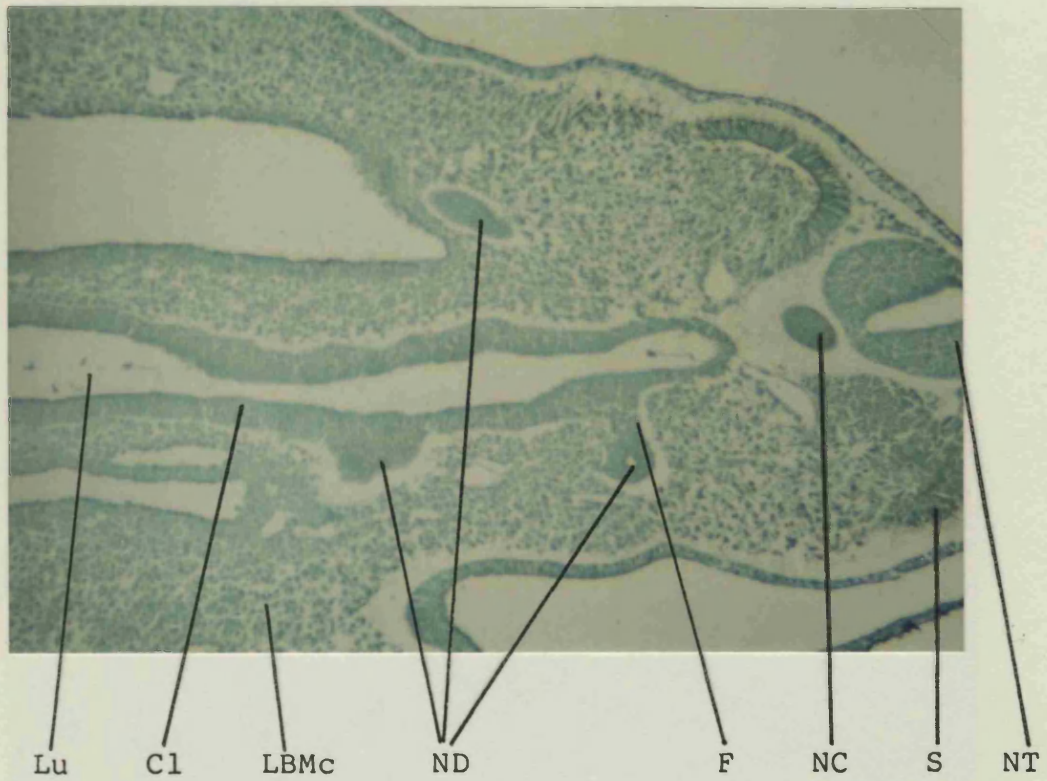


Fig. 52 H&H 18, posterior level, oblique section, stained with SNB10.3B4, X 160

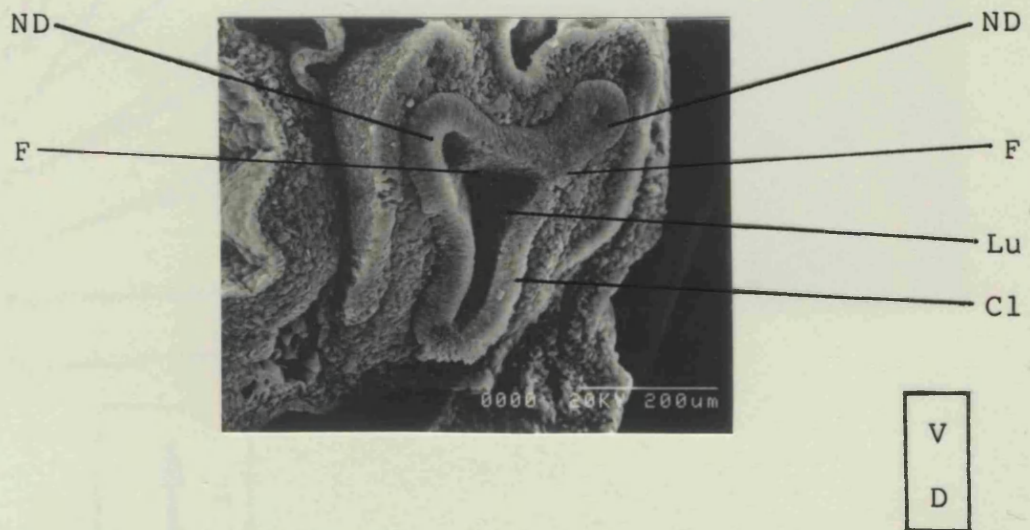


Fig. 53 H&H 16-17, posterior level, transverse freeze-fracture SEM, X150

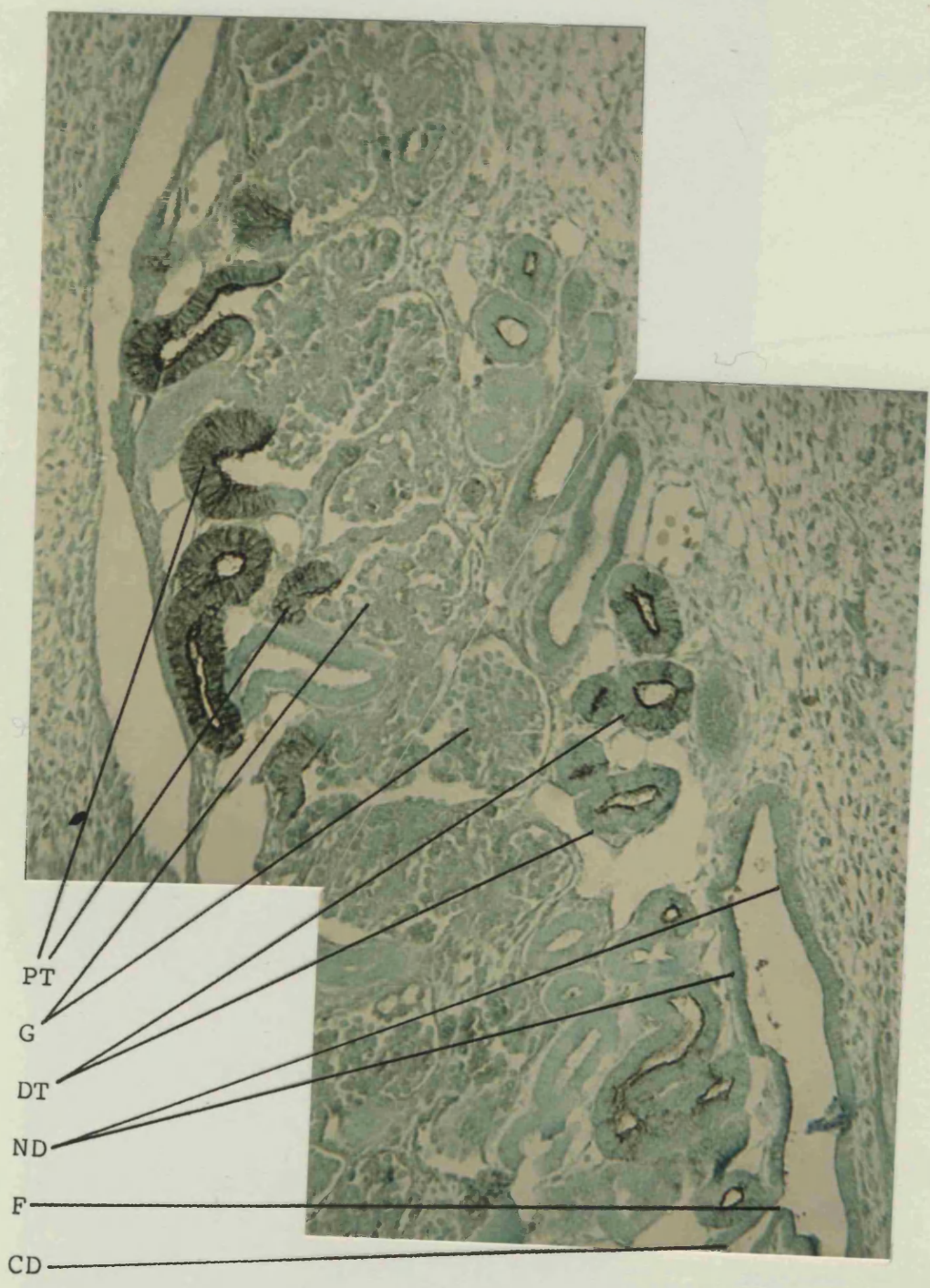


Fig. 55 H&H 27, parasagittal section
stained with S-FC10.2, X 235

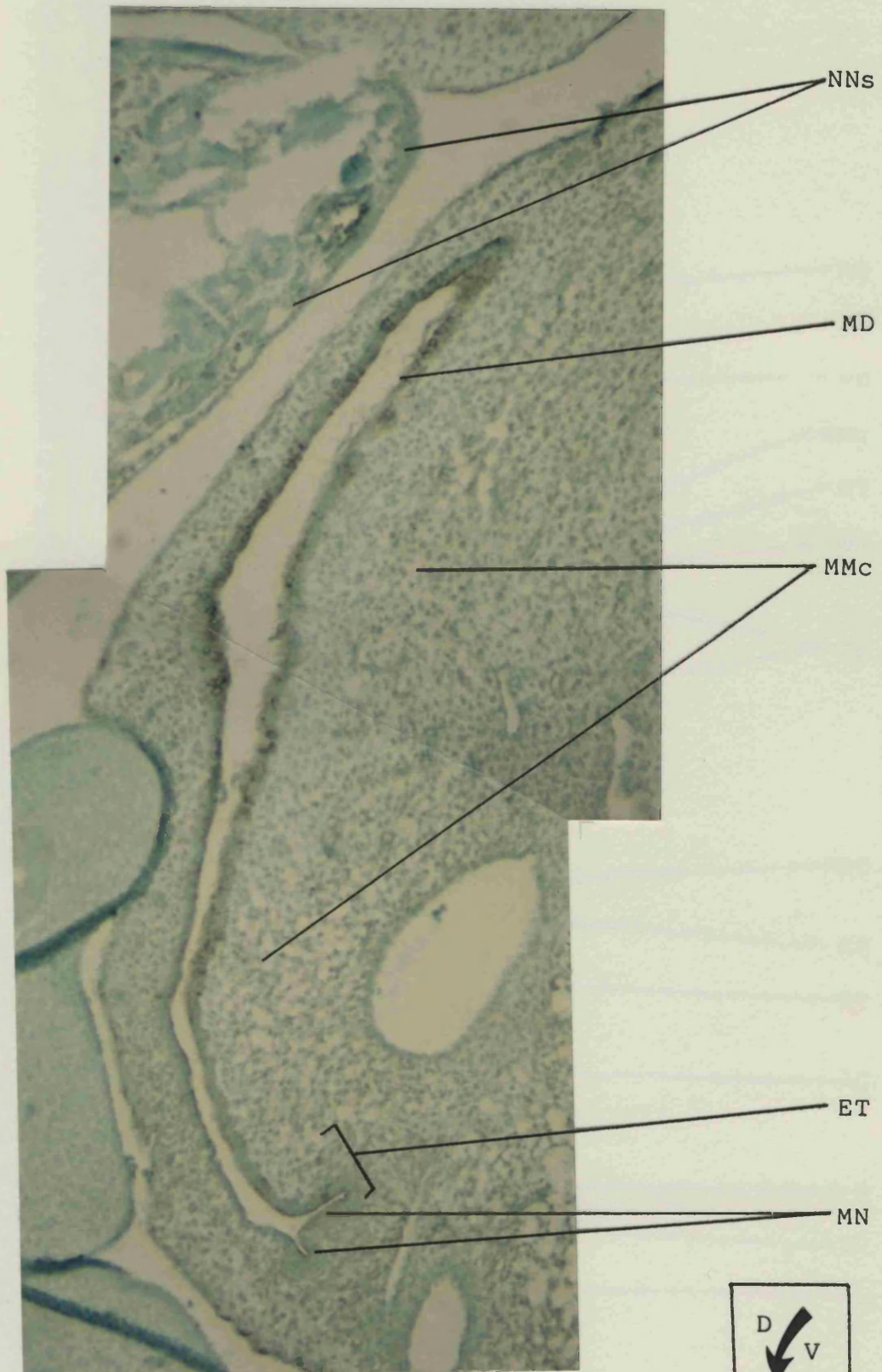


Fig. 56 H&E 27, parasagittal section
stained with S-FC10.2, X190

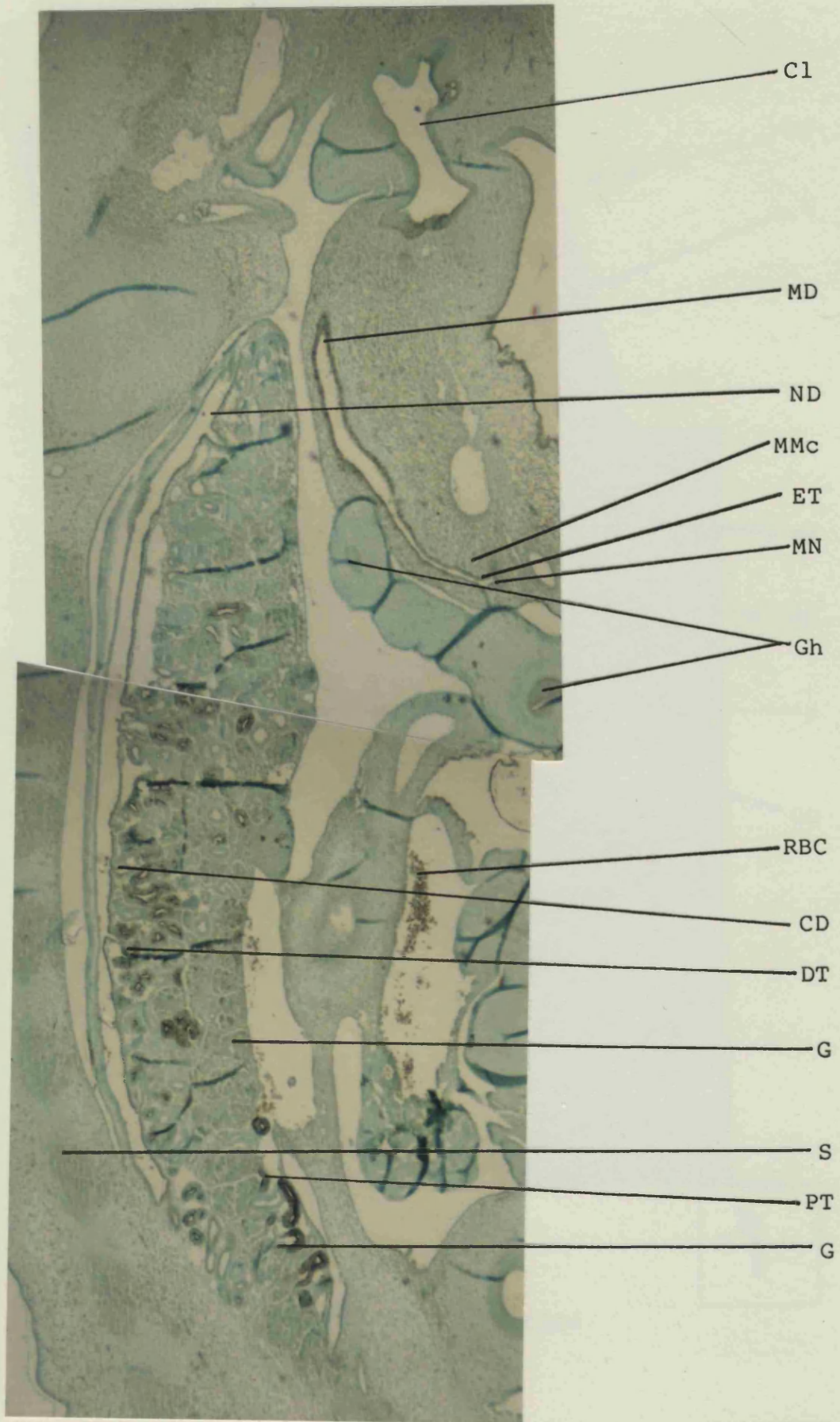
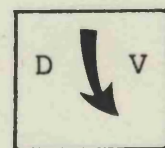


Fig. 57 H&E 27, parasagittal section
 stained with S-FC10.2, X 60



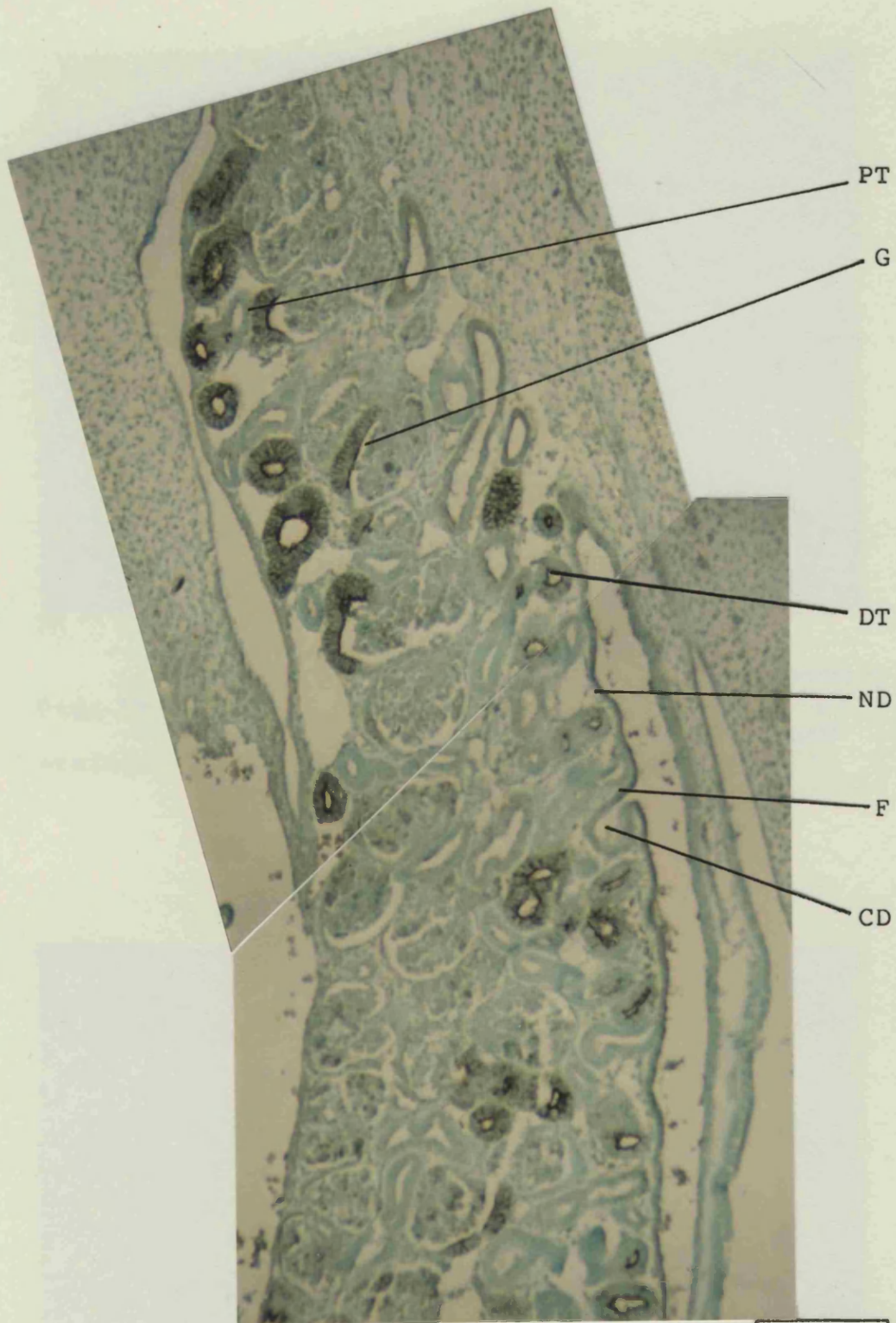
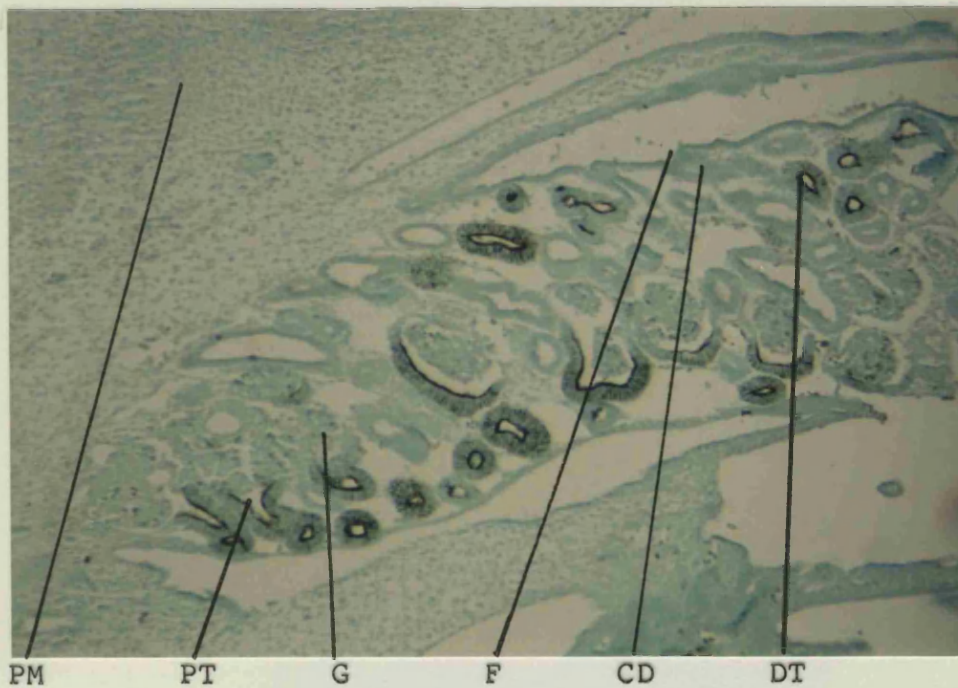
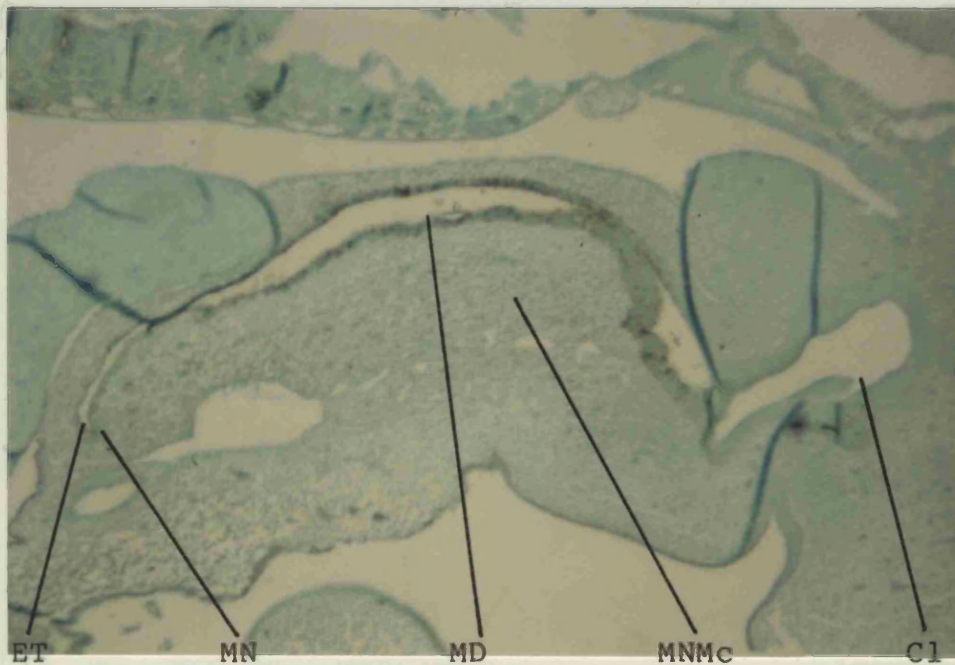
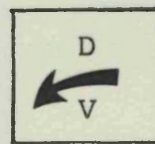


Fig. 58 H&H 27, parasagittal section
stained with S-FC10.2, X160



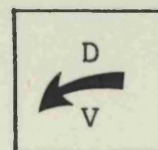
PM PT G F CD DT

Fig. 59 H&E 27, parasagittal section,
stained with S-FC10.2, X 120



ET MN MD MNMc Cl

Fig. 60 H&E 27, parasagittal section,
stained with S-FC10.2, X 80



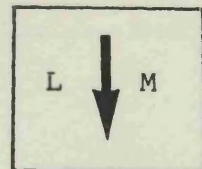
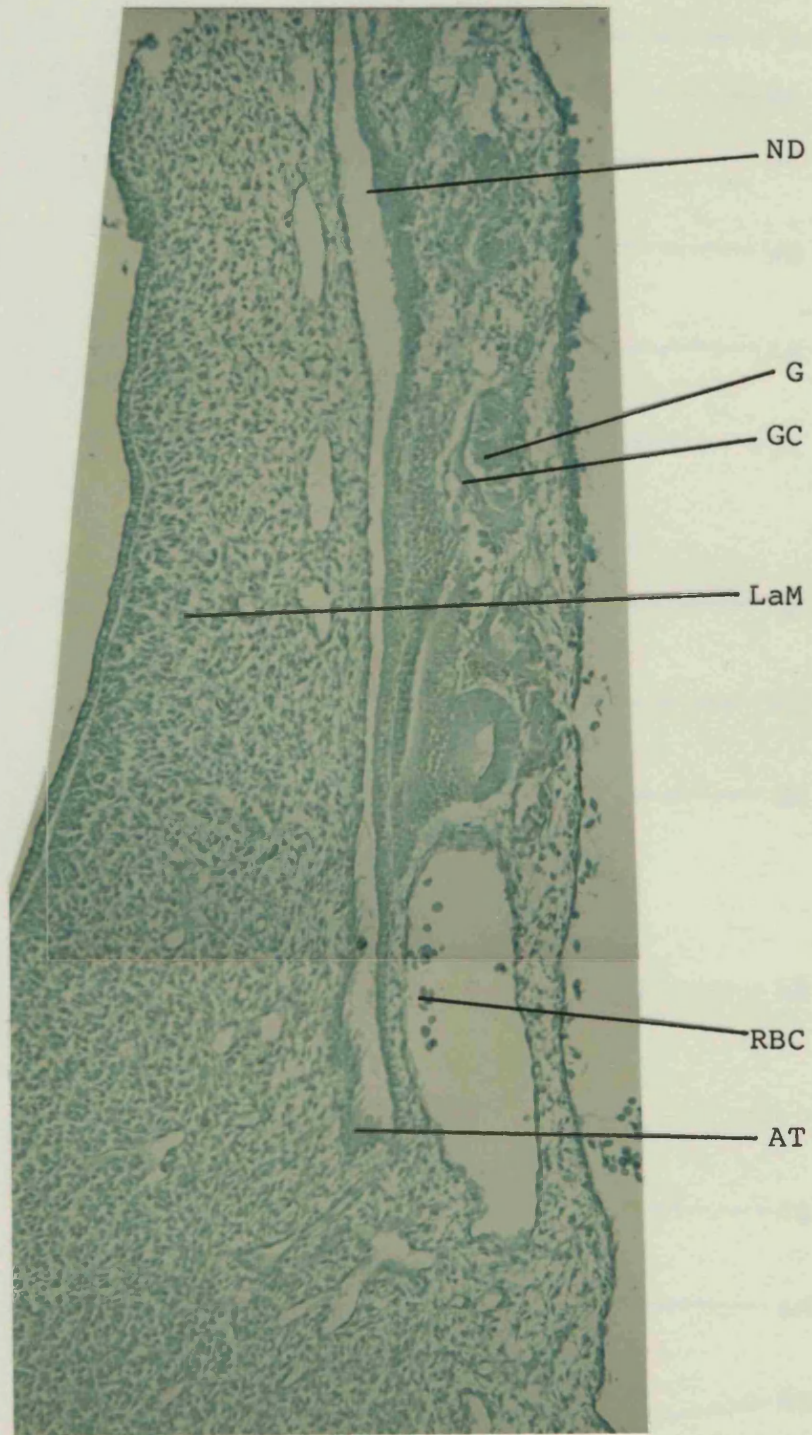


Fig. 61 H&E 20⁻, coronal section,
stained withSNB10.3B4, X190

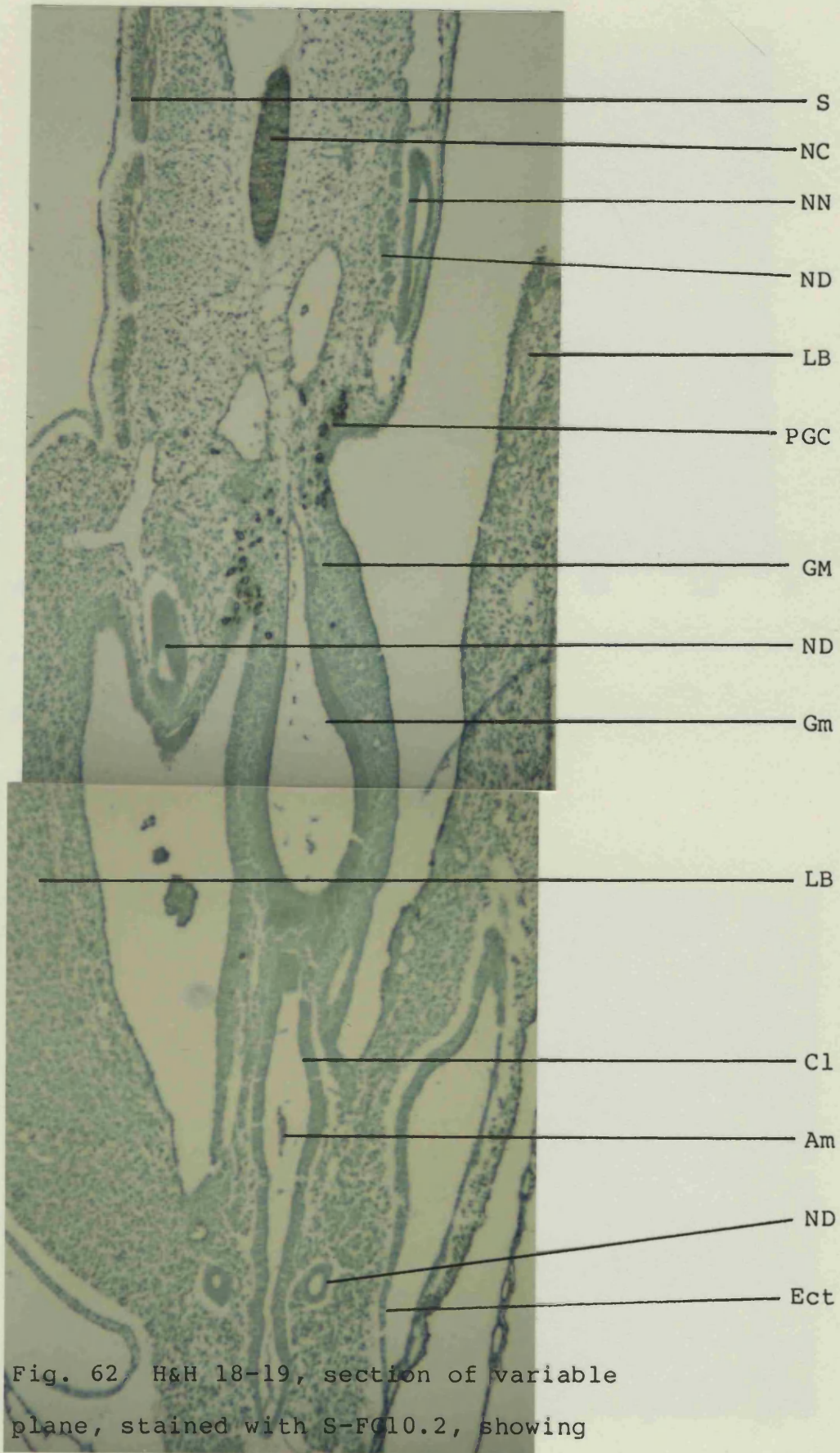


Fig. 62 H&E 18-19, section of variable plane, stained with S-FC10.2, showing tissue relationships, X 120

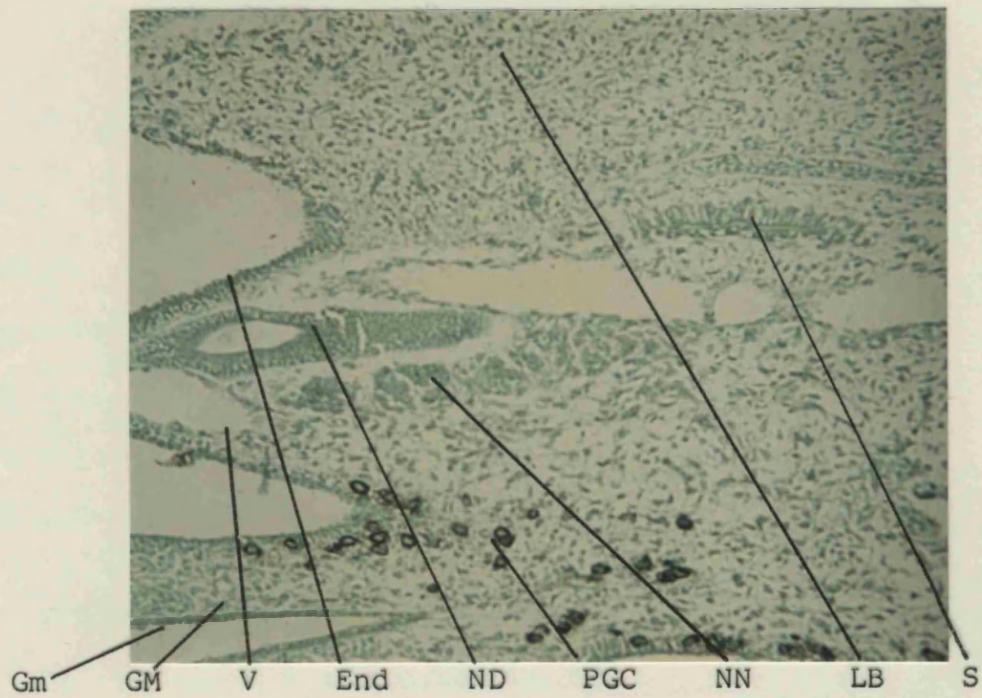


Fig. 63 H&H 18-19, oblique section,
stained with S-FC10.2, X 190

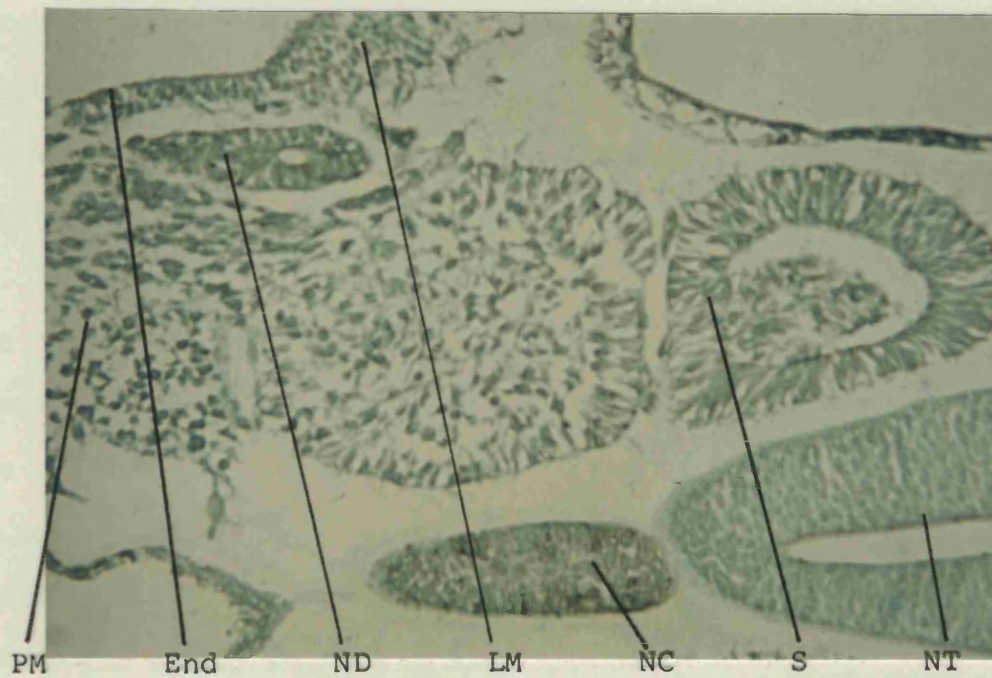


Fig. 64 H&H 15⁺, oblique section, stained with
S-FC10.2, showing tissue relationships, X 300

CHAPTER TWO

DISCUSSION

As described at the beginning of this chapter, the main questions raised by previous nephrogenic analyses at the tissue level concern the nephric tissues and either the events (time and place) or underlying processes (causes) of their morphogenesis. Thus, questions of derivation, sequence and site of formation, and the pattern of epithelialisation of the tissues (1, 2, 4, 6 & 7 on pp. 58-59) directly concern the timing and regions, but not the causes, of particular morphogenetic events. These questions also concern whether events are occurring in situ or distantly and may profitably be expanded to include all nephrogenetic stages. Furthermore, issues of timing and regions should be addressed using morphological approaches.

Therefore at the tissue level, the present study was concerned generally with morphological changes and particularly with the questions previously raised, during development of the nephric and early metanephric tissues (H&H 8-27). The morphological changes observed may be summarised as follows:

- 1 A mesenchymal rudiment first appeared adjacent to somites 5-8 at about H&H 9.
- 2 This, the first nephrogenic rudiment (NR) to appear, extended posteriorly.
- 3 From about H&H 11 a second mesenchymal, nephrogenic rudiment appeared to segregate mediolaterally from the first rudiment and, almost immediately, to segregate anteroposteriorly.
- 4 From H&H 11 the first rudiment began to epithelialise and form a central lumen anteroposteriorly, as the nephric duct rudiment (ND).
- 5 The second rudiment (NN) continued segregating anteroposteriorly from the first, while beginning to epithelialise and itself to divide anteroposteriorly into individual, rosette-shaped nephron rudiments (Ro).

- 6 The duct rudiment and the individual nephron rudiments continued to form in a similar manner until the duct fuses with the lateral wall of the cloaca and simultaneously became fully epithelial with a continuous central lumen at H&H 17⁻.
- 7 The nephron rosettes also extended, though medially and laterally, i.e. perpendicularly in relation to the duct rudiment.
- 8 The metanephric duct rudiment (MD) first appeared as an outgrowth of the anterior wall of the cloaca at H&H 24.
- 9 This rudiment extended anteriorly into the metanephric mesenchyme (MMc), an area slightly ventral to the nephric mesenchyme.
- 10 Almost immediately, the metanephric rudiment epithelialised and formed a central lumen.
- 11 At about H&H 27 when the tip of the metanephric duct had reached an anterior region of the metanephric mesenchyme, it began to bifurcate.
- 12 The metanephrons (MN) began to form as mesenchymal condensations around the metanephric duct bifurcations.
- 13 By H&H 27, the nephric nephrons had elongated considerably and possessed glomeruli. The distal ends of many nephric tubules (DT) could be

seen to have fused with the near-side, lateral wall of the duct, which had widened.

With regard to questions raised by previous nephric analyses concerning events in morphogenesis, the results of the present analysis suggest:

- 1 The first nephric tissue to form is rudimentary. At least the major portion of this tissue develops into the nephric duct, which may therefore be said to have formed before the nephrons.
 - 2 Epithelial differentiation occurs in two phases in both the duct and nephron rudiments. In the first phase, the tissue forms a close lattice. In the second phase, in the presumptive nephron tissue the lattice breaks up into individual rosettes, centred on the 'holes' within the lattice, whereas in the duct tissue, the 'holes' join to form a continuous lumen. Thus in *
 - 3 The first nephric rudiment appeared to form in situ. It could not be resolved from fixed tissue whether progressively posterior formation (extension) of the duct rudiment or what appeared to be segregation of the first nephron rudiment from the duct rudiment were in situ or *
- * the second phase, different adhesion/de-adhesion mechanisms appear to be operating in each tissue.

distant. Hence it was not clear whether the first nephron rudiment (NN) consisted of tissue from the first nephric rudiment (NR). However, the NR at least appeared to play a role in which cellular contact was involved. Formation of the individual-nephron rudiments appeared to be by segregation and hence to involve short-distance migrations. (These may also be involved in epithelialisation.) The presumptive distal tubules of the nephrons appeared to rejoin the nephric duct by short-distance migration.

- 4 Segregation of the first nephron rudiment appeared to be at least approximately segmental, with each segregate corresponding to about one somite-length. Epithelialisation in both the duct and nephron rudiments also appeared to be segmental, with four anteroposteriorly aligned holes in the duct and four nephron rosettes corresponding to one somite-length.
- 5 While there do not appear to be different types of nephric nephron, both nephric and metanephric ducts and nephrons are morphologically distinct.

As discussed in the Introduction to this chapter, the observation of a nephric rudiment forming before either the duct or nephrons and itself developing into the nephric duct supports Jarzem & Meier's (1987) and Jacob & Christ's (1978) SEM

analyses and the earlier work of Balfour and Sedgwick (e.g. 1879), while collectively these data contradict the commonly held view that the duct forms from the 'pro'-nephrons (e.g. fig. 1, p. 24, and Romanoff 1960; see pp. 63-69).

The patterns of epithelialisation and segmentation reported here do not appear to have been previously reported. The present observations were helped considerably by the use of immunohistochemical staining and transverse freeze-fractures in SEM, neither of which appear to have been used previously in the nephric tissues. The branches seen towards the posterior end of the extending nephric duct in the present study, using three-dimensional analysis (stereoscopic SEM) also appear not to have been observed previously, although there have been a relatively large number of SEM analyses of dissected material.

Of the 'primary' morphogenetic processes, it is important to distinguish between those which may be ascertained directly using morphological techniques and those which require experiments. Direct evidence of migration may and should be obtained using dynamic, morphological techniques. Evidence of mitosis and apoptosis is also obtained

morphologically. Induction, however, requires experimentation for its detection.

The standard criteria of induction are: the tissue thought normally to be induced (recipient tissue) should develop normally in the presence of either its normal inductor or a known foreign inductor (embryonic spinal cord is routinely used as an experimental inductor of the metanephrons in vitro). In the absence of such an inductor, the recipient tissue should not develop. Furthermore, the inductor should cause urinary-type development in another mesenchymal tissue and inductive effects must be time-related. Such experiments have demonstrated reciprocal induction between the metanephric duct and metanephrons of the mouse in vitro (see fig. 6, pp. 37-38; pp. 44-45 & 86-87; Saxén 1987).

While it has been accepted from previous ablation and interception experiments that the 'meso'-nephric nephrons are induced by the nephric ducts, all the necessary experiments do not appear to have been carried out. Furthermore, the more anterior 'pro'-nephrons do not appear to have been investigated, apparently because it was considered by the investigators that the earliest nephrons had

formed before the duct. Indirect, morphological evidence from the present study is equally consistent with induction of the earliest and the later nephrons, and therefore provides no reason for distinguishing types of nephric nephrons based on formation. Experiments are therefore needed in all the nephric tissues to ascertain whether induction has occurred and which tissues induce and/or are induced.

It has also been generally accepted from morphological and manipulative investigations that posterior extension of the nephric ducts is achieved by migration. The mesenchymal nature of the caudal part of the nephric duct has frequently been interpreted as indirect evidence of its posterior migration (e.g. Jacob & Christ 1978; Jarzem & Meier 1987). However the results of the present investigation of the earlier stages suggest that a mesenchymal appearance might equally be a characteristic of formation in situ and a semi-epithelial or epithelial appearance might be associated with formation at-a-distance, if only a short distance.

In 1976, Martin formed six chimaeras in which the anterior half of a quail embryo, containing the

nephric ducts including their caudal tips, was grafted onto the posterior half of a chick embryo, in which the duct had not yet formed. In these it was found that the quail nephric duct migrated to the chick cloaca and, furthermore, contributed cells both to the cloaca and the metanephric duct. While this appears to be the only evidence of nephric-duct migration in avians, the possibility that the quail cells divided at a faster rate than the neighbouring chick cells does not appear to have been excluded. (See pp. 82&84).

Projections from single cells are usually associated with their migration because cells migrating in vitro characteristically form lamellipodia and filopodia (e.g. see Bellairs et al 1982). An example of projections from migrating cells in intact embryos is that of nerve axons - themselves as well as their own local projections, including branches (e.g. Becker & Cook 1988). It might therefore seem plausible for the multicellular branches seen in the nephric duct to be associated with its migration.

An alternative role for these branches, however, would be in epithelialisation. The nephric duct branches are not terminal bifurcations, as are

those in the metanephric duct and other tissues which form by epithelial-mesenchymal transformation and 'branching morphogenesis', e.g. lung and salivary gland (figs. 4a&b, pp. 28&29). This difference could be due to the earlier embryonic stages at which the nephric duct is epithelialising. Furthermore, it is clear that the branches are not terminal, as in the case of the epithelial tip of the metanephric duct, but rather are situated behind the tip of the nephric duct, towards the epithelial part.

The mode and mechanism of metanephric duct extension from the cloaca to the metanephric mesenchyme, however, appears not to have been investigated. Yet because it extends in approximately an opposite direction and through a different region of mesenchyme than the nephric duct, there are interesting comparisons to be made between it and the nephric duct, which may help to elucidate the means and mechanisms of both.

The question must be addressed as to how extension of the nephric duct could be achieved without active migration of the caudal end. For extension to occur, processes tending towards regression must be offset by those tending towards

extension. We do not know how the caudal end retains a mesenchymal appearance - why does it not form an epithelium more immediately as do the nephrons at later stages? and why does it not form a lumen more immediately as the metanephric duct does? Probably it is due to the surrounding tissues being at an earlier, i.e. more mesenchymal, stage during nephric duct formation.

Perhaps influences from the surrounding tissues tend to inhibit extension of the duct. This could happen perhaps through degradation at the tip and be a cause of its sometimes more jagged appearance than that of the rest of the mesenchymal part of the duct rudiment. An association between the posterior shape of the nephric duct and cellular death could be investigated and compared with that known to occur in the morphogenesis of other extremities, e.g. shaping of the phalanges (Saunders & Fallon 1966).

The most obvious candidates for non-migratory extension might be epithelialisation together with the formation of a central lumen, and formation of new duct material. In the former, movement of the tip could be brought about passively by cellular elongation in the epithelial region and hence 'pushing' from behind. In the latter, movement might

only be relative, as the formation of the tip in situ though progressively posteriorly. Degradation of the tip, mentioned above, could be an inhibitor of formation in situ, while movement through 'pushing' or 'pulling' (the latter being brought about by active migration of the duct cells) could be counteracted by inhibitors of epithelialisation or migration, respectively. Thus, it must be considered whether epithelialisation, induction, migration, proliferation and degeneration interact to cause extension. (See also Chapter 4).

Segmentation and epithelialisation have both been investigated in the metanephrons, using the mouse in vitro model. Segmentation in mature metanephrons, however, is proximodistal rather than anteroposterior. Any relationship between the two types of segmentation should be investigated at the cellular and sub-cellular levels. Thus it might be found for example that the nephrons form as 'buds' from an anteroposteriorly segmented rudiment and in doing so the segmental pattern within them is rotated to become mediolateral.

FUTURE ANALYSES

It will be apparent from the preceding discussion that a morphological approach to investigating processes on the tissue level can raise more questions than it answers. This is essential for assessing and designing experimental analyses where, as in the nephric tissues, inconsistencies and controversies arising from previous analyses suggest that various, interacting processes are involved in morphogenesis. While it is hoped that the present study has contributed to formulating and addressing nephrogenetic questions, by using SEM, LM and immunohistochemical staining comparatively, it appears that to resolve some of the morphological issues a more dynamic, though still morphological, approach will be necessary. This could involve labelling and following the tissues, as has been done in Xenopus (Lynch & Fraser, 1990). (See Chapter 4, pp. 207-213).

Future, morphological analyses on the tissue-level might also include measuring the lengths of duct rudiment behind the somites and the length of the mesenchymal part of the extending duct as a proportion total duct length and in relation to

length of the nephron rudiments. Previous measurements appear only to have been of the absolute length of duct behind the somites, which has been found to be variable. Proportion of total duct-length could give an indication of a possible tissue-level, biophysical mechanism and nephron-length could indicate a segmental mechanism of duct extension. The speed of extension of nephric and metanephric ducts might also be compared, to assess whether an epithelial duct extends more quickly.

Tissue-level questions should also be further addressed using cellular analyses, for example cell-counting to determine whether epithelialisation contributes to extension, mitotic and apoptotic counting to determine the contributions if any of proliferation and degeneration (see Chapter 4). Tissue-level questions which need to be addressed on a sub-cellular level include the correlation of certain macromolecules with morphogenetic events (see Chapter 3).

CHAPTER THREE

SUB-CELLULAR LEVEL

NEPHROGENESIS

CHAPTER THREE

INTRODUCTION

The majority of recent developmental analyses in both animals and plants has been at the sub-cellular level, while the greatest significance of the sub-cellular level has probably been its association during the last two decades with particular macromolecules. Thus, while in the 1950's transmission electron-microscopy (TEM) initiated modern sub-cellular anatomical analyses, the more recent development of immunological and genetic-recombinant techniques has caused 'sub-cellular' to become almost synonymous with (macro-) 'molecular'.

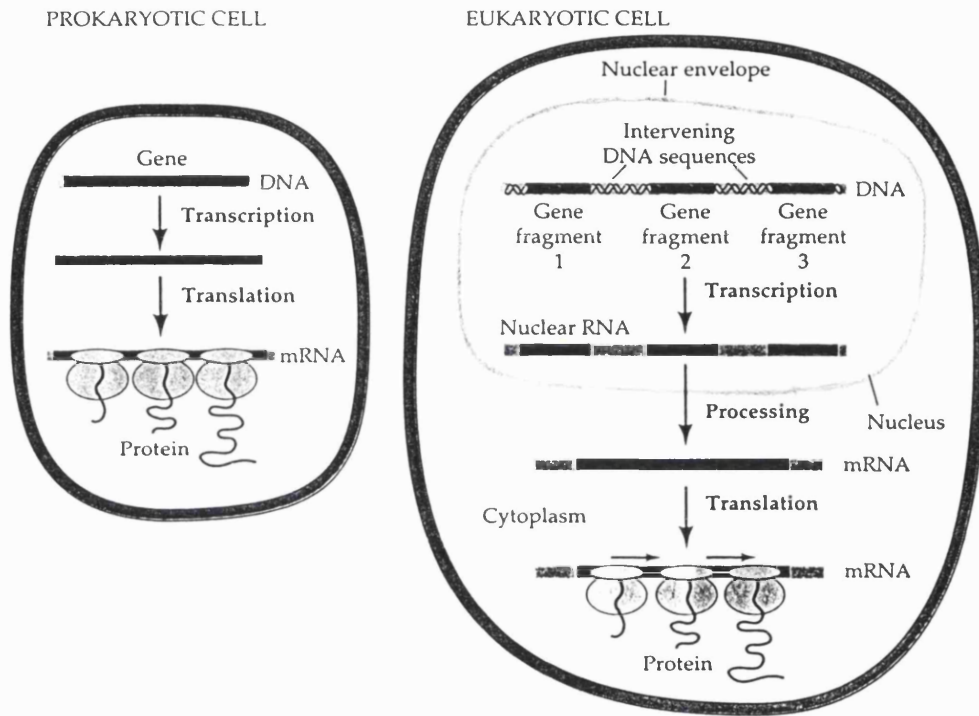


Fig. 65 Summary of gene expression. In prokaryotes (A), the DNA coding regions are co-linear with the protein produced. In eukaryotes (B), the genes are discontinuous along the DNA, which is also separated from the cytoplasm by a nuclear membrane. (From Gilbert 1991).

The results of such molecular analyses have supported the idea that genes (DNA) control patterned development through transcription and translation products (fig 65). Through protein synthesis, mRNA's mediate adhesion and communication within and between cells and/or the micro-environment (see Kerr et al 1992), and hence underly morphogenetic processes (see Akam & Gerhart, 1992; Maniatis & Weintraub 1992; Williams & Hogan 1992). Genes are controlled by genetic and epigenetic factors (e.g. Karin 1990; 1991). Foremost among theories of pattern formation is Wolpert's (1969; 1989), which postulates the existence of 'morphogens', particular molecules instrumental in morphogenesis, e.g. retinoic acid (Thaller & Eichele 1992; Bryant & Gardiner 1992; Tickle 1991; Osmond et al 1991).

In conjunction with sub-cellular morphology, two approaches appear to show the greatest potential for contributing to a general understanding of morphogenesis. The approach of most current developmental research is biochemical, as described above and based on multi-functional macromolecules. There is considerable evidence that many developmental macromolecules are multi-functional, i.e. that interactions may be centred on them (e.g. Schweighoffer & Shaw 1992; Sanders 1989).

For example, growth factors are involved in axis formation (Stern et al 1992), mesoderm induction (e.g. Slack 1991; Ziv et al 1992; Bolce et al 1992), and later inductions, e.g. in 'branching morphogenesis' (fig. 4a, p. 28; e.g. Robinson et al 1991; Coleman & Daniel 1990), as well as in mitotic regulation. This might be due to their controlling synthesis of ECM molecules and receptors (Rogers et al 1992) and interactions with adhesion molecules (Thiery & Boyer 1992; see pp. 173-175). Many known growth factors are related either to fibroblast growth factor (FGF) or transforming growth factor β (TGF β) (Stern 1992b). Onco-developmental antigens and proto-oncogenes also appear to be multi-functional (pp. 170-181).

The second approach is biophysical. Although infrequently used in recent analyses (see Janmey 1991; Ingber 1991), a biophysical approach appears necessary for addressing several morphogenetic issues. For example, at the cellular and tissue-levels, aspects of mechanics are undoubtedly important to migration and perhaps to other processes of tissue-shape change, i.e. mitosis, apoptosis and induction. Investigating biophysical phenomena on the sub-cellular level might help to demonstrate their specificity and thus to promote further investigations at all levels. Subsequently, tissue-level, biophysical analyses might

facilitate an understanding of the roles of tissue-level, biochemical phenomena.

There are probably four reasons for the prevalence of biochemical over biophysical ideas generally in biomedical research. Firstly, biochemicals comprising common 'building blocks' arranged in degrees of varying complexity, according to function, may be readily envisaged - therefore a coherent yet divisible hypothesis may be formed; secondly, as a result, it is easier to develop chemically based techniques and to test the hypothesis; thirdly and partly as a consequence, it is easier to develop chemically based therapeutics; and fourthly, there has been - and promises to be more - progress within this framework.

Sub-cellular biophysical approaches could be developed further from in vitro models of migration of animal cells (for examples, see Bellairs et al 1982, Heaysman et al 1987, Trinkaus 1984 and Bard 1992b); analyses of 'traction' in fixed tissue (e.g. Bard 1990) and in vitro; work involving the effects of microgravity on animal and plant cells (see Bellairs 1993, in press) and analyses of plant morphogenesis (see p. 48). Three-dimensional

computer models (e.g. Selker et al 1992) would be helpful in developing biophysical analyses.

At the sub-cellular level, the present study has a biochemical approach. The present chapter describes the expression pattern of a carbohydrate antigen, S-FC10.2, during nephric and early metanephric development and is closely associated with the tissue-level analysis in the previous chapter.

FC10.2 is of morphogenetic interest for several reasons. Firstly, it is onco-developmental (Feizi 1985): the antibody is raised against a teratocarcinoma cell-line and reacts with fetal tissue (Loveless et al 1990). Secondly, it is found in both mammals and chicks, with the antibody being raised in mouse against human tissue (Loveless et al 1990). Thirdly, it belongs to a class of carbohydrates, the poly-N-acetyllactosamines (fig. 66). These are the antigenic determinants of cell-surface glycoproteins (Feizi & Childs 1987), many of which are expressed with various, characteristic, temporal and tissue-specific patterns during early embryogenesis in the chick, mouse and human embryo; they also form central components of certain, adult human, blood group

Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc/GlcNAc β 1-

SA-Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc/GlcNAc β 1-

Gal α 1-3Gal β -
|
+/- (Fuc α 1-2)

Fig. 66 Oligosaccharide sequences of antigens recognised by FC10.2 (top), S-FC10.2 (middle) and NB10.3B4 (bottom). **Gal** galactose; **Glc** glucose; **GlcNAc** N-acetylglucosamine; **SA** sialic acid; **Fuc** fucose; +/- may be present but does not affect immunoreactivity. (From Loveless et al 1990).

antigens (Thorpe et al 1988; Loveless et al 1990). FC10.2 is of nephrogenetic interest because the nephric tissues express its sialylated form (S-FC10.2) from early stages of their formation (Loveless et al 1990).

Therefore, FC10.2 might be involved in changes in morphology common to both normal development and carcinogenesis, as well as in certain homeostatic or pathological functions, in adults and/or in different amniotes. FC10.2 might also be involved in certain changes specific to nephric morphogenesis, at least in the chick embryo. Furthermore, it may be masked by sialic acid (Loveless et al 1990) or modified by glycosylation, perhaps to control its expression (for glycosylation of proteins, see Feizi 1992; Hart 1992; Ploegh & Neefjes 1990; for lipid-modification of proteins, see Cox & Der 1992; Deschenes et al 1990). A possible morphogenetic role for FC10.2 would be in adhesion, through binding to cell-adhesion molecules and/or lectins (Loveless et al 1990; Feizi 1991). Possible developmental roles of endogenous animal lectins have been reviewed by Zalik (1991); see Pusztai (1991) for the effects of plant lectins on animals.

The concept of adhesion in development is versatile (e.g. Schweighoffer & Shaw 1992). While most of our knowledge of adhesion is based on in vitro analyses, it has been associated with control of differentiation (e.g.

Bellairs et al 1978) and migration (see Bellairs et al 1982 and Heaysman et al 1987). It may also be thought of as both biophysically and biochemically based. Furthermore, the expression patterns and isolation of specific molecules with adhesive properties in vitro has given impetus to the idea that cell-surface adhesion underlies morphogenetic processes. These molecules are the glycoprotein, 'cell-adhesion molecules' (CAM's) and 'substrate-adhesion molecules' (SAM's). CAM's include the calcium-dependent cadherins (e.g. Hynes 1992; Takeichi 1988), e.g. E-cadherin, initially found in liver and also known as L-CAM or uvomorulin, and N-cadherin, also known as A-CAM. N-CAM (initially found on neurons) also has a polysialylated form (Rutishauser 1992).

CAM's are thought to be attached to the cell-surface and involved in the binding of cells within developing tissues (e.g. Rutishauser et al 1988; chick: Thiery et al 1982, 1984). Thiery et al 1982 appears to have been the first morphogenetic analysis of a cell-surface molecule in the early chick nephrogenic tissues (see Hatta et al 1987). In that analysis, it was found that L- and N-CAM are alternately expressed. Thus, the nephric duct expressed L-CAM from H&H 12⁻ (15 somites) and

apparently until after it had fused with the cloaca (31 somites; H&H 17). The presumptive nephrons initially expressed N-CAM but not L-CAM, until from H&H 25, when the nephric tubules had fused with the nephric duct, they expressed L-CAM instead of N-CAM. The metanephric duct also expressed L-CAM; as did the metanephrons after they had formed. Apparently the metanephrons did not express either CAM at the beginning of the morphogenesis.

SAM's appear to be distinct from CAM's. SAM's may be either attached to the cell-surface or integral to the extracellular matrix (ECM) and appear to be involved in the movement of cells relative to each other. SAM's are glycoproteins and include fibronectin (FN; see Duband et al 1990), laminin (LN; see Martin & Timpl 1991) and vitronectin (VN; see Preissner 1991). Other SAM's (see Hortsch & Goodman 1991) are the collagens, tenascin and proteoglycans (Ruoslahti 1988; Wight et al 1992). SAM's are widespread during morphogenesis, including 'branching morphogenesis' (mammalian examples: Roman et al 1991, lung FN; Schuger et al 1991, lung LN; Simo et al 1991, intestinal LN).

SAM's and CAM's are thought to interact and to be closely associated with growth factors (e.g.

Thiery et al 1984; Vainio et al 1992) and differentiation antigens (e.g. Loveless et al 1990) during morphogenesis. The receptors for SAM's are transmembranous integrins (see Hynes 1987; Mecham 1991; Bronner-Fraser et al 1992; Drake et al 1991; for 'disintegrins', see Blobel & White 1992). Some cell-surface locations of molecular function in epithelia are shown in fig. 67; also see Gumbiner (1990).

Transduction of signals across the cell membrane is necessary for an intracellular response, for example by actin filaments within the cytoplasm resulting in movement (see Pollard & Goldman 1992), and for other morphogenetic processes (Damsky & Werb 1992; Janmey 1992; Ingber 1992). In addition to the integrins, the receptor-like protein tyrosine phosphatases (PTPases) have extracellular and intracellular domains (e.g. Tonks 1990) and may be involved in adhesion (e.g. Shattil & Brugge 1991). The widespread existence of protein tyrosine kinases suggests a reciprocal control mechanism (e.g. Pallen et al 1992; Shaw & Thomas 1991). The genes encoding these kinases may be proto-oncogenes (e.g. Ahn et al 1992; Maness et al 1986). Oncogenicity of v-src-transformed avian limb-bud cells may be regulated by the embryonic micro-environment, while PTPases might function as 'anti-oncogenes' (Stoker et al 1990a). In eukaryotic cells, genetic transcription is thought to be

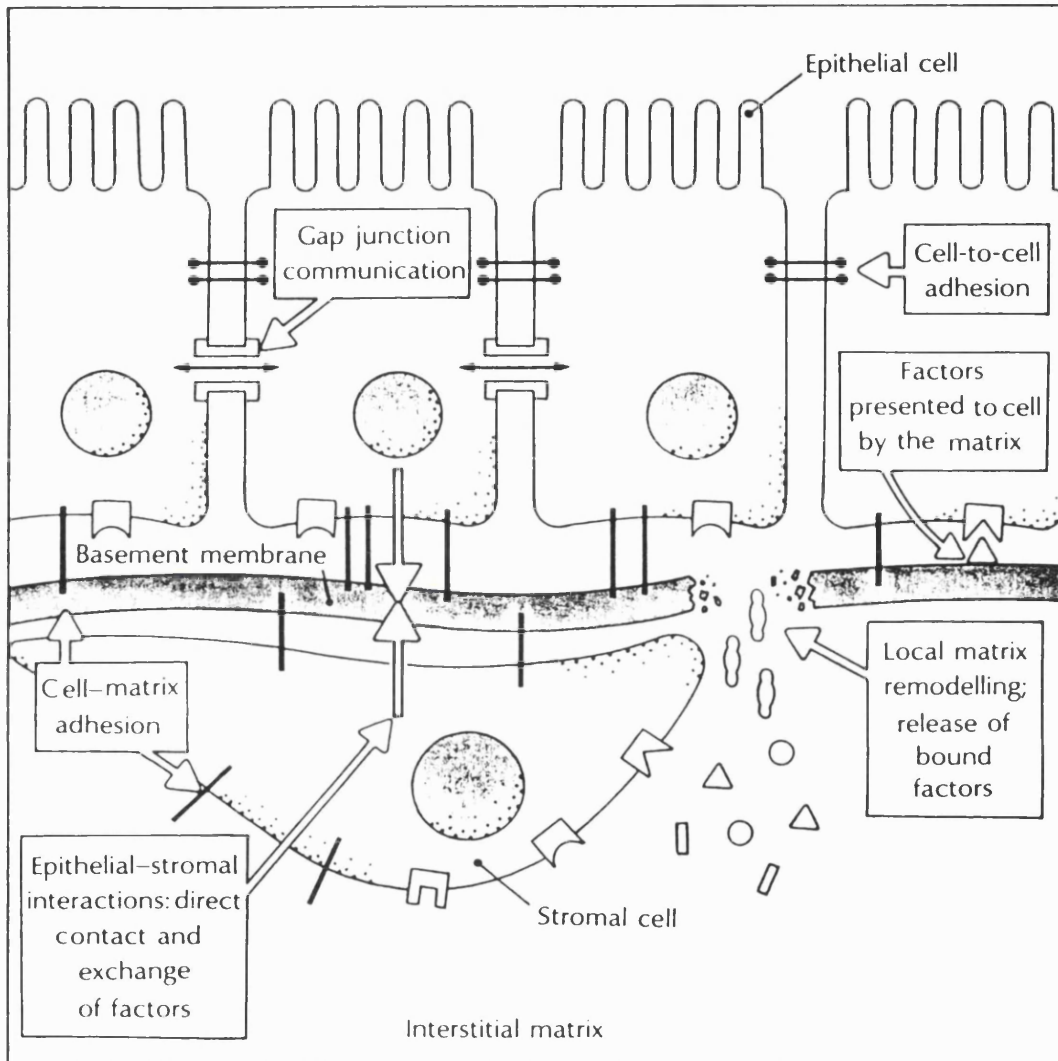


Fig. 67 Sub-cellular (cell-surface) morphology of a typical vertebrate epithelium, showing some locations of molecular function. ●—● cell-adhesion molecule; □ ○ △ cytokines and hormones; ∞ proteases. (From Stoker et al 1990b).

restricted to the nucleus (fig. 65, p.166; see Goodenough & Platt 1992; Kerr et al 1992).

There has been a relatively large number of molecular analyses, both morphological and manipulative, of the metanephric tissues of the mouse and rat (reviewed in Saxén 1987 and Bard 1992a&b). The majority of these have been at stages after the metanephric duct has extended into the metanephric mesenchyme and begun to bifurcate, at day 11 in the mouse or day 12.5 in the rat. In the mouse, morphological examples are: Mugrauer & Ekblom 1991, which describes expression patterns for the c-, L- and N-myc proto-oncogenes; Sainio et al 1992, which describes the expression of gap junction RNA's and proteins; and Harding et al 1991, which describes normal and abnormal expression of the gene for sulphated glycoprotein-2 (SGP-2). In the rat, a morphological example is Lazzaro et al 1992, which describes the sequential expression of homeoproteins LFB 1 and 2.

Several recent manipulative analyses have concerned induction and utilised Grobstein's (1956) transfilter technique (fig. 6, pp. 37-38), e.g. Aufderheide et al 1987, which shows that induction of mouse metanephrons in vitro leads to tenascin

expression; and Rogers et al 1991, which shows that the insulin-like growth factors, IGF I and II, are produced and necessary for rat metanephric induction in vitro.

Bard has been carrying out non-molecular, as well as molecular, analyses at the sub-cellular level, in order to address the phenomenon of cell traction during early condensation of the metanephron rudiments (see fig. 4, pp. 28-30; pp. 80-81; Bard 1990 & 1992b).

There appear to have been very few previous molecular analyses focussing on the earlier nephrogenic tissues. The principal morphological analyses of the nephric tissues appear to be: Smith & Mackay, 1991 (expression of laminin in the duct and nephrons of the mouse) and Jacob et al 1992 (expression of fibronectin and HNK-1 in the duct of the chick). The principal manipulative analyses appear to be: Jacob et al 1991 (competitive inhibition of fibronectin and laminin, using synthetic peptides, in the duct of the chick), Zackson & Steinberg 1988 and 1989 (involvement of cell-surface alkaline phosphatase in duct migration in the axolotl). There is a brief report of disruption of axolotl nephric-duct migration by

tunicamycin, which inhibits glycosylation of N-linked glycoproteins, in Armstrong's review of axolotl development (1989; see Gillespie et al 1985).

Some observations have been made of the rat and mouse 'mesonephric' tissues at the stages of metanephron formation described in the preceding pages. One of the most significant discoveries has been the expression of the Wilm's tumour suppressor genes, WT1 and WT2, in both these tissues in mammalian embryos at metanephric stages (Pritchard-Jones et al 1990; van Heyningen & Hastie 1992). These genes have been cloned and shown to activate a cascade of post-induction events in the metanephrons during normal mouse development (e.g. Call et al 1990). Specific WT1 mutations have been implicated in malignant and non-malignant abnormalities in mouse and human, metanephric morphogenesis (Pelletier et al 1991). Interactions between tumour-suppressor genes and mitosis have been discussed by Sager (1992).

Other nephrogenic analyses to include the nephric tissues have made significant observations concerning Hox and Pax gene products and segmentation in the mouse. Kress et al (1990) found high levels of Hox 2.3 transcripts, using both in situ hybridisation and the lacZ reporter gene linked to β -galactosidase, in 'mesonephric-duct derived' tissue, in both the nephric and metanephric tissues.

The anterior nephric tissues did not express these gene products, reflecting an anteroposterior restriction in the embryo as a whole. Other Hox-gene expression in the more posterior nephric tissues of the mouse at metanephric stages includes that of Hox5.2 and 5.3 (Dollé & Duboule 1989), 1.4 (Galliot et al 1989), 1.5 (Gaunt 1988) and 2.1 (e.g. Holland & Hogan 1988).

Pax2 (Dressler et al 1990) is expressed in all the nephrogenic tissues of the mouse: in the nephric duct and nephrons from the onset of duct extension (day 9) and subsequently in the metanephric duct and metanephrons. It is not expressed in the presumptive metanephrons before they begin to condense and is lost on maturation. Pax8 (Plachov et al 1990) is expressed in the nephric and metanephric tissues, but in contrast to Pax2, only in the mesenchymal condensations of the presumptive nephrons. Thus Pax8 has been associated with induction of both nephric and metanephric nephrons (Plachov et al 1990).

In the chick, 'mesonephric' observations during metanephric-duct outgrowth are those of Benn et al, 1991 (expression of a zinc-finger-protein-encoding gene cKrl) and Roberts et al, 1991 (selective expression of the L5 carbohydrate epitope).

There appear to have been especially few analyses of the beginning of metanephric development, i.e. during outgrowth of the metanephric duct (days 10-11 in the mouse). This could be for two reasons. The first is conceptual: the metanephros, which is the definitive tissue and the one in which urinary filtration occurs, is not seen to develop before the duct has reached its target mesenchyme. As a result of this, 'nephrogenesis' is frequently used to describe development of the metanephros only.

Secondly, the metanephric duct and metanephrons are thought to be derived from different cell-lineages (Mugrauer & Ekblom 1991), the metanephrons may be induced by tissues other than the duct - hence the development of Grobstein's (1956) transfilter technique - and induction alters the behaviour of the presumptive metanephrons. Correspondingly, Mugrauer & Ekblom (1991) have shown different expression patterns of c-, L- and N-myc, with L-myc being strongly expressed in the 'mesonephric' duct and the tissues thought to be derived from it (mesonephrons and metanephric duct); while Sariola et al (1991) have found that the nerve-growth-factor receptor (NGRF) is expressed by

the metanephron-mesenchyme only after the duct has reached it.

Vainio et al (1989) have mapped the expression of the cell-surface proteoglycan, syndecan, from embryonic days 10 to 19 and neonatally in the mouse. Syndecan was expressed by the mesenchyme immediately surrounding the metanephric duct as it extended from the cloaca into the metanephric mesenchyme and subsequently, when the duct began to bifurcate, by all the mesenchyme though most intensely within a distance of ten cells from the duct. The expression of syndecan was correlated with areas of induction and was lost with nephron maturation, throughout metanephric morphogenesis, and was therefore interpreted to be a marker for early inductive changes.

More recently, through the use of double-immunohistochemical staining of fixed tissue, Vainio et al (1992) have found a close correlation between expression of syndecan and increased mitotic rate during metanephric development. Mitosis is thought to be an early response to induction (Saxén 1987). Using the transfilter technique and hanging-drop cultures, Vainio et al (1992) have also shown that syndecan may be expressed both before and in

response to induction. Furthermore, using slot-blot, Northern and in situ hybridization analyses, they have shown that syndecan is initially induced post-transcriptionally and subsequently at the mRNA level.

CHAPTER THREE

METHODS

Forty-five embryos of Hamburger & Hamilton stages 8-27 were incubated, prepared and sectioned at 4 μ m for light microscopy as described in Appendix 4. Selected slides were then stained immunohistochemically with IgM-class antibodies, either to FC10.2 or NB10.3B4 (gifts from Dr. T. Feizi, MRC Glycoconjugates Unit, Northwick Park, Middlesex), and counterstained with Light Green (Gurr). The great majority of slides were pre-treated with neuraminidase (sialidase). The standard immunoperoxidase method was used, in a protocol (Appendix 5) from Dr. W. Loveless, MRC Glycoconjugates Unit, Northwick Park, Middlesex.

The sectioning and staining was carried out in collaboration with Dr. M. Veini, visiting from the Department of Zoology, University of Athens, Greece.

Sections stained in this way were examined using a Zeiss 'Universal' microscope and various bright-field objectives. Photographs were taken using Kodak Ektachrome colour reversal ASA 64 film and a daylight filter (Appendix 5). Films were developed and printed commercially (Colour Processing Laboratories).

CHAPTER THREE

RESULTS

A monoclonal antibody to the carbohydrate antigen, FC10.2, and the standard immunoperoxidase/DAB technique were used to stain sections of the nephric and early metanephric tissues, from H&H 8 to 27. Positive results were obtained only after pre-treatment with neuraminidase (sialidase), indicating that FC10.2 was sialylated (S-FC10.2; Loveless et al 1990). The results of the present chapter, which show a changing pattern of S-FC10.2 expression with morphological development during early nephrogenesis, are summarised in Table 13. The figures are shown in Chapter 2 (pp. 106-149), which should also be referred to for a description of morphological changes (summarised on pp. 151-154).

FOR TABLE 13, SEE OVERLEAF

TABLE 13

MORPHOLOGY AND S-FC10.2 DISTRIBUTION DURING DEVELOPMENT

H&H STAGE	NEPHRIC MORPHOLOGY	
	DUCT	NEPHRONS
8-10	Single 'nephric' rudiment, mesenchymal, without lumen; anteriorly attached at intervals to somites, posteriorly not readily discernable from segmental plate or lateral mesoderm	
10-12	Mainly a single rudiment, broad, mesenchymal and without lumen, attached at intervals to somites; also nephron-shaped rudiment, segregating medially from the first rudiment; both without central lumina	
12-14	Rudiment narrower, longer than at H&H 10-12; wall compact, with lumen forming intermittently in midline	Rudiment or rudiments, with some degree of separation from duct rudiment; wall compact, no lumen
14-16	Rudiment of similar width to H&H 12-14, but longer; wall thinner, lumen more developed, though still intermittent	Rudiments mostly separate from duct rudiment, with lumina forming; some, non-patent, attachments present
16-18	Duct of similar width to H&H 14-16, but at or near final length, fusing with cloaca; lumen continuous along length, wall thinner	Nephrons rosette-shaped and separate from duct; each with central, initially circular lumen, walls thinner
18-20	Duct of similar width, wall of similar thickness, to H&H 16-18; has fused with cloaca; outer face of wall invaginated to complement nephrons	Nephrons still separate from duct, though extended laterally, as are lumina; lumina of greater diameter and walls thinner than at H&H 16-18
20-22	Duct similar to H&H 18-20, but walls fusing with those of nephrons; some sites of fusion non-patent	Nephrons fusing laterally with duct; lumina becoming curved; glomeruli and capsules forming
22-28	Duct continues to fuse with distal ends of nephric tubules; lumen of duct widens	Nephrons elongating proximally and distally, but within a restricted space which forms the boundaries of the nephros

OF THE NEPHRIC TISSUES OF THE CHICK EMBRYO

S-FC10.2 DISTRIBUTION	
a) tissue level	
b) sub-cellular level	
DUCT	NEPHRONS
a) of similar intensity antero-posteriorly and medio-laterally b) pericellularly, of similar intensity on all cell surfaces	
a) punctate (peri-presumptive-luminarly), uniformly in antero-posterior and medio-lateral directions b) pericellularly, especially apically; not cytoplasmically	
a) punctate in midline; outside rudiment b) pericellularly, especially apically; not cytoplasmically; extracellularly outside rudiment	a) punctate in midline, or centrally where rudiments separate from each other b) as for duct rudiment, but not extracellularly outside rudiment(s)
a) peri-presumptive-luminarly; less outside rudiment b) pericellularly, especially apically; less extracellularly outside rudiment	a) peri-presumptive-luminarly; including attachments to duct rudiment b) as in duct rudiment, but not extracellularly outside rudiment(s)
a) less intense in wall; peri-luminar; free within lumen; not seen outside duct b) weaker pericellularly except apically; apparently free within duct lumen	a) peri-presumptive-luminarly and radially b) pericellularly, especially apically and laterally
a) very weak within wall; also weaker peri-luminarly; free within lumen b) pericellularly significant only apically; free within duct	Lateral region of nephron only: a) radially within wall and peri-presumptive-luminarly b) pericellularly, especially apically and laterally
a) weakly peri-luminar; free within lumen b) weakly apical; extracellularly within duct	Lateral region of nephron only: a) radially within wall and peri-presumptive-luminarly b) pericellularly, especially apically and laterally
a) becomes stronger peri-luminarly and lost from within the lumen b) strengthening apically	Extending regions of nephron: a) strongly and uniformly distributed within wall; lost from outer surface, then from within, then inner surface b) pericellularly; lost basally, then laterally, then apically

At the stages examined, only the nephric tissues, metanephric duct, primordial germ cells, notochord and epidermis reacted strongly to the staining, while the mesenchyme of the tail-bud, the sclerotome and parts of the hindgut became weakly stained at some stages. The non-urinary tissues were all readily distinguished from the urinary tissues, while the notochord served as a positive internal control at all stages. Urinary and other tissues stained negatively at all stages examined following treatment with NB10.3B4, a monoclonal antibody used as a control for FC10.2 (figs. 50, 52, 61). Some positive staining was seen in the epidermis and sub-ectodermal material with NB10.3B4.

The mesenchymal nephric rudiments (first nephric, nephric duct and first nephron rudiments: NR, ND and NN respectively) stained fairly uniformly at the beginning of their formation (NR: fig. 14; ND: fig. 17; NN: fig. 19), as did the mesenchymal, posterior tip of the nephric duct in progressively posterior locations (figs. 15, 21). From its appearance, the staining appeared to be pericellular and on all cells within the tissue (figs. 17, 21, 31, 33).

As the mesenchymal nephric tissues began to epithelialise, staining became concentrated where the lumina were about to form ('peri-presumptive-luminarly'), i.e. at the apical surfaces of the cells (figs. 16, 18, 20, 32, 34, 35). This accentuated the punctate appearance of these tissues at these stages.

As epithelialisation progressed in the extending nephric duct rudiment, staining with S-FC10.2 was lost or at least greatly reduced (fig. 30) and weak in comparison to that of the more mesenchymal nephron rudiments (figs. 17, 39-41). There might have been some persistent staining of the the apical cell surfaces of the duct (e.g. fig. 30). At H&H 27 this staining was stronger, but had become restricted to the ventral surface of the duct lumen, i.e. the side on which the nephric tubules opened into the duct (figs. 55, 57).

Also during extension and epithelialisation of the nephric duct rudiment, the extracellular matrix surrounding the duct (where visible) was stained, at least weakly (figs. 16, 31), but quite strongly towards the posterior tip of at least one embryo (fig. 30; the other duct in the same embryo also showed this). More frequently observed was an

amorphous or matrix-like, stained substance which could be seen within the lumen of the duct (figs. 39-41) and of the cloaca (fig. 51; control: fig. 50), at H&H 18-22. This was after the duct had fused with the cloaca and epithelialised (at H&H 17⁻), but before the metanephric duct grew out from the cloaca (at about H&H 24).

Early epithelialisation of the first nephron-rudiment (NN) was accompanied by anteroposterior segregation, first into groups of presumptive nephrons and then into individual, rosette-shaped nephron rudiments (Ro). The changes in the pattern of S-FC10.2 expression which accompanied this morphological change were similar to that in the duct, except that staining in the rosettes could clearly be seen on the lateral as well as apical cell surfaces, which emphasised their rosette-like appearance (LM figs. 34, 39, 40, 42). (In freeze-fracture SEM of these early nephrons, a rosette-like appearance was due to the orientation and shapes of the cells; fig. 36). Strong S-FC10.2 staining persisted in the nephron rosettes after the duct had fused with the cloaca (fig. 34).

Further epithelialisation of the nephron rudiments was accompanied by extension of the

rosettes into more elliptical shapes as the glomerular capsule formed proximally, i.e. on the opposite side of the rosette to the nephric duct. Staining was lost almost immediately in the capsule, but retained quite strongly in the original region of the rosette (figs. 39, 40, 42-45).

From H&H 18 at more anterior levels the original region of the rosette began extending distally, towards the duct. The distal part of the extending rosette (the presumptive distal tubule) appeared to form as a punctate, i.e. partially epithelial, tissue expressing S-FC10.2 as the duct and first nephron rudiments (ND and NN) had previously. However, in contrast to those earlier tissues, the presumptive distal tubule of the nephron was rounded. (Figs. 40-49). As the nephron fused distally with the nephric duct it became fully epithelial and no longer stained.

Although its formation was not specifically investigated, the cloaca was seen to be epithelial by the time the nephric duct fused with its lateral walls. In the majority of cases, neither the duct or cloaca stained during or after fusion; fig. 51 appears to be an aberration in the staining procedure, occasionally observed, in which staining

appeared to be regional rather than tissue-specific. The cloaca only expressed S-FC10.2 consistently during formation and extension of the metanephric duct, when the anterior region from which the duct originated stained (figs. 57, 60).

The metanephric duct expressed S-FC10.2 from its first appearance at H&H 24 and remained staining along its entire length at H&H 27, when it had extended into an anterior region of the metanephric mesenchyme (figs. 56, 57, 60). Not only was this different from the nephric duct, which had ceased staining in an overall anteroposterior direction as it had extended, but there was also a difference in the pattern of epithelialisation and concomitantly in the expression of S-FC10.2 between the two types of duct.

From H&H 24 to 27, the metanephric duct extended anteroventrally from the cloaca into the metanephric mesenchyme, as a canalised but apparently not fully epithelial rudiment. Even at H&H 27, when it had reached its target mesenchyme (indicated by its first bifurcation), the duct appeared only partially epithelial. Towards the cloaca end, the wall of the duct had a thickness of approximately one cell, but stained pericellularly,

fairly uniformly and quite strongly (figs. 56, 57, 60). At the bifurcating end, the duct appeared to be more epithelial, with S-FCl0.2 expression restricted to the apical cell surfaces.

Hence epithelialisation and canalisation appeared to have occurred anteroposteriorly and the pattern of staining appeared to be related to that of epithelialisation as in the nephric tissues. However, in contrast to the nephric tissues, the direction of epithelialisation was opposed to that of anteroposterior extension and a central lumen of considerable width formed before epithelialisation was complete. An ECM or amorphous material were not seen to be associated with the metanephric tissues or the cloaca at these stages.

Also in contrast to the nephric tissues, the mesenchymal condensations (presumptive metanephrons) which had begun to form around the, as-yet short, branches of the metanephric duct rudiment by H&H 27 were not stained (figs. 35, 36).

The nephric nephrons continued extending at these early metanephric stages, ventromedially to form proximal tissue and dorsolaterally, behind the region which had fused with the nephric duct, to

form distal tissue. Apparently associated with the restricted space in these directions, the nephric tubules became curved and even convoluted or spiralled. In contrast both to earlier nephric development and to simultaneous metanephric development, the ends of the tubules appeared to have formed immediately as proper epithelia and expressed S-FC10.2 strongly and pericellularly. Staining appeared to be lost from recently formed regions of the tubules with a similar cellular-surface pattern to that at earlier stages, i.e. basally, then laterally and finally (if at all) apically. (Figs. 55, 57-59.)

CHAPTER THREE

DISCUSSION

The present analysis has shown that there are morphogenetically associated changes in expression of the carbohydrate antigen, S-FC10.2, during early nephrogenesis (H&H 8-27) in the chick embryo. The nephric and metanephric ducts and the nephric nephrons all expressed the antigen from their earliest morphological appearances as mesenchymal rudiments (i.e. at an early stage of phenotypic differentiation). With epithelial differentiation and maturation the expression changed, with slightly different patterns in the various nephrogenic tissues. This appears to be the first description of the expression pattern of a differentiation antigen at these stages of nephrogenesis in an amniote.

Because of its tissue-specific expression (see Results) from the earliest, morphologically detectable stages of formation, the S-FC10.2 antigen may be regarded as a phenotypic marker in the nephric tissues and the metanephric duct. Phenotypic markers are essential for many morphological and manipulative analyses. However, as with all phenotypic markers, care will be needed in the use of S-FC10.2. For example, it should be ascertained whether the antigen, either in its sialylated or un-sialylated form, is still expressed after heterotopic grafting. Furthermore, the intensity of its expression and its degree of specificity to the nephrogenic tissues after manipulations must be established. Both these criteria could probably be met by the use of suitable controls.

A use for the antigen as a phenotypic marker in a morphological analysis would be in tracing cell lineages. Cell-lineage analysis is important in relation to several nephrogenetic questions (see pp. 162 & 210-213). One example is highlighted by one of the tissue-specific differences in expression of the S-FC10.2 antigen. During mesenchyme condensation, the nephric tissues and metanephric duct express this antigen, whereas the metanephrons

do not. Similar patterns are found with L-CAM (Thiery et al 1984) and Hox 2.3 (Kress et al 1990), but not with syndecan (Vainio et al 1989) or Pax8 (Plachov et al 1990), which are expressed by all the nephrogenic tissues at the beginning of mesenchyme condensation. Pax2 (Dressler et al 1990) expression is between the two extremes, as it is expressed in the mesenchymal metanephrons after condensation has begun.

The idea is frequently espoused that the nephric tissues and the metanephric duct are derived from a separate cell-lineage than are the presumptive metanephrons (e.g. Mugrauer & Ekblom 1991). However, there does not appear to have been a comprehensive analysis of cell lineage in the nephrogenic tissues, probably because suitable cellular markers have only recently become available (see pp. 51-53). The evidence from LM supports the idea of a distinct metanephron cell-lineage, because, as shown in the present study, the nephric and metanephric mesenchymes are morphologically different. Expression of S-FC10.2 in the cloaca only at the site of origin of the metanephric duct, together with morphological evidence, in the present study also appears to indicate that, in the chick at

least, although the nephric and metanephric ducts are linked by the cloaca, they are not directly joined to each other as often stated (e.g. Lillie 1952).

Uses of S-FC10.2 in nephrogenetic analyses other than as a phenotypic marker may be either morphological or in the manipulation of processes in which it may be involved. Morphologically, the present analysis has shown that the antigen may be helpful as a specialised histochemical stain. For example in epithelialising nephric tissue, apical expression of the antigen helped to elucidate a 'lattice' pattern, anteroposterior segmentation and lumen formation in both the presumptive nephrons and duct at the beginning of epithelialisation. The present, morphological analyses represent an initial step in addressing possible morphogenetic roles of the antigen, including its masking by sialic acid, or its modification, to form a different antigen and/or to make its detection more difficult (see Schauer 1988; Sanders 1989; pp. 172 and 58-59).

Differences in expression of (putative) transcription factors and antigens between the presumptive metanephrons and other nephrogenic tissues, described in the preceding pages, could be

due to factors other than cell lineage. For example, although from the present analysis in the chick and the previous analyses of Hox and Pax in the mouse, nephric and metanephric nephrons appear to be segmental, they also appear to have different orientations relative to the embryonic axis. Development of the presumptive-metanephron tissue is predominantly radial while that of the other tissues is predominantly linear. Furthermore, where a tissue is extending either in parallel or perpendicularly to the embryonic axis, it expresses the antigen, but where it has become convoluted it (a nephric nephron) no longer does so. Thus perhaps orientation has an effect on segmentation. Such an effect might somehow be mediated by growth factors, whose association with axis formation at earlier stages make their involvement with orientation at later stages not inconceivable.

In the same context, similarities in molecular expression might be correlated with the fact that, although the metanephrons are oriented obliquely with respect to the early embryonic axis, individually their orientations with respect to the nearest core mesoderm are probably perpendicular because this mesoderm is curved.

It would appear that in the nephrogenic tissues in which it is expressed in the chick embryo, the S-FC10.2 antigen is associated primarily with tissue formation and secondarily with epithelial differentiation. Thus:

- 1 In the nephric tissues forming before outgrowth of the metanephric duct - which clearly form via mesenchymal condensation - S-FC10.2 expression is associated with mesenchymal condensation and epithelialisation, but with an altered sub-cellular pattern and less intensity as epithelialisation progresses. After each tissue has become epithelial, S-FC10.2 remains only weakly on the apical cell surfaces. In the nephric nephrons, but not in the nephric duct, this is lost with maturation.
- 2 In the nephric-nephron tissue forming after outgrowth of the metanephric duct has begun [- which appears to form directly as epithelia -] and in the metanephric duct itself [- which appears to form as a semi-epithelium -] S-FC10.2 is expressed with the same sub-cellular pattern (peri-cellularly) as in the nephric tissues which formed previously via mesenchymal condensation. This is so despite the fact that this nephric-

nephron tissue appears to form directly as epithelia and that the metanephric duct forms as a semi-epithelium.

- 3 After the metanephric duct has begun to bifurcate, the nephric duct continues to express S-FC10.2 on its apical cell-surfaces, while the tip of the metanephric duct also expresses S-FC10.2 in this way. In the nephric-nephron tissue which had formed after outgrowth of the metanephric duct began, the pattern of loss of S-FC10.2 expression during maturation is similar to that in the nephric tissues which had formed before outgrowth of the metanephric duct.

Hence, because the antigen is expressed by both the nephric duct and nephrons, it does not appear to be associated with the long-distance migration frequently postulated for nephric-duct but not nephric-nephron tissue. Rather, its pattern of expression appears to be more closely associated with induction, widely accepted as the means of formation of the nephric nephrons, but not of the nephric duct. Induction of these tissues has been previously investigated by ablation, interception and grafting experiments (see Table 8, p. 58; pp. 73-77; Martin 1971 & 1976).

In particular, nephrogenic expression of the antigen might be associated with short-distance migration, including cellular rearrangement, which occurs as an early response to induction and could also lead to extension, growth and shaping of the tissues during formation. With the onset of epithelialisation in the nephric tissues, the antigen is expressed segmentally and in association with lumen formation. Perhaps therefore, migratory distance is limited by segmentation.

The loss of S-FC10.2 expression with maturation of the epithelial tissues would appear to be consistent with cessation of active cellular movement, but not necessarily with passive movement. The latter might be caused by biophysical or biochemical factors in the surroundings and also lead to changes in overall tissue-shape.

Another question to arise in the present analysis is that of the likely causes of the different rates of nephrogenic epithelialisation. In the nephric tissues, this was related to embryonic stage, with the later nephron-tissue epithelialising more quickly, or even forming as an epithelium, as described in the preceding stages.

In the metanephric duct, rate of epithelialisation appeared to be related, though less closely, to embryonic stage, as the duct seemed to form as a semi-epithelial tissue. However, formation of the lumen occurred almost immediately in the metanephric duct, i.e. before it became fully epithelial. This contrasted with the nephric tissues and further suggests that lumen formation is a separate, though related, process to that of epithelialisation.

For a discussion of cellular polarity in epithelial morphology and function, see Gumbiner (1990).

CHAPTER FOUR

CELLULAR LEVEL

NEPHROGENESIS

CHAPTER FOUR

INTRODUCTION

Although the three anatomical levels discussed in the present study - tissue, sub-cellular and cellular - are all interconnected, the cellular level may be the most difficult to define. For example, Godsave & Slack (1991) regard 'cellular' as denoting single cells and criticise many previous, purportedly cellular, analyses of phenotypic commitment for having been carried out on relatively large multicellular grafts, or in a multicellular environment. However, multicellularity does not necessarily equate with tissue status and in the present study the cellular level will be defined as that of whole, though not necessarily single, cells.

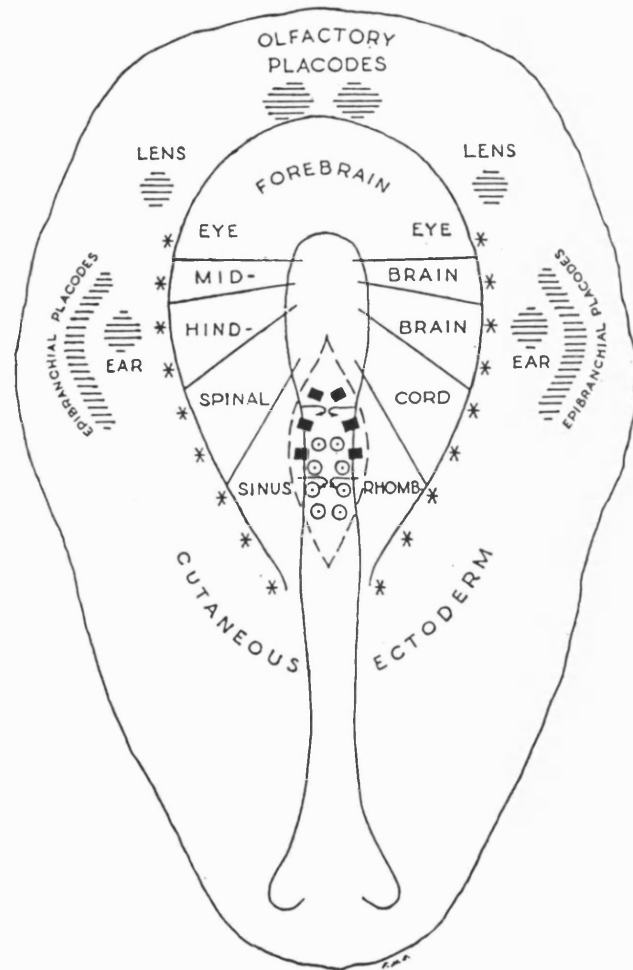
Morphological analyses at the cellular level include those of mitotic and apoptotic rates, and fate-mapping. In vitro analyses of induction have been considered in the previous chapter because of their molecular perspective, although many of these may alternatively be regarded as cellular.

It will be apparent that an understanding of cellular fate is a necessary means of addressing some of the most important morphogenetic questions in the nephrogenic tissues, i.e. the spatial aspects of origin and extension of each tissue, including whether a tissue is entirely mesodermal (see pp. 51; 58-78). A knowledge of origin and fate in the normal embryo is also essential for analyses of phenotypic commitment, or the temporal aspects of a tissue's origin.

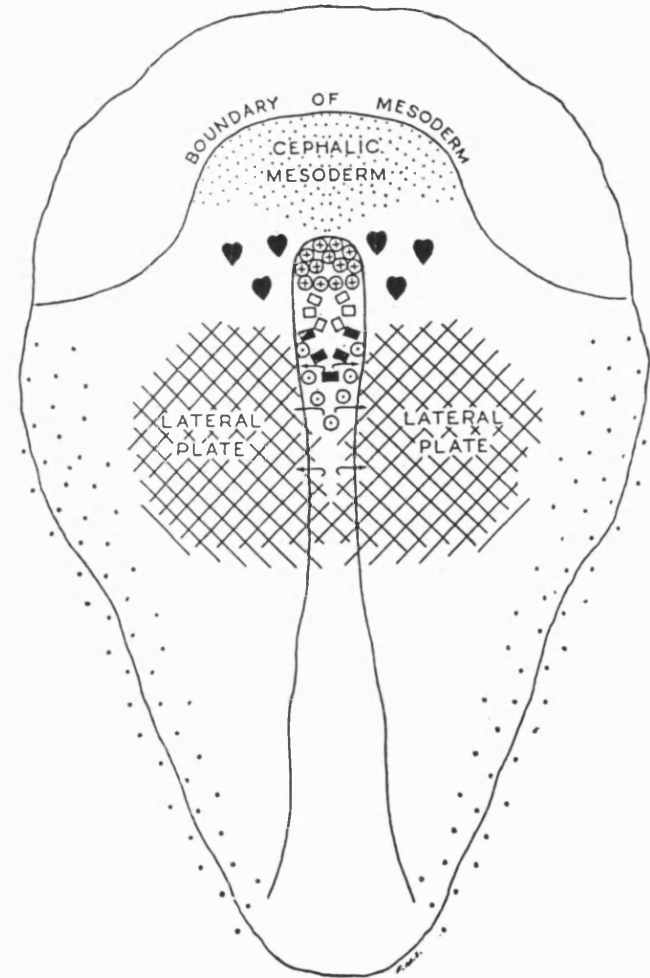
However, there is a dearth of analyses of cellular origin in the nephrogenic tissues. Two reasons for this could be that, until recently, specific cellular markers were not available and the majority of recent nephrogenetic studies have been in the mouse, which is unsuitable for analyses involving the embryo as a whole over a sufficient period (see p. 36).

The majority of previous analyses of nephrogenic origin in the chick embryo have located tissue with nephrogenic or other potentials at mid- and late- primitive-streak stages, in order to construct a general picture of regional potency, e.g. fig. 68. Chorioallantoic- membrane grafting of different regions of the embryo has been used for this (Hunt 1931, Rawles 1936 and Rudnick 1944). Only one analysis (Hoadley 1926) appears to have located regions with nephrogenic potential at earlier stages, i.e. from the unincubated blastoderm to the beginning of formation of the first somite. It should be emphasised that these analyses are not true fate-maps as they have not followed cells in the normal embryo. More recently, using chick- quail chimaeras in New (1955) culture, Vakaet (e.g. 1984) has constructed considerably more normal fate-maps, from the unincubated- blastoderm stage.

Rosenquist constructed an overall map of regional tissue- origin (1966) and appears to have made the only analysis specifically of nephrogenic- tissue origin (1970), using tritiated- thymidine chick chimaeras in New (1955) culture. Rosenquist's (1970) analysis was from the mid- primitive- streak stages and appears to have been the only one of its



A



B

Fig. 68 General map of differentiation potential in the primitive-streak stage chick embryo. **A** in the epiblast; **B** in the mesoderm. * neural crest; ■ somites; ⊙ nephros; ♥ heart; ⊕ notochord; □ endoderm. (Re-drawn from Rudnick 1944 by Patten 1953).

kind to have distinguished duct- from nephron-cells. However, survival was poor, with only two out of a total of twenty-one host embryos surviving to H&H 17-19 and neither of these having received their graft at the earliest stage. In general, 'it was necessary to combine the pathways followed by two or three labelled grafts' (Rosenquist 1970).

Some of Hoadley's (1926) and Rosenquist's (1970) conclusions also need re-examining. For example, Hoadley concluded that the nephric duct forms from fusion of 'pro'-nephrons (Rosenquist assumed this to be the case) (see pp. 64-65), and that the metanephrons form from the same tissue-region as do the nephric tissues and the metanephric duct (but see pp. 197-198), while both Hoadley (1926) and Rosenquist (1970) thought that the 'pronephros' and 'mesonephros' are distinct from one another (see p. 67-69). The metanephric cells appear to have been mapped even less extensively than the nephric cells and their origin remains a common source of question (e.g. Saxén 1987). The general lack of knowledge regarding nephrogenic origins is further underlined by the fact that many general fate-maps do not include the urinary tissues.

Amphibian fate-maps of the nephrogenic cells have also been limited (see Saxén 1987; Burns 1955), although in contrast to analyses in the chick the use of vital dyes has probably produced a more accurate map of the normal embryo. Recently, using the lipophyllic Dye I and assuming this dye is not transferred from a cell to its progeny, Lynch & Fraser (1990) have shown that the nephric duct of Xenopus extends by migration (see pp. 51-52; 78).

Another question frequently asked, but scarcely investigated, is that of phenotypic commitment in the metanephric tissues. Development of the mammalian metanephron is thought to entail permissive induction of the mesenchyme by the metanephric duct (Saxén 1987), leading to the differentiation of at least fourteen epithelial cell types (Herzlinger et al 1992). Herzlinger et al's (1992) transfilter analysis (fig. 6, pp. 37-38) of mouse metanephric tissue appears to be the only recent analysis of phenotypic commitment in the nephrogenic tissues. It has shown that before induction, multipotent stem cells in the mouse metanephric mesenchyme can generate at least three epithelial cell types - glomerular, and proximal- and distal- tubular, whereas after induction,

similar cells can only form one cell type and are therefore restricted to a particular segment of the nephron (fig. 2b, p. 26).

Commitment to nephrogenic phenotypes in the chick appears not to have been investigated specifically since the nephric-tissue analyses of Hoadley (1926), Hunt (1931), Rawles (1936) and Rudnick (1944), discussed in the preceding pages, or those of Boyden (1927), Waddington (1938) and Gruenwald (1942), discussed on pp. 74-77. Boyden (1927) investigated the effect of the nephric duct on both nephric and metanephric formation. In addition to the latter three studies, results from other manipulative analyses (discussed in Chapter 2) may also be interpreted in terms of this potential - although their aim was to ascertain inductive or migratory behaviour in the nephric duct. This further emphasises the necessity of investigating each stage of nephrogenesis in terms of in situ versus distant events. (See Chapter 5).

An analysis of nephrogenic tissue origins in the chick embryo is currently under way. This aims both to elucidate as far as possible the normal events of nephric and metanephric morphogenesis in

an amniote, and to integrate the analysis of commitment with that of fate. In particular, the tracing of single cells (cell-lineage) and grafting experiments should clarify many details, while more general morphogenetic questions, such as how cells become nephrogenic and the role of induction in this type of commitment, may also be addressed.

The present study has addressed the issue of in situ versus distant formation at the cellular level by analysing mitotic and apoptotic indices in the nephric duct. This has been done between H&H 17⁻ and 18, during fusion of the duct with the cloaca, partly to compare the indices with those of the extending duct, as previous counts have been made at H&H 13-14 in the chick (Overton 1959a and Jacob et al 1992). It is also to investigate any correlation between mitosis and/ or apoptosis in the nephric duct during fusion with the cloaca. Apoptosis does not appear to have been analysed previously in the nephric tissues, even though degeneration of these tissues has long been recognised (see also pp. 34, 70, 85; Abdel-Malek 1950; Romanoff 1960). Mitoses have also been counted in amphibian nephric ducts (Overton 1959b) and mouse metanephrons (Vainio et al 1992).

CHAPTER FOUR

METHODS

Fifteen fertilised hens' eggs were incubated for times corresponding to development to H&H stages 17-18. According to the technique of Primmatt et al (1989), the eggs were then windowed and treated with 100 μ l of 0.5 μ g ml⁻¹ solution of bromodeoxyurine (BUDR; BDH) in calcium- and magnesium-free Tyrode's saline (CMF, Appendix 7). The treated eggs were sealed with cellophane tape and re-incubated for two hours. BUDR is incorporated into the chromosomes during S-phase (Primmatt et al 1989).

A trace of Nile Blue powder (Gurr), which is relatively inert, was added to the BUDR solution to improve visibility and doses were injected into the amniotic cavity (fig. 5, pp. 31-32). Controls were injected with CMF/ Nile Blue. The present study was preliminary, owing to the time involved in cell-counting. Eight eggs were treated successfully with BUDR and three with CMF/ Nile Blue alone, while ultimately a single nephric duct from each of three BUDR-treated embryos were counted.

After re-incubation, each embryo was removed from the egg, rinsed thoroughly and trimmed in PBS, fixed in BFS, dehydrated in serial IMS, cleared in cedar wood oil and embedded in paraffin-wax and sectioned coronally at $10\mu\text{m}$, as described in Appendix 4 (see Table 3 for abbreviations).

Selected slides were stained immunohistochemically, using anti-BUDR (Becton-Dickinson) and the standard immunoperoxidase technique (Appendix 8). Slides from three embryos of H&H 17-18, previously treated with BUDR were selected for cell-counting. A Zeiss 'Universal' microscope with an oil-immersion, 100X objective was used. Cells were counted in the left

nephric duct of each embryo, during interphase, at different stages of mitosis, or during pyknosis (putatively apoptosis), and hence as a proportion of total cell number (figs. 69, 70 & 74, p. 218a).

Cells were counted in a posteroanterior direction, dividing the duct into four adjacent regions relative to the cloaca and the nephrons. Cells were not counted in tissues other than the duct itself, with care being taken to exclude cloacal cells at the site of fusion. The regions of the duct were (see fig. 1, p.24):

- 1 The fusing tip (from the cloacal wall to the most posterior, mesenchymal nephron rudiment)
- 2 The region adjacent to the rudimentary nephron tissue (NN)
- 3 The region adjacent to the individual nephron rudiments (Ro) - the main part of the duct
- 4 The most anterior region, where the nephrons had begun to fuse with the duct

Alternate sections were counted. As mesodermal cell diameters at these stages are usually about $10\mu\text{m}$ in the chick, this maximised the chance of counting every cell once but minimised that of counting a cell twice.

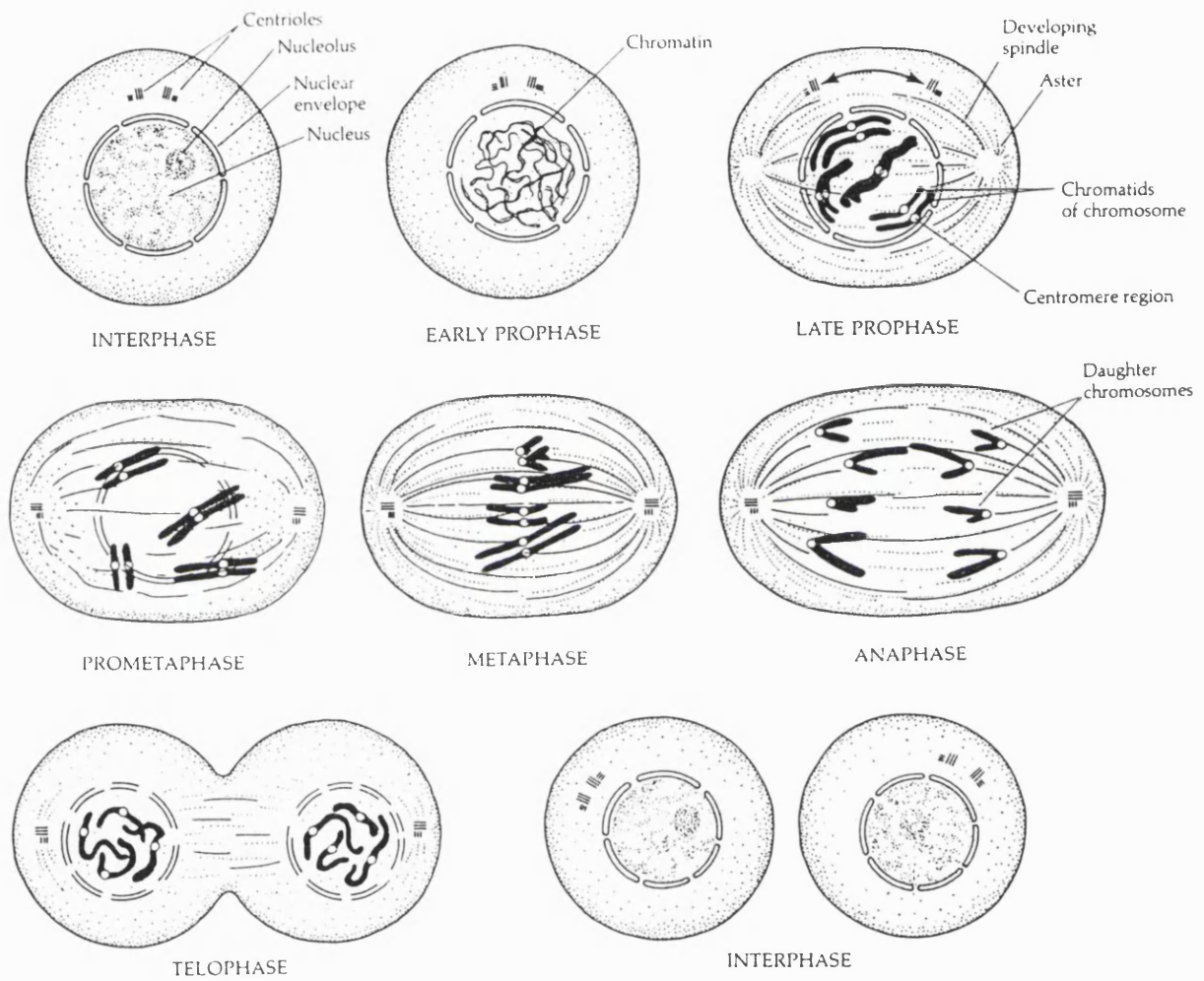


Fig. 69 The main stages of mitotic cell division.
(From Gilbert 1991).

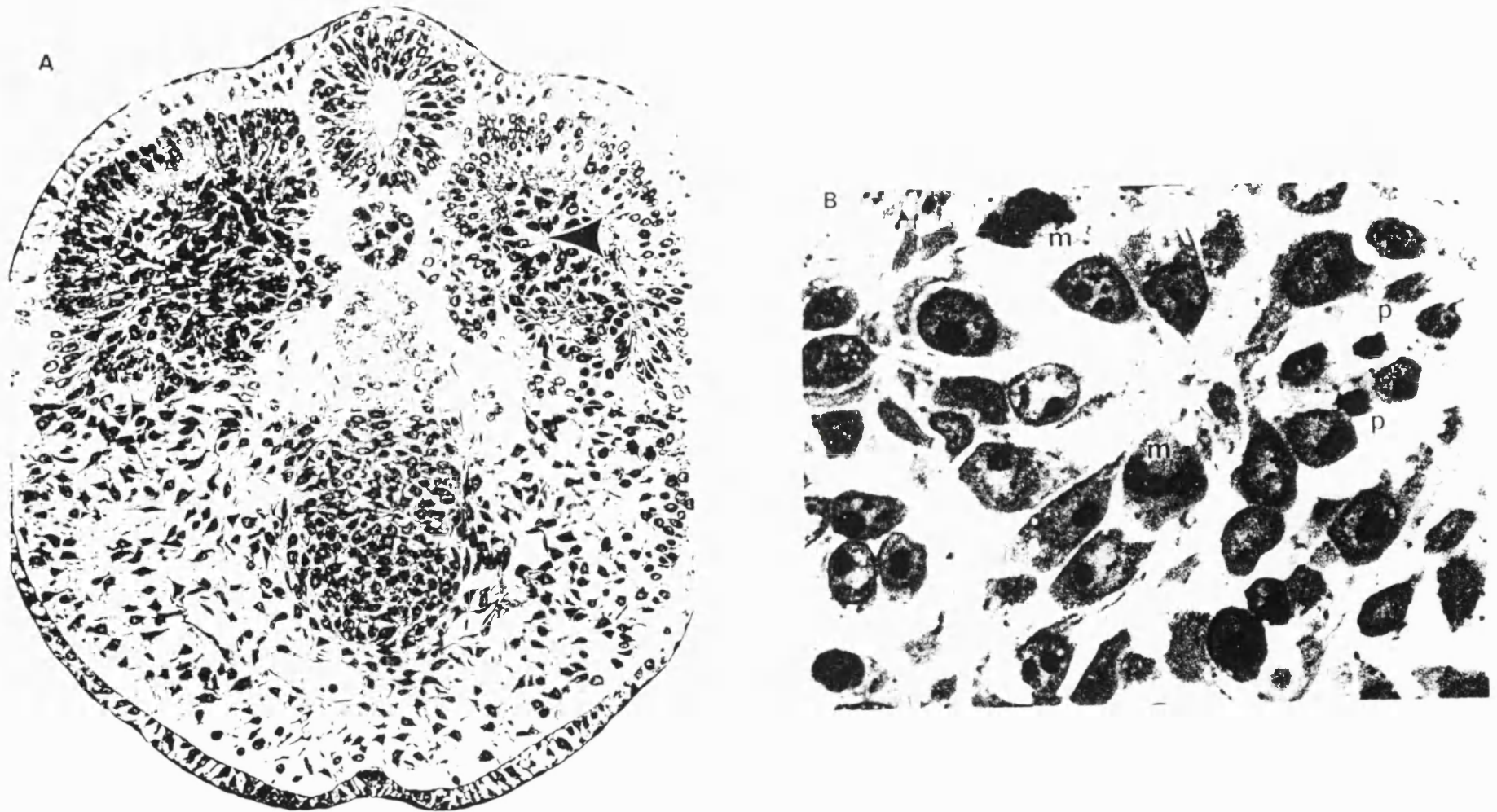


Fig. 70 Mitotic (m) and apoptotic (pyknotic, p) cells in the tail bud mesoderm in a chick embryo. A Transverse section at the level of the most posterior somite at H&H 21⁺ (X 90); arrow indicates region enlarged in B (X 500). (From Sanders et al 1986).

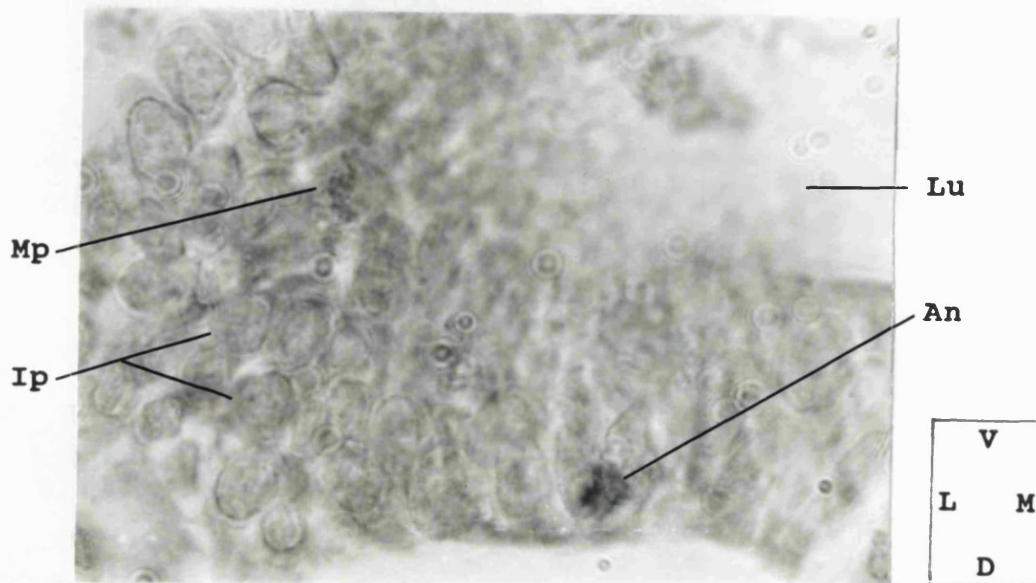


Fig. 74 A Transverse section through the posterior tip of a nephric duct at H&H 18, showing cells during interphase (Ip) metaphase (Mp) and early anaphase (An); X 1800 (stained with anti-BUDR, as described in Appendix 8; for abbreviations see pp. 106-108).

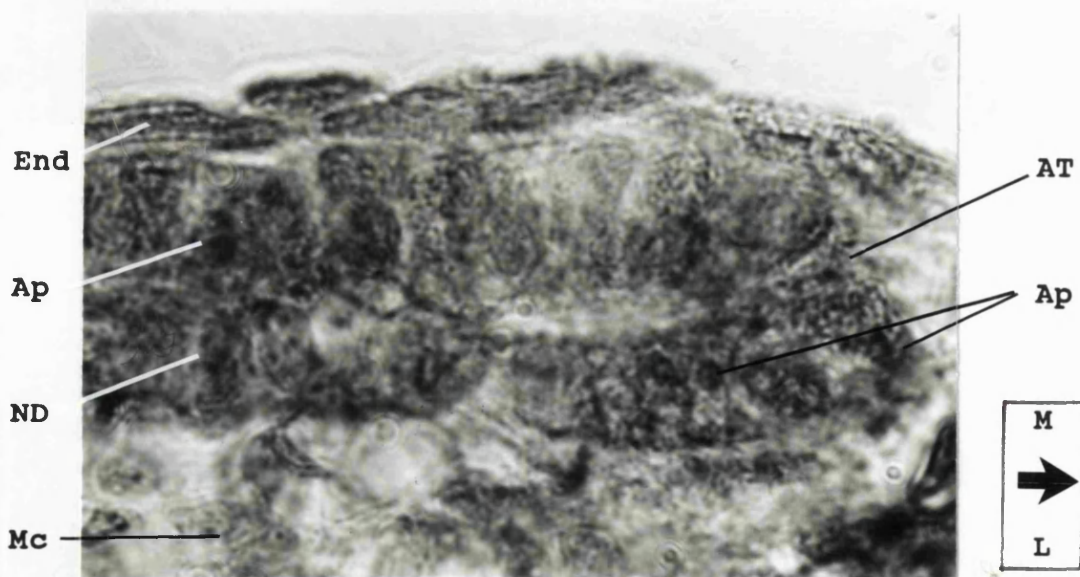


Fig. 74 B Longitudinal section through the anterior tip of a nephric duct at H&H 18, showing cells at various stages of apoptosis (Ap); X 2000 (staining in Appendix 8; abbreviations on pp. 106-108).

CHAPTER FOUR

RESULTS

Embryos were incubated and treated with BRDU (see Methods) as a group, but were at slightly different stages. After two hours of re-incubation, all the embryos appeared to be healthy and the BRDU, as indicated by the presence of Nile blue, appeared both to have been contained within the amniotic cavity and to have penetrated the embryo. After fixing, embedding and sectioning, the left nephric ducts of three embryos at H&H 17⁻, 17 and 18, in which fusion with the cloaca could be clearly seen, were selected for cell-counting.

Mitotic and apoptotic counts were made as a proportion of total cell number along the entire lengths of the selected ducts. The results are shown in Tables 14a-c. Firstly, it will be seen that the criteria listed on pp. 215-216 approximated to total cell numbers. Thus, region 1 corresponded to about 1000 cells, region 2 to 1000-2000 cells, region 3 to 4000-5000 cells and region 4 to about 1000 cells. Each duct comprised about 8000-9000 cells.

In all regions of each duct, the vast majority of cells appeared to be in interphase (approximately 75-95%) (see p. 240). Of the four phases of mitosis, more cells appeared to be in prophase or metaphase than in anaphase or telophase, regardless of anteroposterior level or embryonic stage.

In the following comparisons, cells in the three main phases of the life-cycle - interphase, mitosis and apoptosis - will be compared as proportions of total cell numbers, that is as indices of interphase, mitosis and apoptosis. Table 15 and figs. 71a-d show variations in these indices with anteroposterior region and embryonic stage.

Indices of apoptosis were generally lowest, but of mitosis highest, in region 2, where mitotic index

exceeded apoptotic index. In regions 1 and 3, indices of mitosis were fairly similar, as were indices of apoptosis, but in general apoptosis exceeded mitosis. In region 4, while mitotic indices were similar to those in regions 1 and 3, apoptotic indices were very high.

Although the results were based on one duct at each H&H stage, there appeared to be stage-related variations at different anteroposterior levels. Figs. 71a-d indicate that relative to increasing embryonic stage, in general mitotic indices increased in region 1, decreased in regions 2 and 3, and remained very low in region 4. Apoptotic index generally increased with embryonic stage at all anteroposterior levels, but especially in region 4. Indices of interphase usually decreased as embryonic stage increased, except in region 2. In most of these changes, however, the mid-stage (H&H 17) duct was anomalous.

Stage-related trends are clearer if regions 1&2 and 3&4 are combined, as shown in Table 16 and figs. 72a&b. Especially in the combined regions 3&4 (fig. 72b), stage-related trends may be seen in both mitotic and apoptotic indices. Thus, mitotic indices decreased slightly, while apoptotic indices increased

markedly with increasing stage. Furthermore, there was a steady decrease in the interphase index with increasing embryonic stage in these regions. Fig. 72a shows that there was also a slight decrease in the index of interphase in the combined regions 1&2. With the exception of the mid-stage embryo, there was also a slight decrease in mitotic rate and a slight increase in apoptotic index in these regions.

Figs. 72a&b also show a trend in anteroposterior differences in mitotic and apoptotic indices within individual embryos. Thus in each embryo, except the oldest, mitotic index exceeded apoptotic index posteriorly (combined regions 1&2), and in all the embryos apoptotic index exceeded mitotic index anteriorly (combined regions 3&4).

Table 17 and fig. 73 show that in each duct as a whole, the index of interphase decreased as embryonic stage increased. While overall mitotic and apoptotic indices were similar in the youngest two embryos, apoptotic index was considerably higher than mitotic index in the oldest embryo.

STAGE IN CELL CYCLE	CELLS AT PROGRESSIVELY POSTEROANTERIOR LEVELS OF THE DUCT								DUCT AS A WHOLE	
	1		2		3		4		NO.	%T
	NO.	%T	NO.	%T	NO.	%T	NO.	%T		
INTERPHASE	867	94.6	1624	96.2	4883	94.6	849	89.0	8223	94.3
PROPHASE	5	0.545	43	2.55	63	1.22	4	0.419	115	1.32
METAPHASE	2	0.218	13	0.770	36	0.697	6	0.629	57	0.654
ANAPHASE	1	0.109	5	0.296	17	0.329	1	0.105	24	0.275
TELOPHASE	1	0.109	1	5.921 X10 ⁻²	17	0.329	1	0.105	20	0.229
TOTAL MITOSES	9	0.982	62	3.67	133	2.58	12	1.26	216	2.47
APOPTOSIS	41	4.47	3	0.178	146	2.83	93	9.75	283	3.25
TOTAL	917	100	1689	100	5162	100	954	100	8722	100

TABLE 14a CELLS IN MITOSIS AND APOPTOSIS (PYKNOSIS) IN ONE CHICK NEPHRIC DUCT AT H&H 17

STAGE IN CELL CYCLE	CELLS AT PROGRESSIVELY POSTEROANTERIOR LEVELS OF THE DUCT								DUCT AS A WHOLE	
	1		2		3		4		NO.	%T
	NO.	%T	NO.	%T	NO.	%T	NO.	%T		
INTERPHASE	935	95.5	2001	95.0	4137	96.8	1043	76.6	8116	93.0
PROPHASE	17	1.74	45	2.14	58	1.36	18	1.32	138	1.67
METAPHASE	10	1.02	34	1.61	40	0.94	10	0.73	94	1.11
ANAPHASE	4	0.409	19	0.902	12	0.281	2	0.147	37	0.424
TELOPHASE	5	0.511	4	0.190	2	4.68 $\times 10^{-2}$	2	0.147	13	0.157
TOTAL MITOSES	36	3.68	102	4.84	112	2.62	32	2.35	282	3.36
APOPTOSIS	8	0.817	4	0.190	27	0.631	287	21.1	326	3.94
TOTAL	979	100	2107	100	4276	100	1362	100	8724	100

TABLE 14b

CELLS IN MITOSIS AND APOPTOSIS (PYKNOSIS) IN ONE CHICK NEPHRIC DUCT AT H&H 17

STAGE IN CELL CYCLE	CELLS AT PROGRESSIVELY POSTEROANTERIOR LEVELS OF THE DUCT								DUCT AS A WHOLE	
	1		2		3		4		NO.	%T
	NO.	%T	NO.	%T	NO.	%T	NO.	%T		
INTERPHASE	900	91.4	920	96.4	4546	89.7	732	77.3	7098	89.24
PROPHASE	11	1.12	6	0.629	33	0.651	5	0.528	55	0.692
METAPHASE	5	0.508	5	0.524	20	0.395	4	0.422	34	0.428
ANAPHASE	7	0.711	4	0.419	9	0.178	0	0	20	0.251
TELOPHASE	1	0.102	2	0.210	9	0.178	0	0	12	0.151
TOTAL MITOSES	23	2.44	17	1.78	71	1.40	9	0.950	121	1.52
APOPTOSIS	61	6.20	17	1.78	451	8.90	206	21.8	735	9.24
TOTAL	985	100	954	100	5068	100	947	100	7954	100

TABLE 14c

CELLS IN MITOSIS AND APOPTOSIS (PYKNOSIS) IN ONE CHICK NEPHRIC DUCT AT H&H 18

H&H STAGE	PERCENTAGE OF DUCT CELLS IN DIFFERENT STAGES OF THE LIFE-CYCLE, IN REGIONS 1-4											
	INTERPHASE				MITOSIS				APOPTOSIS			
	1	2	3	4	1	2	3	4	1	2	3	4
17 ⁻	94.6	96.2	94.6	89.0	0.982	3.67	2.58	1.26	4.47	0.178	2.83	9.75
17	95.5	95.0	96.8	76.6	3.68	4.84	2.62	2.35	0.817	0.190	0.631	21.1
18	91.4	96.4	89.7	77.3	2.44	1.78	1.40	0.950	6.20	1.78	8.90	21.8

TABLE 15 REGIONAL RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN THREE CHICK NEPHRIC DUCTS

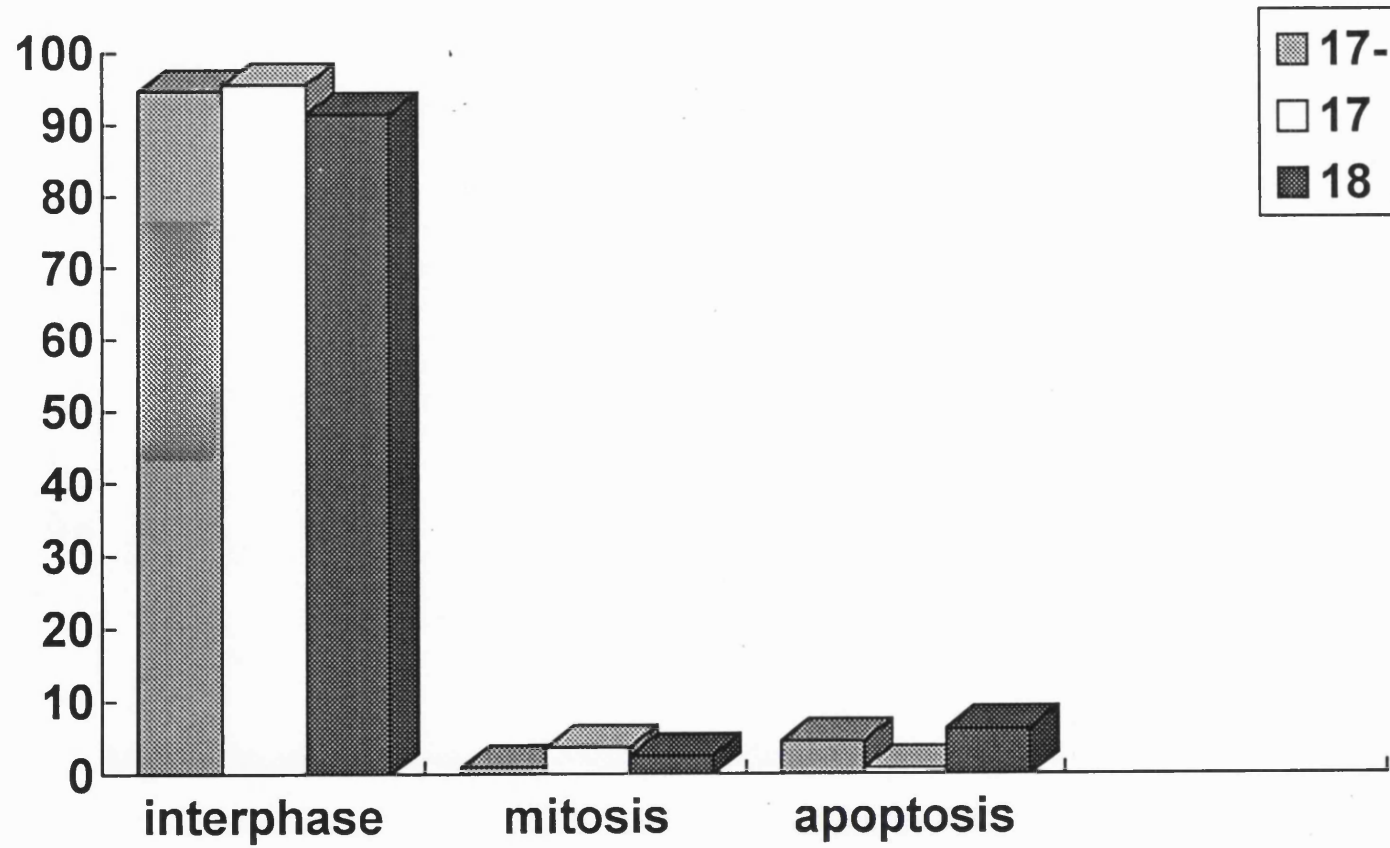


FIG. 71a RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN REGION 1 OF THREE NEPHRIC DUCTS

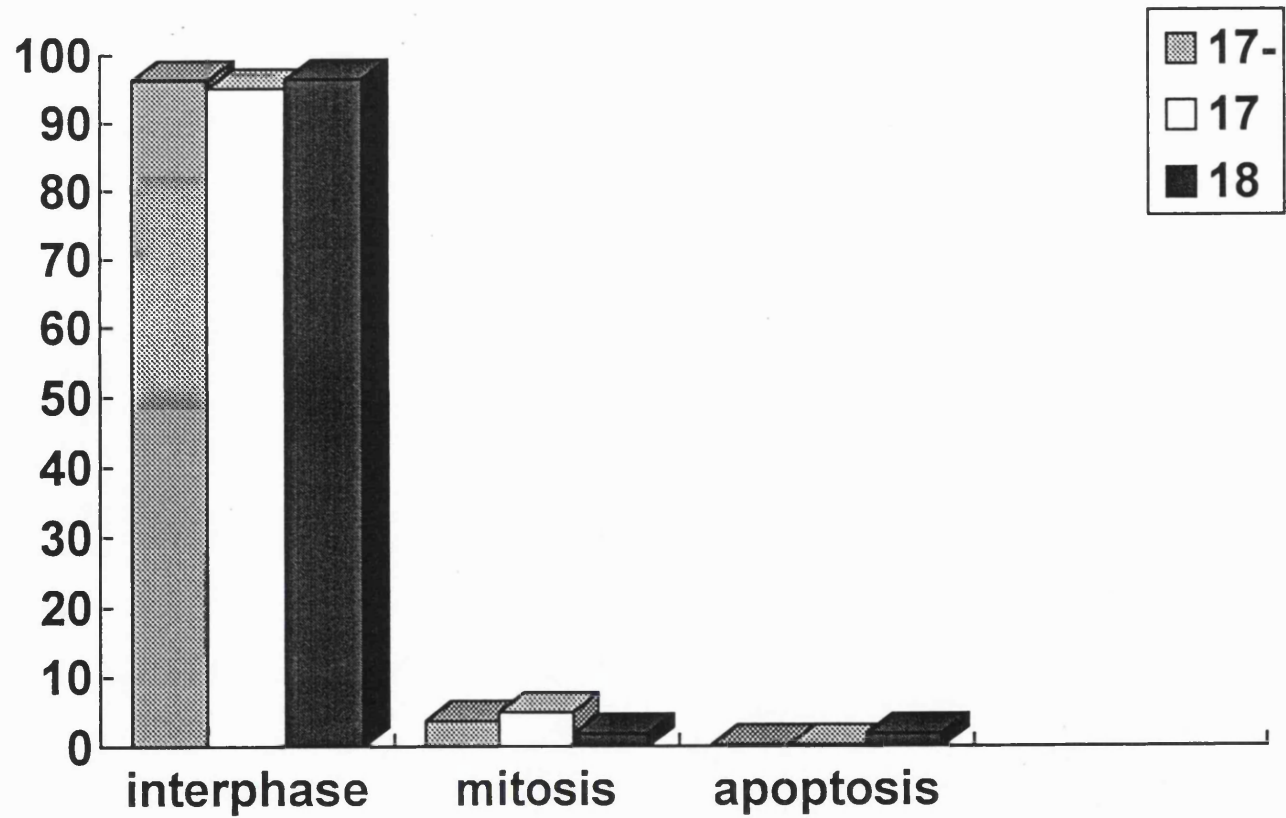


FIG. 71b RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN REGION 2 OF THREE NEPHRIC DUCTS

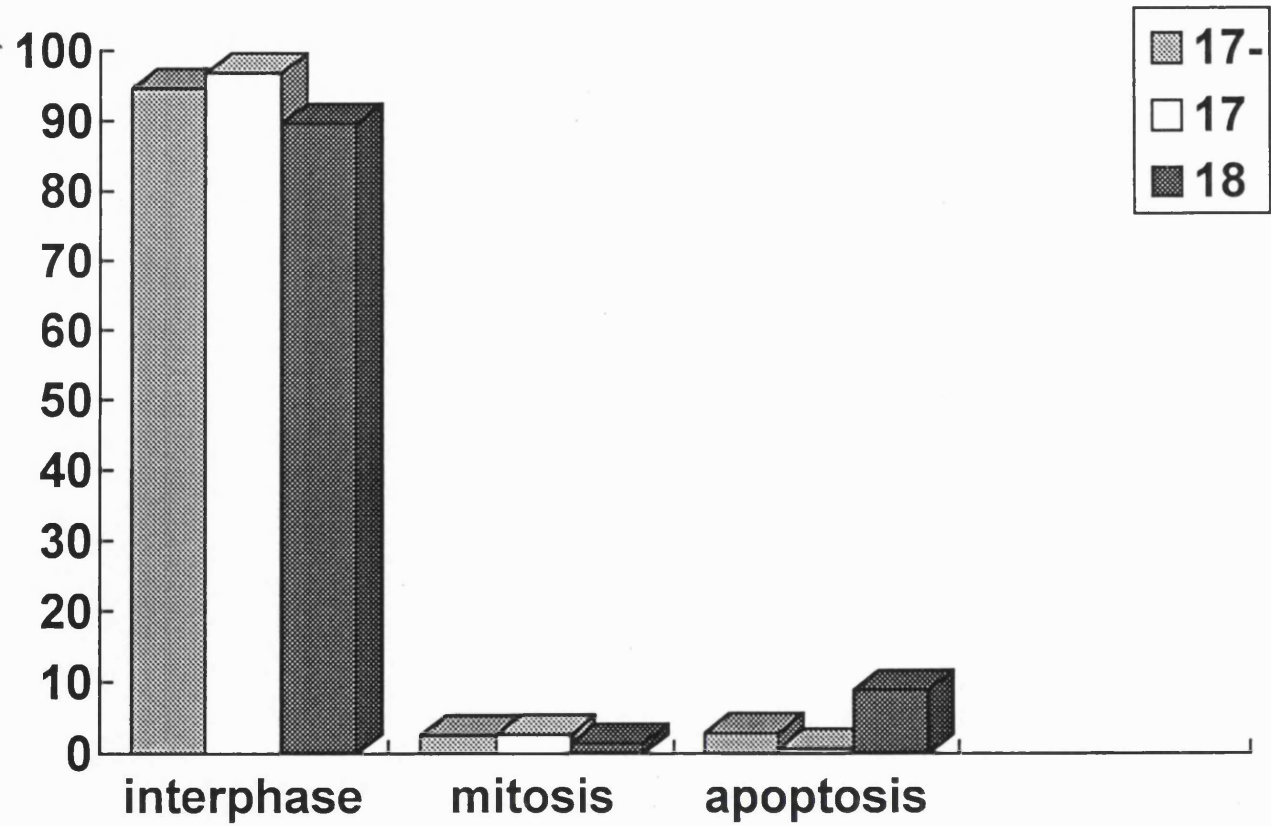


FIG. 71c

RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN REGION 3 OF THREE NEPHRIC DUCTS

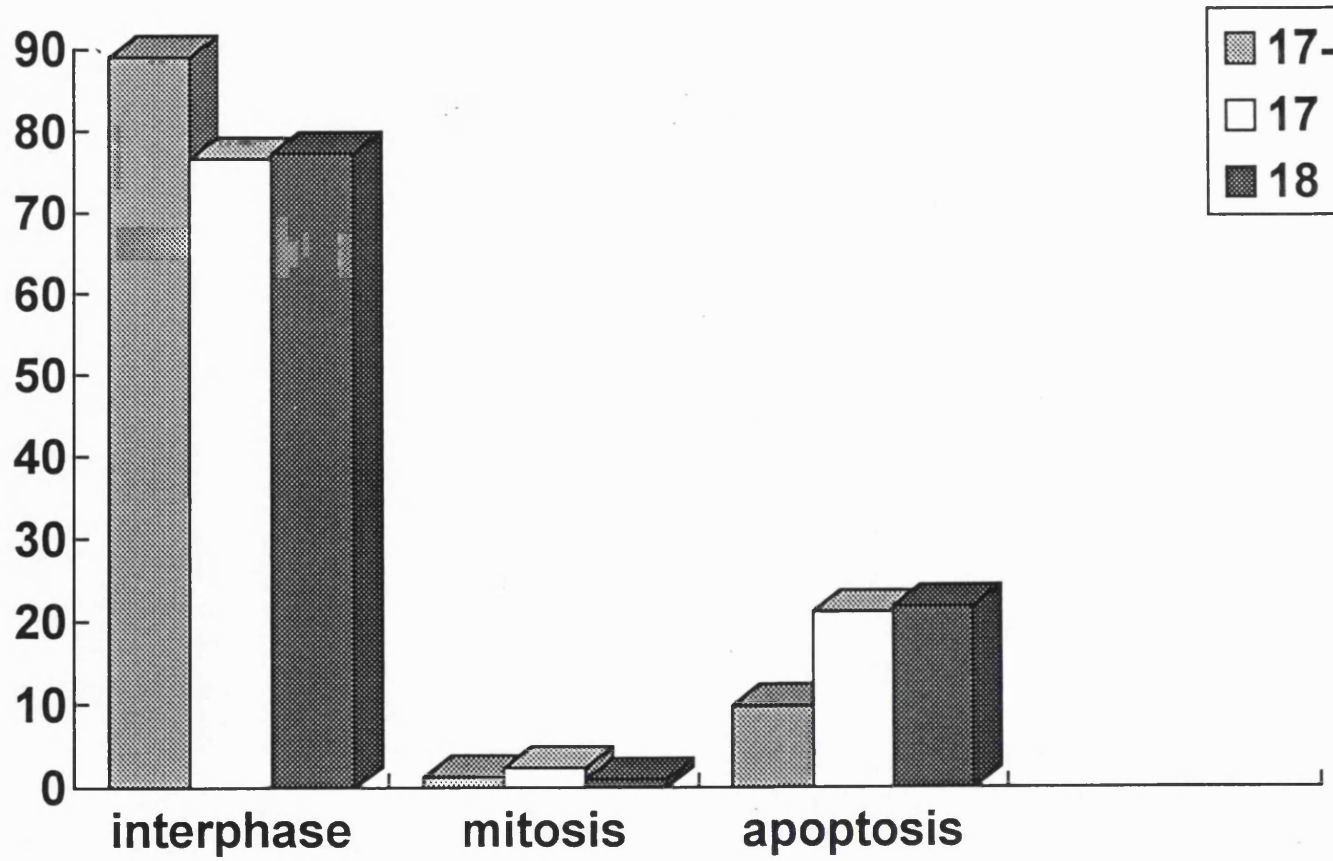


FIG. 71d RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN REGION 4 OF THREE NEPHRIC DUCTS

H&H STAGE	CELLS AT DIFFERENT STAGES IN THE LIFE-CYCLE, IN POSTERIOR (1&2) AND ANTERIOR (3&4) REGIONS OF THE DUCT												TOTAL NUMBERS OF CELLS IN REGIONS	
	INTERPHASE				MITOSIS				APOPTOSIS				1&2	3&4
	1&2		3&4		1&2		3&4		1&2		3&4			
	NO.	%T	NO.	%T	NO.	%T	NO.	%T	NO.	%T	NO.	%T		
17 ⁻	2491	95.6	5732	93.7	71	2.72	145	2.37	44	1.69	239	3.91	2606	6116
17	2936	95.1	5180	91.9	138	4.47	144	2.55	12	3.89 X10 ⁻²	314	5.57	3086	5638
18	1820	93.9	5278	87.7	40	2.06	80	1.33	78	4.02	657	10.9	1939	6015

TABLE 16 ANTERIOR AND POSTERIOR RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN THREE CHICK NEPHRIC DUCTS

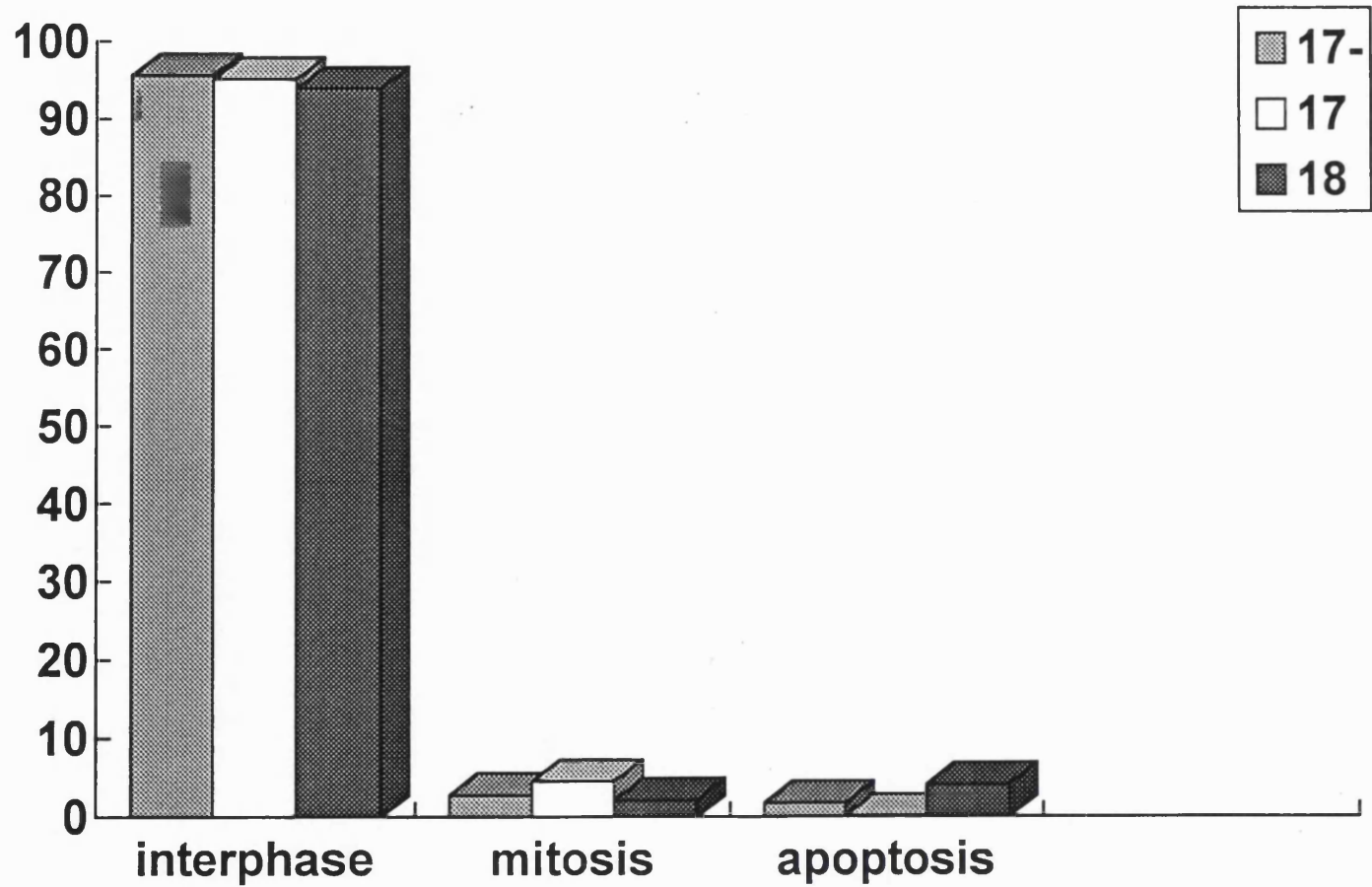


FIG. 72a

RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN COMBINED-REGIONS 1&2 OF THREE
NEPHRIC DUCTS

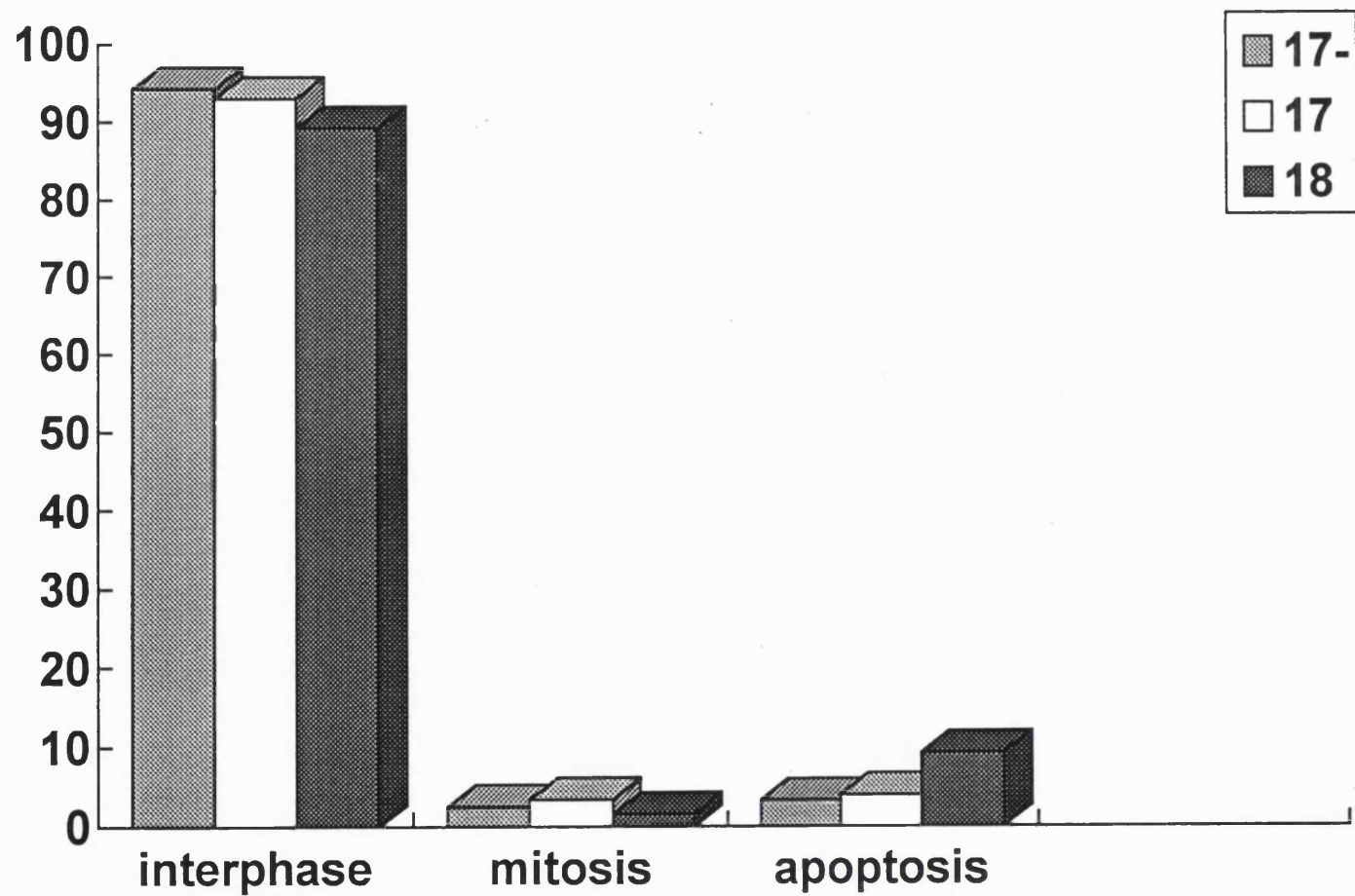


FIG. 72b

RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN COMBINED-REGIONS 3&4 OF THREE
NEPHRIC DUCTS

EMBRYONIC STAGE (H&H)	CELLS AT DIFFERENT STAGES IN THE LIFE-CYCLE IN THE DUCT AS A WHOLE						TOTAL NUMBERS OF CELLS IN DUCT
	INTERPHASE		MITOSIS		APOPTOSIS		
	NUMBER	% TOTAL	NUMBER	% TOTAL	NUMBER	% TOTAL	
17 ⁻	8223	94.3	216	2.48	283	3.25	8722
17	8116	93.0	282	3.36	326	3.94	8724
18	7098	89.24	121	1.52	735	9.24	7954

TABLE 17 OVERALL RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN EACH OF THREE CHICK NEPHRIC DUCTS

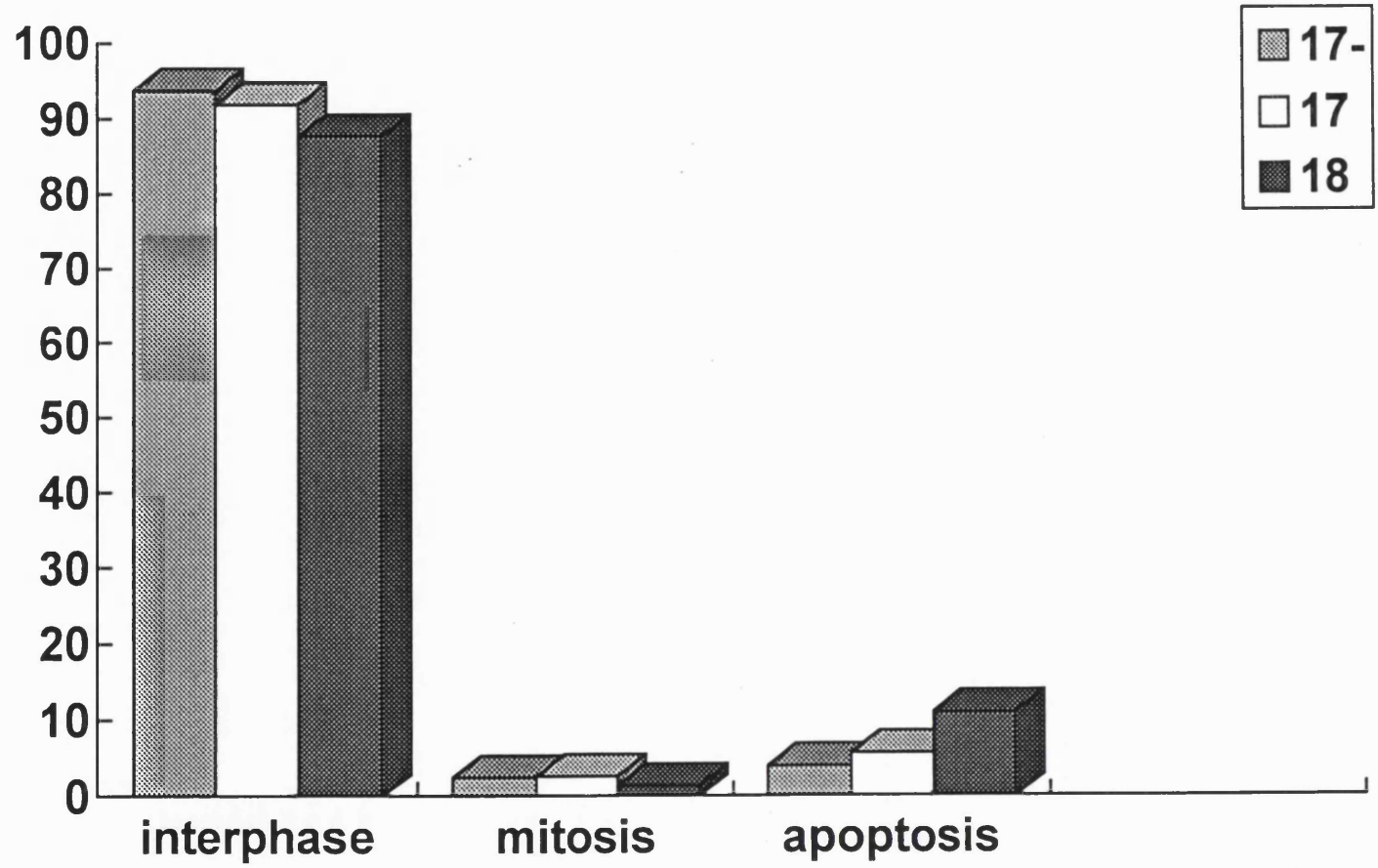


FIG. 73 OVERALL RATES OF MITOSIS, APOPTOSIS AND INTERPHASE IN THREE NEPHRIC DUCTS

CHAPTER FOUR

DISCUSSION

Although the present analysis was preliminary, the results appear to indicate that at H&H 17⁻-18 there are morphogenetically related differences in mitotic and apoptotic indices. Thus, changes in these indices with anteroposterior level may be related to cloacal fusion posteriorly and degeneration anteriorly. While anterior degeneration appeared to be closely associated with apoptosis, cloacal fusion appeared to be associated with both mitosis and apoptosis.

At the site of cloacal fusion (region 1), the general decrease in the index of interphase with increasing embryonic stage indicated that there was an increase in cellular turnover, while the more rapid increase in apoptosis than in mitosis indicated that, unless there was an accompanying, net immigration of cells, the duct was degenerating. If the duct was degenerating in this region, it could have been forming a new site of fusion, although the latter was not observed.

Although in females at later stages the nephric ducts degenerate, at stages before the metanephric tissues have developed the nephric ducts are required for excretion. Furthermore, the Müllerian ducts, which later occupy an anteroposterior region adjacent to that of the nephric ducts, begin to extend posteriorly only after the nephric ducts have reached the cloaca (Abdel-Malek 1950). The sexes of the embryos could not be established morphologically at the stages examined.

The anomalous, mid-stage duct, which had a higher mitotic than apoptotic index at the site of fusion, could have represented the mid-stage of cloacal fusion. Thus at the beginning and end of fusion, inward migration of cells might be more

important than mitosis in duct formation, but at the peak of fusion mitosis might be more important than immigration. This hypothesis is supported by the rate of mitosis exceeding that of apoptosis in the youngest duct in region 2, which, being more anterior, was at a more advanced stage of morphogenesis.

However, in the combined regions 1&2 (fig 72a), it was only the oldest duct which had an apoptotic index greater than its mitotic index. Perhaps in the younger two embryos, cells were dividing in region 2 and migrating into region 1. The oldest duct had the highest apoptotic indices in every region or combined region, although in the most anterior region (4), the mid-stage duct also appeared to be slightly anomalous, as it had an apoptotic index nearly as high as that of the oldest duct.

By comparing figs. 72a and b, it was found that in each duct, apoptotic index was higher anteriorly than posteriorly, while mitotic index was higher posteriorly than anteriorly. Degeneration was clearly associated with an increased apoptotic index, both anteriorly and with increasing embryonic stage (figs. 72b and 73). In the oldest embryo, an

increased apoptotic index was also seen more posteriorly, in region 3 (fig. 71c).

There have been two previous analyses of cellular proliferation in the chick nephric duct, at H&H 12&13 (Jacob et al 1992), and H&H 13-15 (Overton 1959a), i.e. during anteroposterior extension. Using the BRDU/ anti-BRDU method, Jacob et al (1992) found a decrease in the S-phase index - which they took to indicate a decrease in mitotic index - towards the posterior tip of the duct, especially at H&H 12. Overton's (1959a) results, based on twenty-four embryos, indicated that mitotic index increased towards the posterior tip. Both analyses found similar mitotic indices in neighbouring mesoderm and concluded that the duct does not extend as a result of an increased mitotic index at its posterior tip.

Overton (1959a) pre-treated a second group of embryos with the metaphase-arrest agent colchicine. This led to higher numbers of counted mitoses and a much greater increase at the posterior tip. However, it also appeared that extension was inhibited and diameter decreased in the duct as a result of the colchicine, probably because it disrupted tension in the blastoderm (Overton 1959a). Neither Jacob et al (1992) Overton (1959a) nor any other previous

analysis appears to have counted apoptotic indices in the nephric duct.

The present analysis will be extended, firstly to include more ducts of the stages examined in this analysis and, furthermore to include other stages. A future study might divide the duct into smaller regions in order to elucidate any segmental pattern in cellular turnover.

Although the immunohistochemical staining in the present study was consistent and of a reasonable intensity, interphase was potentially the most difficult stage to ascertain, as many of the cells appeared to be in various grades of pre-prophase or pre-pyknosis. Undoubtedly, in part this was due to the relatively short re-incubation time after treatment with BUDR in conjunction with an emphasis on counting mitoses rather than cells in S-phase. In future, more sensitive techniques might be used to identify additional stages in the cellular life-cycle, as well as changes in cellular morphology related to senescence (recently discussed by McCormick & Campisi, 1991).

CHAPTER FIVE

GENERAL DISCUSSION

CHAPTER FIVE

GENERAL DISCUSSION

The significance of the present study is that it appears to be one of very few to have compared development in either nephric and metanephric or nephron- and urinary-duct- tissues. Although recently a considerable amount of attention has been focussed on morphogenetic processes in the metanephric tissues, with the exploitation of molecular techniques (e.g. van Heyningen & Hastie 1992), the same can not be said for the nephric tissues. However, unlike the metanephric tissues, the nephric tissues have the advantage of a tradition of tissue- and cellular level analyses.

On the whole, recent nephrogenic analyses have concentrated either on migration of the nephric duct or induction of the metanephros and, with notable exceptions in the mouse metanephros (e.g. Vainio et al 1992), the interactions of morphogenetic processes have largely been ignored. Clearly, to understand the development of either of these tissues, their analyses need to be co-ordinated, both conceptually and in order to elucidate their likely interactions. (See also Sanders 1989).

The main aim of the present study has therefore been to form an integrated basis for further nephrogenic analyses, in which tissue-level questions may be addressed using precise, cellular and sub-cellular techniques. Some of the main, tissue- and cellular level questions arising from the literature on the nephric tissues (pp.58-59) have been addressed in both nephric and early metanephric tissues. A morphological approach, versatile techniques (LM, SEM and immunohistochemistry) and fixed tissue have been used. An analytical framework has been formulated (Table 6) and the thesis is arranged according to anatomical level (see Chapter 1).

The results of Chapters 2 and 3 (tissue and sub-cellular levels respectively) appear to indicate that in the chick, the earliest urinary duct and nephrons form from a common, mesenchymal rudiment originating adjacent to somites 6-8, at about H&H 9. It appears that the nephrons initially form as segregates of the common rudiment, while the rudiment itself converts into a duct. These results differ from the traditional view, in which the first urinary tissues to form are a small number of 'pro-' nephrons, which fuse posterolaterally to give rise to a duct.

Furthermore, while the accepted view has been that a second type of early nephron, the 'meso-' nephron, forms subsequently and as a result of induction by the duct, it appeared from the present analysis that all the nephrons associated with the first urinary duct formed in a similar manner to those described above. Thus, the general term, 'nephric', has been adopted to refer to the early urinary tissues.

Another important question has been that of the posterior extension of the nephric tissues. While it

is generally thought that successive 'meso-'nephrons are induced, the duct has been thought to extend by migration. However, the question remains as to how duct material is generated anteriorly and whether 'migration' is apparent rather than actual.

The present analysis has shown remarkable similarities between the nephric nephron- and duct-tissues during extension. In particular, both tissues have similar patterns of epithelialisation and appear to be anteroposteriorly segmented, with four presumptive nephrons, or in the duct four presumptive-lumen punctules (e.g. fig. 16, p. 112), corresponding to one somite length (e.g. fig. 34, p. 129). Thus it appeared that epithelialisation and short-range migration (cellular rearrangement), both related to segmentation, might be important in extension. This does not appear to have been previously suggested.

Such a short-range means of extension might be termed 'budding'; which is interesting with regard to the metanephric duct rudiment, often known as the ureteric bud, but whose extension appears not to have

recently been analysed. Such short-range extension might also be involved in anteroposterior formation of the nephric tissues.

Although segmentation of the nephric tissues was a source of debate in the early part of the present century, it does not appear to have been specifically investigated using cell-lineage analysis of the type used by Stern et al (1988).

Expression of the S-FC10.2 antigen did not appear to be associated with long-range duct migration, but rather with formation in all the nephric tissues and the metanephric duct. In those tissues which formed by way of mesenchymal rudiments (nephric duct and nephric-nephron rosettes), expression was considerably weakened or lost with epithelialisation, but in those tissues which formed as epithelia (later parts of the nephric nephrons) or semi-epithelia (the metanephric duct), the antigen was expressed during formation. Thus it did not appear to be as important to epithelialisation as to formation itself.

S-FC10.2 expression might also be cell-lineage-specific, because although it was expressed by the nephric tissues and the metanephric duct, which have frequently been suggested to be derived from the same lineage, it was not expressed during condensation of the metanephrons, which are thought to be derived from a different lineage (e.g. Mugrauer & Ekblom 1991; cf. figs. 56 & 14). Thus, the present analysis has shown that S-FC10.2 expression is consistent with induction in three (nephric duct, nephric nephrons and metanephric duct) of the four nephrogenic tissues (the fourth being the metanephrons). The lack of expression in the metanephrons might further indicate that it is restricted to earlier stages of phenotypic commitment.

There appear to have been few analyses of nephrogenesis at stages of nephric-duct fusion with the cloaca, and up to and including outgrowth of the metanephric duct (H&H 24-27). Preliminary results of an analysis of cellular turnover (Chapter 4) appeared to support the hypothesis of short-range migration described in the preceding pages (also see p. 45). Thus in the chick, fusion with the cloaca occurs from

about H&H 17 to 18 and might depend on short-range migration, from an adjacent region to the tip of the duct (pp. 237-238), together with mitosis and apoptosis.

Future analyses, outlined in Chapters 1-4, should include: a comprehensive fate-map and commitment-schedule of all the nephrogenic tissues; a dynamic, morphological analysis to ascertain the relative importance of *in situ* and migratory events (see Lynch and Fraser 1990); and complementary analyses of molecular expression patterns previously ascertained in other nephrogenic tissues. Using approaches of this type, we may expect to improve our understanding of all stages of nephrogenesis. Thus we may develop a model in amniote development of similar breadth and detail to that in the slime-mould, *Dictyostelium discoideum* (see Cox 1992).

APPENDICES

TABLE 18

APPENDICES

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APPENDIX 1

PANNETT & COMPTON'S (1924) SALINE

Two stock solutions, A and B, were made up, autoclaved and stored in a refrigerator in 250ml medical-flat bottles.

Solution A, in 1000ml distilled water:

121.0g NaCl

15.5g KCl

7.7g CaCl₂

0.4g MgCl₂.6H₂O

Solution B, in 1000ml distilled water:

1.89g Na₂HPO₄

0.15g NaH₂PO₄.H₂O

A physiological solution was made up by adding 40ml of solution A and 60ml of solution B to distilled water (directly mixing A and B causes precipitation) to a final volume of 1000ml.

APPENDIX 2

PREPARATION FOR SCANNING-ELECTRON MICROSCOPY

After removal from the eggs and rinsing (see Chapter 2 Methods, p.89), specimens were transferred into 2.5% glutaraldehyde (Emscope; supplied as a 25% stock solution) in PBS (Oxoid/Unipath; 1 tablet in 100 ml. distilled water). The area opaca of each nephric-stage embryo was stretched immediately but gently under roughened glass rings to prevent distortion of the embryo. Fixation was for approximately two hours. Each embryo was then rinsed three times in PBS, when the area opaca was also removed. At this point they could be kept in a refrigerator for up to five days.

Because of the toxicity and volatility, surgical gloves and a fume cupboard were used to transfer the embryos into 1% osmic acid (osmium tetroxide in 0.067M PBS). These were covered immediately with foil, as the osmic solution is light-sensitive, and allowed to stand for one hour; after this the embryos were again rinsed well in PBS.

Supplementary steps included treatment with tannic acid (Sigma), to increase brittleness in the embryo if it was to be freeze-fractured for examination in transverse section, and maceration in a weak (0.1%) osmic acid solution for about 48 hours, to destroy the

cell and nuclear membranes and thus enhance examination of the remaining intracellular structures. If required, both these treatments were carried out just before standard osmication.

Freeze-fracturing, which was done after osmication, on a metal platform cooled in liquid nitrogen, involved rapidly freezing each specimen in 50% dimethoxy-sulphoxide (DMSO, a cryoprotectant) in PBS and striking a single-edged razor blade held against it in the required position. After freeze-fracturing, specimens were re-osmicated, as the high molecular mass of osmium ions impedes their penetration into the embryo.

Immediately before critical-point drying, a maximum of six embryos was dehydrated in an ascending series of acetone concentrations, allowing about twenty minutes in each solution and changing the final, 100% solution once. The embryos were then transferred into individual, numbered compartments of the specimen-holder of the critical-point drying apparatus within a small plastic container in which there was a residual amount of 100% acetone. The specimen-holder was placed in the chamber of the critical-point dryer, which was then closed by means of a screw-thread, securely but without damaging the thread.

The internal temperature of the chamber was set at 0-2 °C. Carbon dioxide was introduced into the chamber

slowly through a valve until the chamber was full, as seen through one or both of its windows. An internal stirring mechanism was used to circulate the carbon dioxide. Another valve was then opened to allow the carbon dioxide to escape into a beaker containing a solution of sodium bicarbonate in which it dissolved. This was repeated twice, to rinse the acetone from the embryos.

The chamber was filled for a fourth time and the stirrer switched off to prevent turbulence during dessication. Dessication was carried out by re-setting the chamber's temperature at 40°C (above the critical temperature of CO₂ at a pressure of 60-70 Tor) and releasing the carbon dioxide at a rate which allowed the pressure in the chamber not to rise above 70Tor. This is important in order to prevent damage to the valve or an explosion.

After dessication, the embryos were mounted on individual metal stubs to fit the specimen-holder of the microscope. Each specimen was stuck to the platform of a stub using a paste of graphite in organic solvent, which was also used to coat the remaining exposed platform surface. This was done quickly with a Borodaile knife before the solvent evaporated and to prevent unnecessary exposure of the embryo to atmospheric moisture. Each mounted embryo was stored immediately in a dessicator containing dried crystals of silica gel.

A maximum of five stubs could be contained in the 'sputter-coater', which was then used to cover the specimens with atomised gold. In this apparatus, a high voltage (600-700kV) is set up across a diode in a vacuum. The vacuum was set up by first rinsing and filling the chamber with argon gas and then emptying it of the pure gas. The cathode is gold-plated and the specimen-holder acts as the anode; a current of 20 mA and a purple glow of argon were used to indicate the transference of gold onto the specimens. Gold was transferred for 4-6 minutes to form a fine coating. After sputter-coating, up to four stubs at a time were examined in the microscope.

APPENDIX 3

BUFFERED FORMAL SALINE

An aqueous solution of 10% formalin (i.e. 4% formaldehyde) in 0.9% (physiological) sodium chloride was made up using the following proportions:

9g NaCl

100ml 40% formaldehyde in distilled water

900ml distilled water

This fixative can be stored in a stoppered glass bottle at room temperature for at least one year.

APPENDIX 4

PREPARATION FOR LIGHT MICROSCOPY

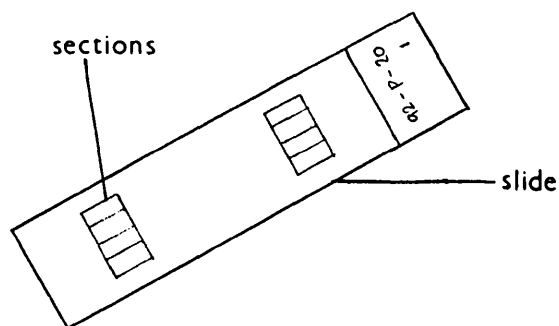
Specimens was transferred from P&C into buffered formal saline (BFS), fixed for 1-2 hours, depending on their size (H&H stage), and then rinsed three times in PBS. (See Chapter 2 Methods, p. 89).

Dehydrating was carried out in an ascending series of aqueous ethanol, with about twenty minutes in each solution and one change in 100% ethanol (the first 100% ethanol contained 0.2% Light Green [Gurr], a relatively inert stain which may be subsequently washed out if required). The embryos were transferred immediately into cedar wood oil, where they were left to clear overnight; they may have been left for up to two weeks in the oil in covered glass petri dishes.

To exchange the cedar wood oil for paraffin-wax, about six cleared embryos of similar size were transferred into individual plastic wax-moulds and each was touched gently with filter paper to remove excess oil. The moulds were filled with melted paraffin-wax and placed immediately in an oven set at 45°C. After about thirty minutes the wax was changed. This was repeated two or three times, depending on the size of the embryos.

To embed the specimens, the wax was changed once more and the tip of a fine metal spatula heated in a Bunsen flame was used to hold each embryo briefly above the bottom of the mould while the latter was submersed in a cold-water bath to allow the wax near the bottom to solidify. The embryo was then released and the upper surface of the wax was blown, to form a skin, before being submersed gently. After about one hour in the water bath, the embedded specimens were stored in a refrigerator.

The wax blocks were trimmed, mounted on chucks and sectioned with a microtome at either 4 or 10 μ m. For immunohistochemical staining, sections were placed on clean, dry, glass slides, in two groups as shown below. The sections were then stretched on the slides, by floating each group on a few drops of distilled water and placing each slide horizontally for about three minutes on a hot-plate set at about 60°C. The water was then drained carefully onto a paper towel, leaving the sections on the slide, which was placed in a rack to dry at room temperature overnight.



APPENDIX 5

IMMUNOPEROXIDASE STAINING FOR S-FC10.2

For abbreviations, see Table 3, p. 10; for stock solutions see p. 262.

1. Score around sections.
2. De-wax and take sections through xylene and graded IMS to 0.9% NaCl (approx. 5 min. in each).
3. Wet with 1% BSA in PBS.
4. Remove excess: wipe the slide with tissue paper, underneath as well as between and around the sections (BSA helps the following solution to spread).
5. Neuraminadase treatment:
Approx. 30 μ l of 100mU/ml neuraminidase (frozen Ependorf's) on each set of sections; incubate 2 hr. at 37°C in humid atmosphere.
N.B. Check after 1 hr. for drying-out.
6. Wash in 0.9% NaCl (as above).
7. Wash in PBS for approx. 15 min. including one change.
8. Wet with 1% BSA (as above and in steps below) and remove excess.
9. Block with 15 X 10⁻³% normal goat serum (Vector) in PBS for 10 min.
10. Remove excess.

11. Treat with 50% supernatant/PBS solution of primary antibody, FC10.2, or control, NB10.3B4 (approx. 30 μ l on each set of sections), for 30 min. (frozen Ependorf's).
12. Prepare avidin-peroxidase (Vector) (about 30 min. before use in step 19): 5ml PBS + 1 drop A + 1 drop B in squeeze-bottle.
13. Wash with PBS (as above and in steps below).
14. Wet with 1% BSA and remove excess.
15. Treat all sections with 5 X 10⁻³% secondary antibody, biotin-labelled anti-mouse IgM (Vector) in PBS for 15 min. (frozen Ependorf's).
16. Remove DAB substrate from freezer:
N.B. HARMFUL - WEAR GLOVES. Handle with care and put vial in a beaker; all instruments etc. coming into contact with DAB in subsequent steps (22, 24, 25) must be de-contaminated with an aqueous solution of bleach and disposable items must be incinerated.
17. Wash with PBS.
18. Wet with 1% BSA and remove excess.
19. Treat with avidin-peroxidase solution for 15 min.
20. Wash with PBS.
21. Wash with distilled water.
22. Add 2.5-3.0 μ l H₂O₂ to DAB.
23. Wet with 1% BSA and remove excess.
24. Treat with DAB for approx. 30 min., in the dark.
25. Wash with distilled water (as above).

26. Counterstain:

- a. 70% IMS (dip)
- b. 0.2% Light Green in 100% IMS (1 min.) (Gurr; to be made freshly)
- c. 100% IMS (dip)
- d. xylene I (dip)
- e. xylene II (dip)

27. Wipe coverslip gently with Vellin tissue and mount in DPX (BDH).

28. Photograph within three days, using Kodak Ektachrome daylight ASA 64 colour reversal film.

Note 1:

Re-staining does not require neuraminidase treatment; coverslips attached with DPX can be removed by soaking overnight in xylene.

Stock solutions:

- a. 500ml physiological PBS: 1 tablet (Oxoid/Unipath)
in 100ml distilled water
- b. 200ml 0.9% NaCl in distilled water
- c. 100ml 1.0% BSA (Sigma, fraction V) in PBS
- d. H₂O₂ (Sigma): use as supplied (30% aqueous
solution)

Frozen aliquots:

- a. Neuraminidase (Boehringer-Mannheim, from *Vibrio cholerae*): 300 μ l of 100mU ml⁻¹ in pH 5.5 acetate
buffer (Appendix 6a, p. 263)
- b. FC10.2 (gift of Dr. T. Feizi): 300 μ l of 50%
supernatant in PBS
- c. NB10.3B4 (gift of Dr. T. Feizi): 300 μ l of 50%
supernatant in PBS
- d. 2° antibody (biotinylated anti-mouse IgM; Vector):
5 X 10⁻³% in PBS
- e. DAB (Sigma): 5ml vials of 0.2mg ml⁻¹ in pH 5.5
citrate buffer (Appendix 6b, p. 263)

Note 2:

This protocol was provided by Dr. Wendy Loveless.

APPENDIX 6

a. ACETATE BUFFER

100mM sodium acetate (ethanoate) was made up in distilled water and brought to the required pH 5.5 by adding acetic (ethanoic) acid. To the buffer were added CaCl_2 (1mM), BSA (0.01%) and Tween 20 (0.02%).

The buffer with additives was frozen in 5ml vials, which were used as needed to dilute neuraminidase. Dilute neuraminidase was also stored frozen.

b. CITRATE BUFFER

50mM sodium dihydrogen phosphate was made up in distilled water and brought to the required pH 5.5 by adding 50mM tri-sodium citrate in distilled water.

APPENDIX 7

CALCIUM- & MAGNESIUM- FREE TYRODE'S SALINE

A stock solution was made up, autoclaved and stored in the refrigerator in 250ml medical-flat bottles. In 900 ml distilled water the 10X stock solution contained:

80.0g NaCl
2.0g KCl
0.5g NaH₂PO₄
10.0g glucose

For 200ml aliquots of a physiological solution, which could be stored in a refrigerator:

- i. 90ml of stock solution were made up to 900ml with sterile, double-distilled water,
- ii. 180ml were dispensed into each of five 250ml bottles and
- iii. 20ml of 1% sodium hydrogen carbonate in distilled water were added to each bottle using a syringe and 0.22 μ m Millipore filter.

APPENDIX 8

IMMUNOPEROXIDASE STAINING FOR BUDR

For abbreviations, see Table 3, p. 10; for stock solutions see p. 267.

1. Score around sections.
2. De-wax in two xylenes and take through 100%, 90% and 70% IMS to distilled water.
3. Hydrolyse in 1.5N hydrochloric acid for 20 min. at room temperature.
4. Wash with distilled water for 5 min., including one change.
5. Wash with PBS for 15 min., including one change.
6. Discard distilled water.
7. Wet with 1% BSA in PBS.
8. Remove excess, by wiping around and between sections and underneath slide.
9. Block with normal goat serum (Vector; 15 X 10⁻³% in PBS) for 10 min.
10. Remove excess.
11. Treat with primary antibody, 10% anti-BUDR (Becton-Dickinson) in PBS, for 1 hour (about 30 μ l on each group of sections) (frozen Ependorf's).
12. Wash with PBS.
13. Wet with BSA and remove excess.

14. Treat with secondary antibody, 2% biotin-labelled anti-mouse IgG for 1 hour (Vector; frozen Ependorf's) (about 30 μ l on each group of sections).
15. Prepare avidin-peroxidase (Vector) about 30 min. before use (in step 19): one drop A + one drop B in 5ml PBS.
16. Remove DAB substrate from freezer - **HARMFUL: WEAR GLOVES. Handle with care** and put vial in a beaker; all instruments etc. coming into contact with DAB in subsequent steps (22, 24, 25) must be decontaminated with an aqueous solution of bleach and disposable items must be incinerated.
17. Wash with PBS.
18. Wet with 1% BSA and remove excess.
19. Treat with avidin-peroxidase for 15 min.
20. Wash with PBS.
21. Wash with distilled water.
22. Add 2.5-3.0 μ l H₂O₂ to DAB.
23. Wet with BSA and remove excess.
24. Treat with DAB for approx. 30 min., in the dark.
25. Wash with distilled water.
26. Counterstain:
 - a. Harris's haematoxylin (Gurr) in tap water (1 min.)
 - b. Rinse in slowly running tap water
 - c. Differentiate in acid alcohol (1% v/v 1N HCl/70% IMS) (dips)
 - d. Rinse in running water a further 10 min.

27. Dehydrate in serial alcohols and clear in two xylenes.
28. Wipe coverslips gently with Vellin tissue and mount in DPX (BDH).

Stock solutions:

- a. 500ml physiological PBS: 1 tablet (Oxoid/Unipath) in 100ml distilled water
- b. 200ml 0.9% NaCl in distilled water
- c. 100ml 1.0% BSA (Sigma, fraction V) in PBS
- d. H₂O₂ (Sigma): use as supplied (30% aqueous solution)

Frozen aliquots:

- a. anti-BUDR (Becton-Dickinson): 300μl of 10% in PBS, with 0.5% BSA (Sigma), 0.05% Tween 20 (Sigma) and 0.1% Triton 100 (BDH).
- b. 2° antibody (biotinylated anti-mouse IgG; Vector): 300μl of 2% in PBS, with 0.5% BSA (Sigma), 0.05% Tween 20 (Sigma) and 0.1% Triton 100 (Sigma)
- c. DAB (Sigma): 5ml vials of 0.2mg ml⁻¹ in pH 5.5 citrate buffer (Appendix 6b, p. 263)

Note:

This protocol was adapted from Primmitt et al (1989), Jacob et al (1992) and Appendix 5.

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